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Original article

Homopterocarpanes as bridged triarylethylene analogues: synthesis and antagonistic effects in human MCF-7 breast cancer cells

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Abstract

A series of new compounds structurally derived from 6a,12a-dihydro-6H,7H-[1]-benzopyran-[4,3-b]-benzopyran (homopterocarpane) was efficiently synthesized by reduction of the corresponding pyrilium salts obtained by treatment of selected flavanones and aldehydes with anhydrous $HClO_4$. Cytotoxic effects on the human breast cancer cell line MCF-7 and antiestrogenic activity (only for compounds which resulted more active than tamoxifen (TAM)) on MCF-7 cells stimulated by 17β-estradiol were evaluated. In vivo antiestrogenic activity and the relative binding affinity were also assessed. Some of the new compounds (**4c**, **4h**, **4i** and **4l**) showed a biological activity in the micromolar range, and were more potent than TAM taken as the reference.

Keywords: Homopterocarpanes; Synthesis; MCF-7 breast cancer cells

1. Introduction

Antiestrogens are a structurally varied group of compounds designed with the aim of contrasting the actions of endogenous estrogens and mainly used in the treatment of breast cancer [1]. The antitumor effect is a consequence of the antagonist action of these compounds at the estrogen receptor (ER), for which they need to show a high affinity. The primary agonist at the ER is the steroid hormone 17β-estradiol **1** (Fig. 1), but many nonsteroidal derivatives are known to bind to the same receptor and exert either an agonist or an antagonist action. Cumestrol **2** is a natural product showing agonist properties [2], while tamoxifen (TAM), a representative triarylethylene antiestrogen [3], is the most used nonsteroidal antiestrogen drug for chemotherapy and chemoprevention of breast cancer [4]. The antiproliferative effects of TAM in estrogen-dependent breast cancer cells are mediated by high-affinity binding to ER [5]. However, TAM inhibits also the growth of ER-negative breast cancer cells and other cell types that lack ER [6–8]. Actually, TAM has been reported to have several physiological effects that are ER independent, including sensitization of resistant tumor cells to many chemotherapeutic agents [9], and several pleiotropic effects both in vivo and in vitro [10].

For a long time, we have been interested in the study of homopterocarpane (6a,12a-dihydro-6H,7H- [1]-benzopyran-[4,3-b]-benzopyran) **3** (Fig. 2) as a carrier in medicinal chemistry [11–13]. Considering the current search for innovative antiestrogen compounds and the efforts devoted to the elucidation of the spatial characteristics of ER antagonists [14–19], we thought it of interest to explore the possibility of properly modifying the structure of **3** to obtain ER ligands.

We selected **4** (Fig. 2) as the parent molecule due to its close resemblance with a series of known compounds of general formula **5** [18], selective ER modulators, which fully antagonize the effects of estrogen on uterine and mammary tissue. Moreover, we decided to probe the effect of substitu-

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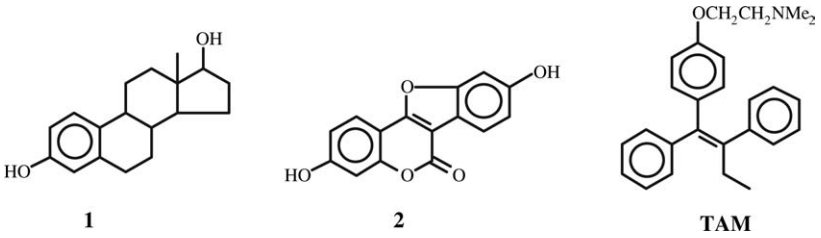


Fig. 1.

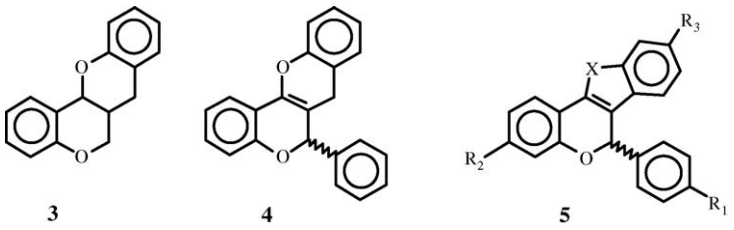


Fig. 2.

ents in the para position of the three phenyl rings, by replacing the H atom with two of the groups most often encountered in TAM derivatives [3], i.e., the methoxy- and the ω-aminoalkoxy-group. Our early attempts to introduce hydroxy groups in the same position failed due to the chemical instability of the resulting compounds (except for **4i**).

The aim of this paper is to describe the synthesis of a series of new compounds related to the general formula **4** (**4a**–**w**, **6**, **7**, **8a**,**b**) listed in Tables 1 and 2, and to report on both their activity on a mammary tumor cell line (MCF-7) and their antiestrogenic activity (Tables 1,2).

2. Chemistry

Compound **4a** was synthesized as described by Andrieux et al. [20].

Compounds **4b**–**w** were prepared as shown in Scheme 1. The selected flavanone and aldehyde were treated with anhydrous $HClO_4$ to yield the pyrilium salts **9b**–**w**. Treatment of these salts with HCOOH in pyridine afforded the desired derivatives **4b**–**w**, which could also be obtained via reduction with $NaBH_4$ in THF (**4i**).

Since compounds **4f** and **4h** were obtained only in low yields, an alternative route was devised: 7,4′-dimethoxyflavanone [21] was condensed with the selected 2-benzyloxybenzaldehyde in EtOH by bubbling HCl (gas) at 0 °C. The benzylidene derivatives (**10f**,**h**) were debenzylated with H_2 over $Pd/CaCO_3$ (**11f**,**h**) and ring closure with P_2O_5 in benzene gave compounds **4f** and **4h** (Scheme 1).

The pyrilium salt **9c** was oxidized with Et_3N in DMSO to afford **7**, while **4c** was hydrogenated over Pd/C to give **6**.

Compounds **8a**,**b** were prepared from 7-methoxy-2,2-dimethylchromanone [22] and 2-hydroxy-5-methoxybenzaldehyde or salicylaldehyde following the procedure outlined in Scheme 1 (steps i,ii) for **9a**–**w** and **4a**–**w**.

The synthesis of 4′-pyrrolidinoethoxy-7-methoxy flavanone was accomplished as shown in Scheme 2. The 4-hydroxybenzaldehyde and 2-bromo-1-chloroethane were refluxed in acetone in the presence of K_2CO_3, and the resulting chloroethoxy derivative was treated with an excess of pyrrolidine. The obtained pyrrolidinoethoxybenzaldehyde **12** was treated with 2-hydroxy-4-methoxyacetophenone in the presence of KOH to yield the chalcone **13**, which was cyclized to flavanone **14** by refluxing with 3% HCl in EtOH.

The synthesis of 7-aminoalkoxy-4′-methoxy flavanones was accomplished as shown in Scheme 3. The 2,4-dihydroxyacetophenone and 1-bromo-ω-chloroalkanes were refluxed in acetone in the presence of K_2CO_3, and the resulting ω-chloroalkoxy derivatives were treated with an excess of the selected amine. The obtained aminoalkoxyacetophenones (**15a**–**g**) were treated with *p*-methoxybenzaldehyde in the presence of KOH to yield the chalcones (**16a**–**g**), which were cyclized to flavanones **17a**–**g** by refluxing with 3% HCl in EtOH.

All the chiral compounds were prepared and tested as racemic mixtures.

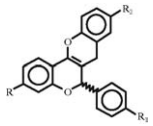
3. Biological assays

Antiproliferation activity of the new derivatives was evaluated on a human breast cancer cell line (MCF-7) using TAM as the reference compound. As the toxicity index, the MTT test was used, which is based on the reduction of tetrazolium salts by mitochondrial enzyme tetrazolium succinate reductase.

The compounds which proved to be more active than TAM (**4c**, **4h**, **4i**, and **4l**) were also tested for their antiestrogenic properties, evaluated by measuring the inhibition of MCF-7 cell proliferation stimulated by 17β-estradiol.

In vivo antiestrogenic activity was assessed by measuring the ability of new selected compounds to block estradiol-stimulated uterine weight gain in immature female Swiss–Webster mice. Moreover, estrogenic activity was also determined by using the method of Magarian et al. [23].

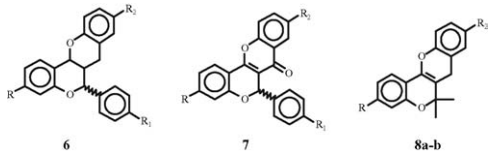
Table 1
Physicochemical and analytical data of the studied compounds (**4a–w**)



Compound	R	R_1	R_2	mp (°C) [a]	Yield (%)	Formula	Analysis
4a	H	H	H	116–118 [b]	80*	$C_{22}H_{16}O_2$	C, H
4b	H	H	OCH_3	165–167	60	$C_{23}H_{18}O_3$	C, H
4c	H	OCH_3	H	95–98	70	$C_{23}H_{18}O_3$	C, H
4d	H	OCH_3	OCH_3	134–138	70	$C_{24}H_{20}O_4$	C, H
4e	OCH_3	H	H	113–115	40	$C_{23}H_{18}O_3$	C, H
4f	OCH_3	OCH_3	H	122–124	10	$C_{24}H_{20}O_4$	C, H
4g	OCH_3	H	OCH_3	122–125	30	$C_{24}H_{20}O_4$	C, H
4h	OCH_3	OCH_3	OCH_3	129–131	30	$C_{25}H_{22}O_5$	C, H
4i	H	OH	H	140–142	50	$C_{22}H_{16}O_3$	C, H
4j	OCH_3	$O(CH_2)_2N$	OCH_3	155–158	10	$C_{30}H_{31}NO_5$	C, H, N
4k	$O(CH_2)_2N$	OCH_3	H	60–63	10	$C_{29}H_{29}NO_4$	C, H, N
4l	$O(CH_2)_2N$	OCH_3	OCH_3	58–60	35	$C_{30}H_{31}NO_5$	C, H, N
4m	$O(CH_2)_3N$	OCH_3	H	53–54	60	$C_{30}H_{31}NO_4$	C, H, N
4n	$O(CH_2)_3N$	OCH_3	OCH_3	75–78	60	$C_{31}H_{33}NO_5$	C, H, N
4o	$O(CH_2)_4N$	OCH_3	H	49–51	60	$C_{31}H_{33}NO_4$	C, H, N
4p	$O(CH_2)_4N$	OCH_3	OCH_3	58–60	60	$C_{32}H_{35}NO_5$	C, H, N
4q	$O(CH_2)_5N$	OCH_3	H	48–50	50	$C_{32}H_{35}NO_4$	C, H, N
4r	$O(CH_2)_5N$	OCH_3	OCH_3	79–81	65	$C_{33}H_{37}NO_5$	C, H, N
4s	$O(CH_2)_2N$O	OCH_3	H	75–81	65	$C_{29}H_{29}NO_5$	C, H, N
4t	$O(CH_2)_2N$O	OCH_3	OCH_3	74–77	35	$C_{30}H_{31}NO_6$	C, H, N
4u	$O(CH_2)_2N$	OCH_3	H	119–120	70	$C_{30}H_{31}NO_4$	C, H, N
4v	$O(CH_2)_2N$N·Ph	OCH_3	H	54–58	72	$C_{35}H_{34}N_2O_4$	C, H, N
4w	$O(CH_2)_2N$N·Ph	OCH_3	OCH_3	155–158	40	$C_{36}H_{36}N_2O_5$	C, H, N

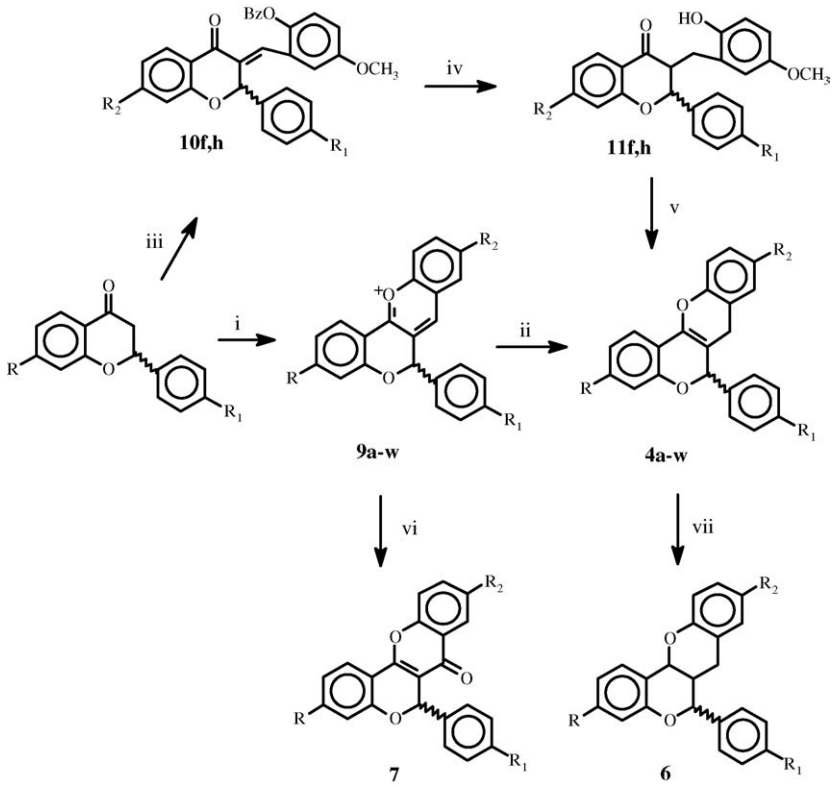
[a] Crystallization solvent: ligroin.
[b] Ref. [20], mp 117 °C.

Table 2
Physicochemical and analytical data of the studied compounds (**6**, **7**, **8a**,**b**)



Compound	R	R_1	R_2	mp (°C) [a]	Yield (%)	Formula	Analysis
6	H	OCH_3	H	143–144	70	$C_{23}H_{20}O_3$	C, H
7	H	OCH_3	H	130–131	20	$C_{23}H_{16}O_4$	C, H
8a	OCH_3	–	H	99–101	70	$C_{19}H_{18}O_3$	C, H
8b	OCH_3	–	OCH_3	96–98	70	$C_{20}H_{20}O_4$	C, H

[a] Crystallization solvent: ligroin.



Scheme 1. Reagents: (i) appropriate substituted benzaldehyde, $HClO_4$, AcOH, 2' reflux; (ii) HCOOH, pyridine, 2' reflux or $NaBH_4$, THF (see Section 5); (iii) selected 2-benzyloxybenzaldehyde, HCl gas, EtOH, 0 °C, 30'; (iv) H_2, $Pd/CaCO_3$, THF; (v) P_2O_5, C_6H_6, **4h**, reflux; (vi) DMSO, $(C_2H_5)_3N$, rt; (vii) H_2, Pd/C, THF.

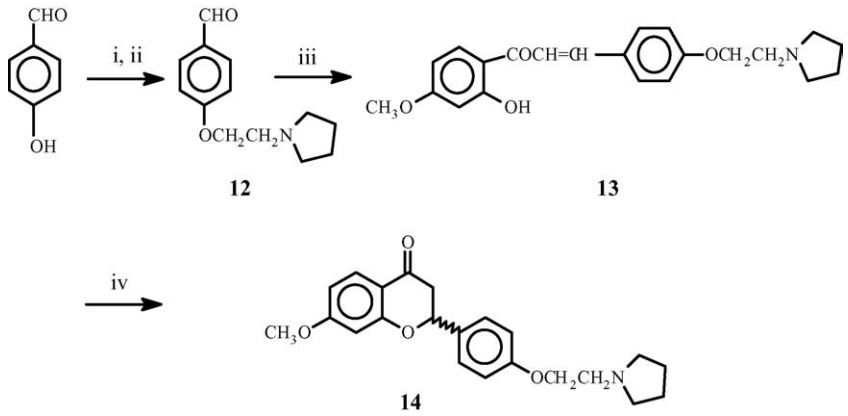
To complete the evaluation of the selected compounds' pharmacological profile, the relative binding affinity to human uterine tissue receptor preparation was assessed as determined by competitive binding assay and displacement of [^{3}H] estradiol.

4. Results and discussion

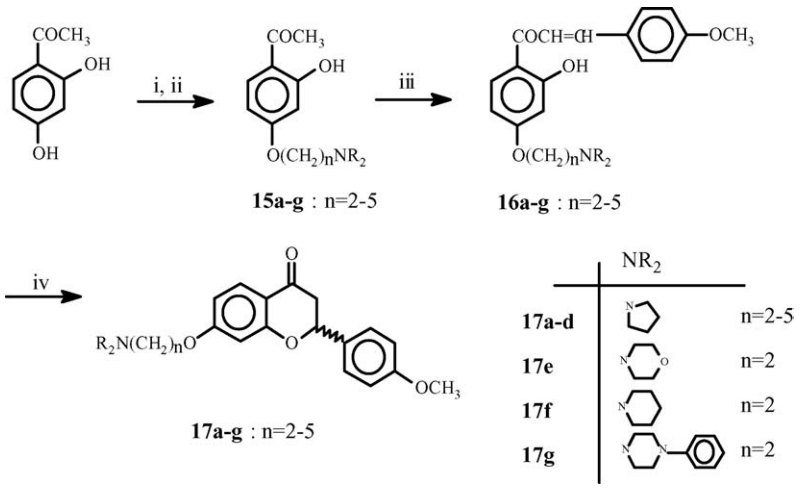
TAM and the novel compounds (**4a–w**, **6**, **7**, **8a**,**b**) were evaluated for their in vitro antiproliferation activity in the MCF-7 human breast cancer cell line, to determine whether the introduction of different functions in the parent compound structure **4a** could improve the biological activity of the derivatives. All the compounds were tested in the absence of estradiol (Table 3) to identify any antiproliferation activity. Then the compounds showing a potency ratio (PR) >1 vs. TAM in antiproliferative assays were tested in combination with 17β-estradiol to determine their in vitro antiestrogenic activity (Table 4).

The IC_{50} value recorded for TAM in this work was in good agreement with IC_{50} values reported in other works utilizing the MTT assay in MCF-7 cells [24].

No antiproliferative effect was observed for compounds **4a**, **4g**, and **4u**, while other derivatives showed a significant antiproliferation activity with IC_{50} varying from 6.4 to 63.6 μM. In particular, compounds **4c**, **4h**, **4i**, and **4l** were



Scheme 2. Reagents: (i) $BrCH_2CH_2Cl$, K_2CO_3, reflux; (ii) pyrrolidine, reflux; (iii) 2-hydroxy-4-methoxyacetophenone, KOH, rt; (iv) 3% HCl, EtOH, reflux.



Scheme 3. Reagents: (i) $Br(CH_2)_nCl$, K_2CO_3, reflux; (ii) selected amine, reflux; (iii) *p*-methoxybenzaldehyde, KOH, rt; (iv) 3% HCl, EtOH, reflux.

more active than TAM, with PR values of 1.36, 1.35, 1.32 and 1.77, respectively (Table 3).

In vitro intrinsic estrogen antagonist activity was determined by measuring the ability of selected compounds to inhibit estrogen-induced proliferation of MCF-7 cells. The results revealed an increase in activity with respect to the non-stimulated cells (antiestrogenic effect). In particular, **4l** exhibited 88.7% inhibition of estradiol-stimulated growth (Table 4) with an increase of 35% with respect to the non-stimulated cells (88.7% vs. 65.7%), followed by **4c**, with an increase of 23% (72% vs. 57.8%), **4h**, with an increase of the 18.3% (80.1 vs. 67.7%), **4i**, with an increase of 16.6% (72.2% vs. 61.9%), and TAM, with an increase of 5.4% (78.4% vs. 74.4%). Consequently, the homopterocarpan derivatives demonstrated better antiestrogenic action in estradiol-stimulated MCF-7 cells than TAM.

Compounds **4c**, **4h**, **4i**, and **4l** showed no uterotrophic activity (estrogenic activity), when tested in the immature mouse at doses of 30, 150 and 750 μg in the absence of estradiol, while TAM elicited a significant estrogenic response at total doses of 150 and 750 μg (data not shown).

The selected compounds **4c**, **4h**, **4i** and **4l** were tested also for in vivo estrogen antagonism at doses of 30, 150 and 750 μg against a stimulating dose of 0.03 μg of estradiol. As expected, TAM had no antiestrogenic activity in the mouse uterus, while all the tested compounds produced a dose-dependent decrease in uterine weight (Table 4). In particular, **4l** elicited the highest antiestrogenic activity (89.2% at 750 μg).

In Table 4, the affinity to human uterine tissue of TAM, **4c**, **4h**, **4i**, and **4l**, and the percent of inhibition in the binding assay are also reported. Only TAM showed detectable affinity with a $K_i = 0.5 \pm 0.003$ μM, whereas the new derivatives were not potent inhibitors of the binding (>10 μM), even if **4i** and **4l** showed a significant inhibitory ability (32–34%).

The biological activity of TAM ranges from full estrogen agonist to partial agonist to full antagonist, depending on the species studied, the target organ assessed, and the end point measured. This range of activity may account for some of the undesirable effects of TAM, such as increased endometrial proliferation, a slightly increased risk of endometrial carcinoma, and tumor flare [25]. The ability to stimulate growth through the ER may be responsible for some cases of TAM resistance [26] that have been demonstrated in preclinical models [27,28]. In this work, we report the synthesis and the biological profile of some homopterocarpan derivatives, to

Table 3
Percent inhibition of MCF-7 cells growth (antiproliferation activity): IC_{50} μM (95% confidence limits), and potency ratio (PR) between TAM and each compound (IC_{50} TAM/IC_{50} compound)

Compound	IC_{50} (μM)	PR
4a	–	–
4b	12.8 (10.96–14.05)	0.87 (0.68–1.12)
4c	8.53 (7.22–9.35)	1.36 (1.08–2.71) *
4d	15.7 (12.97–19.05)	0.71 (0.54–0.93)
4e	13.57 (0.57–32.21)	0.82 (0.034–19.65)
4f	14.18 (12.42–16.19)	0.79 (0.62–0.99)
4g	–	–
4h	8.28 (7.25–9.46)	1.35 (1.06–1.70) *
4i	8.45 (7.57–9.44)	1.32 (1.06–1.65) *
4j	9.90 (8.29–11.83)	1.13 (0.87–1.46)
4k	23.6 (18.3–30.4)	0.47 (0.34–0.60) #
4l	6.36 (4.99–8.10)	1.77 (1.29–2.39) *
4m	26.5 (21.8–32.2)	0.42 (0.32–0.55) #
4n	22.1 (18.1–27.0)	0.55 (0.38–0.66) #
4o	22.2 (18.1–27.2)	0.50 (0.38–0.67) #
4p	23.2 (17.8–30.2)	0.48 (0.35–0.67) #
4q	21.3 (15.7–29.3)	0.52 (0.36–0.76) #
4r	34.1 (23.5–49.6)	0.32 (0.21–0.50) #
4s	29.7 (21.4–41.3)	0.37 (0.25–0.55) #
4t	63.6 (41.7–97.0)	0.17 (0.11–0.28) #
4u	–	–
4v	19.4 (16.3–23.1)	0.57 (0.44–0.75) #
4w	48.5 (32.1–73.1)	0.23 (0.14–0.36) #
6	12.81 (10.31–15.92)	0.87 (0.65–1.16)
7	10.20 (8.98–11.58)	1.09 (0.87–1.38)
8a	46.5 (32.7–66.1)	0.24 (0.16–0.36) #
8b	44.9 (33.4–60.3)	0.24 (0.17–0.35) #
TAM	11.2 (9.24–13.52)	1

* more potent; # less potent

explore the possibility of developing alternative compounds endowed with favorable antitumor properties.

The results shown in Tables 3,4 allow one to draw a general picture of the SAR for this new class of antiestrogens, whose most potent representatives (**4c**, **4h**, **4i**, and **4l**) perform better than the reference TAM in all the biological assays presented, except for the binding to the freshly collected human uterine tissue. Particularly, looking at the data for compounds **4c**, **4h** and **4i**, it appears that the oxyethylamino moiety is not indispensable for the antiestrogenic activity. However, when this side chain is present, best results are obtained when it is in position C3 rather than C6 (**4l** vs. **4j**), and when the amine is pyrrolidine. Also, the number of methylene units in the alkyl chain seems critical, with an optimal length of two C atoms. The presence of oxygenated functions seems important, mostly on the phenyl ring attached to position C6, and in position C9 of the pyrrolidinoethoxy-derivatives (**4l** vs. **4k**). Double bond saturation or introduction of a carbonyl function reminiscent of that carried by cumestrol (**2**) decrease the activity (**6** and **7** vs. **4c**), whereas replacement of the phenyl ring of C6 with methyl groups affords only slightly active compounds (**8a**,**b**).

We present a series of homopterocarpane derivatives that can be considered as analogues of the classical triarylethylene-based antiestrogens. The new compounds were tested in a number of assays to characterize their biological profile, and some of them showed promising characteristics. In particular, compounds **4c**, **4h**, **4i**, and **4l** performed better than TAM as regards the antiproliferative effect.

The antiestrogenic activity shown in vivo and the lack of binding affinity of the homopterocarpanes could be rational-

Table 4
Percent inhibition of MCF-7 cell growth in absence (–E2) and in presence (+E2) of 17β-estradiol (antiestrogenic activity), in vivo antiestrogenic activity, and human uterine cytosolic receptor-binding assay for the selected compounds and TAM

Compound	Antiproliferation activity on MCF-7 cells		Antiuterotrophic activity			Binding inhibition K_i (μM) (% inhibition at 10 μM)
	% inhibition (–E2)	% inhibition (+E2) [a]	Total dose (μg)	Weight of the uterus mg [b]	Reduction [c] (%)	
TAM	74.4	78.4	30	56.28 ± 2.4	1.9	0.50 ± 0.03
			150	55.85 ± 2.9	3.3	
			750	61.40 ± 1.6	–14.3	
4c	58.7	72.0	30	53.60 ± 2.6	10.5	>10 (5)
			150	45.12 ± 3.4	37.4	
			750	36.30 ± 2.7	65.3	
4h	67.7	80.1	30	51.30 ± 2.1	17.7	>10 (4)
			150	42.74 ± 3.2	44.9	
			750	34.88 ± 2.8	71.3	
4i	61.9	72.2	30	50.70 ± 1.8	19.7	>10 (32)
			150	40.00 ± 2.8	53.6	
			750	34.00 ± 2.3	72.7	
4l	65.7	88.7	30	55.60 ± 2.8	4.1	>10 (34)
			150	42.8. ± 3.5	44.8	
			750	28.80 ± 3.0	89.2	

[a] Increase in cell proliferation in the presence of 17β-estradiol only: 32%.
[b] Weight of the uterus of the untreated animals: 25.4 ± 1.7 mg; weight of the uterus of the estradiol-stimulated animals: 56.9 ± 3.2 mg.
[c] $\frac{[(\text{mean estradiol-stimulated}) - (\text{mean control})] - [(\text{mean test compound}) - (\text{mean control})]}{[(\text{mean estradiol-stimulated}) - (\text{mean control})]} \times 100.$

ized by the formation of active metabolites. The elucidation of their structure will be the aim of further investigations.

5. Experimental procedures

5.1. Chemistry

All melting points were determined in open glass capillaries, using a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were recorded in $CDCl_3$ solution on a Varian Gemini 300 spectrometer, with Me_4Si as the internal standard. Mass spectra were recorded on a V.G. 7070 E spectrometer. Silica gel Merck 230–400 mesh was used for purification with flash chromatography. Wherever analyses are only indicated with element symbols, analytical results obtained for those elements were within 0.4% of the theoretical values. Compounds' names were obtained using AUTONOM, PC software for nomenclature in organic chemistry, Beilstein-Institut and Springer.

*3-{1-[2-(Benzyloxy)-5-methoxyphenyl]methylidene}-2-(4-methoxyphenyl)-4-chromanone **(10f)***

A solution of 7,4′-dimethoxyflavanone (1.8 g, 0.0063 mol), 2-benzyloxybenzaldehyde (1.42 g, 0.0067 mol) in anhydrous EtOH (30 ml) was stirred at 0 °C for 30 min, while a stream of HCl was introduced. The reaction mixture was stirred at room temperature for 24 h, poured in H_2O, and extracted with CH_2Cl_2. The organic layer was washed with H_2O and evaporated to dryness. The residue was purified by silica gel column chromatography using toluene–acetone 4:1 as eluent, affording 1.5 g (50%) of **10f**: mp 68–70 °C (ligroin). ^{1}H NMR: δ 3.92 (s, 3H), 3.96 (s, 3H), 5.15 (s, 2H), 5.25 (s, 1H), 6.4–8.1 (m, 17H, Ar + CH=CH). Anal. ($C_{31}H_{26}O_5$): C, H.

*3-{1-[2-(Benzyloxy)-5-methoxyphenyl]methylidene}-7-methoxy-2-(4-methoxyphenyl)-4-chromanone **(10h)***

Using the same procedure and starting from 7,4′-dimethoxyflavanone (2.54 g, 0.01 mol) and 2-benzyloxy-5-methoxybenzaldehyde (2.42 g, 0.01 mol), 3.56 g (70%) of uncrystallizable product were obtained, mp 54–56 °C, which was used in the next step.

*3-(2-Hydroxy-5-methoxybenzyl)-2-(4-methoxyphenyl)-4-chromanone **(11f)***

A solution of **10f** (1.43 g, 0.003 mol) in THF (50 ml) was hydrogenated at room temperature and pressure over $Pd/CaCO_3$. The solution was filtered from catalyst and evaporated to dryness. The uncrystallizable residue (1.21 g, 100%) was used in the next step.

*3-(2-Hydroxy-5-methoxybenzyl)-7-methoxy-2-(4-methoxyphenyl)-4-chromanone **(11h)***

Using the same procedure, and starting from **10h** (2.45 g, 0.005 mol), 1.26 g (60%) of **11h** was obtained, mp 142–144 °C (toluene). ^{1}H NMR: δ 2.45 (d, 1H), 3.05 (m, 1H), 3.25 (m, 1H), 3.65 (s, 3H), 3.82 (s, 3H), 3.9 (s, 3H), 5.22 (d, 1H), 5.9–7.9 (m, 10H, Ar). Anal. ($C_{25}H_{24}O_6$): C, H.

*General method for the preparation of pyrilium salts **9b–w***

To a cold solution of the selected flavanone (0.01 mol) and the selected benzaldehyde (0.012 mol) in acetic acid (20 ml), anhydrous $HClO_4$ (15 g) was added. The reaction mixture was refluxed for 2 min. After cooling, diethyl ether was added and the red pyrilium salt was obtained in quantitative yield. **9b**: mp 238–240 °C. Anal. ($C_{23}H_{17}ClO_7$): C, H. **9c**: mp 198–200 °C. Anal. ($C_{23}H_{17}ClO_7$): C, H. **9d**: mp 188–191 °C. Anal. ($C_{24}H_{19}ClO_8$): C, H. **9e**: mp 175–180 °C. Anal. ($C_{23}H_{17}ClO_7$): C, H. **9f**: mp 210–213 °C. Anal. ($C_{24}H_{19}ClO_8$): C, H. **9g**: mp 196–198 °C. Anal. ($C_{24}H_{19}ClO_8$): C, H. **9h**: mp 180–184 °C. Anal. ($C_{25}H_{21}ClO_9$): C, H. **9i**: mp 156–160 °C. Anal. ($C_{22}H_{15}ClO_7$): C, H. **9j**: mp 171–175 °C. Anal. ($C_{30}H_{30}ClNO_9$): C, H, N. **9k**: mp 119–120 °C. Anal. ($C_{29}H_{28}ClNO_8$): C, H, N. **9l**: mp 184–186 °C. Anal. ($C_{30}H_{30}ClNO_9$): C, H, N. **9m**: mp 155–160 °C. Anal. ($C_{30}H_{30}ClNO_8$): C, H, N. **9n**: mp 185–187 °C. Anal. ($C_{31}H_{32}ClNO_9$): C, H, N. **9o**: mp 155–157 °C. Anal. ($C_{31}H_{32}ClNO_8$): C, H, N. **9p**: mp 144–146 °C. Anal. ($C_{32}H_{34}ClNO_9$): C, H, N. **9q**: mp 148–151 °C. Anal. ($C_{32}H_{34}ClNO_8$): C, H, N. **9r**: mp 153–156 °C. Anal. ($C_{33}H_{36}ClNO_9$): C, H, N. **9s**: mp 178–182 °C. Anal. ($C_{30}H_{30}ClNO_9$): C, H, N. **9t**: mp 171–174 °C. Anal. ($C_{30}H_{30}ClNO_{10}$): C, H, N. **9u**: mp 119–120 °C. Anal. ($C_{30}H_{30}ClNO_8$): C, H, N. **9v**: mp 213–217 °C. Anal. ($C_{35}H_{33}ClN_2O_8$): C, H, N. **9w**: mp 106–110 °C. Anal. ($C_{36}H_{35}ClN_2O_9$): C, H, N.

*4-(2-Tetrahydro-1H-1-pyrrolylethoxy)benzaldehyde **(12)***

A solution of 4-hydroxybenzaldehyde (12.2 g, 0.1 mol), 1-bromo-2-chloroethane (14.3 g, 0.1 mol), K_2CO_3 (13.8 g, 0.1 mol) in acetone (400 ml) was stirred under reflux for 24 h. The reaction mixture was filtered and evaporated to dryness. The residue was crystallized from ligroin to yield 9.2 g (50%) of 4-(2-chloroethoxy)benzaldehyde, mp 96–98 °C.

A solution of the resulting product (9.2 g, 0.05 mol) and pyrrolidine (8.1 g, 0.1 mol) in toluene was refluxed for 10 h. The reaction mixture was washed with H_2O and evaporated to dryness to yield an oil, which was suitable for the next step. ^{1}H NMR: δ 1.9 (m, 4H), 2.3 (m, 4H), 2.45 (t, 2H), 4.1 (t, 2H), 6.2–7.6 (m, 4H, Ar), 13.1 (broad, 1H). A sample was treated with HCl in ethanol to give the hydrochloride salt, mp 228–230 °C. Anal. ($C_{13}H_{18}ClNO_2$): C, H, N.

*1-(2-Hydroxy-4-methoxyphenyl)-3-[4-(2-tetrahydro-1H-1-pyrrolylethoxy)phenyl]-2-propen-1-one **(13)***

To a solution of **12** (2.19 g, 0.01 mol) and 2-hydroxy-4-methoxyacetophenone (1.65 g, 0.01 mol) in EtOH (40 ml), 50% KOH (15 g) was added. The reaction mixture was stirred at room temperature for 3 days, poured into H_2O, and then extracted with CH_2Cl_2. The organic layer was washed with

H_2O, dried, and evaporated. The residue, on crystallizing from ligroin, afforded 2.9 g (80%) of **13**: mp 106–108 °C. ^{1}H NMR: δ 1.8 (m, 4H), 2.6 (m, 4H), 2.95 (m, 2H), 3.75 (s, 3H), 4.2 (m, 2H), 6.4–8.0 (m, 9H, Ar and CH=CH). Anal. ($C_{22}H_{25}NO_4$): C, H, N.

*7-Methoxy-2-[4-(2-tetrahydro-1H-1-pyrrolylethoxy)-phenyl]-4-chromanone **(14)***

A solution of **13** (2 g) in 3% HCl–EtOH was refluxed for 24 h, and then evaporated to dryness. The residue was treated with 10% K_2CO_3 solution and extracted with CH_2Cl_2. The residue was purified by flash chromatography (methanol), affording 1.5 g (75%) of **14** as an oil, which was suitable for the next step. ^{1}H NMR: δ 1.85 (m, 4H), 2.65 (m, 4H), 2.8–3.15 (m, 4H), 3.9 (s, 3H), 4.2 (m, 2H), 5.45 (d, 1H), 6.5–7.9 (m, 7H, Ar). A sample was treated with HCl in ethanol to give the hydrochloride salt, mp 108–110 °C. Anal. ($C_{22}H_{26}ClNO_4$): C, H, N.

*1-[2-Hydroxy-4-(2-tetrahydro-1H-1-pyrrolylethoxy)-phenyl]-1-ethanone **(15a)***

A solution of 2,4-dihydroxyacetophenone (30 g, 0.197 mol), 1-bromo-2-chloroethane (17 ml, 0.197 mol), K_2CO_3 (49 g) in acetone (900 ml) was stirred under reflux for 24 h. The reaction mixture was filtered and evaporated to dryness. The residue was crystallized from ligroin to yield 20.8 g (50%) of 4-(2-chloroethoxy)-2-hydroxyphenyl]-1-ethanone, mp 96–98 °C. ^{1}H NMR: δ 2.55 (s, 3H), 3.8 (t, 2H), 4.25 (t, 2H), 6.4–7.65 (m, 3H, Ar), 12.6 (broad, 1H, OH).

A solution of the resulting product (10.7 g, 0.05 mol) and pyrrolidine (8.1 g, 0.1 mol) in toluene was refluxed for 10 h. The reaction mixture was washed with H_2O and evaporated to dryness to yield **15a** (9.9 g, 80%) as oil, which was suitable for the next step. ^{1}H NMR: δ 1.8 (m, 4H), 2.45–2.65 (m, 9H), 4.1 (t, 2H), 6.4–7.7 (m, 3H, Ar), 12.35 (broad, 1H, OH).

*1-[2-Hydroxy-4-(3-tetrahydro-1H-1pyrrolyl-propoxy)phenyl]-1-ethanone **(15b)***

Using the same procedure, and starting from 2,4-dihydroxyacetophenone and 1-bromo-3-chloropropane, 40% of 4-(3-chloropropoxy)-2-hydroxyphenyl]-1-ethanone was obtained, mp 69–71 °C (ligroin). ^{1}H NMR: δ 2.25 (m, 2H), 2.5 (s, 3H), 3.7 (t, 2H), 4.15 (t, 2H), 6.4–7.65 (m, 3H, Ar), 12.7 (s, 1H, OH).

Starting from the resulting product and pyrrolidine, **15b** was obtained (80%, oil). ^{1}H NMR: δ 1.75 (m, 2H), 1.9 (m, 4H), 2.25 (m, 9H), 3.95 (t, 2H), 6.2–7.8 (m, 3H, Ar), 12.35 (s, 1H, OH).

*1-[2-Hydroxy-4-(4-tetrahydro-1H-1pyrrolylbutoxy)phenyl]-1-ethanone **(15c)***

Using the same procedure, and starting from 2,4-dihydroxyacetophenone and 1-bromo-4-chlorobutane, 35% of 4-(4-chlorobutoxy)-2-hydroxyphenyl]-1-ethanone was obtained, mp 48–49 °C (ligroin). ^{1}H NMR: δ 1.95 (m, 4H), 2.5 (s, 3H), 3.6 (t, 2H), 4.0 (t, 2H), 6.4–7.6 (m, 3H, Ar), 12.7 (s, 1H, OH).

Starting from the resulting product and pyrrolidine, **15c** was obtained (80%, oil). ^{1}H NMR: δ 1.75 (m, 4H), 2.0 (m, 4H), 2.35 (m, 9H), 3.95 (t, 2H), 6.2–7.8 (m, 3H, Ar), 12.4 (s, 1H, OH).

*1-[2-Hydroxy-4-(5-tetrahydro-1H-1pyrrolylpentyloxy)phenyl]-1-ethanone **(15d)***

Using the same procedure, and starting from 2,4-dihydroxyacetophenone and 1-bromo-5-chloropentane, in 50% yield of 4-(5-chloropentyloxy)-2-hydroxyphenyl]-1-ethanone was obtained, mp 49–51 °C (ligroin). ^{1}H NMR: δ 1.65–1.95 (m, 6H), 2.6 (s, 3H), 3.6 (t, 2H), 4.05 (t, 2H), 6.4–7.65 (m, 3H, Ar), 12.5 (s, 1H, OH).

Starting from the resulting product and pyrrolidine, **15d** was obtained (80%, oil). ^{1}H NMR: δ 1.75 (m, 6H), 1.9 (m, 4H), 2.25 (m, 9H), 3.95 (t, 2H), 6.2–7.8 (m, 3H, Ar), 12.35 (s, 1H, OH).

*1-[2-Hydroxy-4-(2-morpholinoethoxy)phenyl]-1-ethanone **(15e)***

Using the same procedure, and starting from 4-(2-chloroethoxy)-2-hydroxyphenyl]-1-ethanone and morpholine, **15e** was obtained (85%, oil). ^{1}H NMR: δ 2.6 (m, 7H), 2.85 (t, 2H), 3.75 (m, 4H) 4.15 (t, 2H), 6.45–7.65 (m, 3H, Ar), 12.75 (s, 1H, OH).

*1-[2-Hydroxy-4-(2-piperidinoethoxy)phenyl]-1-ethanone **(15f)***

Using the same procedure, and starting from 4-(2-chloroethoxy)-2-hydroxyphenyl]-1-ethanone and piperidine, **15f** was obtained (75%, oil). ^{1}H NMR: δ 1.5 (m, 6H), 2.5 (m, 7H), 2.75 (t, 2H), 4.1 (t, 2H), 6.35–7.6 (m, 3H, Ar), 12.7 (s, 1H, OH).

*1-{2-Hydroxy-4-[2-(phenylpiperazino)ethoxy]phenyl}-1-ethanone **(15g)***

Using the same procedure, and starting from 4-(2-chloroethoxy)-2-hydroxyphenyl]-1-ethanone and phenylpiperazine, **15g** was obtained (65%, oil). ^{1}H NMR: δ 2.5 (s, 3H), 2.7 (m, 4H), 2.8 (t, 2H), 3.2 (m, 4H), 4.1 (t, 2H), 6.45–7.6 (m, 8H, Ar), 12.7 (s, 1H, OH).

*1-[2-Hydroxy-4-(2-tetrahydro-1H-1-pyrrolylethoxy)phenyl]-3-(4-methoxyphenyl)-2-propen-1-one **(16a)***

To a solution of **15a** (2.49 g, 0.01 mol) and 4-methoxybenzaldehyde (1.36 g, 0.01 mol) in EtOH (40 ml), 50% KOH (20 g) was slowly added. The reaction mixture was stirred at room temperature for 3 days, poured into H_2O and then extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried, and evaporated. The residue, on crys-

tallizing from ligroin, afforded 2.8 g (80%) of **16a**: mp 118–120 °C. ^{1}H NMR: δ 1.8 (m, 4H), 2.6 (m, 4H), 2.95 (m, 2H), 3.85 (s, 3H), 4.2 (m, 2H), 6.4–8.0 (m, 9H, Ar and CH=CH). Anal. ($C_{22}H_{25}NO_4$): C, H, N.

*1-[2-Hydroxy-4-(3-tetrahydro-1H-1-pyrrolylpropoxyphenyl]-3-(4-methoxy phenyl)-2-propen-1-one **(16b)***

Using the same procedure, and starting from **15b** and 4-methoxybenzaldehyde, **16b** was obtained (67%), mp 93–98 °C (EtOH). ^{1}H NMR: δ 1.8 (m, 4H), 2.05 (m, 2H), 2.55 (m, 4H), 2.65 (m, 2H), 3.9 (s, 3H), 4.1 (t, 2H), 6.5–7.9 (m, 9H, Ar and CH=CH). Anal. ($C_{23}H_{27}NO_4$): C, H, N.

*1-[2-Hydroxy-4-(4-tetrahydro-1H-1-pyrrolylbutoxyphenyl]-3-(4-methoxyphenyl)-2-propen-1-one **(16c)***

Using the same procedure, and starting from **15c** and 4-methoxybenzaldehyde, **16c** was obtained (65%), mp 86–90 °C (EtOH). ^{1}H NMR: δ 1.6–1.9 (m, 8H), 2.55 (m, 6H), 3.8 (s, 3H), 4.05 (t, 2H), 6.45–7.9 (m, 9H, Ar and CH=CH). Anal. ($C_{24}H_{29}NO_4$): C, H, N.

*1-{2-Hydroxy-4-[(5-tetrahydro-1H-1-pyrrolyl-pentyl)oxy]phenyl}-3-(4-methoxy phenyl)-2-propen-1-one **(16d)***

Using the same procedure, and starting from **15d** and 4-methoxybenzaldehyde, **16d** was obtained (68%), oil. ^{1}H NMR: δ 1.5 (m, 4H), 1.8 (m, 6H), 2.4 (m, 6H), 3.8 (s, 3H), 4.0 (t, 2H), 6.5–7.9 (m, 9H, Ar and CH=CH). Anal. ($C_{25}H_{31}NO_4$): C, H, N.

*1-[2-Hydroxy-4-(2-morpholinoethoxy)phenyl]-3-(4-methoxyphenyl)-2-propen-1-one **(16e)***

Using the same procedure, and starting from **15e** and 4-methoxybenzaldehyde, **16e** was obtained (65%), mp 120–125 °C (EtOH). ^{1}H NMR: δ 2.65 (m, 4H), 2.9 (t, 2H), 3.8 (m, 4H), 3.9 (s, 3H), 4.2 (m, 2H), 6.45–7.9 (m, 9H, Ar and CH=CH). Anal. ($C_{22}H_{25}NO_5$): C, H, N.

*1-[2-Hydroxy-4-(2-piperidinoethoxy)phenyl]-3-(4-methoxyphenyl)-2-propen-1-one **(16f)***

Using the same procedure, and starting from **15f** and 4-methoxybenzaldehyde, **16f** was obtained (62%), mp 113–118 °C (EtOH). ^{1}H NMR: δ 1.45 (m, 2H), 1.6 (m, 4H), 2.5 (m, 4H), 2.8 (m, 2H), 3.85 (s, 3H), 4.1 (m, 2H), 6.45–7.9 (m, 9H, Ar and CH=CH). Anal. ($C_{23}H_{27}NO_4$): C, H, N.

*1-{2-Hydroxy-4-[2-(4-phenylpiperazino)ethoxy]phenyl}-3-(4-methoxyphenyl)-2-propen-1-one **(16g)***

Using the same procedure, and starting from **15g** and 4-methoxybenzaldehyde, **16g** was obtained (70%), mp 125–130 °C (EtOH). ^{1}H NMR: δ 2.7 (m, 4H), 2.8 (t, 2H), 3.2 (m, 4H), 3.85 (s, 3H), 4.1 (t, 2H), 6.5–8.0 (m, 14H, Ar + CH=CH). Anal. ($C_{28}H_{30}N_2O_4$): C, H, N.

*2-(4-Methoxyphenyl)-7-(2-tetrahydro-1H-1-pyrrolylethoxy)-4-chromanone **(17a)***

A solution of **16a** (2 g) in 3% HCl–EtOH was refluxed for 24 h and then evaporated to dryness. The residue was treated with 10% K_2CO_3 solution and extracted with CH_2Cl_2. The organic layer was washed with H_2O and evaporated to dryness. The residue was purified by flash chromatography (MeOH), affording **17a** (1.5 g, 75%) as an oil which was suitable for the next step. ^{1}H NMR: δ 1.85 (m, 4H), 2.65 (m, 4H), 2.8–3.15 (m, 4H), 3.9 (s, 3H), 4.2 (m, 2H), 5.45 (d, 1H), 6.5–7.9 (m, 7H, Ar). A sample was treated with HCl in ethanol to give the hydrochloride salt, mp 158–160 °C. Anal. ($C_{22}H_{26}ClNO_4$): C, H, N.

*2-(4-Methoxyphenyl)-7-(3-tetrahydro-1H-1-pyrrolylpropoxy)-4-chromanone **(17b)***

Using the same procedure, and starting from **16b**, **17b** (oil, 80%) was obtained. ^{1}H NMR: δ 1.8 (m, 4H), 2.0 (t, 2H), 2.6 (m, 6H), 2.75–3.1 (m, 2H), 3.85 (s, 3H), 4.1 (t, 2H), 5.4 (m, 1H), 6.5–7.9 (m, 7H, Ar).

*2-(4-Methoxyphenyl)-7-(4-tetrahydro-1H-1-pyrrolylbutoxy)-4-chromanone **(17c)***

Using the same procedure, and starting from **16c**, **17c** (oil, 79%) was obtained. ^{1}H NMR: δ 1.7–1.9 (m, 8H), 2.6 (m, 6H), 2.8–3.1 (m, 2H), 3.9 (s, 3H), 4.05 (t, 2H), 5.4 (m, 1H), 6.5–7.9 (m, 7H, Ar).

*2-(4-Methoxyphenyl)-7-[(5-tetrahydro-1H-1-pyrrolylpentyl)oxy]-4-chromanone **(17d)***

Using the same procedure, and starting from **16d**, **17d** (oil, 75%) was obtained. ^{1}H NMR: δ 1.5 (m, 4H), 1.85 (m, 6H), 2.5 (m, 6H), 2.75–3.1 (m, 2H), 3.85 (s, 3H), 4.0 (t, 2H), 5.4 (m, 1H), 6.45–7.9 (m, 7H, Ar).

*2-(4-Methoxyphenyl)-7-(2-morpholinoethoxy)-4-chromanone **(17e)***

Using the same procedure, and starting from **16e**, **17e** was obtained (76%), mp 138–142 °C (EtOH). ^{1}H NMR: δ 2.55 (m, 4H), 2.75–3.1 (m, 4H), 3.75 (m, 4H), 3.9 (s, 3H), 4.15 (m, 2H), 5.4 (m, 1H), 6.45–7.9 (m, 7H, Ar).

*2-(4-Methoxyphenyl)-7-(2-piperidinoethoxy)-4-chromanone **(17f)***

Using the same procedure, and starting from **16f**, **17f** was obtained (78%), mp 141–145 °C (EtOH). ^{1}H NMR: δ 1.45 (m, 2H), 1.6 (m, 4H), 2.5 (m, 4H), 2.8–3.1 (m, 4H), 3.85 (s, 3H), 4.1 (m, 2H), 5.4 (m, 1H), 6.45–7.9 (m, 7H, Ar).

*2-(4-Methoxyphenyl)-7-[2-(4-phenylpiperazino)ethoxy]-4-chromanone **(17g)***

Using the same procedure, and starting from **16g**, **17g** was obtained (79%), mp 128–131 °C (EtOH). ^{1}H NMR: δ 2.7 (m,

4H), 2.8–3.1 (m, 4H), 3.2 (m, 4H), 3.85 (s, 3H), 4.1 (t, 2H), 5.6 (m, 1H), 6.5–8.0 (m, 12H, Ar).

General method for the preparation of compounds (I) ***4b–e, 4g, 4j–w, 8a,b***

To a cold solution of 98% HCOOH (10 ml) and pyridine (10 ml), 1 g of the selected pyrilium salt was added portionwise. The mixture was refluxed for 2 min, and then poured into ice. The separated solid was collected by filtration, purified by flash chromatography and crystallized from ligroin.

Method for the preparation of compounds ***4f*** *and* ***4h***

To a suspension of **11f** or **11h** (1 g) in benzene (50 ml), P_2O_5 (11g) was added and the mixture was refluxed for **4h**. The mixture was cooled to room temperature, and poured into ice. The organic phase was separated, washed with H_2O, dried, and evaporated. The residue was then purified by flash chromatography and crystallized from ligroin.

General method for the preparation of compounds (II) ***4i, 4k–w***

To a suspension of $NaBH_4$ (2 g) in THF (100 ml), 1.5 g of the selected pyrilium salt was added portionwise under stirring. Hydrogen gas developed and the reaction mixture decolorized. The mixture was then poured into ice and 10 ml HCl and filtered (**4i**). Hydrochloride salts (**4k**–**w**) were resuspended in H_2O, basified with K_2CO_3, extracted with CH_2Cl_2, dried and evaporated to dryness. The residue was then purified by flash chromatography and crystallized from ligroin.

^{1}H NMR and mass spectrum data for compounds ***4b–w, 8a,b***

4b: δ 3.03 (d, 1H), 3.28 (d, 1H), 3.75 (s, 3H), 5.82 (s, 1H), 6.5–7.65 (m, 12H, Ar). MS: *m/z* (rel. abundance): 342 (M^+, 95.2), 265 (100), 221 (6). **4c**: δ 3.08 (d, 1H), 3.30 (d, 1H), 3.78 (s, 3H), 5.78 (s, 1H), 6.7–7.65 (m, 12H, Ar). MS: *m/z* (rel. abundance): 342 (M^+, 100), 235 (69.01), 206 (6.18). **4d**: δ 3.06 (d, 1H), 3.28 (d, 1H), 3.72 (s, 3H), 3.78 (s, 3H), 5.80 (s, 1H), 6.5–7.65 (m, 11H, Ar). MS: *m/z* (rel. abundance): 372 (M^+, 100), 265 (85.06), 186 (12.5). **4e**: δ 3.1 (d, 1H), 3.26 (d, 1H), 3.78 (s, 3H), 5.82 (s, 1H), 6.3–7.6 (m, 12H, Ar). MS: *m/z* (rel. abundance): 342 (M^+, 65.95), 265 (100), 222 (6.71). **4f**: δ 3.08 (d, 1H), 3.28 (d, 1H), 3.72 (s, 3H), 3.78 (s, 3H), 5.81 (s, 1H), 6.4–7.65 (m, 11H, Ar). MS: *m/z* (rel. abundance): 372 (M^+, 100), 265 (85.06), 186 (12.5). **4g**: δ 3.04 (d, 1H), 3.27 (d, 1H), 3.75 (s, 3H), 3.77 (s, 3H), 5.81 (s, 1H), 6.3–7.6 (m, 11H, Ar). MS: *m/z* (rel. abundance): 372 (M^+, 72.25), 295 (100), 186 (5.0). **4h**: δ 3.06 (d, 1H), 3.28 (d, 1H), 3.76 (s, 3H), 3.78 (s, 3H), 3.81 (s, 3H), 5.80 (s, 1H), 6.35–7.55 (m, 10H, Ar). MS: *m/z* (rel. abundance): 402 (M^+, 100), 387 (13.11), 295 (73.71). **4i**: δ (DMSO) 3.08 (d, 1H), 3.31 (d, 1H), 5.85 (s, 1H), 6.7–7.5 (m, 12H, Ar), 9.6 (s, 1H, OH). **4j**: δ 1.8 (m, 4H), 2.65 (m, 4H), 2.90 (m, 2H), 3.08 (d, 1H), 3.2 (d, 1H), 3.7 (s, 3H), 3.8 (s, 3H), 4.02 (m, 2H), 5.78 (s, 1H), 6.35–7.75 (m, 10H, Ar). MS: *m/z* (rel. abundance): 485 (M^+, 42.3), 402 (84.9), 388 (100). **4k**: δ 1.82 (m, 4H), 2.61 (m, 4H), 2.90 (m, 2H), 3.08 (d, 1H), 3.24 (d, 1H), 3.84 (s, 3H), 4.02 (m, 2H), 5.78 (s, 1H), 6.3–7.7 (m, 10H, Ar). MS: *m/z* (rel. abundance): 455 (M^+, 3.6), 98 (14.9), 84 (100). **4l**: δ 1.82 (m, 4H), 2.61 (m, 4H), 2.90 (m, 2H), 3.08 (d, 1H), 3.24 (d, 1H), 3.78 (s, 3H), 3.84 (s, 3H), 4.02 (m, 2H), 5.78 (s, 1H), 6.3–7.7 (m, 10H, Ar). MS: *m/z* (rel. abundance): 485 (M^+, 10.44), 388 (3.2), 98 (45.55), 84 (100). **4m**: δ 1.9 (m, 2H), 2.2 (m, 4H), 2.75 (m, 2H), 2.85–3.1 (m, 6H), 3.8 (s, 3H), 4.0 (t, 2H), 5.8 (s, 1H), 6.3–7.5 (m, 11H, Ar). **4n**: δ 1.9 (m, 2H), 2.2 (m, 4H), 2.75 (m, 2H), 2.85–3.1 (m, 6H), 3.7 (s, 3H), 3.8 (s, 3H), 4.0 (t, 2H), 5.8 (s, 1H), 6.3–7.5 (m, 10H, Ar). MS: *m/z* (rel. abundance): 499 (M^+, 14.94), 418 (25.2), 406 (100). **4o**: δ 1.85 (m, 8H), 2.2 (m, 2H), 2.8 (m, 4H), 3.15 (m, 2H), 3.8 (s, 3H), 4.0 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 11H, Ar). MS: *m/z* (rel. abundance): 483 (M^+, 100), 357 (27.2), 291 (62.08). **4p**: δ 1.85 (m, 8H), 2.2 (m, 2H), 2.8 (m, 4H), 3.25 (m, 2H), 3.7 (s, 3H), 3.8 (s, 3H), 4.0 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 10H, Ar). MS: *m/z* (rel. abundance): 513 (M^+, 44.6), 388 (100), 347 (31.28). **4q**: δ 1.85 (m, 8H), 2.15 (m, 2H), 2.7 (m, 4H), 3.15 (m, 4H), 3.8 (s, 3H), 3.9 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 11H, Ar). MS: *m/z* (rel. abundance): 497 (M^+, 65.16), 376 (23.12), 357 (100). **4r**: δ 1.85 (m, 8H), 2.15 (m, 2H), 2.7 (m, 4H), 3.15 (m, 4H), 3.7 (s, 3H), 3.8 (s, 3H), 3.9 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 10H, Ar). MS: *m/z* (rel. abundance): 527 (M^+, 12.26), 387 (20.48), 211 (100). **4s**: δ 2.55 (m, 4H), 2.8 (m, 2H), 3.08 (d, 1H), 3.25 (d, 1H), 3.75 (m, 4H), 3.9 (s, 3H), 4.1 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 11H, Ar). MS: *m/z* (rel. abundance): 471 (M^+, 9.36), 114 (68.12), 100 (100). **4t**: δ 2.55 (m, 4H), 2.8 (m, 2H), 3.08 (d, 1H), 3.25 (d, 1H), 3.75 (m, 4H), 3.8 (s, 3H), 3.9 (s, 3H), 4.1 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 10H, Ar). MS: *m/z* (rel. abundance): 501 (M^+, 9.44), 114 (85.46), 100 (100). **4u**: δ 1.5–1.85 (m, 6H), 2.85–3.2 (m, 8H), 3.8 (s, 3H), 4.4 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 11H, Ar). **4v**: δ 2.75 (m, 4H), 2.85 (t, 2H), 3.2 (m, 6H), 3.8 (s, 3H), 4.15 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 16H, Ar). MS: *m/z* (rel. abundance): 501 (M^+, 9.44), 114 (85.46), 100 (100). **4w**: δ 2.75 (m, 4H), 2.85 (t, 2H), 3.2 (m, 6H), 3.7 (s, 3H), 3.8 (s, 3H), 4.15 (t, 2H), 5.75 (s, 1H), 6.3–7.5 (m, 15H, Ar). MS: *m/z* (rel. abundance): 576 (M^+, 26.52), 189 (63.46), 175 (100). **8a**: δ 1.45 (s, 6H), 3.45 (s, 2H), 3.8 (s, 3H), 6.4–7.45 (m, 7H, Ar). MS: *m/z* (rel. abundance): 294 (M^+, 5.48), 279 (15.19), 91 (100). **8b**: δ 1.5 (s, 6H), 3.45 (s, 2H), 3.8 (d, 6H), 6.45–7.45 (m, 6H, Ar). MS: *m/z* (rel. abundance): 324 (M^+, 13.78), 309 (100), 294 (6.88).

6-(4-Methoxyphenyl)-6a,12a-dihydro-6H,7H-chromeno [4,3-b]chromene ***(6)***

A solution of **4c** (0.5 g, 0.0015 mol) in THF was hydrogenated at room temperature and pressure over Pd/C. The solvent was evaporated, and the mixture purified by flash chromatography (toluene): 0.24 g (50%) of **6** were obtained, mp 143–144 °C (ligroin). ^{1}H NMR: δ 2.2–2.4 (m, 1H), 2.7–2.8 (m, 2H), 3.8 (s, 3H), 5.5 (s, 1H), 5.8 (m, 1H) 6.7–7.6 (m, 12H, Ar). Anal. ($C_{23}H_{18}O_3$): C, H.

6-(4-Methoxyphenyl)-6H,7H-chromeno[4,3-b]chromen-7-one (7)

To a solution of **9c** (1.5 g, 0.0043 mol) in DMSO (20 ml) at room temperature, dry triethylamine (0.6 ml, 0.005 mol) was added, which caused immediate decoloration of the solution. The mixture was poured into ice, filtered, and purified by flash chromatography (toluene): 300 mg (20%) of **7** were obtained, mp 130–131 °C (ligroin). ^{1}H NMR: δ 3.75 (s, 3H), 6.6 (s, 1H), 6.7–8.2 (m, 12H, Ar). Anal. ($C_{23}H_{16}O_4$): C, H.

5.2. Biological assays

5.2.1. Animals and housing

Virus-free immature Swiss–Webster mice were obtained at 17–19 days of age from Charles River (Milano, Italy) weighing 8–15 g, and were used in the uterotrophic and antiuterotrophic assays. Animals were housed at five animals per cage with the environment controlled at 25 °C and 12 h light/dark cycle. The animals received a diet Altromin R (Rieper S.P.A. Vandoies BZ, Italy) and water *ad libitum*.

5.2.2. Cell culture assay

MCF-7 human breast cancer cells (obtained from Istituto Zooprofilattico Sperimentale di Brescia, Italy) were grown as monolayer culture at 37 °C in T-75 flasks in MEM with Eagle's salts supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin (10,000 U/10.000 μg/ml), 1% pyruvate, and 10% heat-treated calf serum (Seromed-Biochrom KG, Berlin). Cultures were grown at 37 °C in a humid atmosphere of 95% air and 5% CO_2.

The cultures were trypsinized, and 10,000 cells were plated in each well of a 96-well tissue culture plate (Falcon). Two days later, medium in each well was replaced with fresh medium or medium containing various concentrations of the test compounds, and the cells were then incubated for 24 h.

The number of viable cells was measured by a colorimetric technique based on the cleavage of tetrazolium salts added to the culture medium (MTT test) [29] .In this technique, the tetrazolium salt is broken down to the coloured product, formazan, by the succinate-tetrazolium reductase system of the mitochondrial respiratory chain in viable cells. Thus, the amount of formazan dye, measured as absorbance at wavelength of 570 nm ($A_{570\ nm}$) with reference wavelength of 630 nm ($A_{630\ nm}$) directly correlates with the number of metabolically active cells.

During the last 4 h of experimental treatment, 20 μl of MTT solution (5 mg/ml in PBS) were added to each well, then 150 μl of DMSO were added to all wells and mixed thoroughly to dissolve the dark blue crystals, and the $A_{570\ nm} - A_{630\ nm}$ was measured using a micro-culture plate reader (Titertek Multiscan).

The test compounds were dissolved in a mixture of chremophor EL-94% ethanol (1:0.42), diluted 1:100 in saline and finally 1:10 in culture medium [30]. Control sample received vehicle alone at the same concentration. For each compound, three different experiments in triplicate were performed.

The results were reported as the percentage of controls and statistically evaluated by Student's *t*-test [31]. The IC_{50}, 95% confidence limits and the potency ratio between TAM and each compound (IC_{50} TAM/IC_{50} complexes) were estimated using the Lichfield and Wilcoxon method [31].

Antiestrogenic activity was assayed by determining the inhibition of MCF-7 cell proliferation stimulated by 17β-estradiol. Cells were seeded in 96-well tissue plates in complete medium or in complete medium containing 0.01 nM of 17β-estradiol, and treated 48 and 96 h later with 10 μM of TAM and the compounds showing a PR>1 vs. TAM in antiproliferative assays. MTT test was performed after further 48 h. The results were reported as the percentage of reductase activity inhibition with respect to the control in absence (–E2) and in presence (+E2) of 17β-estradiol.

5.2.3. Binding assay

Healthy human tissues of uterus were provided by Obstretic-Gynecologic Clinic, University of Ferrara. Connective tissue was removed by dissection, and the freshly collected uterus was washed in cold isotonic saline, promptly cut into small pieces, and quickly frozen in liquid nitrogen. These tissue preparations were pulverized in a pulverizer dismembrator, and suspended in eight volumes of ice-cold 10 mM phosphate buffer, pH 7.4 containing 1.5 mM EDTA, 1 mM dithiothreitol, 10 mM of sodium molybdate. The samples were centrifuged at 100,000 × *g* for 60 min, and the temperature was maintained at 2 °C throughout the centrifugation [32]. The protein concentration in each cytosol was determined according to a Bio-Rad method [33] with human albumin as reference standard. In competition experiments carried out to determine the IC_{50} values, 1 nM [^{3}H] estradiol was incubated with 40 μl of cytosol (150–200 μg of protein/assay) and at least 8 to 10 different concentrations of the compounds examined. Inhibition experiments were carried out at 5 °C in a thermostatic bath, and incubation time was 24 h according to the results of previous time-course experiments. Non-specific binding was defined as binding in the presence of diethylstilboestrol at a final concentration of 10 μM. Samples were treated for 15 min at 0 °C with 100 μl of dextran coated charcoal and centrifuged at 1000 × *g* for 20 min at 4 °C. Supernatant fluid (100 μl) was removed and mixed with 4 ml of atom light and counted for radioactivity determination in a LS 1800 Beckman scintillation counter. The inhibition binding constant (K_i) values were calculated from IC_{50} values according to the equation [34] $K_i = IC_{50}/1 + [C^*]/K_d$, where [*C**] is the concentration of the radioligand and K_d its dissociation constant. A weighted non-linear least square curved fitting programme LIGAND [35] was used for computer analysis of inhibition experiments.

5.2.4. Antiuterotrophic assay

Antiestrogenic activity was determined by inhibition of estradiol-induced uterine weight gain in immature female

Swiss–Webster mice. Estradiol was dissolved in sesame oil (0.1 μg/ml). Injections were made for 3 consecutive days. The non-stimulated control group received vehicle alone, while the stimulated control group received 0.1 ml of estradiol solution (total dose 0.03 μg). The treatment groups received 0.1 ml of the stimulating dose of estradiol plus 0.1 ml of the test compound solutions at doses of 30, 150 and 750 μg (total dose). Antiestrogenic activity was measured as a decrease in the estradiol-stimulated uterotrophic response (increase in uterine weight) in the group that received both the test compound and estradiol, as compared to the group treated only with estradiol.

5.2.5. Uterotrophic assay

Estrogenic activity of the compounds was determined by using the method of Magarian et al. [23]. Immature female Swiss–Webster mice (8–15 g) at 17–19 days of age were used. The mice were randomly separated into groups of five animals and weighed, and the compounds were administered by s.c. injection of 0.1 ml of the oil solution for 3 consecutive days. Estradiol was administered at doses of 0.02, 0.04, and 0.06 μg (total dose) as the assay standard. Each test compound was examined at doses of 30, 150 and 750 μg (total dose). The animals were anaesthetized by Et_2O and euthanized by cervical dislocation 24 h after the last injection. Body weights were determined and the estrogenic activity was measured as an increase in uterine weight produced by each test compound.

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