Oxidation of 7-dehydrocholesterol and desmosterol by human cytochrome P450 46A1[S]

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Abstract Cytochrome P450 (P450 or CYP) 46A1 is expressed in brain and has been characterized by its ability to oxidize cholesterol to 24*S*-hydroxycholesterol. In addition, the same enzyme is known to further oxidize 24*S*-hydroxycholesterol to the 24,25- and 24,27-dihydroxy products, as well as to catalyze side-chain oxidations of 7α-hydroxycholesterol and cholestanol. As precursors in the biosynthesis of cholesterol, 7-dehydrocholesterol has not been found to be a substrate of P450 46A1 and desmosterol has not been previously tested. However, 24-hydroxy-7-dehydrocholesterol was recently identified in brain tissues, which prompted us to reexamine this enzyme and its potential substrates. Here we report that P450 46A1 oxidizes 7-dehydrocholesterol to 24-hydroxy-7-dehydrocholesterol and 25-hydroxy-7-dehydrocholesterol, as confirmed by LC-MS and GC-MS. Overall, the catalytic rates of formation increased in the order of 24-hydroxy-7-dehydrocholesterol < 24-hydroxycholesterol < 25-hydroxy-7-dehydrocholesterol from their respective precursors, with a ratio of 1:2.5:5. In the case of desmosterol, epoxidation to 24*S*,25-epoxycholesterol and 27-hydroxylation was observed, at roughly equal rates. The formation of these oxysterols in the brain may be of relevance in Smith-Lemli-Opitz syndrome, desmosterolosis, and other relevant diseases, as well as in signal transduction by lipids.—Goyal, S., Y. Xiao, N. A. Porter, L. Xu, and F. P. Guengerich. **Oxidation of 7-dehydrocholesterol and desmosterol by human cytochrome P450 46A1.** ***J. Lipid Res.*** **2014.** 55: **1933–1943.**

Supplementary key words 24-hydroxy-7-dehydrocholesterol • 25-hydroxy-7-dehydrocholesterol • 24*S*,25-epoxycholesterol • 27-hydroxydesmosterol

Cytochrome P450 (P450 or CYP) 46A1, or cholesterol 24-hydroxylase, is a brain-specific enzyme that converts cholesterol to 24*S*-hydroxycholesterol, which can be further oxidized to 24,25- and 24,27-dihydroxycholesterols by the same enzyme (1, 2) (**Fig. 1**). 24*S*-hydroxycholesterol is an important brain oxysterol that regulates cholesterol homeostasis by interacting with liver X receptors (LXRs) and the SREBP2 pathway (3–5). It has been reported that P450 46A1 can oxidize a broad range of substrates with diverse chemical structures, including sterols, Δ^4-steroids, and some drugs (1).

7-Dehydrocholesterol is a biosynthetic precursor to previtamin D_3 in human skin and to cholesterol in tissues (6, 7). Although the level of 7-dehydrocholesterol is normally low in human tissues and fluids, it is significantly elevated in a number of human disorders, including Smith-Lemli-Opitz syndrome (SLOS) and cerebrotendinous xanthomatosis (7–9). SLOS is caused by mutations in the gene encoding the enzyme 3β-hydroxysterol-Δ^7-reductase (DHCR7), which reduces 7-dehydrocholesterol to cholesterol (7, 10, 11). In a previous study, we identified 24-hydroxy-7-dehydrocholesterol in brain tissue of a rat model for SLOS, and we speculated that this oxysterol was derived from the oxidation of 7-dehydrocholesterol by P450 46A1 (12). Although it was previously reported that 7-dehydrocholesterol is not a substrate of P450 46A1 (13), the existence of 24-hydroxy-7-dehydrocholesterol in vivo warranted reexamination of this possibility.

The additional double bond at C7 renders 7-dehydrocholesterol highly reactive toward free radical oxidation (14), a process that leads to a number of biologically active oxysterols (12, 15–19). In addition, we recently reported that the Δ^7-double bond also makes 7-dehydrocholesterol an unusual substrate for P450 7A1, which directly converts 7-dehydrocholesterol to 7-ketocholesterol, with the 7,8-epoxide being a minor product (20). Elevated levels of these two oxysterol products have been observed in tissues and/or fluids of an animal model and patients affected with

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Abbreviations: APCI, atmospheric pressure chemical ionization; HPβCD, 2-hydroxypropyl-β-cyclodextrin; LXR, liver X receptor; NOE, nuclear Overhauser effect; P450 (or CYP), cytochrome P450; SLOS, Smith-Lemli-Opitz syndrome.

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SLOS and/or cerebrotendinous xanthomatosis (12, 21), providing support for such enzymatic transformation in vivo. Desmosterol, an alternate immediate precursor of cholesterol, contains a double bond at C24 and we hypothesized that, analogous to the reactions of 7-dehydrocholesterol and P450 7A1, desmosterol could be a substrate for P450 46A1, leading to 24,25-epoxycholesterol, 24-ketocholesterol, and/or some side-chain hydroxylated product.

The present work was designed to study the metabolic reactions of 7-dehydrocholesterol and desmosterol by cytochrome P450 46A1. In addition to cholesterol, both 7-dehydrocholesterol and desmosterol were found to be substrates of CYP46A1. Both hydroxylated and epoxidized products (but no ketone) were identified from desmosterol, and only hydroxylated products were identified from 7-dehydrocholesterol by LC-MS, NMR, and GC-MS studies (Fig. 1). The rates of formation of these products were compared with cholesterol oxidation to 24-hydroxycholesterol.

MATERIALS AND METHODS

Materials

7-Dehydrocholesterol, cholesterol, 2-hydroxypropyl-β-cyclodextrin (HPβCD), *N,O-bis*(trimethylsilyl)trifluoroacetamide, and dimethylformamide were purchased from Sigma-Aldrich. Desmosterol and a racemic mixture of 24*S*,25-epoxycholesterol and 24*R*,25-epoxycholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). 24*S*,25-epoxycholesterol and L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine were purchased from Enzo Life Science. 24*S*-hydroxycholesterol was purchased from Abcam Biochemicals (Cambridge, UK). All organic solvents (Fisher) were of HPLC grade.

Codon optimization and choice of vector

Codon optimization for P450 46A1 was done using on-line software available through Integrated DNA Technologies (Coralville, IA). A DNA containing an optimized coding sequence (supplementary Fig. I) and a His_6 tag were synthesized by Genewiz (South Plainfield, NJ) and ligated into a pCW vector using NdeI and XbaI restriction sites. The expression vector pCW (22) has been successfully used for the expression of a number of mammalian P450 enzymes (23, 24), including human P450 46A1 (1).

Expression of human P450 46A1

A fresh overnight culture was prepared by adding 20 μl of a glycerol stock of *Escherichia coli* (DH5α) cells containing the plasmid for the P450 46A1 cDNA, plus a plasmid containing the gene for the *E. coli* molecular chaperone GroEL/ES, to 100 ml of Luria-Bertani medium containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). This starter culture was grown overnight at 37°C and 220 rpm. Large-scale expression of P450 46A1 was done with 6 l of Terrific broth medium supplemented with the same antibiotics (see above), trace elements (250 μl/l of culture) (25), 1 mM NaCl, 1 mM thiamine, 2 ml of glycerol per liter of culture, and an overnight starter culture (at a dilution of 1:100, v/v). Cultures were incubated for at least 6 h with shaking at 37°C and 220 rpm, until the optical density at 600 nm reached 0.7. The expression of P450 46A1 was induced by the addition of 1.0 mM isopropyl β-D-1-thiogalactopyranoside, 1.0 mM 5-aminolevulinic acid, and 1.0 mM arabinose. Cultures were incubated for another 42 h at 27°C and 225 rpm in a Multifors incubator, followed by harvesting of cells by centrifugation at 3,500 *g* for 15 min (Sorvall RC 3B Plus centrifuge, H6000A/HBB6 rotor).

Purification of human P450 46A1

All operations were done at 4°C. The recovered cell pellets (from 6 l of culture) were resuspended in 650 ml of TES buffer [100 mM Tris-acetate (pH 7.5) containing 0.5 M sucrose and 0.5 mM EDTA] per liter of cell culture. Cells were mixed with lysozyme (2 mg/l of cell culture) and kept on ice for 60 min. Spheroplasts were obtained by centrifuging the cell suspension at 3,500 *g* for 15 min. The resulting spheroplasts were resuspended in 50 ml of sonication buffer [100 mM potassium phosphate (pH 7.5) containing 16% glycerol (v/v), 9 mM magnesium acetate, 100 μM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, and two protease inhibitor tablets (Roche) per liter of cell culture]. After sonication on ice, the material was centrifuged at 12,000 *g* for 15 min, and the recovered supernatant was further centrifuged at 1.4×10^5 *g* for 60 min. The pellet obtained after ultracentrifugation was collected, homogenized, and solubilized in 600 ml of solubilization buffer [100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 0.1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 M KCl, and 1.0% sodium cholate (w/v)]. The resulting material was centrifuged at 1.4×10^5 *g* for 60 min, and the supernatant was loaded on a 1.5 × 8 cm Ni^{+2}-nitrilotriacetic acid column, which had been pre-equilibrated with 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 10 mM β-mercaptethanol, 0.5 M KCl, and 1.0% sodium cholate (w/v). The column was washed with the same buffer containing 20 mM imidazole, and P450 46A1 was eluted with the same buffer containing 200 mM imidazole. The eluted fractions containing P450 were dialyzed against 2 l of 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v, 24 h, three buffer changes). The concentration of P450 was measured spectrally (26) with an OLIS/Aminco DW2 spectrophotometer (On-Line Equipment Systems, Bogart, GA). A typical yield of P450 46A1 from 6 l of culture was 130 nmol after purification.

Expression of NADPH-P450 reductase

The rat enzyme was expressed in *E. coli* and purified as described elsewhere (27).

LC-MS

LC-MS was done using a Waters Acquity UPLC system connected to a Thermo LTQ mass spectrometer (see below for LC conditions). Analyte (10 μl, otherwise stated) was injected into the system with a partial loop in the needle overfill mode. An atmospheric pressure chemical ionization $(APCI)^+$ source was used in all cases with the following parameters: discharge voltage, 4.1 kV; discharge current, 5.1 μA; vaporizer temperature, 450°C; sheath gas flow rate, 50 arbitrary units; auxiliary gas flow rate, 5 arbitrary units; sweep gas flow rate, 5 arbitrary units; capillary voltage, 18 V; capillary temperature, 275°C; and tube lens voltage, 80 V.

GC-MS

GC-MS analysis was performed using a gas chromatograph (Agilent 6890 GC) coupled with a mass selective detector (Agilent MSD 5973). An Agilent-J and W Scientific GC capillary column (DB-5MS; 25 m length, 0.2 mm inside diameter, 0.33 μm film thickness) was used. Helium was used as the carrier gas, maintained at a constant flow rate of 1.5 ml/min. The GC temperature program was as follows: initial temperature 70°C for 1 min; program from 70 to 130°C at a rate of 50°C/min, hold for 1 min at 130°C; program

from 130 to 300°C at a rate of 15°C/min, hold at 300°C for 15 min. Analyte was injected in a splitless mode. MS conditions were as follows: electron impact mode at an ionization energy of 70 eV, transfer line and ion source temperature at 280 and 230°C, respectively. Mass spectra were recorded from *m/z* 50–700.

NMR

A 600 MHz Bruker NMR spectrometer was used to acquire NMR spectra. The NMR spectrometer was equipped with a 5-mm Z-gradient TCI cryo-probe and Topspin software was used to analyze the data. $CDCl_3$ was used as a solvent. The peak at (δ) 7.26 ppm for $CHCl_3$ was used as a reference in ^1H NMR. 2D NMR was obtained with an optimized 90° pulse width.

Enzymatic assays

Enzymatic assays with 7-dehydrocholesterol and desmosterol were carried out in a 0.5 ml reaction volume containing 1.0 μM P450 46A1, 2.0 μM NADPH-P450 reductase, 100 mM potassium phosphate buffer (pH 7.5), 150 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and (unless indicated otherwise) a final substrate concentration of 15 μM. The stock solutions of substrates were prepared in 310 mM HPβCD [45% HPβCD (w/v)]. A 5 μl aliquot of stock substrate solution was added to the 500 μl final reaction volume. After preincubation for 15 min at 37°C, enzymatic reactions were initiated by the addition of 150 μl of an NADPH-generating system (final concentrations: 20 mM glucose 6-phosphate, 1 mM $NADP^+$, and 2 U/ml yeast glucose 6-phosphate dehydrogenase) (28) and incubated for 15 min at 37°C. The reaction was quenched with CH_2Cl_2 (2 ml), and the products were extracted twice with 2 ml of CH_2Cl_2 and centrifuged at 2,000 *g* for 10 min (23°C). The organic layers were combined and dried under a nitrogen stream. The dried samples were dissolved in CH_3CN and subjected to LC-MS analysis. LC-MS was performed using a Waters Acquity UPLC system and ACQUITY UPLC BEH octadecylsilane (C_{18}) (1.7 μm, 2.1 mm × 100 mm) column at 35°C with a flow rate of 0.3 ml/min. Mobile phase A consisted of 95:5 (v/v) water and CH_3CN with 0.1% HCO_2H (v/v); mobile phase B consisted of 5:95 (v/v) water and CH_3CN with 0.1% HCO_2H (v/v). LC methods were as follows. For 7-dehydrocholesterol products: 0–8 min, 70% mobile phase B (v/v); 8–9 min, 70% mobile phase B to 100% mobile phase B (v/v); 9–20 min, 100% mobile phase B; 20–21 min, 100% mobile phase B to 70% mobile phase B (v/v); 21–30 min, 70% mobile phase B (v/v). For desmosterol products: 0–15 min, 80% mobile phase B (v/v); 15–16 min, 80% mobile phase B to 100% mobile phase B (v/v); 16–30 min, 100% mobile phase B; 30–31 min, 100% mobile phase B to 80% mobile phase B (v/v); 31–40 min, 80% mobile phase B (v/v).

Identification of reaction products

The various products obtained from 7-dehydrocholesterol, desmosterol, and cholesterol assays with P450 46A1 were confirmed either by comparison with authentic standards, by LC-MS and GC-MS (of TMS ethers), or by LC-MS and NMR. The stereochemistry of 24,25-epoxycholesterol was confirmed by comparison with standards of 24*R*,25- and 24*S*,25-epoxycholesterol separated on a Waters Alliance (2695) HPLC system that was coupled to a Thermo LTQ mass spectrometer using a chiral column [CHIRALPAK AD-H column; 5 μm, 4.6 mm × 250 mm; solvent condition: 5% C_2H_5OH in hexanes (v/v); flow rate, 1.0 ml/min; temperature, 23°C]. The MS conditions were the same as described above.

Trimethylsilylation of enzymatic products for GC-MS analysis

The oxidative products of 7-dehydrocholesterol were converted into TMS ether derivatives for GC-MS analysis. The fragmentation patterns of TMS ether derivatives of the products were analyzed for structure elucidation. Derivatization was done using *N,O-bis*(trimethylsilyl)trifluoroacetamide:dimethylformamide in a 1:1 ratio (v/v) at 23°C for 60 min, and reactions were directly subjected to GC-MS analysis.

Large-scale incubation of P450 46A1 with desmosterol for NMR

NMR was used to identify the structure of the other unknown oxidized product of desmosterol. In order to obtain a sufficient amount of the products for NMR analysis, a large-scale incubation was performed. P450 46A1 (50 nmol) was incubated with 100 μM desmosterol in 50 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 50 nmol of NADPH-P450 reductase and 150 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine. After initial incubation for 30 min at 37°C, the enzymatic reaction was initiated by the addition of 15 ml of an NADPH-generating system (28) and incubated for 2 h at 37°C. The reaction was quenched with CH_2Cl_2 and product was extracted twice with 80 ml of CH_2Cl_2, with centrifugation at 2,000 *g* for 10 min. The organic layers were combined and dried under a nitrogen stream. The dried sample was dissolved in CH_3CN:CH_3OH (80:20, v/v) and centrifuged as above to precipitate the suspended particles; the organic layer was collected carefully and subjected to purification. Purification was done with a Waters Acquity UPLC system using an ACQUITY UPLC BEH octadecylsilane (C_{18}) column (1.7 μm, 2.1 mm × 100 mm) at 35°C with a flow rate of 0.6 ml/min and an injection volume of 15 μl. Because of the lack of chromophore in the analyte, LC-MS was used for detection, with an 8:2 (v/v) splitter into the mass spectrometer. Mobile phase A consisted of 95:5 (v/v) water and CH_3CN with 0.1% HCO_2H (v/v); mobile phase B consisted of 5:95 (v/v) water and CH_3CN with 0.1% HCO_2H (v/v). The LC method was as follows: 0–4.0 min, 80% mobile phase B (v/v); 4.0–4.5 min, 80% mobile phase B (v/v) to 100% mobile phase B; 4.5–8.0 min, 100% mobile phase B; 8.0–8.5 min, 100% mobile phase B to 80% mobile phase B (v/v); 8.5–12.5 min, 80% mobile phase B (v/v). Purified fractions were combined, extracted into CH_2Cl_2, dried under nitrogen, and redissolved in $CDCl_3$ for NMR analysis.

Kinetic assays

Kinetic assays with 7-dehydrocholesterol, desmosterol, and cholesterol were performed as described above but with substrate concentrations varying from 1 μM to 100 μM {substrates were dissolved in 310 mM HPβCD [45% HPβCD (w/v)], and 5 μl of stock substrate solution was added to a 500 μl final reaction volume}. The same LC method was used for cholesterol products as in the case of 7-dehydrocholesterol. A product at *m/z* 385 was detected in the cholesterol assay (24*S*-hydroxycholesterol). The k_{cat} and K_M values were calculated using the program Dynafit (29).

Binding assays

For binding studies, a series of stock solutions of varying concentrations (of each substrate) were prepared in 310 mM HPβCD [45% HPβCD (w/v)]. Binding of various substrates was done in a final volume of 0.33 ml containing 100 mM potassium phosphate (pH 7.5) and 2 μM P450 46A1, and substrate was varied from 1–100 μM (3.3 μl of stock solution of substrate was added). The same volume of cyclodextrin without substrate (3.3 μl) was added to a reference cuvette, and spectra were recorded from 350 to 500 nm. The ΔA_{389}-A_{421}, ΔA_{387}-A_{419}, and ΔA_{389}-A_{422} differences for 7-dehydrocholesterol, desmosterol, and cholesterol (respectively) were plotted against increasing concentrations of substrate and binding dissociation constants (K_d) were estimated by using the program Dynafit (29).

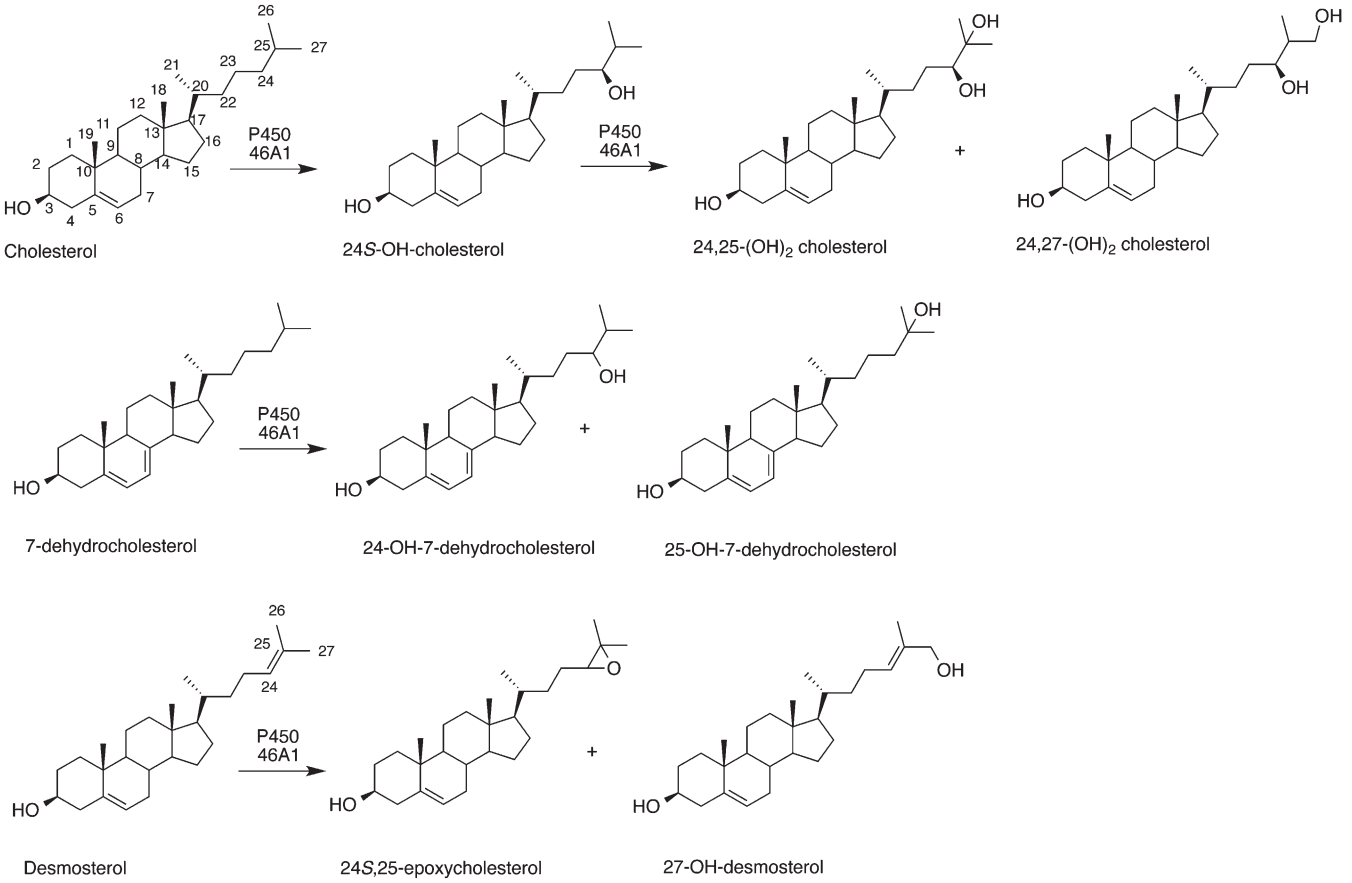

Fig. 1. Oxidation of cholesterol, 7-dehydrocholesterol, and desmosterol to products by P450 46A1.

Data analysis

Steady-state kinetics and binding studies were fit using the program Dynafit (29, 30).

Each substrate was dissolved in 310 mM HPβCD. The final concentration of HPβCD in each reaction mixture was 3.1 mM. The binding constant (K_d) of cholesterol with HPβCD is reported to be 1.05 mM (31), and the same value was used in calculations for 7-dehydrocholesterol and desmosterol, in that the calculated logarithms of the octanol-water partition coefficients (cLogP values) for cholesterol, 7-dehydrocholesterol, and desmosterol are of the same order of magnitude, i.e., 6.52, 6.30, and 6.04, respectively (ChemDraw, Cambridgesoft, Cambridge, MA). The concentration of free substrate was calculated using the cholesterol K_d value of 1.05 mM and a simple binding model (i.e., substrate + HPβCD ⇌ substrate-HPβCD). In the cases of 7-dehydrocholesterol and desmosterol, where the affinity of the steroids for the cyclodextrin would be less due to the lower cLogP values, the effect of using the K_d value of 1.05 mM would be to raise the apparent concentration of the sterol and thus overestimate the K_M or K_d for dissociation of the sterol-P450 46A1 complex.

RESULTS

Protein expression and purification

P450 46A1 was expressed in *E. coli* cells. We were unsuccessful in the expression of the native sequence and did not obtain good expression in this system until we used a DNA sequence optimized for *E. coli* expression (supplementary Fig. I). The purified protein showed a band at 450 nm in the reduced-CO difference spectrum (**Fig. 2**). The Soret band was observed at 417 nm (data not shown), a characteristic feature of ferric low-spin P450. SDS-PAGE gel showed a single major band at 50 kDa, accounting for >94% of the protein as judged by densitometry of the Coomassie staining (**Fig. 3**).

Oxidation of 7-dehydrocholesterol, desmosterol, and cholesterol

Incubation of 7-dehydrocholesterol with P450 46A1 yielded two products, t_R 5.5 and 6.4 min (**Fig. 4B**). Incubation

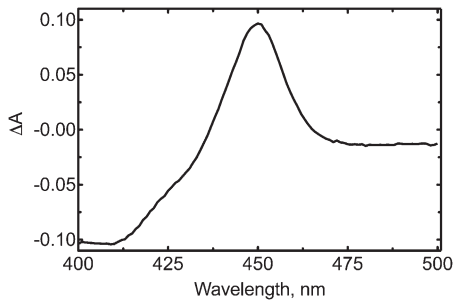

Fig. 2. Fe^{2+}-CO versus Fe^{2+} difference spectrum of purified P450 46A1. The P450 concentration was 1.2 μM.

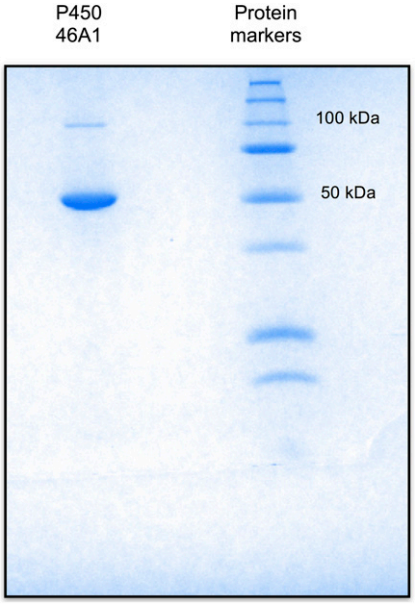

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified P450 46A1. The acrylamide concentration was 12% (w/v). Standard markers are included on the right side of the gel.

of desmosterol with P450 46A1 also yielded two products, t_R 3.9 and 6.4 min, (**Fig. 5B**). No product was observed in control assays without P450 46A1 (Figs. 4A, 5A). All products were observed at *m/z* 383, which corresponds to dehydration of ions with an *m/z* of 401 (7-dehydrocholesterol or desmosterol plus one oxygen atom). The product eluted at t_R 6.4 min in the 7-dehydrocholesterol assay was identified as 24-hydroxy-7-dehydrocholesterol by comparison with an authentic standard (Fig. 4C). Further, the fragmentation pattern obtained by collision-induced dissociation for 24-hydroxy-7-dehydrocholesterol matched that of the standard (i.e., MS^2 at *m/z* 383.3 and MS^3 at *m/z* 365.3, respectively) (**Fig. 6**). The product 24-hydroxy-7-dehydrocholesterol was further confirmed by GC-MS (supplementary Fig. II). Similarly, the product at t_R 6.4 min from the desmosterol assay was identified as 24,25-epoxycholesterol by comparison with a commercial standard (Fig. 5B, C). The stereochemistry of 24,25-epoxycholesterol was confirmed to be 24*S* by comparing it with standards available using a chiral column (supplementary Fig. III). The fragmentation pattern of the product matched with that of a 24*S*,25-epoxycholesterol standard (data not shown). The desmosterol product (t_R 3.9 min) was not 24-ketocholesterol, as judged by comparison with an authentic standard. The reaction with cholesterol yielded only one product, 24*S*-hydroxycholesterol, confirmed with commercially available standard (supplementary Fig. IV). The identification of the unknown products of 7-dehydrocholesterol and desmosterol is presented below.

Characterization of a second product from 7-dehydrocholesterol

In order to characterize the unknown peak eluting at t_R 5.5 min, the collected product was converted to a TMS ether derivative and subjected to GC-MS analysis. GC-MS

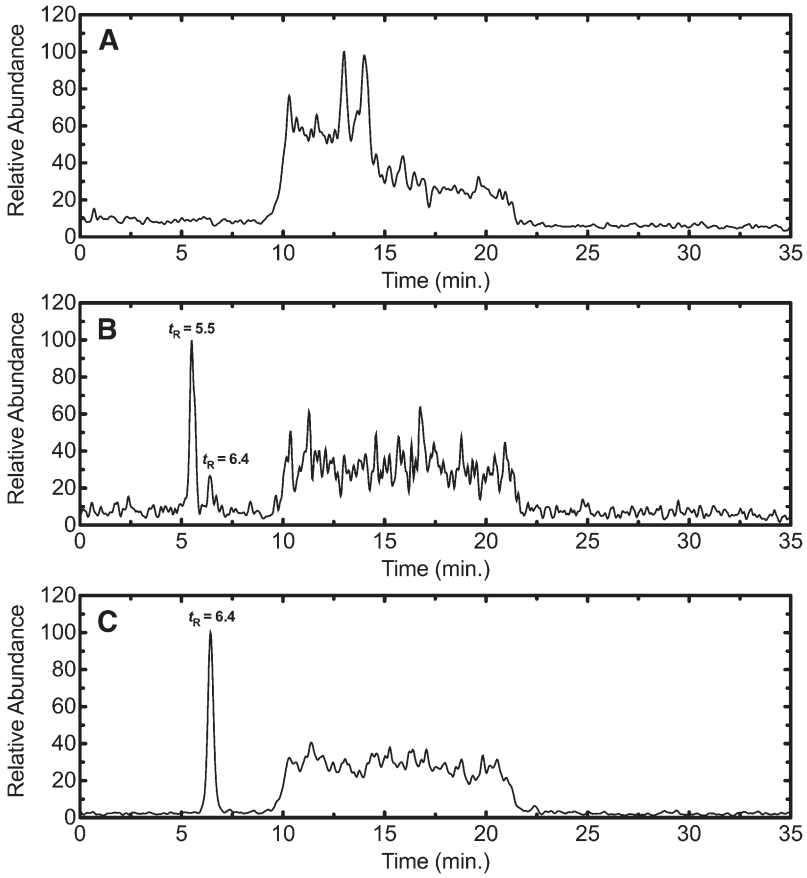

Fig. 4. LC-MS profile of 7-dehydrocholesterol incubation products. Assay without P450 46A1 (A), assay with P450 46A1 (B), and standard 24-hydroxy-7-dehydrocholesterol (C). $APCI^+$ ionization was used, and *m/z* 383 was monitored in each case. The peak at t_R 6.4 min in (B) matched the t_R of the 24-hydroxy-7-dehydrocholesterol standard in (C).

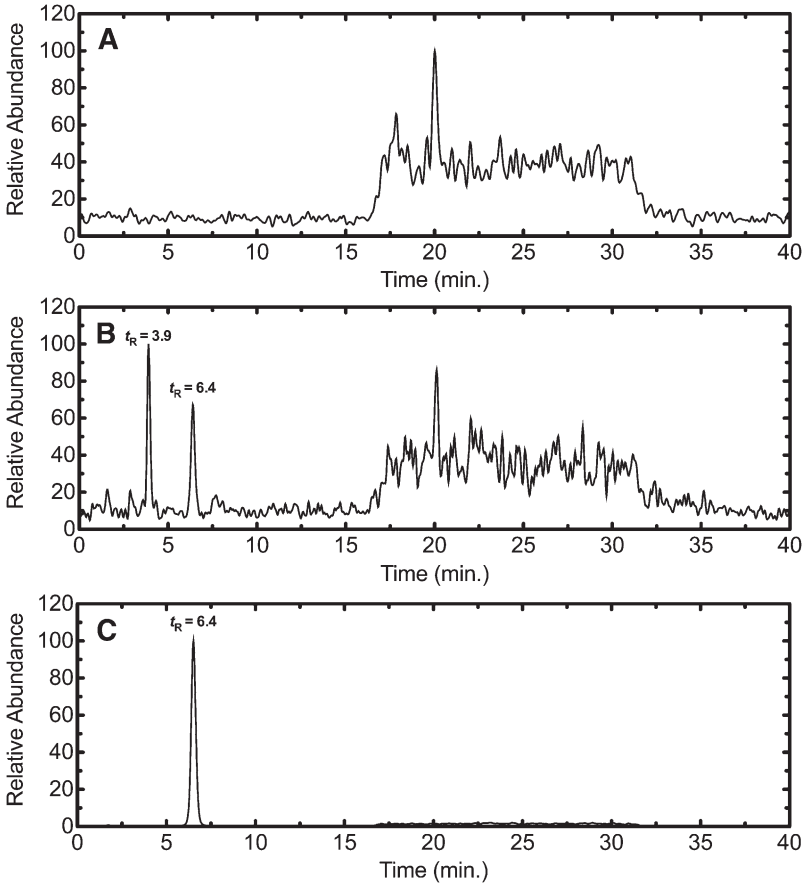

Fig. 5. LC-MS profile of desmosterol incubation products. Assay without P450 46A1 (A), assay with P450 46A1 (B), and standard 24*S*,25-epoxycholesterol (C). $APCI^+$ ionization was used, and *m/z* 383 was monitored in each case. The peak at t_R 6.4 min in (B) matched the t_R of the 24*S*,25-epoxycholesterol standard in (C).

showed two peaks at *m/z* 544 (M^+ for TMS ether derivative of a mono-oxygenation product of 7-dehydrocholesterol) (supplementary Fig. II). One peak was identified as 24-hydroxy-7-dehydrocholesterol from its fragmentation pattern (supplementary Fig. II), confirming the results from LC-MS analysis. Analysis of the fragmentation of the second peak led to its assignment as 25-hydroxy-7-dehydrocholesterol (**Fig. 7**). The assignment is based on the loss of TMS isopropyl ether from the parent ion (α-cleavage).

Characterization of the second product from desmosterol

The unknown desmosterol product was characterized by NMR. We speculated that this product was a hydroxylated product, formed at an allylic position, in that preliminary analyses had ruled out a C24 ketone (see above). In the ^{1}H NMR spectrum, a peak from δ 5.08 ppm in desmosterol (1H, m, C24) was shifted downfield to δ 5.38 ppm in the product (1H, m, C24) with similar splitting patterns, suggesting a more electron-deficient environment of this proton (**Fig. 8**). On the other hand, a new peak appeared at δ 3.99 ppm with an integration of two protons, suggesting that it is a methylene group next to an oxygen atom (Fig. 8; confirmed by heteronuclear single-quantum correlated spectroscopy, supplementary Fig. VB). On the basis of ^{1}H NMR (Fig. 8) and homonuclear correlation spectroscopy, heteronuclear multiple-bond correlation spectroscopy, and heteronuclear single-quantum correlation spectroscopy spectra (supplementary Fig. V), the unknown product was characterized as either 27-hydroxydesmosterol or 26-hydroxydesmosterol. In the nuclear Overhauser effect (NOE) spectroscopy experiment (supplementary Fig. VD), there was a strong NOE between H-24 and a proton in the terminal methylene group, but no NOE between H-24 and the terminal methyl group, indicating the product is 27-hydroxydesmosterol (terminal methylene group is *syn* to H-24 to produce the NOE correlated spectroscopy signal).

Kinetic analysis

The calculated k_{cat} and K_M values for the formation of 24-hydroxy-7-dehydrocholesterol (from 7-dehydrocholesterol) were 24 ± 1 pmol·nmol^{-1}·min^{-1} and 2.8 ± 0.02 μM, respectively (corrected for the binding to HPβCD) (**Table 1**, supplementary Fig. VI). Because a 25-hydroxy-7-dehydrocholesterol standard was not available, the rate for its formation was estimated relative to the rate for 24-hydroxy-7-dehydrocholesterol, assuming an equal MS response, to be ∼110 pmol·nmol^{-1}·min^{-1}. The k_{cat} and K_M values for 24*S*,25-epoxycholesterol (from desmosterol) were 33 ± 1 pmol·nmol^{-1}·min^{-1} and 9.4 ± 1.0 μM, respectively (corrected for the binding to HPβCD) (Table 1, supplementary Fig. VI). Again, because a 27-hydroxydesmosterol standard was not available, the rate for this was estimated relative to the rate for 24*S*, 25-epoxycholesterol, assuming an equal MS response, to be ∼44 pmol·nmol^{-1}·min^{-1}.

Binding analysis

The calculated K_d values for 7-dehydrocholesterol, desmosterol, and cholesterol were 1.4 ± 0.1 μM, 1.0 ± 0.4 μM,

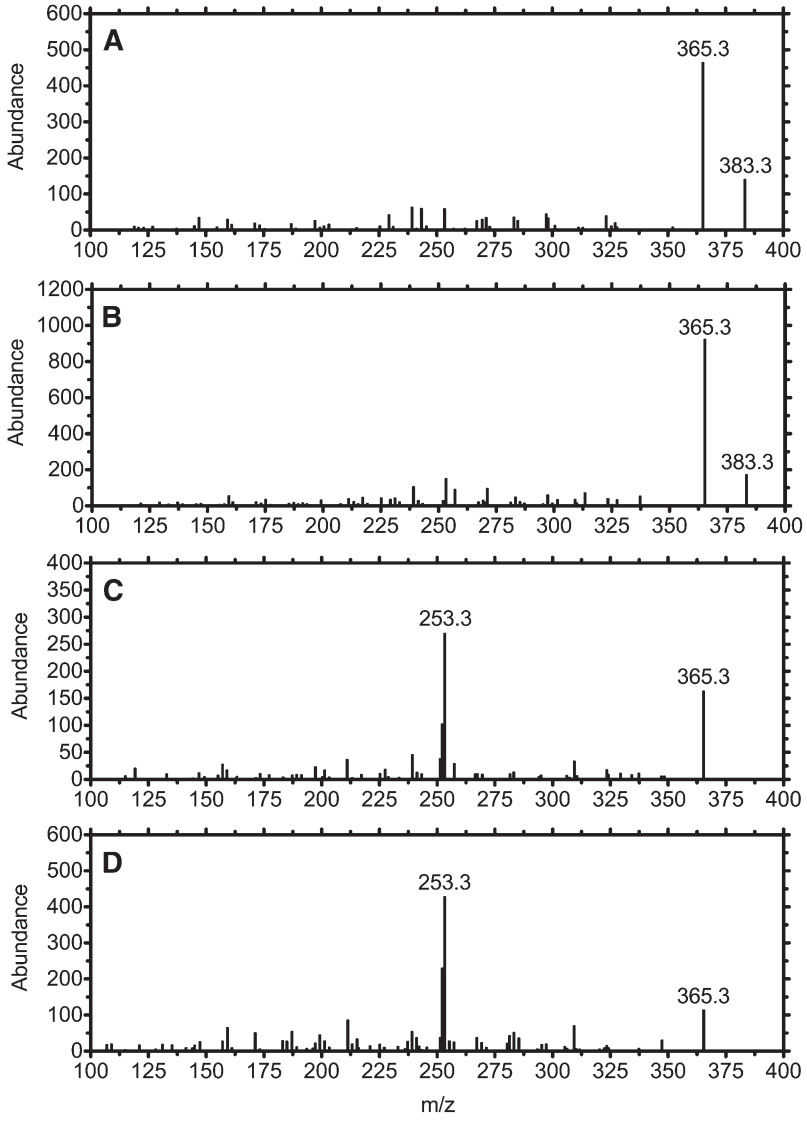

Fig. 6. MS^n fragmentation pattern of 24-hydroxy-7-dehydrocholesterol. A: MS^2 (at *m/z* 383.3) of the 24-hydroxy-7-dehydrocholesterol product obtained from enzymatic reaction with P450 46A1. B: MS^2 (at *m/z* 383) of standard 24-hydroxy-7-dehydrocholesterol. C: MS^3 (at *m/z* 365) of 24-hydroxy-7-dehydrocholesterol product obtained from enzymatic assay with P450 46A1. D: MS^3 (at *m/z* 365) of standard 24-hydroxy-7-dehydrocholesterol.

and 2.2 ± 0.4 μM, respectively (corrected for the binding to HPβCD) (supplementary Figs. VII, VIII).

DISCUSSION

This study demonstrates that 7-dehydrocholesterol and desmosterol are substrates of P450 46A1, leading to 24-hydroxy- and 25-hydroxy-7-dehydrocholesterol and to 24*S*,25-epoxycholesterol and 27-hydroxydesmosterol as the products, respectively. The rate of formation (k_{cat}) of 24-hydroxy-7-dehydrocholesterol was about one-third that of 24*S*-hydroxycholesterol, but the formation of 25-hydroxy-7-dehydrocholesterol was a much more favored reaction (Table 1). The enzyme efficiency (k_{cat}/K_m) was similar for 7-dehydrocholesterol compared with cholesterol but 2–3 times higher than that of desmosterol. We note that although previous work by Björkhem et al. (13) did not find 7-dehydrocholesterol as a substrate of P450 46A1; their work was carried out in HEK293 cells, which presumably would metabolize 7-dehydrocholesterol to cholesterol efficiently with intact cholesterol biosynthesis machinery in them. Alternatively, the expression levels of added P450s are relatively low in such cells, and the conversion rate might have been too low for detection. A third possibility is that the oxidation products of 7-dehydrocholesterol might have been further metabolized by other enzymes in the HEK293 cells.

The binding of all substrates showed typical "type I" difference spectra (supplementary Fig. VII). The estimated dissociation constant (K_d) for 7-dehydrocholesterol was less than that for cholesterol, indicating stronger binding to P450 46A1.The K_d for desmosterol was similar to that of 7-dehydrocholesterol, but it was found to be a less efficient substrate compared with 7-dehydrocholesterol.

Under normal physiological conditions, the cholesterol product 24*S*-hydroxycholesterol is the dominant oxysterol in the adult brain (32–35), modulating cholesterol homeostasis through activation of LXRs (3, 4) and inhibition of the SREBP2 pathway by binding INSIG (5). A reduced level of 24*S*-hydroxycholesterol has been associated with aging and severe neurological diseases (36, 37). A decreased level of 24*S*-hydroxycholesterol has also been reported in plasma of SLOS patients (13), likely due to

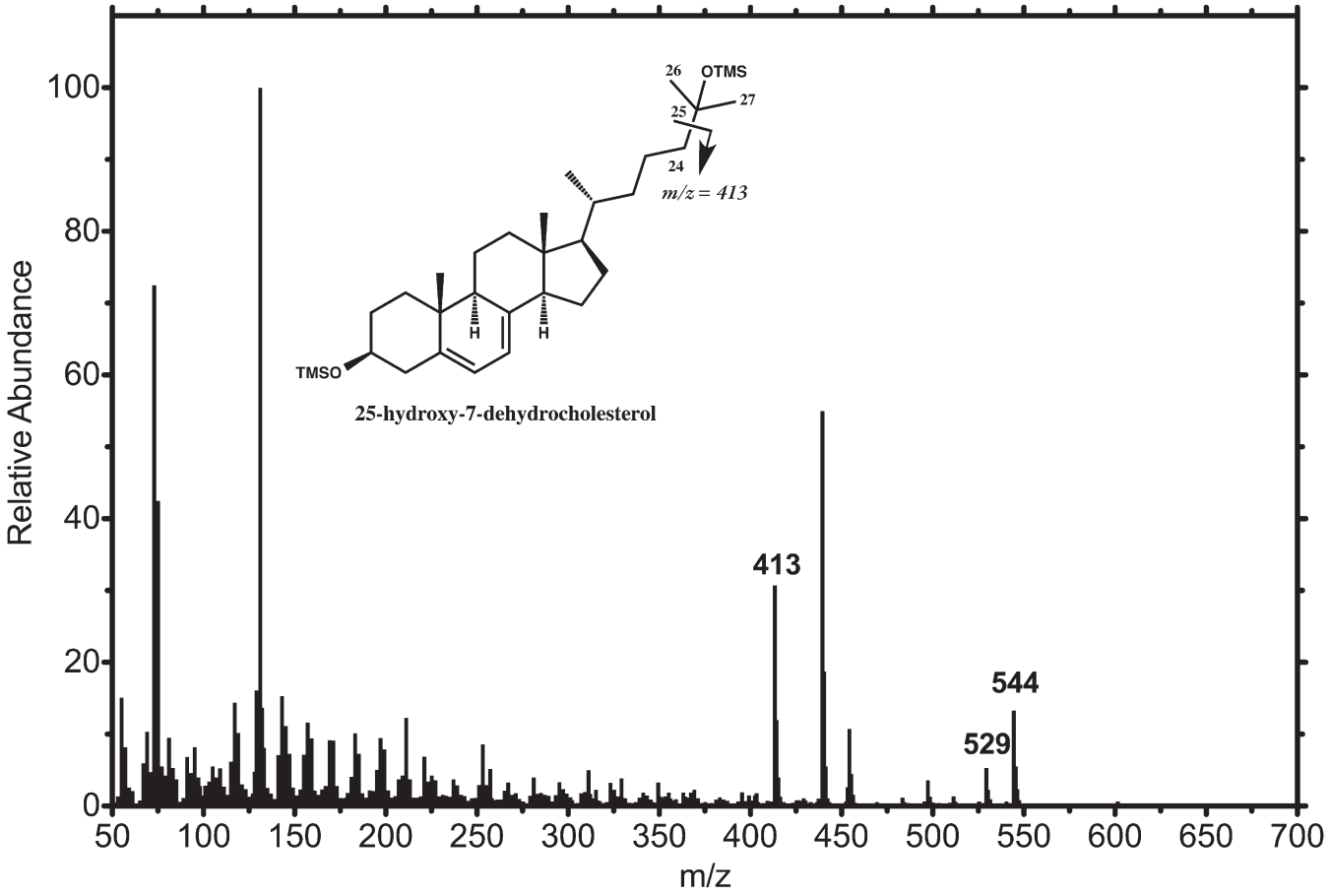

Fig. 7. GC-MS fragmentation pattern of TMS ether product of unknown peak (t_R 5.5 min) in LC-MS profile of 7-dehydrocholesterol assay (Fig. 4B). The fragment at *m/z* 413 confirmed the product to be 25-hydroxy-7-dehydrocholesterol.

deficiency in cholesterol. We have previously identified 24-hydroxy-7-dehydrocholesterol in the brain tissues of SLOS rodent models (12, 38). However, to what extent the 24-hydroxy-7-dehydrocholesterol could compensate for the lack of 24*S*-hydroxycholesterol in terms of its biological functions remains to be elucidated.

It is not entirely unexpected to identify 7-dehydrocholesterol as a substrate of P450. In addition to our findings on this sterol being a substrate of P450 7A1 (20) and P450 46A1 (current study), it has also been reported that P450 27A1 catalyzes the metabolism of 7-dehydrocholesterol to 25-hydroxy-7-dehydrocholesterol and 26/27-hydroxy-7-dehydrocholesterol (13, 39, 40). Interestingly, both oxysterols were found to be activators of LXRs, slightly less potent than their cholesterol analogs (39, 40). Furthermore, 7-dehydrocholesterol-derived steroids have been observed in the urine of SLOS patients, suggesting that 7-dehydrocholesterol is also a substrate of sterol side-chain cleavage enzyme (P450scc, P450 11A1) (41, 42).

The formation of 24*S*,25-epoxycholesterol directly from desmosterol may have significant biological and physiological consequences. Until now, squalene 2,3;22,23-diepoxide was the only known biosynthetic precursor to 24*S*,25-epoxycholesterol (43), but our study reveals a novel pathway for the formation of this biologically important oxysterol. 24*S*,25-epoxycholesterol was found to be the most potent and abundant oxysterol ligand of LXRs in developing mouse brain (significantly higher than the level of 24*S*-hydroxycholesterol), and it promotes neurogenesis and neuronal survival in an LXR-dependent fashion (44, 45). Notably, the levels of desmosterol are also high in developing brains (up to 30% of total brain sterols) (46–48) and

TABLE 1. Kinetic parameters measured for 7-dehydrocholesterol, desmosterol, and cholesterol

Substrate	Product	k_{cat} (pmol·nmol^{-1}·min^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} min^{-1})
7-Dehydrocholesterol	24-hydroxy-7-dehydrocholesterol	24 ± 1	2.8 ± 0.02	8.5 ± 0.4
	25-hydroxy-7-dehydrocholesterol	~110	—	—
Desmosterol	24*S*,25-epoxycholesterol	33 ± 1	9.4 ± 1	3.5 ± 0.4
	27-hydroxydesmosterol	~44	—	—
Cholesterol	24-hydroxycholesterol	59 ± 6	7 ± 1	8.4 ± 1.5

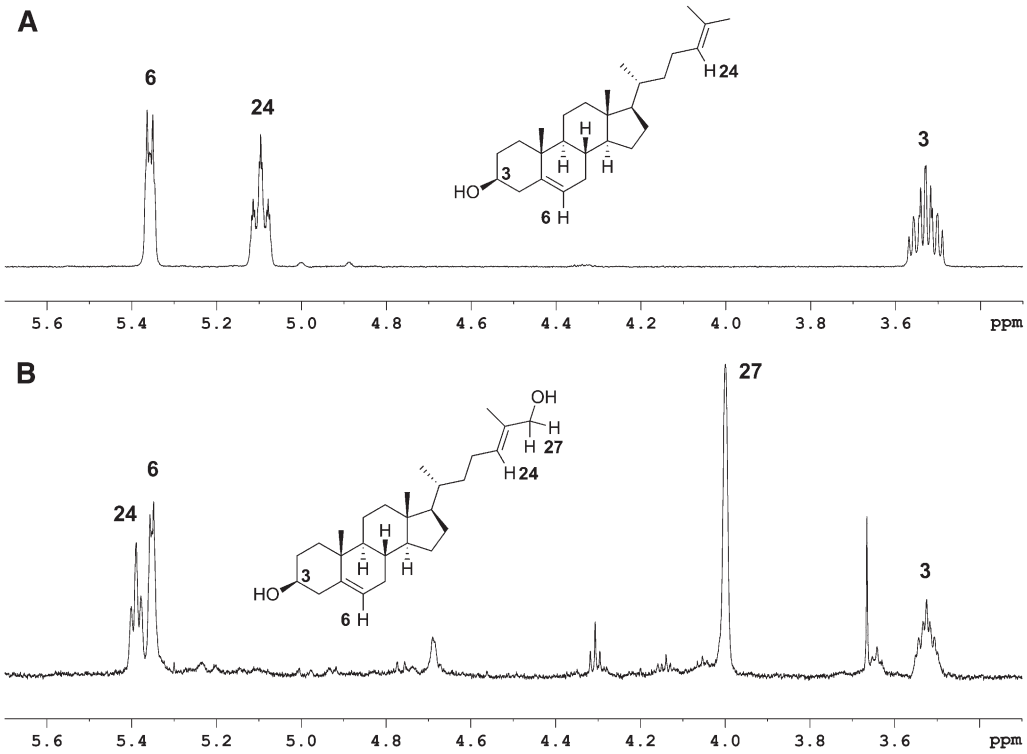

Fig. 8. ^{1}H NMR spectra of desmosterol (A) and purified product derived from desmosterol (B) (t_R 3.9 min in LC-MS profile of Fig. 5B).

low in the adult brain (~0.1% of cholesterol) (49). Thus, our finding suggests a possible link between desmosterol and the production of 24*S*,25-epoxycholesterol during brain development. Interestingly, Griffiths and coworkers recently reported that the level of 24*S*,25-epoxycholesterol was greatly decreased in the brain of *Cyp46a1*-knockout mice (one-fifth of the level in wild-type mice), providing support for the participation of P450 46A1 in the production of this oxysterol in vivo (50). It is of additional interest that 24*S*,25-epoxycholesterol was found to be an inhibitor of 3β-hydroxysterol-Δ^{24}-reductase (DHCR24), leading to the accumulation of desmosterol in a number of cell lines (51).

The substrate selectivity of P450 46A1 is not as limited as once thought. Cholesterol 24*S*-hydroxylation has been the main reaction ascribed to P450 46A1. However, several other sterols are also substrates, including 24*S*-hydroxycholesterol (25- and 27-hydroxylations), 7α-hydroxycholesterol, cholestanol, progesterone, and testosterone (1). Some drugs are also substrates (1). Here we have shown that recombinant human P450 46A1 catalyzes the 24- and 25-hydroxylation of 7-dehydrocholesterol and the epoxidation and hydroxylation of desmosterol. Interestingly, P450 46A1 binds and oxidizes a number of drugs (1, 52). This is an unusual phenomenon in that most of the P450s that appear to be specialized for oxidation of endogenous substrates do not use xenobiotic chemicals as substrates. The overall in vivo contribution of P450 46A1 to the metabolism of these drugs, even in brain, is unknown. Furthermore, P450 46A1 activity (toward cholesterol) is stimulated by binding to some drugs (e.g., efavirenz, acetaminophen, mirtazapine, galantamine), and the in vivo relevance of this effect has been shown in a mouse model (53). We have not examined the effects of any of these drugs on the new reactions we characterized here.

In conclusion, we have identified 7-dehydrocholesterol and desmosterol as substrates of P450 46A1. The oxysterols derived from these enzymatic pathways could have significant biological consequences in related physiological or pathological conditions where the levels of these two cholesterol precursors are high, such as in SLOS (7-dehydrocholesterol) (7, 8), desmosterolosis (desmosterol) (7, 54), and developing brain (desmosterol) (46–48). The direct conversion of desmosterol to 24*S*,25-epoxycholesterol by P450 46A1 represents a distinctly new pathway for the formation of this potent oxysterol ligand of LXRs (4, 44, 45).

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