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X-Ray Crystal Structure of 10β-Hydroxy-4β,5β-epoxyestr-1-en-3,17-dione and Antitumor Activity of its Congeners

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Abstract: Based on the biological properties of epoxyquinols from natural sources, the title compound was synthesised as a potential antitumor agent. Its molecular structure was partially confirmed by NMR studies. The detailed structure was established by X-ray analysis revealing two symmetry independent molecules in the asymmetric unit each consisting of four fused rings with the C(10) β-oriented hydroxy group and β-oriented O atom bridging C(4) and C(5). The conformation of A ring in both conformers **A** and **B** is *boat* ($B_{3,6}$), while rings B and C are *chairs* ($^1C_4$) and the five-membered D ring is in an *envelope* (E_2) conformation. The *in vitro* antitumor activity of title compound and its 17β-acetoxy analogue against HeLa and Fem-x cells revealed IC_{50} values of 5.7 and 7.1 μM, and 2.25 and 1.58 μM, respectively. Corresponding quinols were tested on 47 cell lines with 10β-hydroxy-17β-acetoxyestra-1,4-dien-3-one being most active against leukemia SR cells (GI_{50} = 0.17 μM).

Keywords: Steroids, epoxyquinol, quinol, X-ray crystallography, antitumor activity.

Introduction

A large number of natural as well as synthetic compounds with an epoxyquinol moiety show various biological activities (Figure 1) [1], being at the same time useful intermediates in the synthesis of cyclitols (conduritols, inositols) [2], which are also of biological importance [3].

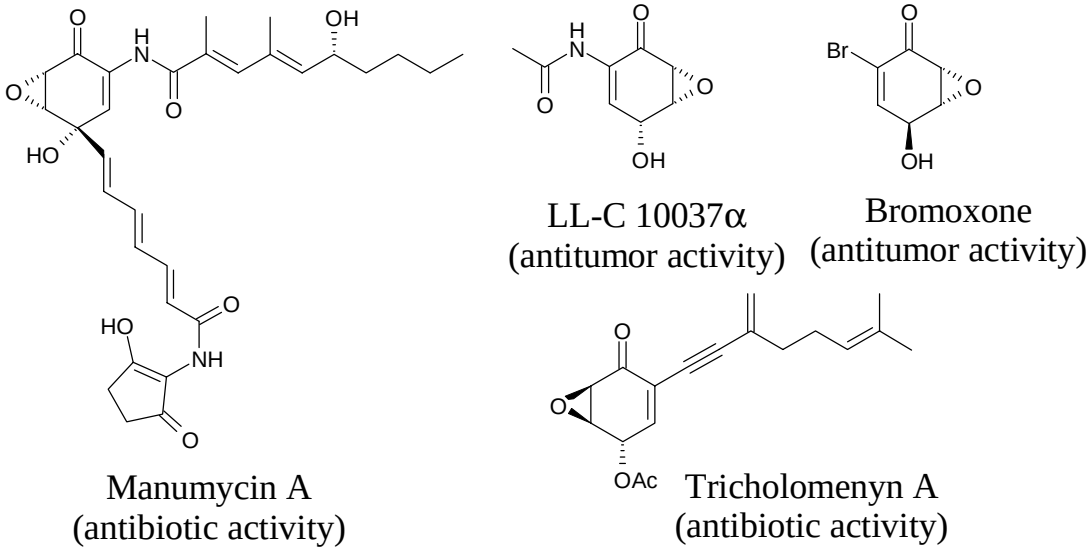
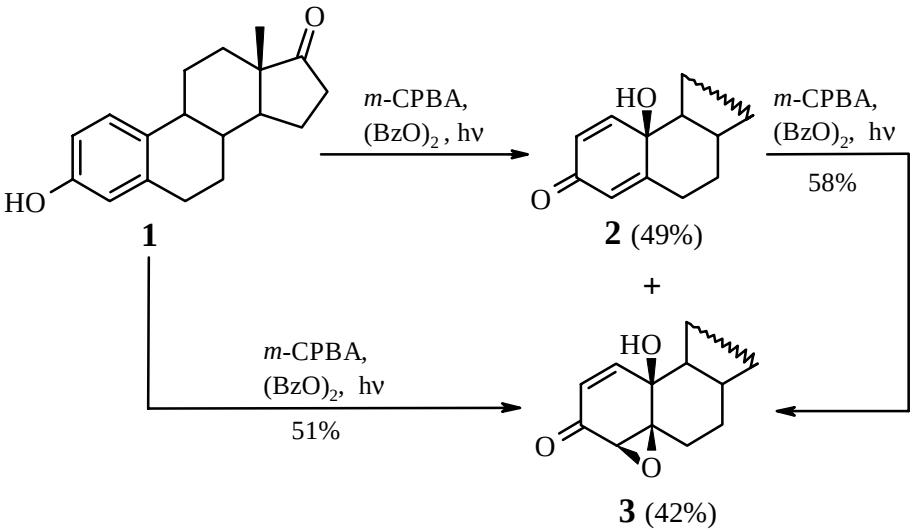


Figure 1.

The incorporation of such substructures into the steroidal framework could lead to synthetically and biologically important compounds [4]. Synthesis of the steroidal epoxyquinol **3** (along with quinol **2**) was achieved in good yield [5], but no direct determination of configuration of the epoxide ring in **3** has been made [6].



Scheme 1.

The determination of the configuration of **3** was undertaken as a part of our program aimed at fully characterising these and related compounds in an attempt to synthetically expand the structural frameworks of natural compounds in an analogous way for further structure-activity studies.

Here we present the structure determination of epoxyquinol **3** and *in vitro* antitumor activity of **3** and of 17β-acetoxy analogue **5** (Figure 5) against HeLa and Fem-x cell lines. The antitumor activity of corresponding quinols **2** and **4** will also be presented.

Results and discussion

Synthesis

The epoxyquinol **3** (Scheme 1) was synthesised either from estrone **1** (two step one-pot reaction) or from quinol **2**, using the oxidation system consisting of *m*-CPBA / $(BzO)_2$ / hν as described in [4], [5], [9].

Structure Elucidation

The constitution of the epoxyquinol **3** (mp = 201-203°C (colourless needles, benzene)) was derived from the following data: $[\alpha]_D^{24}$ = +294 (c = 1.0, chl.). IR (KBr) cm^{-1}: 3359(s), 2955(w), 1725(s), 1686(s), 1152(w). MS (CI, isobutane, m/e): 303 (MH^+). λ_{max}^{MeOH}=234 nm(8200). Anal. calc. for $C_{18}H_{22}O_4 \times 2/3\ C_6H_6$ (354.45): C 74.55, H 7.39, found: C 74.61, H 7.12.

Molecular modelling indicated that the configuration of oxirane ring cannot be determined using NOE difference spectroscopy: in both α and β configuration of the epoxide, the distance between H-C(4) and all other NOE-related protons (O*H*, H_α-C(6) and H_β-C(6) is less than 2.7 Å). Therefore we determined the structure of **3** by X-ray analysis.

X-ray crystallography

Crystals obtained by slow evaporation of a benzene solution at room temperature were studied by X-ray diffraction procedures. Selected geometrical parameters, bond distances, bond angles and torsion angles are listed in Table 6 according to the numbering scheme displayed in Figure 2.

Perspective view of the crystal unit of **3** is depicted in Figure 2 [15]. The structure consists of two symmetry independent molecules in the asymmetric unit, each with four fused rings with β oriented hydroxy group, and β-oriented O atom bridging C(4) and C(5) atoms.

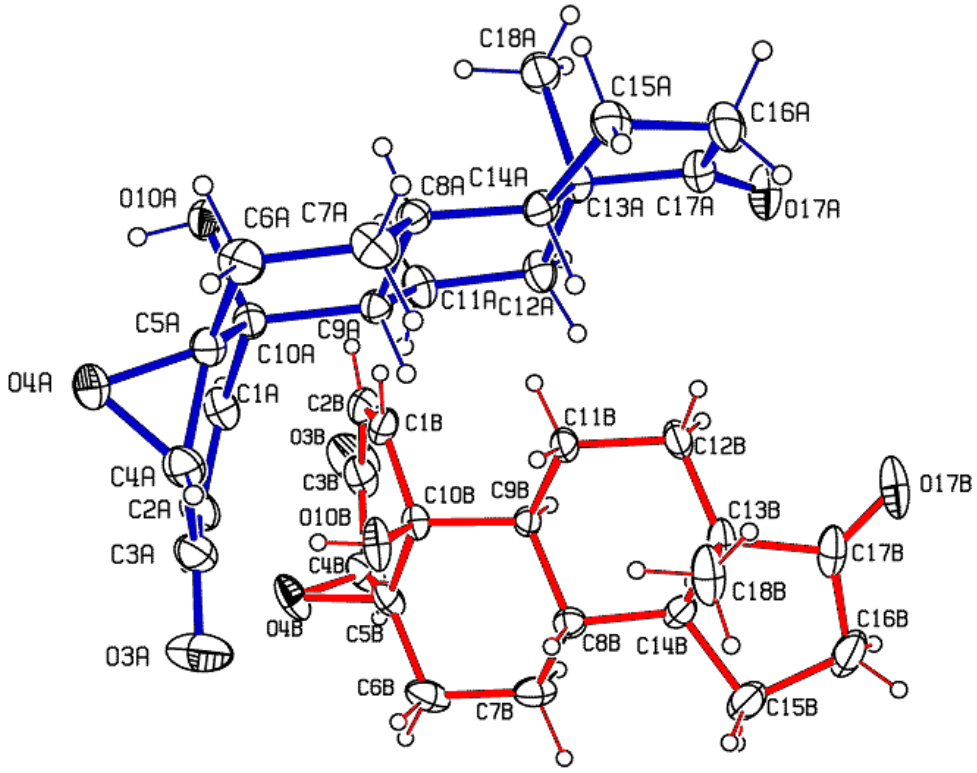


Figure 2. ORTEP [15] drawing of the molecules **A** (blue) and **B** (red) with the non-H atom labeling scheme. The displacement ellipsoids are drawn at 25% probability.

Geometrical analysis confirmed the existence of two C-O double bonds, with distances C(3)-O(3) = 1.217(4)Å (**A**), 1.217(4)Å (**B**) and C(17)-O(17) = 1.212(3) Å (**A**), 1.212(4) Å (**B**) for molecules **A** and **B**, respectively. The analysis of the steroid rings by calculating puckering parameters [7] and asymmetric factors [8] (Table 1) has shown that the symmetry independent molecules **A** and **B** are of very similar conformations. This was also confirmed by fitting the molecules into each other.

The A ring in both molecules is in boat $B_{3,6}$ conformation, B and C steroid rings are chairs with $^1C_4$ conformations, while the five membered D ring is in envelope E_2 conformation. The energetically more stable conformation of steroid rings in the molecule under study, compared to the methoxyquinone derivative [9], indicate a less strained and hence lower energy conformation for this compound. The values of relevant torsion angles [O(10)A(B)-C(10)A(B)-C(5)A(B)-O(4)A(B) = 75.7(3)° (**A**), 74.4(3)° (**B**)] reveal the cis junction between A/B rings, trans junction [H(9)A(B)-C(9)A(B)-C(8)A(B)-H(8)A(B) = -177.7(2)° (**A**), 177.9(2)° (**B**)] between B/C rings and [C(18)A(B)-C(13)A(B)-C(14)A(B)-H(14)A(B) = -179.5(2)° (**A**), 180.0(2)° (**B**)] between C/D steroid rings. The best plane through the rings B and C in the molecule **A** form an angle of 71.35° with the corresponding plane of the molecule **B** (Figure 2).

Table 1. Puckering parameters and asymmetry factors.

ring	mole-cule	Q(Å)	φ(°)	θ(°)	fCs(Å)	fC_2(Å)	$^{c}N_d$
A	A	0.267(3)	117.6(6)	96.3(6)	0.011(1)[C3A] 0.026(1)[C1A-C2A]	0.061(2)[C1A] 0.079(3)[C2A]	$B_{3,6}$
	B	0.215(3)	121.4(8)	102.6(8)	0.0050(1)[C3B] 0.038(2)[C1B-C2B]	0.060(3)[C1B] 0.057(3)[C2B]	$B_{3,6}$
B	A	0.566(3)	203(5)	3.6(3)	0.011(1)[C5A] 0.017(1)[C6A] 0.035(1)[C7A]	0.0030(1)[C5A-C6A] 0.027(1)[C6A-C7A] 0.023(1)[C7A-C8A]	$^{1}C_4$
	B	0.553(3)	151(6)	2.9(3)	0.011(1)[C5B] 0.023(1)[C6B] 0.015(1)[C7B]	0.020(1)[C5B-C6B] 0.020(1)[C6B-C7B] 0.0010(1)[C7B-C8B]	$^{1}C_4$
C	A	0.571(3)	270(3)	5.0(3)	0.041(1)[C8A] 0.021(1)[C9A] 0.032(1)[C11A]	0.035(1)[C8A-C9A] 0.000(1)[C9A-C11A] 0.035(1)[C11A-C12A]	$^{1}C_4$
	B	0.586(3)	316(4)	3.8(3)	0.022(1)[C8B] 0.031(1)[C9B] 0.011(1)[C11B]	0.031(1)[C8B-C9B] 0.023(1)[C9B-C11B] 0.008(1)[C11B-C12B]	$^{1}C_4$
D	A	0.428(4)	29.4(5)		0.050(1)[C14A]	0.065(1)[C16A]	E_2
	B	0.422(4)	32.2(6)		0.028(1)[C14B]	0.080(2)[C16B]	E_2

Table 2. Geometry (Å,°) of hydrogen-bond nets and short contact bridged by an hydrogen atom.

X – H…Y		X– H [Å]	H…Y [Å]	X…Y [Å]	X – H…Y [°]
O(10) – H(10)…O(17)^{i}	**A**	0.818(2)	2.100(2)	2.873(3)	157.5(1)
	B	0.821(2)	1.963(2)	2.779(3)	172.2(2)
C(16) – H(162)…O(4)^{ii}	**A**	0.965(3)	2.521(2)	3.411(4)	153.3(2)
	B	0.972(4)	2.356(3)	3.201(5)	145.0(2)
C(9A) – H(9A)…..O(3B)^{iii}		0.981(2)	2.373(3)	3.342(4)	169.4(1)
C(2A) – H(2A)…..O(17B)^{i}		0.934(3)	2.691(3)	3.406(4)	133.9(2)
C(2B) – H(2B)…..O(10B)^{v}		0.933(4)	2.663(2)	3.447(4)	142.0(2)
C(4) – H(4)……..O(10)^{iv}	**A**	0.980(3)	2.664(2)	3.637(3)	172.0(2)

Symmetry code: (i) x–1, y, z (ii) x+1,y,z (iii) –x,y-1/2,-z (iv) -x,y-1/2,-z+1 (v) -x,y+1/2,-z.

Analysis of the molecular packing in the unit cell revealed a weak C-H…O intermolecular hydrogen contacts (Table 2) and a very strong intermolecular hydrogen bond [O(10) - H(10)…O(17)^{i} = 2.873(3)Å in molecule **A** and 2.779(3) Å in molecule **B**]. The above mentioned strong hydrogen bonds

lead to the creation of the chains consisting of only one type of molecules, **A** or **B**. These chains are stacked alternatively along *a*-axis and bonded by weaker C-H….O type bonds, which results in a layered packing of the molecules parallel to the *ab* plane (Figure 3).

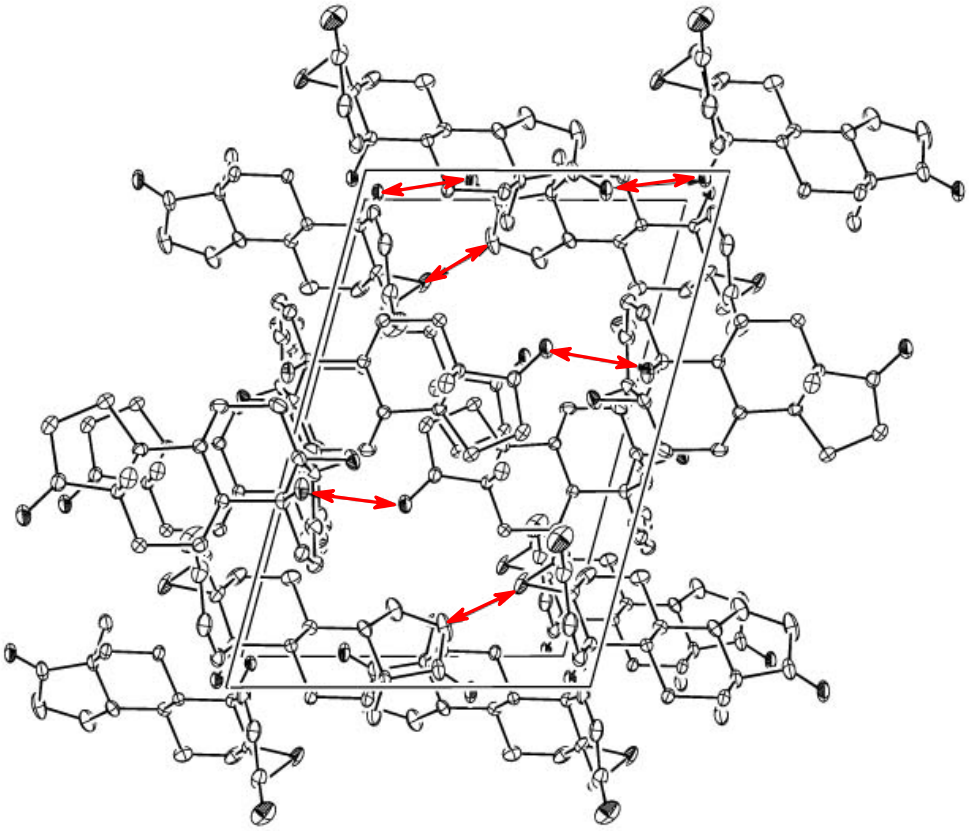


Figure 3. The crystal packing for the unit cell projected along the *b*-axis. The arrows denote hydrogen bonds (two strongest ones).

NMR analysis

Appearance of the signal at 3.33 ppm (d, J = 2.2 Hz) in ^{1}H NMR spectrum indicates the existence of oxirane moiety in compound **3** (H-C(4)). Doublet at 6.68 ppm (J = 10.6 Hz) belongs to H-C(1), which is coupled to H-C(2); consequently, doublet of doublets at 5.77 ppm (J = 10.6, 2.2 Hz) is assigned to H-C(2), and its J-values are a measure of coupling to H-C(1) ("ortho"-H) and H-C(4) (long-range W scalar coupling through 4 σ-bonds including the carbonyl function). In addition, connection of signals at 6.68, 5.77 and 3.33 ppm was confirmed by HOMO decoupling experiments: irr. at 6.68 ppm → 5.77 ppm, d, J = 2.2 Hz; irr. at 5.77 ppm → 6.68 and 3.33 ppm, both s; irr. at 3.33 ppm → 6.68 and 5.77 ppm, both d, J = 10.6 Hz. Singlet at 5.79 ppm, exchangeable with D_2O, belongs to O*H*, and the one at 0.81 ppm to the angular methyl group hydrogens.

a) b)

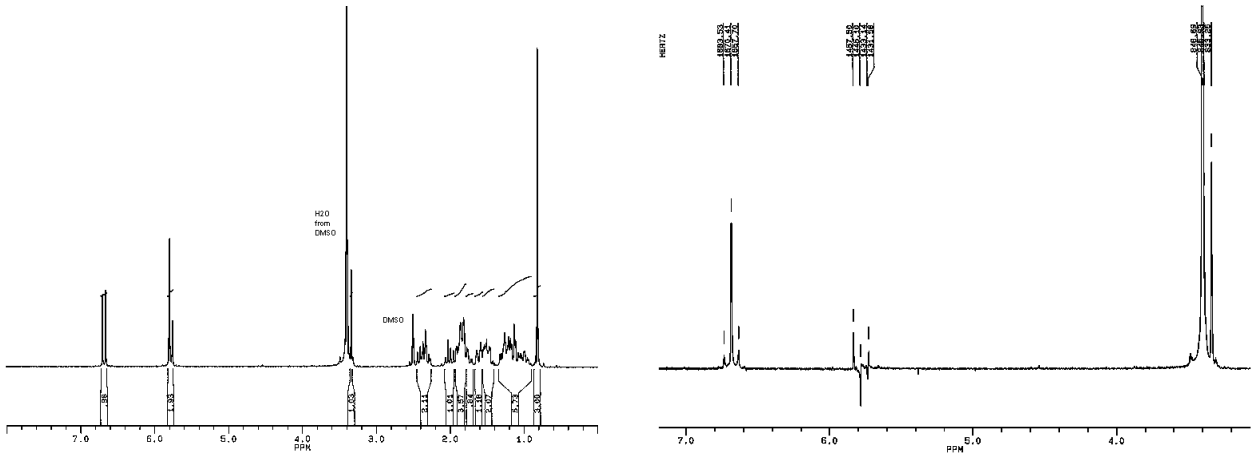


Figure 4. a) ^{1}H NMR spectrum of compound **3**; b) HD ^{1}H NMR spectrum (irradiated signal at 5.77 ppm) of compound **3**.

a) b) c)

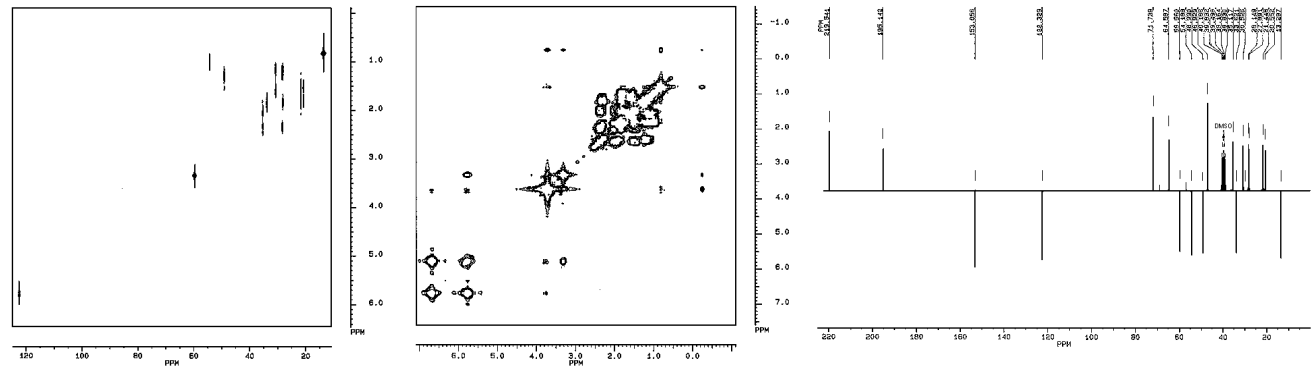


Figure 5. Selected spectra of epoxyquinol **3.**
a) C,H-COSY; b) H,H-COSY; c)^{13}C NMR-DEPT.

Assignation of selected spin-active nuclei is obtained by using comparative analysis of DEPT ^{13}C and 2D correlated NMR spectra (H,H and C,H COSY) (Table 3).

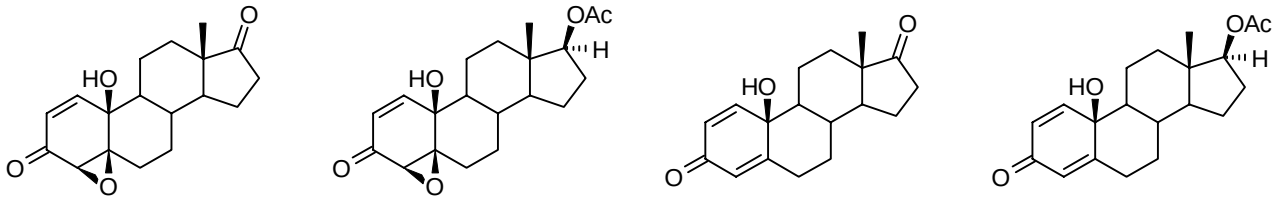
Table 3. C- and H-chemical shifts of 10β-hydroxy-4β,5β-epoxyestr-1-en-3,17-dione (**3**; DMSO-d_6).

Position	δ_C (ppm)	δ_H (ppm)
1	153.06	6.68 (d, *J* = 10.6)
2	122.33	5.77 (dd, *J* = 10.6, 2.2)
3	195.14	-
4	59.56	3.33 (d, *J* = 2.2)
5	64.59	-
6	28.15	2.32[a] (m, H_β), 1.24 (m, H_α)
8	33.62	1.87 (m, H_β)
9	54.19	1.03 (m)
10	71.74	-
13	46.93	-
17	219.54	-
18	13.30	0.81 (s)
O*H*	-	5,79 (s)

[a] Values for presented multiplets are mean of signal in C,H COSY spectrum.

Biochemistry

In this work, the antiproliferative action of synthetic compounds **3** and **5** derived from estrone on two human neoplastic cell lines, HeLa and Fem-x, *in vitro*, was determined. Presented are also the activities [10] of corresponding quinols **2** and **4** (Figure 6).



3 **5** **2** **4**

Figure 5.

The epoxyquinols **3** and **5** expressed the dose-dependent antiproliferative action toward investigated cell lines. In order to compare the extent of the antiproliferative action between neoplastic cell lines of different origin, IC_{50} were determined under the same conditions by MTT test. They were very similar for HeLa and Fem-X cells and mean values from three independently performed experiments for 48 h of continuous agent's action (epoxyquinols **3** and **5**, respectively) were 5.7±1.3 and 2.25±0.35 μM for

HeLa, and 7.1 ± 2.25 and 1.58 ± 0.04 μM for Fem-x cells (Table 4). Morphological examination of target cells by inverted microscope showed that the cytotoxic action resulted in cells rounding in the presence of agent in concentration higher than 6 μM and / or cell ballooning in the concentration higher than 12 μM.

Table 4[a]**.** *In vitro* antitumor activity of epoxyquinols **3** and **5**, and quinols **2** and **4**.

Compound	IC_{50} (μM) [b]		GI_{50} (μM) [c]	
	HeLa	Fem-x	Leukemia SR	Leukemia CCRF-CEM
3	5.73	7.10		
5	2.25	1.58		
2			74.30	26.40
4			0.17 [d]	11.90

[a] 48 h of continuous agent's action; [b] IC = Inhibition concentration; [c] GI = Growth inhibition. Out of 47 cell lines tested, presented are only the ones where highest activity of **2** and **4** was established; [d] TGI (Total GI) = 1.63 μM, LC_{50} > 100 μM.

The title compounds **2** and **4** were *in vitro* tested by NCI at a minimum of five concentrations at 10-fold dilutions. A 48 hours continuous drug exposure protocol is used, and a SRB (sulforhodamine B) protein assay is used to estimate cell survival or growth. The GI_{50} is interpolated value representing concentration at which the percentage growth is +50. Presented results indicate that quinol **2** is moderately active against both leukemia cell lines, while analogue compound **4** is much more selective.

Both compounds, **2** and **4**, were also tested for their antiviral activity. The procedure used in the NCI's test for agents active against HIV virus is designed to detect agents acting at any stage of the virus reproductive cycle. The assay involves the killing of T4 lymphocytes by HIV. Small amounts of HIV are added to cells, and two cycles of virus reproduction are necessary to obtain the required killing. Agents that interact with virions, cells or virus gene-products to interfere with viral activities, will protect cells from cytolysis. All tests are compared with at least one positive (AZT-treated) control performed at the same time under identical conditions. Both quinols are confirmed inactive, having $IC_{50} > 2\times10^{-4}$ M against CEM-SS cell line.

Although the two pairs of compounds, epoxyquinols **3** and **5** and quinols **2** and **4**, were tested on different cell lines, one observation merits comment: In both cases estradiol derived compounds **4** and **5** (possessing 17β-acetoxy group instead of 17-oxo functionality as in **2** and **3**) exhibited stronger activity towards target cell lines. Compound **4** is also highly active against melanoma SK-MEL-5 cell lines with GI_{50} = 3.14 μM (quinol **2**, GI_{50} = 82.0 μM).

Our further investigation in this area will be focused to the synthesis and antitumor activity evalua-

tion of the influence of the oxirane ring position and its stereochemistry, as well as on the influence of C(17) substituent in estrane derived quinols.

Experimental

General

Melting points were determined on a Mikro-Heiztisch Boetius PHMK apparatus and were not corrected. IR and UV spectra were recorded on Perkin-Elmer FT-IR 1725X and Beckman DU-50 spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AM-250 spectrometer. Chemical shifts were expressed as ppm (δ) values and coupling constant (*J*) in Hz. Mass spectra were taken on a Finnigan-MAT 8230 spectrometer.

All compounds were synthesised and the relevant data are presented in refs. [4], [5] and [9].

X-ray crystallography

A colourless plate-like crystal of dimensions 0.5×0.3×0.2 mm was mounted on an STOE four circle diffractometer equipped with CuKα radiation and Ni filter. Cell parameters (Table 5) were determined by least-squares refinement of diffractometer angles for 59 reflections collected in the range $15.0 \leq \theta \leq 31.0^{o}$. Three standard reflections were monitored every 90 min but no considerable intensity variations were recorded. Reflections were recorded with 2θ-ω scan in the range $3.19 \leq \theta \leq 65.04^{o}$ and with Miller indices hmin= -11, hmax= 11, kmin= -1, kmax= 14, lmin= -16, lmax= 16. 2954 independent reflections were merged with R = 0.0155 to 2822 unique reflections. The intensity data were corrected for Lorentz and polarisation effect.

The structure was solved by direct methods using SHELX86 [11] and SIR92 [12] program and refined by anisotropic full-matrix least squares on F^2 (SHELXL93 [13]). The position of the H atoms were generated from assumed geometry, checked on a difference Fourier map and refined isotropically with common displacement parameter U_1=0.056(2) Å^2, U_2=0.066(4) Å^2 and U_3=0.063(2) Å^2, U_4=0.100(7) Å^2 for all H atoms except H atoms attached to C(15)A(B), C(16)A(B) (peripheral atoms) for molecules **A** and **B,** respectively and methyl type atoms C(18)A(B) with isotropic displacement parameters fixed to 1.5 U_{eq} of the parent atoms. Final R-factor was R = 0.0308 for 404 parameters and 2954 reflections. Scattering factors were taken from SHELXL93. Conformational calculations were performed using the program RING [14]. Molecular graphics were drawn using ORTEP-III [15].

Table 5. Crystal data and structure refinement.

Empirical formula	$C_{18}H_{22}O_4$
Formula weight	302.36
Temperature	293(2) K
Wavelength	1.54178 A
Crystal system	Monoclinic
Space group	$P2_1$
Unit cell dimensions	a = 9.745(5Å α = 90 ° b = 12.071(5) Å β = 105.050(5) ° c = 14.342(5) Å γ = 90 °
Volume	1629.2(12) $Å^3$
Z	4
Density (calculated)	1.233 Mg/m^3
Absorption coefficient	0.699 mm^{-1}
F(000)	324
Crystal size	0.5 × 0.3 × 0.2 mm
Theta range for data collection	3.19 to 65.04 °
Index ranges	$-11 \le h \le 11$, $-1 \le k \le 14$, $-16 \le l \le 16$
Reflections collected	5499
Independent reflections	2954 [R(int) = 0.0155]
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2954 / 1 / 404
Goodness-of-fit on F^2	1.094
Final R indices [$I>2\sigma(I)$]	R1 = 0.0308, wR2 = 0.0886
R indices (all data)	R1 = 0.0327, wR2 = 0.0900
Absolute structure parameter	0.1(2)
Extinction coefficient	0.0022(4)
Largest diff. peak and hole	0.147 and -0.122 e $Å^{-3}$

Table 6. Selected bond lengths [Å] and angles [°].

O(3A)-C(3A)	1.217(4)	O(3B)-C(3B	1.217(4)
O(4A)-C(5A)	1.441(3)	O(4B)-C(4B)	1.438(4)
O(4A)-C(4A)	1.454(3)	O(4B)-C(5B)	1.441(3)
O(10A)-C(10A)	1.433(3)	O(10B)-C(10B)	1.424(3)
O(17A)-C(17A))	1.210(3	O(17B)-C(17B)	1.212(4)

Continuation of the Table 6.

C(1A)-C(2A)	1.329(4)	C(1B)-C(2B)	1.328(4)
C(1A)-C(10A)	1.504(3)	C(1B)-C(10B)	1.500(4)
C(2A)-C(3A)	1.455(4)	C(2B)-C(3B)	1.450(5)
C(3A)-C(4A)	1.487(4)	C(3B)-C(4B)	1.478(5)
C(4A)-C(5A)	1.475(4)	C(4B)-C(5B)	1.460(4)
C(5A)-C(6A)	1.501(3)	C(5B)-C(6B)	1.498(4)
C(5A)-C(10A)	1.519(3)	C(5B)-C(10B)	1.516(3)
C(13A)-C(18A)	1.541(4)	C(13B)-C(18B)	1.551(4)
C(14A)-C(15A)	1.533(3)	C(14B)-C(15B)	1.528(4)
C(15A)-C(16A)	1.540(4)	C(15B)-C(16B)	1.541(5)
C(16A)-C(17A)	1.510(4)	C(16B)-C(17B)	1.513(6)
C(5A)-O(4A)-C(4A)	61.3(2)	C(4B)-O(4B)-C(5B)	60.9(2)
C(2A)-C(1A)-C(10A)	124.8(2)	C(2B)-C(1B)-C(10B)	125.8(3)
C(1A)-C(2A)-C(3A)	122.2(2)	C(1B)-C(2B)-C(3B)	121.4(3)
O(3A)-C(3A)-C(2A)	123.7(3)	O(3B)-C(3B)-C(2B)	123.1(3)
O(3A)-C(3A)-C(4A)	120.3(3)	O(3B)-C(3B)-C(4B)	120.1(3)
C(2A)-C(3A)-C(4A)	116.0(2)	C(2B)-C(3B)-C(4B)	116.7(2)
O(4A)-C(4A)-C(5A)	58.9(2)	O(4B)-C(4B)-C(5B)	59.6(2)
O(4A)-C(4A)-C(3A)	113.0(2)	O(4B)-C(4B)-C(3B)	112.7(3)
C(5A)-C(4A)-C(3A)	119.7(2)	C(5B)-C(4B)-C(3B)	120.9(2)
O(4A)-C(5A)-C(4A)	59.8(2)	O(4B)-C(5B)-C(4B)	59.4(2)
O(4A)-C(5A)-C(6A)	114.3(2)	O(4B)-C(5B)-C(6B)	114.7(2)
C(4A)-C(5A)-C(6A)	119.1(2)	C(4B)-C(5B)-C(6B)	119.3(2)
O(4A)-C(5A)-C(10A)	115.8(2)	O(4B)-C(5B)-C(10B)	115.6(2)
C(4A)-C(5A)-C(10A)	119.5(2)	C(4B)-C(5B)-C(10B)	119.3(2)
C(17A)-C(13A)-C(18A)	104.8(2)	C(12B)-C(13B)-C(18B)	111.3(2)
C(12A)-C(13A)-C(18A)	111.6(2)	C(17B)-C(13B)-C(18B)	104.2(2)
C(14A)-C(13A)-C(18A)	113.8(2)	C(14B)-C(13B)-C(18B)	114.0(2)
O(17A)-C(17A)-C(16A)	125.1(2)	O(17B)-C(17B)-C(16B)	126.6(3)
O(17A)-C(17A)-C(13A)	126.5(2)	O(17B)-C(17B)-C(13B)	125.5(3)
O(3A)-C(3A)-C(4A)-O(4A)	-129.1(3)	O(3B)-C(3B)-C(4B)-O(4B)	-121.1(3)
O(3A)-C(3A)-C(4A)-C(5A)	164.7(3)	O(3B)-C(3B)-C(4B)-C(5B)	171.7(3)
O(4A)-C(5A)-C(10A)-O(10A)	75.7(2)	O(4B)-C(5B)-C(10B)-O(10B)	74.4(3)
C(18A)-C(13A)-C(14A)-C(8A)	64.6(2)	C(18B)-C(13B)-C(14B)-C(8B)	64.7(3)
C(18A)-C(13A)-C(17A)-O(17A)	-91.7(3)	C(18B)-C(13B)-C(17B)-O(17B)	-91.4(4)

Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no.CCDC - 132357. Copies of the data can be obtained free of charge on application to The Director, CCDC,12 Union Road, Cambridge CB2 1EZ, UK (fax: int.code +(1223) 336-033, E-mail: teched@chemcrys.cam.ac.uk

Biochemistry

Materials and methods

Stock solution of investigated compounds were made in DMSO at a concentration of 10.58 mM, and it was diluted by nutritient medium (RPMI 1640 medium supplemented with l-glutamine (3 mmol/L), streptomycin 100 μg/mL and penicillin 100 IU/mL, 10% heat inactivated fetal bovine serum, FBS and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution) to various final concentrations. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); RPMI 1640 cell culture medium and fetal bovine serum (FBS), were purchased from Sigma Chemicals (St. Luis, MO, U.S.A.). MTT was dissolved, 5 mg/mL in phosphate buffer saline pH 7.2, and filtered through Milipore filter, 0.22 μm, before use.

Cell culture

Human malignant melanoma Fem-x cells, and human cervix carcinoma HeLa cells were maintained as a monolayer culture in the same nutritient medium The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere.

Treatment of Fem-x and HeLa cells

Target cells were seeded (2000 cells in 100 μL of nutrient medium per well), into 96-well micro-titer flat bottomed plates and twenty hours later, five different concentrations of the investigated compound were added to the wells in triplicate, to various final concentrations, except to the control wells where only a nutritient medium was added to the cells. Nutritient medium with corresponding concentrations of compound, but void of cells was used as blank, in triplicate too. The experiment was repeated three times.

Determination of HeLa and Fem-x cell survival

Cell survival was determined as reported earlier [16] by MTT test 48 h after the drug addition. Briefly, MTT solution (5 mg / mL PBS) was added to each well. Samples were incubated for further four hours at 37 °C in 5% CO_2 and humidified air atmosphere. Then, 10% SDS in 0.01 M HCl was

added to the wells. Optical density (OD) at 570 nm was red the next day. To get cell survival (%), optical density at 570 nm of a sample with cells grown in the presence of various concentration of investigated agent (OD), was divided with control optical density ODc, (the OD of cells grown only in nutritient medium) ×100. Concentration IC_{50} was defined as the concentration of a drug needed to inhibit cell survival by 50%, compared with vehicle-treated control.

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Samples Availability: compounds **2**, **3** and **5** are available from MDPI.