Interaction of Cholesterol with Sphingomyelin in Monolayers and Vesicles†

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Received June 20, 1994®

ABSTRACT: To understand the structural basis for the apparent strong interaction between cholesterol and sphingomyelin (SPM), we have synthesized an analogue of SPM, 3-deoxy-2-*O*-stearoyl-SPM, in which an ester-linked acyl chain replaces the amide-linked acyl chain at C-2 and a hydrogen replaces the hydroxy group at C-3. We have compared the behavior of this analogue with that of 3-deoxy-*N*-stearoyl-SPM in monolayers and vesicles, both as pure phospholipids and in mixtures with cholesterol. The force–area isotherm of 3-deoxy-2-*O*-stearoyl-SPM was similar to that of 3-deoxy-*N*-stearoyl-SPM. The surface potential across the pure SPM monolayer at the air–water interface was larger for 3-deoxy-2-*O*-stearoyl-SPM than for 3-deoxy-*N*-stearoyl-SPM (about 430 mV and 330 mV, respectively, at 50 Å^2). The overall dipole moment of 3-deoxy-2-*O*-stearoyl-SPM was almost constant at 570 mD (between a mean molecular area range of 45–85 Å^2), whereas that of 3-deoxy-*N*-stearoyl-SPM was about 420 mD. Cholesterol appeared to be equally miscible in both SPM monolayers, as determined from the condensing effect cholesterol had on the lateral packing of the two SPMs. The oxidation of monolayer cholesterol by cholesterol oxidase was also determined using both SPMs. The stoichiometry at which free cholesterol clusters disappeared in monolayers, when going from high to low cholesterol content, was 2:1 (mol sterol/mol SPM) for both SPMs. Cholesterol was oxidized faster in 3-deoxy-2-*O*-stearoyl-SPM mixed monolayers than in 3-deoxy-*N*-stearoyl-SPM mixed monolayers; a 5.4-fold difference in rate of oxidation susceptibility was found at an equimolar ratio of SPM to sterol at 25 °C. The rate and extent of enzyme-catalyzed oxidation of cholesterol incorporated into small unilamellar vesicles (SUVs) formed from 3-deoxy-2-*O*-stearoyl-SPM were higher than in SUVs prepared with 3-deoxy-*N*-stearoyl-SPM by factors of 4.8 and 2.6, respectively, at 30 °C. The results of this study indicate that the NH group of SPM is important in allowing a tight interaction between SPM and cholesterol in monolayers and SUVs; however, the amide linkage is not critical for the 2:1 stoichiometry typical of cholesterol oxidation in cholesterol/SPM monolayers.

Surface pressure–molecular area isotherms of monolayers show that the lateral packing density of membranes increases when sphingomyelin (SPM)[1] is incorporated, and that SPM molecules are more closely packed on addition of cholesterol at a given surface pressure compared with phosphatidylcholine molecules of similar acyl-chain saturation and length (Lund-Katz et al., 1988; Kan et al., 1991). Differences in van der Waals attractive forces (Lund-Katz et al., 1988) or in hydrogen bonding (Schmidt et al., 1977; Barenholz et al., 1980; Sankaram & Thompson, 1990) have been proposed to explain the higher lateral cohesion of SPM-containing membranes. SPM contains a long-chain *N*-acyl group at the 2 position of the sphingosine backbone, a hydroxy group at the 3 position, and a *trans* double bond between C-4 and C-5 (Figure 1). In contrast, phosphatidylcholine contains long chains at the *sn*-1 and *sn*-2 positions but no hydroxy or NH groups. Thus, SPM

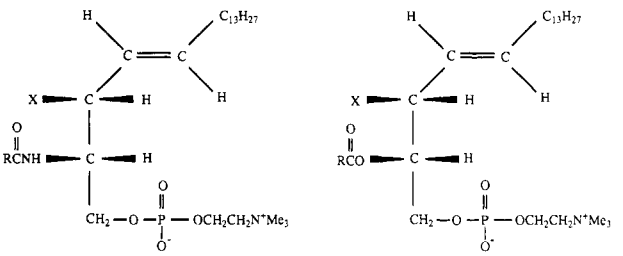

FIGURE 1: Structures of 3-deoxy-2-*N*-stearoyl-SPM, 3-deoxy-2-*O*-stearoyl-SPM, and *N*-stearoyl-SPM.

contains both hydrogen-bond donor and acceptor sites, whereas phosphatidylcholine has only hydrogen-bond acceptor sites. Recent studies with a series of synthetic SPM derivatives showed that the hydroxy group of SPM is not a major stabilizing force in SPM–cholesterol interactions in vesicles and monolayers (Kan et al., 1991; Grönberg et al., 1991). Thus van der Waals forces were considered to be more important in explaining the apparent strong SPM–cholesterol interactions than hydrogen-bonding interactions involving the hydroxy group of SPM (Kan et al., 1991; Grönberg et al., 1991). However, the observation that the molecular areas of SPM derivatives were not directly correlated with the rates of intermembrane cholesterol exchange between SPM-containing vesicles implied that van der Waals interactions are not solely responsible for the high lateral cohesion energy

† This work was supported by NIH Grant HL 16660 and by grants from the Sigrid Jusèlius Foundation, the Borg Foundation, and the Academy of Finland. FAB–mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility, which is supported in part by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for Research Resources, N.I.H.

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® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: DCC, *N*,*N*-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetyl; OTs, tosylate; SPM, sphingomyelin; THF, tetrahydrofuran; Tr, trityl (Ph_3C-).

0006-2960/94/0433-11776$04.50/0

in SPM–cholesterol membranes (Kan et al., 1991). In an effort to obtain additional information about the molecular details of the SPM–cholesterol interactions in membranes, we have synthesized an analogue of SPM in which the long-chain amide group is replaced by a long-chain ester group. This analogue, 3-deoxy-2-*O*-stearoyl-SPM, has been used to examine the role of the NH group of SPM in SPM–cholesterol interactions in monolayers.

MATERIALS AND METHODS

Materials. AD-mix-β, allyl bromide, (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*R*)-(−)-MTPA chloride], dimethylboron bromide, DMAP, DCC, and *n*-butyllithium were obtained from Aldrich Chemical Co. 1-Pentadecyne was purchased from Lancaster Synthesis, Inc., and $LiAlH_4$ was obtained from Sigma Chemical Co. 3-Deoxy-DL-*N*-stearoyl-SPM was synthesized as described previously (Kan et al., 1991). Solvents were dried as follows. THF was refluxed over sodium/benzophenone ketyl for several hours, distilled, and then used immediately. Chloroform and dichloromethane were distilled from calcium hydride and stored over type 3A molecular sieves. Phosphorus oxychloride was distilled before use. Pyridine was distilled over barium oxide. Choline *p*-toluenesulfonate was prepared as described previously and dried under vacuum over P_2O_5 (Rosenthal, 1966). The water used in the monolayer subphase was purified by reverse osmosis and MilliQ UF Plus, to a resistivity better than 15 MΩ/cm.

Silica gel G TLC plates of 0.25-mm thickness (Analtech, Newark, DE) were used to monitor reactions, with visualization of the spots by short wavelength UV light or by charring with 10% H_2SO_4 in ethanol. Column chromatography was carried out with E. Merck silica gel 60 (230–400 ASTM mesh). ^1H NMR spectra were recorded using a 200-MHz IBM Bruker spectrometer, unless noted otherwise, and chemical shifts are given in parts per million downfield from tetramethylsilane as internal standard. Infrared spectra were recorded on a Perkin-Elmer FT-IR-1600 spectrophotometer. Mass spectra were recorded on a HP-5988 GC-mass spectrometer, and FAB mass spectra were recorded at the Michigan State University mass spectrometry facility with *m*-nitrobenzyl alcohol as a matrix. Optical rotations were measured on a JASCO DIP-140 polarimeter using a 1-dm cell of 2-mL capacity. Melting points are uncorrected.

Monolayer Studies. Surface pressure versus mean molecular area isotherms of pure SPM or binary cholesterol/SPM monolayers were obtained with a KSV 3000 surface barostat (KSV Instruments, Helsinki, Finland), as described previously (Slotte, 1992a). Surface potentials across the water–lipid interface were recorded with a KSV vibrating plate surface potential, as described previously (Slotte, 1992b). The overall dipole moment, $\mu_\perp$, was calculated according to

$$\mu_\perp = A\Delta V/12\pi \quad (1)$$

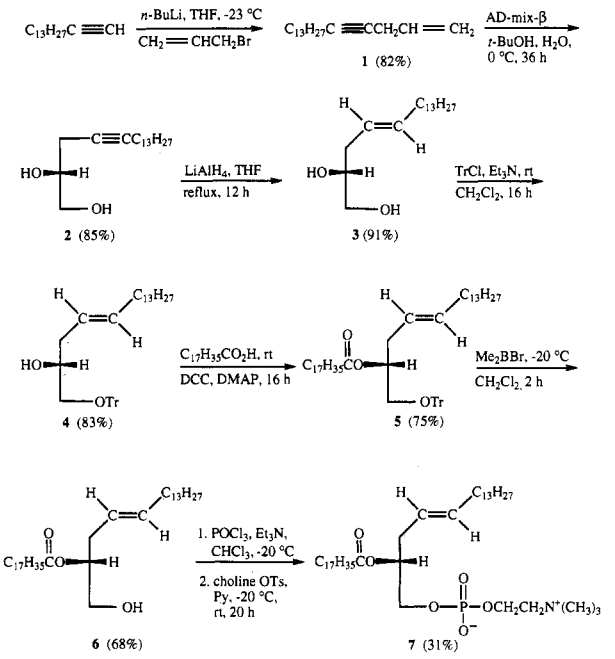
where A is the mean molecular area in Å^2, and ΔV is the potential in mV (Gaines, 1966). The compressibility, k, was calculated from

$$k = -1/A(d\pi/dA) \quad (2)$$

where A is the mean molecular area in Å^2, and π is the surface pressure in mN/m (Ali et al., 1991).

The oxidation of monolayer cholesterol by cholesterol oxidase was performed at 30 °C, as described previously (Slotte, 1992a).

Scheme 1. Synthesis of 3-deoxy-2-*O*-stearoyl-SPM (7)



Assay of Cholesterol Oxidation in Vesicles. SUVs were prepared by ethanol injection of an equimolar cholesterol/phospholipid mixture into phosphate-buffered saline at 25 °C. The final concentration of cholesterol in the vesicles was 10 μM. The vesicles were incubated at 30 °C for 30 min prior to exposure to cholesterol oxidase. The rate and extent of oxidation were estimated by fluorescence as described elsewhere (Slotte, 1992a; Slotte et al., 1994).

Methods of Synthesis. Scheme 1 shows the synthetic route to the 3-deoxy-2-*O*-stearoyl-SPM analogue (**7**).[2] Alkylation of the lithium salt of 1-pentadecyne with allyl bromide gave octadec-1-en-4-yne (**1**) in 82% yield after purification by silica gel chromatography (elution with hexane). Since asymmetric dihydroxylation of terminal olefins using AD-mix-α or -β is known to proceed with good enantioselectivity (Sharpless et al., 1992), we used commercially available AD-mix-β in 1:1 *tert*-butyl alcohol/water at 0 °C to achieve the hydroxylation of the terminal olefinic linkage of enyne **1**. After purification by column chromatography and two recrystallizations from hexane, chiral yne-diol **2** was obtained as a white crystalline solid in 85% yield. An enantiomeric excess of 64% was achieved, as estimated by ^1H NMR analysis of the derived bis-MTPA ester; this corresponds to a ratio of *R*/*S* of 82/18 at the chiral carbon of compounds **2**–**7**. Reduction of the propargylic alcohol with lithium aluminum hydride gave enediol **3**, which was protected at the primary hydroxy group as a *O*-trityl ether. Acylation with stearic acid in the presence of DMAP and DCC gave ester **5**, which was detritylated using conditions that avoid significant acyl migration, i.e., dimethylboron bromide at −20 °C. Alcohol **6** was purified by crystallization in hexane at 0 °C. The phosphocholine head group was introduced using a standard procedure, i.e., phosphorus oxychloride in the presence of triethylamine at −20 °C followed by choline tosylate in pyridine (Witzke & Bittman, 1986). The ester analogue of 3-deoxy-SPM (**7**) was obtained after purification by chromatography on Amberlite

[2] The 3-deoxy-2-*O*-stearoyl-SPM analogue was synthesized instead of 3-hydroxy-2-*O*-stearoyl-SPM because the latter could spontaneously undergo acyl migration, giving a mixture of 3-hydroxy-2-*O*-stearoyl-SPM and 2-hydroxy-3-*O*-stearoyl-SPM.

(elution with THF/water) and on silica gel (elution with chloroform, chloroform/methanol, and methanol) and lyophilization from benzene. The detailed procedures of the steps illustrated in Scheme 1 are presented below.

Octadec-1-en-4-yne (1). To a solution of 1.04 g (5.0 mmol) of 1-pentadecyne in 20 mL of dry THF at −23 °C was added *n*-butyllithium (2.1 mL of a 2.5 M solution in hexane). After the mixture was stirred for 30 min at −23 °C, a solution of 2.4 g (19.8 mmol) of allyl bromide in 10 mL of THF was added over a 10-min period. The mixture was stirred for 6 h at −23 °C and then left overnight at 0–5 °C. The reaction mixture was adjusted to pH 2 with dilute HCl and extracted with ether (30 mL × 2). The organic layer was washed with water (20 mL), dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (elution with hexane) to yield 1.02 g (82%) of octadec-1-en-4-yne (**1**) as an oil. TLC: R_f 0.66 (hexane); IR ($CHCl_3$, cm^{-1}): 2919, 2848, 1642, 1466, 984, 914, 720; ^1H NMR ($CDCl_3$) δ: 0.88 (t, 3H, J = 6.1 Hz, $CH_3(CH_2)_{11}$), 1.26 (m, 22H, $(CH_2)_{11}$), 2.14–2.21 (m, 2H, $C_{12}H_{25}CH_2C{\equiv}C$), 2.93 (t, 2H, J = 2.8 Hz, $C{\equiv}CCH_2CH{=}$), 5.05–5.11 (dd, 1H, J = 1.5 Hz and 10.2 Hz, $CH{=}CH_AH_B$), 5.22–5.35 (dd, 1H, J = 1.6 Hz and 16.7 Hz, $CH{=}CH_AH_B$), 5.72–5.91 (m, 1H, $CH_2CH{=}CH_2$).

(2R)-Octadec-4-yne-1,2-diol (2). A mixture of 2.8 g of AD-mix-β, 10 mL of *tert*-butyl alcohol, and 10 mL of water was stirred at room temperature to produce two clear layers. When the mixture was cooled to 0 °C, some of the dissolved salts precipitated. Octadec-1-en-4-yne (400 mg, 1.6 mmol) was added at once at 0 °C, and the reaction mixture was stirred vigorously for 36 h at 0 °C. Sodium sulfite (3.0 g) was added, and the mixture was allowed to warm to room temperature and stirred for 30 min. Ethyl acetate (30 mL) was added, and the mixture was stirred for 10 min. The aqueous layer was extracted with ethyl acetate (30 mL × 2), and the organic layer was washed with 20 mL of aqueous saturated NaCl solution. The organic phase was dried over sodium sulfate, filtered, and concentrated under vacuum to give a residue that was purified by column chromatography (elution with hexane:ethyl acetate 8:2), yielding **2** as a white solid. Crystallization in hexane (two times) gave 380 mg (85%) of octadec-4-yne-1,2-diol (**2**) as white crystalline flakes; mp 58–60 °C. TLC: R_f 0.49 (hexane: ethyl acetate 8:2); $[\alpha]_D^{23}$ −3.5° (*c* 5.6, $CHCl_3$); IR ($CHCl_3$, cm^{-1}): 3448–3083, 2919, 2837, 1466, 1325, 1260, 1172, 1043, 896, 720; ^1H NMR ($CDCl_3$) δ: 0.88 (t, 3H, J = 6.4 Hz, $CH_3(CH_2)_{11}$), 1.26 (m, 22H, $(CH_2)_{11}$), 2.10–2.19 (m, 4H, $C_{12}H_{25}CH_2C{\equiv}C$), $CHOH$-CH_2OH), 2.38–2.43 (m, 2H, $C{\equiv}CCH_2CHOH$), 3.53–3.62 (dd, 1H, J = 6.4 Hz and 11.5 Hz, $CHOHCH_AH_BOH$), 3.70–3.77 (dd, 1H, J = 3.4 Hz and 11.8 Hz, $CHOHCH_AH_BOH$), 3.80–3.88 (m, 1H, $CHOHCH_2OH$). ^1H NMR ($CDCl_3$) after D_2O exchange) δ: 0.85–0.90 (m, 3H, J = 5.5 Hz, CH_3-$(CH_2)_{11}$), 1.26 (m, 22H, $(CH_2)_{11}$), 2.12–2.15 (m, 2H, $C_{12}H_{25}CH_2C{\equiv}C$), 2.38–2.41 (m, 2H, $C{\equiv}CCH_2CHOH$), 3.52–3.70 (m, 2H, $CHOHCH_2OH$), 3.70–3.77 (m, 1H, $CHOH$). MS, *m/z* 282 (M^+).

Bis-MTPA ester of 2. To a solution of 10 mg (35 μmol) of **2** and 9.5 mg (78 μmol) of DMAP in 1 mL of methylene chloride was added 24 mg (88 μmol) of (*R*)-(−)-MTPA chloride. The mixture was stirred overnight at room temperature, the solvent was removed under reduced pressure, and the residue was suspended in ether. The suspension was filtered through a pad of silica gel, and the filtrate was concentrated to give 6 mg of the crude bis-MTPA ester of **2** as a colorless oil. ^1H NMR ($CDCl_3$) δ: 0.87 (t, 3H, J = 6.2 Hz, ω-CH_3), 1.25–1.44 (m, 22H, $(CH_2)_{11}$), 2.14 (t, 2H, J = 6.6 Hz, $C_{12}H_{25}CH_2C{\equiv}C$), 2.38–2.42 (m, 2H, $C{\equiv}CCH_2$), 3.42 (s, 3H, OCH_3), 3.49 (s, 3H, OCH_3), 4.39–4.45 (ABq, 1H, J = 5.0 Hz and 12.0 Hz, $CHOCH_2O$), 4.57–4.63 (ABq, 1H, J = 2.9 Hz and 12.3 Hz, $CHOCH_2O$), 5.35–5.39 (m, 1H, CH_2CHCH_2), 7.28–7.53 (m, 10H, Ph). The enantiomeric excess was calculated to be 64% by ^1H NMR (400 MHz) from the integrated areas of the signals between δ 4.58 and 4.74.

(2R,4E)-Octadec-4-ene-1,2-diol (3). To a solution of 300 mg (1.06 mmol) of octadec-4-yne-1,2-diol (**2**) in 20 mL of dry THF was added 400 mg (10.5 mmol) of $LiAlH_4$ in 20 mL of dry THF with stirring, and the reaction mixture was refluxed for 12 h under nitrogen atmosphere. The reaction was cooled in an ice bath, and water was added slowly to decompose the excess $LiAlH_4$. The mixture was acidified with cold 6 N HCl to adjust the pH to 2, and then the product was extracted with ethyl acetate (20 mL × 2). The organic phase was washed with water (10 mL), dried over sodium sulfate, and the solvent was removed under reduced pressure. Purification by column chromatography (elution with hexane: ethyl acetate 8:2) gave 275 mg (91%) of **3** as a white crystalline solid; mp 52–53 °C. TLC: R_f 0.78 (ethyl acetate); $[\alpha]_D^{23}$ −0.55° (*c* 11.6, $CHCl_3$); IR ($CHCl_3$, cm^{-1}): 3532–3083, 2907, 2837, 1454, 1084, 1031, 961, 714; ^1H NMR ($CDCl_3$) δ: 0.88 (t, 3H, J = 6.2 Hz, $CH_3(CH_2)_{11}$), 1.25 (m, 22H, $(CH_2)_{11}$), 2.00 (t, 2H, J = 6.5 Hz, $C_{12}H_{25}CH_2CH{=}CH$), 2.08 (s, 2H, $CHOHCH_2OH$), 2.14–2.21 (q, 2H, J = 4.8 Hz, $CH{=}CHCH_2CHOH$), 3.42–3.51 (dd, 1H, J = 6.9 Hz and 10.9 Hz, $CHOHCH_AH_BOH$), 3.63–3.77 (m, 2H, $CH_2CHOHCHHOH$), 5.31–5.63 (m, 2H, J = 14.3 Hz, $CH{=}CH$). MS, *m/z* 284 (M^+).

1-O-Trityl-(2R)-2-hydroxy-(4E)-octadec-4-ene (4). To a solution of 230 mg (0.81 mmol) of (4*E*)-octadec-4-ene-1,2-diol (**3**) in 20 mL of dry methylene chloride were added 245 mg (0.88 mmol) of trityl chloride and 89 mg (0.88 mmol) of triethylamine. The reaction mixture was stirred for 16 h at room temperature. The mixture was washed with water (10 mL × 2), the organic phase was dried over sodium sulfate, and the solvent was removed under reduced pressure. The residue was purified on column chromatography (elution with hexane) to yield 355 mg (83%) of 1-*O*-trityl-2-hydroxy-(4*E*)-octadec-4-ene (**4**) as an oil. TLC: R_f 0.32 (hexane: ethyl acetate 9:1); $[\alpha]_D^{23}$ −0.96° (*c* 16.0, $CHCl_3$); IR ($CHCl_3$, cm^{-1}): 3448, 2919, 2848, 1545, 1495, 1448, 1219, 1072, 973, 761, 741, 702; ^1H NMR ($CDCl_3$) δ: 0.87 (t, 3H, J = 6.1 Hz, $CH_3(CH_2)_{11}$), 1.25 (m, 22H, $(CH_2)_{11}$), 1.91–1.94 (m, 2H, $C_{12}H_{25}CH_2CH{=}CH$), 2.13–2.24 (m, 3H, $CH{=}CHCH_2$-$CHOH$), 2.88 (b, H_2O), 2.97–3.14 (m, 2H, $CHOHCH_2OTr$), 3.70–3.81 (m, 1H, $CHOH$), 5.18–5.51 (m, 2H, J = 13.0 Hz, $CH{=}CH$), 7.13–7.37 (m, 15H, Ph).

1-O-Trityl-(2R)-2-O-stearoyl-(4E)-octadec-4-ene (5). To a solution of 269 mg (0.95 mmol) of stearic acid, 116 mg (0.95 mmol) of DMAP, and 195 mg (0.94 mmol) of DCC in 10 mL of alcohol-free chloroform was added a solution of 327 mg (0.63 mmol) of 1-*O*-trityl-2-hydroxy-(4*E*)-octadec-4-ene (**4**) in 2 mL of alcohol-free chloroform. After the reaction mixture was stirred for 16 h at room temperature, the solvent was removed under reduced pressure, leaving a residue that was purified by flash chromatography (elution with hexane), giving 375 mg (75%) of 1-*O*-trityl-2-*O*-stearoyl-(4*E*)-octadec-4-ene (**5**) as a colorless oil. TLC: R_f 0.88 (hexane: ethyl acetate 9:1); $[\alpha]_D^{23}$ +2.5° (*c* 17.5, $CHCl_3$); IR ($CHCl_3$, cm^{-1}): 2919, 2848, 1736, 1595, 1448, 1172, 1084, 967, 761, 702; ^1H NMR ($CDCl_3$) δ: 0.87 (t, 6H, J = 6.2 Hz, $CH_3(CH_2)_{11}$), $CH_3(CH_2)_{16}$), 1.25 (m, 50H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.54–1.64

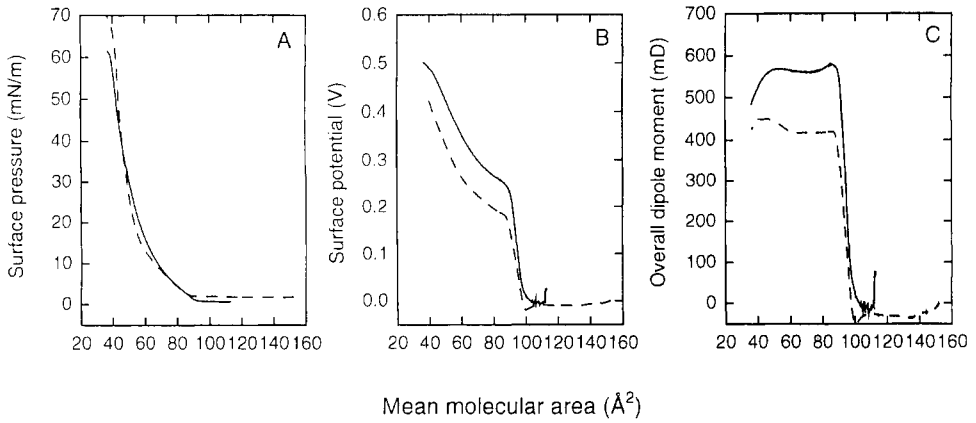


FIGURE 2: Surface pressure and surface potential isotherms of SPMs in monolayers. Pure 3-deoxy-2-*O*-stearoyl-SPM or 3-deoxy-*N*-stearoyl-SPM were spread on the clean surface of pure water at 22 °C. After the film was allowed to stand for 5 min, it was compressed at a speed not exceeding 6 Å² molecule⁻¹ min⁻¹. The force–area isotherms of 3-deoxy-2-*O*-stearoyl-SPM (—) and 3-deoxy-*N*-stearoyl-SPM (- - -) are shown in panel A, the surface potential isotherm in panel B, and the calculated overall dipole moment, $\mu_\perp$, in panel C.

(m, 2H, $COCH_2CH_2$), 1.85–1.88 (m, 2H, $C_{12}H_{25}CH_2$-CH=CH), 2.24–2.36 (m, 4H, $COCH_2$, CH=CHCH_2CH), 3.04–3.18 (m, 2H, $CHCH_2OTr$), 5.03–5.08 (m, 1H, CH_2CH-(OCOR)CH_2), 5.12–5.47 (m, 2H, J = 14.0 Hz, CH=CH), 7.18–7.46 (m, 15H, Ph).

1-Hydroxy-(2R)-2-O-stearoyl-(4E)-octadec-4-ene (6). To a solution of 300 mg (0.37 mmol) of 1-*O*-trityl-2-*O*-stearoyl-(4*E*)-octadec-4-ene (**5**) in 10 mL of dry methylene chloride was added 137 mg (1.13 mmol) of dimethylboron bromide in 5 mL of methylene chloride at −20 °C under nitrogen atmosphere. After 2 h of stirring at −20 °C the reaction was complete, based on the disappearance of starting material (R_f 0.88; hexane–ethyl acetate, 9:1) on TLC. After the reaction mixture was quenched by adding 2 mL of saturated aqueous sodium bicarbonate solution, the mixture was extracted with ether (20 mL × 2). The organic layer was washed with 10 mL of water and dried over sodium sulfate, and the solvent was evaporated under reduced pressure. The residue was dissolved in 3 mL of hexane and kept at 0 °C. Trityl bromide was removed by decantation; this process was repeated two times to remove traces of trityl ether **5**. (Column chromatography was avoided to prevent acyl migration.) The solvent was evaporated to give 140 mg (68%) of 1-hydroxy-2-*O*-stearoyl-(4*E*)-octadec-4-ene (**6**) as a white solid; mp 48–50 °C. TLC: R_f 0.24 (hexane: ethyl acetate 9:1); IR ($CHCl_3$, cm⁻¹): 3636–3142, 2907, 2837, 1725, 1460, 1372, 1049, 961, 755; ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 6H, J = 6.0 Hz, CH_3-$(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.25 (m, 50H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.61 (m, 2H, CH_2CH_2CO), 1.81 (s, 1H, CH_2OH), 1.95–1.99 (m, 2H, $C_{12}H_{25}CH_2CH$=CH), 2.29–2.35 (m, 4H, CH_2CO, CH=CHCH_2CH), 3.58–3.75 (m, 2H, CH(OCOR)CH_2OH), 4.88–4.93 (m, 1H, CH_2CH(OCOR)CH_2), 5.25–5.59 (m, 2H, CH=CH).

1-O-Phosphocholine-(2R)-2-O-stearoyl-(4E)-octadec-4-ene (7). To a solution of 38 mg (23 μL, 25 μmol) of phosphorus oxychloride and 26 mg (35 μL, 25 μmol) of triethylamine in 1.5 mL of alcohol-free chloroform at −20 °C under nitrogen atmosphere was added a solution of 110 mg (200 μmol) of **6** in 1 mL of chloroform over a 10-min period. The reaction mixture was stirred for 45 min at −20 °C. Dry choline tosylate (83 mg, 30 μmol) and pyridine (138 μL) were added at −20 °C, and the mixture was allowed to warm to room temperature. After the reaction mixture was stirred for 20 h, 150 μL of water was added and the mixture was stirred for 30 min. The solvents were removed, and the residue was dissolved in 10 mL of THF–H_2O (9:1) and passed through an Amberlite MB-3 column (elution with THF–H_2O, 9:1).

Table 1: Compressibility of Sphingomyelin Analogues at 30 °C[a]

surface pressure (mN/m)	3-deoxy-*O*-stearoyl-SPM k (m/mN)	3-deoxy-*N*-stearoyl-SPM k (m/mN)
5	0.035 ± 0.002	0.040 ± 0.003
10	0.023 ± 0.002	0.034 ± 0.001
15	0.018 ± 0.001	0.020 ± 0.001
20	0.015 ± 0.001	0.012 ± 0.001
30	0.011 ± 0.0005	0.0075 ± 0.0003

[a] The data represent the average (±SD) of at least three experiments at each pressure.

After removal of the solvents, the residue was purified by column chromatography (elution with chloroform, 1:1 chloroform–methanol, 1:1 and then methanol). The material that eluted with methanol was concentrated, and the residue was dissolved in 20 mL of chloroform and passed through a Metricel filter three times. The filtrate was concentrated, and the residue was lyophilized from benzene (1 mL), yielding 45 mg (31%) of 1-*O*-phosphocholine-2-*O*-stearoyl-(4*E*)-octadec-4-ene (**7**) as a white solid. TLC: R_f 0.45 ($CHCl_3$: MeOH: H_2O 65: 35: 6); $[\alpha]_D^{23}$ −4.45° (*c* 1.2, $CHCl_3$); IR ($CHCl_3$, cm⁻¹): 2907, 2837, 1725, 1460, 1243, 1084, 1055, 961, 756, 720; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 6H, J = 6.3 Hz, CH_3-$(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.25 (m, 50H, $(CH_2)_{11}$), $(CH_2)_{14}$), 1.57–1.68 (m, 2H, CH_2CH_2CO), 1.94–1.96 (m, 2H, $C_{12}H_{25}$-CH_2CH=CH), 2.23–2.30 (m, 4H, CH_2CO, CH=CHCH_2-CH), 3.37 (s, 9H, $N(CH_3)_3$), 3.88 (m, 4H, $CH_2OP(O)(O^-)$, $CH_2N^+(CH_3)_3$), 4.20–4.33 (m, 2H, $P(O)(O^-)OCH_2CH_2$), 5.01 (m, 1H, CH_2CH(OCOR)CH_2), 5.25–5.51 (m, 2H, CH=CH). FAB-MS: 716.7 (M^+); calcd M^+ 716.07.

RESULTS

Characteristics of Pure SPM Monolayers. The force–area isotherms of pure 3-deoxy-2-*O*-stearoyl-SPM and 3-deoxy-*N*-stearoyl-SPM at the water/air interface are similar (Figure 2A). This pattern is also obvious from the compressibility data presented in Table 1, in which the compressibility of the two SPMs is compared as a function of surface pressure.

The surface potential properties of 3-deoxy-2-*O*-stearoyl-SPM and 3-deoxy-*N*-stearoyl-SPM monolayers are given in Figure 2 (panels B and C). The shape of the surface potential curve (Figure 2B) and the overall dipole moment (Figure 2C) of both 3-deoxy-2-*O*-stearoyl-SPM and 3-deoxy-*N*-stearoyl-SPM indicate that the overall dipole orientation of the molecules did not change during compression from about 85 Å² to about 50 Å². The overall dipole of 3-deoxy-*N*-stearoyl-

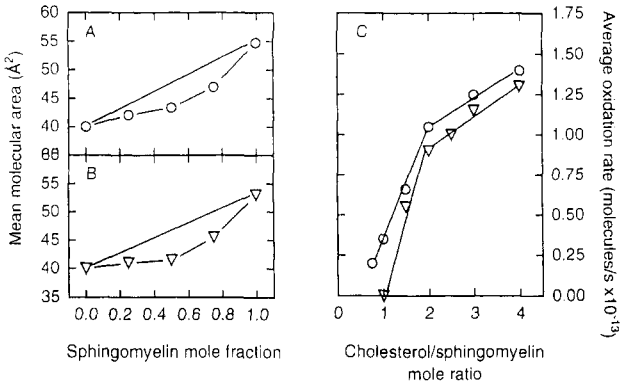


FIGURE 3: Binary mixed monolayers of SPMs and cholesterol. Force–area isotherms of pure or mixed cholesterol/SPM monolayers were obtained as described under Materials and Methods, to give the mean molecular areas at a surface pressure of 15 mN/m. The condensation plot for (A) 3-deoxy-2-*O*-stearoyl-SPM and (B) 3-deoxy-*N*-stearoyl-SPM. (C) Mixed monolayers of cholesterol and SPMs with variable molar ratios of sterol-to-SPM were exposed to cholesterol oxidase in the subphase at 30 °C of 3-deoxy-2-*O*-stearoyl-SPM (○) and 3-deoxy-*N*-stearoyl-SPM (▽) monolayers. The rate of cholesterol oxidation was calculated as described previously (Slotte, 1992a).

SPM was about 150 mD smaller than the corresponding value for the ester derivative (Figure 2C).

Characteristics of Mixed Cholesterol/SPM Monolayers. To test the effects of cholesterol on monolayers from the two SPM analogues, we determined both the condensation effect of cholesterol (De Bernard, 1958; Chapman et al., 1969) and the accessibility of cholesterol to oxidation by cholesterol oxidase at the interface (Paltauf et al., 1971; Grönberg et al., 1991; Slotte, 1992a,b). Cholesterol was able to condense the lateral packing density of 3-deoxy-2-*O*-stearoyl-SPM and 3-deoxy-*N*-stearoyl-SPM to an equal extent (Figure 3A,B).

Previous studies showed that the accessibility of cholesterol in mixed monolayers to oxidation by cholesterol oxidase in the subphase is a sensitive parameter for monitoring the strength of interlipid interaction in monolayer membranes (Grönberg & Slotte, 1990; Slotte, 1992a). Figure 3C shows that the average oxidation rates are higher at all cholesterol/phospholipid molar ratios in monolayers of 3-deoxy-2-*O*-stearoyl-SPM, indicating that cholesterol is more readily accessible to enzymatic oxidation in mixed 3-deoxy-2-*O*-stearoyl-SPM monolayers than in 3-deoxy-*N*-stearoyl-SPM mixed monolayers. This rate difference was most markedly seen in monolayers containing equimolar concentrations of cholesterol and SPMs. At an equimolar ratio, cholesterol was oxidized 5.4-fold more rapidly in 3-deoxy-*O*-stearoyl-SPM than in 3-deoxy-*N*-stearoyl-SPM monolayers at 25 °C.[3] Thus the 2-ester bond in 3-deoxy-2-*O*-stearoyl-SPM appears to destabilize the interaction with cholesterol compared with the interaction between 3-deoxy-*N*-stearoyl-SPM and cholesterol. However, the apparent stoichiometry at which free cholesterol clusters disappear, when the cholesterol concentration is reduced from a high to a low level, remained constant at a 2:1 cholesterol-to-SPM molar ratio for both 3-deoxy-2-*O*-stearoyl-SPM and 3-deoxy-*N*-stearoyl-SPM (Figure 3C). It should be noted that this stoichiometry is 1:1 with diacylphosphatidylcholines (Slotte, 1992a).

Oxidation of Cholesterol in SUVs Prepared from SPMs. Table 2 shows that the initial rate and the extent of cholesterol oxidation at 30 °C are higher by factors of approximately 4.8 and 2.6, respectively, in SUVs prepared from 3-deoxy-2-*O*-stearoyl-SPM than in those prepared from 3-deoxy-*N*-stearoyl-SPM. This result suggests that cholesterol is more accessible for enzymatic oxidation in vesicles formed from the ester-linked SPM analogue.

Table 2: Oxidation of Cholesterol in SUVs Prepared with SPM Analogues[a]

SPM analogue	initial rate[b] (ΔFI s^{-1})	extent of oxidation[b] (FI at 10 min)
3-deoxy-*O*-stearoyl-SPM	20.9 ± 3.6	3067 ± 94
3-deoxy-*N*-stearoyl-SPM	4.4 ± 1.4	1180 ± 263

[a] SUVs were prepared from an equimolar mixture of SPM analogue and cholesterol (15.5 μM total lipid) as described under Materials and Methods. Cholesterol oxidase was used at 200 milliunits/mL. The oxidation was carried out at 30 °C. The data represent the mean ± SD (n = 5 or 6). [b] The fluorescence intensity is measured in arbitrary units.

DISCUSSION

In this study the functional importance of the amide group at the 2 position of the sphingosine backbone of 3-deoxy-SPM for interaction with cholesterol was tested in monolayers and small unilamellar vesicles. The nitrogen atom of the amide group may donate a lone pair of electrons to the hydrogen of the hydroxy group of cholesterol, or the NH function is capable of accepting a lone pair of electrons from the oxygen atom of the hydroxy group of cholesterol. Furthermore, the carbonyl oxygen of the amide linkage is electron rich because of the presence of the NH group. Although the NH group is expected to be solvent accessible at the lipid–water interface, where competition with hydrogen bonding to water would take place, we sought to examine the possible role of the amide linkage of SPM in hydrogen bonding to the cholesterol hydroxy group because our cholesterol exchange kinetic studies indicated that van der Waals forces are not solely responsible for tight interactions between SPM and cholesterol in vesicles (Kan et al., 1991).

We estimated the extent of interaction by measuring the condensing effect of cholesterol on the lateral packing density of the SPMs, and by determining the susceptibility of cholesterol in mixed monolayers and vesicles to oxidation by cholesterol oxidase. In addition, we compared the overall dipole properties of these two SPM analogues at the air–water interface (Figure 2). In mixed monolayers, cholesterol appeared to be equally miscible in 3-deoxy-2-*O*-stearoyl-SPM as well as 3-deoxy-*N*-stearoyl-SPM monolayers, as evidenced by the similar extent to which cholesterol induced the condensation of the lateral packing of the SPMs (Figure 3). Since the condensing effect of cholesterol on phospholipid packing is not a direct function of the apparent mean molecular area in the mixed monolayer, we probed the cholesterol-SPM interaction by using cholesterol oxidase localized in the subphase. The higher average rate of cholesterol oxidation in 3-deoxy-2-*O*-stearoyl-SPM than in 3-deoxy-*N*-stearoyl-SPM monolayers (Figure 3C) and vesicles (Table 2) suggests that cholesterol is more accessible to the enzyme when the amide bond is absent in the SPM analogue. This important finding implies that the amide-linked acyl chain is important in enhancing the molecular interactions between cholesterol and SPM. The stronger interaction between cholesterol and 3-deoxy-*N*-stearoyl-SPM compared with 3-deoxy-2-*O*-stearoyl-SPM could in part be due to the fact that the rotational barrier around the C–O bond of an ester is lower than that around the N–H bond of an amide; therefore, the ester linkage confers

[3] The rate difference is appreciably smaller at higher temperatures; for example, at 32 °C, the initial rate of cholesterol oxidation in SUVs from the ester-SPM analogue was 1.8-fold higher than that found in SUVs from the amide-SPM analogue.

additional flexibility to the acyl chain (Wiberg & Laidig, 1987).

The dipole properties of 3-deoxy-*N*-stearoyl-SPM and 3-deoxy-2-*O*-stearoyl-SPM were different, as expected based on fact that one derivative contains an ester linked carbonyl function whereas the other does not. It has been shown that the C=O dipole contributes significantly to the overall dipole of phospholipids (Paltauf et al., 1971), and therefore sphingolipids which lack ester-linked carbonyl groups also give lower surface potentials and lower overall dipole moments across the air–water interface (Shah & Schulman, 1967; Ali et al., 1991). The finding that the overall dipole moments of both 3-deoxy-2-*O*-stearoyl-SPM and 3-deoxy-*N*-stearoyl-SPM did not change markedly during compression from about 80 to 55 Å^2 suggests that there was no marked reorientation of the head group dipoles during compression within this range (Gaines, 1966; Paltauf et al., 1971).

Interestingly, the stoichiometry of the lateral packing in mixed monolayers containing cholesterol and 3-deoxy-2-*O*-stearoyl-SPM, as detected by cholesterol oxidase, was similar to that of 3-deoxy-*N*-stearoyl-SPM and also to that of other SPMs (Slotte, 1992a), but different from that observed with phosphatidylcholines, which also contain ester-linked acyl chains. The stoichiometry obtained by use of cholesterol oxidase in experiments similar to those presented in Figure 3C should not be interpreted to suggest the formation of stoichiometric complexes between cholesterol and a colipid in mixed monolayers. Instead, our interpretation of the observed stoichiometries is that the oxidation rate is related to the extent of cholesterol solubilization in the mixed monolayer, since the enzyme actually reports the rate of sterol-rich cluster formation within the limits of the experimental setup. In this respect, it appears that both 3-deoxy-2-*O*-stearoyl-SPM and 3-deoxy-*N*-stearoyl-SPM are equally effective in solubilizing cholesterol in monolayers at the air–water interface. This conclusion is supported by the similar condensation results presented in Figure 3A,B.

There has been speculation that intermolecular hydrogen bonding between SPM and cholesterol contributes to the apparent preferential interactions between these compounds in noncocrystallizing lipid mixtures (Schmidt et al., 1977; Barenholz & Thompson, 1980; Boggs, 1980; Sankaram & Thompson, 1990). In order to test the speculation, a variety of structural analogues of SPM are required to examine the contributions of the various substituents of SPM in the interaction with cholesterol. We recently tested the importance of the 3-hydroxy group of SPM in the interaction with cholesterol by replacing the hydroxy group with other substituents (Kan et al., 1991; Grönberg et al., 1991). We concluded that the hydroxy group at the 3 position of SPM is not critically important in this interaction. The results reported here with the ester analogue of 3-deoxy-SPM support the hypothesis that formation of a hydrogen bond between the NH group of SPM and the hydroxy group of cholesterol is important in monolayers and SUVs. Since the present data are insufficient to establish whether the same conclusion applies to bilayers of different curvatures, further biophysical studies of the interaction of cholesterol with the ester analogue of SPM and with other synthetic analogues modified at the 2 position of SPM are being carried out in vesicles.

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