In Vitro Cytotoxic Activity of Novel Protoflavone Analogs – Selectivity Towards a Multidrug Resistant Cancer Cell Line

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Abstract. *Background: Protoapigenone (PA), a natural flavonoid possessing an unusual p-quinol moiety on its B ring, is a prospective novel lead compound against cancer currently in development, together with WYC0209, a potent synthetic PA analog. Structure activity relationships (SAR) concerning different 1'-O-alkyl side-chains were also studied on two sets of derivatives. Materials and Methods: Fifteen 1'-O-alkyl protoflavone derivatives were synthesized from genkwanin or 4'-hydroxy-6-methylflavone, thirteen of which are new compounds. All compounds were tested for their cytotoxic effect on four human cancer cell lines, such as HepG2 and Hep3B (hepatic), A549 (lung) and MDA-MB-231 (breast) cell lines, with doxorubicin as a positive control. All compounds, as well as PA, WYC0209 and fourteen of their previously reported analogs were also tested on a multidrug-resistant (MDR) sub-cell line of L5178 mouse T-cell lymphoma and on its parental counterpart (PAR). Results: In general, derivatives bearing a free hydroxyl group at C-1' exerted the strongest activities, while C-1'-substituted compounds were found to be much weaker. Derivatives of 6-methylflavone exhibited mild, but statistically significant selectivity towards the MDR cell line. Conclusion: The results are in agreement with our previous findings for fundamental SAR of protoflavones. 6-Methylated protoflavones may serve as valuable leads for developing selective compounds against MDR cancer. Identical activity of other derivatives on the PAR and MDR cell lines suggests that cancer cells cannot exhibit resistance to protoflavones by ABCB1 efflux pump overexpression.*

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Flavonoids have long been proposed to exert various bioactivities interfering with cancer cells on several levels, including those connected to their antioxidant and chemopreventive effects, and, for certain derivatives, specific anticancer activities have also been described (1-4).

Protoapigenone is an oxidized derivative of the common flavonoid apigenin, expressing an unusual *p*-quinol moiety in its B-ring; the structures of these two compounds as well as that of WYC0209, the strongest synthetic derivative of protoapigenone obtained to date, are shown in Figure 1.

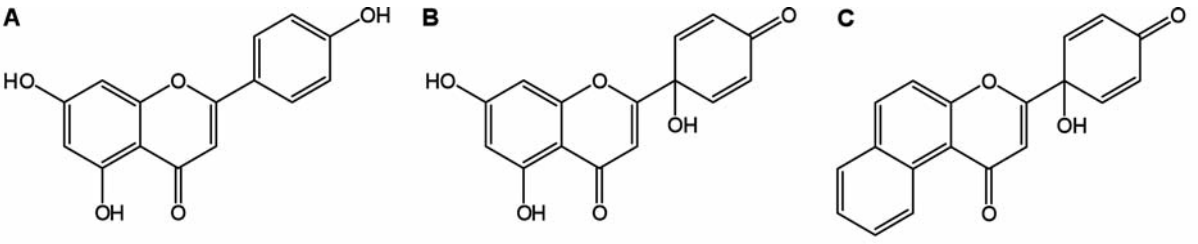

Figure 1. *Structures of apigenin (A), protoapigenone (B) and WYC0209, currently the strongest synthetic protoflavone derivative known (C).*

Isolated for the first time from the fern *Thelypteris torresiana*, protoapigenone was recently discovered as a promising, natural anticancer agent (5) based on its strong antitumor activity against several cancer cell lines of different origin both *in vitro* and *in vivo* (5, 6). It induces apoptosis through the activation of p38 mitogen-activated protein kinase and c-Jun NH_2-terminal kinase 1/2 (7) resulting in a G(2)/m cell cycle arrest (8), in which the role of oxidative stress and glutathione-*S*-transferase π inhibition was also suggested (9). Most recently, a unique mechanism was found for the anticancer action of protoapigenone and WYC0209: these flavonoids were found to affect the ataxia telangiectasia-mutated and Rad3-related (ATR) signaling pathway both *in vitro* and *in vivo* (10). This pathway plays an essential role in maintaining genomic integrity, and it is considered an attractive target for the development of a new class of anticancer drugs (11). Interestingly, as well as its cancer-related effects, protoapigenone was also found to inhibit the lytic cycle of Epstein-Barr virus (12), a well-known risk factor for several malignant diseases.

After the successful total synthesis of protoapigenone and several of its derivatives (13), a very simple semi-synthetic way to obtain this compound directly from apigenin was also reported by our group (14). This work also resulted in the production of several 1'-*O*-alkyl derivatives of both protoapigenone and WYC0209, revealing new side-chain-dependent structure activity relationships within the protoapigenone derivatives but not within those of the WYC0209. Although a 1'-OCH_3 group markedly reduced the cytotoxic activity, increasing the length of an aliphatic side chain restored, or even increased the activity. This effect did not occur in the presence of a branching or unsaturated side chain (14). As a direct continuation of this work, here we report the synthesis and cytotoxicity evaluation of several new protoflavones towards various human cancer cell lines and two mouse lymphoma cell lines: a parental (susceptible, PAR) cell line and its multidrug resistant (MDR) sub-cell line expressing the human ATP Biding Cassette (ABC) B1 efflux pump, which, as the most prevalent ABC transporter, is among the major reasons for failure of cancer chemotherapy (15).

Materials and Methods

Protoflavones. Compounds **1**-**15** were synthesized from 4'-hydroxyflavones (genkwanin, 4'-hydroxy-6-methylflavone and 4'-hydroxy-6-methoxyflavone) obtained from Indofine Chemical Company Ltd. (Hillsborough, NJ, USA), as reported previously (14). Briefly, the starting material was dissolved in a 9:1 (v/v) mixture of acetonitrile and water or the alcohol to be coupled to C-1' as an *O*-alkyl side chain, at a concentration of 1 mg/ml. After this, two equivalents of [*bis*(trifluoroacetoxy)iodo]benzene (Sigma Aldrich, Munich, Germany) were added to the mixture, the container was sealed and 1-min microwave heating was applied at 300 W in a standard domestic microwave oven. After cooling, the reaction mixture was evaporated, re-dissolved in acetone and purified by rotational planar chromatography on a Chromatothron equipment (Harrison Research, Palo Alto, California, USA) with adequately chosen eluents consisting of *n*-hexane–ethyl acetate–acetone on silica. Solvents and silica were purchased from Merck (Darmstadt, Germany). Protoflavones obtained were re-crystallized from solvent mixtures similar to the eluents. Synthesis, bioactivity on the human cancer cell lines and compound characterization data for compounds **16**-**29,** were reported previously (14).

Structure elucidation carried out by means of electrospray ionization tandem mass spectrometry (ESI-MS/MS) and nuclear magnetic resonance (NMR) spectroscopy. MS and MS/MS spectra were taken on an API 2000 triple-quadrupole tandem mass spectrometer (AB SCIEX, Foster City, CA, USA) in ESI+ mode. NMR spectra were obtained on a Bruker Avance DRX-500 spectrometer in acetone-d_6, methanol-d_4, or chloroform-d_1.

Cell lines. *Human cell lines:* Human liver (HepG2 and Hep3B), lung (A549) and breast (MDA-MB-231) cancer cell lines were obtained from the American Type Culture Collection. All cell lines were propagated in RPMI-1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Mouse lymphoma cell lines. PAR and MDR cell lines were L5178 mouse T-cell lymphoma cells and the L5178 cells transfected with pHa MDR1/A retrovirus, respectively. MDR cell lines were selected by culturing the infected cells with 60 μg/l of colchicine (16). Both cell lines were cultured in McCoy's 5A medium, supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics (penicillin and streptomycin), at 37˚C and in an atmosphere with 5% CO_2.

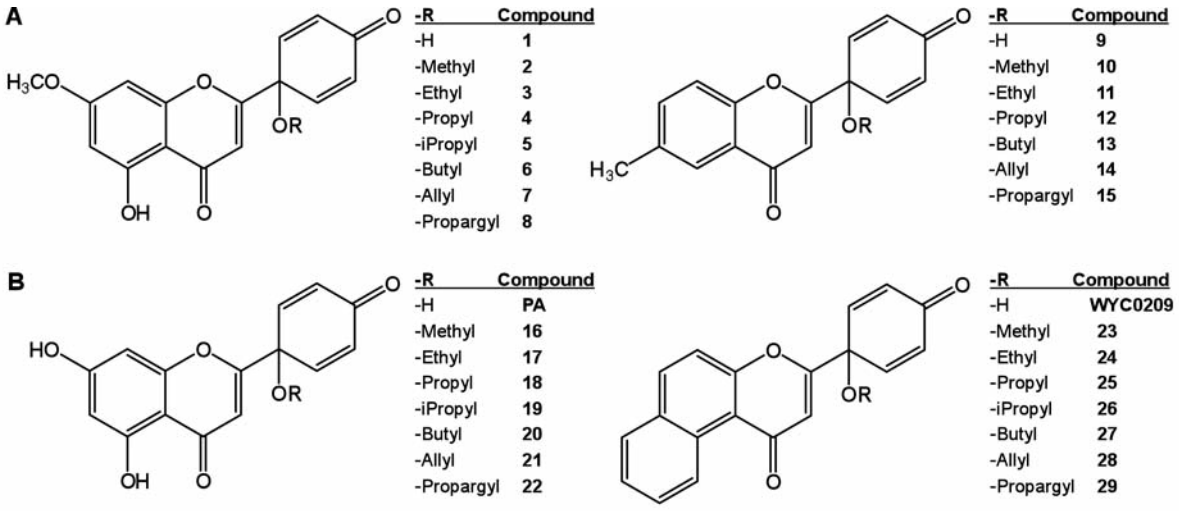

Figure 2. *Structures of the novel protoflavone derivatives 1-15 obtained (A), and those of the analogs of protoapigenone (**16-22**) and WYC0209 (**23-29**) reported previously (14) (B). Substituents at position 1' for the individual compounds are presented next to their corresponding core structures.*

Cytotoxicity assay. Cell viability was measured by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method (17). Human cancer cells were seeded at densities of 5,000 to 10,000 cells/well in 96-well tissue culture plates. On the next day, cells were treated with 10 μl of the test compounds dissolved at different concentrations for another 72 h. After drug treatment, attached cells were incubated with MTT (0.5 mg/ml, for 1 h) and subsequently solubilized in dimethyl sulfoxide (DMSO). The absorption at 550 nm was then measured using a microplate reader.

In contrast to these, the non-adherent PAR and MDR cell lines were used at 20,000 cells/well without being seeded but treated immediately. The cells were incubated for 24 h, after which MTT was added to a final concentration of 10% per well. After 4 h of incubation, sodium dodecyl sulfate (SDS) 10% was also added to a final concentration of 5% per well and cells were incubated overnight. Absorption was read at 540 and 630 nm using an ELISA reader (Multiskan EX, Lab Systems, USA).

Each assay was performed in triplicate. In both protocols, the IC_{50} was recorded as the concentration of the agent that reduced cell viability by 50% under the experimental conditions, as determined by applying log (concentration) *vs.* inhibition variable slope nonlinear regression with automatic outlier elimination at Q=1.0%, by using GraphPad Prism 5.0 (GraphPad Software Inc., California, USA), and 95% confidence intervals of the IC_{50} values were obtained from the curve fitting. Statistical evaluation of data obtained for the PAR and MDR cell lines was performed by using one-way ANOVA followed by Bonferroni's multiple comparison test; in those cases where variances were not significantly different, unpaired *t*-tests were also performed in order to test for occasional differences under less strict statistical conditions.

Results and Discussion

With a microwave-assisted oxidative de-aromatization by a common hypervalent iodine reagent, 15 protoflavones were obtained from genkwanin, 4'-hydroxy-6-methylflavone. The structure elucidation of the synthesized compounds was performed according to the following: As a first step, by means of the ESI-MS and MS/MS spectra, the expected molecular mass of each compound was confirmed. After this, analyzing the ^{1}H-NMR spectrum was sufficient to unambiguously prove successful transformation: the characteristic coupling constants of 8.8 Hz of the two doublets present in the aromatic B-ring of the starting material (representing H-2'/H-6' and H-3'/H-5') changed to ~10.0 Hz, suggesting the formation of a symmetric cyclohexadien-1-one, and aliphatic proton signals, and their multiplicity, together with the practically unchanged A-ring signals and H-3 singlet, proved the presence of the expected side-chain at position C-1'; detailed data are presented in the Experimental section. Structures of the obtained compounds, as well as those of the ones previously reported (14), are presented in Figure 2. Compounds **3**-**15** are described here for the first time.

All derivatives were tested for their cytotoxicity towards HepG2, Hep3B, A549 and MDA-MB-231 human cancer cell lines; results are summarized in Table I.

Structure activity relationships previously reported by our group (14) were confirmed by the present experiments. The presence of 1-*O*-alkyl side-chain increases the IC_{50} in the case of derivatives of the less polar 4'-hydroxyflavones. The cytotoxic activities of compounds **10**-**15** were significantly weaker than that of compound **9**. Interestingly, derivatives of genkwanin had a similar behavior, and cytotoxicity of these compounds was generally also not restored or increased when a **3**-**4** carbon aliphatic O-alkyl side-chain was introduced to C-1' [as found for protoapigenone (14)] with one single exception: compound **6** (1'-*O*-butyl ether) was as

Table I. *Inhibition concentration (IC_{50}) values of **1-15** and, in parenthesis, the corresponding 95% confidence intervals for the six cancer cell lines.*

	HepG2	Hep3B	A549	MDA-MB-231
PA	3.07[a] (2.83-3.34)	1.21[a] (1.19-1.24)	11.29[a] (10.07-12.65)	1.35[a] (1.17-1.55)
WYC0209	1.06[a] (1.04-1.08)	0.25[a] (0.20-0.30)	2.19[a] (2.03-2.37)	0.43[a] (0.41-0.45)
1	1.86 (1.85-1.88)	0.64 (0.55-0.73)	1.77 (1.50-2.08)	0.70 (0.64-0.77)
2	7.65 (7.44-7.87)	1.88 (1.55-2.28)	12.09 (8.95-16.34)	2.08 (1.96-2.22)
3	7.27 (7.05-7.50)	1.82 (1.73-1.91)	10.97 (9.24-13.03)	2.09 (1.99-2.20)
4	8.69 (8.05-9.38)	1.83 (1.33-2.52)	10.27 (9.49-11.11)	2.20 (2.05-2.36)
5	>50	>50	>50	>50
6	12.27 (11.70-12.87)	0.65 (0.61-0.68)	11.83 (10.16-13.77)	5.13 (4.52-5.84)
7	7.85 (7.66-8.04)	1.65 (1.15-2.36)	5.04 (3.48-7.31)	1.87 (1.74-2.01)
8	n. d.	n. d.	n. d.	n. d.
9	2.02 (1.87-2.18)	0.54 (0.53-0.54)	2.00 (1.82-2.19)	0.61 (0.57-0.65)
10	9.79 (9.23-10.39)	3.18 (2.64-3.83)	15.47 (13.15-18.20)	5.46 (5.12-5.82)
11	8.61 (8.46-8.77)	2.17 (1.78-2.66)	7.35 (6.86-7.88)	3.20 (3.03-3.39)
12	14.10 (13.08-15.19)	3.75 (3.12-4.50)	13.45 (11.58-15.61)	5.13 (4.93-5.35)
13	7.47 (7.28-7.65)	3.69 (2.90-4.70)	5.13 (4.92-5.35)	4.85 (4.56-5.16)
14	11.09 (10.47-11.75)	2.41 (1.54-3.78)	14.57 (12.54-16.92)	4.37 (4.22-4.52)
15	9.13 (8.88-9.39)	2.03 (1.50-2.75)	15.30 (14.53-16.12)	4.86 (4.74-4.99)
D	0.79 (0.41-1.55)	1.16 (0.91-1.47)	2.19 (1.76-2.73)	2.60 (2.05-3.30)

[a]Previously published data (14). PA: Protoapigenone; D: doxorubicin, n.d.: not determined; n=3.

potent as compound **1** in the case of the Hep3B cell line. The overall tendency, however, suggests the importance of a free 7-OH group in order to have stronger activity when a longer aliphatic side-chain is present at C-1'.

Compounds (**1-15**), as well as protoapigenone, WYC0209 and their corresponding analogs (**16-29**) were also tested for their cytotoxic activity towards MDR mouse lymphoma cell line expressing the human ABCB1 pump, and against its parental cell line; results are shown in Table II.

The fact that none of the tested compounds exerted weaker activity on the MDR cell line than on the PAR cell line suggests that cancer cells cannot exhibit resistance to protoflavone derivatives by overexpressing the ABCB1 efflux pump. Moreover, for derivatives of 6-methylflavone, selective killing of the ABCB1 overexpressing MDR cancer cells takes place with a rather small (~1.5 fold), yet statistically significant, selectivity. Some selectivity against MDR cell lines overexpressing energy-consuming efflux pump systems could be due to a generally higher susceptibility of these cells whenever they encounter cytotoxic agents that are not ligands of the pumps. However, as from all tested compounds only the 6-methylflavone derivatives were found to be selective towards the MDR cell line, more specific reasons are to be suspected, in which presence of a 6-methyl group seems to be important. Interestingly, such a functional group (although connected to a non-aromatic A-ring) is also present in the unique flavone desmosdumotin B, which was found to exert selective cytotoxic activity towards the ABCB1 expressing MDR cell line KB-VIN, with an IC_{50} value of 2.0 μg/ml in contrast to the >40 μg/ml found towards the parental cell line (19).

In view of our results, 6-methylated protoflavone derivatives may serve as valuable leads toward the development of a potent and selective agent against MDR cancer cells overexpressing the ABCB1 transporter; design and synthesis of further analogs with the aim of exploring relevant structure activity relationships is currently in progress.

Experimental

Chemical characterization data for compounds **1-15** are presented below.

5-Hydroxy-2-(1-hydroxy-4-oxocyclohexa-2,5-dienyl)-7-methoxy-4*H*-chromen-4-one (**1**)

Yield: 57%; ^{1}H NMR (500 MHz, acetone-d_6) δ 12.6 (^{1}H, s, OH-8), 7.03 (2H, d, *J*=10 Hz, H-2' and H-6'), 6.62 (1H, s, H-3), 6.45 (1H, s, *J*=2.1 Hz, H-8), 6.34 (2H, d, *J*=10.0 Hz, H-3' and H-5'), 6.31 (d, *J*=1.85 Hz, H-6), 3.87 (3H, s, OMe-7); ESI-MS (*m/z*): 301 (M^++H); ESI-MS-MS (*m/z*, %): 83.3 (14), 223.3 (27), 256.0 (7), 284.1 (14), 301.2 (100).

5-Hydroxy-7-methoxy-2-(1-methoxy-4-oxocyclohexa-2,5-dienyl)-4*H*-chromen-4-one (**2**)

Yield: 62%; ^{1}H NMR (500 MHz, chloroform-d_1) δ 12.43 (1H, s, OH-8), 6.77 (2H, d, *J*=10.0 Hz, H-2' and H-6'), 6.61 (1H, s, 1H, s, H-3), 6.56 (2H, d, *J*=10.0 Hz, H-3' and H-5'), 6.32 (1H, d, *J*=1.8 Hz, H-8), 6.25 (1H, d, *J*=1.8 Hz, H-6), 3.82 (3H, s, OMe-7), 3.39 (3H, s, OMe"); ESI-MS (*m/z*): 315 (M^++H); ESI-MS-MS (*m/z*, %): 243.3 (2), 256.3 (16), 284.2 (100), 315.2 (47).

2-(1-Ethoxy-4-oxocyclohexa-2,5-dienyl)-5-hydroxy-7-methoxy-4*H*-chromen-4-one (**3**)

Yield: 59.2%; ^{1}H NMR (500 MHz, chloroform-d_1) δ12.53 (1H, s, OH-8), 6.79 (2H, d, *J*=10.1 Hz, H-2' and H-6'), 6.67 (1H, s, H-3), 6.52 (2H, d, *J*=10.1 Hz, H-3' and H-5'), 6.32 (1H, d, *J*=2.1 Hz, H-8), 6.23 (1H, d, *J*=2.1 Hz, H-6), 3.81 (3H, s, OMe), 3.57 (2H, q, *J*=6.9 Hz, H-1"), 1.27 (3H, t, *J*=6.5 Hz, H-2"); ESI-MS (*m/z*, %): 329.1 (M^++H); ESI-MS-

Table II. *Cytotoxic activity of protoapigenone, WYC0209 and compounds 1-29 towards the multidrug resistant cell line (MDR) and its parental cell line (PAR).*

	IC_{50} (μM)				IC_{50} (μM)		
Compound	PAR	MDR	Selectivity[a]	Compound	PAR	MDR	Selectivity[a]
1	1.72 (1.57-1.90)	1.51 (1.28-1.78)	n.s.	PA	0.76 (0.66-0.88)	0.79 (0.71-0.88)	n.s.
2	0.55 (0.45-0.67)	0.57 (0.50-0.65)	n.s.	**16**	3.24 (2.71-3.87)	2.50 (2.04-3.08)	n.s.
3	1.36 (1.20-1.54)	1.41 (1.21-1.64)	n.s.	**17**	3.72 (3.11-4.46)	3.43 (2.83-4.16)	n.s.
4	1.47 (1.26-1.73)	1.63 (1.41-1.87)	n.s.	**18**	4.66 (3.50-6.21)	3.69 (2.57-5.31)	n.s.
5	n.d.	n.d.	n.d.	**19**	7.07 (4.89-10.23)	8.16 (3.41-19.55)	n.s.
6	3.95 (3.17-4.93)	3.98 (3.58-4.42)	n.s.	**20**	1.29 (1.10-1.53)	1.11 (0.92-1.33)	n.s.
7	1.17 (1.00-1.36)	1.00 (0.87-1.15)	n.s.	**21**	1.27 (1.08-1.49)	1.20 (1.09-1.33)	n.s.
8	1.18 (0.96-1.45)	0.97 (0.83-1.12)	n.s.	**22**	1.96 (1.64-2.35)	1.91 (1.62-2.26)	n.s.
9	0.26 (0.23-0.29)	0.16*$^{(T)}$ (0.15-0.18)	1.63	**WYC0209**	0.15 (0.14-0.16)	0.13 (0.11-0.14)	n.s.
10	1.85 (1.64-2.07)	1.35** (1.15-1.59)	1.37	**23**	0.95 (0.81-1.13)	0.82 (0.70-0.95)	n.s.
11	2.38 (2.08-2.73)	1.75** (1.50-2.05)	1.36	**24**	1.46 (1.22-1.75)	1.34 (1.16-1.55)	n.s.
12	4.61 (3.83-5.56)	2.94*** (2.46-3.53)	1.57	**25**	1.71 (1.53-1.92)	1.79 (1.60-2.01)	n.s.
13	3.71 (3.33-4.14)	2.71*** (2.29-3.21)	1.37	**26**	~6.79	8.49 (4.66-15.46)	n.s.
14	2.27 (1.94-2.66)	1.73** (1.50-1.98)	1.31	**27**	1.44 (1.25-1.66)	1.26 (1.12-1.43)	n.s.
15	1.45 (1.29-1.63)	0.98* (0.88-1.08)	1.48	**28**	0.82 (0.70-0.96)	0.74 (0.63-0.87)	n.s.
D	0.61 (0.51-0.74)	8.60*** (7.80-9.47)	0.071	**29**	0.76 (0.67-0.85)	0.81 (0.65-1.01)	n.s.

[a]Selectivity was calculated for those compounds which showed significant differences in the IC_{50} values towards the two cells lines. 95% Confidence intervals are presented for each curve fitting result, ~: ambiguous fitting, no confidence interval available. Selectivity=$IC_{50}^{(PAR)}/IC_{50}^{(MDR)}$. Statistical comparisons were made between values in columns PAR and MDR. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ by one-way ANOVA followed by Bonferroni's multiple comparison test; *$^{(T)}$: $p<0.05$ by unpaired *t*-test, variances are not significantly different; D: doxorubicin, n.d.: not determined, n.s.: no selectivity, n=3.

MS (*m/z*, %): 54.1 (3), 86.3 (4), 243.2 (4), 256.2 (14), 284 (100), 301.1 (4), 329.3 (52).

5-Hydroxy-7-methoxy-2-(4-oxo-1-propoxycyclohexa-2,5-dienyl)-4*H*-chromen-4-one (**4**)

Yield: 68.8%; ^{1}H NMR (500 MHz, chloroform-d_1) δ 12.41 (1H, s, OH-8) 6.76 (2H, d, *J*=10.0 Hz, H-2' and H-6'), 6.66 (1H, s, H-3), 6.52 (2H, d, *J*=10.0 Hz, H-3' and H-5'), 6.32 (1H, d, *J*=1.6 Hz, H-8), 6.23 (1H, d, *J*=1.4 Hz, H-6), 3.81 (3H,s, OMe), 3.45 (2H, t, *J*=6.4 Hz, H-1"), 1.65 (3H, m, H-2"), 0.96 (3H, t, *J*=7.4 Hz, H-3"); ESI-MS (*m/z*, %): 343.3 (M^++H); ESI-MS-MS (*m/z*, %): 54.4 (6), 86.5(10), 243.2 (5), 257.1 (12), 273.1 (2), 284.3 (100), 301.3 (2), 343.2 (40)

5-Hydroxy-2-(1-isopropoxy-4-oxocyclohexa-2,5-dienyl)-7-methoxy-4*H*-chromen-4-one (**5**)

Yield: 49.4%; ^{1}H NMR (500 MHz, chloroform-d_1) δ 12.42 (1H, s, H-8), 6.80 (2H, d, *J*=9.9 Hz, H-2' and H-6'), 6.67 (1H, s, H-3), 6.49 (2H, d, *J*=10.0 Hz, H-3' and H-5'), 6.30 (1H, d, *J*=1.8 Hz, H-8), 6.21 (1H, d, *J*=2.0 Hz, H-6), 3.83 (1H, dd, *J*=12.2, 6.1 Hz, H-1"), 3.80 (3H, s, OMe), 1.21 (6H, d, *J*=6.1 Hz, H-2" and H-3"); ESI-MS (*m/z*): 343.0 (M^++H); ESI-MS-MS (*m/z*, %): 54.3 (6), 86.3(9), 242.5 (4), 256 (13), 272.9 (6), 284.3 (100), 301.3 (8), 343.1 (68).

2-(1-Ethoxy-4-oxocyclohexa-2,5-dienyl)-5-hydroxy-7-methoxy-4*H*-chromen-4-one (**6**)

Yield: 33%; ^{1}H NMR (500 MHz, chloroform-d_1) δ12.44 (1H, s, H-8), 6.77 (2H, d, *J*=10 Hz, H-2' and H-6'), 6.68 (1H, s, H-3), 6.54 (2H, d, *J*=10Hz, H-3' and H-5'), 6.33 (1H, d, *J*=1.85 Hz, H-8), 6.25 (1H, d, *J*=2.0 Hz, H-6). 3.83 (3H, s, OMe), 3.51 (2H, s, H-1"), 1.62 (2H, m , H-2"), 1.43 (2H, m, H-3"), 0.92 (3H, t, *J*=7.4 Hz, H-4"); ESI-MS (*m/z*): 357 (M^++H); ESI-MS-MS (*m/z*, %): 100.5 (1), 243.1 (5), 257.2 (14),273.2 (4), 284.2 (100), 301.1 (8), 329.3 (1), 357.1 (72).

2-(1-(Allyloxy)-4-oxocyclohexa-2,5-dienyl)-5-hydroxy-7-methoxy-4*H*-chromen-4-one (**7**)

Yield: 43.6%; ^{1}H NMR (500 MHz, chloroform-d_1) δ12.39 (1H, s, H-8), 6.79 (2H, d, *J*=10.1 Hz, H-2' and H-6'), 6.67 (1H, s, H-3), 6.53 (2H, d, *J*=10.1 Hz, H-3' and H-5'), 6.31 (1H, d, *J*=2.1 Hz, H-8), 6.23 (1H, d, *J*=1.8 Hz, H-6), 5.91 (1H, m, *J*=5.2 Hz, H-2"), 5.33 (1H, d, *J*=17.2 Hz, H-3", cisz), 5.22 (1H, d, *J*=10.4 Hz, H-3" , trans), 4.04 (1H, m, H-1"), 3.81 (3H, s, OMe); ESI-MS (*m/z*): 341.3 (M^++H); ESI-MS-MS (*m/z*, %): 243.2 (6), 257.2 (16), 272.3 (2), 284.1 (100), 300.1 (2), 341.3 (42).

5-Hydroxy-7-methoxy-2-(4-oxo-1-(prop-2-ynyloxy) cyclohexa-2,5-dienyl)-4*H*-chromen-4-one (**8**)

Yield: 37.2%; ^{1}H NMR (500 MHz, chloroform-d_1) δ 12.38 (1H, s, H-8), 6.85 (2H, d, *J*=9.9 Hz, H-2' and H-6'), 6.66 (1H, s, H-3), 6.55 (2H, d, *J*=9.9 Hz, H-3' and H-5'), 6.32 (1H, d, *J*=1.8 Hz, H-8), 6.24 (1H, d, *J*=1.8 Hz, H-6), 4.21 (2H, d, *J*=2.1 Hz, H1"), 3.81 (3H, s, OMe), 2.52 (1H, t, *J*=2.4 Hz, H-3"); ESI-MS (*m/z*, %): 339.3 (M^++H,); ESI-MS-MS (*m/z*, %): 49.2 (4), 243.3 (6), 256.2 (18), 271.2 (3), 284.3 (100), 300.3 (2), 339.2 (42).

2-(1-Hydroxy-4-oxocyclohexa-2,5-dienyl)-6-methyl-4*H*-chromen-4-one (**9**)

Yield: 48.2%; ^{1}H NMR (500 MHz, chloroform-d_1) δ 7.93 (1H, s, H-7), 7.47 (1H, d, *J*=8.5 Hz, H-8), 7.27 (1H, d, *J*=8.6 Hz, H-5), 6.94 (2H, d, *J*=9.5 Hz, H-2' and H-6'), 6.84 (1H, s, H-3), 6.41 (2H, d, *J*=9.5 Hz, H-3' and H-5'), 2.43 (3H s, CH3-6); ESI-MS (*m/z*): 269.1 (M^++H,); ESI-MS-MS (*m/z*, %): 83.5 (1), 92.4 (5), 107.6 (6), 120.7 (11), 134 (30), 147 (14), 224.2 (1), 241.1 (16), 252 (16), 269.2 (100).

2-(1-Methoxy-4-oxocyclohexa-2,5-dienyl)-6-methyl-4*H*-chromen-4-one (**10**)

Yield: 59.7%; ^{1}H NMR (500 MHz, methanol-d_4) δ 7.87 (1H, s, H-7), 7.57 (1H, d, *J*=8.5 Hz, H-8), 7.33 (1H, d, *J*=8.6 Hz, H-5), 6.97 (2H, d, *J*=9.9 Hz, H-2' and H-6'), 6.71 (1H, s, H-3), 6.57 (2H, d, *J*=9.9 Hz, H-3' and H-5'), 3.44 (3H s, H-1"), 2.43 (3H s, CH3-6); ESI-MS (*m/z*): 283.2 (M^++H,); ESI-MS-MS (*m/z*, %): 133.8 (1), 146.9 (3), 224.2 (20), 252.2 (100), 283.1 (25).

2-(1-Ethoxy-4-oxocyclohexa-2,5-dienyl)-6-methyl-4*H*-chromen-4-one (**11**)

Yield: 20%; ^{1}H NMR (500 MHz, methanol-d_4)) δ 7.88 (1H, s, H-7), 7.59 (1H, d, *J*=8.6 Hz, H-8), 7.33 (1H, d, *J*=8.6 Hz, H-5), 7.00 (2H, d, *J*=10.1 Hz, H-2' and H-6'), 6.75 (1H, s, H-3), 6.54 (2H, d, *J*=10,0 Hz, H-3' and H-5'), 3.64 (2H q , *J*=6.9 Hz, H-1"), 2.44 (3H s, CH3-6), 1.30 (3H, t, *J*=7.0 Hz, H-2"); ESI-MS (*m/z*): 296. 8 (M^++H,); ESI-MS-MS (*m/z*, %): 224.2 (18), 241 (2), 252.1 (100), 269.2 (3), 297.3 (28).

6-Methyl-2-(4-oxo-1-propoxycyclohexa-2,5-dienyl)-4*H*-chromen-4-one (**12**)

Yield: 36%; ^{1}H NMR (500 MHz, methanol-d_4)) δ 7.89 (1H, s, H-7), 7.59 (1H, d, *J*=8.5 Hz, H-8), 7.34 (1H, d, *J*=8.6 Hz, H-5), 7.00 (2H, d, *J*=10.1 Hz, H-2' and H-6'), 6.75 (1H, s, H-3), 6.54 (2H, d, *J*=10,0 Hz, H-3' and H-5'), 3.54 (2H q , *J*=6.3 Hz, H-1"), 2.45 (3H s, CH3-6), 1.75 (sextet, *J*=6.8 Hz H-2"), 1.01 (3H, t, *J*=7.3 Hz, H-3"); ESI-MS (*m/z*): 311. 3 (M^++H,); ESI-MS-MS (*m/z*, %): 54.4 (12), 86.6 (11), 224.1 (20), 241.1 (7), 252.3 (100), 268.8 (3), 311 (28).

2-(1-Butoxy-4-oxocyclohexa-2,5-dienyl)-6-methyl-4*H*-chromen-4-one (**13**)

Yield: 35%; ^{1}H NMR (500 MHz, methanol-d_4)) δ 7.89 (1H, s, H-7), 7.59 (1H, d, *J*=8.5 Hz, H-8), 7.34 (1H, d, *J*=8.6 Hz, H-5), 6.99 (2H, d, *J*=10.0 Hz, H-2' and H-6'), 6.75 (1H, s, H-3), 6.55 (2H, d, *J*=10,0 Hz, H-3' and H-5'), 3.59 (2H, t , *J*=6.2 Hz, H-1"), 2.45 (3H s, CH3-6), 1.66 (2H, m , H-2"), 1.48 (2H, m, H-3"), 0.97 (3H, t, *J*=7.4 Hz, H-4"); ESI-MS (*m/z*): 325. 3 (M^++H,); ESI-MS-MS (*m/z*, %): 225.2 (18), 241.2 (7), 252.2 (100), 269 (9), 325 (4).

2-(1-(Allyloxy)-4-oxocyclohexa-2,5-dienyl)-6-methyl-4*H*-chromen-4-one (**14**)

Yield: 73%; ^{1}H NMR (500 MHz, methanol-d_4)) δ 7.77 (1H, s, H-7), 7.47 (1H, d, *J*=8.5 Hz, H-8), 7.23 (1H, d, *J*=8.6 Hz, H-5), 6.91 (2H, d, *J*=10.0 Hz, H-2' and H-6'), 6.66 (1H, s, H-3), 6.45 (2H, d, *J*=9,9 Hz, H-3' and H-5'), 5.90 (1H, m, *J*=5.3 Hz, H-2"), 5.27 (1H, d, *J*=17.2 Hz, H-3", cisz), 5.12 (1H, d, *J*=10.4 Hz, H-3", trans) 4.02 (2H, d , *J*=5.2 Hz, H-1"), 2.33 (3H s, CH3-6); ESI-MS (*m/z*): 309.1 (M^++H,); ESI-MS-MS (*m/z*, %): 224.1 (14), 240 (18), 252 (7), 268.2 (19), 281.1 (21), 309.1 (100).

6-Methyl-2-(4-oxo-1-(prop-2-ynyloxy)cyclohexa-2,5-dienyl)-4*H*-chromen-4-one (**15**)

Yield: 63%; ^{1}H NMR (500 MHz, methanol-d_4)) δ 7.77 (1H, s, H-7), 7.48 (1H, d, *J*=8.4 Hz, H-8), 7.23 (1H, d, *J*=8.6 Hz, H-5), 6.95 (2H, d, *J*=10.1 Hz, H-2' and H-6'), 6.62 (1H, s, H-3), 6.46 (2H, d, *J*=10,1 Hz, H-3' and H-5', 4.21 (2H, d, *J*=2.2 Hz, H-1"), 2.89 (t, *J*=2.3 Hz, 2H, H-3"), 2.34 (3H s, CH3-6); ESI-MS (*m/z*): 306.9 (M^++H,); ESI-MS-MS (*m/z*, %): 49.2 (1), 224.2 (23), 252.3 (100), 268 (2), 279 (1), 307.2 (37).

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