Eur. J. Med. Chem., **22** (1987) 27—31

Original paper

Synthesis and properties of some new 3-substituted hexestrol derivatives

Michel VILKAS [1], Luzolo MASAMBA [1], Siku MAKANI [1] and Guy LECLERCQ [2]

[1]*Laboratoire de Chimie Organique Biologique, Université de Paris-Sud, Bâtiment 420, 91405 Orsay, France, and* [2]*Laboratoire de Cancérologie Mammaire, Service de Médecine, Institut Jules-Bordet, 1000 Brussels, Belgium*

(Received 5-7-1986, accepted 10-1-1986)

Summary — A series of new monosubstituted hexestrol derivatives were synthesized and the estrogenic and cytotoxic properties of some of them were studied. 3-Acetoxymethyl-hexestrol **8** has a binding affinity for the cytoplasmic estrogen receptor as high as estradiol. It is 100—1000 times lower for the coumarinic derivatives **13**, **14** and **15**. None of these compounds becomes covalently bound to the receptor. They display a 20—1000 times lower affinity for the estrogen receptor in the whole cell test on the MCF-7 mammary tumor cell culture. At low concentrations (10^{-8} M) the three coumarinic derivatives stimulate the growth of these cells (estrogenic effect) as strongly as estradiol does.

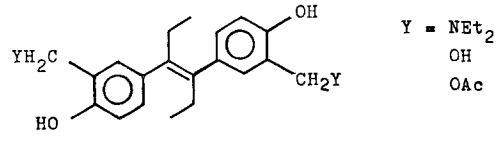
Résumé — Synthèse et propriétés de quelques nouveaux dérivés substitués en 3 de l'hexestrol. *Une série de nouveaux dérivés monosubstitués de l'hexestrol a été synthétisée et leurs propriétés oestrogènes et cytotoxiques étudiées pour quelques-uns d'entre eux. L'acétoxyméthyl-3-hexestrol* **8** *présente une affinité pour le récepteur cytoplasmique d'oestrogènes aussi élevée que celle de l'oestradiol ; celle des dérivés coumariniques* **13**, **14** *et* **15** *est 100—1000 fois plus faible. Aucun de ces composés ne forme de liaison covalente avec le récepteur. Leur affinité pour le récepteur d'oestrogènes de cellules entières de tumeur mammaire en culture (MCF-7), est 20—1000 fois plus faible. A faible concentration (10^{-8} M), les trois dérivés coumariniques exercent un effet oestrogène aussi fort que l'oestradiol sur la croissance de ces cellules.*

hexestrol analogs / estrogen receptor / mammary tumor cells

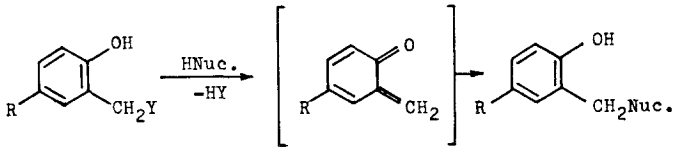
Introduction

Estrogen analogs displaying cytotoxic and/or anti-estrogenic activities are used for the treatment of breast cancer [1, 2]. Synthesis of analogs designed to bind covalently to the cellular estrogen receptor (ER) is a way to obtain anti-estrogenicity. It could also provide insight into the nature of the ER's chemical residues involved in the binding of the hormone.

In an earlier report [3], we described the synthesis and biological properties of a series of *o,o'*-disubstituted diethylstilbestrols **1** designed to alkylate a nucleophile of the ER's binding site *via* a quinone—methide mechanism [4—6]:



1



Their binding affinities were low, ($\sim$ 0.2% E_2 where E_2 = estradiol) probably due to the steric crowding around both phenolic hydroxyls.

In order to overcome this factor, we decided to synthesize monosubstituted hexestrols carrying a CH_2Y group *ortho* to only one phenolic hydroxyl. We could expect that these estrogen analogs would display a comparatively good binding affinity owing to the unmodified moiety, as well as their alkylating properties. Hexestrol was chosen rather than stilbestrol because of the greater flexibility of the molecule, enabling a better fit of the modified portion to the receptor's binding site [7].

Ortho-halomethylphenols are too labile to be isolated. On the contrary *ortho*-acetoxymethylphenols can be

prepared and are a good source of quinone—methides [4, 5]. Therefore, our first goal was the synthesis of 3-acetoxymethylhexestrol **8**. Another series of asymmetrically modified hexestrols are the coumarin derivatives **13**, **14** and **15** which also possess an electrophilic center adjacent to the 3 position. The present paper describes the synthesis of these compounds as well as their binding properties with the estrogen receptor.

Chemistry

Acetoxymethylhexestrol

The starting material was the meso-hexestrol mono-methyl ether which was obtained in 90% yield by a modification of the published procedure [8]. By treatment with aqueous formaldehyde and diethylamine or dimethylamine it yielded the Mannich bases **2** or **3** which were converted into their hydrochlorides and demethylated by means of boron tribromide to give the Mannich bases **4** or **5** with a free phenolic hydroxyl at the other end of the molecule.

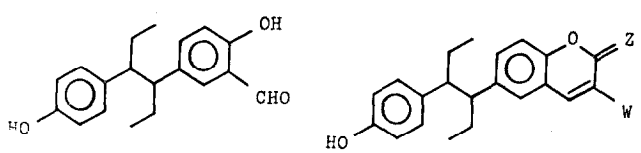
The acetoxymethylhexestrol **8** could be obtained from either of the Mannich bases by two routes. Deamination of **4** by acetic anhydride/acetic acid/anhydrous sodium acetate (10:2:1) yielded the triacetate **6** which was converted into the hydroxymethylhexestrol **7** by $LiAlH_4$ reduction (alcaline hydrolysis of the triacetate gives a partial loss of formaldehyde). Finally the triol **7** was mono-reacetylated under well-defined conditions: acetic acid containing two equivalents of acetic anhydride and 0.02 equivalents of *p*-toluenesulfonic acid, yielding the monoacetate **8**. Its structural assignment as a benzylic acetate rather than a phenolic one is based upon the following criteria: 1) the chemical shift of the benzylic methylene (δ = 5.14 ppm) is closer to the one of the triacetate **6** (5.08) rather than the triol 7 (4.78); 2) phenolic acetates of *o*-hydroxymethylphenols rearrange spontaneously to form the benzylic ones [9]. The overall yield of **8** from **4** was 31%.

The second route to acetoxymethylhexestrol **8** was as follows. Methylation of the Mannich base **5** by methyl iodide in ether gave the quaternary ammonium salt **9** which was converted directly into **8** by boiling in acetic acid containing molten sodium acetate. The overall yield was 70%.

Using sodium methylate in methanol, **9** was transformed into the methyl ether **10**. This kind of substitution, which occurs by an elimination—addition mechanism, has been described for the Mannich base methiodides of some other phenols [10].

Coumarin derivatives

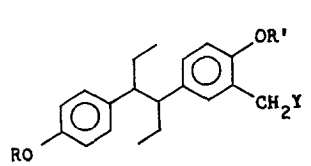
Compounds were synthesized as follows. The Mannich base **5** was converted into the amine—oxide **11** by means of 30% hydrogen peroxide. The amine—oxide was subjected to the Polonovski—Potier reaction [11, 12] using trifluoroacetic anhydride in dichloromethane. After acidic work-up, the aldehyde **12** could be isolated in 73% yield based on **5**. 3-Formylhexestrol **12** was condensed with diethyl malonate under the conditions used with salicylaldehyde [13] to yield the ethyl coumarin-3″-carboxylate **13** which was saponified to hexestrol-coumarin-3″-carboxylic acid **14**. Finally malononitrile and **12** reacted together very easily when a catalytic amount of piperidine was added, as described for salicylaldehyde [14] to yield the hexestrol-3″-imino-2″-cyanocoumarin **15**.



12	Z	W
13	O	CO_2Et
14	O	CO_2H
15	NH	CN

Biological properties

The binding affinity of the compounds to ER was measured by competitive inhibition of the binding of [^{3}H]estradiol to rat uterine cytosol. The acetoxymethylhexestrol **8** displayed a value as high as that of estradiol (relative binding affinity, RBA = 100). This high value cannot be ascribed to an irreversible binding to the receptor, since an exchange experiment revealed that the receptor—bound compound was totally displaced by [^{3}H]estradiol (Table I). The three coumarin derivatives were characterized by a low binding affinity (RBA: **13** = 0.1; **14** < 0.1; **15** = 1.0). Exchange experiments indicated that they were also bound to the receptor in a reversible manner (Table I). For all com-



	R	R′	Y
2	CH_3	H	NEt_2
3	CH_3	H	NMe_2
4	H	H	NEt_2
5	H	H	NMe_2
6	Ac	Ac	OAc
7	H	H	OH
8	H	H	OAc
9	H	H	N^+Me_3, I^-
10	H	H	OMe
11	H	H	$N(O)Me_2$

Table I. Reversibility of binding to estrogen receptor (exchange experiment).

Compounds	% inhibition of [^{3}H]estradiol binding	
	Before exchange	After exchange
8	23	90
13	56	100
14	72	86
15	23	100

The percentage of inhibition is given by the ratio of bound radioactivity after incubation with [^{3}H]estradiol in the absence (100%) or presence of the test compound.

pounds, measurement of binding affinity in growing MCF-7 cells (whole cell assay) gave lower values (**8** = 6; **13** = 0.02; **14** < 0.01; **15** = 0.3). Recent data from one of our laboratories (G.L.) strongly suggest that this property could be indicative of a defect in the ability of a compound to stabilize the active form of the receptor, *i.e.*, the conformation which promotes the transcription of estrogen-induced products (S. Stoessel and G. Leclercq (1986) *J. Steroid Biochem.*, 25, 677—682). Logically, a reduction of cell permeability may also account for this difference between whole cell and cytosol assays. Whatever the explanation may be, it appeared that the three coumarin derivatives (sharing the lowest values in the whole cell test) were characterized by a strong estrogenicity in regard to mammary tumor growth. Indeed, as for estradiol, all these derivatives stimulated the growth of the MCF-7 cells at the concentration of 10^{-8} M (Table II). This stimulatory effect always disappears with the increase of concentration giving no significant stimulation at 10^{-6} M. Estradiol at 10^{-8} **M** was unable to reverse this inhibitory effect indicating that it was not mediated by the receptor (data not shown).

All these data indicate that the present chemical modifications of hexestrol fail to produce irreversible binding to ER. As expected, these modifications often reduce the binding affinity for the receptor, although to a lower extent than previously observed with disubstituted derivatives of diethylstilbestrol [3]. Finally, the fact that compounds **13**, **14** and **15** share estrogenic activity in the MCF-7 mammary tumor model indicates that the substitution of one of the two phenolic groups of hexestrol by a coumarin ring does not suppress the endocrinological property of the compound even if its binding affinity is greatly decreased.

Experimental protocols

Syntheses of compounds

Mp's are uncorrected. Analyses indicated by elemental symbols were within ± 0.4% of the theoretical values unless otherwise indicated and were performed by the Service Central de Microanalyse du C.N.R.S. Division de Gif-sur-Yvette. NMR spectra were recorded at 90 MHz on a Perkin—Elmer R32 apparatus with TMS as the internal standard. IR spectra were recorded on a Perkin—Elmer Infracord 257 apparatus. Commercial meso-hexestrol (Sigma) was used without purification.

Hexestrol mono-methyl ether

5.40 g (0.02 mol) of hexestrol were dissolved by warming in 70 ml of 1 N NaOH diluted with 100 ml water. After cooling to 0°C, 1.9 ml (0.02 mol) of dimethyl sulfate were added dropwise and the stoppered vessel was mechanically shaken for 90 min. A white solid separated almost immediately. A second 1.9 ml dose of dimethyl sulfate was added and the mixture shaken for 90 min more. The reaction mixture was taken up in ether, acidified with aqueous HCl and washed with water and with 3 × 40 ml 5% KOH. From the alkaline extract, 550 mg of hexestrol were recovered upon acidification. The ethereal solution was washed with water containing NaCl and with saturated bicarbonate, dried and evaporated, yielding 5.13 g (90%) of hexestrol mono-methyl ether, mp 117—118°C; 119—121°C according to [8].

3-Diethylaminomethyl-hexestrol O'-methyl ether **2**

3.96 g (0.014 mol) of hexestrol mono-methyl ether, 0.84 g (0.028 mol) of paraformaldehyde, 5.8 ml (0.056 mol) of diethylamine and 4 drops of conc. HCl in 30 ml of 95% ethanol were refluxed for 5 h. Ether

Table II. Effect of 10^{-8} M coumarin derivatives on MCF-7 cell growth.

Plating density	Optical density[a]				
	Controls		Coumarin derivatives		
($n \times 10^3$ cells/ml)	—	+ 10^{-8} M estradiol	**13**	**14**	**15**
5	.037	.240	.265		
10	.177	.337	.429		
20	.273	.357	.445		
5	.040	.166		.144	
10	.156	.312		.317	
20	.265	.305		.348	
5	.060	.186			.190
10	.191	.303			.382
20	.299	.276[b]			.422

At each plating density, coumarin derivatives produce an increase in optical density, exactly as estradiol. This phenomenon, which corresponds to an increase of cells, is indicative of a stimulatory effect.

[a] Each value corresponds to the mean of 4 optical densities.

[b] Artifactual low value due to the detachment of a too large number of cells (*cf.* ref. [19]).

extraction, washing with water, drying (Na_2SO_4) and evaporation gave a solid residue which was crystallized from methanol to yield 3.88 g of **2**. From the mother—liquors a second crop of 0.35 g of practically pure **2** was obtained. Total yield: 82%. mp 101—102°C. Anal. $C_{24}H_{35}NO_2$ (C, H, N, O). ^1H NMR [$CDCl_3$—DMSO-d6 (3:1)]: δ 0.53 (6H, *t*, CH_3CH_2CH); 1.10 (6H, *t*, CH_3CH_2N); 1.40 (4H, *m*, CH_3CH_2CH); 2.45 (2H, *m*, CHEt); 2.63 (4H, *q*, CH_3CH_2N); 3.78 (2H, *s*, $ArCH_2$); 3.84 (3H, *s*, CH_3O); 6.75—7.20 (7H, *m*, arom.); 7.40 (1H, *s*, OH).

3-Dimethylaminomethyl-hexestrol O'-methyl ether **3**
The same procedure was used with an aqueous 40% solution of dimethyl amine in a pressure bottle with magnetic stirring. Yield: 83%. mp 102—103°C. Anal. $C_{22}H_{31}NO_2$ (C, H, N, O). ^1H NMR [$CDCl_3$—DMSO-d6 (3:1)]: δ 0.56 (6H, *t*, CH_3CH_2); 1.35 (4H, *m*, CH_3CH_2); 2.30 (6H, *s*, CH_3N); 2.45 (2H, *m*, CHEt); 3.60 (2H, *s*, $ArCH_2$); 3.82 (3H, *s*, CH_3O); 6.75—7.20 (7H, *m*, arom.); 9.30 (1H, *s*, OH).

3-Diethylaminomethyl-hexestrol **4**
The hydrochloride of the Mannich base **2** was prepared from 5.77 g (0.0156 mol) of **2** dissolved in methanol and a 10% excess 1 N aqueous HCl. Evaporation to dryness in a rotating evaporator gave a solid residue which was reacted with 8.6 g (2.2 mol. equiv.) of BBr_3 in CH_2Cl_2 for 2 h at — 78°C external and then allowing the temperature to rise to +20°C overnight. The reaction mixture was stirred for 20 min with aqueous HCl to decompose the borate esters, alkalinized, washed with $NaHCO_3$ and dried. Evaporation of the solvent left a solid residue which was crystallized from pentane to yield 5.49 g (93%) of **4**. mp 127—128°C. Anal. $C_{23}H_{33}NO_2$ (C, H, N, O). ^1H NMR: disappearance of the methoxyl signal at 3.84 ppm, 2 OH exchangeable with D_2O at 8.15 ppm.

3-Dimethylaminomethyl-hexestrol **5**
By the same procedure, the Mannich base **3** yielded 91% **5**. mp 135—136°C (CH_2Cl_2—pentane). Anal. $C_{21}H_{29}NO_2$ (C, H, N, O). ^1H NMR: disappearance of the methoxyl signal at 3.82 ppm, 2 OH at 7.85 ppm exchangeable with D_2O.

3-Acetoxymethyl-hexestrol diacetate **6**
Acetylative deamination of **4** was carried out by a modification of the published procedure for phenolic Mannich bases [15, 16]. An acetylating mixture was prepared by dissolving at 130°C 2 g of anhydrous sodium acetate in 20 ml of acetic anhydride and 4 ml of acetic acid. After cooling, 860 mg of **4** were added and heated for 6 h at 120—130°C under nitrogen. Ether extraction, Na_2CO_3 washing, drying and evaporation of the solvent left a product which was crystallized in methanol giving 800 mg of **6** (yield: 78%). mp 88—88.5°C. Anal. $C_{25}H_{30}O_6$ (C, H, O). IR (film): $\nu_{C=O}$ 1740, 1750 and 1770 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.55 (6H, *t*, CH_3CH_2); 1.38 (4H, *m*, CH_3CH_2); 2.07 (3H, *s*, CH_3-$COOCH_2$); 2.16 and 2.19 (6H, two *s*, CH_3COOAr); 2.60 (2H, *m*, CHEt); 5.08 (2H, *s*, $ArCH_2$); 6.98—7.30 (7H, *m*, arom).

3-Hydroxymethyl-hexestrol **7**
To a stirred slurry of 0.6 g (15.8 mmol) of $LiAlH_4$ in dry ether, cooled in an ice bath, an ethereal solution of 3.0 g (7 mmol) of **6** was added dropwise. After 1 h at 20°C, the excess hydride was destroyed with ethyl acetate and the reaction mixture was acidified with dilute HCl and extracted with ether. After washing with water, solvent evaporation leaves a quantitative yield of **7**, mp (benzene) 122—123°C. Anal. $C_{19}H_{24}O_3$ (C, H, O). IR (film): ν_{OH} 3500—3300 (broad) cm^{-1}, disappearance of the C=O bands. ^1H NMR [$CDCl_3$—DMSO-d6 (3:1)]: disappearance of the signals CH_3CO; δ 4.78 (2H, *s*, $ArCH_2$); 8.2 (3H, large, OH, exchangeable with D_2O).

Methiodide of 3-dimethylaminomethyl-hexestrol **9**
1.25 g of the Mannich base **5** in dry ether were reacted with 3 ml of methyl iodide overnight at room temperature with magnetic stirring. The white precipitate was filtered and washed with dry ether. Yield: 1.7 g (94%). mp (dec.) 210°C. ^1H NMR [$CDCl_3$—DMSO-d6 (3:1)]: δ 3.15 (9H, *s*, $^+NMe_3$); 4.55 (2H, *s*, $ArCH_2$).

3-Acetoxymethyl-hexestrol **8**
By acetylation of 3-hydroxymethyl-hexestrol **7**. A solution of 0.5 g (1.66 mol) of **7** in 10 ml of glacial acetic acid was treated with 0.3 ml (2 mol. equiv.) of acetic anhydride and 5 mg (0.02 mol. equiv.) of *p*-toluene sulfonic acid monohydrate overnight at room temperature. Ether extraction, bicarbonate washing, chromatography on silicagel and crystallization from benzene yielded 230 mg (40%) of **8**. mp 130—131°C. Anal. $C_{21}H_{26}O_4$ (C, H, O). IR (film): $\nu_{C=O}$ 1700; ν_{OH} 3450—3200 cm^{-1}. ^1H NMR (CD_3COCD_3): δ 0.52 (6H, *t*, CH_3CH_2); 1.35 (4H, *m*, CH_3CH_2); 2.03 (3H, *s*, CH_3CO); 2.50 (2H, *m*, CHEt); 5.14 (2H, *s*, $ArCH_2$); 6.75—7.15 (7H, *m*, arom); 8.07 and 8.32 (2H, *s*, OH).

By deamination of the methiodide **9**. 1.4 g of **9** and 2 g of anhydrous sodium acetate in 20 ml of glacial acetic acid were refluxed for 6 h. Usual work-up gave 0.74 g (74%) of **8**, identical (mp, CCM, IR, NMR) to the above preparation.

3-Methoxymethyl-hexestrol **10**
450 mg of the methiodide **9** were refluxed overnight with 10 mol. equiv. of sodium methoxide in methanol. Ether extraction and crystallization from benzene yielded 200 mg (69%) of **10**, mp 146—147°C. Anal. $C_{20}H_{26}O_3$ (C, H, O). ^1H NMR [$CDCl_3$—DMSO-d6 (3:1)]: disappearance of the $^+NMe_3$ signal; δ 3.35 (3H, *s*, OCH_3); 4.55 (2H, *s*, $ArCH_2$).

3-Dimethylaminomethyl-hexestrol N-*oxide* **11**
3.13 g of the Mannich base **5**, dissolved in 45 ml of methanol, were reacted with 6 ml of 30% H_2O_2 for three days. The excess of hydrogen peroxide was destroyed by stirring with palladium on charcoal and the product was extracted with chloroform. Evaporation of the solvent and crystallization from isobutanol—pentane yielded 2.62 g (81%) of **11**: mp 172—174°C; Anal. $C_{21}H_{29}NO_3$ (C, H, N, O). ^1H NMR [$CDCl_3$—DMSO-d6 (3:1)]: δ 0.53 (6H, *t*, CH_3CH_2); 1.32 (4H, *m*, CH_3CH_2); 2.42 (2H, *m*, CHEt); 3.20 (6H, *s*, CH_3N); 4.55 (2H, *s*, $ArCH_2$); 6.65—7.12 (9H, *m*, arom and OH exchangeable with D_2O).

3-Formyl-hexestrol **12**
To 1 g of **11** dissolved in 20 ml of dichloromethane and cooled in an ice bath 3.7 g (6 mol. equiv.) of trifluoroacetic anhydride were added dropwise. The mixture was left for 2 h at room temperature. The iminium trifluoroacetate and excess reagent were hydrolyzed by adding iced sat. $NaHCO_3$ to pH 8 and stirring overnight. CH_2Cl_2 extraction and crystallization from ethanol—water yielded 780 mg (90%) of **12**. mp 144—145°C. Anal. $C_{19}H_{22}O_3$ (C, H, O). ^1H NMR [$CDCl_3$—DMSO-d6 (3:1)]: δ 0.57 (6H, *t*, CH_3CH_2); 1.35 (4H, *m*, CH_3CH_2); 6.78—7.38 (7H, *m*, arom); 9.90 (1H, *s*, ArCHO); 10.8 (2H, *m*, OH exchangeable with D_2O).

Ethyl hexestrol-coumarin-3''-carboxylate **13**
500 mg (1.67 mmol) of **12**, 320 mg (2.0 mmol) of ethyl malonate, 40 μl of piperidine and 4 μl of glacial acetic acid in 2 ml of absolute ethanol were refluxed for 3 h. After cooling the product was precipitated with water and recrystallized from ethanol—water, yielding 625 mg (95%) of **13**. mp 183—184°C. Anal. $C_{24}H_{26}O_5$ (C, H, O). ^1H NMR [$CDCl_3$—DMSO-d6(3:1)] δ 0.57 (6H, *t*, CH_3CH_2CH); 1.42 (7H, *m*, CH_3CH_2CH + CH_3CH_2O); 2.58 (2H, *m*, CHEt); 4.40 (2H, *q*, CH_3CH_2O); 6.76—7.02 (4H, 2*d*, disubst. arom ring); 7.27—7.54 (3H, *m*, trisubst. arom ring); 8.58 (1H, *s*, ArCH=).

Hexestrol-coumarin-3''-carboxylic acid **14**
300 mg (0.76 mmol) of **13** were hydrolyzed by a 5 fold excess of NaOH, 0.2 N (final concentration), in ethanol—water (4:1) for 1 h in a boiling water bath. Acidification of the yellow solution by HCl N precipitated 234 mg of pure **14**, mp 201—202°C. ^1H NMR [$CDCl_3$—DMSO-d6 (3:1)]: disappearance of the CH_3CH_2O and CH_3CH_2O signals; δ 8.5 (2H, broad, OH exchangeable with D_2O); 8.78 (1H, *s*, ArCH=).

Hexestrol-3''-imino-2''-cyanocoumarin **15**
600 mg (2 mmol) of 3-formyl-hexestrol **12** were dissolved in 3 ml anhydrous ethanol and one drop of piperidine was added. To this stirred solution cooled in an iced water bath, 160 mg (2.4 mmol) of malononitrile dissolved in 2 ml of anhydrous ethanol were added dropwise. After 2 h of stirring at 0—5°C, the precipitate was filtered and washed with a small amount of anh. ethanol, yielding 460 mg (67%) of **15**, mp 141—142°C. No satisfactory elemental analysis could be obtained for this compound. ^1H NMR showed the presence

of impurities. Mass spectrum (desorption/chemical ionization (NH_4^+)): *m/z* (relative abundance, fragment ion) 365 (16, $MH^+ + NH_4^+$); 347 (100, MH^+); 212 (17, $C_{13}H_{11}N_2OH^+$); 197 (4, $C_{13}H_{10}NOH^+$); 135 (16, $C_9H_{11}O^+$); 107 (7, $C_7H_7O^+$).

Binding to estrogen receptor

Relative binding affinity (RBA)

Biochemical assay [2, 18]. Immature rat uterine cytosol was incubated at 18°C for 30 min with 5×10^{-9} M [^{3}H] estradiol in the absence and presence of increasing amounts (10^{-8}—10^{-5}M) of a test compound (**8**, **13**, **14** or **15**) or unlabeled estradiol (control). Unbound compounds were then removed by a dextran-coated charcoal treatment and the amount of remaining radioactivity was measured. The relative concentrations of unlabeled estradiol and test compound required to achieve 50% inhibition of [^{3}H] estradiol binding gave the RBA:

$$\text{RBA} = \frac{(I_{50})\ \text{estradiol}}{(I_{50})\ \text{test compound}} \times 100$$

Whole cell assay [17]. MCF-7 cells were incubated at 37°C for 50 min with 10^{-9} M [^{3}H]estradiol in the absence and presence of increasing amounts (10^{-10}—10^{-5} M) of the test compound or unlabeled estradiol (control). Bound compounds were then extracted with ethanol and the amounts of estrogen receptor bound [^{3}H]estradiol were measured. The RBA values were calculated as for the biochemical assay.

Reversibility of binding [2]. Immature rat uterine cytosol was incubated overnight at 18°C with 5×10^{-9} M [^{3}H]estradiol in the absence and presence of a 20-fold excess of a test compound. After removal of unbound ligands by a dextran-coated charcoal treatment, bound radioactivity was measured. Fractions of these labeled cytosols were then incubated at 18°C with [^{3}H]estradiol to allow [^{3}H]estradiol to exchange with the reversibly bound ligands. After 7 h of incubation, unbound ligands were again removed by a dextran-coated charcoal treatment and the remaining radioactivity measured.

Effect on MCF-7 cell growth [19]

MCF-7 cells were plated in three 96-multiwell Falcon dishes (plating densities: 5, 10 or 20 $\times$ 10^3 cells/ml). After 24 h of culture, a test compound (**13**, **14** or **15**) was added to one of these dishes. Estradiol was added to some wells to evaluate its potential agonistic or antagonistic effect. Compounds were at the following final concentrations: estradiol: 10^{-8} M; test compound: 10^{-8}, 10^{-7}, 10^{-6} M. After 5 days of culture, the monolayer was fixed and stained with hematoxylin. The intensity of the coloration, which is a measure of the cell number, was then assessed with a multiscan spectrophotometer at $\lambda = 540$ nm (Flow Laboratories Inc.).

Acknowledgements

Biological studies were supported by a grant from the Fonds Cancérologique de la Caisse générale d'Epargne et de Retraite de Belgique.

References

1 Leclercq G., Devleschouwer N. & Heuson J. C. (1983) *J. Steroid Biochem.* 19, 75—85 (and references therein)
2 Leclercq G., Devleschouwer N., Legros N. & Heuson J. C. (1980) *in: Cytotoxic Estrogens in Hormone Receptive Tumors* (Raus J., Martens H. & Leclercq G., eds.), Academic Press, London, pp. 165—181
3 Vilkas M., Epsztein R., Sainton J. & Leclercq G. (1982) *Eur. J. Med. Chem. - Chim. Thér.* 17, 191—192
4 Turner A. B. (1964) *Quart. Rev.* 18, 347—360
5 Wakselman M. (1983) *Nouv. J. Chim.* 7, 439—447
6 Vilkas M. (1978) *in: Enzyme-activated Irreversible Inhibitors* (Seiler N., Jung M. J. & Koch-Weser J., eds.), Elsevier, Amsterdam, pp. 323—335
7 Vilkas M. & Masamba L. (1984) *C. R. Acad. Sci.* 299, II, 1137—1139
8 Bretschneider H. & Ajtai N. (1941) *Chem. Ber.* 74, 571—583
9 Wakselman M. (1966) *C. R. Acad. Sci.* 262, C, 770—773
10 Gardner P. D., Rafsanjani H. S. & Rand L. (1959) *J. Am. Chem. Soc.* 81, 3364—3366
11 Ahond A., Cavé A., Kan-Fan C., Husson H.-P., de Rostolan J. & Potier P. (1968) *J. Am. Chem. Soc.* 90, 5622—5623
12 Michelot R. (1969) *Bull. Soc. Chim. France* 4377—4385
13 Horning E. C., Horning M. G. & Dimmig D. A. (1955) *Org. Synth. Coll.* Vol. 3, pp. 165—167
14 Baker W. & Howes C. S. (1953) *J. Chem. Soc.* 119—124
15 Bruson H. A. & McMullen C. W. (1941) *J. Am. Chem. Soc.* 63, 270—272
16 Miller J. B., Fields D. L. & Reynolds D. B. (1965) *J. Org. Chem.* 30, 247—251
17 Olea-Serrano N., Devleschouwer N., Leclercq G. & Heuson J. C. (1985) *Eur. J. Cancer Clin. Oncol.* 21, 965—973
18 Leclercq G., Deboel M. C. & Heuson J. C. (1976) *Int. J. Cancer* 18, 750—756
19 Maddedu L., Roy F. & Leclercq G. (1986) *Anticancer Res.* 6, 11—16