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SHORT COMMUNICATION

SYNTHESIS AND BIOLOGICAL ACTIVITY OF 4-METHYLESTRADIOL

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Summary—The synthesis of 4-methylestradiol (4-ME_2) was carried out by reductive aromatization of 4-methyl-1,4-androstadiene-3-one-17β-ol. The relative binding affinity of 4-ME_2 was found to be 10 and 25% of estradiol at 0 and 25°C, respectively. 4-ME_2 had considerably weaker uterotrophic activity relative to estrone and was found to have no antiuterotrophic activity.

INTRODUCTION

Recent studies [1–3] indicate that although 2-methylestradiol and 4-methylestradiol exhibit appreciable estrogen receptor binding affinities compared to estradiol, these compounds have considerably lower estrogenic activity than estradiol in terms of stimulating uterine growth in ovariectomized rats. These results seem to suggest that the 2- and 4-methylated estradiols might antagonize the estrogenic effect of estradiol. Indeed, 2-methylestradiol has been shown to have antiestrogenic activity as it can antagonize the effect of estradiol in stimulating uterine growth in immature mice. However, 4-methylestradiol (4-MeE_2) has not been studied for its antiestrogenic activity. In this communication, we report the synthesis and the biological activity of 4-methylestradiol as a potential antiestrogen.

EXPERIMENTAL

Chemical synthesis

4-Methyl-androsta-4-ene-3-one-17β-ol ***(2)***. To a solution of testosterone (**1**, 1.5 g, 5.2 mmol) and potassium *t*-butoxide (640 mg) in *t*-butyl alcohol (150 ml) was added, over a period of 150 min, methyl iodide (0.37 ml, 5.7 mmol) in *t*-butyl alcohol (100 ml). The reaction mixture was refluxed under N_2 gas for an additional 30 min, cooled, neutralized with 6 N HCl and extracted with $CHCl_3$. The organic layer was dried and evaporated, and the residue applied to a silica gel column. Elution with hexane and ethyl acetate/benzene (1:3) gave **2** (600 mg, 1.98 mmol, 38% yield).

4-Methyl-androsta-4-ene-3-one-17β-ol-acetate ***(3)***. To a solution of **2** (300 mg, 1 mmol) in pyridine (10 ml) was added acetic anhydride (1 ml). After standing at room temperature for 12 h, the reaction mixture was poured into 6 N HCl (250 ml) and extracted with ethyl acetate. The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. Recrystallization from hexane/ethyl acetate gave product **3** (320 mg, 0.94 mmol, 94% yield). m.p. 150–152°C; [^{1}H]NMR ($CDCl_3$) δ 0.84 (s, 3H, C–18–CH_3), 1.18 (s, 3H, C–19–CH_3), 1.78 (s, 3H, C–4–CH_3), 2.04 (s, 3H, C–17–$OOCCH_3$); IR (KBr) 2952 (aliphatic CH), 1733 (17–acetate C=0), 1659 (3–C=0), 1610 (C=C).

4-Methyl-androsta-1,4-diene-3-one-17β-ol-acetate ***(4)***. To a solution of **3** (3.8 g, 11.0 mmol) in dry dioxane (30 ml) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (3.2 g, 14.1 mmol) and the solution was refluxed under N_2 for 12 h. After filtering through a pad of celite, the reaction mixture was diluted with $CHCl_3$ (250 ml) and washed thoroughly with 5% aqueous NaOH until the aqueous layer had no yellow color. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure, and the residue applied to a silica gel column. Elution with hexane/$CHCl_3$ afforded **4** (2.1 g, 6.1 mmol) in 55% yield. m.p. 170–172°C; [^{1}H]NMR ($CDCl_3$) δ 0.87 (s, 3H, C–18–CH_3), 1.22 (s, 3H, C–19–CH_3), 1.91 (s, 3H, C–4–CH_3), 2.04 (s, 3H, C–17–$OOCCH_3$), 6.25 (d, 1H, J = 10.75 Hz, C–1–H), 7.02 (d, 1H, J = 10.75 Hz, C–2–H); IR (KBr) 2938 (aliphatic CH), 1734 (17–acetate C=0, 1658 (3–C=0), 1621, 1604 (C=C).

4-Methyl-estra-1,3,5(10)-triene-3,17β-diol ***(5)***. To a mixture of lithium (30% suspension in wax, 160 mg), bisphenyl (430 mg) and diphenylmethane (300 μl) in THF (20 ml) was added, dropwise, a solution of **4** (300 mg, 0.87 mmol) in THF (10 ml) over a period of 30 min. The reaction mixture was refluxed for an additional 15 min. After the excess lithium was destroyed by methanol (5 ml), the reaction mixture was evaporated under reduced pressure. The resultant mixture was taken up by $CHCl_3$ and washed with 3 N HCl, 10% $KHCO_3$ and water, sequentially. After the organic phase was dried over $MgSO_4$ and evaporated under reduced pressure, the crude product was purified by flash chromatography over a dry silica gel column using benzene/ethyl acetate (3:1) as eluting solvent. Recrystallization from acetone/hexane gave **5** (45 mg, 0.16 mmol, 19% yield). m.p. 217–219°C; [^{1}H]NMR ($CDCl_3$ δ 0.77 (s, 3H, C–18–CH_3), 2.15 (s, 3H, C–4–CH_3), 6.61 (d, 1H, J = 8.9 Hz, C–1–H), 7.05 (d, 1H, J = 8.9 Hz, C–2–H); IR (KBr) 3458 (C–3–OH), 3254 (C–17–OH), 2938 (aliphatic CH), 2595 (aromatic C=C).

Biological methods

Estrogen receptor binding assay [4, 5]. Uteri freshly dissected from 5 immature female Sprague–Dawley rats (Biolab Corporation, St Paul, Minn., 22–24-days-old, average body wt 42 $\pm$ 4.4 g) were rinsed with TED buffer. All the following procedures were carried out at 4°C. The washed uteri (net weight 0.15–0.25 g) were homogenized in TEMG buffer (10 nM Trizma base, 1.5 mM EDTA, 0.1%

monothioglycerol, 10% glycerol, pH adjusted to 7.4 with HCl) with a motor-driven glass-Teflon homogenizer. The homogenate was centrifuged at 100,000 *g* for 35 min. After removing the fat floating on the surface, the supernatant (cytosol fraction containing estrogen receptor) was further diluted with TED buffer to a protein concentration of 3.5 mg/ml. Diluted cytosol (150 μl) was added to each tube (12 × 75 mm) containing [1,2,4,6-^{3}H]estradiol (50 μl, 4 nM, SA 91 Ci/mmol) and assay solution of test compounds or nonradioactive estradiol (50 μl, concentration varied from 0 to 3 μM at 9 levels). Each concentration of the assay solution was run in duplicate and the final volume of each mixture was 250 μl. After incubation for 2 h at 4°C or 3 h at 25°C, 300μl DCC solution (dextran-coated charcoal solution: 10 mM Trizma base, 1.0 mM EDTA, 250 mM sucrose, 0.05% dextran, 0.5% charcoal) was added to each incubation tube, vortexed and allowed to stand in ice for 15 min with occasional shaking. After centrifugation at 2000 *g* for 5 min at 0°C, 200 μl supernatant was pipetted into vials containing 2 ml scintillation solution (0.055% PPO, 0.001% POPOP, 66.7% toluene, 33.3% Triton X-100) and the radioactivity (cpm) determined. The relative binding affinity of each test compound was determined by comparing the concentration of test compound required to reduce the specific binding of tritium-labeled estradiol to estrogen receptor by 50% to the concentration of unlabeled estradiol required to achieve the same reduction.

Uterotrophic and antiuterotrophic assay. For uterotrophic and antiuterotrophic studies, compounds were prepared in olive oil immediately before injection; the desired dosage was administered in 0.1 ml oil. Control group received only 0.1 ml oil injection. Groups of immature rats (22 days-of-age; from Biolab Co., St Paul, Minn.) received s.c. injections for 3 days, and animals were killed 24 h after the last injection. The uteri were excised, slit longitudinally, blotted, and weighed. The uterotrophic and antiuterotrophic activities were determined as described previously [6].

Statistical analysis

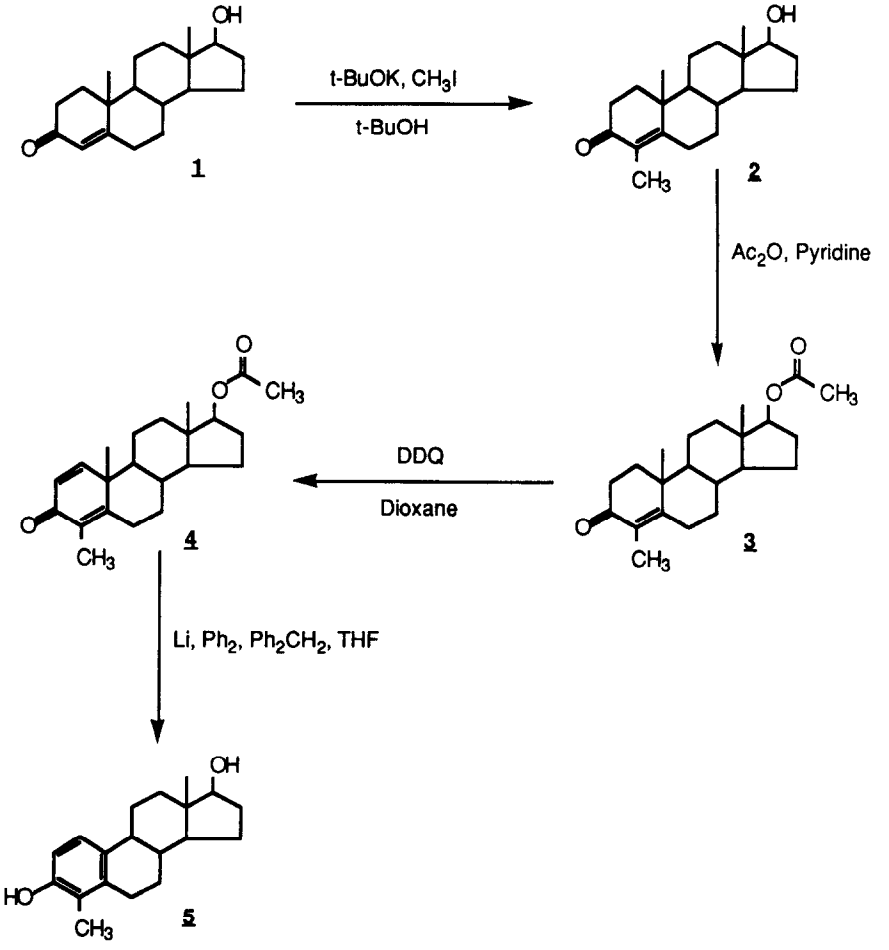
Student's *t*-test was used, and the level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

4-Methylestradiol was synthesized by well-established procedures as outlined in Scheme 1. Testosterone was methylated at the 4-position by treatment with potassium *t*-butoxide and methyl iodide. Protection of the 17β-hydroxy group by acetylation followed by DDQ dehydrogenation gave compound **4** which was reductively aromatized by lithium in tetrahydrofuran to give 4-methylestradiol in 25% yield.

The relative binding affinity data of 4-methylestradiol indicated that 4-methylsubstituted estradiol bound to the estrogen receptor with RBA of 10% or 30% of estradiol at 0 or 25°C, respectively. However, it showed considerably weaker uterotrophic activity at doses up to 100μg (Fig. 1), confirming previous results [1].

The ability of 4-methylestradiol to antagonize the effect of estradiol in immature rats is shown in Fig. 1. 4-Methylestradiol at daily doses of 5 and 25 μg exhibited antiestrogenic activity of 14 and 17%, respectively, while at a daily



Scheme 1. Synthesis of 4-methylestradiol.

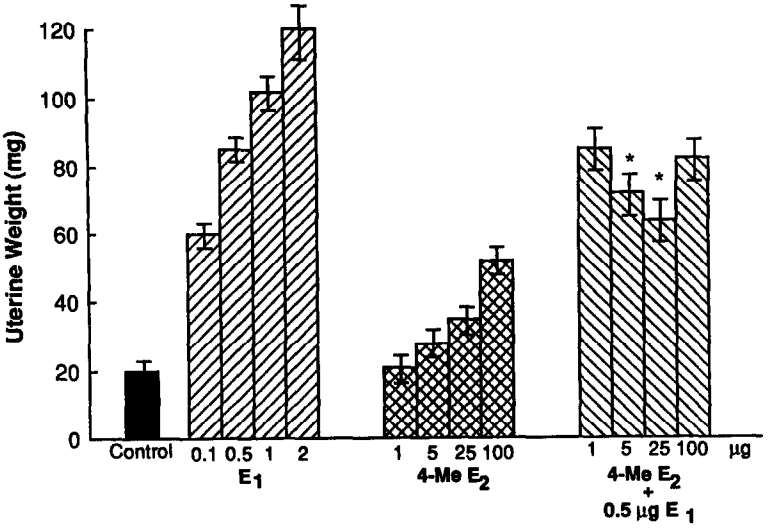


Fig. 1. Effects of 4-MeE_2 and E_1 alone or in various combination on uterine weight. Immature 22-day-old rats were injected with the indicated daily dose of compound(s) once daily for 3 days, and uterine weight was determined 24 h after the last injection. Values are the mean ± SEM, with ten rats in each treatment group.* $P < 0.05$ vs E_1 (0.5 µg) control.

dose of 100 µg no antiestrogenic activity was observed. These results are not too surprising if one takes into consideration the appreciable receptor binding affinity for 4-MeE_2 and its low estrogenic activity. Thus, at 5 and 25 µg doses 4-MeE_2 inhibits binding of E_2 to its receptor, and since this compound has low estrogenic activity the net effect is a small but significant decrease in uterine tissue stimulation. However, with the 100 µg dose the 4-MeE_2 seems to have appreciable estrogenic activity, resulting in no antiuterotrophic acivity.

Ball *et al.*[2] proposed that 4-hydroxylation of estradiol *in vivo* constitutes an essential step in the expression of estrogenicity of estradiol. When this step is blocked, estradiol will not induce its physiological estrogenic activity *in vivo* although it still can bind strongly to the estrogen receptor in the target cell. The results from this study seem to be in agreement with this hypothesis.

REFERENCES

1. Kirchoff J., Wang X., Ghraf R., Ball P. and Knuppen R.: Interactions of methylestrogens with cytoplasmic and nuclear estrogen receptors in rat pituitary gland, hypothalamus and uterus. *Brain Res.* **295** (1984) 354–358.
2. Ball P., Emons G., Haupt O. and Knuppen R.: Pharmacological effects of 2- and 4-methyloestradiol as a probe to test the biological importance of 2- and 4-hydroxylation of oestrogens (catecholoestrogen formation). *Acta Endocr.* **102** (1983) 150–152.
3. Raynaud J. P., Ojasoo T., Bouton M. M., Bignon E., Pons M. and Crastes de Paulet A.: Structure-activity relationships of steroid estrogens. In *Estrogens in the Environment* (Edited by J. A. McLachlan). Elsevier, New York (1985) pp. 24–41.
4. EORTC Breast Cancer Cooperative Group. Standards for the assessment of estrogen receptors in human breast cancer. *Eur. J. Cancer* **9** (1973) 379–381.
5. Martucci C. and Fishman J.: Uterine estrogen receptor binding of catechol estrogens and of estetrol[1,3,5(10)-estratriene-3,15α,16α,17β-tetrol]. *Steroids* **27** (1976) 325–333.
6. Qian X. D. and Abul-Hajj Y. J.: Synthesis and biological activity of 17β-substituted estradiol. *J. Steroid Biochem.* **29** (1988) 657–664.