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Synthesis of Ring B Unsaturated Estriols. Confirming the Structure of a Diagnostic Analyte for Smith–Lemli–Opitz Syndrome

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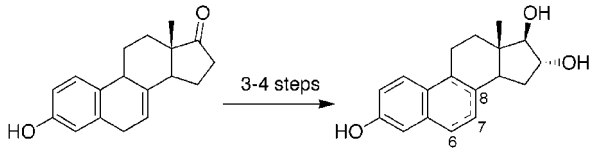
ABSTRACT



8 Δ^7, Δ^8, and $\Delta^{6,8}$ estriols

Brief partial syntheses are described for ring B unsaturated estriols, which are candidate metabolites diagnostic for Smith–Lemli–Opitz syndrome prenatally. These steroids are also likely metabolites of the Premarin preparation used in estrogen replacement therapy. Equilin (8) was converted in three steps to 7-dehydroestriol, which was isomerized to 8-dehydroestriol. The simplicity of the transformations belies the lability of these previously inaccessible metabolites and their synthetic precursors.

The Smith−Lemli−Opitz syndrome[1] (SLOS) is an autosomal recessive disorder of cholesterol biosynthesis caused by a defect in the enzymatic conversion of 7-dehydrocholesterol to cholesterol (Scheme 1). This defect results in an abnormal accumulation of 7- and 8-dehydrocholesterol (**1** and **2**), which can be detected by GC/MS analysis of blood from affected individuals.[2] Prenatal detection is also important because SLOS is a serious birth defect characterized by mental retardation, multiple developmental malformations, and a high carrier frequency.[3] Prenatal diagnosis presently necessitates amniocentesis or chorionic villus sampling.[4] We are developing less invasive diagnostic methods that target aberrant metabolites recently found in urine and serum during pregnancy.[5] Validation of these methods requires authentic standards of estrogen metabolites unsaturated in ring B (e.g., **3** and **4**). These dehydroestriols are also of interest as candidate metabolites of equine steroids contained in Premarin, which is widely used in estrogen replacement therapy.[6]

Equilin (**8**) and estriol (**6**) have been known since the seminal isolation studies of estrogens in the 1930s,[7] but definitive syntheses of dehydroestriols have not been reported. Although an early patent describes the Birch reduc-

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(1) (a) Kelley, R. I.; Hennekam. R. C. M. *J. Med. Genet.* **2000**, *37*, 321−335. (b) Waterham, H. R.; Wanders, R. *Biochim. Biophys. Acta* **2000**, *1529*, 340−356. (c) Porter, F. D. *Mol. Genet. Metab.* **2000**, *71*, 163−174. (d) Battaile, K. P.; Steiner, R. D. *Mol. Genet. Metab.* **2000**, *71*, 154−162.

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(4) Kratz, L. E.; Kelley, R. I. *Am. J. Med. Genet.* **1999**, *82*, 376−381.

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(7) Fieser, L. F.; Fieser, M. *Steroids*; Reinhold Publishing Co.: New York, 1959; pp 452−465.

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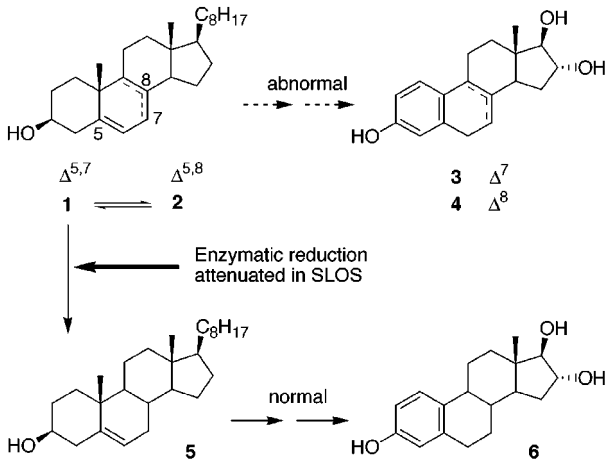
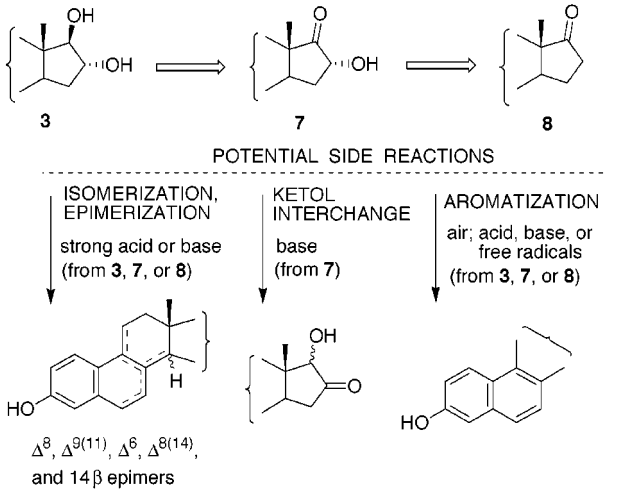
Scheme 1. Metabolism of Fetal $\Delta^{5,7}$ and $\Delta^{5,8}$ Sterols to Estriols



Scheme 2



tion of 6,8-didehydroestriol **15** to **3** (identified based on its UV spectrum),[8] reductions of similar equilenins generally give mixtures of olefins.[9] Other synthetic efforts have been thwarted by the facile aromatization of equilins.[10] This Letter describes the first synthesis of homogeneous samples of dehydroestriols.

Our primary target was the preparation of compounds **3** and **4**. Considering the modest amounts of material required for bioanalytical purposes, we focused on partial synthesis from available estrogens, such as equilin, equilenin (**10**), and the Torgov diene.[11] In a standard synthetic approach to estriols, the 16-hydroxyl is introduced by acid hydrolysis of a 16α,17α-epoxide formed from the enol acetate of estrone.[12] However, application of this method to equilin resulted in aromatization to 16α-hydroxyequilenin.[10] Another approach to 16-hydroxylation entails 16-bromination of estrone, followed by hydrolysis in DMF to the ketol.[13] Although many unsaturated 17-ketosteroids can be selectively brominated at C-16 with $CuBr_2$ in refluxing methanol,[13,14] this reaction was reported to give a complex mixture for equilin.[10] Aiming to overcome these problems, we set out to devise conditions for implementing the simple ring D manipulations shown in the retrosynthetic analysis (Scheme 2) without triggering the indicated side reactions, namely, aromatization of ring B,[15] epimerization at C-14,[7] double-bond isomerization,[7,16] and ketol rearrangement.[13a]

In our hands, bromination of equilin with $CuBr_2$ in MeOH[10] also gave complex mixtures. 1D and 2D NMR analysis of standards and crude reaction mixtures led to identification of 10 bromosteroids, which, together with equilenin and equilin, accounted for >95% of the steroids observed in most reactions. Knowledge of reporter signals for the numerous bromosteroids facilitated byproduct identification and optimization of reaction conditions. Thus, use of 3 equiv of $CuBr_2$ in methanol[13] led mainly to bromination in ring A, dehydrogenation to equilenins, and rapid conversion of the desired 16-bromosteroids **9a** and **9b** to dibromides and equilenins (Table 1, entries 1 and 2). Shorter reaction times and a smaller excess of $CuBr_2$ resulted in large amounts of unbrominated steroids (**8** and **10**) and low conversion to the desired products (Table 1, entries 3−5). In THF, the yield of 16-bromoequilins doubled to 21%, but the product still consisted mainly of equilenins and ring A brominated equilins (Table 1, entry 6). However, reaction in $CHCl_3$−EtOAc[17] gave >70% conversion to 16-bromoequilins (Table 1, entry 7), and these conditions were sufficiently reproducible to afford gram quantities of the desired products as a 2:1 mixture of 16α- and 16β-bromo epimers. Under specific reaction conditions,[13a] this mixture was cleanly hydrolyzed to 16α-hydroxyequilin (**7**) without formation of 16-keto byproducts.[18] Reduction of **7** with $NaBH_4$ led to the target 7-dehydroestriol (**3**).[19]

With the intention of synthesizing 8-dehydroestriol (**4**) by a parallel bromination−hydrolysis−reduction scheme, we prepared 8-dehydroestrone (**12**) by isomerizing equilin with $LiNHCH_2CH_2NH_2$ in ethylenediamine (Scheme 3).[20] However, refluxing **12** with $CuBr_2$ in $CHCl_3$−EtOAc resulted in virtually no bromination at C-16 or in ring A, the product consisting of a 1:1:2 mixture of **10**, **12**, and 9(11)-dehydroestrone. An alternative attempt to prepare **13** by $LiNHCH_2CH_2NH_2$ isomerization of **7** gave none of the expected products.[21] However, isomerization of triol **3**, which lacks the potentially labile[13] 16,17-ketol functionality of **7**, was

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(9) Marshall, D. J.; Deghenghi, R. *Can. J. Chem.* **1969**, *47*, 3127−3131.
(10) Ikegawa, S.; Kurosawa, T.; Tohma, M. *Steroids* **1990**, *55*, 250−255.
(11) **3-Methoxyestra-1,3,5(10),8,14-pentaen-17-one.** For leading references, see: Tanaka, K.; Nakashima, H.; Taniguchi, T.; Ogasawara, K. *Org. Lett.* **2000**, *2*, 1915−1917.
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(13) (a) Numazawa, M.; Nagaoka, M. *J. Org. Chem.* **1982**, *47*, 4024−4029. (b) Numazawa, M.; Kimura, K.; Nagaoka, M. *Steroids* **1981**, *38*, 557−565.
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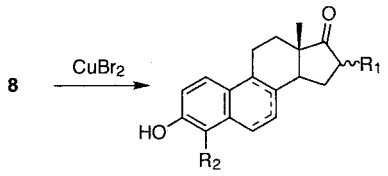
(15) Junghans, K. *Chem. Ber.* **1975**, *108*, 2824−2826.
(16) Jacquesy, J. C.; July, G.; Gesson, J. P. *C. R. Acad. Sci., Ser. C* **1972**, *274*, 969−971 and references therein.
(17) King, L. C.; Ostrum, G. K. *J. Org. Chem.* **1964**, *29*, 3459−3461.

Table 1. Bromination of Equilin (**8**) with $CuBr_2$: Effects of Reaction Conditions on Product Distribution[a]



	Δ^7			$\Delta^{6,8}$		
8	R_1 = H	R_2 = H	**10**	R_1 = H	R_2 = H
9a	R_1 = αBr	R_2 = H	**11a**	R_1 = αBr	R_2 = H
9b	R_1 = βBr	R_2 = H	**11b**	R_1 = βBr	R_2 = H
9c	R_1 = αBr	R_2 = Br	**11c**	R_1 = αBr	R_2 = Br
9d	R_1 = βBr	R_2 = Br	**11d**	R_1 = βBr	R_2 = Br
9e	R_1 = H	R_2 = Br	**11e**	R_1 = H	R_2 = Br

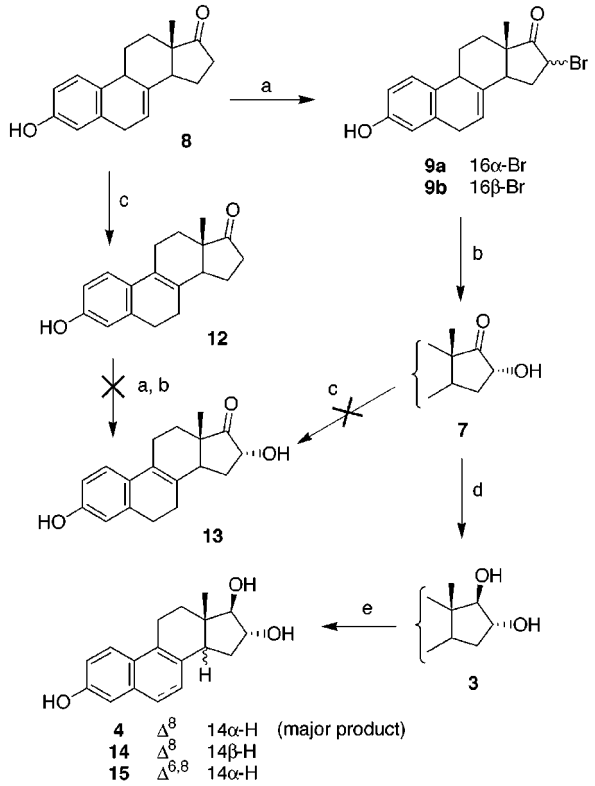
entry	1	2	3	4	5	6	7
solvent	MeOH					THF	C-EA[b]
$CuBr_2$[c]	3.0	3.0	1.6	1.6	1.6	1.0	1.2
t (min)	30	90	5	15	30	80	90
%**9a**	10	0	6	9	10	16	48
%**9b**	2	0	2	2	1	5	24
%**9c**	9	4	8	5	4	3	0
%**9d**	11	24	1	2	2	1	2
%**9e**	2	4	0	0	0	0	1
%**11a**	0	0	2	4	5	1	7
%**11b**	0	0	0	0	1	1	3
%**11c**	35	32	19	18	15	16	1
%**11d**	21	30	1	2	2	5	4
%**11e**	5	6	0	1	0	2	2
%**8**	7	0	34	20	15	42	6
%**10**	0	0	17	22	28	1	1

[a] Product distributions were determined by ^1H NMR. Desired products (**9a** and **9b**) are highlighted. [b] Chloroform−ethyl acetate 1:1. [c] Molar ratio of $CuBr_2$ to **8**.

more promising. Despite the poor solubility of **3** and its sluggish rate of isomerization, reaction conditions were found to give **4** as the major product.[22,23] Semipreparative reverse-phase HPLC afforded **4**, **14**, and **15**, which were characterized by 2D NMR and NOE difference spectroscopy to confirm the regio- and stereochemical structure assignments.

With availability of authentic samples of the dehydroestriols, we compared their GC mobilities and mass spectral fragmentation with those of the SLOS urinary metabolites[24]

Scheme 3[a]



[a] (a) $CuBr_2$, $CHCl_3$−EtOAc 1:1, reflux; (b) NaOH, DMF−H_2O 3:1, rt, 1.5 h; (c) $LiNHCH_2CH_2NH_2$, $NH_2CH_2CH_2NH_2$, 30 °C, 1 h; (d) $NaBH_4$, MeOH, 0 °C, 2 h; (e) $LiNHCH_2CH_2NH_2$, NH_2CH_2-CH_2NH_2, 40 °C, 48 h.

(18) Equilin (101 mg) and freshly ground $CuBr_2$ (166 mg) were heated in $CHCl_3$−EtOAc (25 mL each) for 2 h under vigorous reflux (to remove HBr). The crude product (140 mg; 48% **9a**, 24% **9b**) was stirred for 1.5 h at room temperature in DMF−water (3:1, total 10 mL) containing 2 equiv of NaOH. MPLC on silica gel (EtOAc−hexane 3:7) gave **7** (78 mg, containing some $\Delta^{6,8}$ material). Attempts to purify **7** by reverse-phase HPLC (MeOH−H_2O 35:65) gave a 19:1 mixture of **7** and its $\Delta^{6,8}$ analogue. Consequently, the equilenins formed during bromination and hydrolysis were removed after reduction of **7** to **3**. NMR ($CDCl_3$, 25 °C): **9a** δ 0.814 (s), 4.589 (d, 7.3 Hz), 5.449 (m); **9b** δ 0.995 (s), 4.263 (t, 8 Hz), 5.500 (m); **7** δ 0.865 (s), 4.420 (d, 8.3 Hz), 5.509 (m).

(19) Ketol **7** (446 mg) was reduced with $NaBH_4$ (47 mg) in MeOH (25 mL) for 2 h at 0 °C. Methanol was removed at <20 °C in a stream of N_2 (higher temperatures resulted in formation of **15**). Addition of cold saturated NH_4Cl (10 mL) followed by extraction with EtOAc gave **3** (452 mg, 81% purity). HPLC purification (250 × 21.2 mm C_{18} column, MeOH−H_2O 45:55) of a 50-mg sample gave **3** (35 mg, 99% purity).

(20) Raijmakers, P. H. U.S. Patent 5,739,363, 1998.

(21) Reaction of **7** (50 mg) with 0.3 M Li in ethylenediamine (3 mL) gave mainly unreacted **7**, whereas 1 M Li led to a complex mixture.

(22) Dehydroestriol **3** (100 mg) was heated at 40 °C for 48 h in ethylenediamine (3.4 mL) containing $LiNHCH_2CH_2NH_2$ (prepared by adding 13.4 mmol of MeLi−LiBr in ether to ethylenediamine, followed by evaporation of the ether at 55 °C). NMR of the crude product (104 mg) indicated a 9:5:3:3 mixture of **4**, **3**, **14**, and **15**. Preparative HPLC (250 × 21.2 mm C_{18} column, MeOH−H_2O 35:65, 7 mL/min) gave **14** (t_R 146 min), **15** (t_R 159 min), **3** (t_R 165 min), and **4** (t_R 172 min). NMR (CD_3OD, 25 °C): **3** δ 0.636 (s), 3.578 (d, 5.4 Hz), 4.043 (ddd, 9.1, 5.4, 2.0 Hz), 5.358 (br d, 3.4 Hz); **4** δ 0.757 (s), 3.549 (d, 5.3 Hz), 4.119 (ddd, 9.0, 5.3, 1.8 Hz); **14** δ 0.946 (s), 3.560 (d, 6.9 Hz), 3.999 (ddd, 8.8, 7.9, 6.9 Hz); **15** δ 0.667 (s), 3.668 (d, 5.6 Hz), 4.228 (ddd, 9.1, 5.6, 2.1 Hz).

(23) The forcing isomerization conditions resulted in partial epimerization of **4** to **14** and prompted a thorough structure of determination of all products by NMR. In contrast, byproducts were negligible in the preparation of **12** (3% **3**, 1% 6-dehydroestrone, and 1−2% 14β steroids) and are frequently absent in base-catalyzed olefin isomerizations: Pines, H.; Stalick, W. M. *Base-catalyzed reactions of hydrocarbons and related compounds*; Academic Press: New York, 1977; Chapter 2.

(24) **Isolation of dehydroestriols:** Urine from a pregnant woman carrying an SLOS fetus was processed by our standard methods for analyzing urinary steroids: Shackleton, C. H. L. *J. Steroid Biochem. Mol. Biol.* **1993**, *45*, 127−140. Briefly, steroid sulfates and glucuronides from a C_{18} solid-phase extraction (SPE) of the urine sample were hydrolyzed with *Helix pomatia* (Roman snail) digestive juice (Sigma-Aldrich). The resulting unconjugated steroids were reextracted by SPE and fractionated on Sephadex LH-20 (100 × 10 mm column; cyclohexane−ethanol 4:1) as described: Setchell, K. D.; Shackleton, C. H. L. *Clin. Chim. Acta* **1973**, *47*, 381−388. GC/MS analysis of individual 5-mL fractions (as TMS ethers) revealed that dehydroestriol was eluted between 140 and 165 mL. The only two steroids found in this fraction were dehydroestriol and didehydroestriol **15**.

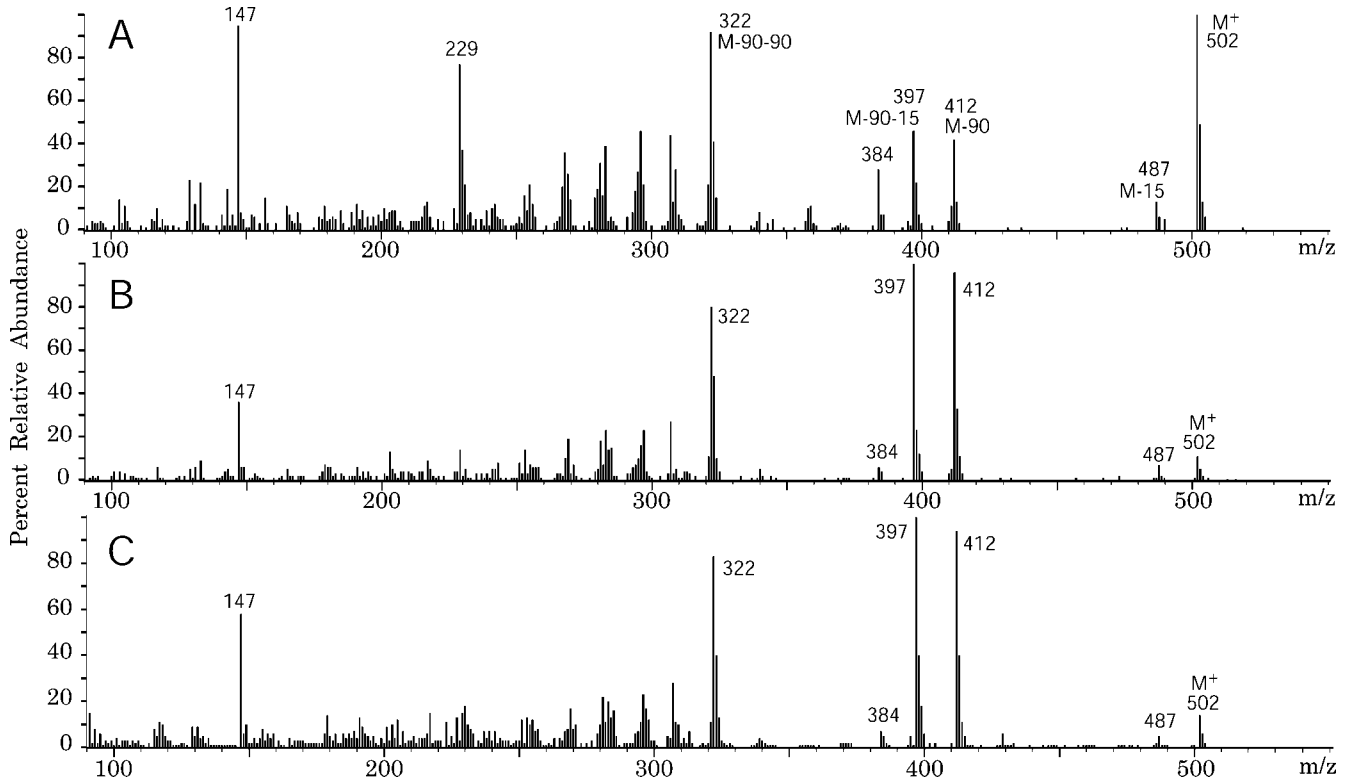


Figure 1. Comparison of GC/MS spectra of tris-TMS derivatives of authentic 7-dehydroestriol (A), 8-dehydroestriol (B), and the dehydroestriol isolated from urine (C). The molecular ions are at *m*/*z* 502, and major fragments are formed by losses of trimethylsilanol (−90) and methyl groups (−15). GC retention times for A, B, and C were 18.77 ± 0.03 min.

(Figure 1). Although the TMS ethers of 7- and 8-dehydroestriols coeluted on the nonpolar column used and shared the same parent and fragment ions, the isomers could be distinguished by the relative abundance of these ions. The steroid isolated from urine had the abundance profile of 8-dehydroestriol. This finding does not completely exclude production and excretion of 7-dehydroestriol by SLOS patients since only a few affected individuals have so far been studied. In addition, 7-dehydroestriol is less stable and may undergo aromatization to the didehydroestriol (**15**) found in urine.

In conclusion, we have developed simple and efficient methods for preparing estrogen metabolites unsaturated in ring B. The availability of these reference dehydroestriols will facilitate the establishment of routine noninvasive prenatal diagnosis for SLOS.

Acknowledgment. This work was supported by Grants HL-49122 and R03HD-39707 from the National Institutes of Health. We are grateful to Dr. Richard Kelley for providing the urine samples and to Ms. Esther Roitman for valuable technical assistance.

Supporting Information Available: Tables of NMR signal assignments, NOE difference results, HR-MS data, conformational analysis of **4** and **14**, and ^{1}H NMR spectra of **3**, **4**, **14**, and **15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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