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Synthesis and cytotoxic activity of new 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones on MCF-7 cells

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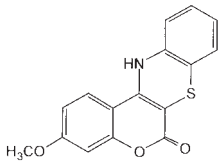
A series of eight halogenated 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones have been synthesized, characterized and their stereochemistry determined. In a second stage of our work, the reported molecules were tested for their antiproliferative activity on MCF-7 breast carcinoma cells. Pharmacological results were compared with those of diethylstilbestrol (DES), an estrogen, as well as ICI 182,780, a pure antiestrogen. Then, these derivatives were evaluated for their capacity to activate the transcription of a reporter gene and for their affinity for human recombinant estrogen receptors α (hERα). These results were compared with those of coumestrol, a phytoestrogen structurally close to 2,4-diaryl-4*H*,5*H*-pyrano-[3,2-*c*]benzopyran-5-ones, and with RU 58668, a pure antiestrogen. Although these derivatives exhibit a significant antiproliferative activity higher than that of ICI 182,780, neither of them displayed a significant estrogenicity or an affinity for hERα. Such results may suggest that their antiproliferative activity is not dependent of an antiestrogenic response.

1. Introduction

During these last 30 years, a great number of heterocyclic coumarins have been synthesized and their biological activities were extensively evaluated. Indeed, heterocyclic coumarins display some interesting pharmacological activities, particularly as anticoagulants [1], photosensibilizing drugs [2], estrogenic derivatives [3–5] or as antiproliferative compounds [6–9]. For this last activity, we can hold up the case of psoralidin as an example, a coumestan isolated from the seeds of *Psoralea corylifolia* and active against stomach cancer cell lines [10].

Moreover, as part of our work on coumarins with pharmacological activities on MCF-7 breast carcinoma cells, we synthesized some new 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]-benzopyran-5-ones, structurally closed to 6,12-dihydro-3-methoxy-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-one, a new synthetic xenoestrogen of which we have discovered a promising pharmacological profile [5].



6,12-Dihydro-3-methoxy-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-one

The ability of the new diarylpyranocoumarins to inhibit the proliferation of MCF-7 cells was extensively studied compared to ICI 182,780. The ability of these new derivatives to activate the expression of a gene under the control of ERs as well as their binding affinity for hERα were compared to that of coumestrol and RU 58668. The new molecules reported in the present work clearly show an ER-independent antiproliferative activity. Nevertheless, it is interesting to underline the activity of this group of molecules twice more important than that of ICI 182,780 making these new 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones potential candidates for antiproliferative drug development.

2. Investigations, results and discussion

2.1. Chemistry

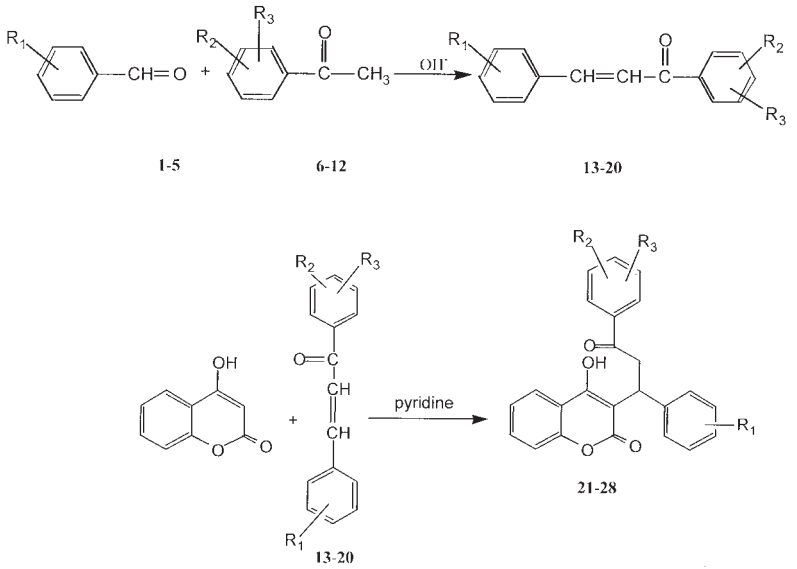
Substituted 4-hydroxy-3-(1,3-diaryl-3-oxopropyl)coumarins **21**–**28** were easily synthesized in satisfactory yields as racemic mixtures by an 1,4-Michael addition in refluxing pyridine [11, 12]. Compounds **21**–**28** were obtained from starting material 4-hydroxycoumarin on one equivalent of the appropriate chalcone **13**–**20**. The chalcones used in the present work were obtained from commercially available aldehydes **1**–**5** and acetophenones **6**–**12** by applying the previously reported and validated Claisen-Schmidt reaction [13, 14] (Scheme 1).

The third stage of this synthesis consisted of an heterocyclization of the substituted 4-hydroxy-3-(1,3-diaryl-3-oxopropyl)coumarins **21**–**28** (Table 1) by $POCl_3$ into the desired 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones **29**–**36** (Table 2) as shown in Scheme 2.

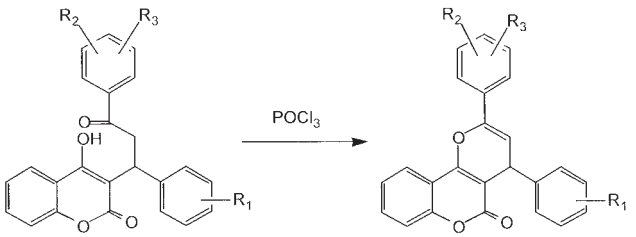
In this third step, $POCl_3$ presents the advantage to be both the cyclizing agent and the dehydrating agent. For this reason, $POCl_3$ represents an interesting alternative compared to the alcoholic/hydrochloric acid procedure [15] or the perchloric acid method [16].

The structure of each compound was precisely determined by FTIR and NMR experiments. The ^1H NMR spectrum of the 4-hydroxy-3-(1,3-diaryl-3-oxopropyl)coumarins **21**–**28** showed diastereotopic protons for the CH_2 group of the 1,3-diaryl-3-oxopropyl substituent. Protons attribution for the CH_2 group was carried out using coupling values of the ABX system and the Karplus relation. Compared to H_x (or H_1 from 4.90 ppm to 5.20 ppm) ^1H NMR data show a marked shilding (from 3.40 ppm to 3.85 ppm) characterizing the proton in *cis* position (H_a or $H_{2\,(cis)}$) when the marked deshilding (between 4.15 and 4.55 ppm) was attributed to the proton in *trans* position (H_b or $H_{2\,(trans)}$) as shown in Table 2. We can note that our investigations concerning the stereochemistry of the intermediates **21**–**28** were very easy. Actually, an intramolecular hydrogen bond between the carbonyl of the diaryloxopropyl substituent at the position 3 and the hydroxyl at the position 4 of the coumarin as shown by NMR experiment, contributes to stabilize the system.

Scheme 1



Scheme 2



We were equally confronted to the determination of the position of the pyranic double bond. The HMBC spectrum clearly showed a correlation between two non aromatic protons associated with the pyran and the carbon 5 of the lacton's carbonyl. Following this finding we came to the conclusion that such correlation can only be implicated by coupling constants ^3J and ^4J in agreement with the presence of a 2–3 double bond.

Physicochemical characteristics and NMR data of compounds **29–36** are resumed in Tables 3 and 4.

2.2. Pharmacology

The biological profile of the synthesized molecules was evaluated by their capacity to modulate the proliferation of MCF-7 breast carcinoma cells. The antiproliferative effect of these compounds was compared to the proliferative activity observed with DES at 10^{-7} M and with ICI 182,780 at 10^{-6} M. The stimulation of MCF-7 cells with compounds **29–36** was performed for 4 days in Dulbecco's Modified Eagle's Medium (DMEM) with 10% of Fetal Bovine Serum (FBS). At the end of this period an estimation of the cell proliferation was performed using tetrazolium salt WST-1. Our pharmacological results clearly show that diarylpyranocoumarins **29–36** produce a significant antiproliferative activity at 1 μM which surpasses the inhibitory effect of ICI 182,780. As expressed, DES produced stimulatory effect. The results are shown in Table 5.

Table 1: Physicochemical characteristics of compounds 21–28

Compd.	R1	R2	R3	M.p (°C)	Yield (%)	Formula (molecular mass)	IR (cm^{-1})
21	H	H	4-F	157	63	$C_{24}H_{17}O_4F$ (388)	3242; 1683, 1695
22	H	H	4-Br	217	35	$C_{24}H_{17}O_4Br$ (449)	3360; 1660, 1685
23	H	3-Cl	4-Cl	226	35	$C_{24}H_{16}O_4Cl_2$ (439)	3211; 1664, 1670
24	H	H	4-SC_6H_5	205	31	$C_{30}H_{22}O_4S$ (478)	3200; 1681, 1683
25	4-Cl	H	H	174	80	$C_{24}H_{17}O_4Cl$ (404.5)	3200; 1683, 1685
26	4-Br	H	H	194	71	$C_{24}H_{17}O_4Br$ (449)	3320; 1675, 1690
27	2-Br	2-F	H	212	56	$C_{24}H_{16}O_4BrF$ (467)	3162; 1689, 1691
28	4-OCH_3	H	4-F	164	40	$C_{25}H_{19}O_5F$ (418)	3235; 1683, 1700

Table 2: ^{1}H NMR data of 4-hydroxy-3-(1,3-diaryl-3-oxopropyl) coumarins 21–28 in DMSO-d_6 (300 MHz)

Compd.	H_1	$H_{2\ (cis)}$	$H_{2\ (trans)}$	Ar–H	OH
21	5.05 (dd) $^3J_{cis} = 2.5$ $^3J_{trans} = 9.1$	3.85 (dd) $^3J_{cis} = 2.5$ $^2J_{gem} = 18.9$	4.55 (dd) $^3J_{trans} = 9.1$ $^2J_{gem} = 18.9$	7.05–8.40 (m)	9.55 (s)
22	5.10 (dd) $^3J_{cis} = 4.7$ $^3J_{trans} = 8.8$	3.85 (dd) $^3J_{cis} = 4.7$ $^2J_{gem} = 18.0$	4.35 (dd) $^3J_{trans} = 8.8$ $^2J_{gem} = 18.0$	7.00–8.25 (m)	11.25 (s)
23	5.05 (dd) $^3J_{cis} = 4,7$ $^3J_{trans} = 8.9$	3.85 (dd) $^3J_{cis} = 4,7$ $^2J_{gem} = 18.4$	4.20 (dd) $^3J_{trans} = 8.9$ $^2J_{gem} = 18.4$	7.15–8.40 (m)	11.70 (s)
24	5.00 (dd) $^3J_{cis} = 2.8$ $^3J_{trans} = 10.0$	3.40 (dd) $^3J_{cis} = 2.8$ $^2J_{gem} = 18.9$	4.40 (dd) $^3J_{trans} = 10.0$ $^2J_{gem} = 18.9$	7.05–7.95 (m)	10.05 (s)
25	4.90 (dd) $^3J_{cis} = 2.3$ $^3J_{trans} = 10.1$	3.75 (dd) $^3J_{cis} = 2.3$ $^2J_{gem} = 19.1$	4.45 (dd) $^3J_{trans} = 10.1$ $^2J_{gem} = 19.1$	7.10–8.15 (m)	11.20 (s)
26	5.10 (dd) $^3J_{cis} = 4.7$ $^3J_{trans} = 8.9$	3.85 (dd) $^3J_{cis} = 4.7$ $^2J_{gem} = 18.1$	4.30 (dd) $^3J_{trans} = 8.9$ $^2J_{gem} = 18.1$	6.95–8.20 (m)	11.55 (s)
27	5.20 (dd) $^3J_{cis} = 6.9$ $^3J_{trans} = 9.1$	3.55 (dd) $^3J_{cis} = 6.9$ $^2J_{gem} = 13.9$	4,55 (dd) $^3J_{trans} = 9.1$ $^2J_{gem} = 13.9$	6.85–8.30 (m)	12.05 (s)
28*	5.05 (dd) $^3J_{cis} = 6.4$ $^3J_{trans} = 8.8$	3.80 (dd) $^3J_{cis} = 6.4$ $^2J_{gem} = 17.6$	4.15 (dd) $^3J_{trans} = 8.8$ $^2J_{gem} = 17.6$	6.70–8.20 (m)	11.60 (s)

* The methoxy group of the compound **28** exhibits as a singlet at 3.70 ppm

Table 3: Physicochemical data of 2,4-diphenyl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones 29–36

Compd.	R_1	R_2	R_3	M.p (°C)	Yield (%)	IR (cm^{-1})
29	H	H	4-F	208	60	1714; 1628
30	H	H	4-Br	210	55	1714; 1629
31	H	3-Cl	4-Cl	232	47	1680; 1616
32	H	H	4-SC_6H_5	250	70	1714; 1625
33	4-Cl	H	H	210	66	1715; 1627
34	4-Br	H	H	184	68	1716; 1624
35	2-Br	2F	H	256	67	1740; 1625
36	4-OCH_3	H	4F	204	52	1732; 1629

Table 4: ^{1}H NMR data of 2,4-diphenyl-4*H*,5*H*-pyrano[3,2-*c*]-benzopyran-5-ones 29–36 in $CDCl_3$ (300 MHz)

Compd.	H_3	H_4	Ar–H
29	5.85 (d) $^3J = 4.9$	4.75 (d) $^3J = 4.9$	7.10–8.15 (m)
30	5.55 (d) $^3J = 5.0$	4.45 (d) $^3J = 5.0$	6.85–7.70 (m)
31	5.9 (d) $^3J = 5.0$	5.7 (d) $^3J = 5.0$	6.15–8.05 (m)
32	5.80 (d) $^3J = 5.0$	4.70 (d) $^3J = 5.0$	7.20–8.05 (m)
33	5.30 (d) $^3J = 4.9$	4.20 (d) $^3J = 4.9$	6.70–7.65 (m)
34	5.50 (d) $^3J = 4.8$	4.35 (d) $^3J = 4.8$	6.80–7.75 (m)
35	6.00 (d) $^3J = 4.6$	5.20 (d) $^3J = 4.6$	7.10–8.00 (m)
36*	5.80 (d) $^3J = 4.9$	4.75 (d) $^3J = 4.9$	6.80–8.00 (m)

* The methoxy group of the compound **36** exhibits as a singlet at 3.85 ppm

To evaluate the eventual implication of ERs in the antiproliferative activity of these new 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones **29**–**36**, the binding affinity measurement for ERs was carried out on ER+ human breast cancer cytosols. Cytosol preparations from pools of ER+ breast cancer cells were incubated overnight at 0–4 °C with 5 nM of [^{3}H]E_2 in the presence or in the absence of increasing amounts of either unlabelled E_2 (control) or in the presence or in the absence of increasing amounts of test compounds (1 nM to 1 μM). Unbound ligand was then removed by a dextran-coated charcoal (DCC) treatment and the residual radioactivity measured by scintillation counting. None of the compounds investigated produced a significant inhibition of the binding of [^{3}H]E_2 to our human breast cancer cytosol preparations (two independent experiments) even when in a very large excess (synthesized compounds at 1 μM versus [^{3}H]E_2 at 5 nM). Moreover, unlabelled E_2 produced its expected inhibition validating our experimentation ($I_{50} \sim 5$ nM).

Hence, this study clearly shows that all these compounds are devoid of a significant binding affinity for ERs as shown in Table 6.

Table 5: Antiproliferative activity of compounds 29–36 on MVLN cells

Compd.	Proliferation (%)
Control	0
DES (10^{-7} M)	100.0
ICI 182,780 (10^{-6} M)	−18.0
29	−25.8
30	−40.5
31	−31.6
32	−41.3
33	−22.0
34	−41.8
35	−26.1
36	−39.2

* Increase/decrease: expressed in % of control value. 100% corresponds to the absorbance of DES stimulated cells at 10^{-7} M (= 1.228 U of absorbance) ($p < 0.01$)

Table 6: Binding affinity of compounds 29–36 for hERα

Compd.	R^1	R^2	R^3	Binding affinity (%)*
E2				6
29	H	H	4-F	109
30	H	H	4-Br	92
31	H	3-Cl	4-Cl	96
32	H	H	4-SC_6H_5	96
33	4-Cl	H	H	117
34	4-Br	H	H	107
35	2-Br	2-F	H	101
36	4-OCH_3	H	4-F	101

* Percent of residual tritiated E2 binding in competition assay ($p < 0.01$)

The last stage of our pharmacological study concerned ERE-regulated luciferase expression to evaluate a possible indirect estrogenic mechanism. We used MVLN cells [17] (tk-vit ERE stably transfected MCF-7 cells) cultured during 3 to 4 days in 35 mm ∅ Falcon dishes (plating density 80.000 cells/dish) in 10% serum depleted of endogenous steroid (DCC treatment). Investigated compounds were subsequently added to the medium and the culture pursued for 4 days. For that purpose, medium was removed and cells were washed twice with a saline phosphate buffer solution (PBS). Then, a minimal volume (250 µl) of a 5-fold diluted lysis solution (Promega E153A) was added to the dishes and maintained under mild agitation for 20 min to extract luciferase. Lysed cells were subsequently detached with a scraper (Costar 3010) and centrifuged for 5 s at 12,000 g to clarify the extracts. Finally, 20 µl of these extracts were mixed at room temperature with 100 µl of a luciferase reactant medium (Promega E151A/E152A) prepared according to the manufacturer's protocol. Induced light was measured with a Berthold luminometer (Lumat LB 9507) and luciferase induction expressed in arbitrary units with regard to the light measured with a blank (Relative Light Unit, RLU). To compare RLU data protein content of each extract, induction was measured by the Coomassie method (PIERCE) and data expressed per mg of protein. The lack of significant binding affinity of compounds **29–36** for ERs was reflected upon a very weak tendency to induce luciferase (2 independent experiments). At 1 µM, each compound increases luciferase at a level less than 30% of the control activity (Table 7). This behaviour contrasts with that of coumestrol for which an estrogenicity activity was significant at 0.1 nM (Table 8). Note that 0.1 µM E_2 always increases the luciferase activity by a factor near or above 2, while the pure antiestrogen RU 58668 [18, 19] at 0.1 µM produces a clear inhibition. Hence, the lack of decrease of luciferase activity at 1 µM of each compound investigated suggests an ER-independent cytotoxic pharmacological mechanism.

Table 7: Estrogenic activity of compounds 29–36 on MVLN cells

Compd.	R^1	R^2	R^3	Luciferase Induction (%)*
29	H	H	4-F	123–117
30	H	H	4-Br	129–131
31	H	3-Cl	4-Cl	117–114
32	H	H	4-SC_6H_5	129–131
33	4-Cl	H	H	111–108
34	4-Br	H	H	110–108
35	2-Br	2-F	H	117–142
36	4-OCH_3	H	4-F	104–114

* Control = 100%. Two independent experiments ($p < 0.01$)

Table 8: Estrogenic activity of coumestrol on MVLN cells

	Induction of luciferase by coumestrol in MVLN cells (2 independent experiments) (% of control activity)*	
E_2 (0.1 nM)	243	215
RU 58668 (0.1 nM)	48	41
Coumestrol		
0.1 nM	115	118
1 nM	123	127
10 nM	137	136
0.1 µM	217	219
1 µM	287	267

* RLU/mg prot, 100% corresponds to 3,130,952 (#1) and 3,811,506 (#2) ($p < 0.01$)

In conclusion, a good yielding synthesis of 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*] benzopyran-5-ones (**29–36**) was developed providing access to the desired compounds in a racemic mixture utilizing a three-step route. The insaturation of the pyran ring was precisely localized by 2D-NMR experiments. These 2,4-diaryl-4*H*,5*H*-pyrano-[3,2-*c*]benzopyran-5-ones **29–36** were screened on MCF-7 breast carcinoma cells. The results reported in Table 5 show that these compounds have a strong cytotoxic activity twice higher than that of ICI 182,780 at the same concentrations (10^{-6} M). Because of such an interesting activity some other pharmacological investigations were oriented to precise the possible ERs mediated pharmacological mechanism. Binding studies and ERE-dependent transcriptional activities rejected this possibility. Moreover, some arguments led us to justify the lack of affinity for ERs. Actually, 2,4-diaryl-4*H*,5*H*-pyrano[3,2-*c*]benzopyran-5-ones (**29–36**) mainly differ from coumestrol not only by the lack of phenolic hydroxyls but also by the presence of two aromatic rings at positions 2 and 4, critical for ER binding. In addition, since the compounds **29–36** are relatively planar, the steric effects could disrupt the plasticity of the LBD into directions not allowed by the ER. Because of the interesting antiproliferative activity of compounds **29–36** at 10^{-6} M, further studies should clarity their mechanism of action. Nevertheless, we believe that such derivatives could be candidates for *in vivo* testing and open the door to promising pharmacomodulation.

3. Experimental

Mp's were determined with a Kofler Heizbank Reichert 18.43.21 and are uncorrected. IR spectra were run on a Shimadzu FTIR-8201PC spectrometer (KBr pellets, ν in cm^{-1}). NMR experiments were recorded on a 300 MHz Bruker AC 300 spectrometer. The chemical shift values were reported in parts per million (ppm, δ units) and spin-spin coupling *J* were exposed in Hz. Thin-layer chromatography (TLC) was carried out on an Alugram Sil G/UV_{254} plate with appropriate solvents. Microanalysis were carried out by the Service Central d'Analyses, Centre National de la Recherche Scientifique, Vernaison (France). All the results were in an acceptable range.

3.1. 4-Hydroxy-3-(1,3-diaryl-3-oxopropyl)coumarins **21**–**28**

4-Hydroxycoumarin (0.01 mol) and 0.01 mol of substituted chalcone were added to pyridine (5 times in weight of 4-hydroxycoumarin) and refluxed during 3 h. Once cooled at room temperature, the mixture was rapidly added to 100 ml of cold water and vigorously stirred. Concentrated hydrochloric acid was then added drop by drop to pH = 1 under stirring. The liquid phase was eliminated and the residual gum was recrystallised from EtOH as a pure powder corresponding to the expected product.

3.2. 2,4-Diaryl-4H,5H-pyrano3,2-cbenzopyran-5-ones **29**–**36**

4-Hydroxy-3-(1,3-diaryl-3-oxopropyl)coumarins **21**–**28** (0.01 mol) were added to 20 ml of $POCl_3$ and the mixture was refluxed during 30 min. Once cooled at room temperature, the mixture under stirring was carefully hydrolyzed in an ice bath. The mixture was then cooled to 20 °C and the product was extracted with 20 ml of CH_2Cl_2. The organic phase was dried over Na_2SO_4 and CH_2Cl_2 was eliminated under reduced pressure. The product obtained was a pure and amorphous powder.

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