Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 1259–1261. Pergamon Press. Printed in Great Britain

TWO DIMERS, 4:4'- AND 2:2'-DI[ESTRADIOL], OBTAINED BY CHEMICAL OXIDATIVE COUPLING OF ESTRADIOL

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(*Received* 10 *February* 1977)

SUMMARY

Oxidative coupling of estradiol with potassium ferricyanide in an alkaline medium gave the same results as those obtained with peroxidase. The two dimers 4:4'-di[Estra-1,3,5(10)-trien-3,17β diol] and 2:2'-di[Estra-1,3,5(10)-trien-3,17β diol] were isolated from the reaction products. The structures were determined by mass spectrometry and n.m.r.

INTRODUCTION

Horseradish peroxidase (HRPO EC 1.11.1.7) in the presence of hydrogen peroxide catalyses the reaction[1]:

$$\text{Estradiol} + H_2O_2 \xrightarrow{\text{HRPO}} \text{products} + H_2O$$

The study of this *in vitro* reaction is of interest because estrogens can be metabolized by phenol-oxidising enzymes [2, 3] and can also react *in vivo* in the presence of NADH-linked oxidases with other organic compounds [4]. The products of these reactions have little or no estrogenic activity [4].

The reaction was carried out in a heterogeneous water–organic-solvent system (P. Cremonesi, private communication), but even so the low yield of the reaction made it difficult to characterize the products.

We decided to reproduce this reaction by non-enzymatic means to increase the yield so as to obtain amounts of products sufficient for characterisation. Of the several possible oxidants for this reaction, we elected to use the most frequently used system; potassium ferricyanide in alkaline medium [5–7].

EXPERIMENTAL

Melting points were obtained using a Kofler hot-plate microscope and are uncorrected. U.V. data were recorded using a Beckman DU2; I.R. spectra using a Perkin-Elmer 237 (nujol). The n.m.r. spectra were obtained using a Perkin-Elmer R12 (60 MHz) with tetramethylsilane as internal reference, and the mass spectra using a Perkin-Elmer 270 Mass-Spectrometer with an electron potential of 80 eV. The elementary analysis were performed using a CHN mod. 185 F. & M. apparatus. Purity was tested by t.l.c. on Merck silica gel F_{254} plates; eluants: acetone–*n*-hexane, (1/1, v/v), benzene–ethyl acetate, (1:1, v/v), benzene–ethyl acetate–acetone, (5:4:1,; by vol.) detection by spraying with 9M-H_2SO_4 in water and heating at 120°C for 10 min.

4:4'-di[Estra-1,3,5(10)-trien-3,17β diol] (I) *and 2:2'-di[Estra-1,3,5(10)-trien-3,17β diol]* (*II*)

Estradiol (2.72 g) was dissolved in 50 ml tetrahydrofuran; 30 ml of a solution of $K_3Fe(CN)_6$(0.37 M) and NaOH(0.1 M) were added and stirred at room temperature for 30 min.

After acidification with HCl and extraction with ethyl acetate the organic phase was evaporated to dryness.

The residue was chromatographed. Silica gel (Type 60, Merck) and Celite 535 (Johns–Manville) were used as supplied. Equal weights of silica gel and celite were thoroughly mixed, and 150 g were transferred to a glass column (3.8 cm, i.d.) as a slurry in ethyl acetate–benzene–chloroform (3:2:1, by vol.) to yield a column 80 cm high.

The residue was applied to the column in the same solvent and the solvent collected at a rate of 2 ml per min.

Unreacted estradiol (1.9 g) was found after 4.4 l had passed through the column: compounds I (183 mg) and II (232 mg) were eluted after 5.1 l. and 5.35 l. respectively. Other compounds (260 mg) were eluted from the column with larger volumes of solvent, but these were not investigated.

Compounds I and II were acetylated with pyridine and ethyl acetate to give 180 mg of IA and 226 mg of IIA. These were recrystallized from methanol.

IA: m.p. 98–100°; I.R. 1775, 1740, 1615, 1580, 1245 cm^{-1}; n.m.r. ($CDCl_3$) δ 0.95 (s,3), 2.00 (s), 2.10 (s), 4.65 (m,1), 6.63 (*d*,1,J = 8 Hz), 7.05 (*d*,1,J = 8 Hz). Mass spectrum M^+ = 710. (Calc. for $C_{44}H_{54}O_8$: C = 74.34%, H = 7.66%. Found: C = 74.22%, H = 7.83%).

IIA: m.p. 111–113°; I.R. 1770, 1740, 1615, 1580, 1245 cm^{-1}; n.m.r. ($CDCl_3$) δ 0.93 (s,3), 2.01 (s), 2.03

(s), 4.65 (m,1), 6.73 (s,1), 7.05 (s,1). Mass spectrum $M^+ = 710$. (Calc. for $C_{44}H_{54}O_8$: C = 74.34%, H = 7.66%, Found: C = 74.15%, H = 7.38%).

Reaction between HRPO and estradiol

The reaction catalyzed by HRPO was performed by shaking 400 ml of ethyl acetate containing 4.25 g of estradiol and 1 l. of buffer solution pH 7, containing 15 ml of HRPO (lyophilized powder). 0.5 ml Of H_2O_2 (30%) in 125 ml buffer pH 7 was added simultaneously at a rate of 50 ml/h. After 12 h the organic layer was separated, evaporated and the residue was chromatographed.

Equal weights of silica gel and celite were thoroughly mixed, and 230 g were transferred to a glass column (4.3 cm, i.d.) as a slurry in ethyl acetate–benzene–chloroform (3:2:1, by vol.) to yield a column 95 cm high. The residue was applied to the column in the same solvent and the solvent collected at a rate of 2.5 ml per min. Unreacted estradiol (3.4 g) was found after 5.8 l. had passed through the column: compounds I (16 mg) and II (20 mg) were eluted after 6.3 l. and 6.5 l. respectively. Other compounds (290 mg) were eluted from the column with larger volumes of solvent, but these were not investigated. The identity of compounds obtained by the two oxidative reactions was demonstrated by in all cited systems. Also mixed melting points of acetylated compounds were not depressed.

RESULTS AND DISCUSSION

Under the experimental conditions used only 30% of the estradiol reacted. When the ferricyanide–estradiol molar ratio was increased, the yield of the compounds with $R_F \simeq 0$ in the used systems increased. These are probably polymers formed by further oxidation. Analogous results were obtained by increasing the reaction time. Temperature (from 4° to 50°C), O_2 and light, did not affect the reaction significantly.

The structure of compounds IA and IIA was deduced essentially from mass and n.m.r. data. All the products of oxidative coupling that have been characterized and reported in the literature[8–15] present a C—C or C—O bond, through phenoxy free radicals [16, 17], exclusively in the *ortho* and *para* positions with respect to the phenolic hydroxyl.

The mass spectrum of both compounds showed a molecular ion at $M^+ = 710$ corresponding to the molecular weight of a dimer of estradiol diacetate with C—C bond. Confirmation of the presence of C—C bond comes from the n.m.r. spectra for both the acetylated compounds (IA and IIA) which show a 2:1 ratio between the aromatic protons and those at 4.65 ppm (C*H*-17), while this ratio is 3:1 in estradiol.

The possible structures of compounds IA and IIA are shown in Fig. 2.

Compound IA

The NMR spectrum shows two doublets centred at 6.63 and 7.05 ppm (δ) in the region of the aromatic protons, having equal areas and coupling constant $J = 8$ Hz. These signals can respectively be attributed to protons in the *ortho* and *meta* positions with respect to the acetylated hydroxyl (the *para* position is occupied). The equal area values of these two signals indicates a 1:1 ration between protons in the

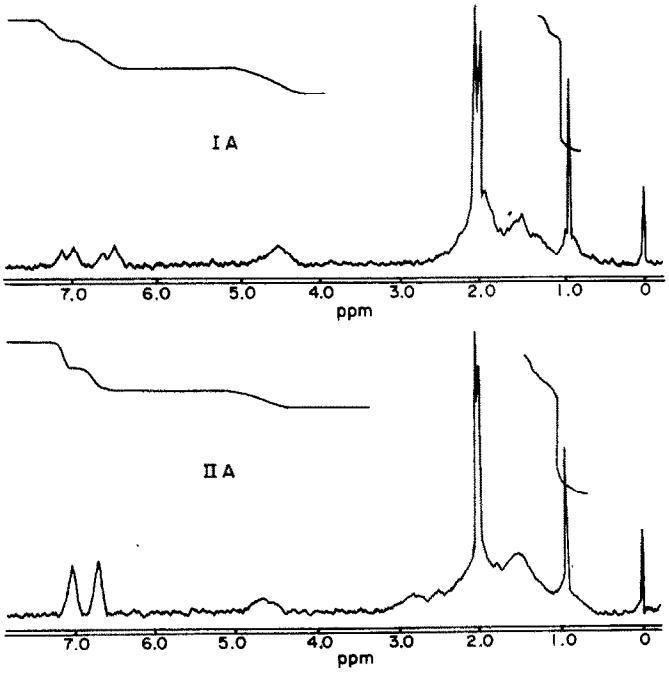

Fig. 1. ^{1}Hmr spectra at 60 MHz of the acetylated compounds IA and IIA. The areas of signals at 4.65 and 6.6–7.1 ppm allow the selection among the possible dimers of estradiol.

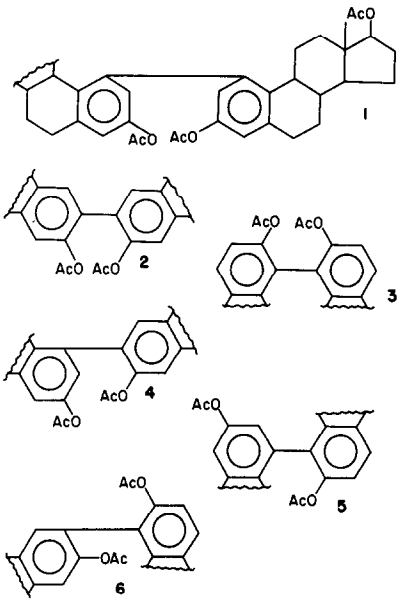

Fig. 2. The 6 possible structures of a dimer of estradiol.

ortho and *meta* positions. Formulae 1, 4 and 5 of Fig. 2 can therefore be excluded. The value $J = 8$ Hz indicates an *ortho*-type coupling between the protons in both aromatic rings [18]. This excludes formulae 2 and 6, and compound IA must have structure 3.

Compound IIA

The n.m.r. spectra shows two singlets of equivalent area in the aromatic-proton zone at 6.73 and 7.05 p.p.m. These can be attributed respectively to protons in the *ortho* and *meta* positions with respect to the acetylated phenolic hydroxyls. The same considerations that were applied to the spectrum of compound IA lead to the exclusion of formulae 1, 4 and 5 because of the equivalence of the areas of the two signals. Furthermore the absence of any appreciable coupling between the two signals is consistent only with formulae 2, in which there can only be coupling between protons in the *para* position with respect to each other, with $J \simeq 0$. Structures 2 and 3 for the compounds agree both with the radical mechanism which had been shown in earlier finding for analogous oxidation products [19] obtained with the same reaction from tetralols with various substitutions.

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