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Estrogenic and Anti-Inflammatory Activities of a Steroidal Indoxyl

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The estrogenic and anti-inflammatory activities of 3-methoxy-16,17-seco-16-norestra-1,3,5-trien-15-(2'-indoxyliden)-17-oic acid is reported. After intraperitoneal administration, the dose of this compound required to reduce swelling of the rat paw by 50% (ED_{50}) was 14.1 mg/kg using the carrageenan-induced rat paw oedema anti-inflammatory assay method. Indomethacin had an ED_{50} of 3.2 mg/kg in this assay while dexamethasone had an ED_{50} of 1.7 mg/kg. The estrogenic activity of the compound after intramuscular administration in rats was 0.72 relative to diethylstilbestrol, when the two compounds were assayed at three dose levels of 1.0, 0.3 and 0.1 mg/kg.

Key Words: Steroidal indoxyl, synthesis, estrogenic, anti-inflammatory

INTRODUCTION

Estrogens show acute anti-inflammatory effects and even protect against tissue damage induced by snake venoms where antihistamines, corticosteroids, prostaglandin antagonists and protease inhibitors prove ineffective [1]. Estrogens also inhibit adjuvant arthritis in rats [2]. Several mechanisms have been proposed for the anti-inflammatory activity of estrogens [3]. A steroidal indoxyl, 3-methoxy-16,17-seco-16-norestra-1,3,5,-trien-15-(2'-indoxyliden)-17-oic acid **1** (Figure 1) has some of the structural features found in the naturally occurring estrogens, such as estrone, of which it is a derivative. Compound **1** also shows structural similarities to the acidic non-steroidal anti-inflammatory drugs (NSAID). This group of drugs generally has two hydrophobic moieties, comparable to the steroidal and indoxyl moieties of **1**, in addition to a carboxylic acid function [4]. These structural similarities prompted us to investigate the estrogenic and anti-inflammatory activities of **1**.

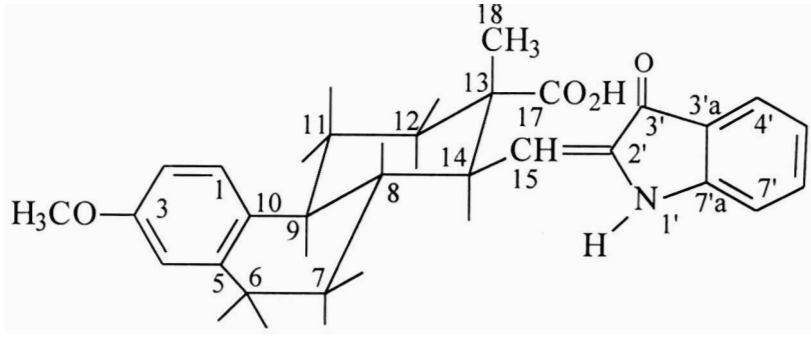

Figure 1: Structure of compound 1

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Compound **1** was synthesized by a base-catalyzed condensation reaction between estrone-3-methyl ether and 2-nitrobenzaldehyde and its identity and purity was established using physical, chromatographic and spectroscopic methods. The synthesis of structurally similar steroidal indoxyls from 17-ketosteroids has been described previously [5].

EXPERIMENTAL

Estrone-3-methyl ether, indomethacin, dexamethasone, diethylstilbestrol, 2-nitrobenzaldehyde and carrageenan Type 1 were all obtained from Sigma Chemical Company (St. Louis, MI, U.S.A.). The rest of the reagents were laboratory reagent grade, from BDH (Poole, UK).

Synthesis

3-Methoxy-16,17-seco-16-norestra-1,3,5,-trien-15-(2'-indoxyliden)-17-oic acid, **1**, was synthesized as follows: to a solution of estrone-3-methyl ether (3.0 g, 10.0 mmol) in ethanol (250 ml) was added a solution of 2-nitrobenzaldehyde (1.6 g, 10.6 mmol) in ethanol (50 ml) followed by aqueous KOH (3 g in 3 ml H_2O). The solution was allowed to stand at room temperature for 72 h and then concentrated *in vacuo* to about 50 ml followed by the addition of dilute HCl with vigorous stirring. The precipitated solid was filtered, washed with water and dried. Recrystallization from methanol gave (**1**) (2.32 g, 52 %) as orange needles m.p. 245-246 °C (uncorrected).

IR spectrum was run as KBr matrix on a Perkin Elmer 727 B IR recording spectrophotometer (Perkin Elmer, Buckinghamshire, UK). UV spectrum in methanol as solvent was run on a Philips PU8741 UV/VS scanning spectrophotometer (Philips, Eindhoven, the Netherlands). Liquid chromatography was carried out using an LC system consisting of a L-6200 intelligent pump (Merck, Darmstadt, Germany), an Autosampler Spectra Series AS 100 equipped with a 100 µL loop (Thermo Separation Products, Freemont, USA) a variable wavelength Spectra 100 UV-VIS detector set at 262 nm and an integrator model HP 3396A series (Hewlett Packard, Avondale, PA, USA). An XTerra™ RP C_{18} column (250 x 4.6 mm ID) (Waters, Milford MA, USA) was immersed in a waterbath at 45 °C. The mobile phase used was acetonitrile-water-0.2 M ammonium acetate buffer pH 6.5 (40:55:5, v/v) and the sample was dissolved in methanol.

The LC-MS spectrum was obtained using an LCQ Ion Trap Mass Spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization source operated in the negative ion mode. The LC apparatus consisted of a Spectra System P1000XR quaternary pump, a Spectra Series AS 100 Autosampler equipped with a 20 µL loop, a variable wavelength Spectra 100 UV-VIS detector set at 262 nm, all from Thermo Separation Products (Freemont, CA, USA). The UV data were processed with ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK). The XTerra™ RP C18 column (3.2 µm, 100 x 2.1 mm ID) was immersed in a waterbath at 45 °C. The mobile phase used was acetontrile-water-0.2 M ammonium acetate buffer pH 6.5 (40:55:5, v/v) and the sample was dissolved in methanol.

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian (Palo Alto, USA) Unity 500 spectrometer operating at 499.193 MHz for ^{1}H and at 125.534 MHz for ^{13}C, and using an inverse 5 mm broad-band probe with $\pi/2$ pulses of 5.5 and 18.5 µs, respectively and equipped with pulsed magnetic field gradient coils. The standard Varian software Vnmr version 6.1b was used throughout. The measurements were performed in deuterated DMSO solution at 27 °C with tetramethylsilane as internal standard set at 0 ppm for ^{1}H spectra, and using the solvent multiplet set at $\delta = 39.6$ ppm for the ^{13}C-NMR spectra. Spectral assignments (s = singlet, d = doublet, t = triplet, q = quadruplet, br = broad, overl = overlapped signal, a = axial, e = equatorial) were based not only on chemical shift rules and coupling patterns (using first order analysis), but also on APT (Attached Proton Test) experiments for ^{13}C and especially on routine 2D-correlations such as COSY45-, GHSQC- (single bond or ^{1}J C,H-correlations), and GHMBC-experiments (multiple bond or ^{3}J/^{2}J C,H-correlations).

IR (cm^{-1}): 3450 (OH), 3425 (NH), 2750-2400 (COOH), 1680, 1630 (C=O and C=C of α, β-unsaturated carbonyl).

UV λ_{max} (nm): 230, 261, 275 (sh) 300 (sh) and 450.

% Purity: (LC) 99.65 in partially resolved isomeric mixture (54.67; 44.98). $[M-H]^-$ (LC-MS): 416
(100 %).

^{1}H NMR (DMSO-d_6): δ 1.210 (s, 3H, 18-H_3), 1.385 (overl m, 2H, 7-H_a and 11-H_a), 1.500 (dq, *1H, J_d=2.0 Hz and J_q=11.1 Hz, 8-H), 1.683 (ddt,* 1H, ^2J_d=12.8 Hz, J_d=5.4 Hz and J_t= 2.6 Hz, 7-H_e), 1.811 (dt, 1H, ^2J_d=13.2 Hz and J_t=3.2 Hz, 12-H_e), 2.031 (dt, 1H, J_d=3.6 Hz and J_t=13.4 Hz, 12-H_a), 2.404 (overl m, 2H, 9-H and 11-H_e), 2.720 (m, 2H, 6-H_2), 2.976 (t, 1H, J=11.4 Hz, 14-H), 3.690 (s, 3H, OMe), 5.660 (d, 1H, J=11.7 Hz, 15-H), 6.600 (d, 1H, J=2.4 Hz, 4-H), 6.706 (dd, 1H, J=2.4 Hz and J=8.8 Hz, 2-H), 6.799 (t, 1H, J=7.6 Hz, 5'-H), 6.998 (d, 1H, J=8.3 Hz, 7'-H), 7.220 (d, 1H, J=8.8 Hz, 1-H), 7.451 (dt, 1H, J_d=1.2 Hz and J_t=7.8 Hz, 6'-H), 7.505 (br d, 1H, J=7.4 Hz, 4'-H), 9.366 (s, 1H, NH), 12.195 (s, 1H, COOH) ppm.

^{13}C NMR (DMSO-d_6): δ 15.4 (CH_3, C18), 25.3 (CH_2, C11), 27.4 (CH_2, C7), 29.3 (CH_2, C6), 36.2 (CH_2, C12), 38.8 (CH, C8), 42.3 (CH, C9), 46.01 (C, C13), 46.03 (CH, C14), 54.9 (CH_3, OMe), 111.7 (CH, C7'), 111.8 (CH, C2), 112.9 (CH, C4), 113.3 (CH, C15), 118.5 (CH, C5'), 120.2 (C, C3'a), 124.2 (CH, C4'), 126.2 (CH, C1), 131.4 (C, C10), 136.4 (CH, C6'), 137.6 (C, C5), 138.7 (C, C2'), 154.1 (C, C7'a), 157.3 (C, C3), 178.4 (COOH, C17), 184.8 (CO, C3') ppm.

ESTROGENIC ACTIVITY

The estrogenic activity of (**1**) was screened with diethylstilbestrol (DES) as standard using the 4-day uterine assay method [6]. Immature female albino rats, 21-25 days old, weighing between 40-60 g, were used. DES and (**1**) were each administered intramuscularly in olive oil as vehicle in volumes of 0.2 ml at three dose levels of 1.0, 0.3 and 0.1 mg/kg daily for three consecutive days. Six rats were used for each dose level and six control rats received 0.2 ml of the vehicle. Twenty-four hours after the last dose, the uterine horns were excised under ether anaesthesia, emptied of any fluid present and weighed.

Anti-Inflammatory Activity

The carrageenan-induced rat paw oedema method [7] was used. Indomethacin, dexamethasone and compound **1** were suspended in 2 % carboxymethylcellulose whereas carrageenan 1 % was prepared in normal saline. The compounds were administered intraperitoneally in dose volumes of 0.5 ml into male albino rats (180-210 g), one hour before injection of 0.1 ml of carrageenan into the subplantar area of the left hind paw. The doses employed were: *indomethacin 1.67, 3.00, and 5.00 mg/kg;* dexamethasone 1.00, 2.00 and 4.00 mg/kg and compound **1** 3.00, 10.00, 25.00 and 50.00 mg/kg, body weight. Control animals received 0.5 ml of the vehicle, 1 h before the injection of carrageenan. Six rats were used at each dose level as well as the for the controls. Before carrageenan injection, the initial paw volume (Vi) was measured using mercury displacement method. Three hours after carrageenan administration, the final paw volume (V_f) was measured.

The change in volume of the rat paw for the control (ΔVc) and test (ΔVt) was obtained as follows:

$$\Delta Vc = Vf - Vi$$
$$\Delta Vt = Vf - Vi$$

and the % inhibition of oedema was calculated from the equation:

$$\% \text{ inhibition} = 100\left[1 - \frac{\Delta Vt}{\Delta Vc}\right]$$

RESULTS AND DISCUSSION

Chemical structure

From the NMR spectra it was easily deduced that compound **1** had a molecular formula of $C_{26}H_{27}NO_4$ which corresponds with the data of the mass spectrum showing a $[M-H]^-$ at 416. Indeed, the ^{13}C-NMR spectrum showed two carbonyl peaks above 170 ppm typical for a conjugated ketone and a carboxyl group. In the aromatic region, there were six quaternary and eight methine carbons at the shifts as expected for a methoxy-substituted annealed phenyl and an indoxyl group connected via an exocyclic double bond. The substitution pattern in the two phenyls was readily deduced from the coupling information in the aromatic region of the ^{1}H NMR spectrum. In the aliphatic region of the carbon spectrum, ten signals were found, namely two methyls corresponding to a methoxy group and a ring methyl-substituent, four signals typical for methylene carbons, three methine peaks, and an almost obscured signal for a non-protonated carbon at 46.01 ppm. All these signals were nicely correlated

with each other via 2D COSY- and GHSQC-experiments, and the whole analysis was then finally checked by a GHMBC or multiple bond C,H-correlation experiment. In this way, not only was the proposed overall structure of the synthesized compound (Figure 1) strongly confirmed, but also its conformation could be deduced on the basis of the coupling constants in the ^{1}H-NMR spectrum.

Estrogenic Activity

The uterine weight assay is one of the most commonly used methods of determination of estrogenic activity [8]. This uterotropic assay uses the weight increase of the uterus of immature intact rats as an endpoint [6]. Potency comparison can be determined from the linear region of the log dose-response curves. Figure 2 shows the plot of net weight of uterine horns against log dose obtained from our assay. Compound **1** had 72 % the estrogenic activity of DES when computed from linear regression plots.

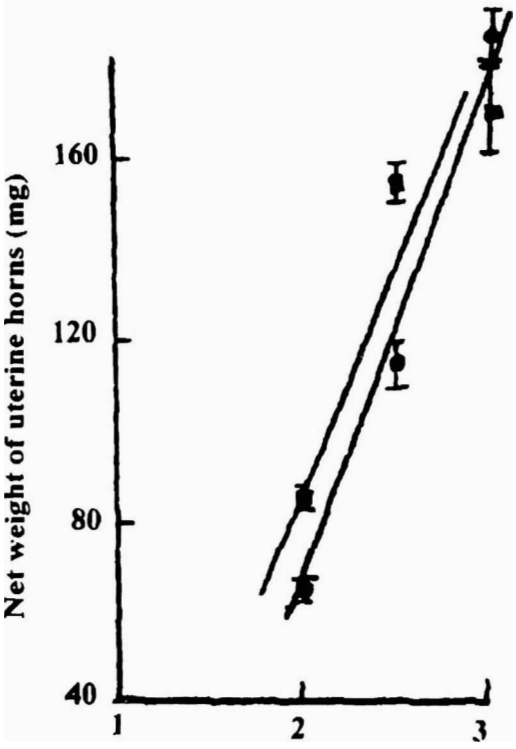

Figure 2: Effect of intramuscular administration of different doses of diethylstilbestrol (DES) and (1) for 3 consecutive days on the net weight of uterine horns of the rat. The log dose is for the daily dose in µg/kg.

■ **diethylstilbestrol**
● **Compound 1**

Anti-Inflammatory Activity

Table 1 shows the percentage inhibition of carrageenan rat paw oedema produced by various doses of indomethacin, dexamethasone and compound **1**. A plot of percentage inhibition of oedema against log dose gave ED_{50} values (50 % inhibition) of 3.2, 1.7 and 14.1 mg/kg for indomethacin, dexamethasone and compound **1** which is equivalent to 8.9 x 10^{-3}, 4.3 x 10^{-3} and 33.7 x 10^{-3} mMol/kg, respectively.

Table 1: Inhibition of carrageenan oedema produced by indomethacin, dexamethasone and compound 1 after intraperitoneal administration

Test Substance	Dose (mg/kg)	Mean % Inhibition (± s.e.m.)
Indomethacin	5.0	65.1 ± 4.1
Indomethacin	3.0	50.1 ± 2.7
Indomethacin	1.7	27.0 ± 6.9
Compound (1)	50.0	67.4 ± 6.9
Compound (1)	25.0	65.5 ± 7.7
Compound (1)	10.0	45.2 ± 4.9
Compound (1)	3.0	22.6 ± 7.7
Dexamethasone	4.0	76.8 ± 5.2
Dexamethasone	2.0	64.1 ± 7.8
Dexamethasone	1.0	7.2 ± 2.2

s.e.m.: Standard error of the mean

The ED_{50} for indomethacin in the carrageenan test in 27 studies has been reported to be 3 mg/kg [9] which is in agreement with the value obtained in the present study.

In addition to the several mechanisms proposed for the anti-inflammatory effect of estrogens, compound **1** may also act in a similar manner to the NSAID due to its structural similarity to these drugs, which are potent inhibitors of prostaglandin synthesis [10]. Compound **1**, an estrone derivative, is structurally related to the estrogens. It also shows partial structural resemblance to the acidic non-steroidal anti-inflammatory drugs (NSAID), and the activities noted for this compound could be a result of these structural similarities.

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