Synthesis of Deuterium-Labeled Plant Sterols and Analysis of Their Side-Chain Mobility by Solid State Deuterium NMR

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The plant sterols sitosterol and stigmasterol exert very different effects on plant model membranes, the first one being a "reinforcer" like cholesterol, the second one not. 25-^{2}H-Stigmasterol has been synthesized by coupling of the 22-aldehyde derived from stigmasterol by ozonolysis, with the proper sulfone labeled in position 25. The configuration of the ethyl side chain at C-24 was controlled by separation of the diastereomers introduced via a chiral sulfoxide. This synthetic scheme allowed the introduction of a labeled side chain in plant sterols in eight steps for stigmasterol and nine for sitosterol (overall yield ca. 15%). Using both diastereomers, the 24-epimers of sitosterol (clionasterol) and stigmasterol (poriferasterol) have also been synthesized. Deuterium NMR on oriented lipid bilayers made of soybean phosphatidylcholine and containing these four labeled plant sterols clearly reveals the difference of orientation and mobility of the four side chains.

In animal cell membranes, cholesterol (cholest-5-en-3β-ol) is the major sterol present. However, plant cell membranes contain a complex mixture of sterols in which sitosterol ((24*R*)-24-ethylcholest-5-en-3β-ol), stigmasterol ((24*S*)-24-ethylcholesta-5-(*E*)-22-dien-3β-ol), campesterol ((24*R*)-24-methylcholest-5-en-3β-ol) and 22,23-dihydrobrassicasterol ((24*S*)-24-methylcholest-5-en-3β-ol) usually predominate.[1]

The 24-ethylsterols (sitosterol and stigmasterol) have the same α-configuration for the ethyl C-24 substituent, and there is a mixture of both 24-α and 24-β epimers for the 24-methylsterols. Moreover, stigmasterol differs from sitosterol by a supplementary *trans* 22(23) double bond (Figure 1). In addition to these sterols, poriferasterol and clionasterol, respectively the C-24 epimers of stigmasterol and sitosterol, are also present in certain algae, and ergosterol is the major sterol in fungi.[2]

These sterols comprise a 3β hydroxyl group, a quasi-planar sterol skeleton, and a side chain, which are essential requirements for reinforcing phospholipid−sterol interactions in membranes.[3] All these sterols are indeed concentrated in the plasma membrane;[4] they play a structural role and act as regulators of some membrane properties, but they are also precursors of effectors of a variety of other biological functions. These sterols have been incorporated into model membrane bilayers and tested for their ability to regulate either membrane fluidity as demonstrated by polarization fluorescence[5] and deuterium NMR of properly labeled phospholipids,[6]

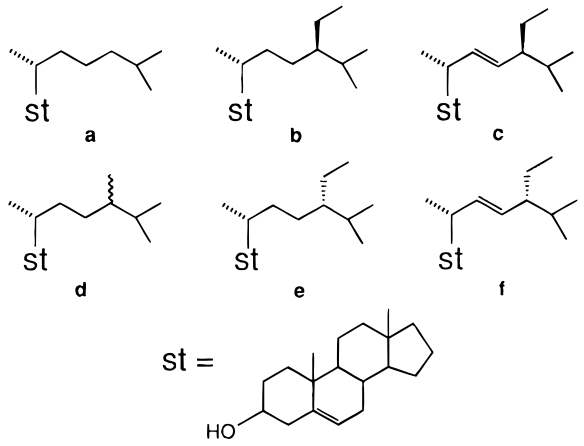

Figure 1. Structures of cholesterol (a), sitosterol (b), stigmasterol (c), 24ξ-methyl cholesterol (d), clionasterol (e), poriferasterol (f).

or water permeability.[7] In particular, deuterium NMR is a very efficient technique for the analysis of the average orientation and of the dynamics of the *n*-alkyl chains of deuterium-labeled phospholipids in the biomembranes.[6−12] By using this technique with membrane model systems prepared from soybean phosphatidylcholine (PC), a representative phospholipid of plant membranes with a high content of unsaturated fatty acyl chains,[5] we have compared the effect of a variety of plant sterols and analogues on the lipid dynamics and demonstrated the surprising specificity of the sterol−lipid interaction.[6,7] Contrary to expectation, sitosterol and

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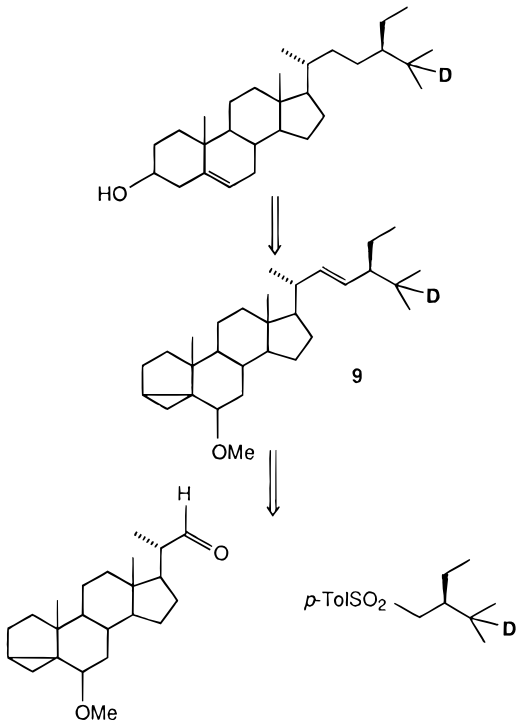


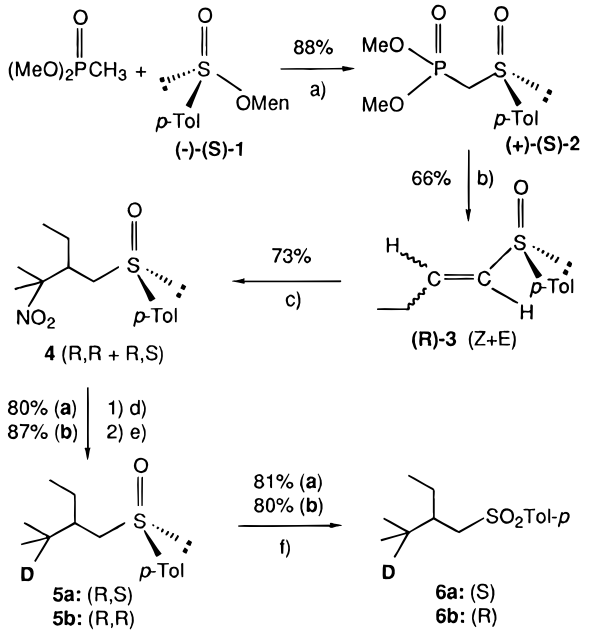
Figure 2. Retrosynthetic scheme.

stigmasterol had opposite effects: while sitosterol was found to be very efficient in diminishing membrane fluidity and permeability in a manner similar to cholesterol in animal cell membranes, stigmasterol appeared to be a poor membrane reinforcer.[5−7]

In order to understand the molecular mechanism of this specificity, we have now compared the orientation and the dynamic behavior of the side chains of both sterols in phospholipid bilayers. The dynamic properties of the sterols themselves in membranes have been hardly studied, except in the case of cholesterol itself, which can be labeled at specific positions on rings A and B; this had allowed the determination of its orientation and molecular order parameter.[10] However, since the difference between stigmasterol and sitosterol is localized on the side chain, it appeared of greater interest to analyze the dynamics of the side chain, using specifically D-labeled sterols. We have thus synthesized 25-^{2}H-stigmasterol and 25-^{2}H-sitosterol as well as their epimers (25-^{2}H-poriferasterol and 25-^{2}H-clionasterol) in order to measure the order parameter of these sterols incorporated in oriented bilayers.

The stereospecific introduction of the 24-*R* or the 24-*S* ethyl group into a steroidal chain presents a significant challenge. In 1970s, Sucrow and co-workers[13] developed an elegant stereoselective synthesis of (24*R*)- and (24*S*)-ethylsterols by a Claisen rearrangement to generate a specific chiral center on the steroidal side chain. However, this requires eight consecutive steps from the starting i-methyl ether aldehyde to stigmasterol. We now report a novel method controlling the chirality of the C-24 side chain, which was developed for the synthesis of stigmasterol, but which should also be applicable to the synthesis of other natural compounds. Our strategy is illustrated in the retrosynthetic scheme (Figure 2). 25-

Scheme 1. Synthesis of the Chiral Synthon[a]



[a](a) n-BuLi, THF, −78 °C; (b) n-BuLi, C_2H_5CHO, THF, −78 °C; (c) DBU, $(CH_3)_2CHNO_2$; (d) separation of the diastereomers on chromatography; (e) AIBN, Bu_3SnD, benzene, 85 °C; (f) m-CPBA, dichloromethane, 40 °C.

^{2}H-Sitosterol derives from the i-methyl ether of deuterated stigmasterol **9** by hydrogenation of the 22-double bond, followed by recovery of the 3β-hydroxyl group and the Δ^5double bond. We have chosen the stereospecific synthesis of a chiral deuterated synthon coupled with a steroidal aldehyde, in order to obtain finally phytosterols with both natural configurations of the asymmetric C-24 on the side chain.

Synthesis of Synthon 6. We wanted to synthesize two diastereomers containing two chiral centers: an asymmetric carbon bearing the ethyl group and a chiral sulfoxide[14] (Scheme 1); this would allow the separation of the two diastereomers and the determination of their configuration by X-ray crystallography.

The first step of the stereospecific synthesis of synthon **6** is the coupling of the commercial diastereomerically pure (−)-(*S*)-menthyl *p*-toluenesulfinate **1** with dimethyl methylphosphonate.[15] This typical nucleophilic substitution takes place with complete inversion of configuration at sulfur to give the (+)-(*S*)-sulfinyl phosphate **2**.[16] The Horner−Wittig reaction[15] of the lithio derivative of sulfoxide **2** with propionaldehyde gives a mixture of *E* and *Z* isomers of (*R*)-sulfoxide **3**. Their structures were determined by ^{1}H NMR spectroscopy. The *E*- and *Z*-sulfoxides **3** were used without separation in the following reaction. The production of the deuterated sulfoxide consists of a sequence of two steps: a Michael addition[17] of 2-nitropropane on the α,β-unsaturation and a substitution of the nitro group to deuterium. The first step catalyzed by DBU[17] produced two diastereomers of the nitro sulfoxide **4** (*RR*+*RS*) which could be separated by

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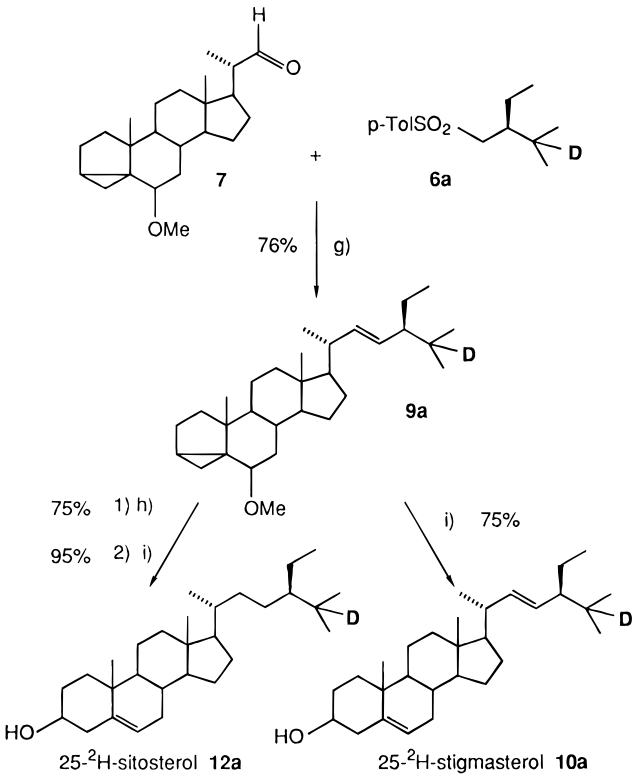
medium pressure chromatography. The Michael addition of secondary nitro compounds to α- or *β*-substituted alkenes or to α,*β*-unsaturated sulfoxides is rather difficult.[17] Finally, we could resolve this problem by using 2-nitropropane as the solvent (as well as the reagent) and by employing 2 equiv of DBU. The separation of the diastereomers appeared to be easier at the level of the nitro synthon than after its reduction by Bu_3SnD. The purity of each diastereomer was checked by ^{1}H NMR. The less polar diastereomer was crystallized, and its X-ray analysis allowed us to deduce the configuration of the asymmetric carbon, (*S*), from that of the sulfoxide (*R*).[18] This analysis showed that the crystallized nitro sulfoxide **4a** (*R*,*S*) has on the asymmetric carbon the correct configuration required to obtain the natural 24-ethyl side chain (Scheme 1). The replacement of the nitro group by a deuterium in the nitrosulfoxides **4a** (*R*,*S*) and **4b** (*R*,*R*) was performed using tributyltin deuteride in the presence of AIBN at 80 °C.[17] In addition to ^{1}H NMR analysis, the IR spectrum revealed the disappearance of the nitro bands at 1537 and 1347 cm^{-1} and the appearance of the two specific C−D stretching vibrations 2172 and 2110 cm^{-1}.

The deuterated sulfoxides **5a** (*R*,*S*) and **5b** (*R*,*R*) thus obtained were then oxidized by m-CPBA to give the deuterated sulfones **6a** (*S*) an **6b** (*R*). Two specific bands for the sulfone group were present on the IR spectrum at 1304 and 1146 cm^{-1}. The products **6** contain now only one asymmetric center: the chiral carbon bearing the ethyl group with the (*S*) configuration in **6a** will become C-24 on the side chain of stigmasterol (configuration *S*) and of sitosterol (configuration *R*).

Synthesis of Sitosterol, Stigmasterol, and Their 24 Epimers. These syntheses require coupling of the deuterated chiral synthon **6a** and the steroidal i-methyl ether aldehyde **7** (Scheme 2), prepared from natural stigmasterol according to the method described in the literature.[19] The coupling of the sulfone **6a** with the i-methyl ether aldehyde **7** was performed in the presence of BuLi. To favor the formation of the 22-double bond in the following procedure, the hydroxyl group is converted *in situ* into a mesylate.[20] The mixture of coupling products is reduced with 6% sodium amalgam in presence of disodium hydrogen phosphate according to Julia's method.[21] This method allowed the formation of the trans olefin in the 25-deuterated i-methyl ether **9a**. The ^{1}H NMR reveals resonances around 5 ppm with $J = 15$ Hz for the trans ethylenic protons on the lateral chain. The presence of deuterium on the 25 position is confirmed by the IR spectrum and by the comparison of the ^{1}H NMR of the deuterated stigmasterol and that of the commercial stigmasterol: the specific doublets observed for the two terminal methyls at C-26 and C-27 on the side chain of stigmasterol are replaced by two broad singlets in the deuterated compound because of the replacement of the hydrogen in C-25 position by a deuterium. The third confirmation of the structure is the observation of a triplet at 32.1 ppm ($J = 19$ Hz, C-25) in ^{13}C-NMR. One part of compound **9a** was deprotected by treatment with *p*-toluenesulfonic acid in aqueous dioxane to give the 25-^{2}H-stigmasterol **10a**.[22] The other part of **9a** was submitted to catalytic hydrogenation with palladium on carbon (under control by GC as the i-methyl ether of stigmasterol and sitosterol have very close R_f on TLC) to give the deuterated i-methyl ether of sitosterol **11**, which was transformed to 25-^{2}H-sitosterol **12a**. The absence of ethylenic protons in the ^{1}H NMR spectrum of **11** confirms the total hydrogenation of the Δ^{22} double bond to produce sitosterol.

Scheme 2. Synthesis of 25-^{2}H-Stigmasterol and 25-^{2}H-Sitosterol[a]



[a] (g) (1) n-BuLi, THF, −78 °C; (2) $MeSO_2Cl$, THF, −10 °C; (3) Na_2HPO_4, Na−Hg 6%, methanol/ethyl acetate, 4 °C; (h) H_2, Pd−C, methanol/ethyl acetate; (i) *p*TsOH, dioxane/water, 80 °C. The same scheme was used with the other diastereomer **6b** in order to obtain 25-^{2}H-clionasterol and 25-^{2}H-poriferasterol. The yields were (g) 81%, (h) 60%, (i) 80% for **12b**, and (i) 60% for **10b**.

The other diastereomers in position 24, 25-^{2}H-poriferasterol, **10b**, and 25-^{2}H-clionasterol, **12b**, have been also obtained from the more polar oily diastereomer **4b** (*R*,*R*) by the same procedure as for **10a** and **12a** (yields indicated in the legend of Scheme 2).

The approach presented here has allowed the synthesis of stigmasterol and poriferasterol (respectively sitosterol and clionasterol), deuterium labeled at C-25 in eight steps (respectively nine steps) with an overall yield of about 15%. The configuration at C-24 (the ethyl chain) has been controlled by the use of an optically pure synthon which had been prepared from a chiral sulfoxide in five steps including the separation of diastereomers by medium pressure chromatography.

^{2}H NMR Analysis. Deuterated stigmasterol, sitosterol and their 24-epimers have been incorporated into oriented bilayers. Four samples have been prepared in conditions identical to those of previous studies.[7] Samples were composed of 16 mol % sterol, 10 mol % dimyris-

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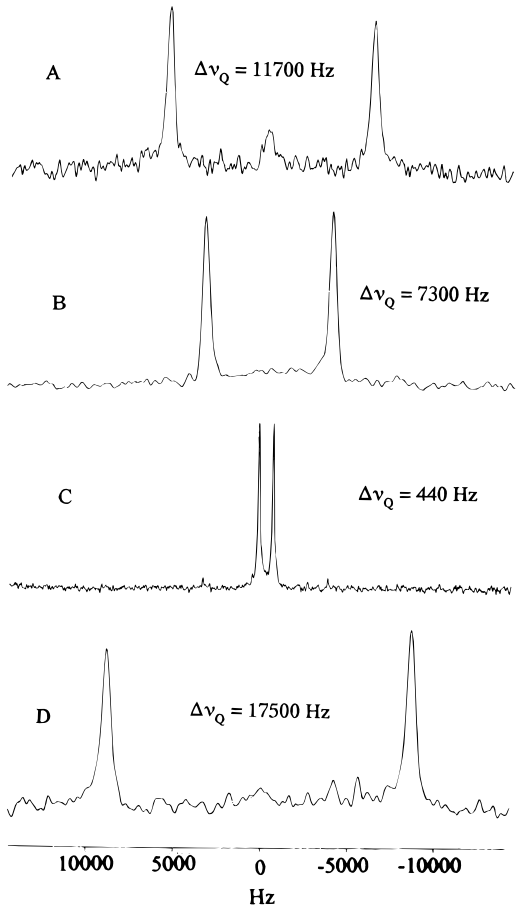

Figure 3. Proton-decoupled deuterium NMR spectra of 90°-oriented multibilayers consisting of 10 mol % DMPC/73 mol % SPC/1 mol% BHT/16 mol % sterol; stigmasterol (A), sitosterol (B), poriferasterol (C), clionasterol (D). $\Delta\nu_Q$, the quadrupolar splitting of the C−D bond, is characteristic of the amplitude of motion and average orientation of this bond. Spectrum C was acquired under proton decoupling in order to resolve the small doublet coupling.

toylphosphatidylcholine (DMPC), 1 mol % butylated hydroxytoluene (BHT) as an antioxidant, and 73 mol % soybean phosphatidylcholine (SPC).

In Figure 3 are shown the ^{2}H NMR spectra recorded for 25-^{2}H-stigmasterol (3A), for 25-^{2}H-sitosterol (3B), for 25-^{2}H-poriferasterol (3C), and for 25-^{2}H-clionasterol (3D). In each case, one doublet is observed, from which the deuterium quadrupolar splitting is measured: 7.3 kHz for sitosterol, 11.7 kHz for stigmasterol, 0.44 kHz for poriferasterol, and 17.4 kHz for clionasterol. This splitting is proportional to the order parameter of the C−D bond: $S_{CD} = \langle 3\cos^2\theta - 1\rangle/2$, where θ is the angle between the C−D bond and the bilayer normal and where $\langle 3\cos^2\theta - 1\rangle$ denotes a time and ensemble average on all the possible orientations of the C−D bond.

These data show immediately that the four sterols behave very differently when they are incorporated into a lipid bilayer. A simple, intuitive, analysis of the data fails to explain why stigmasterol is a less efficient sterol than sitosterol, since one would expect to correlate a good sterol efficiency with a high rigidity of the molecule. The double bond of stigmasterol is expected to increase the side chain mobility of the sterol. Indeed, although the double bond itself is rigid, the dihedral angles on each side of it have much more rotational freedom than in the case of a saturated chain. According to the quadrupole splittings that we observe, the unsaturated side chain appears to be more rigid than the saturated one; however, a smaller quadrupolar splitting may be obtained even with a more rigid molecule, if the average orientation of the CD bond is different. Therefore, an exact interpretation of these data in terms of a model for the molecular motions of the sterols in a lipid bilayer would require more extensive NMR experiments to be performed; in particular, the behavior of the tetracyclic ring system may be analyzed by labeling on ring A and B.[10] T_1 measurements have to be performed in order to extract correlation times for the movements responsible for the averaging of the quadrupolar interaction. Molecular dynamics simulations of the four sterols may indicate preferential orientation of each side chain, the number of accessible conformers, and the amplitude of fluctuation around each of them. These simulations may be performed either in vacuum or in a force field describing the cooperative van der Waals interactions with the surrounding lipids. All these experiments are now being performed and will be published later.

One obvious and unexpected result is that the 24-diastereomers of sitosterol and stigmasterol display an even stronger difference in their side-chain mobility than the natural ones. We had not determined previously the influence of these unnatural epimers on water permeability and on the fatty acid order parameters of lipid bilayers. This needs now to be performed to understand better the specificity of plant sterols−plant lipids interactions.

Experimental Section

Chemicals. General Procedure. All reactions were run under argon. All solvents and reagents used were reagent grade and used as obtained except as noted below. THF was distilled from $LiAlH_4$, dry benzene and dry ethyl acetate were obtained by distillation from CaH_2, CH_2Cl_2 was distilled from P_2O_5, and dry methanol was obtained by distillation from sodium. 2-Nitropropane was purified by distillation from $MgSO_4$, and pyridine from KOH. Dioxane was used without further purification. All the reactions were followed by TLC, on plates of silica gel 60 F_{254} (Merck). "Extractive workup" refers to extraction of the material into the indicated solvent; the organic layer was dried over anhydrous Na_2SO_4, or $MgSO_4$, and the solvent was removed under reduced pressure. Chromatography was performed for all reaction products on silica gel 60 (Merck), 230−400 mesh. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 500 MHz and 125 MHz, respectively; significant chemical shifts are reported in ppm and coupling constants (J values) are given in hertz (Hz).

(+)-(*S*)-(Dimethylphosphoryl)methyl *p*-Tolyl Sulfoxide (2). This compound was prepared from (−)-(*S*)-menthyl *p*-tolyl sulfinate according to the procedure described in the literature.[15]

(*E*)- and (*Z*)-(*R*)-(*p*-tolylsulfinyl)butenes 3. (+)-(*S*)-(Dimethylphosphoryl)methyl *p*-tolyl sulfoxide (3 g , 11.4 mmol) was dissolved in 34 mL of THF and cooled to −78 °C; a solution of *n*-butyllithium (8.1 mL, 13 mmol) in hexane was then added. After 30 min at −78 °C, a solution of propionaldehyde (661 mg, 11.4 mmol) in THF (26 mL) was added to this mixture. Stirring at this temperature was continued for 30 min. After having returned to room temperature, the solution was washed with saturated NH_4Cl (3 × 30 mL) and then purified by chromatography, eluting with ether/cyclohexane (8:2), to give 1.47 g (66%) of a mixture of *E* and *Z* isomers : $[\alpha]^{25}_D = -168$ (*c* 1.88, $CHCl_3$); IR (NaCl) 3069, 2968, 1618, 1454, 1083, 1038, 810 cm^{-1}; ^{1}H NMR (200 MHz) δ 1.04 (t, J = 7.3 Hz, 3H) and 1.11 (t, J = 7.6 Hz, 3H), 2.25 (m, 2H) and 2.58 (m, 2H), 6.19 (m, 2H (Z), 1H (E)), 6.62 (dt, 1H (E)), 7.29 and 7.47 (AB q, $\Delta\nu$ = 36.8 Hz, J = 8.2 Hz, 4H); ^{13}C NMR ($CDCl_3$, 200 MHz) δ 13.5, 21.2, 22.6, 123.8, 129.8, 134.1, 136.1, 140.9, 141.1, 142.2, 143.0.

(*R,S*)- and (*R,R*)-1-(*p*-tolylsulfinyl)-2-ethyl-3-nitro-3-methylbutanes (4a, 4b). DBU (2.7 mL,18 mmol) was added to a solution of (*R*)-*p*-tolyl sulfinyl butene (1.4 g, 7.2 mmol) in 2-nitropropane (3.26 mL, 36 mmol). The mixture was stirred in darkness at room temperature. After 28 h, 40 mL of ether and 20 mL of water were added. The organic phase was washed with 2 N HCl (2 × 40 mL) and then with water (2 × 40 mL). The mixture of the pure diastereomeric nitro sulfoxides, (*R,S*) and (*R,R*), obtained after the chromatography (ether/cyclohexane (8:2)) represented 1.48 g (73%). The separation of the diastereomers by medium pressure chromatography (30 g of Lichroprep silicagel 60 (Merck, 5−10 μm), column pressure, 8 bars) gave 710 mg of the pure (*R,S*) diastereomer (less polar) and 601 mg of the pure (*R,R*) diastereomer (more polar).

The (*R,S*) diastereomer **4a** was crystalline: mp 89−95 °C; $[\alpha]^{25}_D = +255$ (*c* 0.57, $CHCl_3$); IR (NaCl) 3020, 2917, 1537 (ν_{as} NO_2), 1450, 1406, 1345 (ν_{ss} NO_2), 1086, 1038 (ν_s SO), 805 cm^{-1}; ^1H NMR (200 MHz) δ 0.99 (t, $J = 6$ Hz, 3H), 1.48 (m, 1H), 1.55 (s, 3H), 1.58 (m, 1H), 1.62 (s, 3H), 2.43 (s, 3H), 2.51 (m, 1H), 2.55 (m, 1H), 2.90 (dd, $J = 10$ Hz, $J = 2$ Hz, 1H), 7.35 and 7.55 (ABq, $\Delta\nu = 40$ Hz, $J = 8$ Hz, 4H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 13.2, 22.2, 24.1, 24.5, 25.8, 44.3, 61.2, 91.8, 124.7, 130.8, 141.7, 142.7.

The (*R,R*) diastereomer **4b** was oily: $[\alpha]^{25}_D = +106$ (*c* 0.76, $CHCl_3$); IR (NaCl) 3020, 2969, 1537 (ν_{as} NO_2), 1461, 1400, 1347 (ν_{ss} NO_2), 1086, 1044 (ν_s SO), 810 cm^{-1}; ^1H NMR (200 MHz) δ 1.07 (t, $J = 7.3$ Hz, 3H), 1.28 (m, 1H), 1.44 (s, 3 H), 1.50 (m, 1H), 1.58 (s, 3H), 2.38 (m, 1H), 2.43 (s, 3H), 2.79 (dd, $J = 4.3$ Hz, 2H), 7.34 and 7.55 (ABq, $\Delta\nu = 42$ Hz, $J = 8.3$ Hz, 4H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 13.1, 22.1, 24.4, 24.8, 44.5, 60.6, 91.9, 125.0, 130.8, 141.5, 142.9. Anal. Calcd for $C_{14}H_{21}NSO_3$: C, 59.4; H, 7.41; N, 4.94; S, 11.32. Fd: C, 59.58; H, 7.50; N, 4.86; S, 11.59.

(*R,S*)- and (*R,R*)-1-(*p*-tolylsulfinyl)-2-ethyl-3-deuterio-3-methylbutanes (5a, 5b). To a solution of (*R,S*)-**4a** (290 mg, 1.02 mmol) [or 120 mg, 0.42 mmol of (*R,R*)-**4b**] in dry benzene (14 mL) [or 5.5 mL] were added 84.1 mg (0.51 mmol) [or 35 mg, 0.21 mmol] of AIBN and then 1.38 mL (4.9 mmol) [or 534 μL, 2.1 mmol] of tributyltin deuteride. The mixture was heated at 85 °C for 6 h and cooled slowly to room temperature. Chromatography, eluting with ether/cyclohexane (7:3), gave 196 mg (80%) of deuterated (*R,S*) sulfoxide **5a** and 88.2 mg (87%) of deuterated (*R,R*) sulfoxide **5b**, both as a white solids.

(*R,S*) Diastereomer: mp 50.3−51.4 °C; $[\alpha]^{25}_D = +186$ (*c* 0.6, $CHCl_3$); IR (NaCl) 3018, 2917, 1445, 1043, 815; ^1H NMR (200 MHz) δ 0.76 (s, 3H), 0.84 (s, 3H), 0.88 (t, $J = 7.6$ Hz, 3H), 2.40 (s, 3H), 2.63 and 2.77 (AB qd, $\Delta\nu = 28$ Hz, $^2J = 12.9$ Hz, $^3J = 6.2$ Hz), 7.31 and 7.53 (AB q, $\Delta\nu = 44$ Hz, $J = 8.5$ Hz, 4H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.4, 19.1, 19.3, 22.1, 24.0, 29.0 (t, $J = 19.3$Hz), 41.3, 61.5, 125.0, 130.6, 142.3.

(*R,R*) diastereomer: mp 63−65 °C; IR (NaCl) 3020, 2958, 2172 (ν C−D), 2110 (ν C−D), 1461, 1083, 1036 (ν_s SO), 808 $cm^{-1;}$ ^1H NMR (200 MHz) δ 0.78 (s, 3H), 0.86 (s, 3H), 0.9 (t, $J = 7.4$ Hz, 3H), 1.44 (m, 3H), 2.42 (s, 3H), 2.66 and 2.79 (AB qd, $\Delta\nu = 26.1$ Hz, $^2J = 13.1$ Hz, $^3J = 6.2$ Hz, 2H), 7.32 and 7.55 (AB q, $\Delta\nu = 46$ Hz, $J = 8.1$ Hz, 4H).

(*R*)- and (*S*)-1-(*p*-tolylsulfonyl)-2-ethyl-3-deuterio-3-methylbutanes (6a, 6b). A 163 mg (0.68 mmol) amount of (*R,S*)-**5a** [or 82 mg, 0.32 mmol of (*R,R*)-**5b**] was dissolved in dry CH_2Cl_2 (8.2 mL) [or 4.1 mL] and cooled to −15 °C. A solution of *m*-CPBA (470 mg, 2.7 mmol) [or 237 mg, 1.37 mmol] in dry CH_2Cl_2 (41 mL) [or 20 mL] was added under stirring at this temperature. After returning to room temperature, the mixture was heated at 40 °C for 2.5 h, cooled, washed (3 × 20 mL saturated $NaHCO_3$), dried over anhydrous $MgSO_4$, and chromatographed, eluting with ether/cyclohexane (2:8) to give the pure deuterated (*S*) sulfone **6a** (140 mg, 81%) and the (*R*) sulfone **6b** (70.2 mg, 80%) as oils.

(*S*) Sulfone: $[\alpha]^{25}_D = +4$ (*c* 1.27, $CHCl_3$); IR (NaCl) 3020, 2953, 1456, 1301 (ν_{as} SO_2), 1141 (ν_{ss} SO_2), 1082, 815 $cm^{-1;}$ ^1H NMR (200 MHz) δ 0.75 (s, 6H), 0.82 (t, $J = 7.4$ Hz, 3H), 1.25 (m, 1H), 1.42 (m, 2H), 2.46 (s, 3H), 2.86 and 3.04 (AB q, $^2J = 13.6$ Hz, $^3J = 1.3$ Hz, 2H), 7.36 (d, $J = 8.3$ Hz, 2H) and 7.80 (d, $J = 8.4$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.1, 18.6, 19.5, 22.3, 24.0, 28.6 (t, $J = 20$ Hz), 41.1, 58.1, 128.7, 130.5, 137.8, 145.1.

(*R*) sulfone: $[\alpha]^{25}_D = -5$ (*c* 1.21, $CHCl_3$); IR (NaCl) 3020, 2960, 2171 (ν C−D), 2108 (ν C−D), 1463, 1304 (ν_{as} SO_2), 1146 (ν_{ss} SO_2), 1083, 818 cm^{-1}; ^1H NMR (200 MHz) δ 0.76 (s, 6H), 0.82 (t, $J = 7.4$ Hz, 3H), 1.45 (m, 2H), 1.74 (m, 1H), 2.45 (s, 3H), 2.86 and 3.04 (AB qd, $\Delta\nu = 45$ Hz, $^2J = 14.4$ Hz, $^3J = 6.3$ and 4.4 Hz, 2H), 7.35 (d, $J = 7.9$ Hz, 2H) and 7.79 (d, $J = 7.9$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 11.8, 18.3, 22.1, 23.8, 28.5 (t, $J = 20$ Hz), 41.0, 58.0, 128.5, 130.5, 137.3, 145.2. Anal. Calcd for $C_{14}H_{21}DSO_2$: C, 65.89; H, 8.23; S, 12.56. Fd: C, 64.80; H, 8.73; S, 12.12.

(*S*)- and (*R*)-6β-Methoxy-3α,5-cyclo-25-deuteriostigmast-22(*E*)-enes 9a, 9b. The deuterated (*S*) sulfone **6a** (140 mg, 0.56 mmol) [or the deuterated (*R*) sulfone **6b**: 70 mg, 0.29 mmol] was dissolved in 6 mL [or 3.4 mL] of dry THF. A solution of nBuLi in hexane (360 μL, 0.57 mmol) [or 200 μL, 0.31 mmol] was added to the mixture cooled at −78 °C. After 10 min, 190 mg (0.55 mmol) [or 101 mg, 0.29 mmol] of the i-methyl ether aldehyde **7** in 6 mL [or 3.5 mL] of dry THF was added dropwise under stirring. The mixture was kept at −78 °C during 4 h, and then, after returning to room temperature (1 h), methanesulfonyl chloride (51 μL, 0.66 mmol) [or 27 μL, 0.35 mmol] in THF (340 μL) [180 μL] was added at −10 °C. After a reflux at 80 °C for 25 min, THF was removed and the residue was extracted with ether. The organic layer was washed with aqueous saturated $NaHCO_3$, dried, and evaporated to yield an oily product. This mixture of diastereomers **8** (273 mg of (*S*)) [and 202 mg of (*R*)] was dissolved in methanol/ethyl acetate (2:1, 5.9 mL) [or 7.9 mL], and 257 mg (1.82 mmol) [or 191 mg, 1.3 mmol] of Na_2HPO_4 was added. Under stirring at room temperature, then at 4 °C, freshly prepared 6% sodium amalgam (3.1 g) [or 2.4 g] was added and the stirring continued for 5 h. Water was added and the extraction was performed with dichloromethane (6 × 35 mL). Chromatography of the crude product [cyclohexane/ethyl acetate (97:3)] yielded as colorless oils the (*S*) diastereomer **9a** (131 mg, 76%) and the (*R*) diastereomer **9b** [100 mg, 81%].

(*S*) diastereomer **9a**: $[\alpha]^{25}_D = +30$ (*c* 0.71, $CHCl_3$); IR (NaCl) 3050, 2930, 2141 (ν C−D), 1595, 1451, 1376, 1093, 1018 cm^{-1}; ^1H NMR (200 MHz) δ 0.41 (dd, $J = 8.2$ Hz, $J = 5.3$ Hz, 1H), 0.63 (t, $J = 5$ Hz, 1H), 0.71 (s, 3H), 0.77 (s, 3H), 0.79 (t, $J = 7.04$ Hz, 3H), 0.82 (s, 3H), 0.99 (d, $J = 5.3$ Hz, 3H), 2.75 (t, $J = 2.6$ Hz, 1H), 3.31 (s, 3H), 5.0 and 5.12 (AB qd, $\Delta\nu = 24$ Hz, $^2J = 15$ Hz, $^3J = 8.5$ Hz and 7.9 Hz, 2H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.9, 13.1, 13.8, 19.6, 20.0, 21.7, 21.9, 22.2, 23.5, 25.0, 25.7, 26.1, 29.7, 31.2, 32.1 (t, $J = 19.2$ Hz), 34.1, 35.8, 36.0, 40.9, 41.2, 43.4, 44.1, 48.8, 51.8, 56.8, 57.3, 83.1, 130.0, 139.1.

(*R*) diastereomer **9b**: $[\alpha]^{25}_D = +30$ (*c* 1.15, $CHCl_3$); IR (NaCl) 3060, 2932, 2857, 2141 (ν C−D), 1638, 1606, 1451, 1376, 1093, 1024 cm^{-1}; ^1H NMR (200 MHz) δ 0.41 (dd, $J = 7.9$ Hz, $J = 5$ Hz, 1H), 0.63 (t, $J = 4.7$ Hz, 1H), 0.72 (s, 3H), 0.77 (s, 3H), 0.80 (t, $J = 7.3$ Hz, 3H), 0.82 (s, 3H), 1.00 (d, $J = 6.5$ Hz, 3H), 1.01 (s, 3H), 2.75 (t, $J = 3$ Hz, 1H), 3.31 (s, 3H), 5.00 and 5.14 (AB qd, $\Delta\nu = 28$ Hz, $^2J = 15.3$ Hz, $^3J = 8.5$ Hz and 7.9 Hz, 2H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 13.1, 13.8, 19.5, 20.0, 21.5, 21.9, 22.2, 23.5, 24.9, 25.7, 26.1, 29.6, 31.2, 32.0 (t, $J = 20$ Hz), 34.0, 35.8, 36.0, 40.9, 41.2, 43.4, 44.1, 48.8, 51.8, 56.8, 57.3, 83.1, 129.9, 139.0.

25-Deuteriostigmasterol (10a). A solution of **9a** (40 mg, 94 μmol) in dioxane/water (7:3, 2.6 mL) was stirred at 80 °C for 5 h in presence of a catalytic amount of *p*-toluenesulfonic acid. The mixture was poured into aqueous saturated NaCl (10 mL), and the crude product was extracted three times with 10 mL of ether. Column chromatography, eluting with cyclohexane/ethyl acetate (8:2), afforded the pure deuterated stigmasterol **10a** as a white solid (28.5 mg, 75%): mp = 164.2−166 °C; $[\alpha]^{25}_D = -50$ (*c* 0.57, $CHCl_3$); IR (NaCl) 3348, 2942, 2857, 2141 (ν C−D), 1659, 1638, 1446, 1376, 1045 cm^{-1}; ^1H NMR (500 MHz) δ 0.74 (s, 3H), 0.82 (s, 3H), 0.84 (t, $J = 7.4$ Hz, 3H), 0.88 (s, 3H), 1.05 (s, 3H), 1.06 (d, $J = 6.6$ Hz, 3H), 1.74 (m, 1H), 1.88 (m, 2H), 2.27 (m, 1H), 2.32 (m, 1H), 3.57 (m, 1H), 5.06 and 5.19 (AB qd, $\Delta\nu = 65$ Hz, $^2J = 15.2$ Hz, $^3J = 8.7$ Hz and 9.1 Hz, 2H), 5.39 (m, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.7, 12.9, 19.5, 20.1, 21.7, 21.8, 21.9, 25.1, 26.1, 29.6,

32.1 (t, J = 18.9 Hz), 32.4, 32.6, 37.2, 37.9, 40.4, 41.2, 42.9, 43.0, 50.8, 51.8, 56.6, 57.6, 72.5, 122.4, 129.9, 139.0, 141.4.

25-Deuterioporiferasterol (10b). With 35 mg of deuterated i-methyl ether **9b**, 20 mg (60%) of pure deuterated porifera sterol **10b** was obtained: mp = 154.0−155.0 °C; $[\alpha]^{25}_D$ = −42 (*c* 0.50, $CHCl_3$); IR (NaCl) 3362, 2928, 2854, 2135, 1665, 1613, 1449, 1375, 1047, 962 cm^{-1}; ^1H NMR (500 MHz) δ 0.70 (s, 3H), 0.79 (s, 3H), 0.81 (t, J = 7 Hz, 3H), 0.83 (s, 3H), 1.01 (s, 3H), 1.02 (d, J = 6.5 Hz, 3H), 1.99 (m, 1H), 2.05 (m, 1H), 2.23 (m, 1H), 2.30 (m, 1H), 3.53 (m, 1H), 5.02 and 5.16 (AB qd, $\Delta\nu$ = 70 Hz, ^2J = 15 Hz, ^3J = 8.5 Hz and 9.5 Hz, 2H), 5.35 (dd, J = 3 Hz, J = 2.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 11.9, 12.3, 18.7, 19.3, 20.7, 21.0, 21.1, 24.2, 25.3, 28.7, 29.6, 30.8, 31.2 (t, J = 20 Hz), 31.6, 36.4, 37.2, 39.6, 40.3, 42.1, 42.2, 50.1, 51.0, 55.8, 56.8, 71.7, 121.6, 129.2, 138.1, 140.6.

(*S*)- and (*R*)-6β-Methoxy-3α,5-cyclo-25-deuteriositosterol (11a, 11b). To a solution of (*E*)-6-methoxy-3, 5-cyclo-25-deuteriostigmast-22-ene (**9a**) (37 mg, 87 μmol) [or **9b**: 29 mg, 68 μmol] in methanol/ethyl acetate (1:1, 3.8 mL) was added 7.5 mg [or 5.8 mg] of activated Pd−C. The mixture was stirred at room temperature in a hydrogen atmosphere during 2 h. After filtration on Celite and washing with ethyl acetate, the filtrate was evaporated in vacuo. The crude product was chromatographed on silica gel [cyclohexane/ethyl acetate (98:2)] to give 28 mg of **11a** (75%) and 18 mg (60%) of **11b** as pure colorless oils.

(*R*) Diastereomer **11a**: $[\alpha]^{25}_D$ = +50 (*c* 1.4, $CHCl_3$); IR (NaCl) 2934, 2868, 2141, 1633, 1451, 1381, 1098, 1029 cm^{-1}; ^1H NMR (500 MHz) δ 0.43 (dd, J = 8 Hz, J = 5.1 Hz, 1H), 0.64 (t, J = 4.9 Hz, 1H), 0.71 (s, 3H), 0.80 (s, 3H), 0.82 (s, 3H), 0.84 (t, J = 7.4 Hz, 3H), 0.91 (d, J = 6.5 Hz, 3H), 1.02 (s, 3H), 2.77 (t, J = 2.7 Hz, 1H), 3.32 (s, 3H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.7, 13.0, 13.8, 19.5, 19.6, 20.0, 20.4, 22.2, 23.5, 23.8, 24.9, 25.7, 26.8, 29.0, 29.3 (t, J = 18.8 Hz), 31.2, 34.1, 34.6, 35.8, 36.0, 36.9, 41.0, 43.5, 44.1, 46.4, 48.7, 57.0, 57.2, 83.2.

(*S*) Diastereomer **11b**: IR (NaCl) 2933, 2888, 2141, 1643, 1456, 1376, 1093, 1018 cm^{-1}; ^1H NMR (500 MHz) δ 0.44 (dd, J = 8 Hz, J = 5.1 Hz, 1H), 0.66 (t, J = 5.1 Hz, 1H), 0.73 (s, 3H), 0.81 (s, 3H), 0.83 (s, 3H), 0.86 (t, J = 7.4 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H), 1.03 (s, 3H), 2.78 (t, J = 2.8 Hz, 1H), 3.34 (s, 3H).

25-Deuteriositosterol (12a). A catalytic quantity of *p*-toluenesulfonic acid was added to a solution of **11a** (37 mg, 86.2 μmol) in dioxane/water (7:3, 2.6 mL). After stirring at 80 °C during 1.5 h, the mixture was poured into an aqueous saturated NaCl solution (20 mL). The crude product was extracted three times with ether and chromatographed [cyclohexane/ethylacetate (8:2)] to give a white solid (34 mg, 95%): $[\alpha]^{25}_D$ = −30 (*c* 1, $CHCl_3$); mp 131.0−133.4 °C; ^1H NMR (500 MHz) δ 0.72 (s, 3H), 0.84 (s, 3H), 0.87 (s, 3H), 0.88 (t, J = 7.5 Hz, 3H), 0.96 (d, J = 6.5 Hz, 3H), 1.05 (s, 3H), 1.89 (m, 1H), 2.28 (m, 1H), 2.33 (m, 1H), 3.57 (m, 1H), 5.39 (m, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.6, 12.7, 19.5, 19.6, 20.1, 20.4, 21.8, 23.8, 25.0, 26.7, 28.9, 29.3 (t, J = 19.7 Hz), 30.4, 32.4, 32.6, 32.6, 34.6, 36.8, 37.2, 37.9, 45.5, 43.0, 46.4, 50.8, 56.7, 57.5, 72.5, 122.4, 141.5.

25-Deuterioclionasterol 12b. With 17 mg of deuterated methyl ether **11b**, by the above procedure, 13 mg (80%) of pure deuterated clionasterol was obtained: $[\alpha]^{25}_D$ = −42 (*c* 0.56, $CHCl_3$); mp 137.0−138.5 °C; IR (NaCl) 3380, 2932, 2868, 2141, 1643, 1456, 1376, 1104, 1045 cm^{-1}; ^1H NMR (500 MHz) δ 0.72 (s, 3H), 0.84 (s, 3H), 0.86 (s, 3H), 0.89 (t, J = 7.5 Hz, 3H), 0.96 (d, J = 6.5 Hz, 3H), 1.05 (s, 3H), 1.88 (m; 1H), 2.27 (m, 1H), 2.33 (m, 1H), 3.56 (m, 1H), 5.39 (m, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.5, 13.0, 19.5, 20.0, 20.2, 21.8, 23.7, 25.0, 27.0, 28.9, 30.4, 32.4, 32.6, 32.6, 34.6, 37.0, 37.2, 37.9, 40.5, 43.0, 46.6, 50.8, 54.1, 56.7, 57.4, 72.5, 122.4, 141.4.

^2H NMR Experiments. Lipid mixtures were prepared with one of the sterols (2 mg, 16 mol %), DMPC (2 mg, 10 mol %) (Sygena, Inc., Liestal), SPC (16 mg, 73 mol %) (Sigma), and 1 mol % BHT (Sigma) in 1.4 mL of 2-propanol and deposited onto 40−50 circular coverslips (diameter 4.5 mm, Polylabo). The plates were placed under vacuum overnight and stacked into tubes (diameter 7 mm). The lipids were hydrated with ^2H-depleted water (Aldrich) at 37 °C overnight, and then 18 μL of this water was added to the tube. The stacks of bilayers thus obtained were oriented with their normal at 90° with respect to the magnetic field.

Proton decoupled ^2H-NMR spectra were acquired at 30 °C in a 7 mm coil doubly tuned for deuterium (76.77 MHz) and proton with a Bruker AMX 500 spectrometer equiped for solid state NMR. Quadrupolar echos[8] were obtained by using two 90° pulses of 3.5 μs, width separated by 30 μs, the sweepwidth was 250 kHz, proton decoupling was performed during acquisition (decoupling power 15 W), the repetition rate was 1 s to allow for thermal dissipation of the decouping power, and the acquisition time was 4 ms.

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