DEUTERIUM LABELING OF DIETHYLSTILBESTROL AND ANALOGUES

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Received 12-28-82

ABSTRACT

E-2,2,3',3",5,5,5',5"-octadeuteriodiethylstilbestrol (DES-d8) and Z-2,3',3",4,5,5,5',5"-octadeuterio-3,4-bis(p-hydroxyphenyl)-2-hexene (Ψ-DES-d8) were synthesized from E-diethylstilbestrol (DES) by hydrogen/deuterium exchange in a mixture of methanol-d and deuterium chloride in deuterium oxide. The structures, isotopic purity, and positions of uptake of deuterium were determined by nuclear magnetic resonance (NMR) and mass spectrometry (MS). Additional confirmation of the positions of deuterium exchange in stilbestrols was obtained from an analysis of the oxidation of DES-d8 to Z,Z-2,3',3",5,5',5"-hexadeuteriodienestrol (β-DIES-d6) and of the hydrogen/deuterium exchange reaction of hexestrol (HEX) to 3',3",5',5"-hexestrol (HEX-d4). Structural analysis and the determination of isotopic purity of the latter two compounds were also carried out by NMR and MS. The uptake of eight deuterium atoms by DES is postulated to proceed via two different reactions occurring simultaneously: 1. acid catalyzed deuteration of all four phenolic ortho-positions (3',3",5',5"); 2. acid catalyzed deuteration of the olefin bridge with subsequent formation of deuterated Ψ-DES (3 or 4). Due to the equilibration between DES, Ψ-DES, and Z-diethylstilbestrol (cis-DES) in the acidic reaction mixture at 85°C, the deuterated Ψ-DES is thought to rapidly rearrange to deuterated DES. Repeated deuteration will eventually form DES-d8 fully labeled in the 2,2,5,5 methylene positions.

INTRODUCTION

The established carcinogenic activity of DES in humans and in rodents (1,2) has prompted thorough investigations of its in vivo and in vitro metabolism and an intensive search for a carcinogenic reactive metabolic intermediate [reviewed by Metzler (3)]. Furthermore, the extensive use of DES in human medicine and as a growth promoting agent in poultry and cattle [reviewed in "Estrogens in the Environment" (4)] necessitated the development of highly sensitive methods of detection and quantitation of this synthetic estrogen and its metabolites. Most assays for DES from biological samples involved a gas chromatographic (5,6) or high pressure liquid chromatographic procedure (7-11).

Analyses of the metabolic fate of DES, however, required gas chromatography-mass spectrometry (GC-MS) using labeled DES or analogs as internal standards (3,12-14). A deuterium labeled DES-d5 for use as an internal standard in GC-MS determinations of DES had been synthesized (12) by Metzler from perdeuterioethanol following a procedure of Dodds et al. (15) and Kuwada and Sasagawa (16). We now report a simplified H/D exchange procedure for the preparation of deuterated DES and analogues to be used as internal standards in GC-MS analyses of DES and metabolites.

EXPERIMENTAL

Chemicals. DES and HEX were purchased from Sigma Chemical Co., St. Louis, Missouri; lead tetraacetate, methanol-d (CH_3OD, 99.5 atom % D), and deuterium chloride (20 wt. % solution in D_2O, 100.0 atom %) were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. cis-DES was a gift of Dr. P. Murphy, Eli Lilly and Co., Indianapolis, Indiana. Unlabeled β-DIES was prepared by oxidation of DES using lead tetraacetate (17). N,O-bis(trimethylsilyl) trifluoroacetamide was purchased from Pierce Chemical Co., Rockford, Illinois.

Instrumentation. MS was carried out using a Finnigan, Model 3200, GC-MS system combined with an Incos data system. Spectra of underivatized samples were recorded using the direct inlet system. For gas chromatographic analyses, samples were trimethylsilylated using pyridine and N,O-bis(trimethylsilyl) trifluoroacetamide (1:10; v:v) at 60°C for 30 min. GC-MS analyses were done using the following conditions: 6 ft x 2 mm glass column packed with 3% OV-1 on Gas Chrom Q 100/120, column temperature programmed from 200-290°C at a rate of 10°C/min. ^{1}H-NMR spectra were obtained using a JEOL, Model FX 90Q, 90 MHz NMR spectrometer.

Deuterium labeling of DES. DES (1.0 g) was dissolved in a mixture of 15 g of methanol-d and 6 g of deuterium chloride. This mixture was heated in a tightly capped vessel for three days at 85°C. After the solution was cooled to room temperature, the mixture was concentrated and dried in vacuo. The exchange reaction was then repeated twice with the same amounts of deuterating reagents under the same conditions. After the third exchange reaction, an aliquot of the mixture was dried, trimethylsilylated, and analyzed by GC-MS.

DES-d8. The products of the isotope enrichment reaction were separated using the method of Airy and Sinsheimer (18). Dimethylsulfoxide (8 ml) was added to the dried reaction products and the mixture was warmed on a steam bath for 3 minutes. The contents were diluted with water (16 ml) and filtered immediately. The precipitate collected was dissolved in ether, and the organic phase was washed with water twice. The organic phase was concentrated in vacuo, the residue was dried and recrystal-

lized from acetone/water and dried. Yield and analytical data are listed in Tables 1 and 2.

Ψ-DES-d8. The filtrate from the separation described above was diluted with water and extracted three times with diethyl ether. The organic phase was concentrated and the residue was recrystallized from acetone/water. Yield and analytical data are described in Tables 1 and 2.

HEX-d4. Hexestrol (200 mg) was dissolved in a mixture of 0.5 ml of deuterium chloride/deuterium oxide and 1.7 ml of methanol-d. The mixture was heated to 110°C for two days. After the mixture was cooled to room temperature, the solvents were evaporated in a stream of nitrogen, the residue was dried in vacuo, and the deuteration procedure was then repeated. After evaporation of the solvents and drying, the deuterated hexestrol was recrystallized from methanol. Analytical data are given in Tables 1 and 2.

β-DIES-d6. DES-d8 (30 mg) was dissolved in a mixture of 0.4 ml of ether and 0.7 ml of chloroform. Lead tetraacetate (51 mg) was added in small portions to the mixture which was stirred for 30 minutes at room temperature. After filtration and evaporation of the solvents, the residue was dissolved in a solution of sodium carbonate in water-methanol and extracted with ether. After evaporation of the ether the product was purified by TLC (silica; ether/petroleum ether, 6:4). Yield and analytical data are listed in Tables 1 and 2.

Equilibration Reactions. DES-d8 (2 mg) was dissolved in methanol-d (75 µl) and deuterium chloride/deuterium oxide (10 µl). The mixture was kept at 85°C for 24 hours. An aliquot of the reaction mixture was dried in vacuo, trimethylsilylated and analyzed by GC-MS. The following products were observed: 7% of cis-DES-d8, 39% of Ψ-DES-d8, and 54% of DES-d8. Ψ-DES-d8 (2 mg) dissolved in the deuterating mixture (using the same amounts) was also kept at 85°C for 24 hours. An aliquot of this reaction mixture was isolated and analyzed in the same way using GC-MS. The products were: 7% of cis-DES-d8,36% Ψ-DES-d8, and 57% DES-d8.

RESULTS AND DISCUSSION

In analogy to the acid catalyzed hydrogen/deuterium exchange at the ortho-positions of phenols (19) and, in particular, of estradiol and estrone (20), deuterium labeling of DES in a mixture of methanol-d and DCl in deuterium oxide was attempted. The mixture was kept at 85°C for three days and after back exchange of the phenolic deuteroxy to hydroxy groups in cold methanol the reaction product was analyzed by MS. The mass spectrum showed evidence for an uptake of 8 D atoms into the

molecule (MW 276), rather than the expected deuterium labeling of only the four phenolic ortho-positions (3',3",5',5"). The GC-MS analysis of the reaction product (after trimethylsilylation) identified three components of the reaction mixture: DES-d8 (56%), Ψ-DES-d8 (31%), and cis-DES-d8 (13%). After three exchange reactions, the isotopic purity of each of the three compounds was high: 92% d8; 8% d7; <1% d6).

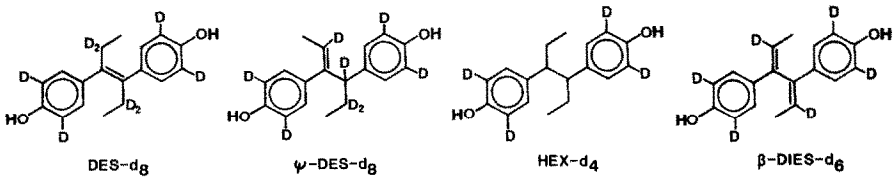

Fig. 1: Structures of compounds

The separation of the products of the deuterium exchange reaction was carried using the method of Airy and Sinsheimer (18) for separating DES-d8 from the mixture as a complex with dimethylsulfoxide. After extraction of the filtrate with ether and recrystallization of the residue from acetone/water, this compound was identified as Ψ-DES-d8 on the basis of its spectral characteristics. Analytical data for all deuterium labeled compounds are given in Table 1.

The precipitate obtained in the filtration step above, was dissolved in ether and washed with water to break up the DMSO-DES complex. After concentration in vacuo, the residue was recrystallized from acetone/water. The compound isolated was identified as DES-d8 by comparison with unlabeled DES (yield: 73%).

The structural confirmation and the determination of isotopic purity were carried out by NMR and MS. The mass spectra of underivatized DES-d8 and also of the trimethylsilyl derivative clearly showed an uptake of eight deuterium isotopes into the molecule with high isotopic

Table 1: Analytical characteristics of deuterated stilbestrols

Compound	yield (%)[a]	retention time[b]	ret. time of unlabeled parent[b]	isotope enrichment (%) d8	d7	d6	d5	d4	d3	chemical purity (%)[c]
DES-d8	73	6:18	6:18	90	10	<1				94
Ψ-DES-d8	10	6:00	n.d.[d]	86	14	<1				91
HEX-d4	>98	7:39	7:36					98	2	>98
β-DIES-d6	92	6:33	6:30			91	8	1		95

Table 2: Spectral data of deuterium labeled stilbestrols

Compound	partial mass spectra [m/z (% rel. int.)] underivatized	trimethylsilylated	^{1}H-NMR spectra[e] (acetone-d6)
DES-d8	276(12)M; 261(4)M-CH_3; 245(12)M-CH_3CD_2; 230(13); 214(7); 149(50); 110 (100).	420(100)M; 405(18)M-CH_3; 389(15)M-CH_3CD_2.	0.73(6H,s,-CH_3), 7.03(4H,s, aromatic H), 8.22 (2H,s,-OH).
Ψ-DES-d8	276(15)M; 245(100)M-CH_3CD_2; 230(5); 215(6); 149(22); 148(24); 136(21); 110(19).	420(32)M; 405(3)M-CH_3; 389(100)M-CH_3CD_2.	0.83(3H,s,6-CH_3), 1.45(3H,s, 1-CH_3), 6.70(2H,s, arom. H), 6.91(2H,s,arom. H), 8.06(1H, s,-OH), 8.18(1H,s,-OH).
HEX-d4	274(1)M; 137(94) m/2; 122(26) m/2-CH_3; 109 (100) m/2-C_2H_4; 93(15); 79 (14).	418(0.1)M; 403(0.2)M-CH_3; 209(100) m/2.	0.52(6H,t,CH_3), 1.32(4H,m, -CH_2-), 2.50(2H,t,CH), 7.03 (4H,s,arom. H), 8.13(2H,s,-OH).
β-DIES-d6	272(70)M; 257(42)M-CH_3; 242(42)M-CH_3CHD; 148(90); 136(91) m/2; 124(94); 110(100).	416(100)M; 401(67)M-CH_3; 386(15)M-CH_3CHD.	1.66(6H,s,-CH_3), 7.22 (4H,s, s, arom. H), 8.22 (2H,s,-OH).

a isolated yield of pure compounds.

b retention time (minutes: seconds) of trimethylsilylated compounds as determined from total ion chromatograms. Gas chromatography conditions are outlined in the description of instrumentation.

c as determined from the total ion chromatogram.

d not determined.

e Signals are given in ppm downfield from tetramethylsilane (relative number of protons, multiplicity, designation). Abbreviations are: s= singlet, t= triplet, m= multiplet.

purity. In the spectra of the derivatized and of the underivatized samples, ions $[M-CH_3]^+$ and $[M-31]^+$ were observed resembling $[M-CH_3]^+$ and $[M-CH_3CH_2]^+$ fragments generated from side chain fragmentation of DES (3). The elimination of methyl indicated that the terminal carbons were not deuterium enriched. Ions $[M-31]^+$ likely were generated by elimination of $CH_3CD_2^{\bullet}$ radicals from the molecular ion. This spectral evidence thus suggested that both methylene units of the ethyl side chains of DES were deuterated. In the NMR spectrum of DES-d8, the signal arising from absorption by methyl had the same chemical shift as that of the methyl group of DES (21). However, it appeared as a singlet due to the deuterium labeling in the neighboring methylene position. The signals of the methylene protons, observed (21) at 2.17 ppm in DES, were absent in the spectrum of DES-d8. The singlet in the aromatic region (7.03 ppm) confirmed partial deuteration in the aromatic region. The chemical shift value suggested that the protons remaining were in positions meta to hydroxyl.

The mass spectra of Ψ-DES-d8 also were characterized by an enrichment with eight deuterium atoms (Table 2). With the exception of mass shifts due to the deuteration, the mass spectrum resembled that reported for undeuterated Ψ-DES (18). The elimination of ethyl from the molecular ion of Ψ-DES (18) was shifted to an elimination of $CH_3CD_2^{\bullet}$ from the molecular ion of Ψ-DES-d8. Other ions characteristic of Ψ-DES were also shifted to higher m/z ratios because of the deuterium enrichment in Ψ-DES-d8. In the NMR spectrum of Ψ-DES-d8, both methyl groups reported for Ψ-DES (18) were present with similar chemical shift values (Table 2), but differing coupling constants. The signals found were singlets due to deuterium substitution in the neighboring positions

[δ 0.83 (s,6-CH_3), 1.45 (s, 1-CH_3)]. Other non-aromatic hydrogens were absent. The aromatic hydrogen signals appeared as singlets supporting the proposed H/D exchange ortho to the phenolic hydroxy groups. Both NMR and mass spectra supported the proposed structure for Ψ-DES-d8 shown in Fig. 1.

Two additional experiments were carried out to confirm the proposed structures of the deuterated stilbestrols:

1.) The unique deuterium enrichment pattern of DES-d8 was confirmed by oxidation of this material to a deuterated dienestrol (17,21). The oxidation was carried out using lead tetraacetate (17). The reaction product was a material cleanly enriched with six deuterium atoms. The mass spectrum (Table 2) resembled that of β-DIES (22) except that the molecular ion and fragments were correspondingly shifted by 6 amu or less respectively. In the NMR spectrum of β-DIES-d6, a singlet at 1.66 ppm confirmed the structural assignment (Fig. 1). The chemical shift of this signal was similar to that of the methyl groups in unlabeled β-DIES (21). The deuterium enrichment of the aromatic rings remained unaltered. The specific loss of two deuterium atoms in the oxidation of DES-d8 to β-DIES-d6 confirmed the proposed deuteration of DES in the 2-and 5-positions.

2.) HEX, a structural analog of DES with a saturated hexyl chain, was subjected to a similar deuteration procedure. Without the stilbene bridge, deuterium enrichment was expected to occur only in the 3',3",5',5"-positions of the aromatic ring but not in the hexyl chain. The mass spectra (Table 2) showed a de-

fined uptake of four deuterium isotopes with high isotopic purity (Table 1). The NMR spectrum of HEX-d4 was not different from that of HEX in the aliphatic region. Deuterium enrichment at the sites ortho to the phenolic hydroxy groups was confirmed by the singlet [δ 7.03, (s, arom. H)] with a chemical shift characteristic of hydrogens in the meta position. The deuteration of HEX to HEX-d4 indicated that a normal enrichment reaction in the ortho positions of phenols was operating in stilbestrols. The deuterium uptake in aliphatic positions of DES, unusual under the reaction conditions employed (19,20), must therefore have been facilitated by interactions with the bridging double bond.

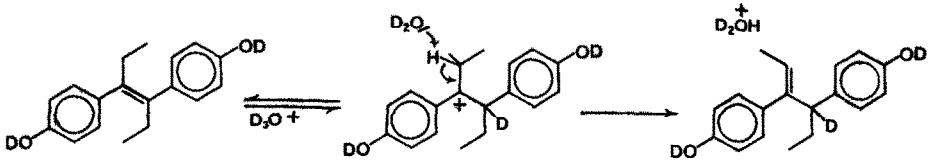

Fig. 2: Formation of deuterated ψ-DES

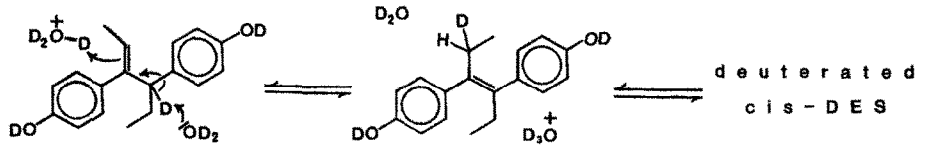

Fig. 3: Equilibrium of deuterated ψ-DES, DES, and cis-DES

The uptake of eight deuterium isotopes into DES and the concomitant formation of deuterated Ψ-DES may be explained in the following way:

In a methanol-d/deuterium chloride/deuterium oxide solvent, electrophilic attack by D^+ in the ortho positions of DES resulted in the expected uptake of four deuterium atoms in the 3',3",5',5"-positions (19,20). Electrophilic attack at the double bond led to a deuteron addition. The ion formed, shown in Fig. 2, may eliminate D^+ and revert back to DES or eliminate a proton and form partially deuterated Ψ-DES.

Under the reaction conditions of deuteration, cis-DES, Ψ-DES, and DES were shown to equilibrate. Pure Ψ-DES-d8 or pure DES-d8, when heated in methanol-d, deuterium chloride, and deuterium oxide to 85°C, equilibrated to a mixture of approximately 7% cis-DES-d8, 38% Ψ-DES-d8, and 56% DES-d8. Therefore, deuterated Ψ-DES will rapidly be converted (Fig. 3) to deuterated DES or deuterated cis-DES and will then continue to take up deuterium as shown in Fig. 2. It is thus postulated that deuterated Ψ-DES is an intermediate in the repeated isotope enrichment of DES. The rapid interconversions in this deuterating mixture will eventually lead to a mixture of completely labeled compounds: DES-d8, Ψ-DES-d8, and cis-DES-d8 (Fig. 1). These highly deuterium enriched compounds may then be separated and isolated as described above.

The equilibrium obtained between DES, Ψ-DES, and cis-DES under acidic conditions affords an improved synthesis of Ψ-DES from DES (10% yield) over the method reported by Airy and Sinsheimer (5% yield) (18).

ACKNOWLEDGMENTS

The authors would like to thank Mrs. Tsing-Ying Fan for her skillful operation of the mass spectrometer. This research was supported by a grant from the National Cancer Institute (CA 27539).

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