CHEMISTRY & BIODIVERSITY

Accepted Article

Title: Synthesis and Spectral Characterization of Asymmetric Azines Containing a Coumarin Moiety: The Discovery of New Antimicrobial and Antioxidant Agents

Authors: Milenko N. Ristić, Niko S. Radulovic, Biljana R. Dekić, Vidoslav S. Dekić, Novica R. Ristić, and Zorica Stojanović-Radić

To be cited as: *Chem. Biodiversity* 10.1002/cbdv.201800486

Link to VoR: http://dx.doi.org/10.1002/cbdv.201800486

Chem. Biodiversity

Synthesis and Spectral Characterization of Asymmetric Azines Containing a Coumarin Moiety: The Discovery of New Antimicrobial and Antioxidant Agents

Milenko N. Ristić,[a] Niko S. Radulović,*[,b] Biljana R. Dekić,[a] Vidoslav S. Dekić,[a] Novica R. Ristić,[a] and Zorica Stojanović-Radić,[c]

[a] Department of Chemistry, Faculty of Science and Mathematics, University of Priština, Lole Ribara 29, 38220 Kosovska Mitrovica, Serbia

[b] Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia, e-mail: nikoradulovic@yahoo.com

[c] Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia

Nine unsymmetrical azines containing a coumarin moiety were prepared by the reaction of the hydrazone of 4-hydroxy-3-acetylcoumarin with differently substituted aromatic aldehydes. The azines were fully spectrally characterized, including a complete assignment of ^{1}H- and ^{13}C-NMR resonances, and assessed for their acute toxicities in the *Artemia salina* model. Their free radical scavenging activities were tested in the DPPH$^{\cdot}$ assay, and *in vitro* antimicrobial activities were determined against seven bacterial and two fungal strains. The azines containing a *p*-hydroxyphenyl group were shown to be the most effective antimicrobial agents, and in the case of resistant strains of *Staphylococcus aureus* and *Acinetobacter baumannii*, the activity was comparable to that of chloramphenicol. The derivative having a 3,5-dimethoxy-4-hydroxyphenyl group exhibited pronounced antioxidant power reacting rapidly and in 1 : 1 mol ratio with the DPPH radical.

Keywords: unsymmetrical azines • coumarins • *Artemia salina* • antioxidant • antimicrobial activity

Introduction

Azines were in the focus of organic chemists due to their wide range of interesting chemical properties ("conjugation blockers"), arising from the opposite orientation of two polar electron-acceptor groups joined by an N–N bond, in addition to numerous biological and therapeutic actions.[1] In addition to having a possible application in analytical chemistry, they are considered as good synthons in a number of synthetic transformations, especially the ones leading to heterocyclic systems,[2][3] but those having aromatic moieties also have been utilized for the preparation of conducting materials, dye lasers, image-recording materials, dosimeters of ultraviolet radiation, and other optical devices.[4] Azines have been used as versatile ligands for the synthesis of novel complexes with different metal cations.[5–8] The interaction of azines with biological systems is also well investigated, and they are known to possess antimicrobial,[9] anticonvulsant,[10] antidepressant,[11] anti-inflammatory,[12] and antitumor activities.[12][14] Likewise, they behave as platelet[15] and aldose reductase inhibitors.[16]

Coumarins (2*H*-1-benzopyran-2-ones) represent a class of synthetically useful heterocyclic compounds[17][18] that have been accredited with a broad range of biological/pharmacological action including antimicrobial,[19–24] antifungal,[25][22] antiviral,[26–29] anticoagulant,[30] antitumor[31–33] and anti-inflammatory[34] activities. They have been used as additives in food and cosmetics,[35] brightening agents and dyes for tuning lasers.[36] Among numerous derivatives, those possessing the 4-hydroxy substituent have been long known for their effect as competitive inhibitors of vitamin K in the biosynthesis of prothrombin (oral anticoagulants), with warfarin, dicoumarol, and bromadiolone as examples of coumarin derivatives used as rodenticides with this *modus operandi*.[37–39] The mentioned activity of 3-substituted 4-hydroxycoumarins is known to be dependent on the nature of the substituent in position 3.[17][40][41] There are examples of poorly water-soluble antibiotics (such as novobiocin and clorobiocin), containing the substituted 4-hydroxycoumarin core and that act as potent inhibitors of bacterial DNA gyrase 2 and topoisomerase IV, but that have not found successful pharmaceutical application so far due to: a) low activity against Gram-negative bacteria, b) toxicity and side effects, c) rapid emergence of coumarin-resistant bacterial strains.[42]

It appears natural to expect that 4-hydroxycoumarin derivatives 3-substituted with an aromatic moiety linked with an azine tether would display a similar range of activities where both the azine and coumarin functionalities would contribute to the overall activity. Such unsymmetrical (mixed) azines of 3-acetyl-4-hydroxycoumarin are known, albeit not in great number, and have been proven to inhibit cyclin-dependent kinases.[43] Reports on these compounds contain only limited or largely unassigned spectral data, particularly the NMR spectra.[43] Prompted by all of the above mentioned and our interest in antimicrobial coumarins,[24][44–46] we decided to contribute to the structural diversity of these derivatives by preparing a series of

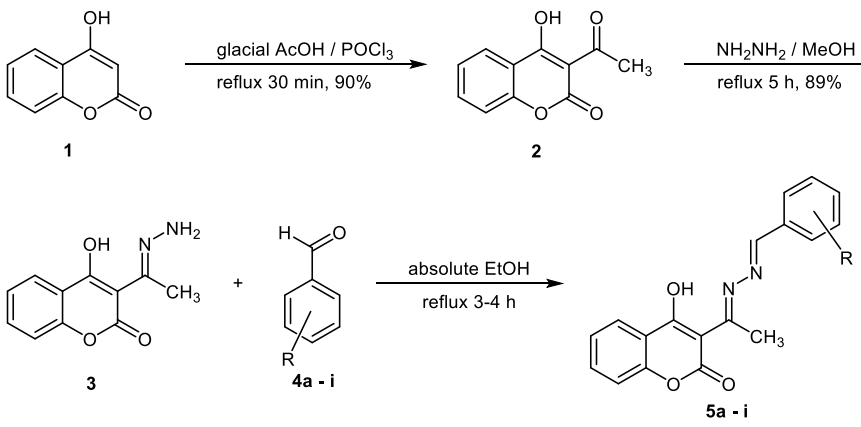
unsymmetrical azines baring the 3-substituted-4-coumarin core and a range of differently substituted phenyl rings in a three-step reaction sequence starting from the commercially available 4-hydroxycoumarin. The prepared compounds would be spectrally characterized in detail, including the full assignment of ^{1}H- and ^{13}C-NMR resonances with the aid of extensive 1D- and 2D-NMR experiments. In order to locate the most pharmacologically interesting ones, with respect to the shortcomings given above, these were envisaged to be tested against a range of Gram-positive and Gram-negative bacteria, and fungal strains, of differing resistance to antibiotics. Their acute toxicity would be evaluated in the simple *Artemia salina* model. Furthermore, wishing to probe the non-existence of extended conjugation in azines, and gaining an insight into their possible mode of action, the prepared compounds would also be screened for radical scavenging activity (DPPH$^{\cdot}$).

Results and Discussion

Chemistry

The starting coumarin ketone, compound **2**, was obtained by the acetylation of 4-hydroxycoumarin performed with glacial acetic acid in the presence of $POCl_3$ as the catalyst. We considered two possible ways to reach the target asymmetric azines: either directly in a single condensation step involving no control over the formation of the initial hydrazone and subsequently of the azine from a mixture of two carbonyl compounds, or in two separate steps where the hydrazone would be isolated and purified and then subjected to the reaction with one more equivalent of a carbonyl compound. Expectedly, the first approach gave mixtures of products that mostly consisted of the symmetric azine of the aromatic aldehydes, the hydrazone of 3-acetyl-4-hydroxycoumarin **3**, and a varying amount of the asymmetric azines. Practically no symmetrical azine of the coumarin derivative could be detected. This reactivity of compound **2**, i.e. the formation of the hydrazone **3** and no symmetric azine, and its less pronounced reactivity when compared to (more electrophilic) aldehydes, can be possibly explained in two ways. The reduced nucleophilicity of the NH_2 nitrogen in the hydrazone **3** due to the formed intramolecular hydrogen bond with the phenolic group in position 4 of the coumarin core could retard the formation of the azine or the lower solubility of the formed hydrazone (again due to the intramolecular hydrogen bonding) in the reaction medium (as evidenced by the formation of precipitate during the condensation step) would shift the reaction equilibrium in that direction.

For this reason, we opted for the two-step approach. Although there were two conceivable combinations (to form the hydrazone of the aldehydes or that of compound **2**), the above-mentioned results and the general shortening of the synthetic pathway encouraged us to choose the approach involving hydrazone **3**, i.e. asymmetric azines were obtained starting from the purified hydrazone **3** which was condensed with an equivalent amount of an aromatic aldehyde in solution. We also assayed the other combination (reacting **2** with a hydrazone of an aromatic aldehyde) but found it to produce lower yields of the azines which were contaminated with the symmetric azines of the aldehydes. Thus, a series of asymmetric azines **5a – i** of 3-acetyl-4-hydroxycoumarin and different aromatic aldehydes **4a – i** was prepared as presented in *Scheme 1*. Optimal conditions for the preparation of compound **3** involved the reaction of compound **2** with hydrazine hydrate in 1 : 1 molar ratio in methanol. The target azines **5a – i** were synthesized in absolute ethanol in moderate-to-good yields. Seven of the obtained compounds (**5a, 5d – h,** and **5i**) are new and two (**5b** and **5c**) are previously known.[43] However, the available NMR spectral data for the two known compounds and other related ones are scarce or limited. In the case of their ^{13}C-NMR spectral data, they are reported here for the first time.



	R	yield, %
5a	4'-OMe	85
5b	3',4'-diOMe	81
5c	4'-NMe_2	89
5d	3'-OMe, 4'-OH	83
5e	2'-OMe	80
5f	4'-OH	87
5g	3',5'-OMe, 4'-OH	88
5h	4'-Me	85
5i	4'-$CHMe_2$	82

Scheme 1. Synthesis of unsymmetrical azines **5a – i**

The structures of the synthesized compounds were confirmed by HR-MS(EI), IR, 1D (^{1}H- and ^{13}C-NMR, including DEPT90/135 and a series of ^{1}H homonuclear decoupling experiments) and 2D (^{1}H-^{1}H COSY, NOESY/ROESY, HSQC, HMBC) NMR spectroscopy, and they are in complete agreement with the proposed structures. In the following part of this section, we will illustrate the structural elucidation of these compounds in the specific case of the azine **5a**, with an emphasis on the total assignation of NMR signals with the aid of 2D NMR experiments.

High-resolution mass spectrometric analysis HR-MS(EI) confirmed the molecular formula of the synthesized compound **5a** – $C_{19}H_{16}N_2O_4$ ([M^+] at *m/z* 336.1121, Δ = +1.1 mmu). IR spectrum of **5a** showed a sharp absorption band at 3434 cm^{-1} corresponding to an intramolecularly hydrogen bonded OH group, and Ar-H bond vibrations at 3085 cm^{-1}. The strong absorption at 1697 cm^{-1} could be attributed to the lactonic C=O group of the coumarin moiety. The IR spectrum also showed intense vibrations at 1602, 1568, 1170 and 1113 cm^{-1}, most probably arising from the presence of C=C, C=N, C–O, C–N bonds. ^{1}H-NMR spectrum of compound **5a** consisted of 10 signals, six of which had chemical shifts characteristic for methine protons on an aromatic core (*Table S1*). With the information from the ^{1}H-^{1}H COSY and NOESY spectra these signals could be separated into two groups (*Figure 1*, *Supplementary material, Figure S5*).

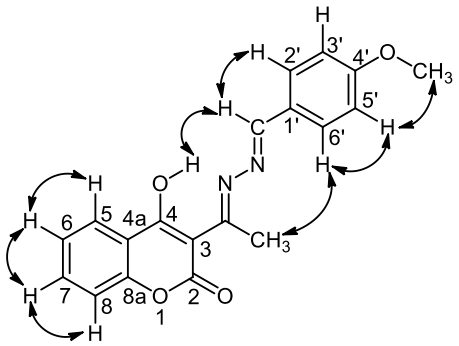


Figure 1. Crucial nOe correlations (↔) observed in the NOESY spectrum of compound **5a**

The first group included two higher order multiplets at 7.06 – 7.11 and 7.81 – 7.86 ppm that appeared to originate from a *para*-substituted aromatic core (one electron donating and one electron accepting substituent). The second group represented a four-resonance spin system belonging to an *ortho*-disubstituted (condensed) benzene ring with two sets of doublets of doublets at 7.98 and 7.31 ppm, one doublet of doublets of doublets at 7.68 ppm, and one doublet of triplets at 7.34 ppm. Integration of the signals confirmed that the two groups consisted of 4 protons each.

Based on HMBC (*Figure 2*, *Supplementary material, Figure S4*) and HSQC (*Supplementary material, Figure S3*) data, and the structure of compound **5a**, the two groups could be easily allocated to the protons of the phenyl group present in the starting benzaldehyde (*para*-substituted) and of the coumarin core (four-spin group) and. The assignment of these signals was performed from the observed HSQC and HMBC correlations and supplemented with the occurrence of vicinal and long-range proton-proton couplings.

The signal at 7.98 ppm was placed at position 5 since it displayed three-bond correlations (HMBC) with two non-protonated carbons at 180.4 (C–4) and 153.7 (C–8a) ppm, as well as with one methine carbon (C–7) at 134.9 ppm (*Figure 2*). It is interesting to note that the remaining doublet of doublets from at 7.32 ppm (H–8), besides showing correlations through three bonds with C–6 (124.4 ppm) and C–4a (120.2 ppm), coupled through two bonds with the non-protonated C–8a as previously noted in related coumarin systems.[44][45]

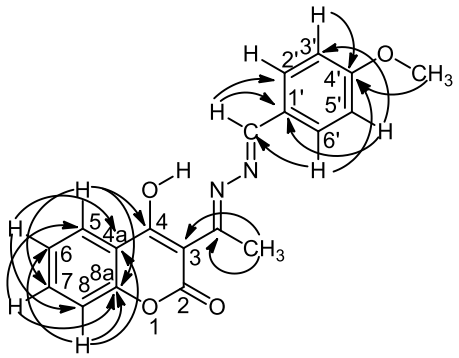


Figure 2. Crucial ^{1}H-^{13}C correlations (→) through 2 and 3 bonds observed in the HMBC spectrum of compound **5a**

Two upfield proton signals at 2.95 and 3.84 ppm corresponded to the more shielded protons of the methyl and methoxy groups. A NOESY correlation of the signal at 3.84 ppm with the multiplet at 7.06 – 7.11 ppm assigned the protons of the methoxy group and H-3'/5', respectively. The chemical shift of the carbon atoms to which these protons were bonded are readily determined from the HSQC spectrum (C–2'/6' at 130.9 ppm, C–3'/5'

at 115.1 ppm, and O<u>C</u>H$_3$ at 55.9 ppm), and further corroborated by HMBC correlations of the proton signal at 8.71 ppm assigned to N=C<u>H</u> (part of the azine bridge) with the C–2'/6' (130.9 ppm). The HMBC correlations of H–3'/5' with 125.7 ppm, and simultaneous correlations of H–2'/6' and methoxy protons with the signal at 162.8 ppm assigned C–1' and C–4', respectively. The methyl protons showed a three-bond correlation with the carbon at 95.9 ppm, assigned to C–3, and a two-bond interaction with 171.9 ppm, assigned to the carbon atom of the remaining imine group (CH$_3$<u>C</u>=N).

The only remaining unassigned signal in the ^{13}C-NMR spectrum, at 161.9 ppm, then belongs to C–2, and this is in agreement with the observed chemical shift and the lack of interactions in both HSQC and HMBC spectra. The comparison of this value with the one from analogous signals in related compounds corroborated this assumption.[44][45] The most deshielded signal observed in the ^{1}H-NMR spectrum appeared as a broad singlet at 16.44 ppm and was assigned to the OH proton at position 4 of the coumarin moiety. This signal showed a NOESY cross-peak with a proton of the azomethine group at 8.71 ppm. This correlation confirms the existence of the diimine bridge between the coumarin core and the aryl side of the molecule, and together with the cross-peak of the azomethine proton with H–2'/6'.

The signal of the OH resonates at significantly lower field (ca. 16 ppm) compared to an intramolecularly hydrogen-bonded phenol group (10 – 12 ppm) in the analogous azines or hydrazone of salicylaldehyde.[47 – 50] This could be interpreted as an exceptionally strong intramolecular hydrogen bond that imparts the molecule with the zwitterionic character (R–O$^-$H$^+$NR'R'') close to the other possible tautomer (ketone-enamine). The highly deshielded carbon at C–4 (180.4 ppm) also agrees with this assumption. The mentioned key nOe interaction between OH and <u>H</u>C=N is only possible if the conformation of that part of the molecule is near planarity, i.e. when OH is the closest and *syn* to the hydrogen from <u>H</u>C=N fragment. This again brings the CH$_3$C=N methyl group in close proximity to the *ortho*-hydrogens of the phenyl core of the starting aldehyde, resulting in the planar *s-trans* conformation of the azine moiety. The rotation around the N=CH–C^{Ar} single bond disconnects the aryl group from the extended conjugation of the planar azine-coumarin hybrid. However, as mentioned in the introductory section, many azines were found not to be planar, but to exist in a gauche conformation. This was the consequence of an unfavorable 4-electron repulsion of the free electron pairs on the two adjacent nitrogen atoms. In our case, as in the case of the azine of salicylaldehyde,[48] this repulsive interaction either does not exist, or is converted to an attractive one, by the formation of strong intramolecular hydrogen bonds which changes the polarity (nature) of these lone pairs (*Figure 3*). This can be envisaged as a favorable orbital interaction of the anomeric type, in this specific case with antiperiplanar $n_N \rightarrow \sigma^*_{N\text{-}H}$, as depicted in *Figure 3*. In a similar manner, the spectra of the remaining synthesized azines **5b-i** were assigned.

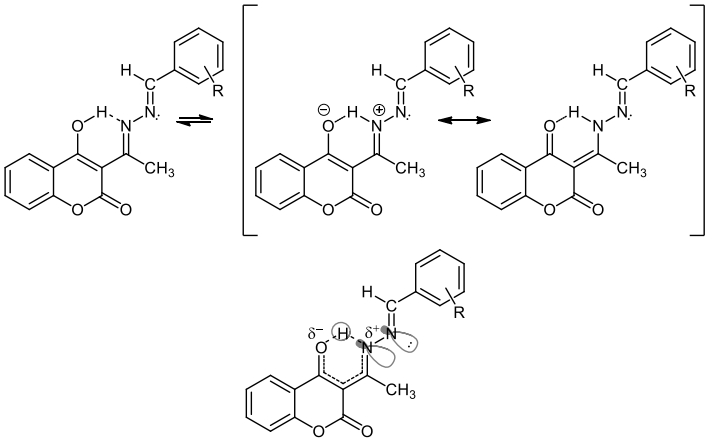


Figure 3. Structures of the possible tautomeric equilibrium existing for the azine-coumarin hybrids and a qualitative depiction of an orbital interaction leading to an *s-trans* conformation of the planar azine moiety.

Antimicrobial activity

Compounds **5a** – **i** were tested for growth inhibitory activity against a panel of ATCC microbial strains consisting of four Gram-positive and three Gram-negative bacteria and two fungi (*Table 1*). The compounds displayed a very wide spectrum of activity in the concentration range 0.16 – 6.03 μmolml^{-1} which was in particular cases even comparable to the observed activity of one of the used positive controls (chloramphenicol). Practically all compounds were effective in reducing the microbial growth of all tested strains with the only exception **5i** against *C. albicans*, which was resistant even to the highest tested concentration of this compound. The lowest *MIC* value was determined for **5f** that displayed antistaphylococcal activity at 160 nmolml^{-1}. When mutually compared, among the tested coumarin derivatives, **5a** and **5f** exhibited the highest antimicrobial effect with average *MIC* values of 1.32 and 1.46 μmolml^{-1}, respectively. On the other hand, compounds **5i**, **5g** and **5e** were the least efficient antimicrobial agents that were active in the ranges 0.75 – 6.03 (with the highest *MIC*s against *C. albicans*), 1.31 – 5.23 and 2.68 – 5.36 μmolml^{-1}, respectively. There was no notable selectivity observable towards any of the tested microbial groups (Gram-positive and negative bacteria, or fungi). Among the tested strains, *S.*

epidermidis and *P. aeruginosa* exhibited the highest resistance to the action of the tested coumarin derivatives, which was also visible from their susceptibility to the tested reference antibiotics (*Table 1*). Although *A. baumannii* was quite resistant to both applied positive controls (154.74 and 21.49 nmolml^{-1} for chloramphenicol and streptomycin, respectively), compounds **5a – i** showed significant activity towards this bacterium in the lower tested range 0.60 – 3.01 μmolml^{-1}. It is interesting to note that against this particular strain, **5d** and **5i**, compounds with medium-to-low activity against other strains, strongly inhibited *A. baumannii* at low concentrations – 0.60 and 0.75 μmolml^{-1}, respectively. These values are only around four times higher than the *MIC* of chloramphenicol against the same bacterium. Since this bacterial species represents a very important human opportunistic pathogen, primarily significant as the causative agent of multiresistant hospital-acquired infections, these results are thus very promising and urge further research in this direction. Among the tested compounds, **5c** was the most potent antifungal agent, while other compounds showed more or less the same activity against both the tested mold and yeast.

Table 1. Minimal inhibitory concentrations (*MIC*) of compounds **5a – i** (μmolml^{-1}) and antibiotics (nmolml^{-1}) against a panel of ATCC strains

Compound	5a	5b	5c	5d	5e	5f	5g	5h	5i	CHL[a]	STR[b]	NYS[c]
Gram-positive												
S. aureus	0.74	2.46	3.01	0.28	5.36	0.16	5.23	1.56	6.03	19.34	0.67	/
B. cereus	0.74	1.23	1.49	2.41	5.36	0.31	5.23	3.12	1.49	9.66	0.15	/
S. lutea	0.74	2.46	1.49	0.28	2.68	0.31	1.31	1.56	1.49	2.41	2.68	/
S. epidermidis	1.49	2.46	3.01	1.19	5.36	2.48	5.23	1.56	6.03	4.83	2.68	/
Gram-negative												
P. aeruginosa	1.49	5.19	6.01	1.19	5.36	1.24	5.23	3.12	6.03	38.68	5.36	/
E. coli	0.74	1.23	3.01	2.41	5.36	2.48	5.23	1.56	1.49	4.83	0.67	/
A. baumannii	1.49	2.46	3.01	0.60	2.68	1.24	5.23	3.12	0.75	154.7	21.5	/
Fungi												
C. albicans	2.98	2.46	0.72	2.41	2.68	2.48	5.23	3.12	> 6.03	/	/	2.53
A. brasiliensis	1.49	2.46	0.37	2.41	5.36	2.48	2.62	3.12	3.02	/	/	0.33

[a] CHL – chloramphenicol. [b] STR – streptomycin. [c] NYS – nystatin

We made an effort to further (statistically) analyze the obtained results and, as previously done with other coumarin derivatives,[24] agglomerative hierarchical clustering (AHC) analysis was performed to determine the similarities and selectivity of the mentioned compounds. The AHC analysis was applied utilizing *MIC* values against seven bacterial and two fungal strains as variables and the results are presented in the dendrogram shown in *Figure 4*. As it can be seen, three groups/classes of compounds (C1–C3) were clearly separated.

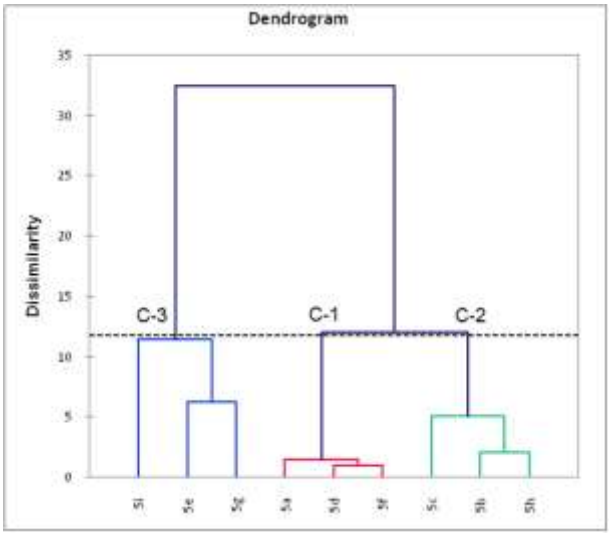


Figure 4. The dendrogram (AHC analysis) - dissimilarity in the antimicrobial activity (*MIC* values of compounds **5a – i** used as variables against seven bacterial and two fungal strains) relationships of the coumarin-azine hybrids (*Euclidean* distance dissimilarity, the aggregation criterion-the *Ward* method). Three groups of compounds were found: C1–C3.

Group C–1 (**5a**, **5d,** and **5f**) displayed the lowest *MIC* values for most of the tested microorganisms. The antimicrobial activities of these compounds were nonselective in nature but, as it can be seen from *Figure 5*, the fungi were slightly more resistant. The group was composed of electron rich only oxygenated derivatives with an obligatory OH or OMe at C–4′. Although slightly lower in inhibitory activity but still largely unselective, compounds **5b**, **5c**, and **5h**, which belonged to group C–2, only significantly differed from group C–1 compounds in their weaker effect against *P. aeruginosa*. The phenyl rings of compounds belonging to C-2 were also electron rich and possessed a C–4′ substituents, but this time along one derivative with an OMe (**5b**), there was a methyl group (**5h**) and a Me_2N- group (**5c**) on this ring. The last group (C–3) showed the least similarity with the activities observed from groups C–1 and C–2. Compounds from this group (**5i**, **5e**, and **5g**) were the least active ones against the tested strains but demonstrated greater selectivity by affecting the growth of *S. lutea* and *A. baumannii* in almost the same concentrations as the compounds from groups C–1 and C–2.

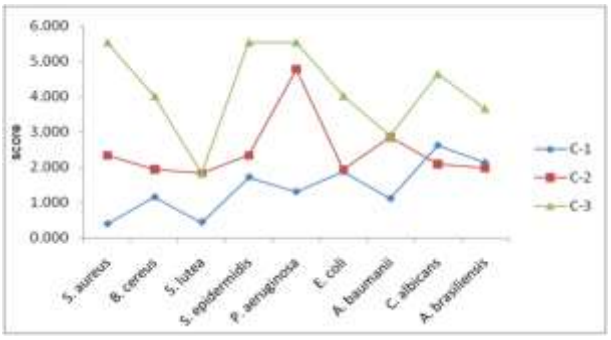


Figure 5. Graphical representation of the scores (the lowest activity is the highest value of *MIC*) of the centroids (classes C1–C3) from the AHC analysis

Most of the derivatives that possessed a phenolic group on the phenyl ring were among the most active ones (**5d** and **5f** from C–1). This appears to be an expected outcome since phenolics are known to be efficient antimicrobials (among the natural products thymol and carvacrol are good examples).[51] As these monoterpene phenols are known to interact with cell membranes, making them more permeable,[51] our coumarin derivatives (**5a** – **i**) might also have a similar mode of action (as already mentioned the OH at C–4 could be regarded as a phenolic group). Thus, additional phenolic groups at the phenyl ring would be expected to enhance the overall activity. The lower activity of **5g** from C–3 seems to be the result of steric hindrance of the two neighboring methoxy groups. It is also worth mentioning that the derivative that did not possess a C–4′ substituent (**5e**) was the least active of all tested compounds. This implies that a C–4′ substituent is likely to be required for the more subtle tuning of antimicrobial activity in these derivatives. However, the *ortho*-OMe in **5g** might have influenced the conformation of this part of the molecule and exerted its effect in this way.

Acute toxicity assay – Artemia salina *model*

As some of the derivatives were demonstrated to possess interesting antimicrobial effect, we were naturally interested whether they were also toxic (a shortcoming of some of the mentioned coumarin antibiotics mentioned in the introductory section). The most convenient test for this, that provides the first glimpse into the toxicological profile of these compounds, is the *Artemia salina* assay. The results of the acute toxicity testing in this model are presented in *Table 2*. Fortunately, the tested compounds did not show any significant toxicological effect at a concentration that corresponded to *MIC*. We were unable to test higher concentrations of **5a** – **i** due to their low solubility in artificial seawater. At the highest tested concentration after 24 h, the highest mortality rate of 18.3% was observed for compound **5a**. After 48 h of incubation, compounds **5b** (48.3%), **5e** (35.0%), **5f** (35%) and **5h** (36.7%) showed a more significant degree of toxicity. At the concentrations of 0.20 and 0.020 $mgml^{-1}$, after 24 h, compound **5a** did not show any toxicity, while compounds **5e**, **5c**, **5f** and **5h** (24 h) displayed a comparable mortality rate of 11.7, 10.0, 10.0 and 8.3%, respectively at 0.20 $mgml^{-1}$.

Table 2. Acute toxicity of compounds **5a – i** in the *A. salina* model

Concentration ($mgml^{-1}$)	Observation period (after 24 and 48 h)	Mortality rate (%) for the tested compounds								
		5a	5b	5c	5d	5e	5f	5g	5h	5i
2.00	24	18.3	11.7	11.7	10.0	13.3	13.3	10.0	15.0	10.0
	48	30.0	48.3	23.3	25.0	35.0	35.0	31.7	36.7	18.3
0.20	24	0.0	6.7	10.0	5.0	11.7	10.0	5.0	8.3	8.3

	48	16.7	35.0	23.3	15.0	18.3	16.7	30.0	16.7	15.0
0.02	24	0.0	5.0	5.0	0.0	10.0	6.7	3.3	3.3	5.0
	48	13.3	11.7	16.7	10.0	16.7	11.7	18.3	11.7	13.3

DPPH radical scavenging activity

The free radical scavenging activity of compounds **5a-c**, **5e**, **5g**, and **5i**, dissolved in methanol, was determined from their reaction with the stable, deep violet 1,1-diphenyl-2-picrylhydrazyl radical (DPPH).[52] The reduction of DPPH is accompanied by a change in color change since the yellow hydrazine is formed and can be straightforwardly tracked by the decrease in the absorbance at 517 nm. Along with the coumarin derivatives, ascorbic acid, a known powerful antioxidant, was assayed and the EC_{50} values, antioxidant reducing power (ARP), and stoichiometric relations were calculated for all of them.[53]

In the case of the highest concentration tested (0.1 mM) only a single compound displayed a scavenging activity, and luckily a prominent one, thus *Table 3* presents only the mentioned antiradical parameters for compound **5g** and ascorbic acid. The reaction between **5g** and DPPH radical was practically instantaneous, and based on the values from *Table 3*, one molecule of **5g** was sufficient to scavenge one DPPH radical, while ascorbic acid, as expected, displayed a 1 : 2 stoichiometry of the reaction with DPPH radical. The observed stoichiometry, expected from strong antioxidants, suggests that the primarily formed radical by proton abstraction is a stable one, both kinetically (sterically most probably) and thermodynamically (at least more stable than the DPPH radical). At first, the results concerning the coumarin-azine hybrids were unexpected since all of the molecules contained the same phenolic type OH (an acidic site) in the position 4 of the coumarin core. Thus, this OH-group does not seem to contribute the antiradical properties, probably due to the mentioned strong hydrogen bonding that would be lost upon proton abstraction from this position. It follows that the observed activity of **5g** must be connected to the phenolic group present on the aryl moiety in position 4′. An electron-rich aryl core seems to be beneficial for the formation of a stable radical species by proton abstraction from 4′-OH (Figure 6). Although the azine bridge is expected to be coplanar with coumarin core due to intramolecular hydrogen bonding, the contribution of resonance structures V – VII is questionable since the aryl group is known to freely rotate around the C–1′–C=N bond and not reside in the coplanar orientation due to steric reasons.

Table 3. Free radical scavenging activity of compound **5g** and ascorbic acid. EC_{50} (mole of antioxidant/1 mol of DPPH), antioxidant reducing power (ARP), stoichiometric ratio antioxidant/DPPH (mole of antioxidant/1 mol of DPPH), stoichiometric ratio DPPH/antioxidant (mole of DPPH/1 mol of antioxidant)

Sample	EC_{50}	ARP	Stoichiometric ratio antioxidant/DPPH	Stoichiometric ratio DPPH/antioxidant
5g	0.53	1.89	1.06	0.94
ascorbic acid	0.25	4.00	0.50	2.00

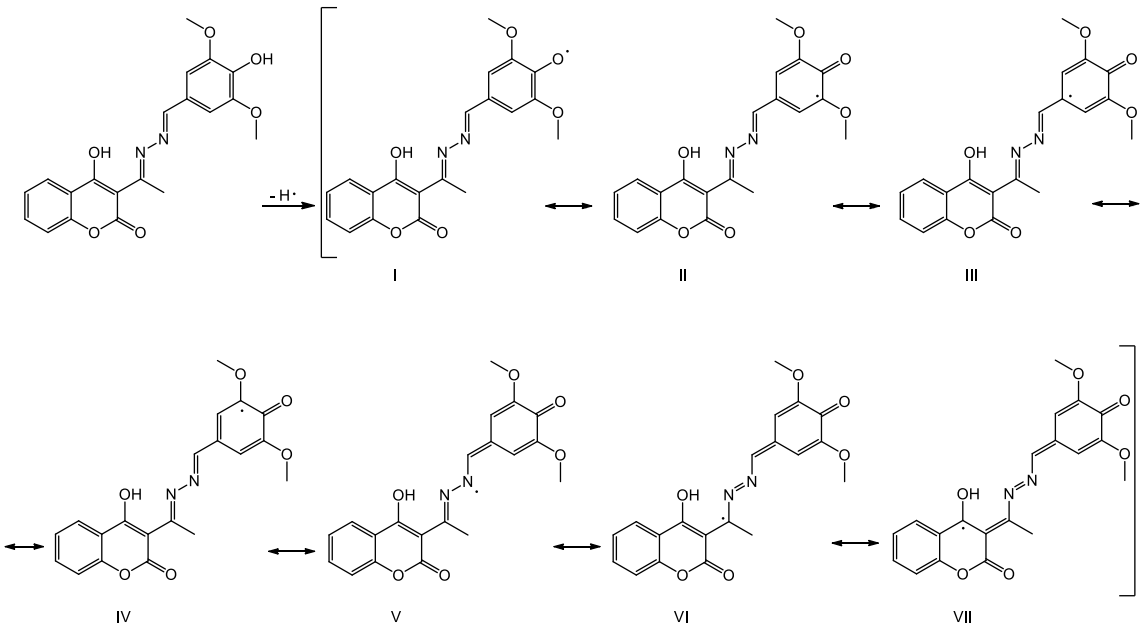


Figure 6. Possible resonance stabilization of the free radical formed in the reaction of **5g** with DPPH radical

Conclusions

Chem. Biodiversity

A series of mixed azines with a coumarin moiety were synthesized and evaluated for their acute toxicity, antimicrobial and antioxidant activities. An optimized two-step method for the synthesis of this small library of coumarin-azine hybrids was developed and represents an efficient, simple and rapid procedure, where the target azines were obtained in moderate to good yields the reaction of the hydrazone of 3-acetyl-4-hydroxycoumarin and differently substituted aromatic aldehydes. The library was intended to provide first insights into the chemical, spectral and biological properties of such hybrids. The prepared azines were fully spectrally characterized, including a complete assignment of ^{1}H- and ^{13}C-NMR resonances with the aid of extensive 1D- and 2D-NMR experiments. All of the spectral data pointed to a strong intramolecular hydrogen bond formed between OH in the position 4 of the comparing core and the adjacent N-atom of the azine bridge. We believe that this strong interaction keeps the coumarin ring and the azine bridge coplanar. Most of the synthesized azines, especially those possessing a *p*-hydroxyphenyl moiety, possessed a wide spectrum of significant antimicrobial activities, especially compound **5f** against *S. aureus*, as well as **5d** and **5i** against *A. baumannii*. The determined *MIC* values were comparable to that of chloramphenicol tested in parallel. The toxicity assay (*A. salina* model) showed that the tested compounds do not have a pronounced effect on the survival of brine shrimps. The derivative having the mentioned phenolic group and additional electron-donating groups (e.g. two OMe) exhibited a pronounced antioxidant power reacting instantly and stoichiometrically (mol ratio 1 : 1) with the DPPH radical. Thus, in this work, we discovered coumarin derivatives that might have a pharmaceutical application as antimicrobial agents with antioxidant properties while being of low toxicity. Also, these results urge further studies in this direction, with the promise of locating even more potent derivatives of this class of hybrids.

Experimental Section

General section

All chemicals were obtained from commercial sources (*Sigma-Aldrich*, USA; *Acros Organics*, Belgium; *Fluka* and *Merck*, Germany) and used as received, except that the solvents were purified by distillation. Melting points were determined on *MPM-HV2* melting point meter (*Paul Marienfeld GmbH & Co. KG*, *Lauda-Königshofen*, Germany). The purity of the synthesized compounds was checked by thin layer chromatography (TLC) on precoated aluminum silica gel plates (Kiesel 60 F_{254}, 0.2 mm, *Merck*, Darmstadt, Germany). Visualization was achieved by UV light and/or spraying the plates with 1:1 (v/v) aqueous sulfuric acid and then heating. Purification of the synthesized compounds was performed by column chromatography (Silica-gel 60, particle size 0.040-0.063 mm, *Fluka*). HR-MS(EI) spectra were recorded on a *JEOL Mstation JMS 700* instrument (*JEOL*, Germany). Elemental microanalysis for carbon, hydrogen, nitrogen, and oxygen were carried out on a *Carlo Erba 1106* microanalyzer (Milan, Italy); their results agreed favorably with the calculated values.

The IR spectra were recorded on *Thermo Nicolet* model *6700 FT-IR* (Waltham, USA) instrument and the vibration frequencies are reported in wave numbers (cm^{-1}). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100.6 MHz) were recorded on *Bruker Avance III* spectrometer (Fällanden, Switzerland) at 25 °C in $CDCl_3$ and (D_6)DMSO as the solvents, using TMS as the internal standard. Chemical shifts are reported in ppm (δ) values relative to TMS (0 ppm) in ^{1}H-NMR spectra or signals of residual solvents, $CDCl_3$: δ_H 7.26, δ_C 77.36 and (D_6)DMSO: δ_H 2.54, δ_C 40.45, in the heteronuclear 2D spectra (gradient ^{1}H-^{1}H COSY, HSQC and HMBC, and NOESY/ROESY). Scalar couplings (*J*) are reported in Hertz. In addition to the mentioned spectra, selective ^{1}H homonuclear decoupling experiments and DEPT90/135 spectra were acquired to aid signal assignment. All NMR spectra were recorded using standard built-in pulse sequences from the software *Topspin* (*Bruker*). UV spectra were measured using a *UV-1800 Shimadzu* spectrophotometer (Tokyo, Japan).

Synthesis

3-acetyl-4-hydroxy-2*H*-chromen-2-one (2). Phosphorus oxychloride (5.6 ml, 60 mmol) was slowly added to a solution of 4-hydroxy-2*H*-chromen-2-one (**1;** 3g, 18.6 mmol) in acetic acid. The reaction mixture was refluxed on an oil bath for 35 minutes, and then gradually cooled to room temperature and eventually in an ice bath. The precipitated solid was collected by filtration. Recrystallization from ethanol yielded white needle-like crystals of compound **2** (m.p. 135 – 136 °C).

3-(1-hydrazonoethyl)-4-hydroxy-2*H*-chromen-2-one (3). 3-Acetyl-4-hydroxy-2*H*-chromen-2-one (**2**; 4.1 g, 20 mmol) was suspended in methanol (20 ml). After stirring at room temperature for 10 minutes, hydrazine hydrate (1g, 20 mmol) was added and the resulting mixture was refluxed for 5 h on a water bath. The progress of the reaction was monitored by TLC, and after the completion of the reaction, the solution was cooled to room temperature. The formed precipitate was filtered and washed with methanol. After drying, the crude solid product was recrystallized from ethanol to give a pure light-greenish yellow compound **3** (m.p. 222 – 224 °C).

General procedure for the synthesis of azines **5a – i**

A solution of 3-(1-hydrazonoethyl)-4-hydroxy-2*H*-chromen-2-one (**3**; 1.1 g, 5 mmol) and the appropriate aromatic aldehyde (**4a – i**; 5 mmol) ((4-methoxybenzaldehyde **4a**, 3,4-dimethoxybenzaldehyde **4b**, 4-(dimethylamino)benzaldehyde **4c**, 4-hydroxy-3-methoxybenzaldehyde **4d**, 2-methoxybenzaldehyde **4e**, 4-hydroxybenzaldehyde **4f**, 4-hydroxy-3,5-dimethoxybenzaldehyde **4g**, 4-methylbenzaldehyde **4h**, and 4-

isopropylbenzaldehyde **4i**) in absolute ethanol (10 ml) was refluxed for 3-4 h. The consumption of the reagents was monitored by TLC (ethyl acetate/hexane 3:2, *v/v*). After the completion of the reaction, the mixture was allowed to cool to room temperature. The formed solid was collected by filtration and washed with chloroform-methanol (1:1, *v/v*). The obtained products were purified by flash SiO_2 column chromatography (ethyl acetate/hexane 1:1, *v/v*).

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(4-methoxybenzylidene)hydrazine (5a). Light yellow crystals. M.p. 190 – 193 °C. IR (KBr): 1113, 1170, 1568, 1602, 1697, 2817, 3085, 3434. ^{1}H-NMR (400 MHz, (D_6)DMSO): 16.44 (*brs*, HO–C(4)); 8.71 (*s*, N=CH); 7.98 (*dd*, *J* = 8.0, 1,6, H–C(5)); 7.86 – 7.81 (*m*, H–C(2'/6')); 7.68, (*ddd*, *J* = 8.8, 8.0, 1.6, H–C(7)); 7.34 (*td*, *J* = 8.0, 0.8, H–C(6)); 7.31 (*dd*, *J* = 8.8, 0.8, H–C(8)); 7.11 – 7.06 (*m*, H-C(3'/5')); 3.84 (*s*, OMe–C(4')); 2.95 (*s*, Me). ^{13}C-NMR (100.6 MHz, (D_6)DMSO): 180.4 (C(4)); 171.9 (C=N); 162.8 (C(4')); 161.9 (C(2)); 156.8 (N=CH); 153.7 (C(8a)); 134.9 (C(7)); 130.9 (C(2'/6')); 126.1 (C(5)); 125.7 (C(1')); 124.4 (C(6)); 120.2 (C(4a)); 116.9 (C(8)); 115.1 (C(3'/5')); 95.9 (C(3)); 55.9 (OMe); 17.8 (Me). HR-MS(EI): 336.1121 ([*M* + H]$^+$, $C_{19}H_{16}N_2O_4^+$; calc. 336.1110). Anal. calc. for $C_{19}H_{16}N_2O_4$: C 67.85, H 4.79, N 8.33, O 19.03; found: C 67.98, H 4.72, N 8.41, O 18.89.

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(3,4-dimethoxybenzylidene)hydrazine (5b). Yellow powder. M.p. 220 – 222 °C. IR (KBr): 1104, 1134, 1574, 1608, 1695, 2841, 3065, 3443. ^{1}H-NMR (400 MHz, (D_6)DMSO): 16.45 (*brs*, HO–C(4)); 8.64 (*s*, N=CH); 7.99 (*dd*, *J* = 7.6, 1,6, H–C(5)); 7.68, (*ddd*, *J* = 8.4, 7.6, 1.6, H–C(7)); 7.48 (*d*, *J* = 2.0, H–C(2')); 7.39 – 7.28 (*m*, H-C(6/8/6')); 7.08 (*d*, *J* = 8.4, H–C(5')); 3.84 (*s*, OMe–C(4')); 3.83 (*s*, OMe–C(3')); 2.98 (*s*, Me). ^{13}C-NMR (100.6 MHz, (D_6)DMSO): 180.4 (C(4)); 171.8 (C=N); 161.9 (C(2)); 157.0 (N=CH); 153.7 (C(8a)); 152.1 (C(4')); 149.6 (C(3')); 135.0 (C(7)); 127.1 (C(1')); 126.1 (C(5)); 124.4 (C(6)); 124.0 (C(6')); 120.2 (C(4a)); 116.9 (C(8)); 111.9 (C(5')); 109.5 (C(2')); 95.9 (C(3)); 56.0 (OMe–C(4')); 55.9 (OMe–C(3')); 17.8 (Me). HR-MS(EI): 366.1234 ([*M* + H]$^+$, $C_{20}H_{18}N_2O_5^+$; calc. 366.1216). Anal. calc. for $C_{20}H_{18}N_2O_5$: C 65.57, H 4.95, N 7.65, O 21.84; found: C 65.03, H 5.02, N 7.83, O 22.12.

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(4-dimethylaminobenzylidene)hydrazine (5c). Brick red powder. M.p. 268 – 270 °C. IR (KBr): 1106, 1179, 1591, 1612, 1697, 2812, 3100, 3451. ^{1}H-NMR (400 MHz, $CDCl_3$): 16.40 (*brs*, HO–C(4)); 8.23 (*s*, N=CH); 8.07 (*dd*, *J* = 7.6, 1,6, H–C(5)); 7.67 – 7.63 (*m*, H-C(2'/6')); 7.56, (*ddd*, *J* = 8.0, 7.2, 2.0, H–C(7)); 7.27 – 7.23 (*m*, H-C(6/8)); 6.73 – 6.68 (*m*, H–C(3'/5')); 3.06 (*s*, NMe_2–C(4')); 2.80 (*s*, Me). ^{13}C-NMR (100.6 MHz, $CDCl_3$): 181.1 (C(4)); 171.2 (C=N); 162.2 (C(2)); 155.3 (N=CH); 153.8 (C(8a)); 152.8 (C(4')); 133.8 (C(7)); 130.3 (C(2'/6')); 125.9 (C(5)); 123.5 (C(6)); 120.3 (C(4a)); 119.9 (C(1')); 116.6 (C(8)); 111.7 (C(3'/5')); 96.1 (C(3)); 40.1 (NMe_2–C(4')); 17.5 (Me). HR-MS(EI): 349.1417 ([*M* + H]$^+$, $C_{20}H_{19}N_3O_3^+$; calc. 349.1426). Anal. calc. for $C_{20}H_{19}N_3O_3$: C 68.75, H 5.48, N 12.03, O 13.74; found: C 68.59, H 5.52, N 12.18, O 13.71.

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(4-hydroxy-3-methoxybenzylidene)hydrazine (5d). Dark yellow powder. M.p. 232 – 234 °C. IR (KBr): 1083, 1162, 1599, 1610, 1710, 2832, 3078, 3431. ^{1}H-NMR (400 MHz, (D_6)DMSO): 16.46 (*brs*, HO–C(4)); 10.01 (*s*, HO–C(4')); 8.61 (*s*, N=CH); 7.96 (*dd*, *J* = 7.6, 1,6, H–C(5)); 7.66, (*ddd*, *J* = 8.8, 7.6, 1.6, H–C(7)); 7.43 (*d*, *J* = 1.6, H–C(2')); 7.34 – 7.26 (*m*, H-C(6/8/6')); 6.90 (*d*, *J* = 8.4, H–C(5')); 3.84 (*s*, OMe–C(3')); 2.94 (*s*, Me). ^{13}C-NMR (100.6 MHz, (D_6)DMSO): 180.3 (C(4)); 171.5 (C=N); 161.9 (C(2)); 157.2 (N=CH); 153.6 (C(8a)); 151.4 (C(4')); 148.5 (C(3')); 134.9 (C(7)); 126.1 (C(5)); 124.7 (C(6')); 124.4 (C(1')); 124.3 (C(6)); 120.2 (C(4a)); 116.8 (C(8)); 116.1 (C(5')); 110.7 (C(2')); 95.8 (C(3)); 56.0 (OMe); 17.8 (Me). HR-MS(EI): 352.1050 ([*M* + H]$^+$, $C_{19}H_{16}N_2O_5^+$; calc. 352.1059). Anal. calc. for $C_{19}H_{16}N_2O_5$: C 64.77, H 4.58, N 7.95, O 22.70; found: C 64.65, H 4.61, N 7.92, O 22.82.

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(2-methoxybenzylidene)hydrazine (5e). Yellow needles. M.p. 204 – 206 °C. IR (KBr): 1105, 1168, 1557, 1609, 1705, 2840, 3053, 3450. ^{1}H-NMR (400 MHz, (D_6)DMSO): 16.39 (*brs*, HO–C(4)); 8.83 (*s*, N=CH); 7.99 (*dd*, *J* = 7.6, 1.6, H–C(5)); 7.95 (*dd*, *J* = 7.6, 1.6, H–C(6')); 7.69, (*ddd*, *J* = 8.4, 7.6, 1.6, H–C(7)); 7.56, (*ddd*, *J* = 8.0, 7.6, 1.6, H–C(4')); 7.35 (*td*, *J* = 7.6, 0.8, H–C(6)); 7.31 (*dd*, *J* = 8.4, 0.8, H–C(8)); 7.19 (*d*, *J* = 8.0, H-C(3')); 7.08 (*t*, *J* = 7.6, H-C(5')); 3.93 (*s*, OMe); 2.97 (*s*, Me). ^{13}C-NMR (100.6 MHz, (D_6)DMSO): 180.5 (C(4)); 172.3 (C=N); 161.9 (C(2)); 159.4 (C(2')); 153.7 (C(8a)); 151.9 (N=CH); 135.0 (C(7)); 134.4 (C(4')); 127.1 (C(6')); 126.2 (C(5)); 124.4 (C(6)); 121.4 (C(5')); 120.9 (C(1')); 120.2 (C(4a)); 116.9 (C(8)); 112.7 (C(3')); 96.1 (C(3)); 56.4 (OMe–C(2')); 17.9 (Me). HR-MS(EI): 336.1097 ([*M* + H]$^+$, $C_{19}H_{16}N_2O_4^+$; calc. 336.1110). Anal. calc. for $C_{19}H_{16}N_2O_4$: C 67.85, H 4.79, N 8.33, O 19.03; found: C 67.93, H 4.72, N 8.29, O 19.06.

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(4-hydroxybenzylidene)hydrazine (5f). Yellow powder. M.p. > 300 °C. IR (KBr): 1110, 1136, 1572, 1603, 1696, 3197, 3471. ^{1}H-NMR (400 MHz, (D_6)DMSO): 16.40 (*brs*, HO–C(4)); 10.31 (*s*, HO–C(4')); 8.58 (*s*, N=CH); 7.94 (*dd*, *J* = 7.6, 1,6, H–C(5)); 7.73 – 7.69 (*m*, H–C(2'/6')); 7.63, (*ddd*, *J* = 8.4, 7.6, 1.6, H–C(7)); 7.29 (*td*, *J* = 7.6, 0.8, H–C(6)); 7.25 (*dd*, *J* = 8.4, 0.8, H–C(8)); 6.91 – 6.86 (*m*, H-C(3'/5')); 2.90 (*s*, Me). ^{13}C-NMR (100.6 MHz, (D_6)DMSO): 181.3 (C(4)); 171.5 (C=N); 161.8 (C(2)); 161.7 (C(4')); 156.9 (N=CH); 153.6 (C(8a)); 134.8 (C(7)); 131.1 (C(2'/6')); 126.1 (C(5)); 124.1 (C(6/1')); 120.2 (C(4a)); 116.8 (C(8)); 116.5 (C(3'/5')); 95.8 (C(3)); 17.7 (Me). HR-MS(EI): 322.0970 ([*M* + H]$^+$, $C_{18}H_{14}N_2O_4^+$; calc. 322.0954). Anal. calc. for $C_{18}H_{14}N_2O_4$: C 67.07, H 4.38, N 8.69, O 19.86; found: C 67.01, H 4.32, N 8.73, O 19.94.

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine (5g). Yellow powder. M.p. 228 – 230 °C. IR (KBr): 1114, 1163, 1595, 1619, 1707, 2842, 3115, 3465. ^{1}H-NMR (400 MHz, (D_6)DMSO): 16.45 (*brs*, HO–C(4)); 9.30 (*s*, HO–(C(4')); 8.50 (*s*, N=CH); 7.92 (*dd*, *J* = 7.6, 1.6, H–C(5)); 7.61, (*ddd*, *J* = 8.4, 7.6, 1.6, H–C(7)); 7.27 (*t*, *J* = 7.6, H–C(6)); 7.22, (*d*, *J* = 8.4, H–C(8)); 7.12 (*s*, H–C(2'/6')); 3.81 (*s*, OMe–(C(3'/5')); 2.90 (*s*, Me). ^{13}C-NMR (100.6 MHz, (D_6)DMSO): 180.3 (C(4)); 171.4 (C=N); 161.8 (C(2)); 157.1 (N=CH); 153.6 (C(8a)); 148.6 (C(3'/5')); 140.4 (C(4')); 134.7 (C(7)); 126.0 (C(5)); 124.2 (C(6)); 123.1 (C(1')); 120.1 (C(4a)); 116.7 (C(8)); 106.6 (C(2'/6')); 95.8 (C(3)); 56.4 (OMe–C(3'/5')); 17.7 (Me). HR-MS(EI): 382.1150 ([*M* + H]$^+$, $C_{20}H_{18}N_2O_6^+$; calc. 382.1165). Anal. calc. for $C_{20}H_{18}N_2O_6$: C 62.82, H 4.74, N 7.33, O 25.11; found: C 62.93, H 4.83, N 7.25, O 24.99.

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(4-methylbenzylidene)hydrazine (5h). Light yellow. M.p. 194 – 196 °C. IR (KBr): 1101, 1173, 1555, 1605, 1695, 2922, 3115, 3468. ^{1}H-NMR (400 MHz, (D_6)DMSO): 16.41 (*brs*, HO–C(4)); 8.74 (*s*, N=CH); 7.99 (*dd*, *J* = 7.2, 1,6, H–C(5)); 7.80 – 7.76 (*m*, H-C(2'/6')); 7.68, (*ddd*, *J* = 8.4, 7.2, 1.6, H–C(7)); 7.37 – 7.29 (*m*, H-C(6/8/3'/5')); 2.97 (*s*, Me); 2.38 (*s*, Me–C(4')). ^{13}C-NMR (100.6 MHz, (D_6)DMSO): 180.5 (C(4)); 172.4 (C=N); 161.9 (C(2)); 157.0 (N=CH); 153.7 (C(8a)); 142.8 (C(4')); 135.0 (C(7)); 130.5 (C(1')); 130.2 (C(3'/5')); 129.0 (C(2'/6')); 126.2 (C(5)); 124.4 (C(6)); 120.2 (C(4a)); 116.9 (C(8)); 96.1 (C(3)); 21.7 (Me–C(4')); 17.8 (Me). HR-MS(EI): 320.1149 ([*M* + H]$^+$, $C_{19}H_{16}N_2O_3^+$; calc. 320.1161). Anal. calc. for $C_{19}H_{16}N_2O_3$: C 71.24, H 5.03, N 8.47, O 14.98; found: C 71.36, H 5.11, N 8.62, O 14.91.

1-[-1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)ethylidene]-2-(4-isopropylbenzylidene)hydrazine (5i). Light yellow needles. M.p. 197 – 199 °C. IR (KBr): 1110, 1176, 1566, 1605, 1698, 2958, 3150, 3472. ^{1}H-NMR (400 MHz, (D_6)DMSO): 16.41 (*brs*, HO–C(4)); 8.74 (*s*, N=CH); 7.99 (*dd*, *J* = 7.6, 1,6, H–C(5)); 7.82 – 7.78 (*m*, H–C(2'/6')); 7.68, (*ddd*, *J* = 8.4, 7.6, 1.6, H–C(7)); 7.43 – 7.39 (*m*, H–C(3'/5')); 7.34 (*td*, *J* = 7.6, 0.8, H–C(6)); 7.30 (*dd*, *J* = 8.4, 0.8, H–C(8)); 2.98 – 2.93 (*m*, Me, HC–(C(4')); 1.23 (*d*, *J* = 6.8, Me_2–C–(C(4')). ^{13}C-NMR (100.6 MHz, (D_6)DMSO): 180.5 (C(4)); 172.4 (C=N); 161.9 (C(2)); 157.1 (N=CH); 153.7 (C(8a/4')); 135.0 (C(7)); 130.9 (C(1')); 129.1 (C(2'/6')); 127.6 (C(3'/5')); 126.2 (C(5)); 124.4 (C(6)); 120.2 (C(4a)); 116.9 (C(8)); 96.1 (C(3)); 34.0 (CH–(C(4'))); 24.0 (Me_2–C–(C(4'))); 17.8 (Me). HR-MS(EI): 348.1489 ([*M* + H]$^+$, $C_{21}H_{20}N_2O_3^+$; calc. 348.1474). Anal. calc. for $C_{21}H_{20}N_2O_3$: C 72.40, H 5.79, N 8.04, O 13.78; found: C 72.25, H 5.88, N 8.12, O 13.75.

Biological evaluation

Test microorganisms. The synthesized compounds were tested against the panel of microbial strains belonging to the American Type Culture Collection reference strains: Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus cereus* ATCC 11778 and *Sarcina lutea* ATCC 9431), Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739 and *Acinetobacter baumannii* ATCC 19606), the yeast *Candida albicans* ATCC 10231 and the mold *Aspergillus brasiliensis* ATCC 16404. Bacterial strains were maintained on *Nutrient Agar* (NA) at 37 °C, while the fungal strains were maintained on *Sabouraud Dextrose Agar* (SDA) at 30 °C at the Microbiology Laboratory, Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš.

Antimicrobial screening. Antimicrobial activity was evaluated using a broth microdilution method in microtiter plates as described earlier.[24] Briefly, cell suspensions of adjusted turbidity (*DEN-1*, *Biosan*) corresponding to McFarland standard No. 0.5 were made using an overnight culture (18 h) of the test microorganisms. Stock solutions of the prepared coumarin derivatives were made in pure DMSO and further diluted with the appropriate sterile broth (*Sabouraud Dextrose* or *Mueller Hinton* broth) in order to prepare the solutions of the target starting concentrations. The final highest concentration of DMSO (10%, *v/v*) in the prepared solutions was verified not affect bacterial nor fungal growth. These solutions were further serially diluted (the diluting factor 2) in the concentration range of 0.005 – 2.10 mgml^{-1}. After preparing the dilutions of the test substances, the inoculum was added to all wells and the plates were incubated at 37 °C during 24 h in the case of bacteria or at 30 °C during 48 h (fungi). Streptomycin, chloramphenicol, and nystatin served as positive controls, while one non-inoculated well, free of any antimicrobial agent, was also included to ensure medium sterility. Bacterial growth was determined by adding 20 µl of 0.5% (*w/v*) 2,3,5-triphenyltetrazolium chloride (TTC) aqueous solution. *MIC* was defined as the lowest concentration of the test compound that inhibited visible growth (red colored pellet on the bottom of the wells after the addition of TTC). All experiments were done in triplicate and repeated twice.

Acute toxicity testing: Artemia salina *model*. A spoon (about 8 g) of lyophilized brine shrimp cysts (*A. salina*) was suspended in 1 l of artificial seawater. The suspension was thermostated at 25 °C, slowly aerated, and constantly illuminated. After 48 h most of the cysts underwent transformation to nauplii. The tested compounds were dissolved in DMSO and diluted with artificial seawater, so that their final concentrations were 2.00, 0.20 and 0.020 mgml^{-1}, while the final concentration of DMSO was less than 1% (*v/v*). Thereafter, 20 hatched nauplii were transferred to a Petri dish containing the prepared solution of the test compounds **5a – i**; brine shrimps were not fed during the test. DMSO did show any effect on the brine shrimps under these conditions as demonstrated by the results of a negative control. Different concentrations of sodium dodecyl sulfate were used as a positive control. The Petri dishes were kept at room temperature under a constant light source and were not aerated. Counting of dead brine shrimps was performed after 24 and 48 hours. The results are expressed as % of dead nauplii after the appropriate time point. All tests were performed in triplicate and repeated twice.

DPPH radical scavenging activity. The free radical scavenging activity of the synthesized compounds was determined in the case of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and compared with ascorbic acid. Stock solutions (0.1 mM) of the test compounds **5a – c**, **5e**, **5g,** and **5i**, and ascorbic acid were prepared in methanol. This solution was further serially diluted to obtain a range of concentrations: 25, 50, and 100 µM. A methanol solution of DPPH (1 ml, 0.1 mM), was mixed with of solutions of varying concentrations of the test compounds or ascorbic acid (2 ml) in a test tube. The mixture was shaken vigorously and allowed to stand at room temperature while at regular time intervals the absorbance of the mixtures was measured at 517 nm (against methanol as a blank) until it became constant (three consecutive measurements that did not differ mutually significantly). A negative control containing no antioxidant was treated in the same way as described above. The free radical scavenging activity was calculated by using the following equation:

$$\text{Scavenging activity (\%)} = ((Abs_{control} - Abs_{sample})/Abs_{control}) \times 100$$

Each sample was assayed in triplicate and the mean values were calculated. The EC_{50} value was defined as the amount of an antioxidant necessary to decrease the absorbance of DPPH to 50% of the initial value and was calculated by linear regression from the antiradical activity percentage against the concentration plots. Antioxidant reducing power (ARP) represents the inverse of EC_{50}. A stoichiometric relation presents the amount of the antioxidant required to quantitatively reduce DPPH radicals, and it can be expressed as the number of moles of the antioxidant required to reduce 1 mol of DPPH. The stoichiometric value is determined by multiplying the EC_{50} value by 2. The inverse value of the stoichiometric relation presents the number of DPPH moles reduced by one mole of the antioxidant.[53]

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbdv.2018xxxxx.

Acknowledgements

This work was supported by the Ministry of Education, Science and Technological Development of Serbia [Projects No. 172061 and 45022].

Author Contribution Statement

N. S. R. initiated, conceived and designed this research. V. S. D. and N. R. R. synthesized the compounds while M. N. R. and Z. S. R. performed all other experiments. B. R. D. and N. S. R. analyzed the data, summarized the results and wrote the manuscript.

References

[1] J. Safari, S. Gandomi-Ravandi, 'Structure, synthesis and application of azines: a historical perspective', *RSC Adv.* **2014**, *4*, 46224 - 46249.

[2] M. D. Reddy, F. R. Fronczek, E. B. Watkins, 'Rh-Catalyzed, Regioselective, C–H Bond Functionalization: Access to Quinoline-Branched Amines and Dimers', *Org. Lett.* **2016**, *18*, 5620 - 5623.

[3] M. D. Reddy, D. Uredi, E. B. Watkins, 'A general method for the metal-free, regioselective, remote C–H halogenation of 8-substituted quinolines', *Chem. Sci.* **2018**, *9*, 1782 - 1788.

[4] J. Safari, S. Gandomi-Ravandi, M. Monemi, 'Novel and selective synthesis of unsymmetrical azine derivatives via a mild reaction', *Monatsh. Chem.* **2013**, *44*, 1375 - 1380.

[5] B. L. Shaw, N. Iranpoor, S. D. Perera, M. Thornton-Pett, J. D. Vessey, 'Complexes of the (1*R*)-(+)-camphor azine diphosphines *Z,Z*-3,3'-$Ph_2P^{\chi}C_{10}H_{15}$=N–N=$C_{10}H_{15}P^{\chi}Ph_2$ and *Z,Z*-3,3'-$Ph_2P^{\chi}C_{10}H_{15}$=N–N=$C_{10}H_{15}P^{\chi}Ph_2$ (x = *exo*, n = *endo*) with Group 6 metal carbonyls: crystal structures of the ligands and *fac*-[$W(CO)_3$(*E,Z*-$Ph_2P^{\chi}C_{10}H_{15}$= N–N=$C_{10}H_{15}P^{\chi}Ph_2$)]', *J. Chem. Soc., Dalton Trans.* **1998**, 1885 - 1892.

[6] K. S. Singh, Y. A. Mozharivskyj, C. Thöne, M. R. Kollipara, 'New mononuclear ruthenium complexes of η^5-cyclichydrocarbon containing azine ligands: Syntheses, spectral and structural studies', *J. Organomet. Chem.* **2005**, *690*, 3720 - 3729.

[7] G. Albertin, S. Antoniutti, M. Bortoluzzi, J. Castro, F. Sibilla, E. Trave, 'Preparation of metalated azine complexes of iridium(III)', *New J. Chem.* **2017**, *41*, 12976 - 12988.

[8] A. Sadimenko, O. Okoh, 'Organometallic Complexes of Azines', *Adv. Heterocycl. Chem.* **2018**, *124*, 51 - 120.

[9] J. Lasri, M. M. Alyb, N. E. Eltayeb, B. A. Babgi, 'Synthesis of symmetrical and asymmetrical azines from hydrazones and/or ferrocenecarboxaldehyde as potential antimicrobial–antitumor agents', *J. Mol. Struct.* **2018**, *1164*, 1 - 8.

[10] H. I. Gul, U. Calis, J. Vepsalainen, 'Synthesis of some mono-Mannich bases and corresponding azine derivatives and evaluation of their anticonvulsant activity', *Arzneim. Forsch.* **2004**, *54*, 359 - 364.

[11] N. Ergenç, N. S. Günay, 'Synthesis and antidepressant evaluation of new 3-phenyl-5- sulfonamidoindole derivatives', *Eur. J. Med. Chem.* **1998**, *33*, 143 - 148.

[12] H. Suleyman, H. I. Gul, M. Asoglu, 'Anti inflammatory activity of 3-benzoyl-1-methyl-4-phenyl-4-piperidinol hydrochloride', *Pharmacol. Res.* **2003**, *47*, 471 - 475.

[13] J. Easmon, G. Pürstinger, G. Heinisch, T. Roth, H. H. Fiebig, W. Holzer, W. Jäager, M. Jenny, J. Hofmann, 'Synthesis, cytotoxicity, and antitumor activity of copper(II) and iron(II) complexes of ^{4}*N*-Azabicyclo[3.2.2]nonane thiosemicarbazones derived from acyl diazines', *J. Med. Chem.* **2001**, *44*, 2164 - 2171.

[14] C. Liang, J. Xia, D. Lei, X. Li, Q. Yao, J. Gao, 'Synthesis, *in vitro* and *in vivo* antitumor activity of symmetrical bis-Schiff base derivatives of isatin', *Eur. J. Med. Chem.* **2014**, *74*, 742 - 750.

[15] L. M. Lima, F. S. Frattani, J. L. Dos Santos, H. C. Castro, C. A. Fraga, R. B. Zingali, E. J. Barreiro, 'Synthesis and anti-platelet activity of novel arylsulfonate-acylhydrazone derivatives, designed as antithrombotic candidates', *Eur. J. Med. Chem.* **2008**, *43*, 348 - 356.

[16] N. A. Meanwell, H. R. Roth, E. C. R. Smith, D. L. Wedding, J. J. K. Wright, 'Diethyl 2,4-dioxoimidazolidine-5-phosphonates: Horner-Wadsworth-Emmons reagents for the mild and efficient preparation of C-5 unsaturated hydantoin derivatives', *J. Org. Chem.* **1991**, *56*, 6897 - 6904.

[17] H. R. Vekariya, D. H. Patel, 'Recent Advances in the Synthesis of Coumarin Derivatives via Knoevenagel Condensation: A Review', *Synth. Commun.* **2014**, *44*, 2756 - 2788.

[18] Y. Zheng, L. Qiu, K. Hong, S. Dong, X. Xu, 'Copper- or Thermally Induced Divergent Outcomes: Synthesis of 4-Methyl 2*H*-Chromenes and Spiro-4*H*-Pyrazoles', *Chem. Eur. J.* **2018**, *24*, 6705 - 6711.

[19] T. Patonay, G. Y. Litkei, R. Bognar, J. Erdei, C. Misztic, 'Synthesis, antibacterial and antifungal activity of 4-hydroxy-coumarin derivatives, analogues of Novobiocin', *Pharmazie* **1984**, *39*, 86 - 91.

[20] O. O. Ajani, O. C. Nwinyl, 'Microwave-assisted synthesis and evaluation of antimicrobial activity of 3-{3-(s-aryl and s-heteroaromatic)acryloyl}-2*H*-chromen-2-one derivatives', *J. Heterocycl. Chem.* **2010**, *47*, 179 - 187.

[21] M. A. Al-Haiza, M. S. Mostafa, M. Y. El-Kady, 'Synthesis and biological evaluation of some new coumarin derivatives', *Molecules* **2003**, *8*, 275 - 286.

[22] R. M. Zaki, Y. A. Elossaily, A. M. Kamal El-Dean, 'Synthesis and antimicrobial activity of novel benzo[f]coumarin compounds', *Russ. J. Bioorg. Chem.* **2012**, *38*, 639 - 646.

[23] A. S. Al-Ayed, N. Hamdi, 'A new and efficient method for the synthesis of novel 3-acetyl coumarins oxadiazoles derivatives with expected biological activity', *Molecules* **2014**, *19*, 911 - 924.

[24] N. Radulović, Z. Stojanović-Radić, N. Stojanović, P. Stojanović, V. Dekić, B. Dekić, 'A small library of 4-(alkylamino)-3-nitrocoumarin derivatives with potent antimicrobial activity against gastrointestinal pathogens', *J. Serb. Chem. Soc.* **2015**, *15*, 315 - 327.

[25] A. A. Al-Amiery, H. K. Abdul Amir, M. Abu Bakar, 'Antifungal activities of new coumarins', *Molecules* **2012**, *17*, 5713 - 5723.

[26] S. Kirkiacharian, D. T. Thys, S. Sicsic, R. Bakhchinian, R. Kurkjian, T. Tonnaire, 'Structure-activity relationships of some 3-substituted-4-hydroxycoumarins as HIV-1 protease inhibitors', *Farmaco* **2002**, *57*, 703 - 708

[27] I. Kostova, S. Raleva, P. Genova, R. Argirova, 'Structure-activity relationships of synthetic coumarins as HIV-1 inhibitors', *Bioinorg. Chem. Appl.* **2006**, *2006*, 1-9.

[28] C. X. Su, J. F. Mouscadet, C. C. Chiang, H. J. Tsai, 'HIV-1 integrase inhibition of biscoumarin analogues', *Chem. Pharm. Bull.* **2006**, *54*, 682 - 686.

[29] D. Yu, M. Suzuki, L. Xie, S. L. Moriss-Natschke, K. H. Lee, 'Recent progress in the development of coumarin derivatives as potent anti-HIV agents', *Med. Res. Rev.* **2003**, *23*, 322 - 345.

[30] O. Danis, B. Yuce-Dursun, C. Gunduz, A. Ogan, G. Sener, M. Bulut, A. Yarat, 'Synthesis of 3-amino-4-hydroxy coumarin and dihydroxy-phenyl coumarins as novel anticoagulants', *Arzneim. Forsch.* **2010**, *60*, 617 - 620.

[31] P. Diana, A. Carbone, P. Barraja, A. Martorana, O. Gia, L. Dalla Via, G. Cirrincione, '3,5-Bis(3'-indolyl)pyrazoles, analogues of marine alkaloid nortopsentin: synthesis and antitumor properties', *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6134 - 6137.

[32] Z. M. Nofal, M. I. El-Zahar, S. S. Abd El-Karim, 'Novel coumarin derivatives with expected biological activity', *Molecules* **2000**, *5*, 99 - 113.

[33] G. Raju, R. Srinivas, M. D. Reddy, C. R. Reddy, N. Nagesh, 'Studies on Non-Covalent Interaction of Coumarin Attached Pyrimidine and 1-Methyl Indole 1,2,3 Triazole Analogues with Intermolecular Telomeric G-Quadruplex DNA Using ESI-MS and Spectroscopy', *Nucleosides, Nucleotides Nucleic Acids* **2014**, *33*, 489 - 506.

[34] A. A. Emmanuel-Giota, K. C. Fylaktakidou, K. E. Litinas, D. N. Nicolaides, D. J. Hadjipavlou-Litina, 'Synthesis and biological evaluation of several 3-(coumarin-4-yl)tetrahydroisoxazole and 3-(coumarin-4-yl)dihydropyrazole derivatives', *J. Heterocycl. Chem.* **2001**, *38*, 717 - 722.

[35] F. Borges, F. Roleira, N. Milhazes, L. Santana, E. Uriarte, 'Simple coumarins and analogues in medicinal chemistry: occurrence, synthesis and biological activity', *Curr. Med. Chem.* **2005**, *12*, 887 - 916.

[36] M. Zahradnik, 'The Production and Application of Fluorescent Brightening Agent', John Wiley & Sons Ltd., Chichester, 1992.

[37] A. Zivelin, L. V. Rao, S. I. Rapaport, 'Mechanism of the anticoagulant effect of warfarin as evaluated in rabbits by selective depression of individual procoagulant vitamin K-dependent clotting factors', *J. Clin. Invest.* **1993**, *92*, 2131 - 2140.

[38] M. E. Marshall, J. L. Mohler, K. Edmonds, B. Williams, K. Butler, M. Ryles, L. Weiss, D. Urban, A. Bueschen, M. Markiewicz, G. Cloud, 'An updated review of the clinical development of coumarin (1,2-benzopyrone) and 7-hydroxycoumarin', *J. Cancer Res. Clin. Oncol.* 120 (Suppl. 1), **1994**, S39 - S42.

[39] R. O'Kennedy, R. D. Thornes, 'Coumarins: Biology, Applications and Mode of Action', John Wiley & Sons Ltd., Chichester, 1997.

[40] I. Manolov, N. D. Danchev, 'Synthesis, toxicological and pharmacological assessment of some 4-hydroxycoumarin derivatives', *Eur. J. Med. Chem.* **1995**, *30*, 531 - 535.

[41] J. J. Chul, P. O. Sook, 'Synthetic approaches and biological activities of 4-hydroxycoumarin derivatives', *Molecules* **2009**, *14*, 4790 - 4803.

[42] P. Laurin, D. Ferroud, M. Klich, C. D. Hamelin, P. Mauvais, P. Lassaigne, A. Bonnefoy, B. Musicki, 'Synthesis and *in vitro* evaluation of novel highly potent coumarin inhibitors of gyrase', *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2079 - 2084.

[43] A. N. Abdel Latif, R. Z. Batran, M. A. Khedr, M. M. Abdalla, '3-Substituted-4-hydroxycoumarin as a new scaffold with potent CDK inhibition and promising anticancer effect: Synthesis, molecular modeling and QSAR studies', *Bioorg. Chem.* **2016**, *67*, 116 - 129.

[44] V. Dekić, N. Radulović, R. Vukićević, B. Dekić, D. Skropeta, R. Palić, 'Complete assignment of the ^{1}H and ^{13}C NMR spectra of antimicrobial 4-arylamino-3-nitrocoumarin derivatives', *Magn. Reson. Chem.* **2010**, *48*, 896 - 902.

[45] V. Dekić, B. Dekić, N. Radulović, ^{1}H and ^{13}C NMR spectral assignments of an amino acid-coumarin hybrid', *Facta Univ., Ser.: Phys., Chem. Technol.* **2013**, *11*, 101 - 107.

[46] N. Radulović, G. Bogdanović, P. Blagojević, V. Dekić, R. Vukićević, 'Could X-ray analysis explain for the differing antimicrobial and antioxidant activity of two 2-arylamino-3-nitro-coumarins?', *J. Chem. Crystallogr.* **2011**, *41*, 545 - 551.

[47] J. Hu, Y. Sun, J. Qi, Q. Li, T. Wei, 'A new unsymmetrical azine derivative based on coumarin group as dual-modal sensor for CN^- and fluorescent "OFF–ON" for Zn^{2+}', *Spectrochim. Acta, Part A* **2017**, *175*, 125 - 133.

[48] W. Tang, Y. Xiang, A. Tong, 'Salicylaldehyde azines as fluorophores of aggregation-induced emission enhancement characteristics', *J. Org. Chem.* **2009**, *74*, 2163 - 2166.

[49] R. Sheng, P. Wang, W. Liu, X. Wu, S. Wu, 'A new colorimetric chemosensor for Hg^{2+} based on coumarin azine derivative', *Sens. Actuators, B* **2008**, *128*, 507 - 511.

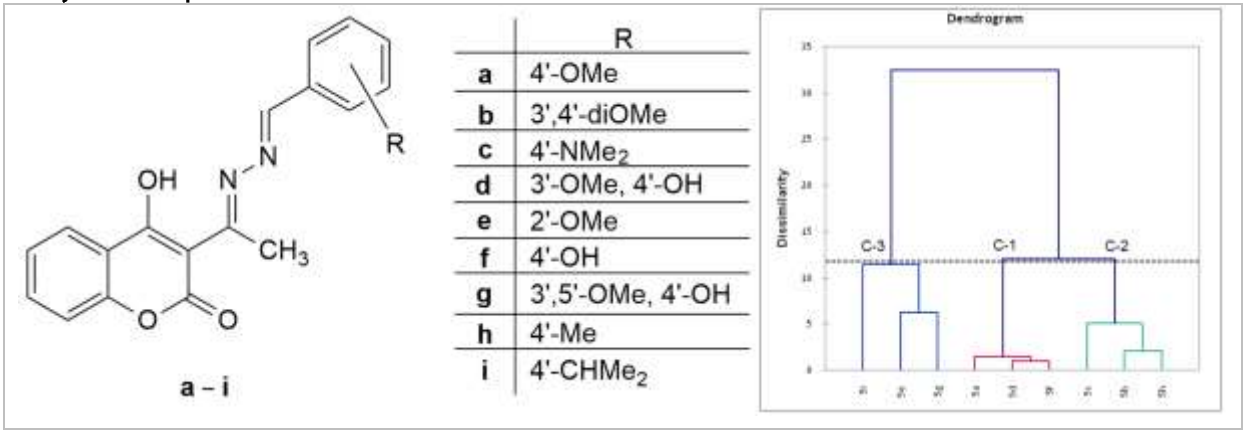
[50] C. Cordier, E. Vauthier, A. Adenier, Y. Lu, A. Massat, A. Cosse-Barbi, 'Salicylaldehyde benzoyl hydrazone: isomerization due to water. A structural analysis using a combination of NMR, IR, and theoretical investigations', *Struct. Chem.* **2004**, *15*, 295 - 307.

[51] N. Radulović, P. D. Blagojević, Z. Z. Stojanović-Radić, N. M. Stojanović, 'Antimicrobial plant metabolites: structural diversity and mechanism of action', *Curr. Med. Chem.* **2013**, *20*, 932 - 952.

[52] Y. Lu, T. J. Khoo, C. Wiart, 'Antioxidant activity determination of citronellal and crude extracts of *Cymbopogon citrates* by 3 different methods', *Pharmacol. Pharm.* **2014**, *5*, 395 - 400.

[53] E. Brand-Williams, M. E. Cuvelier, C. Berset, 'Use of a free radical method to evaluate antioxidant activity', *LWT-Food Sci. Technol.* **1995**, *28*, 25 - 30.

Entry for the Graphical Illustration



Twitter Text

A non-toxic mixed azine possessing 4-hydroxycoumarin and 3,5-dimethoxy-4-hydroxyphenyl moieties is a potent antioxidant and a strong antimicrobial agent.