ChemPubSoc Europe

DOI: 10.1002/ejoc.201501278

EurJOC European Journal of Organic Chemistry

Full Paper

Drug Discovery

One-Pot Synthesis of Benzopyran-4-ones with Cancer Preventive and Therapeutic Potential

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Abstract: A one-pot synthesis of novel benzopyran-4-ones is described. In a tandem reaction, organobase-catalysed Michael addition of $R^1COCH_2COR^2$ on chromone-3-carboxylic acid led to decarboxylation and pyran-4-one ring opening of the latter. This was followed by chromone- and/or chromanone ring closure of the resulting Michael adducts when R^1 is an *ortho*-hydroxyaryl group. Antioxidant testing of 14 derivatives identified strong antiradical properties of chromanones **3o**–**r** (2.1–3.1 μmol Trolox equiv./μmol compound in the DPPH assay). Chromanones **3p** and **3r** and 2-styrylchromone **3k** were also most potent in inducing the cytoprotective Keap1-Nrf2 signalling pathway in a reporter gene assay (fivefold induction at concentrations <3 μM). Of the seven compounds evaluated for antiproliferative activities, **3k** and **3r** were the most active, inhibiting leukaemia K562 cell proliferation by 50 % after 72 h at concentrations of 4.5 and 7.9 μM, whereas normal peripheral blood mononuclear cells were not affected.

Introduction

Recent research has revealed a multitude of biological activities attributed to benzopyran-4-one-based compounds, including chromones, chromanones, flavones and 2-styrylchromones.[1] Derivatives with a basic chromone structure are ubiquitously found in the plant kingdom. Their antimicrobial, antiviral, anticancer, anti-inflammatory, and antioxidant effects are profitable to human health.[2] Further representatives of the benzopyran-4-one nucleus are the chromanones, among which, several are in use for the treatment of various ailments such as hypertension, asthma, ischemia, and urinary incontinence.[3,4] The flavone class of compounds represents the most abundant class of phytochemical components of plants, fruit and vegetables.[4] These compounds are associated with a wide range of biological activities and nutritional benefits, and are mostly recognised as anticancer, anti-inflammatory and antioxidant agents.[5] In parallel, 2-styrylchromones, although scarce in nature, represent one of the most useful chromone-based scaffolds due to their antiviral, antitumor, antimitotic, anti-inflammatory and antioxidant activities.[6,7]

Due to the growing medicinal interest in benzopyran-4-one-based compounds, several multistage approaches for the synthesis of novel derivatives with new biological profiles and/or enhanced bioactivities have been developed. For example, in the last decade, several research groups have been involved in the synthesis of 3-substituted-flavones and -2-styrylchromones in an effort to find compounds with improved antioxidant and antitumor activities.[7,8] Moreover, an interesting series of potent antioxidant agents, 6-hydroxy-7-methoxy-4-chromanones and chroman-2-carboxamides, have been described,[9] thus turning these scaffolds more attractive for biological applications. Nevertheless, better knowledge on structure-activity relationships (SAR) is required to design benzopyran-4-one frameworks that are suitable for testing of in vitro and in vivo biological interac-

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tions. Accordingly, convenient synthetic routes for novel benzopyran-4-one-based compounds are still of urgent need in the medicinal chemistry field.

In 2002, Tuskaev et al. described a molecular engineering approach to increase the antiallergic activity and decrease the toxicity of chromones.[10] They inserted a (2-hydroxyphenyl)-3-oxoprop-1-enyl- (HOPO) moiety as a Michael acceptor group into the chromone structure to design chalcone-like derivatives. Nowadays, chalcones and other natural flavonoids sharing HOPO moieties are widely recognised as compounds of pharmacological interest. As the best example, the chalcone xanthohumol (Figure 1), which is extracted from hop, is one of the most important prenylated flavonoids, offering a broad spectrum of biological activities, including antioxidant activity, potentiation of nerve growth factor action, induction of NAD(P)H:quinone oxidoreductase, inhibition of diacylglycerol acyltransferase, and inhibition of human cytochrome p450 enzymes.[11] Chalcones are also cytotoxic and promote cell death through induction of apoptosis.[12] Importantly, they also possess cancer chemopreventive properties, for example by inducing the cytoprotective Nrf2 signalling pathway, which has been suggested to be mainly due to the presence of the HOPO group.[13] Another natural compound with a Michael acceptor moiety is curcumin (Figure 1), which is one of the most potent natural anticancer compounds. This polyphenol with two cinnamoyl (CINA) substitutions shows an accentuated Michael acceptor character, which could be involved in the inhibition of tumour development in animal models of oral, gastric, intestinal, colonic, hepatic and cutaneous carcinogenesis.[14]

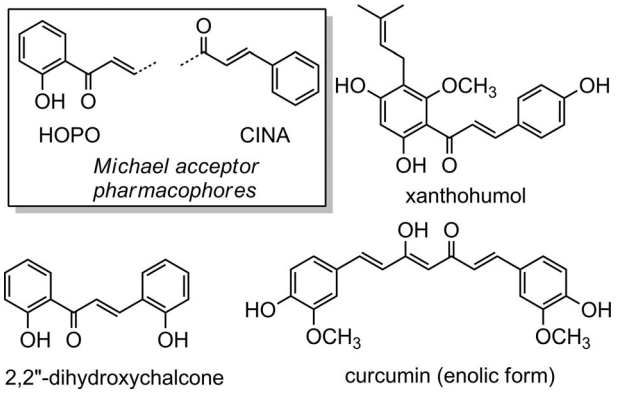


Figure 1. Natural cancer preventive compounds containing a HOPO- or CINA-pharmacophore.

These few examples indicate that the Michael acceptor functionality could be an important structural feature for the biological activities of curcuminoids and chalcones to protect against chronic degenerative diseases in animal models of carcinogenesis, cardiovascular disease and neurodegeneration. Mechanistically, it has been postulated that at least some of the effects exerted by HOPO and CINA compounds in biological systems can be attributed to the electrophilic properties of the Michael acceptor pharmacophore. Hence, sulfur- and nitrogen-based cellular nucleophiles can potentially react with the electrophilic β-carbon of HOPO and CINA compounds. The biological responses of cells exposed to electrophilic compounds may range from the activation of cytoprotective mechanisms to the stimulation of death-promoting signalling.[15]

The present study aimed to provide a simplified synthesis of 17 novel benzopyran-4-ones **3a**–**g**, **3j**–**r** and **3o′**, bearing the HOPO moiety in the 3-position of chromones, flavones and 2-styrylchromones along with 2,3-disubstituted chromanones incorporating the curcuminoid-type moiety (CINA) as an important scaffold for biological activities. To compare their biological properties and deduce SAR correlations, the compounds were tested for antioxidant activity using ferric ion reducing FRAP and DPPH free radical scavenging tests. Anticarcinogenic potential was evaluated by measuring induction of cytoprotective Keap1-Nrf2 signalling,[16] and cytostatic potential in human leukemia cells.[17] The combined approach identified 2-styrylchromone **3k** and chromanone **3r** as promising novel benzopyran-4-one derivatives, which are now available for further investigation of the mechanisms underlying their biological activities.

Results and Discussion

Chemistry

The electron-withdrawing effect of both the 3-carboxylic acid and 4-ketone functions of **1** is useful to generate a strong electrophilic site at the C-2 position. Only recently, synthetic efforts have focussed on the Michael addition on similar C-2 electron-deficient chromones, giving rise to novel functional polycyclic chromones[18] and functionalized 2-hydroxybenzophenones[19] through tandem reaction processes. Peng et al.[20] reported the synthesis of 4-substituted 3,4-dihydrocoumarins using a 1,4-conjugate addition/double decarboxylation cascade reaction of a β-ketocarboxylic acid on the coumarin-3-carboxylic acid.

The reaction of chromone-3-carboxylic acid **1** with primary and secondary amines, hydrazines, cyanoacetohydrazide, cyanoacetamide and malononitrile in different media has mainly led to ring transformation through pyran-4-one-ring opening and decarboxylation.[21] In the same study, 3-(HOPO)chromone **3a** was prepared through intermolecular dimerisation and decarboxylation of chromone-3-carboxylic acid **1** in a sodium hydroxide solution.[21]

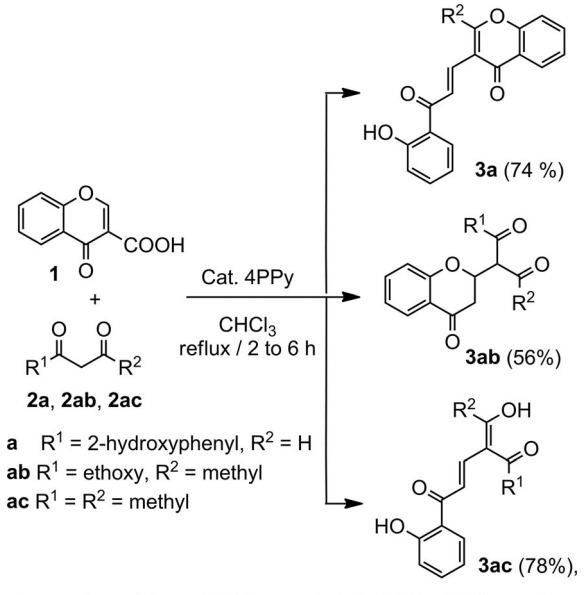
In the present study, the behaviour of acid **1** under organobase catalysis was fully studied. Initially, a catalytic amount of 4-pyrrolidinopyridine (4-PPy) as a tertiary amine organic base was used to convert acid **1** into the dimeric product **3a** with an optimal yield (67 %), involving dimerisation and decarboxylation reactions. A small amount of the unsubstituted chromone **3aa** was also formed. Compound **3a** was readily isolated after recrystallisation from ethanol, whereas **3aa** remained dissolved in ethanol and was subsequently isolated by chromatography. Under these mild conditions, the organobase-promoted dimerisation of **1** gave better results than the inorganic base procedure (NaOH in the procedure developed by Ibrahim[21]), regardless of the longer reaction time (Table 1). Here, the use of an organobase catalyst 4-PPy (or TEA, DBU) allowed the dimerisation of **1** with higher yields (up to 67 %; Table 1, entry 1).

Table 1. Dimerisation of chromone-3-carboxylic acid **1** under organobase catalysis.

Entry	Solvent	Organo-base	Temp. [°C]	Time [h]	Yield [%]
1	CH_2Cl_2	4-PPy	room temp.	48	67[a,b]
2	$CHCl_3$	4-PPy	reflux	3	51[a,b]
3	CH_2Cl_2	DBU	room temp.	24	25[a–c]
4	CH_2Cl_2	TEA	room temp.	48	55[a–c]
5	CH_2Cl_2	pyridine	room temp.	> 48	–[b,c]
6	CH_2Cl_2	2,6-lutidine	room temp.	> 48	–[b,c]

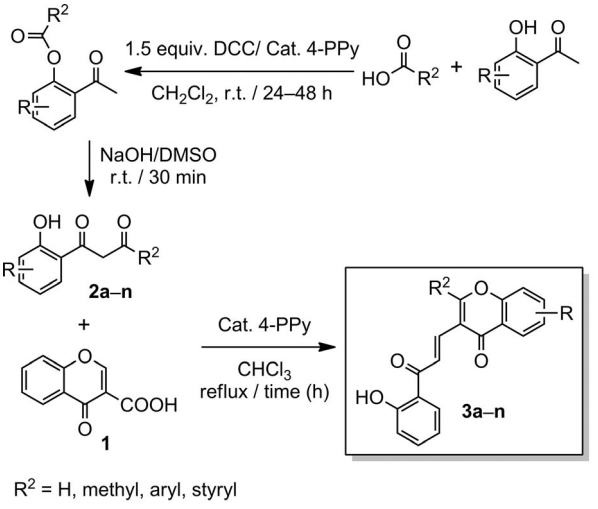
[a] Isolated yield after recrystallisation. [b] Formation of by-product **3aa** was confirmed by analytical TLC using an authentic sample. [c] Remaining starting material **1** was identified by TLC.

Similar to the dimeric product **3a** obtained through Michael addition of the ω-formyl-2′-hydroxyacetophenone intermediate **2a** on acid **1** (Scheme 1), other 1,3-dicarbonyl reagents **2a**–**r** could be used as a Michael donor towards **1** to afford **3**, bearing the desired HOPO moiety. We started to study the C-2 Michael acceptor character of **1** under the action of carbon nucleophiles derived from the 1,3-dicarbonyl compounds **2a**, **2ab** and **2ac**, in the presence of a catalytic amount of 4-PPy in refluxing chloroform. As a result, the action of ω-formyl-2′-hydroxyacetophenone **2a** produced the dimeric compound **3a** in higher yield (74 % compared with the dimerisation procedure of **1** which yielded 67 % of **3a**). The use of different diaryl-1,3-dicarbonyl compounds ($R^1COCH_2COR^2$) sharing mainly the key function 2-hydroxyaryl group **2a**–**n**, synthesised by using the Baker–Venkataraman method,[22] was found to give an additional intramolecular cyclisation event of the linked β-diketone portion into the benzopyran-4-one ring, producing a variety of 3-(HOPO)chromones **3a**–**d**, 3-(HOPO)flavones **3e**–**g**, **3j** and 3-(HOPO)-2-styrylchromones **3k**–**n** and **3o′**, in a one-pot organobase-catalysed tandem reaction (Scheme 2, Table 2).



General conditions: **1** (5.26 mmol, 1 g), **4-PPy** (0.26 mmol, 0.05 equiv., 0.04 g), **2a**, **2ab**, **2ac** (5.26 mmol, 1 equiv.) in chloroform (10 mL)

Scheme 1. Michael addition of 1,3-dicarbonyl compounds **2a**, **2ab** and **2ac** on acid **1**.

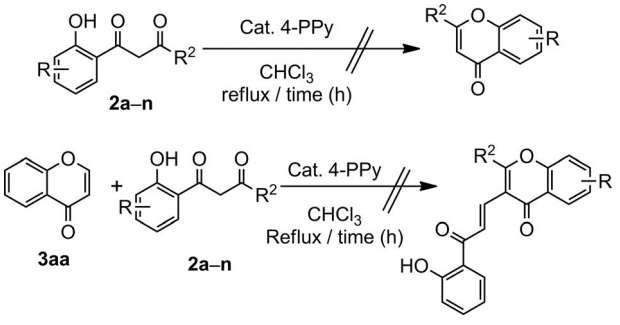


R^2 = H, methyl, aryl, styryl

General conditions: **1** (5.26 mmol, 1 g), **4-PPy** (0.26 mmol, 0.05 equiv., 0.04 g), **2a**–**n** (5.26 mmol, 1 equiv.) in chloroform (10 mL)

Scheme 2. Michael addition of 1,3-dicarbonyl compounds **2a**–**n** on acid **1**.

Cyclisation of 1,3-dicarbonyls (or β-diketones) **2a**–**n** into their corresponding benzopyran-4-one nucleus failed to occur under the operating condition of organobase catalysis (using 4-PPy) in the absence of acid **1** (Scheme 3). Moreover, the unsubstituted chromone **3aa** does not behave as a good Michael acceptor when tested for this type of 1,4-conjugate addition reaction (Scheme 3). Hence, our new synthetic method for the construction of benzopyran-4-one-based compounds combines both the Baker–Venkataraman rearrangement with the tandem Michael addition on chromone-3-carboxylic acid **1** (Scheme 2).



Scheme 3. Effect on the starting materials of the Michael addition of 1,3-dicarbonyl compounds **2a**–**n** on acid **1**.

An exception was found in the reactivity of 1-(mono-, or dimethoxy-2-hydroxyphenyl)-3-styryl-1,3-dicarbonyl compounds **2o**–**r**, for which the Michael addition on the acid **1** led to the selective production of novel 2,3-disubtituted chromanone-based compounds **3o**–**r** incorporating the curcumin-type CINA moiety and bearing an asymmetric (C-2) centre (Scheme 4, Table 2, entries 15–19). In the case of **2o**, reaction with acid **1** leads to the formation of a minor amount of 3-(HOPO)-2-styrylchromone **3o′** (yield 7 %, Table 2, entry 15) along with the major product of 2,3-disubstituted chromanone **3o** (yield 35 %, Table 2, entry 16).

Table 2. Michael addition of 1,3-dicarbonyl **2a**–**r** on acid **1**.

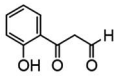
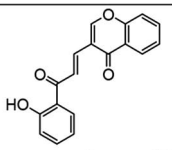
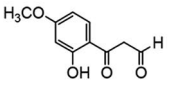
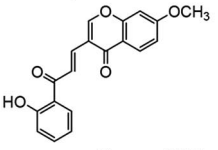
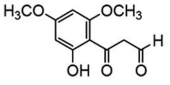
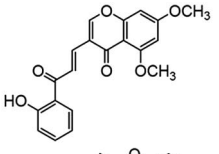
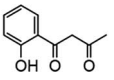
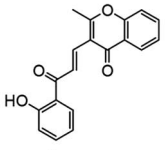
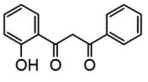
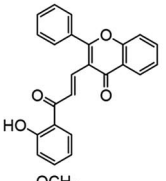
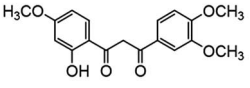
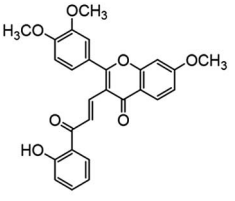
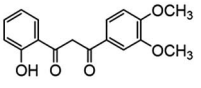
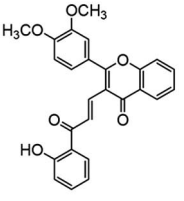
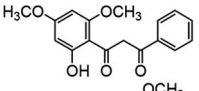
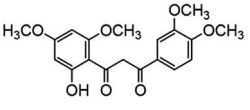
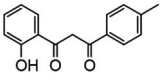
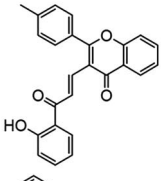
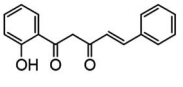
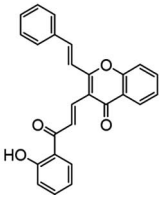
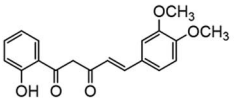
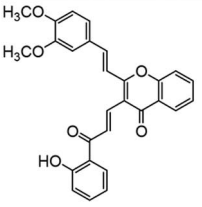
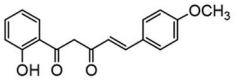
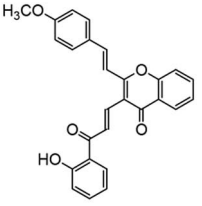
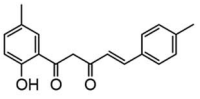
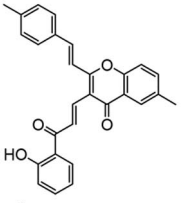
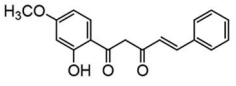
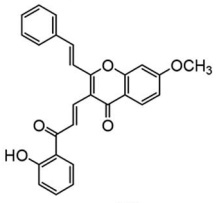
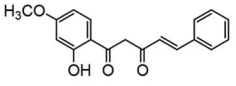
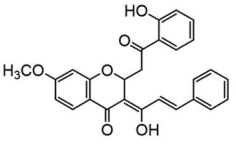
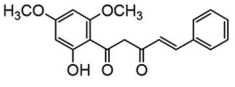
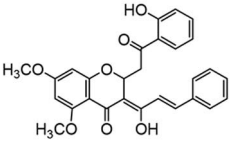
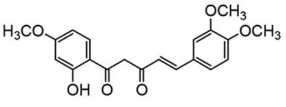
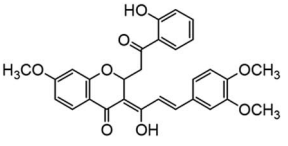
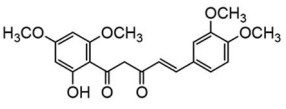
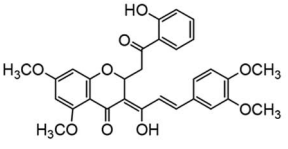
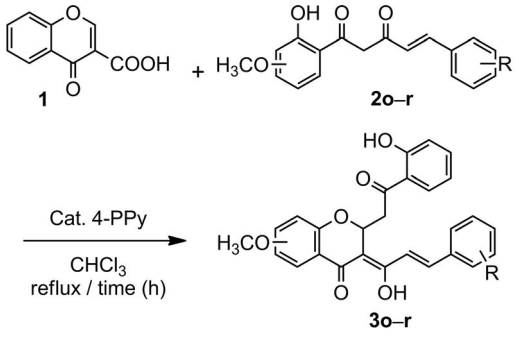
Entry	Substrate	Product	Time [h]	Yield [%][a]
1	**2a** 	**3a** 	6	74[b]
2	**2b** 	**3b** 	6	67[b]
3	**2c** 	**3c** 	24	43[b–d]
4	**2d** 	**3d** 	6	63[b]
5	**2e** 	**3e** 	24	51[b,d]
6	**2f** 	**3f** 	24	44[b,d]
7	**2g** 	**3g** 	24	52[b,d]
8	**2h** 	**3h** no product	>72	–[b,c]
9	**2i** 	**3i** no product	>72	–[b,c]
10	**2j** 	**3j** 	24	75[b,d]
11	**2k** 	**3k** 	24	43[b,d]

Table 2. *(Continued)*.

Entry	Substrate	Product	Time [h]	Yield [%][a]
12	**2l** 	**3l** 	24	68[b,d]
13	**2m** 	**3m** 	24	72[b]
14	**2n** 	**3n** 	24	46[b,d]
15	**2o** 	**3o'** 	24	7[b–d]
16	**2o** 	**3o** 	24	35[b–d]
17	**2p** 	**3p** 	24	31[b–d]
18	**2q** 	**3q** 	48	42[b–d]
19	**2r** 	**3r** 	48	37[b–d]

[a] Isolated yield after recrystallisation. [b] The formation of chromone **3aa** was confirmed by analytical TLC using an authentic sample. [c] The formation of the dimeric product **3a** was confirmed by TLC. [d] The remaining starting materials **1** and **2a**–**r** were determined by TLC.

With the above experimental findings in hand, a global mechanism can be proposed for the synthesis of benzopyran-4-one-based compounds, which offers several advantages, but also presents some limits in exceptional cases (Scheme 5). For instance, 1,3-dicarbonyl compounds **2h** and **2i** did not show reactivity (Table 2, entries 8 and 9), which could be a consequence of lower acidity of their activated methylene protons and/or a bulky structure due to their high degree of methoxy-substitution, thereby hindering access of the tertiary amine 4-PPy. In addition, the use of benzyl-OH-protected 1,3-dicarbonyl compounds with free 2-hydroxyaryl failed to yield the expected products (data not shown). Instead, the dimeric compound **3a**



Scheme 4. Michael addition of 1,3-dicarbonyl **2o**–**r** on acid **1**.

and the chromone **3aa** by-product were formed, indicating that structural hindrance might indeed play a role. 4-PPy, the most efficient tertiary amine used, allowed the formation of only negligible quantities of the by-product chromone **3aa** during the course of the dimerisation reaction compared with the use of DBU and TEA (Table 1).

The structural characterisation of the new benzopyran-4-one-based products was established on the basis of extensive 2D NMR studies, including HSQC, HMBC and NOESY experiments and further supported by X-ray crystallographic studies (fully discussion in the Supporting Information). ^{1}H NMR analysis was used to delineate several stereochemical features that were present in all the synthesised polyphenolics **3a**–**g**, **3j**–**n** and **3o′**, namely the (*E*)-configuration of the C2′=C1′ double bond [*J* = 15.1–15.2 Hz] of the 3-(2-hydroxyphenyl)-3-oxoprop-1-enyl (HOPO) moiety.

Further information on the conformational and stereochemical structural features of the new benzopyran-4-one-based compounds were gained from NOESY experiments. Compound **3a** must exist in its C3-C1′ *s-trans*-conformation according to NOESY spectra analysis, which clearly shows that H-1′ is spatially close to H-2, and H-2′ to H-6″ of the *ortho*-hydroxyphenyl group; this fact is also consistent with the (*E*)-configuration of the C1′=C2′ double bond of HOPO (Figure 2). The same occurred for flavones **3e**–**g**, as shown for compound **3f** (Figure 2).

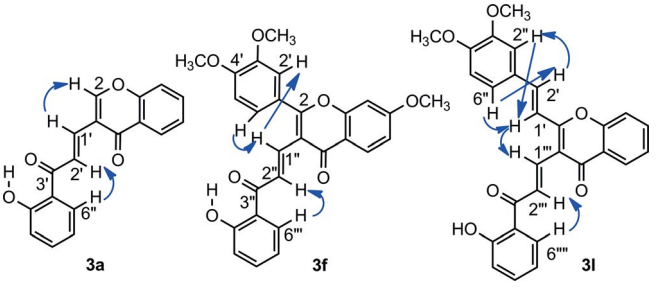


Figure 2. Main NOE effects observed in the NOESY spectra of compounds **3a**, **3f** and **3l**.

The crystallographic studies of **3a** confirmed the stable C3-C1′ *s-trans* conformation of the molecule as inferred from the NOESY spectrum. In the solid state, the very strong hydrogen bond [$d_{D\cdots A}$ = 2.546(3) Å] between the 2″-OH and the C-3′ carbonyl group is clearly visible (Figure 3).

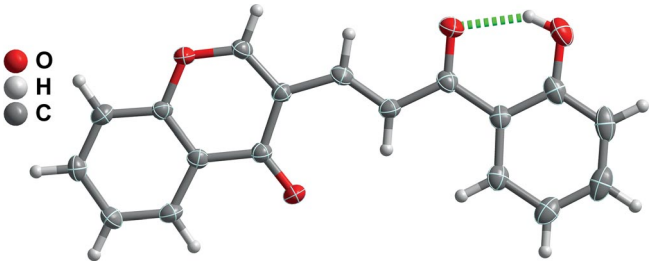
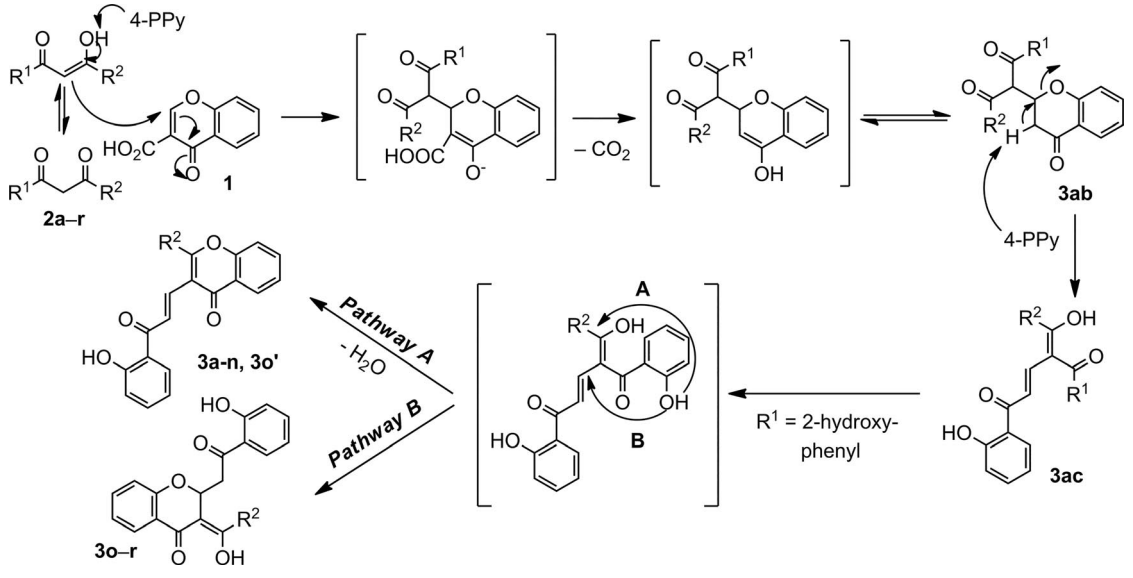


Figure 3. Schematic representation of the molecular unit present in the crystal structure of **3a**. Non-hydrogen atoms are represented as thermal ellipsoids drawn at the 50 % probability level; hydrogen atoms are depicted as small spheres with arbitrary radii.

In the more complex structures, the data obtained from the NOESY experiment supported the ^{1}H NMR spectral analysis for



Scheme 5. Proposed reaction mechanism of Michael addition of 1,3-dicarbonyl **2a**–**r** on chromone-3-carboxylic acid **1**.

the multiple double bond conjugated systems. The C-1′-C3/C1‴-C3 presents an *s-trans* conformation of the HOPO fragment in all the scaffolds **3a**–**g**, **3j**–**n** and **3o**′ (Figure 2 presents the case of **3l**).

Finally, in the case of chromanones **3o**–**r**, the NOESY spectra did not allowed us to unequivocally assign its 3D structure, however X-ray diffraction analysis of chromanone **3o** showed centrosymmetric triclinic $P\bar{1}$ crystals with the asymmetric unit being composed of a whole molecular unit, as depicted in Figure 4. Due to the centrosymmetric nature of the crystal, the bulk material is formed as a racemic mixture of both *R* and *S* enantiomers. In this context, one can immediately infer that the employed Michael protocol under organobase catalysis does not allow an enantioselective production of 2,3-disubstituted chromanones **3o**–**r**.

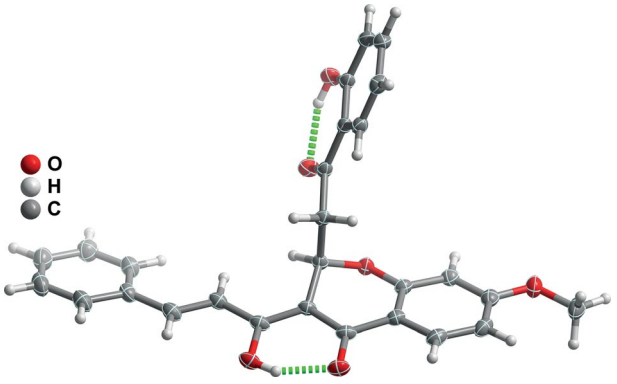


Figure 4. Schematic representation of the molecular unit present in the crystal structure of compound **3o**. Non-hydrogen atoms are represented as thermal ellipsoids drawn at the 50 % probability level; hydrogen atoms are depicted as small spheres with arbitrary radii.

Biological Screening

In vitro Antioxidant Activities of 3a–g, 3k–m and 3o–r

First we evaluated the antioxidant activity of benzopyran-4-ones **3a**–**g**, **3k**–**m** and **3o**–**r** by using the ferric reducing antioxidant power (FRAP) technique[23a] and DPPH methods.[23b] Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as a reference.

Of the 14 tested compounds, all except **3e** were able to reduce ferric ions in the FRAP assay, however, with about 3.9-fold lower efficiency than Trolox (see the Supporting Information, Figure S7). In the DPPH scavenging assay, chromones **3a**–**3d** and all chromanones **3o**–**r** demonstrated DPPḢ scavenging activity, whereas flavones **3e**–**g** and 2-styrylchromones **3k**–**m** were inactive (Figure 5). We identified chromone **3a** as the most potent benzopyran-4-one derivative, with an antiradical activity of 3.7 ± 0.3 µmol Trolox equivalents/µmol compound. SAR analyses within the chromone class of compounds revealed that addition of methoxy **3b**–**c** or methyl **3d** groups on the chromone moiety of **3a** strongly reduced the antiradical activity. Likewise, addition of a phenyl group on the benzopyran-4-one leading to flavone structures **3e**–**g** or a styryl group leading to 2-styrylchromones **3k**–**m** lowered the activity of **3a**. On the other hand, the chromanone derivatives **3o**–**r** showed strong antiradical properties in the range of 2.1–3.1 µmol Trolox equivalents/µmol compound. In a previous study, the 6-hydroxy-7-methoxy-4-chromanone displayed a radical scavenging activity comparable to the activity of Trolox.[9] Strong radical scavenging activity of our newly synthesised chromanones **3o**–**r** may derive from the addition of the CINA moiety. The degree of methoxy substitution did not strongly influence their antiradical capacity.

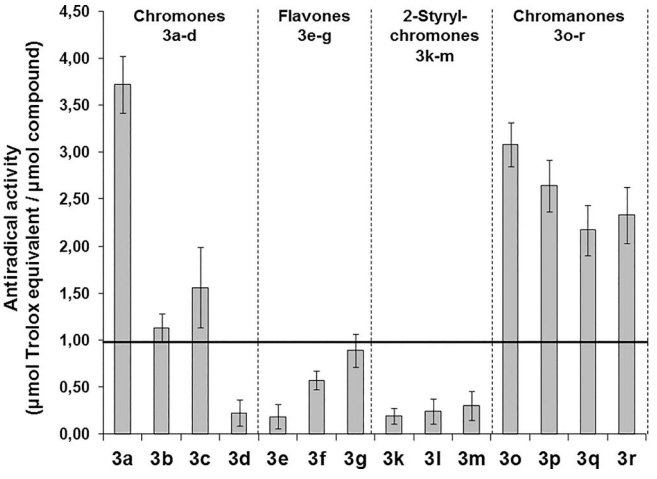


Figure 5. Evaluation of antiradical activity of benzopyran-4-ones **3a**–**g** and **3k**–**r** (DPPH assay).

Benzopyran-4-ones 3a–g and 3k–r Activate the Cytoprotective Keap1-Nrf2 Pathway in AREc32 Cells

Given that benzopyran-4-ones **3a**–**g** and **3k**–**r** contain a Michael acceptor centre, either in the form of a HOPO group in compounds **3a**–**g** and **3k**–**m** or in the form of a CINA group in chromanone derivatives **3o**–**r**, we addressed the question of whether they activate the Keap1-Nrf2 pathway compared to xanthohumol (XN) and sulforaphane (SFN), which were used as positive controls.

To monitor the Nrf2 signalling induction, we utilised the AREc32 cell line treated with the 14 benzopyran-4-ones **3a**–**g** and **3k**–**r** and positive controls SFN and XN, all used in a concentration range of 1.56–100 µM.[16] All compounds induced Nrf2 activity in a dose-dependent manner. With an approximate 91-fold increase above the vehicle control, chromanone **3r** was identified as the most potent inducer (see the Supporting Information, Figure S8). The concentrations required to increase luciferase activity by fivefold (C5 value, Table 3) in relation to solvent control were in the range of 2.5–15.2 µM for chromanones **3o**–**r**, 2.9–18 µM for 2-styrylchromones **3k**–**m**, 4.7–32.8 µM for chromones **3a**–**d**, and >53 µM for flavones **3e**–**g**. Overall, chromanones were the most potent class of Nrf2 inducers. Compounds **3p** and **3r** showed C5 values of 2.5 µM, which were lower than the C5 values achieved with the positive control compounds XN (7.8 µM) and SFN (4.8 µM). Inducing activity was accompanied by weak antiproliferative effects. Compounds **3p** and **3r** reduced cell numbers with half-maximal inhibitory concentrations (IC_{50}) of 49.9 and 41.9 µM, respectively (Table 3). We also identified Nrf2-inducing potential for the chromanone **3o**,

Table 3. Nrf2 inducing potential of benzopyran-4-ones **3a**–**g** and **3k**–**r** in AREc32 cells.

Compound	C5 [μM][a]	Tox. IC_{50} [μM][b]	Number & position of OCH_3
3a	12.4	40.3	0
3b	20.1	> 100 (73)	1 (C-7)
3c	32.8	> 100 (58)	2 (C-5,7)
3d	4.7	> 100 (78)	0
3e	52.9	> 100 (86)	0
3f	> 100 (3.2)	> 100 (100)	3 (C-7,3′,4′)
3g	> 100 (4.5)	> 100 (100)	2 (C-3′,4′)
3k	2.9	> 100 (68)	0
3l	18.0	> 100 (100)	2 (C-3″, 4″)
3m	10.2	> 100 (86)	1 (C-4″)
3o	3.4	> 100 (56)	1 (C-7)
3p	2.5	49.9	2 (C-5,7)
3q	15.2	> 100 (89)	3 (C-7,3‴,4‴)
3r	2.5	41.9	4 (C-5,7,3‴,4‴)
XN	7.8	27.5	1 (C-2)
SFN	4.8	44.5	n.a.

[a] C5 value, concentration causing a fivefold induction of luciferase activity (= Nrf2 activity). Values in parentheses indicate the fold induction vs. solvent control at the indicated concentration. [b] Tox, cytotoxic or antiproliferative activity; IC_{50}, half-maximal inhibitory concentration of cell viability. Values in parentheses indicate the percentage of cell viability compared with the control at the indicated concentration. Data presented correspond to one representative experiment. A repetition of the experiment demonstrated a similar trend of compound activities (see the Supporting Information, Table S1).

2-styrylchromone **3k** and chromone **3d**, with C5 values of 2.9 to 4.7 μM and only minor effects on cell growth at a concentration of 100 μM. Potent induction of the Keap1-Nrf2 pathway at low and possibly physiologically achievable concentrations makes compounds **3p**, **3r**, **3o**, **3k** and **3d** promising agents for further studies as chemopreventive activities.

SAR analyses revealed that both the number and position of methoxy substituents in the tested benzopyran-4-ones significantly influences their Nrf2 induction capacity, as represented by the C5 value. In the chromone group **3a**–**d**, compounds **3a** and **3d**, without methoxy group(s) in ring A, showed more potent Nrf2 induction potential than substituted chromones. We observed a similar SAR for flavones **3e**–**g** and 2-styrylchromones **3k**–**m**: addition of methoxy group(s) to the aromatic rings reduced the capacity to induce the Nrf2 pathway when compared with **3e** and **3k** lacking methoxy substituents. Finally, in the most active group of chromanones **3o**–**r**, both the degree and the position of methoxy substitution influenced inducing potential. 5-Methoxy-substitution on ring A increased the potency of Nrf2 activators **3p** and **3r** when compared with the activity of compounds **3o** and **3q** with a methoxy substitution at position C-7 (ring A). The most active compound **3r** represented the highest level of methoxy substitutions, with four groups placed on two aromatic rings at positions C-5, C-7, C-3‴ and C-4‴ (Table 3).

Antileukemic Activity of Benzopyran-4-ones 3a, 3e–g, 3k–l and 3r

Chromone **3a**, flavones **3e**–**g**, 2-styrylchromones **3k**, **3l** and chromanone **3r** were then selected for analysis of their impact on viability and proliferation of human K562 leukemia cells (Figure 6, Table 4). Chromanone **3r** strongly reduced cell viability at 100 μM after 72 h (IC_{50} 7.9). Styrylchromone **3k** showed a strong cytostatic effect when used at a concentration of 10 μM after 72 h (IC_{50} 4.5 μM).

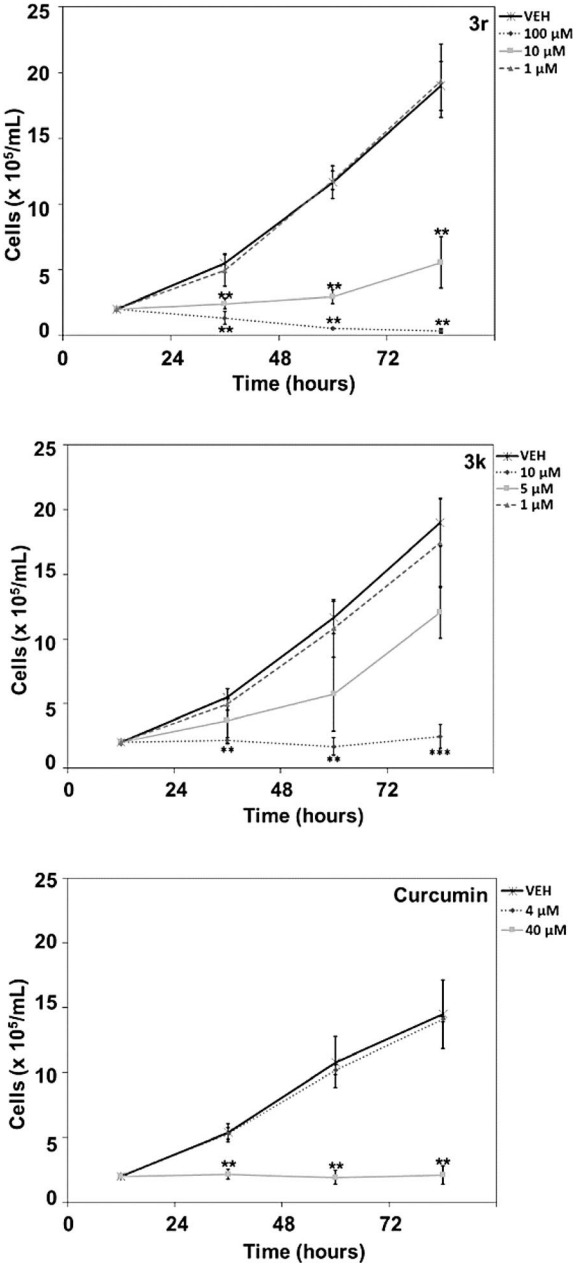


Figure 6. Impact of **3r** (top), **3k** (middle) and curcumin (bottom) on human K562 leukemia cell viability and proliferation. DMSO was used as a vehicle control (VEH); ** $p < 0.01$, *** $p < 0.001$.

Among the benzopyran-4-ones, 2-styrylchromone **3k** was the most active, followed by chromanone **3r**; none of the other compounds including **3a**, **3e**–**g** and **3l** affected either cell proliferation or induced death. Compound **3r** presents functional similarities to curcumin; hence, the CINA pharmacophore could be a useful tool to further investigate cytotoxic and antiproliferative activities.

Most significantly, chromanone **3r** and 2-styrylchromone **3k** did not affect the viability of peripheral blood mononuclear

Table 4. IC_{50} values of selected benzopyran-4-ones for antileukemic activity vs. K562 cells.[a]

Compound	Tox. IC_{50} [μM]		
	24 h	48 h	72 h
3a		> 100	
3e		> 100	
3f		> 100	
3g		> 100	
3k	8.6 ± 4.1	4.0 ± 1.2	4.5 ± 1.9
3l		> 100	
3r	13.1 ± 4.5	7.5 ± 1.0	7.9 ± 2.6
curcumin	28.3 ± 7.2	14.3 ± 3.4	15.0 ± 1.0

[a] Tox, cytotoxic or antiproliferative activity; IC_{50}: half-maximal inhibitory concentration of cell viability.

cells (PBMC) from healthy donors (IC_{50} > 50 μM) (Figure 7). Both curcumin and chromanone **3r** become cytotoxic in a comparable range of concentrations. These data confirm the selective bioactivity of **3r** and **3k** against cancer cells and underline the potential of both compounds as future lead structures.

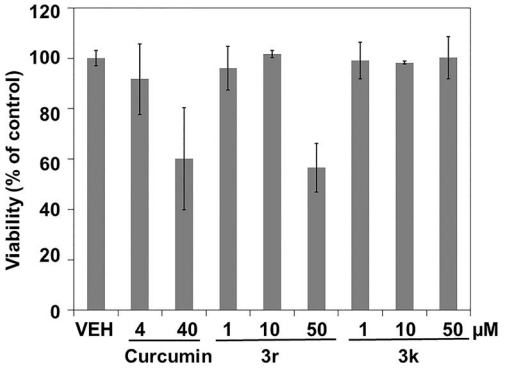


Figure 7. Impact of **3k**, **3r** and curcumin on viability of peripheral blood mononuclear cells (PBMC) from health donors treated for 24 h. DMSO was used as a vehicle control (VEH).

Conclusions

Novel benzopyran-4-one-based compounds (chromone, flavone, 2-styrylchromone and chromanone derivatives) have been prepared by a Baker–Venkataraman synthesis of 1,3-dicarbonyl compounds followed by a Michael addition tandem reaction of these compounds on chromone-3-carboxylic acid under organobase catalysis. The designed benzopyran-4-ones sharing Michael acceptor functionalities were screened for radical scavenging potential and cytoprotective properties by inducing the Nrf2 pathway, and antileukemic activity. These biological effects were most potently achieved by two classes of compounds, chromanones **3o**–**r**, sharing the curcuminoid bioactivator CINA moiety, and 2-styrylchromones **3k**–**m**, sharing the HOPO bioactivator function. The best radical scavengers were chromanones **3o**–**r**, with a very high activity, probably due to their CINA moiety. Moreover, chromanones **3p** and **3r** and 2-styrylchromones **3k** potently activated the Nrf2 response in the AREc32 cell line at low micromolar concentrations (C5 <3 μM), which might be physiologically achievable. At slightly higher concentrations, compounds **3r** and **3k** reduced viability and proliferation of the leukemic cell line K562, whereas PBMCs from healthy donors were not affected. The spectrum of activities revealed by **3r** and **3k** in vitro and their selective toxicity toward cancer cells supports further investigations of both their chemopreventive and therapeutic potential. With our synthetic approach in hand, these promising bioactivities also allow the design of additional structurally related benzopyran-4-ones, as minor modifications in substituents appear to affect the anticancer potential.

Experimental Section

1,3-Dicarbonyl compounds **2a**–**r** were prepared by using the Baker–Venkataraman method.[22]

General Procedure for Michael Addition of 1,3-Dicarbonyl Compounds 2a–r on Chromone-3-carboxylic Acid 1: Synthesis of 3-(HOPO-1)chromones, -flavones, -2-styrylchromones 3a–g, 3j–n and 3o′, and 2,3-Disubstituted Chromanones 3o–r: Chromone-3-carboxylic acid **1** (5.26 mmol, 1 g), was added to 1,3-dicarbonyl compounds **2a**–**r** (5.26 mmol) and a catalytic amount of 4-PPy (0.26 mmol, 0.04 g, 0.05 equiv.), the reaction mixture was brought to reflux in chloroform (10 mL). During the required reaction time (see Table 2), gradual consumption of the starting materials **1** and **2a**–**r** was monitored by TLC (CH_2Cl_2 or CH_2Cl_2/light petroleum), which showed, in most of the cases, several spots, including the desired product accompanied by the formation of by-products **3a** and **3aa** as verified by authentic samples and remaining starting materials (especially, 1,3-dicarbonyl compounds **2a**–**r**, observations are made in Table 2). The required reaction time was determined as the point at which no further change was observed according to TLC analysis. The reaction was then stopped and the solvent was removed by evaporation to give a dark-red resinous solid, which was either purified by silica gel column chromatography and then recrystallised or recrystallised directly from an appropriate solvent to afford compounds **3a**–**g**, **3j**–**n**, **3o′** and **3a**–**r** (indications are mentioned with each compound analytical data). The by-products **3a** (dimeric-product) and **3aa** (chromone) (when formed, see Table 2) were only compared to authentic samples by TLC but never recovered, except in the case of **3h** and **3i** reactions, for which the dimeric-compound **3a** was produced as the major product.

Ferric Ion Reducing Ability Assessment: The antiradical ability of **3a**–**r** was measured by assessing their ferric reducing power against ferric complexes. The FRAP assay[23a] depends upon the reduction of a ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex to the ferrous tripyridyltriazine (Fe^{2+}–TPTZ) by a reductant at low pH. Trolox was used as a reference. Compounds were tested at 25 μM. Working FRAP reagent was prepared as required by mixing acetate buffer (300 mM, 25 mL, pH 3.6), TPTZ solution (10 mM in HCl, 2.5 mL, 40 mM), and $FeCl_3 \cdot 6H_2O$ (20 mM, 2.5 mL). Freshly prepared FRAP reagent was warmed at 37 °C for 30 min and added to the samples. Absorbance of (Fe^{2+}–TPTZ) was measured at 593 nm after 30 min. Each sample was tested two times with three measures in each experiment.

DPPH Scavenging Activity Measurement: The antiradical activity of **3a**–**r** was measured by assessing their DPPḢ (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging activity. DPPḢ is a stable radical displaying an intense violet colour with a maximum absorbance at 515 nm. In the presence of an antioxidant compound, DPPḢ is neutralised to a compound with slight yellow colour, which causes an absorbance decrease.[23b] This modification is proportional to the antioxidant capacity of the compound and is measured by spectrophotometry. The degree of discoloration induced by a tested

sample is related to that induced by Trolox, allowing its antioxidant potent to be expressed as Trolox equivalents. DPPH was used at a final concentration of 120 μM in 1 mL. Compounds were tested at 10 μM. The range of Trolox concentrations for calibration spread from 5 to 70 μM. The optical density at 515 nm was read after incubation in the dark for 30 min. The absorbance of pure compounds suitably diluted was subtracted. Each sample was tested two times in triplicate.

AREc32 (Human Mammary Epithelial Adenocarcinoma MCF-7) Cells: AREc32 (kindly provided by Prof. Roland Wolf, University of Dundee) is a reporter cell line derived from the human mammary epithelial adenocarcinoma MCF-7 cells. It responds strongly and selectively to Nrf2, which, upon activation, binds to the ARE sequence present in eight copies in the promoter region of luciferase gene. This drives luciferase expression and can be assessed by measuring enzyme activity.[16] AREc32 cells were cultured at 37 °C and 5 % CO_2 in a humidified atmosphere in complete DMEM medium supplemented with 10 % heat-inactivated FCS, 2 mM glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (medium and all supplements from Gibco, US), and maintained under antibiotic selection (0.8 mg mL^{-1} G418; Sigma, Germany).

Luciferase Reporter Assay on AREc32 Cells Stimulated with Benzopyran-4-one-Based Polyphenolics 3a–r: For the detection of Nrf2 induction, the luciferase activity in AREc32 cells was measured. AREc32 cells were seeded at a density of 9×10^3 per well in 50 μL growth medium in 384-well plates. After 26 h, compounds were added to obtain final concentrations in the range between 1.56–100 μM. Each concentration was tested in quadruplicate. An equal volume of vehicle was added to the control wells (0.016–1 % DMSO). The reporter assay was performed as described previously.[16] For normalisation, all values of luciferase activity were divided by vehicle control values, yielding the fold change relative to the control. Furthermore, the luciferase activity was normalised to biomass content. Concentration leading to increase of luciferase activity by fivefold (C5) was determined by fitting a logistic dose response curve to the experimental data (Table Curve v1.0, Jandel Scientific).

Cytotoxicity Assay on AREc32 Cells Stimulated with Benzopyran-4-one-Based Polyphenolics 3a–r: Biomass content was assessed by sulforhodamine B (SRB, Sigma, Germany) staining.[16b] Briefly, AREc32 cells were seeded and treated as for the luciferase reporter assay. Detached cells in the supernatant were discarded and attached cells were fixed with 10 % trichloroacetic acid for 30 min at 4 °C. The plate was then rinsed with distilled water, dried and stained for 15 min with a 0.4 % solution of SRB in 1 % acetic acid, washed several times with 1 % acetic acid and dried again. The dye was dissolved with 10 mM TRIS base followed by the measurement of OD at 490 and 515 nm (SpectraMax M5e Microplate reader, Molecular Devices, US). The concentrations of compounds giving cell staining below 50 % of control cells were considered as cytotoxic. The concentration leading to a decrease of cell numbers to 50 % (IC_{50}) relative to solvent treated cells was determined by fitting a logistic dose response curve to the experimental data (Table Curve v1.0, Jandel Scientific).

Cell Culture, Treatments and Viability Assay on K-562: The human K-562 (chronic myeloid leukaemia) cell lines were obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen". PBMCs were obtained as previously reported.[24] Cells were cultured in RPMI 1640 (Lonza) supplemented with 10 % heat-inactivated foetal calf serum (Lonza) and 1 % antibiotic-antimycotic (Lonza) at 37 °C in humid atmosphere and 5 % CO_2. Compounds **3a**, **3e**–**g**, **3k**–**l** and **3r** were dissolved at 50 mM in DMSO (Sigma–Aldrich), aliquoted and stored at –20 °C upon treatment. Cells in exponential growth phase were treated at a concentration of 200,000 cells/mL. Control cells were treated with the same volume of DMSO as the one required for compounds. Cell proliferation and viability as well as cell cycle analyses were measured as previously reported.[24]

Acknowledgments

The authors thank the Fundação para a Ciência e a Tecnologia (FCT), Portugal (grant numbers UID/QUI/00062/2013, UID/CTM/50011/2013) the European Union (EU), the Quadro de Referência Estratégico Nacional (QREN), the Fundo Europeu de Desenvolvimento Regional (FEDER) and the Programa Operacional Factores de Competitividade (COMPETE) for funding the Organic Chemistry Research Unit, the Associate Laboratory Centro de Investigação em Materiais Cerâmicos e Compósitos – CICECO and the Portuguese National NMR Network (RNRMN). European Community's Seventh Framework Programme (FP7/2007-20139, grant agreement number 215009) is also acknowledged for financial support. The General Directorate for Scientific Research and Technological Development-DGRSDT of Algeria is thanked for financial support. The authors further wish to thank FCT for specific funding toward the purchase of the single-crystal X-ray diffractometer. O. T. appreciates support for his post-doctoral position through the project New Strategies Applied to Neuropathological Disorders (CENTRO-07-ST24-FEDER-002034), co-funded by QREN, Mais Centro-Programa Operacional Regional do Centro and the EU, FEDER. B. O. thanks RSL, Luxembourg, for her post-doctoral grant. Thanks are also due to the Fondation de Recherche Cancer et Sang, the Recherches Scientifiques Luxembourg association, the Een Haerz fur Kriibskrank Kanner association, the Action Lions Vaincre le Cancer association, Interreg IVA project Corena, and the Télévie Luxembourg for funding LBMCC. M. D. and B. O. also thank the Research Institute of Pharmaceutical Sciences by the NRF of the MEST of Korea for the Tumor Microenvironment GCRC 2012-0001184 grant and by the Brain Korea (BK21) PLUS program for funding. The AREc32 cell line used in this study was kindly provided by Professor Roland Wolf (University of Dundee, UK) and the Cancer Research UK.

Keywords: Medicinal chemistry · Drug discovery · Anticancer agents · Oxygen heterocycles · Domino reactions · Michael addition · Antioxidants

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Received: October 4, 2015
Published Online: January 15, 2016