ISSN 2321-807X

Scope of Non-estrogenic Steroidal Congeners against Breast Cancer

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Abstract

Various novel non-estrogenic steroidal D-ring substituted analogs were synthesized and evaluated for their breast cancer activity against human breast cancer cell lines. The good cytotoxic results obtained against the breast cancer cell lines throws new insights into the field as the analogs are non 17β –Hydroxy estrogenic derivatives which are considered to be pivotal for the potent estrogenic activity. The synthesized steroidal analogs provide a very good platform for mechanistic studies of the interaction between non-esterogenic steroids and estrogen receptors.

Key Words: Estrogen receptor; Ligand binding domain; Agonist; Antagonist, SERMs

Council for Innovative Research

Peer Review Research Publishing System

Journal: Journal of Advances in Chemistry

Vol. 5, No. 1

editor@cirworld.com

www.cirworld.com, member.cirworld.com

ISSN 2321-807X

Introduction

Cancer, a fatal and devastating disease, needs no introduction. It will be hard to find a person who has not been traumatized by cancer amongst his family and friends. Amongst the women, breast cancer is one of the most common cancers. It is now well established that variour factors lead to the over-expression of estrogen receptors in breast tumour cells and, hence, estrogenicity is enhanced by many folds leading to excessive proliferation [1]. As steroids can regulate a variety of biological processes, they have the potential to be developed as drugs for the treatment of a large number of diseases including cancer [2]. Amongst the anticancer steroids, estrogens hold a potential for breast cancer treatment because of their antagonistic interaction with the Ligand binding domain (LBD) of the estrogen receptors ER-α and ER-β. The ligand receptor complex regulates the transcription of certain genes by binding to response elements in the promoter regions of the genes. The receptor protein activates the transcription machinery by a complex mechanism through the activating functions AF-1 and AF-2 in the estrogen receptor [1]. Broadly there are three types of ligands which bind to LBD but show different pharmacological activities. The three are full agonists such as estradiol, selective estrogen receptor modulators (SERMs) such as raloxifen and the full antagonists such as ICI 182, 780. It is however the last class of ligands i,e the full antagonists which are especially useful for the treatment of breast cancer [3]. The mechanistic studies related to differential ligand receptor interactions of agonists, SERMs and antagonists have been carried out through X-ray crystallography [4, 5].

Known estrogenic compounds contain a hydroxyl group or a hydroxy derivative at the 17-position, particularly 17β –Hydroxy. This is considered to be essential to obtain high binding affinity. The non 17β –Hydroxy analogs have been shown to have low binding affinity or low estrogenic agonistic potency. However various recent reports have concluded that even some non-estrogenic compounds with different D-ring substitution pattern than that mentioned above can have higher binding affinity for the estrogen receptor and can thus eventually be used as antagonists against breast cancers [6]. Thus to check the activity of non estrogenic, non 17β –Hydroxy analogs , we, in continuation of our programme on the synthesis of D-ring substituted steroidal ananlogs for various pharmacological concerns [7], screened series of various novel D-ring substituted analogs for their breast cancer activity against MCF-7 breast cancer cell lines. The objective of the present work is thus to look for non-estrogenic non 17β –Hydroxy analogs which can be used for the treatment of breast cancers either through their binding affinity with the ER's or through some other mechanism.

2. Experimental:

2.1. General methods

Melting points were recorded on Buchi Melting point apparatus D-545; IR spectra (KBr discs) were recorded on Bruker Vector 22 instrument. NMR spectra were recorded on Bruker DPX200 instrument in $CDCl_3$ with TMS as internal standard for protons and solvent signals as internal standard for carbon spectra. Chemical shift values are mentioned in δ (ppm) and coupling constants are given in Hz. Mass spectra were recorded on EIMS (Shimadzu) and ESI-esquire 3000 Bruker Daltonics instrument. The progress of all reactions was monitored by TLC on 2x5cm pre-coated silica gel 60 F254 plates of thickness of 0.25mm (Merck). The chromatograms were visualized under UV 254-366 nm and iodine.

2.2.1. Chemical synthesis

(a) General procedure for the synthesis of chalconyl derivatives: To a solution of pregnenolone 1 (0.316g, 1mmol, 1eq.) in ethanol (10ml) was added a conc. aq. solution of KOH (2 eq.). Then aldehyde 2 (1.2eq.) was charged into the reaction mixture to get the corresponding benzylidine derivative **3** (**Scheme 1**). After completion as revealed by thin layer chromatography (TLC) in an average span of around 1 hr, the reaction mixture was precipitated using water because of the limited solubility. The precipitate was filtered, dried and monitored through TLC for the purity. Thin layer chromatography revealed just a single spot which proved the presence of a single product. For further purification, the product was recrystallized from EtOAc:Hexane to give product as solid white powder. It is to be mentioned that when non-aromatic aldehydes were used, the product was formed in a very minor quantity and that too not stable enough at ambient conditions. Thus the study was restricted to the use of aromatic aldehydes only.

(b) General procedure for the synthesis of pyrazoline derivatives: The benzylidine derivative **3** (1.0 g, 2.4 mmol) was refluxed in ethanol in the presence of hydrazine hydrate (0.24g, 4.8 mmol) so as to yield the desired pyrazolines. However the products thus obtained were very unstable and they decomposed even at ambient temperature conditions probably because of the inherent instability associated with pyrazolines. The solvent thus used was replaced by acetic acid so as to ensure the formation of N-acetyl pyrazoline **4** (0.99 g, 2.2 mmol, 90%) which was highly stable. The product was precipitated by charging the reaction mass into excessive amounts of ice-cold water (**Scheme 2**). After filtration under suction, the product was obtained in high yields as colorless powder which was later dried in vaccuo. The same procedure was followed for the synthesis of all other analogs.

(c) General procedure for the synthesis of 1, 2, 3-triazolyl Pregnanes: To a solution of 21-Bromo pregnenolone **5** (1eq.) in a mixture of tert. butanol and water was charged sodium azide (1.2 eq.) and the reaction was stirred for 30 min. Appropriate terminal alkyne **6** (1 eq.) was charged into the reaction mixture along with copper sulphate (1.2 eq.) and stirring was continued for further 10 min. so as to allow complexation to occur. Sodium ascorbate (5 eq.) was added to the above mixture and the whole reaction mass allowed to stir at ambient conditions for 8-10 hrs. After the usual workup, the product was precipitated using hexane: ethylacetate mixture. Solid white triazolyl pregnanes **7 (Scheme 3**) obtained were characterised using various spectral techniques.

The spectral data of representative compounds of each series are given below. For the detailed spectra of all compounds, our previous publications may be consulted [6].

(1) **(2E)-1-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3(b)-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17(b)-yl)-3-phenylprop-2-en-1-one (3a)**: White powder (86%). M.p: 128–131 °C; IR (KBr, cm-^{1}): 3425, 2938, 1804, 1637, 1403, 1041, 689; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.63 (s, 3H), 1.00 (s, 3H), 1.61–1.90 (m, 6H), 2.20–2.38 (m, 3H), 2.82 (t, J = 8.80, 1H); 3.51 (m, 1H); 6.78 (s, J = 16.00, 1H), 7.39 (m, 3H), 7.55 (m, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 13.33, 19.26, 21.07, 22.67, 24.62, 31.13, 31.78, 31.99, 37.22, 41.81, 45.11, 48.61, 48.78, 48.95, 49.12, 49.29, 50.04, 57.14, 61.97, 71.22, 121.24, 126.69, 128.29, 128.90, 130.41, 134.62, 140.85, 141.96, 201.32; ESI-MS: 405 (M+H); Anal. Calcd. for $C_{28}H_{36}O_2$: C, 83.12; H, 8.97; Found C, 83.37; H, 8.83.

(2) **1-(4,5-dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-5-phenylpyrazol-1-yl) ethanone (4a):** Colourless solid powder. Yield 76%. M.p: 123-125 °C. $[\alpha]_D^{25}$ – 16.9 (*c* 0.20, $CHCl_3$). IR (KBr, cm-^{1}): 3384, 2926, 1717, 1646, 1404, 1042, 699. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.63 (s, 3 H), 1.06 (s, 3H), 1.82-1.90 (m, 6H), 2.17 (s, 3H), 2.65 (t, 1H, *J* =8.8), 2.79 (m, 2H), 3.26 (m, 1H), 3.49 (m, 1H), 5.33 (s, 1H), 5.44 (m, 1H), 7.15 (d, 2H, *J =6.5*), 7.22-7.32 (m, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.85, 20.85, 22.40, 23.32, 25.83, 31.13, 33.04, 33.48, 37.99, 38.72, 39.94, 43.68, 45.30, 47.69, 51.53, 53.18, 57.94, 60.56, 73.11, 122.80, 126.78, 128.84, 130.28, 142.29, 160.63, 164.62. ESI-MS: 483 (M^+ + Na). Anal. Calcd. For $C_{30}H_{40}N_2O_2$: C, 78.22; H, 8.75; N, 6.08; Found C, 78.47; H, 8.83; N, 6.21.

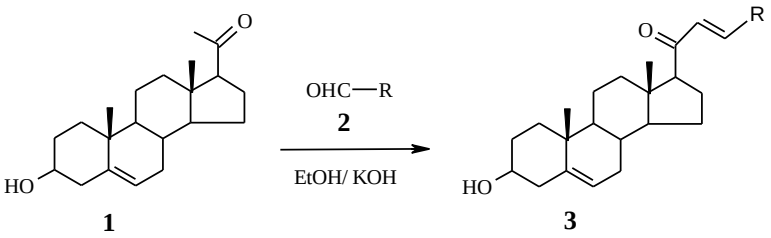
(3) **2-[4-(4-acetyl-phenoxy)-[1,2,3]triazol-1-yl]-1-(3-hydroxy-10-13-dimethyl-2,3,4,7,8,9, 10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-ethanone (7e)**: M.p: 107°C; $[\alpha]_D^{25}$: (+)70; IR (KBr, cm-^{1}): 772, 1092, 1283, 1312, 1465, 1590, 1682, 2745, 2863, 2954, 3388; ^{1}H NMR: δ 0.57 and 0.97 (2s, 6H), 3.35 (m, 2H), 5.19 (s, 1H), 5.31 (s, 2H), 7.04 (d, 2H, J=9.80), 7.73 (s, 1H),7.95 (d, 2H, J=9.80); ^{13}C NMR (500 MHz**,** $CDCl_3$): δ 13.28, 19.44, 21.13, 22.87, 24.53, 31.81, 36.56, 37.30, 38.87, 42.23, 44.09, 50.01, 52.98, 56.36, 63.77, 71.73, 118.51,120.12, 122.638, 124.81, 128.97, 130.65, 134.30, 135.34, 136.97, 137.14 140.81, 148.84 and 209.99; ESI-MS: 554 (M^+ + Na); Anal. Calc. for $C_{32}H_{41}N_3O_4$: C, 72.29; H, 7.77; N, 7.90. Found: C, 72.34; H, 7.74; N, 7.93.

2.2.2. Biology: The human cancer cell lines against which the different series of steroidal analogs were screened were MCF-7 and T-47D (both Breast cancer cell lines) which were obtained from National cancer institute (NCI), biological testing branch, Federick Reserch and Development centre, USA. Cellular viability in the presence and absence of experimental agents was determined using the standard Sulforhodamine B assay. Briefly, cells in their log phase of growth were harvested, counted and seeded (10^4 cells/well in 100 µL medium) in 96-well microtitre plates. After 24 h of incubation at 37° and 5% CO_2 to allow cell attachment, cultures were treated with varying concentrations (0.1–100 µM) of test samples made with 1:10 serial dilutions. Four replicate wells were set up for each experimental condition. Test samples were left in contact with the cells for 48 h under same conditions. Thereafter, cells were fixed with 50% chilled trichloroacetic acid (TCA) and kept at 4°C for 1h, washed and air dried. Cells were stained with Sulforhodamine B dye. The adsorbed dye was dissolved in Tris-Buffer and plates were gently shaken for 10 min on a mechanical shaker. The optical density (OD) was recorded on ELISA reader at 540 nm. The cell growth was calculated by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated. Finally the IC_{50} values (**Table IV**) were calculated using Microsoft Office Excel. The different steroidal derivatives (test material) were dissolved in a mixture of DMSO:Water (1:1) and then introduced into the medium containing the cancer cell lines.

2.3. Results and discussion:

2.3.1. Introduction of biologically potent substitutions in the core structures of physiologically important molecules has since long been a fascinating subject of scientific investigation. This is especially true with respect to the rational modification of steroids. Though the importance of different steroidal D-ring heterocycles is now well validated, only few efforts have been reported for their efficient synthesis. This specially refers to the chalcone, pyrazoline and triazolyl based heterocycles at the D-ring of steroid. We have since long been in the area of designing various steroids based analogs with special focus towards their anticancer activities [7]. In continuation of the same program, we herein want to report the activity of various such analogs exclusively towards the breast cancer cell lines. The encouraging results have made it mandatory to go for the mechanistic studies which are currently in progress. The preparation of the various analogs has already been reported from time to time [7] with some different concerns as objectives. Here a special focus has been made towards the screening of these various non estrogenic analogs for their breast cancer activities.

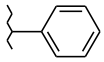
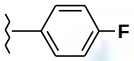
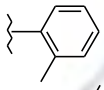
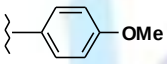
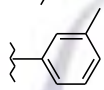
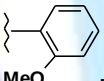
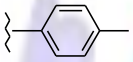
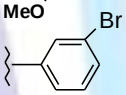
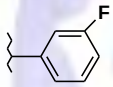
ISSN 2321-807X

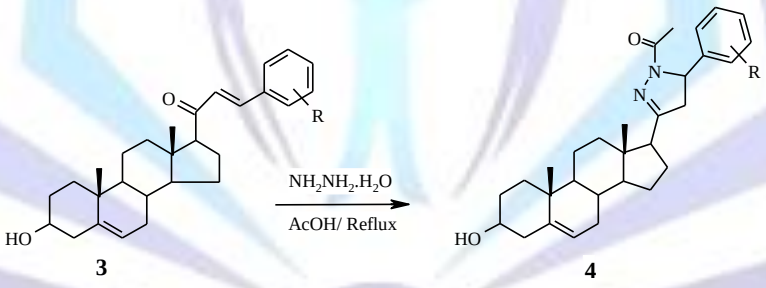


1 **3**

Scheme 1: Synthesis of D-ring substituted steroidal chalcones.

Table 1: Nature of group "R" in the compounds 3a-3j

Entry	Nature of R	Entry	Nature of R
3a		3f	
3b		3g	
3c		3h	
3d		3i	
3e		3j	

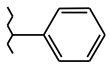
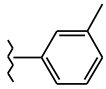
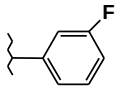
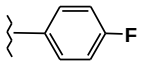
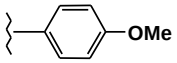
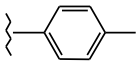
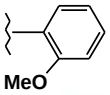
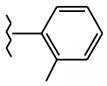
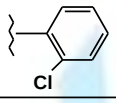


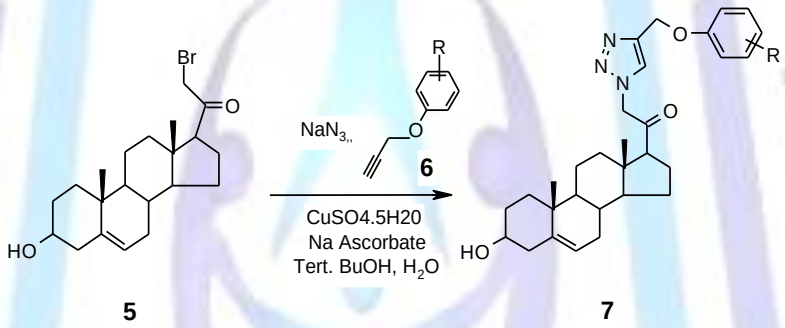
3 **4**

Scheme 2: Synthesis of D-ring substituted steroidal pyrazolines.

ISSN 2321-807X

Table II: Nature of group "R" in the compounds 4a-4j

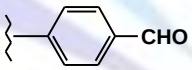
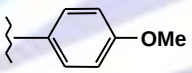
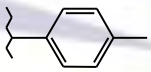
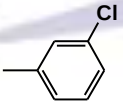
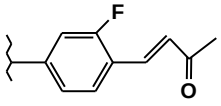
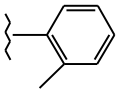
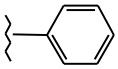
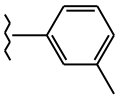
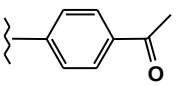
Entry	Nature of R	Entry	Nature of R
4a		4f	
4b		4g	
4c		4h	
4d		4i	
4e		4j	



5 7

Scheme 3: Synthesis of D-ring substituted steroidal triazoles.

Table III: Nature of benzene ring with group "R" in the compounds 7a-7i

Entry	Nature of R	Entry	Nature of R
7a		7f	
7b		7g	
7c		7h	
7d		7i	
7e			

ISSN 2321-807X

2.3.2: Biology. The following table gives the cancer cell inhibitory data obtained after treating breast cancer cell lines with test doses of the different steroidal derivatives and the values are reported in terms of **IC_{50}**.

Table IV. IC_{50} values (μM) of various non-estrogenic steroidal congeners against human breast cancer cell lines.

Entry	MCF-7	T-47D	Entry	MCF-7	T-47D	Entry	MCF-7	T-47D
3a	4.33	3.31	4a	0.43	11.0	7a	2.69	1.26
3b	1.44	1.53	4b	1.60	1.42	7b	2.79	1.50
3c	2.53	1.86	4c	1.84	1.67	7c	3.86	4.18
3d	3.35	4.11	4d	4.84	8.11	7d	4.63	ND
3e	0.62	1.34	4e	0.79	0.64	7e	0.62	13.0
3f	0.31	2.35	4f	1.60	0.56	7f	0.67	2.67
3g	1.08	20.4	4g	ND	48.1	7g	10.6	10.0
3h	2.27	3.35	4h	1.91	0.7	7h	3.44	2.81
3i	12.7	11.8	4i	23.1	29.2	7i	9.05	12.9
3j	2.34	2.45	4j	17.5	32.2	7j	20.2	5.10

ND = not determined;

Cell lines: MCF-7 (Breast) and T-47D (Breast)

It is clear from the IC_{50} values, that the compounds 3e, 3f, 4e, 4f, 7e and 7f show significant cytotoxic activity against both the cancer cell lines. It is evident from the data that even the position of substituent on the aromatic ring influences the relative toxicity which can be attributed to their differences in either the bioavailability or the protein binding properties. The above compounds are being further modified for attaining better analogs.

Conclusion: Three series of novel non-estrogenic steroidal congeners were synthesized and screened for anticancer activity against human breast cancer cell lines. From the data it was found that most of the compounds are having promising anticancer activity and the compounds **3e, 3f, 4e** and **4f** were found to be the most active against both the cell lines.

Acknowledgement: The author thanks *Principal ICSC* and *Dr. F. A. Malik*, for their encouragement and support.

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