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SYNTHESIS OF DIETHYLSTILBESTROL METABOLITES

ω-HYDROXY-DIENESTROL AND DERIVATIVES

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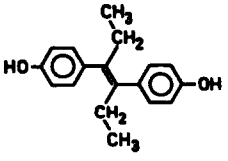
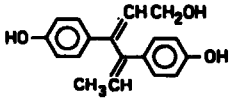
Abstract—ω-Hydroxy-β-dienestrol and some of its derivatives have been synthesized from α-dienestroldiacetate via the ω-bromo- and ω-acetoxy-β-dienestroldiacetate. The NMR and mass spectra of these compounds and their stereochemistry are discussed. Thin layer chromatography, gas chromatography and mass spectrometry show that synthetic ω-hydroxy-β-dienestrol is identical with a major metabolite of diethylstilbestrol. The identity is further substantiated by reverse isotope dilution analysis of the acetylated metabolite. ω-Acetoxy-β-dienestroldiacetate is shown to have alkylating potential in the 4-(p-nitrobenzyl)pyridine test.

Diethylstilbestrol (DES) is a synthetic estrogen which has received much attention recently due to its transplacental carcinogenicity (see review[1]). The biotransformation of DES in several species has been studied in order to evaluate the role of metabolic activation for the toxicity of this drug.[2-5] A major metabolite of DES in humans, nonhuman primates and rodents is ω-hydroxy-dienestrol, and it has been considered a potential activation product with alkylating properties.[4]



DES



ω-hydroxy-dienestrol

The structure of ω-hydroxy-dienestrol has initially been derived from its mass spectrum.[4] In order to substantiate the structural assignment of this metabolite and to study its toxicity, synthetic ω-hydroxy-dienestrol was required. In this paper we report the syntheses and the mass spectrometric characteristics of ω-hydroxy-dienestrol and several of its derivatives. Evidence is presented that the synthetic ω-hydroxy-dienestrol is identical with the DES metabolite. In addition, the alkylation of 4 - (p - nitrobenzyl)pyridine with some of the compounds is described.

RESULTS AND DISCUSSION

Syntheses. The synthetic routes used to prepare some ω-substituted dienestrols are shown in Fig. 1. Reaction of α - dienestroldiacetate **1** with a slight molar excess of N - bromosuccinimide gave, via isomerisation of the dienestrol, to the mono- and dibromo-derivatives, together with some non-brominated material. This mixture, without purification, reacted with silver acetate in glacial acetic acid, and the products were separated by chromatography on a silica gel column. Pure crystalline β-dienestrol diacetate **2**, ω - acetoxy - β - dienestrol diacetate **3** and ω,ω' - diacetoxy - β - dienestroldiacetate **4** were obtained.

Reaction of the crude ω - bromo - dienestrol diacetate with methyl mercaptan in alkaline solution gave ω-methylthio - β - dienestrol **5**, which was also isolated by column chromatography.

The acetates **2–4** can be hydrolysed to the free phenols with methanolic potassium hydroxide. Thus, β-dienestrol **6** was obtained in good yield and pure form from its diacetate **2**.

The ω - acetoxy - β - dienestroldiacetate **3** in methanolic potassium hydroxide gave a mixture of the allylic alcohol, ω - hydroxy - β - dienestrol **8**, and its methyl ether, ω - methoxy - β - dienestrol **7**. The formation of the methyl ether **7**, however, can be avoided by using sodium hydroxide in aqueous dioxane for hydrolysis, and pure **8** was obtained from **3** under these conditions.

Stereochemistry. The geometrical structure of compounds **2–8** can be derived from their NMR data (Table 1). The NMR spectra of α - dienestroldiacetate **1** and β - dienestroldiacetate **2** differ considerably with respect to the chemical shifts of the methyl and methine protons (Table 1). Both spectra, however, indicate a symmetrical molecular structure. Since α - dienestrol has been shown by X-ray crystallography to have the E,E configuration,[6] β - dienestrol must have the Z,Z configuration. This reasoning is confirmed by the NMR spectrum of the third isomer, γ - dienestrol, which is in agreement with an unsymmetrical E,Z configuration.[7] According to their NMR data (Table 1), compounds **3–8** should have the configuration of β - dienestrol. The isomerisation of the starting material, α-dienestroldiacetate, must take place during the bromination reaction and is possibly catalyzed by bromine radicals.

Mass spectra. The mass spectra of the ω-substituted dienestrols as obtained by GC–MS analysis of their TMS derivatives are shown in Fig. 2. All spectra contain a prominent fragment ion arising through loss of the ω-substituent. In the case of the bromo- and methylthio-dienestrol, only the substituent is split off, leaving an ion with *m/e* 409, whereas the methoxy- and trimethyl-

Abbreviations: DES, diethylstilbestrol; NBP 4 - (p - nitrobenzyl)pyridine; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; GC, gas liquid chromatography; MS, mass spectrometry; TMS, trimethylsilyl.

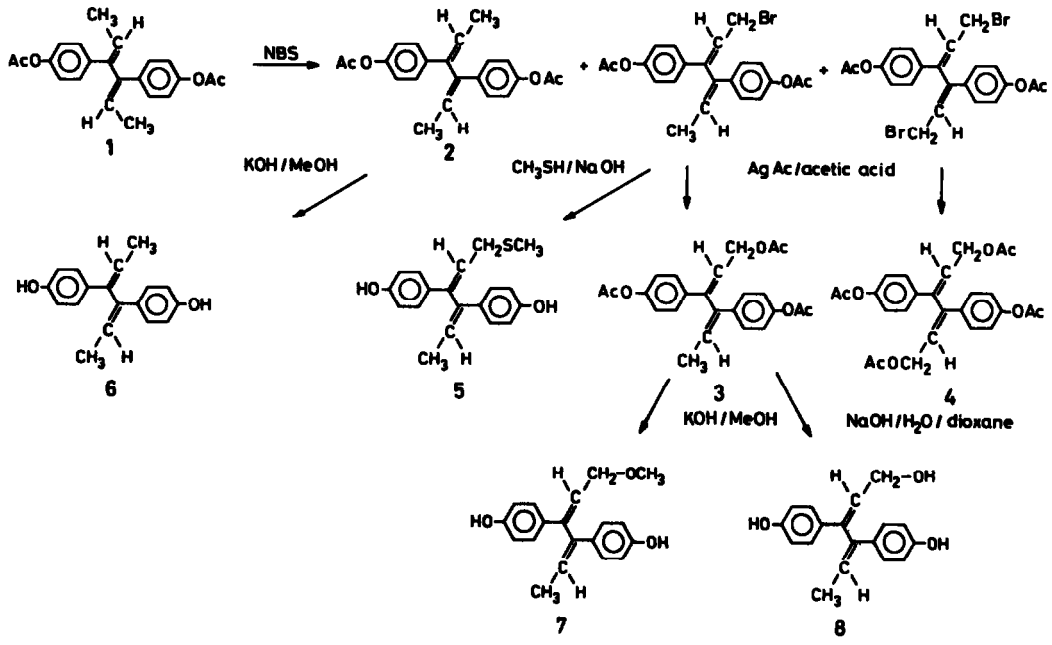


Fig. 1. Synthesis of ω-hydroxy-dienestrol and some derivatives.

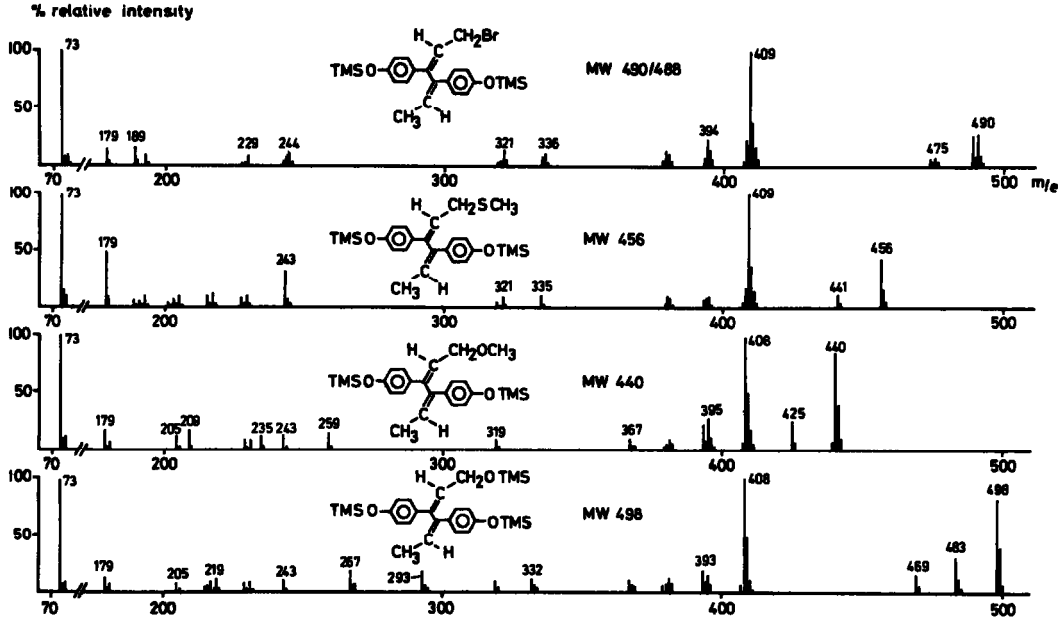


Fig. 2. Mass spectra of ω-hydroxy-dienestrol and derivatives.

silyloxy - dienestrol suffer the loss of an additional H-atom resulting in ions at *m/e* 408. No plausible explanation for this substituent-dependent difference in the fragmentation can be offered at the moment.

Comparison of synthetic ω - hydroxy - β - dienestrol with the metabolic product. The metabolite of ^{14}C-labelled DES assumed to be ω - hydroxy - dienestrol was isolated from human urine and purified by TLC (Experimental). The mass spectrum of the metabolite was obtained by GC–MS and found to be identical with that of the synthetic compound (Fig. 2). Furthermore, the radioactive metabolite cochromatographed with synthetic ω - hydroxy - β - dienestrol in TLC and GC under the conditions given in Table 1.

The identical GC retention time is also indicative for stereochemical identity. Conclusive evidence that the DES metabolite is indeed ω - hydroxy - β - dienestrol was obtained by a reverse isotope dilution analysis. Since ω - hydroxy - β - dienestrol itself does not crystallize well, the acetyl derivative of the radioactive metabolite was prepared and crystallized with synthetic ω - acetoxy - β - dienestrol diacetate 3. 82% of the radioactive were found to cocrystallize with 3, and constant specific radioactivity was already obtained after the first crystallization (Experimental).

Alkylating potential. Allylic esters contain leaving groups attached to an electrophilic C atom. They can therefore be expected to have alkylating potential. This

Table 1. TLC-, GC- and NMR-data of ω-hydroxy-β-dienestrol and derivatives

Compound	Rf in the system[a] A	B	GC retention time (min)[b]	NMR data[c] (signal in ppm downfield from tetramethylsilane, relative number of proton, multiplicity, designation)
1 α-dienestrol-diacetate	0.46	0.60	13.2	1.45 (6H,d,methyl), 2.27 (6H,s,acetyl), 5.28 (2H,q, methine, 7.15 (8H,s,aromatic).
2 β-dienestrol-diacetate	0.39	0.60	17.9	1.70 (6H,d,methyl), 2.23 (6H,s,acetyl),6.30 (2H,q, methine), 6.86-7.43 (8H,m,aromatic).
3 ω-acetoxy-β-dienestrol-diacetate	0.24	0.54	-	1.74 (3H,d,methyl), 1.98 (3H,s,acetyl), 2.19 (6H,s, acetyl), 4.60 (2H,d,methylene), 6.40 (1H,t,methine), 6.42 (1H,t,methine), 6.86-7.50 (8H,m,aromatic).
4 ω,ω'-diacetoxy-β-dienestrol-diacetate	0.11	0.48	-	2.00 (6H,s,acetyl), 2.22 (6H,s,acetyl), 4.65 (4H,d, methylene), 6.50 (2H,t,methine), 6.95-7.56 (8H,m, aromatic).
5 ω-methylthio-β-dienestrol	0.14	0.53	15.4	1.70 (3H,d,methyl), 2.02 (3H,s,methylthio), 3.23 (2H,d,methylene), 6.28 (1H,t,methine), 6.28 (1H,q, methine), 6.67-7.47 (8H,m,aromatic), 8.40 (2H,s broad, hydroxy).
6 β-dienestrol	0.13	0.49	7.8	1.66 (6H,d,methyl), 6.18 (2H,q,methine), 6.62-7.33 (8H,m,aromatic), 8.18 (2H,s,hydroxy).
7 ω-methoxy-β-dienestrol	0.06	0.42	11.5	1,67 (3H,d,methyl), 3.22 (3H,s,methoxy), 3.90 (2H,d, methylene), 6.13 (1H,q,methine), 6.18 (1H,t,methine), 6.57-7.33 (8H,m,aromatic), 8.26 (2H, s broad,hydroxy).
8 ω-hydroxy-β-dienestrol	0.00	0.24	10.5	1.67 (3H,d,methyl), 4.13 (2H,d,methylene), 6.17 (1H, q,methine), 6.27 (1H,t,methine), 6.58-7.33 (8H,m, aromatic), 8.23 (3H,s broad,hydroxy).

[a]system A: benzene–ethylacetate 9:1 v/v; B: benzene–ethylacetate 1:1 v/v.
[b]compounds 5–8 as TMS-derivatives; GC conditions see Experimental.
[c]compounds 1 and 2 were dissolved in $CDCl_3$, compounds 3 to 8 in d_6-acetone. Abbreviations for expressing multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

was tested by incubating 4 - (*p* - nitrobenzyl)pyridine with *ω* - acetoxy - *β* - dienestroldiacetate (3) and *ω,ω'* - diacetoxy - *β* - dienestroldiacete (4). Both compounds alkylate 4 - (*p* - nitrobenzyl)pyridine, the mono - *ω* - substituted compound 3 at half the rate of the di - *ω* - substituted 4 (Fig. 3). Since *β* - dienestroldiacetate (2) did not give any alkylation (Fig. 3), the alkylating potential must be due to the allylic ester function.

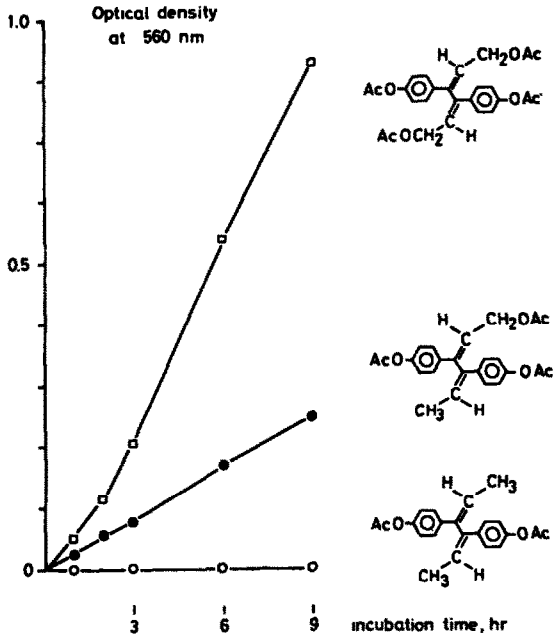


Fig. 3. Reaction with 4 - (*p* - nitrobenzyl)pyridine.

EXPERIMENTAL

M.ps were taken on a Kofler hot-block apparatus and are not corrected. Silica gel 0.05–0.2 mm (70–325 mesh ASTM, E. Merck, Darmstadt, West Germany) was used for column chromatography. TLC was carried out on precoated 250 *μ*m silica gel HF_{254} glass plates (E. Merck, Darmstadt). For GC–MS analysis, a Varian 2700 gas chromatograph coupled to a Varian CH 7 mass spectrometer was used. The GC column (glass, 6 ft × 2 mm i.d.) was packed with 3% OV-225 on GasChrom Q 100/120 mesh and operated at 200–260° with 4°/min and a helium flow of 30 ml/min. Temps were 260° for the injection port, separator and capillary connecting tubes (all-glass system). Samples analyzed by GC–MS were trimethylsilylated with N,O - bis - (trimethylsilyl) acetamide. All mass spectra were taken at an electron energy of 70 eV, and were handled with a Varian SS 100 MS data system.

NMR spectra were recorded on a Varian EM 360 Spectrometer. $CDCl_3$ and d_6-acetone containing 2% TMS as internal standard were used as solvents.

α - Dienestroldiacetate was generously supplied by Kali-Chemie (Hannover, West Germany). N-bromosuccinimide and *α,α'* - azoisobutyronitrile were obtained from Merck AG (Darmstadt, West Germany).

ω - Bromination of α - dienestroldiacetate. *α* - Dienestroldiacetate (1, 30 mmol), N - bromosuccinimide (40 mmol) and *α,α'* - azoisobutyronitrile (0.5 g) were refluxed in dry CCl_4 (100 ml) under N_2, and illuminated with a regular light bulb. After 30 hr, no more starting material was found by TLC analysis. Succinimide was removed by filtration, and the solvent evaporated under reduced pressure to yield a yellow oil, which was used for the subsequent reactions without purification. It contained approximately 22% *β* - dienestroldiacetate, 53% *ω* - bromo - *β* - dienestroldiacetate and 23% *ω,ω'* - dibromo - *β* - dienestroldiacetate, as estimated from the products of the reaction with silver acetate.

β - Dienestroldiacetate 2, *ω - acetoxy - β - dienestroldiacetate* 3 *and ω,ω' - diacetoxy - β - dienestroldiacetate* 4. The crude bromo compound (20 mmol) was dissolved in glacial AcOH (70 ml) and added to the suspension of silver acetate (25 mmol) in AcOH (30 ml). After stirring for 20 hr in the dark, the mixture was filtered and the filtrate concentrated *in vacuo*. The residue was dissolved in benzene (100 ml) and concentrated again (to evaporate the AcOH). The product, containing three compounds according to TLC, was chromatographed on a column (50 × 4 cm) filled with silica gel and eluted with benzene–EtOAc 93:7, v/v. The first compound eluted was crystallized from EtOH to yield 3.6 mmol of 2, m.p. 147/8° (lit.[8] 147/8°). Calc. for $C_{22}H_{22}O_4$ (350.18): C, 75.39; H, 6.33%). Found: C, 75.34; H, 6.36.

The next zone eluted from the column was crystallized from EtOH and gave 8.6 mmol 3, m.p. 100/2°. (Found: C, 70.41; H, 6.07. Calc. for $C_{24}H_{24}O_6$ (408.19): C, 70.55; H, 5.93%).

The third zone after crystallization from EtOH afforded 4.0 mmol 4, m.p. 123°. (Found: C, 67.19; H, 5.84. Calc. for $C_{26}H_{26}O_8$ (466.21): C, 66.92; H, 5.62%).

ω - Methylthio - β - dienestrol 5. The crude bromo compound (5 mmol) in acetone (25 ml) was added dropwise with magnetic stirring to the mixture of 0.2 N NaOH (80 ml), acetone (40 ml) and methyl mercaptan (3 g) at 5°. Dioxane (25 ml) was added and the stirring continued for 20 min. After adjusting the pH to 6 with 1 N HCl, the organic solvents were removed under reduced pressure and the remaining phase extracted twice with ether (100 ml). The ether extract was washed (water), dried (Na_2SO_4), and the residual product chromatographed on a silica gel column with benzene-EtOAc 95:5, v/v. 2.8 mmol of 5 was obtained as a yellow oil, which contained 16% of *β*-dienestrol according to GC–MS analysis. The structure of 5 is substantiated by its mass spectrum (Fig. 2) and NMR spectrum (Table 1).

β - Dienestrol 6. *β* - Dienestroldiacetate (2, 2 mmol) was dissolved in 1 N methanolic KOH (100 ml) and neutralized with conc. HCl after 20 min. The ppt of KCl was removed by filtration and the filtrate concentrated *in vacuo*. Ether (100 ml) and water (50 ml) were added and the ether phase dried (Na_2SO_2) and evaporated. Crystallization from EtOH/water yielded 1.3 mmol *β*-dienestrol of m.p. 184/5° (lit.[8] 184/5°). (Found: C, 81.18; H, 7.05. Calc. for $C_{18}H_{18}O_2$ (266.14): C, 81.16; H, 6.82%).

ω - Methoxy - β - dienestrol 7 *and ω - hydroxy - β - dienestrol* 8. Compound 3 (2 mmol) was hydrolyzed with 1 N methanolic KOH as described for *β* - dienestroldiacetate. The product according to TLC contained two compounds, which were separated by column chromatography on silica gel with benzene–EtOAc 7:3 v/v. The first compound eluted was 7, obtained as a yellow oil in 32% yield. The second compound, 8 (53% yield) was a slightly yellow, crystalline material, m.p. 58/60°. No suitable solvent for recrystallization could be found. The structures of both compounds are based on their NMR- and mass spectra (Table 1, Fig. 2).

Pure 8 without 7 was obtained, when compound 3 (0.2 mmol) was dissolved in 0.5 N NaOH in dioxane–water 1:1 v/v (15 ml) for hydrolysis. After 20 min, the soln was brought to pH 6 with 1 N HCl, half the solvent evaporated *in vacuo*, and the remaining phase extracted twice with ether. Evaporation of the ether after washing (water) and drying (Na_2SO_4) left a quantitative yield of 8, which was homogeneous in TLC and as TMS-derivative in GC.

Reverse isotope dilution analysis. The DES metabolite assumed to be *ω* - hydroxy - dienestrol was obtained as previously described[4] from the urinary glucuronide fraction of humans who had ingested ^{14}C-DES of specific radio-activity 0.25 mCi/mmol. The ether extract of the glucuronide fraction after enzymic hydrolysis was subject to TLC in system B (Table 1) and the radioactive zone corresponding to *ω* - hydroxy - dienestrol eluted with EtOAc. An amount of metabolite containing 700×10^3 dpm ^{14}C (corresponding to approximately 350 *μ*g material) was reacted with Ac_2O (100 *μ*l) and pyridine (20 *μ*l) for 30 min at 60°. EtOH (5 ml) was added and the mixture concentrated to dryness under reduced pressure. Part of the acetylated product was analyzed by TLC and migrated like 3 in system B (Table 1). 100 mg of synthetic 3 was then added to the acetylated metabolite and the mixture crystallized from EtOH. The specific

radioactivities of the material obtained from two consecutive crystallizations were 4750 and 4790 dpm/mg. Quantitative analysis showed that 82% of the radioactivity extracted from the TLC zone is ω - hydroxy - β - dienestrol.

Alkylation of 4 - (p - *nitrobenzyl)pyridine* (*NBP*). The test compound (2 μmol) was dissolved in 3 ml acetone, and 6 ml 0.1 M Tris/HCl puffer pH 7.4 and 10 ml NBP soln (2% in ethylene glycol) were added. Incubation was carried out in a water bath at 70°. Aliquots (3 ml) were taken at various times, cooled to 0°, and mixed with 2.5 ml triethylamine-acetone 1:1, v/v. The absorption at 560 nm was measured exactly 2 min after adding the amine.

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