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FACILE SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL MODIFIED AMINO-STEROIDS

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ABSTRACT

The objective of this study was to elucidate the efficient synthesis of novel modified steroids containing aminopyrane, aminopyridine or aminothiophene rings. 17β-Acetoxy-5α-androstan-3-one **2** reacted with ethyl cyanoacetate **3** to afford the ethoxycarbonylacetonitrile-5α-androstane derivative **4**. The scope and limitation of compound **4** was studied to form the aminopyranoandrostane derivative **7a.b** and the aminopyridoandrostane derivative **10a.b**. Also compound **2** reacted with cyanoacetamide **11a** or cyanothioacetamide **11b** to afford the aminothienoandrostane derivative **12a.b**. *in vitro* cytotoxic activity of compound **12a** was evaluated against hepatocellular carcinoma ($HepG_2$ cell) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay. Also the *in vivo* anti-tumor activity was evaluated against Ehrlich ascites carcinoma (EAC).

Key words: Anti-tumor, Steroids, Heterocycles, Hepatoma (Hep-G_2), Ehrlich ascites carcinoma (EAC).

1. INTRODUCTION

A variety of steroids with unusual and interesting structures have been synthesized and evaluated for their antitumor activities **[Thibeault et al., (2008) and Evgenija et al., (2008)]**. Also the antitumor activities

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of many compounds containing heterocyclic ring have been reviewed **[Preobrazhenskaya (1985) and Kidwai et al., (2002)]**. The biological and medical activities of steroidal heterocyclic compounds have stimulated considerable interest in the chemistry of steroids **[Jindal et al., (2001)]**. These compounds have been also tested successfully against several types of cancer **[Handratta et al., (2005)]**. Recently, our research group has developed several novel efficient syntheses of biologically active modified steroids **[Abdelhalim et al., (2007); Elmeged (2005) and Hana et al., (2008)]**. The main purpose of the present study was the facile synthesis of novel promising anticancer steroidal heterocyclic derivatives. Furthermore, this study was undertaken to shed light on the antitumor activity of some of these derivatives against hepatocellular carcinoma ($HepG_2$ cells) *in vitro* and against Ehrlich ascites carcinoma (EAC) *in vivo*.

2. MATERIALS AND METHODS

2.1. Synthesis

Starting steroid 17β-hydroxy-5α-androstan-3-one (androstanolone) was purchased from Sigma Company, USA. All solvents were dried by distillation prior to use. All melting points were measured using an electrothermal apparatus and are uncorrected. The IR spectra were recorded in (KBr discs) on a shimadzu FT-IR 8201 PC spectrometer and expressed in cm^{-1}. The ^1H NMR and ^{13}C NMR spectra were recorded with Jeol instrument (Japan), at 270 and 125 MHz respectively, in DMSO-d_6 or $CDCl_3$ as solvent and chemical shifts were recorded in ppm relative to TMS. Mass spectra were recorded on a GCMS-QP 1000 Ex spectra mass spectrometer operating at 70 ev. Elemental analyses were carried out by Microanalytical Data Unit at National Research Center, Giza, Egypt. Reactions were monitored on Merck aluminum thin layer chromatography (TLC) plates and visualized by UV light (254 nm). For the nomenclature of steroid derivatives, we used the definitive rules for the nomenclature of steroids published by the Joint Commission on the Biochemical Nomenclature (JCBN) of IUPAC **[IUPAC, (1989a) and IUPAC, (1989b)]**.

2.1.1. 17β-Acetoxy-3-ethoxycarbonylacetonitril-5α-androstane (4)

To a mixture of 17β-acetoxy-5α-androstan-3-one **2** (0.33 g, 1mmol) and ammonium acetate (0.5g), equimolar amounts of ethyl

cyanoacetate **3** (0.11 g, 1mmol) was added. The reaction mixture was heated in an oil bath at 130°C for 15 min. The solid product formed upon cooling at room temperature, was triturated with ethanol, collected by filtration and crystallized from dioxane to form pale yellow crystals from compound **4**: yield 0.29 g (70%), mp 153-155 °C. IR (KBr, cm^{-1}): υ = 2925, 2847 (CH_3, CH_2), 2223 (CN), 1738 (ester-C=O), 1725 (acetate-C=O), 1598 (C=C). ^{1}H NMR ($CDCl_3$, ppm): δ= 0.82 (s, 3H, CH_3-19), 1.02 (s, 3H, CH_3-18), 1.35 (t, 3H, J=6.07, ester-CH_3), 2.08 (s, 3H, $COCH_3$), 3.28-3.50 (m, 1H, C_5-αH), 4.25 (q, 2H, J=6.07, ester-CH_2). MS (EI): m/z (%): 427 (M^+, 18), 367 (M^+-CH_3COOH, 22), 316 (M^+-$C_5H_5NO_2$, 27), 273 ($C_{19}H_{29}O$, 100). Calc. for $C_{26}H_{37}NO_4$ (427.576): C, 73.03; H, 8.72; N, 3.27; found: C, 73.21; H, 8.51; N, 3.10.

2.1.2. Synthesis of 17β-Acetoxy-6'-amino-2'H-pyrano [3',4':2,3] androstane derivatives (7a,b)

General procedure

To a solution of compound **4** (0.85 g, 2mmol) in absolute ethanol (30ml) containing sodium acetate (1.0g) either benzaldehyde **5a** (0.21 g, 2mmol) or anisaldehyde **5b** (0.27 g, 2mmol) was added. The reaction mixture in each case was heated under reflux for 3-5 h until all the reactant had disappeared as indicated by TLC. Sodium acetate was isolated on hot by filtration and the filtrate was concentrated. The isolated product upon cooling over night at room temperature, in each case, was collected by filtration, dried and crystallized from the appropriate solvent.

2.1.2.1. Ethyl 17β-acetoxy-6'-amino-2'-phenyl-2'H-pyrano[3',4':2,3]-5α-androstan-5'-carboxylate (7a)

Pale white crystals from ethanol, yield 0.77 g (73%), mp 116-117°C, IR (KBr, cm^{-1}): υ =3450 (NH_2), 3020 (CH-aromatic), 2935, 2849 (CH_3, CH_2), 1735 (ester, C=O), 1718 (acetate, C=O), 1565 (C=C). ^{1}H NMR (DMSO-d_6 , ppm): δ = 0.77 (s, 3H, CH_3-19), 1.03 (s, 3H, CH_3-18), 1.30 (s,3H, J=6.02, ester-CH_3), 2.07 (s, 3H, $COCH_3$), 3.36-3.57 (m, 1H, C_5-αH), 4.27 (q, 2H, J=6.02, ester-CH_3), 4.59 (s, 2H, NH_2, D_2O-exchangeable), 7.23-7.52 (m, 5H, C_6H_5). MS (EI): m/z (%): 533 (M^+, 38), 473 (M^+-CH_3COOH), 272 ($C_{19}H_{28}O$, 100). Calc. for $C_{33}H_{43}NO_5$ (533.698): C, 74.27; H, 8.12; N, 2.62; found: C, 74.50; H, 8.32; N, 2.83.

2.1.2.2. Ethyl 17β-acetoxy-6'-amino-2'-(p-methoxypheny)-2'H-pyrano[3',4':2,3]-5α-androstan-5'-carboxylate (7b)

Yellow crystals from ethanol, yield 0.76 g (68%), mp 103-105°C, IR (KBr, cm^{-1}): υ = 3549 (NH_2), 3035 (CH-aromatic), 2921, 2851 (CH_3, CH_2), 1728 (ester C=O), 1710 (acetate C=O), 1595 (C=C). ^1H NMR (DMSO-d_6 , ppm): δ = 0.70 (s, 3H, CH_3-19), 0.98 (s, 3H, CH_3),1.38 (s, 3H, J=6.08, ester-CH_3), 2.12 (s, 3H, $COCH_3$), 3.32-3.50 (m, 1H-C_5-αH), 3.80 (s, 3H, OCH_3), 4.25 (q, 2H, J=6.08, ester-CH_2), 5.28 (s, 2H, NH_2, D_2O-exchangeable), 7.25 (dd, 2H-aromatic, J_{HH} 8Hz), 7.48 (dd, 2H-aromatic, J_{HH} 9Hz). MS (EI): m/z (%): 562 (M^+-1, 35), 503 (M^+-CH_3COOH, 72), 456 [M^+-(C_6H_4-OCH_3), 43], 107 (C_6H_4-OCH_3, 100). Calc. for $C_{34}H_{45}NO_6$ (563.724): C, 72.44; H, 8.05; N, 2.48; found: C, 72.26; H, 7.87; N, 2.23.

2.1.3. Synthesis of 17β-Acetoxy-6'-aminopyrido[3',4':2,3]androstane derivatives (10a,b)

General procedure

To a solution of compound **4** (0.85g, 2mmol) in dimethylformamide (20ml) containing a catalytic amount of triethylamine (1ml) either phenyl isothiocyanate **8a** (0.27g, 2mmol) or phenyl isocyanate **8b** (0.23g, 2mmol) was added. The reaction mixture, in each case, was heated under reflux for 5-7 h until all the reactants had disappeared as indicated by TLC. The reaction mixture poured over an ice/ water mixture and neutralized with dilute hydrochloric acid. The solid product that formed, in each case, was filtered off, dried and crystallized from the appropriate solvent.

2.1.3.1. Ethyl 17β-acetoxy-6'-amino-1'-phenyl-2'-thioxopyrido [3',4':2,3]-5α-androstan-5'-carboxylate (10a)

Brown crystals from dioxane, yield 0.78 g (70%), mp 168-170°C , IR (KBr, cm^{-1}): υ = 3385 (NH_2), 3032 (CH-aromatic), 2935, 2840 (CH_3, CH_2), 1738 (ester C=O), 1725 (acetate C=O), 1605 (C=C), 1198 (C=S). ^1H NMR ($CDCL_3$, ppm): δ = 0.82 (s, 3H, CH_3-19), 1.16 (s, 3H, CH_3-18), 1.30 (t, 3H, J=6.12, ester-CH_2), 2.01 (s, 3H, $COCH_3$), 3.32-3.50 (m, 1H, C_5-αH), 4.20 (q, 2H, J=6.12, ester-CH_2), 4.52 (s, 2H, NH_2, D_2O-exchangeable), 6.46-7.01 (m, 5H, C_6H_5). MS (EI): m/z (%): 561 (M^+-1, 29), 502 (M^+- CH_3COOH, 52), 273 ($C_{19}H_{29}O$, 100), 77 (C_6H_5, 35). Calc. for $C_{33}H_{42}N_2O_4S$ (562.762): C, 70.43; H, 7.52; N, 4.98, S, 5.70; found: C, 70.30; H, 7.23; N, 4.75; S, 5.56.

2.1.3.2. Ethyl 17β-acetoxy-6'-amino-1'-phenyl-2'-oxopyrido[3',4':2,3]-5α-androstan-5'-carboxylate (10b)

Yellow crystals from ethanol, yield 0.74 g (68%), mp 177-178°C, IR (KBr, cm^{-1}): υ = 3385 (NH_2), 3030 (CH-aromatic), 2942, 2840 (CH_3, CH_2), 1735 (ester C=O), 1728 (C=O), 1695 (C=O), 1610 (C=C). ^1H NMR ($CDCL_3$, ppm): δ = 0.82 (s, 3H, CH_3-19), 1.16 (s, 3H, CH_3-18), 1.32 (t, 3H, J=6.12, ester-CH_2), 2.20 (s, 3H, $COCH_3$), 3.32-3.50 (m, 1H, C_5-αH), 4.22 (q, 2H, J=6.12, ester-CH_2), 4.52 (s, 2H, NH_2, D_2O-exchangeable), 7.64-7.84 (m, 5H, C_6H_5). MS (EI): m/z (%): 545 (M^+-1, 30), 486 (M^+- CH_3COOH, 59), 273 ($C_{19}H_{29}O$, 100), 77 (C_6H_5, 63). Calc. for $C_{33}H_{42}N_2O_5$ (546.696): C, 72.50; H, 7.74; N, 5.12; found: C, 72.32; H, 7.59; N, 4.90.

2.1.4. 17β-Acetoxy-5'-amino-4'-amidothieno[2',3':2,3]-5α-androstane (12a), 17β-Acetoxy-5'-amino-4'-thioxamido[2',3':2,3]-5α-androstane (12b)

General procedure

Equimolar amounts of 17β-acetoxy-5α-androstan-3-one **2** (1.66 g, 5mmol), sulfur (0.16 g, 5mmol) and cyanoacetamide **11a** (0.42 g, 5mmol) or cyanothioacetamide **11b** (0.50 g, 5mmol) in absolute ethanol (30ml) containing a catalytic amount of triethylamine (0.5ml) were heated under reflux for 3 h until all the starting materials had disappeared as indicated by TLC. The reaction mixture left to cool over night at room temperature. The solid product that formed, in each case, was collected by filtration and crystallized from the proper solvent.

Compound **12a**: Yellow crystals from methanol, yield 1.76 g (82%), mp 113-115°C, IR (KBr, cm^{-1}): 3486, 3340 ($2NH_2$), 2935, 2851 (CH_3, CH_2), 1728,1695 (2C=O), 1575 (C=C). ^1H NMR (DMSO-d_6 , ppm): δ = 0.78 (s, 3H, CH_3-19), 1.03 (s, 3H, CH_3-18), 2.07 (s, 3H, $COCH_3$), 3.45-3.65 (m,1H, C_5-αH), 4.65, 4.87 (2s, 4H, $2NH_2$, D_2O-exchangeable), ^{13}C NMR ($CDCL_3$, ppm). δ = 35.7 (C-1), 140.5, 127.2 (fused C-2, C-3), 20.2 (C-4), 43.7 (C-5), 26.7 (C-6), 29.2 (C-7), 36.0 (C-8), 50.7 (C-9), 37.6 (C-10), 22.5 (C-11), 34.9 (C-12), 43.0 (C-13), 51.8 (C-14), 24.2 (C-15), 27.8 (C-16), 82.0 (C-17), 20.7 (C-18), 15.7 (C-19), 170.8 (C=O), 21.0 (CH_3, acetate), 117.5 (C-4'), 163.8 (C-5'), 168.2 (C=O, amide). MS (EI): m/z(%): 430 (M^+, 32), 370 (M^+-CH_3COOH), 262 [M^+-($C_7H_8N_2OS$, retro-Diels Alder fragment), 26], 168 ($C_7H_8N_2OS$, retro-Diels Alder fragment (**14a**), 100). Calc. for $C_{24}H_{34}N_2O_3S$ (430.603):

C, 66.94; H, 7.96; N, 6.51; S, 7.45; found: C, 66.71; H, 7.70; N, 6.29; S, 7.27.

Compound **12b**: Yellow crystals from ethanol, yield 1.67 g (75%), mp 96-98°C, IR (KBr, cm^{-1}). 3435-3337 ($2NH_2$), 2933, 2852 (CH_3, CH_2). 1727 (C=O), 1565 (C=C), 1190 (C=S). ^1H NMR (DMSO-d_6, ppm): δ = 0.73 (s, 3H, CH_3-19), 0.97 (s, 3H, CH_3-18), 2.28 (s, 3H, $COCH_3$), 3.38-3.58 (m, 1H, C_5-αH), 4.77, 4.92 (2s, 4H, $2NH_2$, D_2O-exchaneable), MS (EI): m/z(%): 446 (M^+, 52), 386 (M^+-CH_3COOH,18), 262 [M^+-$C_7H_8N_2S_2$ (retro-Diels Alder fragment), 37], 184 ($C_7H_8N_2S_2$, retro-Diels Alder fragment (**14b**), 100). Calc. for $C_{24}H_{34}N_2O_2S_2$ (446.668): C, 64.53; H, 7.67; N, 6.27; S, 14.36; found: C, 64.76; H, 7.84; N, 6.41; S, 14.53.

2.2. In vitro cytotoxic activity

2.2.1. Materials

RPMI-1640, Trypsin, Fetal calf serum (FCS), L-glutamine and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Penicillin G sodium and streptomycin sulfate were obtained from Bio-Waste Co. (Wexford, Ireland). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT was purchased from Duchefa-Biochemie (Haarlem, Amsterdam, Netherlands). Sodium bicarbonate was obtained from Merck Co. Inc (USA).

2.2.2. Cell culture

Human hepatocellular carcinoma cell line ($HepG_2$), was supplied by Naval American Research Unit, Egypt (NAMRU). Cells were propagated and maintained in RPMI-1640 medium with L-glutamine and supplemented with 10% fetal calf serum for growth and 2% of the maintenance medium [1% of 4% sodium bicarbonate and 1% antibiotic mixture (1,000,000 units of penicillin G sodium and 1,000,000 µg streptomycin sulfate in 100 ml deionized water)] in 75 cm^2 tissue culture flasks.

2.2.3. Growth inhibition assay

The cytotoxic effect of the compound **12a** was investigated using MTT assay **[Mosmann et al., (1983)]**. The Human hepatocarcinoma ($HepG_2$ cell lines) at approximately 80% confluence (i.e. logarithmically growing cells) were selected for trypsinization, and then counted using trypan blue dye. The percentage of cells that resisted staining ought to be

above 97%. Cells were seeded in 96-well microplates, after the cell concentrations were adjusted to (3 X 10^3 cells / well) in 100 µl RPMI-1640 culture medium and incubated at 37 °C and 5 % CO_2 over night. The cells were treated with compound **12a** which was dissolved individually in olive oil and in dimethylsulfoxide (DMSO), in three different concentrations (10, 50 and 100 µM/ml) and re-incubated for 24, 48 and 72h. Then the cells were washed with sterile phosphate buffer saline (PBS) and 100 µl of the tetrazolium dye (MTT) (0.5mg/ml) solution was added to each well, and the cells was incubated for an additional 4h. The medium was discarded; 100µl of DMSO was added to dissolve the purple formazan crystals formed. The optical density (OD) of solubilized formazan was measured at 570 nm (reference filter 690 nm) using an automatic microplate reader (Wako, Japan). The results were expressed as percent of cell growth inhibition compared with the control.

The effect of compound **12a** on the morphology of treated Hepatocellular carcinoma cells was investigated by the light inverted microscope and then photographed by SONY CYBER-SHORT **[Theiszova et al., (2005)]**.

2.3. In vivo antitumor activity

2.3.1. Tumor transplantation.

A line of Ehrlich Ascites Carcinoma (EAC) was supplied from the National Cancer Institute, Cairo, Egypt. The EAC cells were there after propagated by weekly intraperitoneal (IP) injections of $3x10^6$ cells, freshly drawn from a donor mouse bearing 7-9 day-old ascites tumor suspended in 0.3 ml sterile saline solution **[Mady (2002)]**.

2.3.2. Experimental animals.

Twenty five Swiss albino female mice weighting 20 ± 2 g were obtained from the Animal House Colony of the National Research Center, Cairo, Egypt. Five mice in each cage were housed in plastic cages of dimensions of 42L x 26W x 22H centimeters. Animals were maintained under controlled conditions of humidity, temperature, and diurnal environment of light and dark. The mean ambient temperature in the housing facility was 28°C. The animals were randomly assigned to 5 groups (n=5) as follows: Group 1(vehicle control) was left without any treatment for 14 day. Group 2 (negative control) injected intrapertioneal (i.p.) with 0.2 ml of EAC, which contain 2×10^6 cells for tumor induction

and left for 14 day. Group 3 (positive control) injected (i.p.) with 0.2 ml of EAC and treated with a dose of 20 mg/kg b.wt. i.p. day after day of reference drug 5-flurouracil (as standard antitumor agent) for 14 days. Group 4 received the tested compound **12a** at dose 25 mg/kg b.wt. (i.p.) dissolved in dimethyl sulfoxide (DMSO) day after day for 14 day which is period of the study of antitumor activity and simultaneous alterations in the hematological profile were estimated as well as tumor volume was measured **[Gupta et al., (2004)]**.

In order to detect the influence of effect of compound **12a** on the hematological status of EAC-bearing mice, after administration of the last dose followed by 18 h fasting, all mice were then sacrificed a comparison was made among five groups (n= 5) of mice on the 14^{th}. Blood was drawn from each mice by the retro orbital plexus method and the white blood cell count (WBC), hemoglobin and haematocrit % were determined **[D'Amour et al., (1965) and Lowry et al., (1951)]**. Blood collected from mice should be immediately placed in a tube containing anticoagulant, for routine hematologic testing is ethylene diamine tetra-acetic acid (EDTA). The reports of results from standard hematological evaluation is called complete blood count (CBC) which was determined using automated hematology analyzers (cell-Dyn,3500- USA) **[Hedrich et al., (2004)]**.

2.4. Statistics

Data were assessed by the method of analysis of ANOVA followed by t-test. $P<0.05$ was considered as statistically significant, $P<0.01$ was considered as statistically high significant.

3. RESULTS AND DISCUSSION

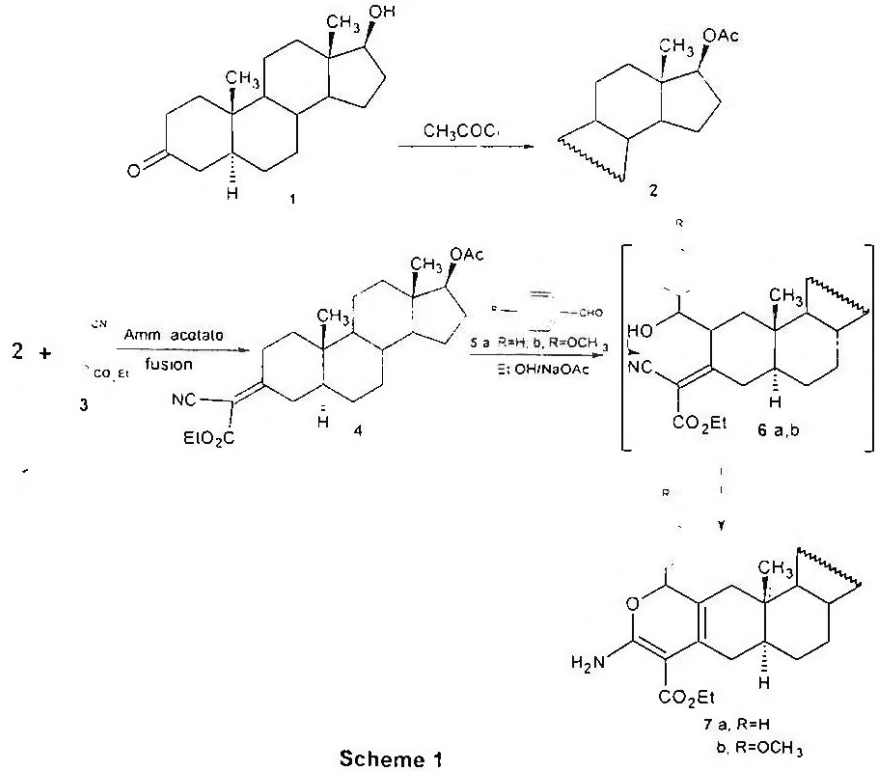
3.1. Chemistry

Pyrane, pyridine and thiophene rings represent molecular frameworks that serve as a platform for developing pharmaceutical agents for various applications. Many derivatives of pyrane, pyridine and thiophene proved as antituomr agents **[Cocco et al., (2007); Amr et al., (2006) and Brault et al., (2005)]**. These observations encouraged us to undertake the synthesis of pyrano, pyrido and thieno-steroid.

Simple acetylation of 17β-hydroxy-5α-androstan-3-one (androsrtanlone **1**) afforded 17β-acetoxy-5α-androstan-3-one **2**. Compound **2** fused with ethyl cyanoacetate **3** in the presence ammonium

acetate to form the Knoevenagel condensed product, 3-ethoxycarbonylacetonitril-5α-androstane derivative **4** in 70% yield (Scheme 1). The mass spectrum of compound **4** revealed the presence of molecular ion peak at m/z = 427 (18 %) and the ^{1}H NMR spectrum showed, beside the expected signals of androstane moiet, the presence of triplet signal at δ =1.35 (3H) and a quartet signal at δ = 4.25 (2H) which are characteristic for the ethyl ester group.

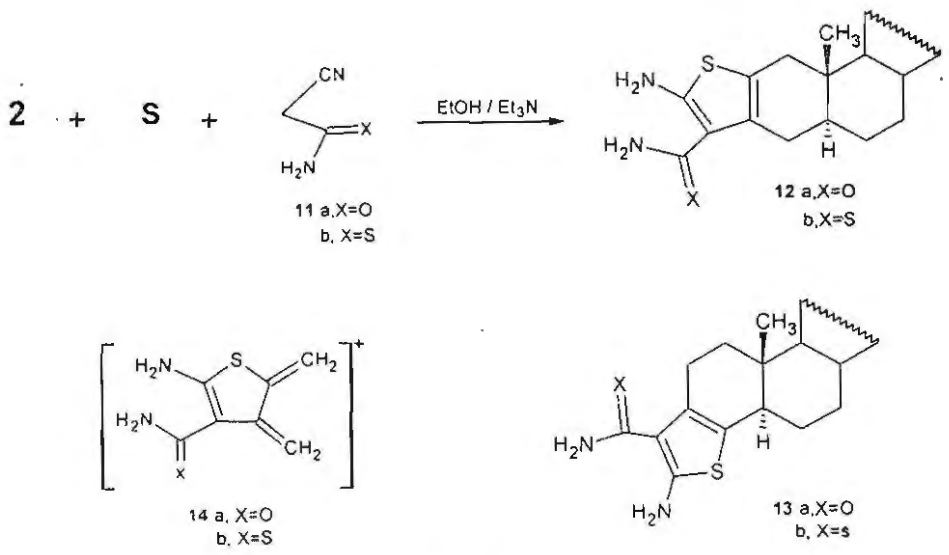
The presence of the α,β-unsaturated nitrile moiety in compound **4** showed interesting activity towards several chemical reagents. The reaction of compounds **4** with either benzaldhyde **5a** or anisaldhyde **5b** in refluxing ethanol in the presence of sodium acetate did not give the corresponding arylidine but afforded compounds containing amino groups which were identified, according to the analytical and spectral data *(cf. Materials and Methods)*, as 6'-amninopyrano[3',4':2.3] androstane derivatives **7a,b** respectively (Scheme 1). The formation of these compounds was assumed to proceed via the aldol adduct intermediates **6a,b** followed by cyclization (Scheme 1).



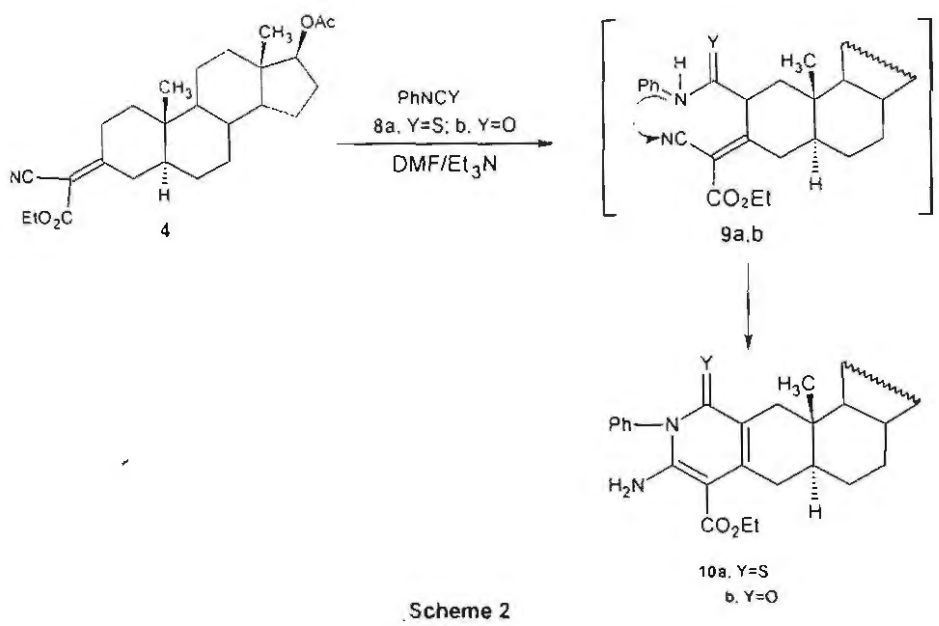
Scheme 1

The reaction of compound **4** with either phenyl isothiocyanate **8a** or phenyl isocyanate **8b** in dimethyl formamide using triethylamine as catalyst afforded the corresponding intermediates **9a,b** which readily underwent intramolecular cyclization to give the 6'-aminopyrido [3`,4`:2,3]androstane derivatives **10a,b** respectively (Scheme 2). Elucidation of the proposed structures **7a,b** and **10a,b** was based on their correct elemental analyses and compatible IR, ^{1}H NMR and mass spectral data *(cf. Materials and Methods)*.

The reaction of 3-keto steroids with cyanomethylene reagents and sulfur under Gewald`s conditions has been reported **[Roy, (1973) and Elmegeed et al., (2004)]**. Thus, the reaction of compound **2** with cyanoacetamide **11a** or cyanothioacetamide **11b** and sulfur in ethanolic triethylamine solution afforded the corresponding aminothieno [2`,3`:2,3]androstane derivatives **12a** and **12b** (Scheme 3) in 82% and 75% yield respectively. Identical mass spectra of the latter products indicate that the compounds are free from the angular isomer aminothieno[2`,3`:4,3]androstane derivatives **13a,b**. The mass spectra showed the molecular ion peaks at m/z = 430 (32 %) for compound **12a** and at m/z = 446 (52 %) for compound **12b** besides a base peak at m/z =168 and m/z =184 respectively for the retro-Diels Alder fragments **14a,b** (Scheme 3). All the analytical and spectral data of compounds **12a** and **12b** are in accordance with the proposed structures *(cf. Materials and Methods)*.



Scheme 3



Scheme 2

3.2. *In vitro* evaluation of the cytotoxic activity of compound 12a.

In this study, the most structurally promising, compound **12a** was investigated as anti-tumor agent against hepatoma ($HepG_2$) cell line. The

inhibition of proliferation of $HepG_2$ cells was determined using MTT assay. The usage of olive oil or DMSO as a solvent at a volume of 100µl (the maximum volume used to dissolve the tested compounds) had no significant effect on the viability of $HepG_2$ cells when treated for 24, 48 and 72h (figure1). Data in table 1 are expressed as percentage of cell growth inhibition of treated cells versus controls ± S.D. calculated on the average of the experiments performed in triplicate. The proliferation (celgrowth) of $HepG_2$ cells when treated with compound **12a** was significantly inhibited in a dose and time dependent manner, especially at 48 and 72h incubation time when olive oil used as solvent and at 24 and 48h incubation time when DMSO used as solvent. Significantly, there was no growth inhibition effect observed at 24h when olive oil or DMSO used as solvents. After 72h incubation when DMSO used as solvent the growth inhibition was 52% at 50µM and decreased to 29% at 100µM, the best results obtained at 72h when olive oil used as solvent at 50 and 100µM where the growth inhibition rate was 67% and 70% respectively.

The results expressed as IC_{50} values in µM were reported in table 2. The IC_{50} of compound **12a** decreased with the increasing of the incubation time when olive oil used as solvent. On the other hand, the IC_{50} increased with the increasing of the incubation time when DMSO used as solvent.

In comparison with data published about the cytotoxic activity of some steroids [**Yoshida et al., (2003)**] and from the structure activity relationship viewpoint, we postulate that the addition of diaminothiophene ring to the steroid moiety increased the cytotoxic activity especially at a concentration of 50µM and at 72h incubation period. In general the results at 72h when olive oil used as solvent is better than that of DMSO, this revealed that olive oil is suitable vechile for antitumor drug **(Kunihiro (1987)]**. These results are confirmed by the morphological study and its photographic data of light microscope at photograph 1.

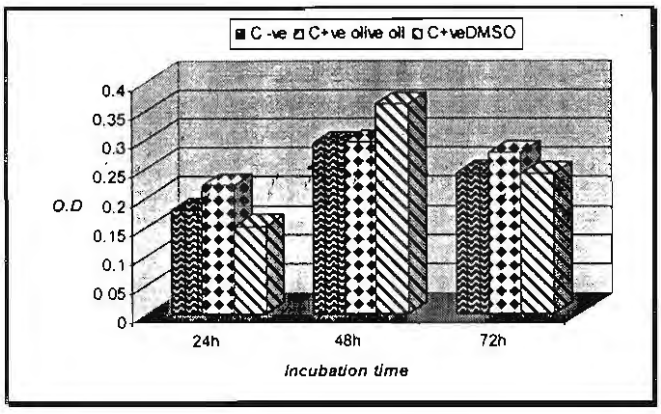


Fig.(1): Effect of DMSO and olive oil (100 µl) on growth of ($HepG_2$) cells, at different time intervals, C= control.

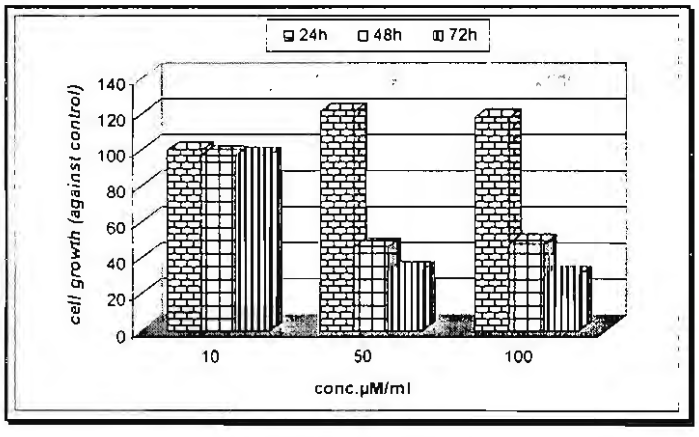


Fig. (2): Effect of compound **12a** dissolved in olive oil at 24, 48 and 72h on the proliferation of $HepG_2$ cells *in vitro*

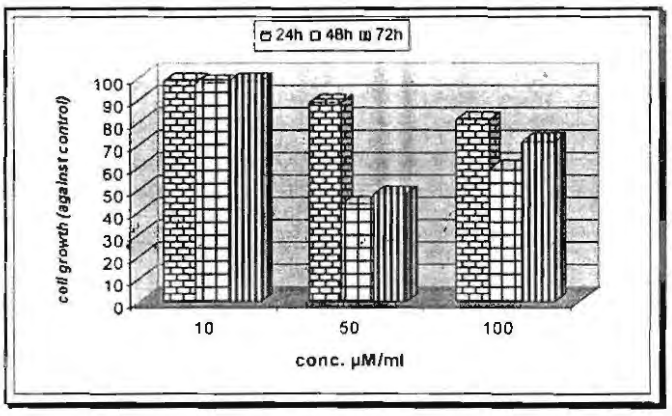


Fig. (3): Effect of compound **12a** dissolved in DMSO at 24, 48 and 72h on the proliferation of $HepG_2$ cells *in vitro*

Table (1): Effect of tested compound (**12a**) using olive oil or DMSO as solvent on hepatoma cell line proliferation, data expressed as percent of cell growth inhibition ± SD. $P < 0.05$ was considered as statistically significant*, $P < 0.01$ was considered as statistically high significant **.

Treatement	24 hr			48 hr			72 hr		
Conc.	10µM	50 µM	100 µM	10µM	50 µM	100 µM	10µM	50 µM	100 µM
12a (in olive oil)	99.78± 0.02	121.36 ±0 08	117.72 ±0.04	97.02±0. 00	46.44± 0.00**	48.13± 0 00**	96.67± 0.00	33.09± 0.00**	30.57± 0.00**
12a (in DMSO)	98.078 ±0 09	88.66± 0 00	81.33± 0 01	97.63±0 00	43.64±. 02**	59.11± 0.00**	98.27± 0 00	48 13± 0.03**	71.36± 0.00**

Table (2): The *in vitro* cytotoxic activity of compound **12a** (IC_{50} in µM).

Compound	Olive oil		DMSO	
12a	48 h	72 h	48 h	72 h
	47	36	43	50

3.3. *In vivo* study

The antitumor activity was evaluated on EAC bearing mice using animal model. The the novel steroid derivative **12a**, at dose level of (25mg/kg), completely inhibited the tumor growth in the experimental model and showed zero tumor volume at the end of *in vivo* experiment, while EAC control showed 4.56±0.37 mL. Our novel compound **12a** was considered as good as standard drug 5-flurouracil (20mg/kg) (table 3). It is well known that 5-flurouracil is one of the most commonly used drugs to treat cancer clinically. In cancer chemotherapy one of the major problem is anemia which mainly due to reduction in RBCs or hemoglobin percentage. Treatment with the novel compound **12a** remained the hemoglobin content, body weight, hematocrit % and WBC cells count near to normal values and similar to values observed with 5-flurouracil (table 3, fig.4). Furthermore, none of the treated mice with compound **12a** exhibited any abnormal behavior or any toxicity symptoms of dose used during this study. Compound **12a** was active as antitumor agent with no side effects, with no loss of appetite and showed no dizziness or erection of hairs or hypothermia. This indicates the safety of using it at specified low dose as chemotherapeutic agent.

Table (3): Effect of the tested compound **12a** on hematological parameters and on body weight of EAC bearing mice. n=5. mean±SD. $^{*}P< 0.05$ vs normal group, $^{**}P< 0.01$ vs normal group.

Parameters	Normal	EAC control (2x 10^6Cells/ mice)	12a (25mg/kg)+ EAC	Standard 5-flurouracil (20mg/kg)+ EAC
Hemoglobin /g%	13.63±0.75	6.86±2.25**	12.77±0.035	10.73±0.15**
Haematocrit /%	42.36±1.00	25.9±2.29**	38.32±0.17*	40.86±2.17
Total WBC/10^3-mm^{-3}	9.16±0.70	11.16±0.25**	6.58±0.1**	6.40±0.52**
Body weight /gm	22.66±0.57	27.33±2.51**	18.27±0.38*	23.0±1.0
Tumor Volume /mL	Nil	4.56±0.37	Nil	Nil

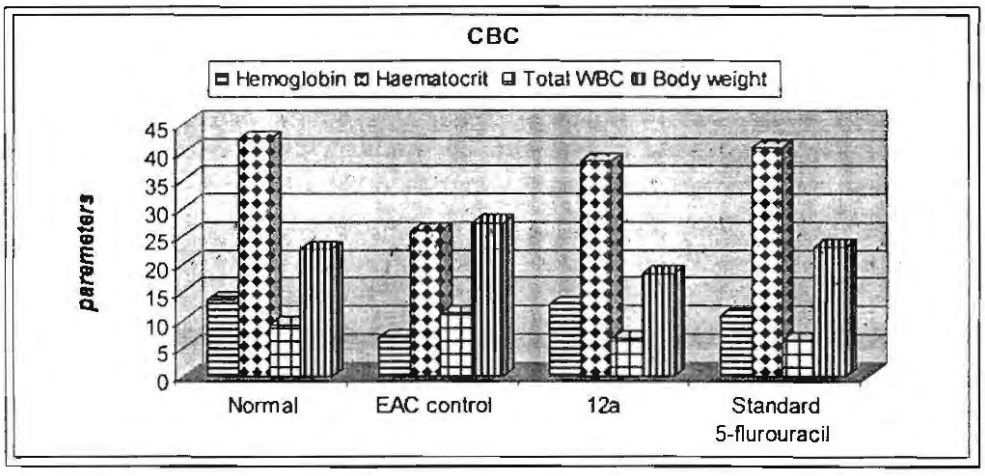
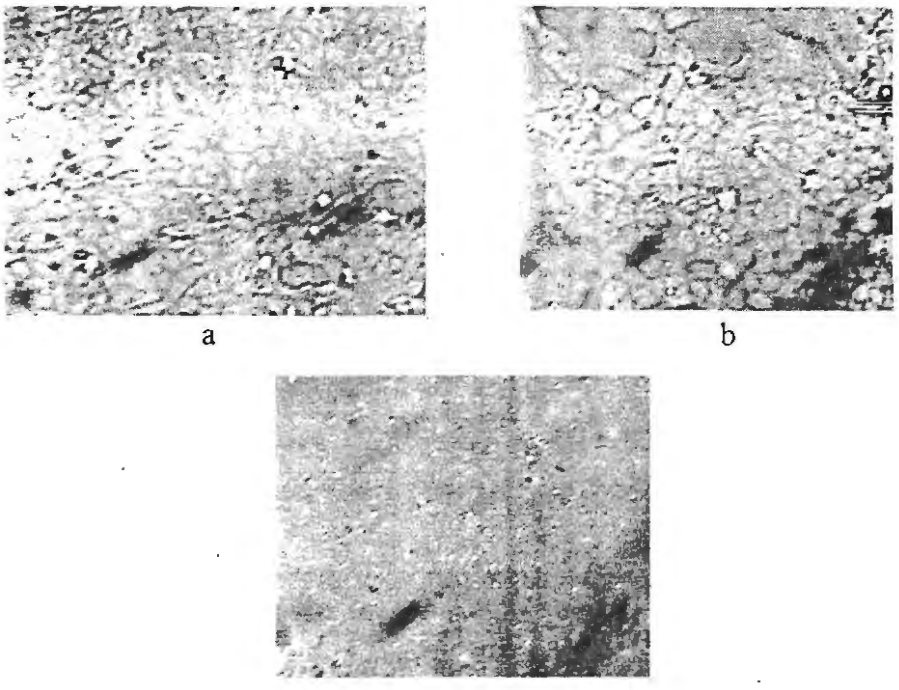
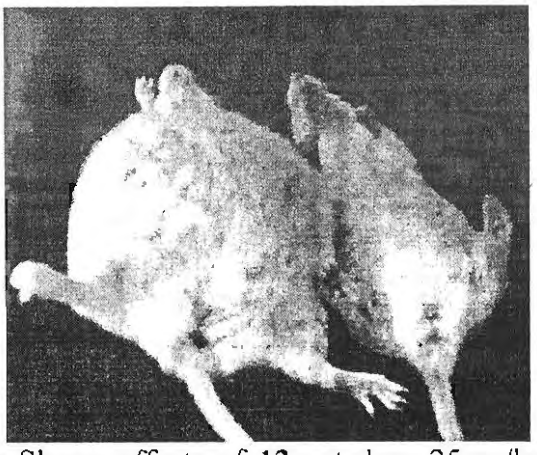


Fig. (4): Effect of the tested compound **12a** on hematological parameters and on body weight of EAC bearing mice.



Photograph I: The morphology of $HepG_2$ cells after 72 hour incubation: a) control without any treatment, b) conc. 100 μM of **12a** using DMSO as solvent, c) conc. 100 μM of **12a** using olive oil as solvent.



Photograph II: Shows effects of **12a** at dose 25mg/kg b.wt. on mice with EAC (right), compared to an EAC control -non treated- animal (left). Substantial difference observed on treated animals indicating complete response.

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تشيد سريع وتقييم النشاط البيولوجى لعدد من مشتقات الأمينواستيرويد المعدله

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الهدف الرئيسى من هذه الدراسة هو توضيح بعض الطرق الفعالة والسريعة لتشيد عدد من المركبات الاستيرودية المعدلة والتى تحتوى على حلقات الامينو-بيران، الامينو-بيريدين والامينو- ثيوفين بتفاعل مركب الاسيتوكسى اندروستين 2 مع كاشف سيانواسيتات الايثيل أعطى مشتق الاندروستان المقابل ٤. تم دراسة مقدرة المركب ٤ على تكوين المشتقات الاستيرودية الجديدة التى ترتبط بحلقات غير متجانسة وهى مركبات **7a,b** و**10a,b**، ايضا بتفاعل مركب ٢ مع مشتق السيانواسيتاميد **11a** ومشتق السيانوثايواسيتاميد **11b**، أعطى مشتقات واعدة كمضادات للاورام وهى مشتقات الامينوثيانواندروستين **12a,b**. تناول البحث ايضا دراسة دور المحتمل للمركب **12a** ضد خلايا اورام الكبد خارج جسم الكائن الحى وتاثيره على الخلايا السرطانية (الايرليخ) داخل جسم الكائن الحى.