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Research Paper

SYNTHESIS OF NOVEL STEROIDAL 17r-TRIAZOLYL DERIVATIVES VIA CU(I)-CATALYZED AZIDE-ALKYNE CYCLOADDITION AND THEIR EVALUATION AS POTENTIAL PROGESTATIONAL AGENTS

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Many progestins have been developed for use in contraception, Hormone Replacement Therapy (HRT), menopausal hormone therapy and treatment of gynecological diseases. Progestins are also highly efficacious in decreasing the occurrence of endometrial hyperplasia and carcinoma caused by unopposed estrogens. Click reaction was adapted for the condensation of the terminal ethynyl group of norethindroneenanthate (NET-EN)3 and levonorgestrel (LNG)5 with substituted phenyl azides2a-c to prepare 1, 4-disubstituted-1, 2, 3-triazole derivatives 4a-c and 6a-c. The assigned structures were confirmed by spectroscopic and element microanalytical data. Biological screening of the prepared compounds was undertaken to evaluate possible progestational activity on rat uterus in vivo and ex vivo on the isolated rat uterus. The histopathologic changes of the uterus associated with the new compounds and the reference drugs NET-EN and LNG encompass a variety of morphologic features that were interpreted. All the prepared compounds conserve their progestational activity, significantly much higher than vehicle. Compound 4c showed activity parameters higher than NET-EN, while 6b matched those displayed by LNG. Compound 4b displayed 200 fold the uterolytic effect of NET-EN. On the other hand, LNG derivatives showed uterolytic effect much weaker than the parent drug. Molecular modeling studies were performed using MOE software (V.10.2010) to further corroborate the biological assay results acquired for this new group of compounds and gain insight into their plausible mode of interaction(s) within the progesterone receptor.

Keywords: Click reaction, Steroidal azoles, Progestational activity, Uterolytic effect, Molecular modeling, Copper catalyzed azide-alkyne cycloaddition

INTRODUCTION

Progesterone (Prog), the natural agonist of

Progesterone Receptor (PR), is critical for female reproductive function (Djerassi, 1995). Increased

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Prog levels in plasma are responsible for the lack of ovulation during pregnancy. Effective inhibitory effect of Prog is the basis of oral contraceptives, which comprise also synthetic progesterone analogues called progestins. Progestins are a class of synthetic compounds structurally distinct, but functionally similar to Prog, with longer biological half-lives (Speroff and Darney, 1996). Synthetic steroidal progestins are widely used as therapeutic agents such as contraceptives, combination Hormone Replacement Therapy (HRT), and a variety of other therapeutic applications such as treatment of gynecological disorders and in cancer therapy (Collins, 1994; Mishell, 1996; De Ziegler and Fanchin, 2000; Schiff, 1982; Lundgren, 1992; Schneider and Jackisch, 1998).

However, a number of side-effects have been reported with the clinical use of progestins such as their effect on bone density, blood pressure, immune function, neurological effects and even minor effects as mood swings, weight gain, hot flushes and loss of libido (Goodman and Gilman's, 2006; Mueck and Sitruk, 2011). Recently, considerable interest has been focused on steroidal azoles; the azole moiety often shows some special biological activity when introduced to biologically active compounds (Levine et al., 2012; Conner et al., 2012; Corrales, 2011; Kadar, 2011; Banday, 2010). Although a number of triazolyl derivatives have been reported, steroids containing this kind of structural moiety have received less attention from both synthetic and pharmacological aspects.

The aforementioned findings about the importance of steroidal azoles coupled with the reported enhancement in progestational activity and selectivity by larger chemical moieties introduced at the 17α position of the steroid backbone (Wanga et al., 2008), prompted us to plan and synthesize novel steroidal 17a-triazoles via an in situ one-pot "click chemistry" approach (Kolb and Sharpless, 2003; Kolb et al., 2001). The premier example of click reaction is Huisgen 1, 3-dipolar cycloaddition of azides and terminal alkynes yielding 1,2,3-triazoles (Tornoe et al., 2002). Cu(I)-catalyzed azide-alkyne 1, 3-dipolar cycloaddition (CuAAC) regioselectively produces 1,4-disubstituted-1*H*-1,2,3-triazoles (Rostovtsev et al., 2002). This reaction has found numerous applications in many aspects of drug discovery (Meldal and Tornoe, 2008; Hein et al., 2008, Bock et al., 2006). Thus, our scope of investigation was to boost the progestational activity through enhancement of anticipated physicochemical binding forces (HBD, HBA, lipophilicity, Polar Surface Area (PSA) and ionic interaction) and to study in silico the binding mode of the new compounds to the PR. These potentialities were attachment challenged via of norethendroneenanthate and levonorgestrel terminal acetylene to functionalized phenyl moiety through 1,2,3-triazole ring linker.m-orp-Nitrophenyl group was prepared to probe the strong electron withdrawing character on the binding mode of the new steroid-triazolesubstituted phenyl molecule to PR. Following the same objective acetic acid moiety was added as substituent on the benzene ring to reveal the impact of added bulkiness and enhancement of flexibility of the molecule on the binding to the receptor. The presence of the carboxyl affords the potential of formation of a water soluble salt with the suitably selected counter base.

MATERIALS AND METHODS

Chemistry

General Methods

All solvents were commercially available. NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrophotometer 300 MHz. DMSO d_{a} was used as a solvent, the chemical shift (δ scale) are reported in (ppm) relative to (TMS) as internal standard. IR spectra were obtained using potassium bromide disc on a Schimadzu 435 IR spectrophotometer. Melting points were obtained on Griffin apparatus in open capillary tubes and are uncorrected. Column chromatography was performed on silica gel (60-120 mesh, E. Merck). The azides employed in the synthesis of the new compounds were successfully prepared, 2-(4azidophenyl)acetic acid (2a), Yield (%): 83, m.p.(°C): 90-92; IR (KBr): 2800-3400 (OH), 2119 (N₃), 1695 (CO-OH) (Chamni, 2011; Settimo, 1979); 1-azido-3-nitrobenzene (2b), Yield (%): 65; m.p.(°C): 54–56; IR (KBr): 2125 (N₃), 1525, 1351(NO₂) (Yang, 2011; Appl, 1959); and 1-azido-4-nitrobenzene (2c), Yield (%): 80; m.p.(°C): 71-74; IR (KBr): 2124 (N₂), 1525, 1351(NO₂) (Smith, 1937; Adamo, 1958).

4-[4-(17S-Heptanoyloxy-3-oxo-19norandrost-4-ene-17r-yl)-1H-1, 2, 3-triazol-1-yl] phenylacetic acid (4a)

Norethindroneenanthate 3 (3 mmol, 1.23 g) and 4-azidophenylacetic acid 2a (3 mmol, 0.53 g) were suspended in a 1:1 mixture of water and *tert*-butyl alcohol (12 mL). Freshly prepared sodium ascorbate (0.7 mmol, 0.14 g) was added, followed by copper (II) sulphate pentahydrate (0.28 mmol, 0.07 g). The heterogenous mixture was stirred 24 h, (monitored by TLC). The reaction mixture was diluted with water (50 mL), cooled in ice, the residue was purified by column chromatography over 60-120 silica gel using EtOAc : hexane : MeOH mixture (4:6:1). A brown sticky mass was obtained after evaporation of solvent in 34% yield. IR (KBr):3400 (OH), 1712 (CO-OR), 1700 (CO-OH), 1656 (CO), ¹HNMR (CDCl₃): δ 7.08(d, 2H, Ph-H₂,Ph-H₆), 7.2 (d, 2H, Ph-H₃,Ph-H₅), 7.9 (s, 1H, triazole-H), MS (m/z,%): Calcd. for C₃₅H₄₅N₃O₅, 587.75, found 587 (M⁺, 1.16), 458 (M⁺ - C₇H₁₃O₂, 4.28), 410 (M⁺ -C₈H₇N₃O₂, 1.48), 385 (M⁺ - C₁₀H₈N₃O₂, 6.66), 256 (M⁺ -C₁₇H₂₁N₃O₄, 6.02), Anal. (%) :Calcd.: C, 71.52; H, 7.72; N, 7.15. Found: C, 71.73; H, 7.89; N, 7.41.

17r-[1-(3-Nitrophenyl)-1H-1, 2, 3-triazol-4yl]-3-oxo-19-norandrost-4-ene-17sylheptanoate(4b)

The titled compound was prepared following the procedure adapted for 4a synthesis, using Norethindroneenanthate3 (3 mmol, 1.23 g) and 1-azido-3-nitrobenzene 2b (9 mmol, 1.48 g). The heterogenous mixture was stirred vigorously for 96 h, (monitored by TLC). The residue was purified by column chromatography on silica gel using hexane. A brown powder was obtained after evaporation of solvent in 29% yield. M.P. = 66-68 °C, IR (KBr) :1830 (CO-OR), 1670 (CO), 1534, 1342 (NO₂), ¹HNMR(CDCl₃): δ 0.7 (t, 3H, CH₃heptanoate), 7.58 (d, 1H, Ph-H₆, J = 8.4), 7.65-7.71 (m, 1H, Ph-H_s), 7.85 (s, 1H, triazole-H), 8.00 $(s, 1H, Ph-H_2), 8.03 (d, 1H, Ph-H_4, J = 8.4), MS$ (m/z,%): Calcd for $C_{33}H_{42}N_4O_5$, 574.7, found 575 (M+ 1, 0.01), 385 (M⁺ - C₈H₅N₄O₂, 0.04), 445 (M⁺ - C₇H₁₃O₂, 0.02), Anal. (%) :Calcd.: C, 68.97; H, 7.37; N, 9.75. Found: C, 69.13; H, 7.40; N, 10.11.

17r-[1-(4-Nitrophenyl)-1H-1, 2, 3-triazol-4yl]-3-oxo-19-norandrost-4-ene-17⊱ylheptanoate(4c)

The titled compound was obtained following the procedure described under 4a, using

Norethindroneenanthate 3 (3 mmol, 1.23 g) and 1-azido-4-nitrobenzene 2c (9 mmol, 1.48 g). The residue was purified by column chromatography on silica gel using EtOAc: hexane mixture (2:8). A brown sticky mass was obtained after evaporation of solvent in 58% yield. IR (KBr) :1734 (CO-OR), 1666 (CO), 1524, 1344 (NO₂), ¹HNMR(CDCl₃): δ 0.7 (t, 3H, CH₃- heptanoate), 5.65 (s, 1H, ethylene), 8.22 (d, 2H, Ph-H₂, Ph-H₆, *J* = 9), 8.43 (d, 2H, Ph-H₃, Ph-H₅, *J* = 9), 8.82 (s, 1H, triazole-H), MS (m/z,%): Calcd. for C₃₃H₄₂N₄O₅, 574.7, found 576 (M+ 2, 0.02), 411 (M⁺ - C₆H₄N₄O₂, 4.63), 256 (M⁺ - C₁₅H₁₈ N₄O₄, 0.06), Anal. (%) :Calcd.: C, 68.97; H, 7.37; N, 9.75. Found: C, 69.13; H, 7.49, N, 10.14.

4-[4-(18, 19-dinor-18-ethyl-17S-OH-3-oxoandrost-4-ene-17r-yl)-1H-1, 2, 3-triazolyl] phenyl acetic acid (6a)

Levonorgestrel5 (3 mmol, 0.93 g) and 4azidobenzoic acid 2a (3 mmol, 0.53 g) were suspended in a 1:1 mixture of water and tert-butyl alcohol (12 mL). Freshly prepared sodium ascorbate (0.7 mmol, 0.14 g) was added, followed by copper (II) sulphate pentahydrate (0.28 mmol, 0.07 g). The heterogenous mixture was stirred for 24 h, (monitored by TLC). The reaction mixture was diluted with water (50 mL), cooled in ice, the residue was purified by column chromatography on silica gel using EtOAc: hexane mixture (7:3) in 27.29 % yield.IR (Kbr):3324 (OH), 1700 (CO-OH), 1654 (CO), ¹HNMR(CDCl₂): δ 0.96 (t, 3H, CH₂CH₂), 1.2 (q, 2H, CH₂CH₂), 5.65 (s, 1H, ethylene), 7.38 (d, 2H, PhH₂, PhH₂), 7.78 (d, 2H, PhH₃, PhH₅), 8.56 (s, 1H, triazole-H), MS (m/z,%): Calcd. for C₂₀H₃₅N₃O₄, 489.26, found 488 (M- 1, 0.99), 312 (M⁺ - C₈H₇N₃O₂, 2.02), Anal. (%) :Calcd.: C, 71.14; H, 7.21; N, 8.58. Found: C,

71.39; H, 7.15, N, 8.87.

17s-hydroxy-17r-[1-(3-Nitrophenyl)-1H-1, 2, 3-triazol-4-yl]-18,19-dinor-18-ethylandrost-4ene-3-one(6b)

Levonorgestrel5 (3 mmol, 0.93 g) and 1-azido-3nitrobenzene 2b (3 mmol, 0.49 g) were suspended in a 1:1 mixture of water and tert-butyl alcohol (12 mL). Freshly prepared sodium ascorbate (0.7 mmol, 0.14 g) was added, followed by copper (II) sulphate pentahydrate (0.28 mmol, 0.07 g). The heterogenous mixture was stirred vigorosly for 50 h, (monitored by TLC). The reaction mixture was diluted with water (50 mL), cooled in ice, the residue was purified by column chromatography over 60-120 silica gel using EtOAc: hexane mixture (1:9). Yellow crystals were obtained after evaporation of solvent in 28.19% yield. M.P. : 120-122 °C, IR (KBr):3345 (OH), 1651(CO), 1450, 1369 (NO₂), ¹HNMR(CDCl3): δ 0.98 (t, 3 H, CH₂CH₂), 1.4 (q, 2H, CH₂CH₂), 5.25 (s, 1H, OH), 5.71 (s, 1 H, ethylene), 7.61 (d, 1 H, PhH_s), 7.61-7.71(m, 1H, PhH_s), 7.69 (s, 1 H, triazole-H), 7.86 (s, 1 H, PhH₂), 8.03 (d, 1H, PhH₄), MS (m/z,%): Calcd. for $C_{27}H_{32}N_4O_4$, 476.57, found 476 (M⁺, 0.99), 287 (M⁺ - C₈H₈N₃O₂, 1.41), Analysis (%) : Calcd.: C, 68.05; H, 6.77; N, 11.76. Found: C, 68.14; H, 6.83, N, 12.19.

$17s-hydroxy-17_{#}-[1-(4-Nitrophenyl)-1H-1, 2, 3-triazol-4-yl]-18,19-dinor-18-ethylandrost-4-ene-3-one(6c)$

Levonorgestrel 5 (3 mmol, 0.93 g) and 1-azido-4-nitrobenzene 2c (3 mmol, 0.49 g) were reacted as previously mentioned under the preparation of 6a. The residue was purified by column chromatography on silica gel using EtOAc: hexane mixture (2:8). An Orange powder was obtained after evaporation of solvent in 60 % yield. M.P. :115-117 °C, IR (KBr):3350 (OH), 1652(CO), 1515, 1340 (NO₂), ¹HNMR(CDCl₃): δ 0.97 (t, 3H, CH₂CH₃), 1.4 (q, 2H, CH₂CH₃), 5.30 (s, 1H, OH), 5.71 (s, 1H, ethylene), 7.37 (d, 2H, PhH₂, PhH₆, *J* = 9 Hz), 8.27 (d, 2H, PhH₃, PhH₅, *J* = 9 Hz), 8.4 (s, 1H, triazole-H), MS (m/z,%): Calcd. for C₂₇H₃₂N₄O₄, 476.57, found 477 (M+ 1, 12.31), 164 (C₆H₄N₄O₂, 12.22), Anal. (%) :Calcd.: C, 68.05; H, 6.77; N, 11.76. Found: C, 68.21; H, 6.72, N, 11.94.

In-vivo Progestational Agonist Activity

The assessment of the progestational of the newly synthesized compounds were performed at the pharmacology department, National Research Centre, Cairo, Egypt.

Animals: Adult female Wistar rats (weighing 120-140 g) were housed 6 per cage at 20-22°C room temperature under controlled conditions of light, with free access to rat chow and tap water. Rats showing regular estrous cycle length (4–5 days). The phases of estrous cycle were determined by observing the vaginal smear in the morning according to the reported procedure (Cooper *et al.*, 1993) . Only those rats showing at least two consecutive 4-day estrous cycles were used.

Drugs and Treatments: Rats received s.c. injections of test compounds daily, for 8 days. Norethindroneenanthate 3 and its synthesized triazole derivatives 4a-c were dissolved in DMSO at a concentration of 0.018 mg/animal/day (Paget and Saranes, 1964). Rats received s.c. injections of 1 mL daily. Levonorgestrel 5 and its synthesized triazole derivatives 4a-c were dissolved in DMSO at a concentration of 0.1 mg/ animal/day (Muhn *et al.*, 1995). Rats received s.c. injections of 1 mL daily. For all experiments the treatment was started when the animals were in estrus phase (Murthy *et al.*, 1997). Initial body weight before treatment and final body weight at

the time of sacrifice were recorded. 24 h after the final dose, rats were killed, and their uteri were carefully excised, trimmed of extraneous tissue, blotted filter paper to remove excess fluid, weighed on electronic balance, fixed, and stained. Paraffin sections of fixed uteri were evaluated for endometrial gland. Morphometric measurements were calculated using LiecaQwin 500 Image Analyzer in Pathology Department, National Research Centre. The thickness of endometrium, myometrium and the uterine epithelial cell heights were measured using an objective lens of magnification 10, and eye lens 10 the total magnification was 100. Ten fields were chosen in each specimen and the mean values were recorded.

Ex-vivo Uterine Relaxant Effect

Animals: non-pregnant female Wistar rats weighing 120-140 g were used in this study. The animals were obtained from the animal house; National Research Centre, Giza, Egypt. Standard laboratory food and water are provided *ad libitum*. Animal procedures are performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institute of Health's Guide for Care and Use of Laboratory Animals (National Institute of Health, 1985).

The animals in the estrus stage (as detected by vaginal smears) were sacrificed. The abdomens were opened and the two uterine horns from each rat were exposed avoiding stretching of uterine smooth muscles. One horn from each rat was freed from its surrounding fat and mesenteric attachments. A myometrial strip of length about 3 cm was cut longitudinally and transferred to a petri dish containing Krebs solution (chemical composition pH and temp. gas

Dalia H Soliman et al., 2014

bubbling). The strips were placed in automatic bath organs of 10 mL capacity in accordance with the reported procedure (Alvarez et al., 1988; Daly et al., 1981). The Krebs-Henseleit buffer (NaCI-118 mM; KCl – 4.7 mM; CaCl₂ – 2.5 mM; MgSO₄ - 1.6 mM; NaHCO₃ - 24.3 mM; KH₂PO₄- 1.18 mM; glucose 5.6 mM) was used as an incubation environment (Coruzzi et al., 1988). The incubation of strips was conducted at the temperature of 37 °C, loaded with 1 g and the oxygen and carbon dioxide gas mixture (95% O₂ and 5% CO₂) was added so that its pH remained within 7.3-7.5. The whole preparation was allowed to equilibrate for 30 minutes according to the method described (Calixto et al., 1991)1X10⁻⁴M concentration of acetylcholine (Ach) was added to the bath and contractile activity was then measured using an isometric force transducer (Grass Model 7E Polygraph, USA). The bath solution was drained completely and washed two to three times and filled with fresh solution. Isotonic contractions of the uterine muscle with different test drug concentrations were recorded, and concentration-response curves were constructed. The test drug additions were cumulative.

STATISTICAL ANALYSIS

The data were expressed as mean± SEM and analyzed using SPSS statistical software. One way analysis of variance (ANOVA) was used to assess the variation of the means among the treatments. If the variation was greater than expected by chance alone, multiple comparison tests was performed to compare each treatment group with the control and standard groups. Significance was established when the p value was less than 0.05. PRISM, versions 5.0 (GraphPad, Inc., San Diego, CA), was used for determination of IC50 value for inhibition using nonlinear regression.

Molecular Modeling

Docking and molecular modeling studies were carried out at the Department of Medicinal Chemistry, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

Software and Hardware

All the molecular modeling calculations and docking simulation studies were performed using Molecular Operating Environment (MOE[®], 2010) version 10.2010, Chemical Computing Group Inc., Montreal, Canada. The computational software operated under "Windows XP" installed on an Intel Pentium IV PC with a 1.6 GHz processor and 512 MB memory.

Target Compounds Optimization

The target compounds were constructed into a 3D model using the builder interface of the MOE program. The stereo chemical configurations of the steroidal nucleus were constrained as in norethindrone. After checking their structures and the formal charges on atoms by 2D depiction, the following steps were carried out:

- The target compounds were subjected to a conformational search.
- All conformers were subjected to energy minimization, all the minimizations were performed with MOE until a RMSD gradient of 0.01 Kcal/mole and RMS distance of 0.1 Å with MMFF94X force-field and the partial charges were automatically calculated.
- The obtained database was then saved as MDB file to be used in the docking calculations.

Optimization of the Enzymes Active Site

The three-dimensional structure of progesterone receptor complexed with norethindrone (PDB Id: 1SQN) was obtained from the Protein Data Bank through the internet.

Hydrogen atoms were added to the system with their standard geometry

- The atoms connection and type were checked for any errors with automatic correction.
- Selection of the receptor and its atoms potential were fixed.
- MOE Alpha Site Finder was used for the active site search in the enzyme structure using all default items. Dummy atoms were created from the obtained alpha Spheres.

Docking of the Target Molecules to the PR Active Site

Docking of the conformation database of the target compounds was done using MOE-Dock software. The following methodology was generally applied:

- The enzyme active site file was loaded and the Dock tool was initiated. The program specifications were adjusted to:
- Dummy atoms as the docking site.
- Triangle matcher as the placement methodology to be used.
- London dG as Scoring methodology to be used and was adjusted to its default values.
- The MDB file of the ligand to be docked was loaded and Dock calculations were run automatically.
- The obtained poses were studied and the poses showed best ligand-receptor interactions were selected and stored for energy calculations

RESULTS AND DISCUSSION Chemistry

The development of copper (I)-catalyzed 'triazole click chemistry' by Sharpless and co-workers has led to synthesis of highly functional molecules from simple building blocks, namely azides and an alkynes which in turn has led to many interesting applications (Kolb and Sharpless, 2003; Kolb *et al.*, 2001; Tornoe *et al.*, 2002; Rostovtsev*et al.*, 2002).

The attractive characteristics of this reaction are its excellent regiospecificity, reliability, mild conditions, good yields and the biocomptability of the generated 1,2,3- triazole nucleus (Rostovtsev et al., 2002). We herein, report the synthesis of novel steroid-triazoles, α -[1-(substitutedphenyl)-1H-1,2,3-triazol-4-yl]-3-oxo-19-nor-androst-4-ene-17β-yl heptanoates (4a-c) and 17β -hydroxy- 17α -[1-(substitutedphenyl)-1*H*-1,2,3-triazol-4-yl]-18,19-dinor-18-ethylandrost-4ene-3-one (6a-c) outlined in schemes (1). The targeted sterod-triazoles 4a-c and 6a-c were prepared according to the reported procedure (Rostovtsevet al., 2002), where norethindrone enanthate (NET-EN) 3 or Levonorgestrel (LNG)5 and the aryl azides 2a-c were reacted at ambient temperature in the presence of CuSO₄ as a precatalyst and sodium ascorbate using t-BuOH/H₂O as a solvent. The reaction time ranged from 24-96 h was monitored by TLC.

It is worthy to comment on the noticeddifference in the reaction time and yields of the steroid-triazoles synthesized by the "click reaction" between the*m*- and *p*-nitrophenylazides 2b and 2c with NET-EN3 and LNG 5 (scheme 1). Table 1 reveals the evidence that 1-(*m*- Scheme 1: Reagents and Conditions (i) NaNO₂, HCI, NaN₃, 0°C (ii) CuSO₄, 5H₂O, Ascorbic Acid or Sodium Ascorbate, H2Ot-Bu OH 1:1 rt, 24-96 hrs. as Specified in Experiment



Table 1: Effect of the Position Of The Nitro Group I n The Nitro- Phenyl Azides on The Yield and Time of Click Reaction with NET-EN and LNG				
Entry	$\mathrm{NO_2(\dagger_x}$ Value)(Hansch and Taft, 1991)	Reaction Time (hr)	Yield(%)	
4 b	0.71(<i>x</i> = <i>m</i>)	96	29	
6 b	0.71(<i>x</i> = <i>m</i>)	50	28	
4 c	0.78 (<i>x=p</i>)	24	58	
6 c	0.78 (<i>x</i> = <i>p</i>)	24	60	



65

nitrophenyl)-1*H*-1,2,3-triazole derivatives 4b and 6b needed more than double the time to give practically half the percentage yield of 1-(p-nitrophenyl)-1H-1,2,3-triazole analogues 4c and 6c.

The evident differences may be attributed to the strong electron-withdrawing character of the NO₂ group expressed by its σ *m*- and *p*- values Table 1. Proximity of the positive charge in the resonating structure of *p*-nitrophenylazide 2c Figure (1a) might stabilize the negative charge of the directly attached nucleophilic nitrogen N1 of the azide group rendering it more localized and available for coordination with Cu(I)-acetylidein the rate limiting step in the mechanism of CuAAC (Fomin et al., 1999). On the other hand the mnitro resonating structures 2b Figure (1-b) are lacking intimacy between opposite charges that allows more facile delocalization of the negative charge over the N1 and hence, impedes smooth coordination with Cu(I) acetylide.

On the other side, 4b took longer time on carrying the "click reaction" than 6b (96 h, 50 h, respectively), that can be attributed to the bulky effect of the enanthate ester moiety geminal to the alkyne center hindring the attack by the azide group. It was also observed that the yield of 4c and 6c increased five folds on carrying the reaction under N_2 using freshly prepared sodium ascorbate.

The IR spectra of 4a-c revealed 4-ene-3-one carbonyl stretchings in the range of 1656-1670 cm⁻¹, and the ester carbonyls at 1712-1830 cm⁻¹ in addition of a third carbonyl stretching band at 1700 cm⁻¹ assigned to the free carboxyl in 4a. Two peaks at 1524, 1344 cm⁻¹ attributed to NO₂ group were shown by 4b,c wile 4a showed a broad band at 3400 cm⁻¹ corresponding to

carboxylic OH. ¹HNMR spectra of 4a-c revealed a singlet at 7.9, 7.85 and 8.82 ppm attributed to the triazole 5-H proton. Compound 4a showed the benzene protons as two doublets one at 7.2 ppm (J=8.7) assigned to (3-H, 5-H) and a second at 7.08 ppm (J=8.9) assigned to (2-H, 6-H) protons. Compound 4c benzene protons showed the same pattern as 4a with more downfield shifted doublets of (3-H, 5-H) at 8.43 ppm (J=7.9) and (2-H, 6-H) at 8.22 ppm (J= 7.9). The m- NO₂ derivative 4b showed the benzene2-H proton as singlet at 8.0 ppm, 4-H as doublet at 8.03 (J=8.1), 5-H as multiplet at 7.65-7.7 ppm and 6-H as doublet at 7.58 ppm (J=9.3). Members of the series 4a-c showed the pattern concerning the steroid nucleus; ring A, α- methyne CO-CH proton at 5.65 ppm, a triplet integrated by two protons assigned for the α -methylenic CH₂-CO at 2.7, 2.49 and 3.0 ppm. A triplet in the range of 0.9-0.7 ppm assigned to the CH₂ of the heptanoate radical and a broad multiplet that occupy the range 0.8-2.45 integrated by 31 protons assigned collectively to twelve CH₂ groups, four CH groups and 19-CH₃ group. Compound 4a showed an additional signal at 3.4 ppm integrated by two protons assigned to the methylenic CH₂COOH.

The IR spectra of 6a-c revealed a common pattern: strong stretching band at 1651-1654 cm⁻¹ attributed to 4-ene-3-one carbonyls and a broad band in the range 3324- 3350 cm⁻¹ assigned to 17-OH stretching. In addition to the assigned absorption bands compounds 6b,c showed the two prominent peaks of NO₂ group at 1517, 1344 cm⁻¹ while 6a showed a carbonyl stretching band at 1700 cm⁻¹ assigned to the COOH group. ¹HNMR spectra of 6a-crevealed a singlet at 8.56, 7.69 and 8.4 ppm attributed to the triazole 5-H proton. Compound 6a showed the benzene *p*-

Dalia H Soliman et al., 2014

substituted pair of doublets one assigned to (3-H and 5-H) protons at 7.78 ppm (J= 8.9), and a second assigned to (2-H and 6-H) protons at 7.38 pm (J=8.7). Compound 6-c showed the same aromatic pattern as 6a with more downfield shifted doublet of (3H- and 5-H) protons at 8.27 ppm (J=9) and (2-H and 6-H) doublet at 7.37 ppm (J=9.3). The m- NO₂ derivative 6b showed the benzene 6-H as doublet at 7.61 ppm (J=8.1), 5-H as multiplet at 7.61-7.71 ppm, 2-H as singlet at 7.86 ppm and 4-H as doublet at 8.03 ppm (J = 8.4). Members of the 6a-c series showed the pattern concerning the steroid nucleus; ring A, a singlet at 5.65, 5.71 or 5.71 ppm assigned to the α methyne CO-CH proton, triplet at 2.8, 2.6 and 2.65 ppm assigned to methylenic α - CH₂-CO in the three derivatives. Methylenic and methyl protons of C13-Et protons appeared as quartet at 1.19-1.20 ppm and triplet at 0.88-0.98 ppm, respectively. A multiplet integrated by 18 protons in the range 1.20 - 2.59 ppm was collectively assigned to seven CH₂ and four CH of the steroid nucleus. Compound 6a showed a singlet at 3.48 ppm assigned to the methylenic CH₂-COOH integrated by two protons. All members of 4a-c and 6a-c series showed molecular ion peaks and elemental analyses complying with the calculated values of the target structures.

PROGESTATIONAL ACTIVITY

In-vivo Progestationalagonist Activity

The progestational activity of the newly synthesized compounds and the parent steroids was assessed on rats by evaluation of the effect on body and uterine weights, histopathologic changes of the endometrium, myometrium and epithelial cell height illustrated in Table 2. In all rats, there was an observed increase in the five measured parameters compared to the control group. The most pronounced effect was elicited by norethindrone enanthate derivative 4c which showed a significant increase ranging from 1.3 up to 2.3 times in all parameters compared to the control. In case of LNG derivatives, the best result was elicited by 6b which showed a significant increase in all the five parameters compared to control rats ranging from 1.3 up to 3 times. Most of the values of LNG derivatives were matching with those shown by Levonorgestrel itself. No signs of toxicity were observed for all experimental groups following treatment with all test compounds during the experiment.

The histopathological sections obtained from the treated uteri showed that the weight gain of the uterus was significantly attributed to an increase in both endometrial and myometrial widths. An impressive increase in epithelial cell height was also observed for all these compounds, Table 2. Endometrial uterine crosssections of the rats exposed to the most effective norethindroneenanthate analogue 4c represented by Figure (2b) demonstrated an evident increase of the uterus diameter and lumen with healthy myometrium and elongation of uterus epithelium, compared to the control groups Figure 2a. As for the Levonorgestrel analogues, compound 6b was the most effective of this series, it showed wide endometrium and an increase in epithelial glands as shown in Figure 2c.

Ex-vivo Uterine Relaxant Effect:

It is well known that compounds possessing progestational activity should suppress the myometrial rhythmic contractions (Fomin *et al.*, 1999; Papka *et al.*, 1999; Dutta and Sanyal, 1969). Therefore, an *in vitro* study was carried out to evaluate the uterolytic effect of the newly synthesized steroidal derivatives. Norethindrone

Table 2: Changes Induced By The Prepared Derivatives on Body Weight and Uterus of Rats ^a						
Entry	Daily Dose (mg/day)	%Body Weight	Relative Uterus Weight (kg)	Endometrial Width (µm)	Myometrial Width (μm)	Epithelial Cell Height (μm)
Vehicle	20%DMSO	22±2	1.5±0.03	151±6	121±3	58±1
3(NET-EN)	0.018	28±1	1.7±0.02	300±2*	225±7*	67±1*
4a	0.018	36±2	1.75±0.10	316±1.5*	239±4*	71±2*
4b	0.018	34±2	1.73±0.02	310±4*	229±1*	69±0.8*
4c	0.018	49±2* ^b	2.2±0.08 *	347±24*	241±3*	78±1* ^b
5(LNG)	0.100	49±4*	2.0±0.10*	402±5*	264±7*	73±1*
ба	0.100	36±3	1.8±0.10	341±28*	251±8*	71±2*
6b	0.100	50±4*	2.1±0.20*	453±45*	325±21*°	76±1*
6c	0.100	41±3*	1.9± 0.04	392±14*	256±3*	72±1

Note: **Significantly different from control group at P < 0.05.

Figure 2(a): A Light Micrograph Of The Uterus Of Control Rat Showing Normal Architecture (B)A Light Micrograph Of The Uterus Of Rat Treated With 4c. (C) A Light Micrograph Of The Uterus Of Rat Treated With 6b



enanthate derivatives induced marked decrease in uterine contraction, that was proportional to their increased concentrations. The IC_{50} values of the tested compounds on Ach-induced contractions were shown in Table 3. Norethindrone enanthate derivative 4b (IC_{50} =11 µMoI) showed the most potent relaxant effect at concentration 200 times less than norethindrone enanthate (IC_{50} = 2.3 mMoI). While, Levonorgestrel derivatives possessed relaxant activity less than that observed for Levonorgestrel. It can be concluded that the prepared norethindrone enanthate derivatives possess potent progestational activity in addition to an evident uterolytic effect which may be of value in certain cases of threatening abortion.

MOLECULAR MODELING

Molecular Operating Environment (MOE[®], 2010) version 10.2010 as a flexible docking program



enables us to predict favorable protein-ligand complex structures with reasonable accuracy and speed. Norethindrone, levonorgestrel and the prepared ligands 4a-c and 6a-c were docked into progesterone (PDB code: 1SQN) active site using X-ray crystal structure data for Prog to further corroborate the biological assays results acquired for the new group of compounds and gain insights into their plausible mode of interaction(s) within the PR. Insights into the PR binding site, revealed that the Ligand Binding Domain (LBD) contains 11α -helices (H1, H3-H12) and two short β -sheets, organized into three layers. Within the ligand binding cavity, the Gln725 and the Arg766 residues anchor the C3 ketone function via hydrogen bonds and make contact with water molecule. Most of the remaining PR-ligand interactions were hydrophobic as steroids



Table 4: Ligand/ PR interaction groups, CLogP and PSA calculated values of the docked molecules							
Entry	dG (Kcal/mol)	Ligand function	Am Hydrogen bonding	nino acid residues Hydrophobic	of PR van der Waals	CLog <i>P</i> * (O/W)	PSA* (A°) ²
NET	-9.9436	3-C=O; 17β-OH	Arg766 Gln725	Leu 887,778, 718,797, 715, 763; Met756, 759,801;	Cys 891, Tyr 890, Phe778, 794	5.9500	70.6659
4a	-11.3238	Carboxylat e	Arg 766 Gln 725	Leu 887,778, 718,797,715, 763; Met756,759 801; Val760	Cys891, Tyr890,Thr8 94, Asn719, Phe778	6.1320	199.438 4
4b	-10.3847	C3=0	Arg 766 Gln 725	Leu715, 718, 797, 887; Met756,759 909; Val760	Cys891, Tyr890, Asn719, Phe794, 905	6.3390	175.473 5
4c	-12.0897	C3=0	Arg 766 Gln 725	Leu715, 718, 797,721, 887; Met756,759 909; Val760	Cys891, Tyr890,Thr8 94, Asn719, Phe778, 905	6.3020	177.424 5
LNG	-9.5554	3-С=О; 17β-ОН	Arg766 Gln725	Leu887,778,7 18,797,715, 763; Met756,759,8 01; Val760	Cys 891, Tyr 890, Thr 894, Asn719, Phe778	3.5600	77.6721
6a	-10.5160	Carboxylat e C ₃ =0	Arg 766 Gln 725 Asn 719	Leu715, 718, 797,721, 887; Met756,759; Val 903	Cys891, Tyr890,Thr8 94, Phe778, 905	3.7420	211.817 8
6b	-12.6830	C3=0	Arg 766 Gln 725	Leu 715, 718, 797,721, 887; Met756,759, 909; Val760,903	Cys891, Tyr890,Thr8 94, Phe778, 905	3.9490	199.752 9
6c	-11.6712	C3=0	Arg 766 Gln 725	Leu715, 718, 797,721, 887; Met756,759, 909; Val760,903	Cys 891, Tyr 890,Thr 894, Phe778, 905, 794	3.9120	195.289 5

are completely buried into a mainly hydrophobic Ligand Binding Proteins (LBP) inside globular protein structure (Alvarez *et al.*, 2011; Petit-Topin *et al.*, 2009; Wanga *et al.*, 2008).

The target compounds were constructed into a 3D model using the builder interface of the MOE program. The stereo chemical configurations of the steroidal nucleus in the ligands were constrained as in norethindrone (NET). After running the conformational search all conformers were subjected to energy minimization by MOE until a RMSD gradient of 0.01 Kcal/mole and RMS distance of 0.1 Å with MMFF94X force-field and the partial charges were automatically calculated.

The PR receptor was prepared for docking studies by adding hydrogen atoms to the system with their standard geometry and checked for any errors with automatic correction then selection of the receptor and its atoms potential were fixed. MOE Alpha Site Finder was used for the active

site search in the enzyme structure using all default items. Dummy atoms were created from the obtained alpha spheres. Docking of the target molecules to the PR active site was then followed and LondondG scoring methodology was adjusted to its default values and Dock





calculations were run automatically. Poses showed best ligand-receptor interactions were selected and stored for energy calculations. Examination of the bindings between the synthesized ligands and the receptor revealed that the active site amino acids are Leu-715, Cys-

891, Thr-894, Tyr-890, Leu-797, Leu -887, Met-756, Met-801, Met-759, Phe-778, Arg-766, Gln-725, Leu-721, Leu-718, Trp-755, Asn-719, Met-909 and Phe-905. As observed in other PR/steroid complexes, a hydrogen bond was observed between oxygen atom of C_3 carbonyl of the steroid A-ring of norethindrone and the side chain of GIn725 and Arg766. Phe778 made van der Waals contact with the steroid A-ring while Asn719 and Try890 with Ring D substituents. Most of the remaining PR-ligand interactions were



hydrophobic, but some polar interactions involving the D-ring may be responsible for molecular recognition and increased affinity, Figure 3.

The intermolecular docking interactions of the parent drugs 3 and 5 and the synthesized derivatives 4a-c and 6a-c are listed in Table 4.

In our work, we are interested to explore the effects of the planned 17α -molecular extension tactic, as well as the electronic effect on the aromatic nucleus on the binding interactions with PR. All derivatives showed enhanced hydrophobic and van der Waals interactions binding mode to PR relative to the reference hormones. In case of compounds 4a and 6a, the introduction of COOH group flipped the orientation inside the active site so COOH is oriented towards Arg766 and Gln 725 Figure 4. On the other hand*m*- and *p*- NO₂ substituted compounds 4b, 4c, 6b and 6C showed equal placement and orientation in the active site as the references 3 and 5, however differently affected dG values Figures 5 and 6.

CONCLUSION

Our docking scores of dG are directly correlated with the measured in vivo and ex vivo progestational activity parameters. Under our experimental conditions, the synthesized compounds displayed on the µM dose level either comparative or more potent progestational action than the reference drugs. Compound 4b was distinguished by the displayed uterine relaxation potential at much lower IC₅₀ relative to the reference. Revealed progestational activity enhancement can be correlated with the visual binding hydrophobic and van der Waals modes of interactions of the prepared compounds at the active site of PR or due to increased lipohilicity indicated by CLog P values. These modifications are expected to modify in some way the ADMET requirements which needs more detailed investigations. The two compounds 4a and 6a offer steroidal derivatives carrying a free aromatic carboxyl group pave the way to explore the preparation of water soluble salt with a suitable base. This study may provide some insights into the development of novel potent steroid-17afunctionalized progestational lead molecules.

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APPENDIX .: SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version.at

List of Abbreviations	Leu=Leucine		
Acetvlcholine =Ach	LNG = Levonorgestrel		
ADMET = Absorption, Distribution,	Lys = Lysine		
Metabolism, Elimination, Toxicity	Met = Methionine		
Arg = Arginine	MOE = Molecular Operating Environment		
Asn = Asparagine	NET = Norethindrone		
BW = Body weight	NET-EN = Norethindrone enanthate		
CUAAC = Copper-catalyzed azide-alkyne	PDB = Protein Data Bank		
cycloaddition	PR= Progesterone Receptor		
DBD = DNA-binding domain	Prog = Progesterone		
GIn = Glutamine	PSA = Polar surface area		
Gly = Glycine	QSAR = Quantitative structure-activity relationship		
His = Histidine			
HRT= Hormone Replacement Therapy	RMS = Root mean square		
LBD= Ligand Binding Domain	SAR = Structure-activity relationship		
LBP = Ligand Binding Pocket			