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# Bimane-labelled thiocholesterol, a new fluorescent lipid probe. Spectral properties and interactions with lipid vesicles

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**S-Bimanylthiocholesterol, a new fluorescent lipid, was synthesized from thiocholesterol and monobromobimane. Fluorescence excitation and emission spectra were measured in some organic solvents and after incorporation into unilamellar lipid vesicles. In the vesicles, excitation was at 389 nm and emission was at 459 nm. Very similar values were seen in methanol and in water containing 0.5% (v/v) Triton X-100. These spectral properties suggested that the bimane fluorophore was located in the lipid vesicles in a polar environment, close to the surface. The molar extinction coefficient at the excitation wavelength in methanol was 5400 $M^{-1} \cdot cm^{-1}$. Up to 7 mol% could be incorporated without self-quenching of the fluorescence. The exchange of *S*-bimanylthiocholesterol between vesicles was demonstrated, with an apparent first-order rate constant of 0.48 $h^{-1}$. The extent of exchange at equilibrium indicated that lipid transfer was restricted to the probe molecules in the outer leaflets of the bilayers.**

## Introduction

Fluorescent lipids are valuable reagents for probing the structures and functions of biological and model membranes [1–3]. Although some lipids are intrinsically fluorescent, many probes have been made by the chemical modification of naturally-occurring membrane components to introduce a fluorescent group. This approach allows considerable flexibility in design, as the same fluorophore, such as 7-nitrobenzo-2-oxa-1,3-diazol-4-yl (NBD), can be placed at different positions in the host molecule. Thus, Pagano and his colleagues [3,4] have used NBD-lipids in many elegant studies of the mechanisms of phospholipid transfer and metabolism.

Many of the properties of the NBD group which make it so useful are shared by the bimane fluorophore, discovered by Kosower [5]. Bimane (3,6,7-trimethyl-4-methylene-1,5-diazabicyclo [3,3,0]octa-3,6-diene-2,8-dione), a small amphiphilic molecule, is highly fluorescent in water and in organic solvents and the labelling reaction is straight forward. By contrast to NBD, however, bimane is resistant to fading during irradiation in the fluorescence microscope [6].

We report here the synthesis and some spectral properties of a novel bimane-labelled lipid, *S*-bimanylthiocholesterol. It is shown that the probe may be readily incorporated into unilamellar lipid vesicles and that the probe in the outer leaflets of the bilayers is rapidly exchanged.

## Materials and Methods

Thiocholesterol, dioleoylphosphatidylcholine and sodium cholate were purchased from Sigma. Monobromobimane (Thiolyte MB) was supplied

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by Calbiochem-Behring. Egg phosphatidic acid was from Lipid Products. [1α,2α(n)-$^3$H]Cholesterol (1.7 GBq/μmol), cholesteryl [1-$^{14}$C]oleate (79 MBq/μmol) and sodium [*carboxyl*-$^{14}$C]cholate (2.06 GBq/mmol) were obtained from Amersham. Spectrophotometric grade dioxane and heptane were purchased from Aldrich. Spectrophotometric grade octan-1-ol was from Fisons. Other solvents were analytical grade and were dried and distilled before use. Silica gel preparative layer chromatography (PLC) plates (Merck 5717) and thin-layer chromatography (TLC) sheets (Merck 5735) were supplied by B.D.H. Sephadex G-50 (fine), Sepharose CL-4B and DEAE-Sephacel were Pharmacia products.

Absorption spectra were recorded with a Perkin-Elmer Lambda 3 spectrophotometer. Fluorescence excitation and emission spectra (uncorrected) were recorded on a Perkin-Elmer LS3 spectrofluorimeter. All measurements were made at 22 ± 1°C.

Radioactivity was counted in a Packard Tri-Carb 300 liquid-scintillation spectrometer. Corrections for quenching and for channel overlap were applied. The scintillation fluid was Pico-fluor 30 supplied by Packard. Aqueous samples up to 0.6 ml in volume were miscible with 4 ml of fluid.

*Synthesis of S-bimanylthiocholesterol*

Monobromobimane (30 mg, 0.11 mmol), thiocholesterol (50 mg, 0.12 mmol) and $NaHCO_3$ (10 mg, 0.12 mmol) were dissolved in chloroform/methanol/water (6 ml, 2:3:1, by vol.). After stirring for 19 h at 21°C in the dark, the mixture was diluted with diethyl ether (50 ml) and extracted with 10% (w/v) citric acid (50 ml), saturated $NaHCO_3$ (50 ml) and saturated NaCl (2 times 25 ml). The ether was dried ($MgSO_4$) and evaporated at 30°C in vacuo. The residue was dissolved in chloroform (2 ml), applied at a PLC plate (20 cm × 20 cm) and developed with chloroform/acetone (5:1, v/v). The major yellow band ($R_f$ 0.48–0.62) was scraped off and eluted with acetone (10 ml). The solid remaining after evaporation was dissolved in chloroform and filtered to remove silica. After recrystallization from hot acetone, *S*-bimanylthiocholesterol (48 mg, 74%) had m.p. 192–193°C and was homogeneous by TLC ($R_f$ 0.48 chloroform/acetone, 9:1, v/v). Calculated composition for $C_{37}H_{56}N_2O_2S$ ($M_r$ = 592.9): C = 74.95, H = 9.52, N = 4.72%. Found: C = 74.76, H = 9.64, N = 4.84%.

*S-Bimanylthio[$^3$H]cholesterol*

Thio[$^3$H]cholesterol was prepared from [1α, 2α(n)-$^3$H]cholesterol (42 mg, 0.34 MBq/μmol) as described [7] and made to react with monobromobimane. After two PLC runs, *S*-bimanylthio[$^3$H]cholesterol (4.9 MBq, 13%) ran as one fluorescent spot on TLC, which contained 97% of the radioactivity.

*Preparation of lipid vesicles*

Unilamellar vesicles were made without sonication by gel filtration of mixed cholate-lipid micelles [8]. Briefly, dioleoylphosphatidylcholine (15 mg, 19 μmol in 0.75 ml chloroform) was mixed with *S*-bimanylthiocholesterol (8.2 mM in toluene), *S*-bimanylthio[$^3$H]cholesterol (16 kBq) and phosphatidic acid (7.3 mM in chloroform/methanol, 1:1, v/v) to give the required molar ratios. Some preparations contained a trace (0.04 mol%, 4 kBq) of cholesteryl [$^{14}$C]oleate. The solvents were removed at 35°C in vacuo and the lipids were dispersed in 10 mM Tris-HCl buffer (pH 7.3) containing 0.1 M NaCl and 0.02% $NaN_3$ (1 ml). Sufficient sodium cholate (0.8 M in water) was added to give a clear solution, which was applied to a column (1.5 cm × 26 cm, 46 ml) of Sephadex G-50 and eluted with buffer (12 ml/h). A portion (0.1 ml) of each fraction (1 ml) was taken for the counting of radioactivity. Fractions corresponding to the peak of *S*-bimanylthio[$^3$H]cholesterol were assayed for phospholipid [9]. After elution of the vesicles, the column was washed with 1% (v/v) Triton X-100 (50 ml) and reequilibrated with the buffer (150 ml). The vesicles were characterized by gel permeation chromatography on a column (1.5 cm × 27 cm, 48 ml) of Sepharose CL-4B eluted with the Tris-HCl buffer under the same conditions.

*Exchange of S-bimanylthio[$^3$H]cholesterol between vesicles*

Exchange was measured between donor and acceptor vesicles that differed in their content of phosphatidic acid [10]. Donor vesicles were made with dioleoylphosphatidylcholine (19 μmol), phos-

phatidic acid and *S*-bimanylthio[$^3$H]cholesterol (16 kBq) in the molar ratio 97.5 : 1.5 : 1.0 and also contained a trace (0.04 mol%, 4 kBq) of the non-exchangeable marker cholesteryl [$^{14}$C]oleate. Acceptor vesicles were made with dioleoylphosphatidylcholine, phosphatidic acid and *S*-bimanylthiocholesterol in the molar ratio 92.6 : 6.4 : 1.0. The concentrations of *S*-bimanylthiocholesterol in the donor and acceptor pools were measured by fluorescence, after dilution into 0.5% (v/v) Triton X-100. Equal amounts (16 nmol) of the probe in donor and acceptor vesicles were mixed at 37°C in 12.5 mM phosphate buffer (pH 7.4) containing 0.02% $NaN_3$ (7.5 ml). At intervals, portions (0.8 ml) of the mixture were applied to columns (0.6 cm × 2 cm, 0.6 ml) of DEAE-Sephacel and rapidly eluted (0.8 ml/min) with 5 ml of the phosphate buffer, containing 0.1 mg/ml of bovine serum albumin. Fractions (0.6 ml) were collected, scintillation fluid was added and the $^3$H and $^{14}$C radioactivities were counted.

*Kinetic analysis*

Kinetic data were analysed by assuming an exchange reaction of the type

$$AX + DX^* \rightleftharpoons AX^* + DX$$

where A represents the acceptor, D the donor, X the unlabelled species and X* the labelled species. According to this scheme, the fractional transfer of label at time $t$, $X_t$, is given by [11,12]

$$X_t = X_\infty[1\text{-}\exp(-kt/X_\infty)] \quad (1)$$

where $X_\infty$ is the fractional transfer at equilibrium and $k$ is the frequency of transfer of a lipid molecule from a donor bilayer to an acceptor vesicle. $X_t$ was calculated as $1 - [(^3H/^{14}C)$ of donor vesicles after incubation$/(^3H/^{14}C)$ of incubation mixture]. Data (42 pairs, five experiments) were fitted to Eqn. 1 by non-linear regression, using the program developed by Duggleby [13]. It was assumed that the apparent value of $t$, the independent variable, was in error by an unknown constant increment $\Delta t$, which represented the sampling and separation time. The analysis gave the best estimates of $k$, $\Delta t$ and $X_\infty$.

## Results and Discussion

*Spectral properties*

The structure of *S*-bimanylthiocholesterol is shown in Fig. 1. The compound was highly fluorescent under long wavelength ultraviolet light (365 nm) and as little as 10 ng was detectable on TLC sheets. Fluorescence excitation and emission wavelengths in some organic solvents are given in Table I. The molar extinction coefficients at the excitation wavelengths are also given. Due to insolubility, values in water could be obtained only in the presence of detergent or after incorporation into vesicles. Apart from the aliphatic hydrocarbon solvents, the excitation wavelength was relatively unaffected by solvent polarity, although it tended to increase slightly in the more polar solvents. A more marked trend was observed in the emission wavelengths, which had the highest values in the most polar solvents. The molar extinction coefficients tended to decrease with increasing solvent polarity. The wavelengths of fluorescence emission from *S*-bimanylthiocholesterol in methanol, in unilamellar vesicles and in 0.5% (v/v) Triton X-100 were very similar. These results suggested that the membrane-bound fluorescent moiety was in a polar environment, but that it was not fully exposed to the aqueous phase. The thiol group of thiocholesterol in lipid vesicles lies close to the surface of the bilayer [14] and the spectral properties of *S*-bimanylthiocholesterol suggest that this preferred orientation is unaltered by bimane labelling.

*Incorporation into unilamellar vesicles*

When mixed micelles of dioleoylphosphatidylcholine, *S*-bimanylthio[$^3$H]cholesterol and sodium cholate were eluted on Sephadex G-50, a peak of radioactivity and phospholipid was eluted

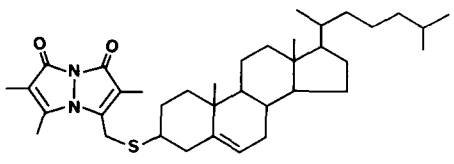


Fig. 1. Structure of *S*-bimanylthiocholesterol.

TABLE I

SPECTRAL PROPERTIES OF *S*-BIMANYLTHIOCHOLESTEROL

| Solvent | Dielectric constant [a] | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | Molar extinction coefficient [b] ($M^{-1} \cdot cm^{-1}$) |
|---|---|---|---|---|
| Heptane | 1.9 | 358 | 412 | 6,100 |
| Cyclohexane | 2.0 | 358 | 412 | 6,100 |
| Dioxane | 2.2 | 384 | 427 | 5,500 |
| Carbon tetrachloride | 2.2 | 382 | 420 | 6,000 |
| Toluene | 2.4 | 384 | 427 | 5,800 |
| Chloroform | 4.8 | 387 | 436 | 5,400 |
| Ethyl acetate | 6.0 | 382 | 426 | 5,600 |
| Dichloromethane | 9.1 | 387 | 436 | 5,500 |
| Octan-1-ol | 10.3 | 387 | 447 | 5,600 |
| Butan-1-ol | 17.8 | 387 | 449 | 5,500 |
| Ethanol | 24.3 | 387 | 452 | 5,600 |
| Methanol | 32.6 | 388 | 459 | 5,400 |
| Triton X-100 [c] | | 389 | 459 | n.d. [d] |
| Lipid vesicles | | 389 | 459 | n.d. |

[a] Values from Handbook of Chemistry and Physics (59th Edn.), pp. E-56–E-57, CRC Press.

[b] At the excitation wavelength.

[c] 0.5% (v/v) in 0.01 M Tris-HCl, pH 7.4, containing 0.1 M NaCl.

[d] Not determined.

at the void volume. On the leading edge of this peak the ratio of radioactivity to phospholipid was constant and these fractions were presumed to contain unilamellar vesicles. Fractions comprising the trailing edge showed an increasing amount of radioactivity, especially in those mixtures containing the highest levels of *S*-bimanylthiocholesterol. The separation was not improved when a longer column (1.6 cm × 70 cm, 140 ml) of Sephadex G-50 was used. These fractions were discarded as we concluded that they contained the fluorophore in cholate micelles.

When vesicles made with 1 mol% *S*-bimanylthio[$^3$H]cholesterol and a trace of cholesteryl [$^{14}$C]oleate were run on an column of Sepharose CL-4B, both radioactive markers were eluted in the same ratio over a symmetrical peak (Fig. 2), as would be expected for a unilamellar vesicle preparation [8,15], although a trace of radioactivity was eluted at the void volume, probably as multilamellar liposomes.

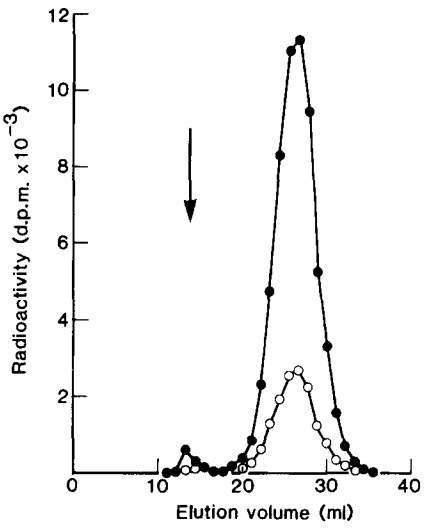


Fig. 2. Elution profile on Sepharose CL-4B of dioleoylphosphatidylcholine vesicles containing 1.5 mol% phosphatidic acid, 1 mol% *S*-bimanylthio[$^3$H]cholesterol (●) and 0.04 mol% cholesteryl [$^{14}$C]oleate (○). The arrow shows the elution position of Blue dextran 2000.

Lipid vesicles were also made with a trace of [$^{14}$C]cholate in order to measure the level of detergent contamination. We found that up to 4 mol% of cholate remained associated with the vesicles. Brunner et al. [8] have reported that contaminating cholate can be decreased to negligible levels by dialysis or by preparation of the vesicles on a longer column of Sephadex G-50, but neither method reduced the cholate content of our vesicle preparations. This suggests that the residual cholate may be present as micelles or entrapped within the vesicles.

Measurements of phospholipid and *S*-bimanylthiocholesterol concentrations showed that only 60% of the fluorophore in the initial lipid mixture was incorporated into the vesicles. The efficiency of incorporation was constant up to a level of 7 mol% in the vesicles. We were not able to measure the saturating level of *S*-bimanylthiocholesterol, as at higher ratios the unincorporated fluorophore became difficult to separate from the vesicles, but 7 mol% may be about half of the saturating concentration. Up to 19 mol% of thiocholesterol can be incorporated into egg yolk phosphatidylcholine vesicles [16] and Rando et al.

[17] incorporated 15 mol% of an NBD-labelled cholesterol. The fluorescence of *S*-bimanylthiocholesterol vesicles was independent of the mole fraction incorporated (data not shown).

*Exchange of S-bimanylthiocholesterol between vesicles*

An important property of any lipid analogue is the extent to which it transfers between membranes. Both exchangeable and non-exchangeable markers have proved to be valuable tools [3,4]. For these experiments we synthesized *S*-bimanylthio[$^3$H]cholesterol and used cholesteryl [$^{14}$C]oleate as a non-exchangeable marker. Changes in the concentration of *S*-bimanylthiocholesterol were followed by dual-label scintillation counting. Exchange was measured between vesicles that contained equal amounts of fluorophore, but differed in phosphatidic acid content. The difference in surface charge allowed the two populations to be separated on DEAE-Sephacel [10], only those vesicles with the lowest surface charge being eluted from the gel. In control experiments we found negligible leakage of the acceptor vesicles.

When dioleoylphosphatidylcholine vesicles containing *S*-bimanylthio[$^3$H]cholesterol, cholesteryl [$^{14}$C]oleate and 1.5 mol% phosphatidic acid were mixed at 37°C with acceptor vesicles containing 6.4 mol% phosphatidic acid, the ratio of $^3H/^{14}C$ in the donors decreased over a period of 2.5 h. At this time about 25% of the $^3$H-labelled fluorophore had been lost (Fig. 3). Experiments at longer time intervals suffered from poor recovery of the donor vesicles, but there appeared to be no additional exchange. These data were fitted to Eqn. 1 by non-linear regression [13] and gave $X_\infty = 0.248 \pm 0.004$ and $k = 0.48 \pm 0.03$ h$^{-1}$. The sampling time error $\Delta t$ was $0.052 \pm 0.017$ h.

The exchange properties of *S*-bimanylthiocholesterol differ from those of cholesterol and thiocholesterol in two respects. The rate of exchange is slightly faster (cf. Ref. 12) and there is a marked difference in the equilibrium extent of exchange. Parkes et al. [16] found cholesterol and thiocholesterol to be freely exchangeable and Dawidowicz and Backer demonstrated rapid rates of transbilayer movement (flip-flop) for both compounds [18,19]. If this were the case with *S*-bimanylthiocholesterol, 50% would have been exchanged at equilibrium in our experiments. We observed half of that value, which indicated that exchange was limited to the outer monolayers of the vesicles. We presume that the hydrophilic nature of the bimane moiety prevented its translocation, perhaps because of hydrogen-bonding to adjacent polar headgroups. It appeared that the cholate contamination of the vesicles did not increase the rate of flip-flop. It is possible, however, that the slightly enhanced rate of exchange compared to cholesterol reflected a solubilizing effect of cholate.

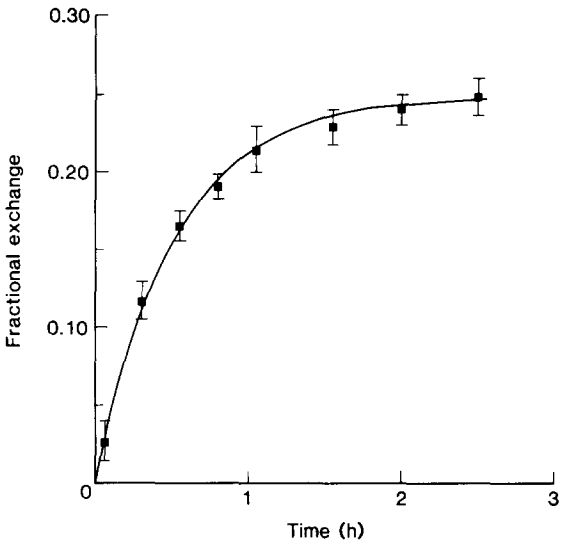


Fig. 3 *S*-Bimanylthiocholesterol exchange between unilamellar vesicles at 37°C. Transfer of the $^3$H-labelled probe from donor vesicles containing cholesteryl [$^{14}$C]oleate to an equal concentration of acceptor vesicles was followed as described in the text. Each point is the mean ± S.E. of five experiments. The data were fitted to Eqn. 1 by non-linear regression and the line has been drawn with $X_\infty = 0.248$ and $k = 0.48$ h$^{-1}$. The apparent sampling time was increased by 0.052 h to allow for the separation of the donors.

*Applications*

*S*-Bimanylthiocholesterol was synthesised as a photostable fluorescent probe to allow the visualization of cell-associated liposomes in the fluorescence microscope (Page-Thomas, D.P. and Knight, C.G., unpublished observations). As observed by Kosower et al. [6], the fading of the fluorescence was slow provided the appropriate filters were used [20]. Recently, a bimane-labelled phosphati-

dylcholine analogue was proposed as a resonance energy transfer probe [21], as the NBD absorption band overlapped the fluorescence emission band of the bimane group. *S*-Bimanylthiocholesterol has similar spectral properties and may provide an additional tool for such studies.

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