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Incorporation of fluorophore–cholesterol conjugates into liposomal and mycobacterial membranes

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ABSTRACT

Fluorescently-labeled steroids that emit intense blue light in nonpolar solvent (λ_{em} (CH_2Cl_2) ≈ 440 nm, $\Phi_F = 0.70$) were prepared by treating cholesteryl chloroformate with 4-amino-1,8-naphthalimides. The lipid portion of the conjugates embeds into liposomal membrane bilayers in minutes, leaving the fluorophore exposed to the external aqueous environment. This causes a 40-nm red-shift in λ_{em} and significant quenching. DFT optimizations predict the conjugates to be about 30 Å long when fully extended, but rotation about the linker group can bring the compounds into an 'L'-shape. Such a conformation would allow the cholesteryl anchor to remain parallel to the acyl chains of a membrane while the fluorescent group resides in the interfacial region, instead of extending beyond it. When incubated with *Mycobacterium smegmatis* mc2 155, a bacterial species known to use natural cholesterol, the labeled steroids support growth and can be found localized in the membrane fraction of the cells using HPLC. These findings demonstrate stable integration of fluorescent cholesterols into bacterial membranes in vivo, indicating that these compounds may be useful for evaluating cholesterol uptake in prokaryotic organisms.

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1. Introduction

Numerous species of bacteria extract cholesterol from the cells of their hosts.[1] Mycobacterial species such as *Mycobacterium tuberculosis* (*Mtb*) use the sterol as a primary source of carbon and energy during the latent stage of infection,[2] catabolizing the tetracyclic core and the side-chain via multiple pathways.[3] Initial import of cholesterol is performed by the Mce4 protein complex, which also recognizes other steroids with nonpolar alkyl groups present on the D-ring.[4] A fuller understanding of mycobacterial utilization of such compounds could lead to new therapies for *Mtb* infection. Studies of cholesterol dynamics and trafficking are commonly carried out using fluorescence spectroscopy. For uptake in the mycobacterial system, the presence of a polar fluorescent group at C22 or C25[5] of the side-chain might interfere with the site(s) of Mce4 recognition. For this reason, the conjugates presented here are labeled far from the alkyl chain, at the 3-position of the A-ring, via a carbamate linker that is resistant toward non-enzymatic hydrolysis.[6] Steroids with labels on ring A or B have been previously described,[6a,7] but unlike those systems, the fluorescent unit in the present work was selected for its potential antimicrobial activity. We envisioned that such conjugates might serve one of two roles, depending on whether the toxicity of the fluorophore was retained in the conjugates: as reporters for following in vivo uptake/catabolism of cholesterol, or as 'Trojan horse' antibiotics.[8] Spectroscopic techniques as well as bacterial viability studies were employed to evaluate how the modified cholesterols localize in model membrane bilayers, and whether they are harmful to live cells. The strain *Mycobacterium smegmatis* mc2 155 was chosen as the model for evaluation of our compounds, because of its demonstrated ability to take up cholesterol, and its relatively rapid growth.

2. Results and discussion

2.1. Synthesis

Two known 4-amino-1,8-naphthalimides[9] (**1**, Scheme 1) were attached to cholesterol. The antibacterial properties of naphthalimides are well-established.[10] Fluorophores like **1** are relatively compact (MW < 300) yet brightly luminescent (yellow-green) in organic solution, and are employed in a variety of sensing applications.[11] Their —NH_2 groups are part of an environmentally-sensitive

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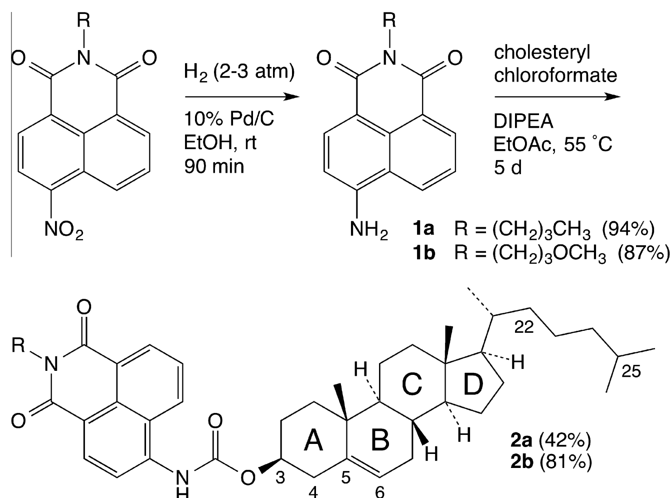

Scheme 1. Synthesis of cholesterol–naphthalimide conjugates. Products **2a** and **2b** are isolated as mixtures of alkene isomers at C5–C6 (shown) and C4–C5 that are inseparable by TLC or flash column chromatography.

electronic 'push–pull' system.[12] As such, nucleophilic addition to a cholesterol scaffold, or hydrolytic removal therefrom, was expected to change the emission wavelength. To prepare them, sonochemically-derived[13] 4-nitro-1,8-naphthalimides were reduced in ~90% yield by hydrogenation over Pd/C catalyst. The subsequent reactions of **1** with cholesteryl chloroformate were readily monitored by silica gel TLC, as the desired products **2** appear blue when visualized with a 365 nm UV lamp. Isolation of the conjugates required column chromatography to remove unreacted amines, which persisted after five days of heating in ethyl acetate at 55 °C. Proton NMR of purified **2a** and **2b** showed each to be a mixture of alkene isomers at C5–C6 and C4–C5.[14] The latter, unnatural isomers presumably arise from deprotonation at the allylic C4 position during the lengthy reaction period. ^{1}H NMR was also used to confirm fluorophore attachment, with the N*H* resonances of the carbamate linkers of **2a** and **2b** appearing at 7.38 ppm in $CDCl_3$. The carbamates were found to be stable toward hydrolysis at pH 7.4 and 37 °C for at least one week.

2.2. Spectroscopic behavior in organic and aqueous solution

Electronic absorption and emission spectra were acquired in low- and high-polarity media (Table 1). Moving from dichloromethane to 0.10 M aqueous phosphate buffer (pH = 7.4), the longest-wavelength absorptions for **2a** and **2b** (~370 nm) undergo modest red-shifts. The same trend is observed computationally. Time-dependent density functional theory (DFT) calculations[15] upon a truncated model of **2a**, in which the cholesteryl moiety has been replaced with methyl, predict the HOMO → LUMO transition to occur at 369.3 nm in the gas phase, with an oscillator strength of 0.28. With two explicit water molecules hydrogen-bonded to each of the imide C=O groups, the calculated absorption appears at 374.8 nm. The shifts in the *emission* bands are more pronounced (>40 nm), in accord with a high degree of charge separation in the excited state.[11c] Other acylated 4-aminonaphthalimides have previously been found to emit in the 480-nm range in aqueous solution.[11b]

Table 1
Photophysical properties of steroid–fluorophore conjugates[a]

	λ_{abs}, nm (CH_2Cl_2)	λ_{abs}, nm (aq buffer)[b]	λ_{em}, nm (CH_2Cl_2)	λ_{em}, nm (aq buffer)[b]	λ_{em}, nm (liposomes)[c]
2a	373	378	443 (Φ_F = 0.70)[d]	487	484
2b	371	378	441 (Φ_F = 0.70)[d]	495	483

[a] For all fluorescence measurements, [**2**] = 3.0 × 10^{-6} M, λ_{ex} = 375 nm.
[b] [NaH_2PO_4] = 0.023 M, [Na_2HPO_4] = 0.077 M, pH = 7.4.
[c] Small unilamellar vesicles comprised of POPC and cholesterol (7:3, mol:mol), [POPC + cholesterol] = 2.9 × 10^{-3} M, 0.10 M phosphate buffer.
[d] Relative to 7-amino-4-methylcoumarin (Φ_F = 0.63 in CH_3CN).

2.3. Localization in liposomal model membranes

To explore how the labeled steroids might interact with mammalian (host) cells, the fluorescence properties of **2** were also assessed in the presence of liposomal model membranes. Small unilamellar vesicles were constituted in pH 7.4 phosphate buffer from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol, in a 7:3 molar ratio. Conjugate **2a** or **2b** was introduced to the gently stirring lipid suspension as a solution in DMSO–dioxane (1:1, v:v) such that the concentration of **2** was approximately one-thousandth of [POPC + cholesterol]. An emission spectrum was immediately acquired (λ_{ex} = 375 nm, λ_{em} = 390–600 nm), and additional spectra were recorded at five-minute intervals until the integrated intensities became constant. Equilibrium was reached after about 15 min. Final emission intensities were higher than those found in plain buffer (Fig. 1), but were still an order of magnitude lower than observed in dichloromethane. Thus, a significant fraction of the naphthalimide units are located outside the hydrophobic core of the lipid bilayer. Proton NMR data are consistent with tight binding of **2** to the membrane, despite the presence of conjugates with C4–C5 double bonds, which are known to render cholesterol less effective at membrane incorporation.[14] Liposomes of POPC (0.010 M) and **2b** (0.0043 M) in pure D_2O display only three broad ^{1}H NMR signals at 0.84, 1.24, and 3.19 ppm, all of which are assigned to CH_n of the phospholipid. The absence of other peaks suggests that there is practically no free **2b** present in the bulk solution.

Data from fluorescence titrations with acids show that the fluorophore of liposome-bound **2a** retains some ability to report on the properties of the bulk aqueous environment. When 1 M HCl was used to lower the pH of buffered POPC/cholesterol/**2a** suspensions from 7.1 to 3.0, the emission intensity of **2a** was unchanged (Fig. 2). An equivalent pH drop brought about by glacial acetic acid

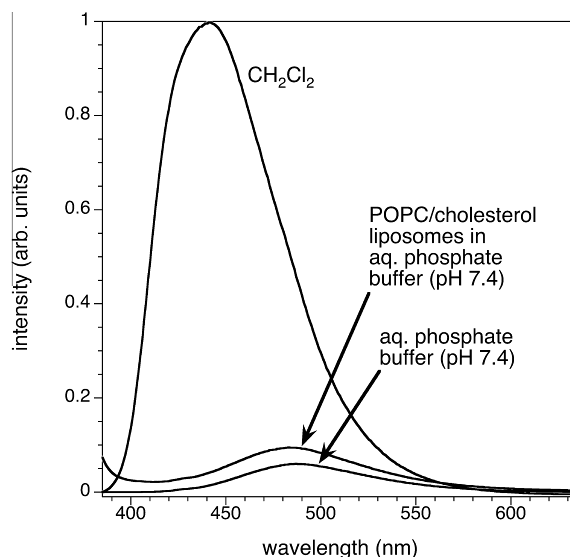

Figure 1. Emission spectra of compound **2a** at a concentration of 3.0 × 10^{-6} M in different milieu. λ_{ex} = 375 nm.

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