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Design of new fluorescent cholesterol and ergosterol analogs: Insights from theory

Lina J. Nåbo [a,*], Nanna H. List [a], Sarah Witzke [a], Daniel Wüstner [b], Himanshu Khandelia [c], Jacob Kongsted [a,**]

[a] Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark
[b] Department of Biochemistry and Molecular Biology, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark
[c] MEMPHYS — Center for Biomembrane Physics, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark

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ABSTRACT

Cholesterol (**Chol**) and ergosterol (**Erg**) are abundant and important sterols in the plasma membrane of mammalian and yeast cells, respectively. The effects of **Chol** and **Erg** on membrane properties, as well as their intracellular transport, can be studied with use of fluorescence probes mimicking both sterols as closely as possible. In the search for new and efficient **Chol** and **Erg** probes, we use a combination of theoretical methods to explore a series of analogs. The optical properties of the analogs (i.e. excitation energies, emission energies and oscillator strengths) are examined using time-dependent density functional theory (TDDFT) and their ability to mimic the effects of **Chol** and **Erg** on membranes is investigated with molecular dynamics (MD) simulations of each analog in a 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) bilayer. From the set of analogs we find two probes (**3a** and **3b**) to display favorable electronic transition properties as well as strong condensing abilities. These findings can lead to the use of new efficient probes and aid in the understanding of the structural features of **Chol** and **Erg** that impart to them their unique effects on lipid membranes.

1. Introduction

Cholesterol (**Chol**) and related sterols, such as ergosterol (**Erg**) found in fungi and other organisms, fulfill a variety of important functions in cellular membranes. Structurally, these sterols condense the fluid lipid bilayer by restraining the molecular motion of the phospholipid fatty acyl chains with the additional effect of increased bending rigidity and resistance against area dilation [1,2]. These properties are very important for maintenance of a permeability barrier against ions and small solutes, retaining at the same time enough flexibility of cellular membranes for ongoing membrane traffic in cells. At high sterol mole fractions in fluid-phase bilayers, **Chol**, **Erg** and similar sterols induce a liquid-ordered (lo) phase, which is likely of physiological relevance in the plasma membrane of cells [3–5]. Inserted into gel-phase model membranes, i.e., in which the phospholipids are below their respective phase transition temperature, these sterols increase lateral diffusion and decrease bending rigidity, both being characteristics of a fluid lo phase [3–7]. As a consequence, **Chol**, **Erg** and related sterols mediate fluid–fluid immiscibility in ternary mixtures containing a low- and a high-melting phospholipid. Importantly, even apparently negligible alteration of the molecular structure can affect such sterol properties dramatically, as amply documented in experimental and simulation studies [5,8–12].

Unrestrained **Chol** accumulation is deleterious for cells and associated with a number of diseases like atherosclerosis or lysosomal storage disorders [13]. Despite intense research within this field, the intracellular transport pathways of cholesterol and related sterols are today largely unknown. A key aspect in this relation is that understanding intracellular transport and membrane dynamics of sterols requires suitable analogs that perturb the original structure minimally, but can be tracked with high sensitivity and specificity. However, most known cholesterol analogs have a covalently linked fluorophore attached and as a result perturb membrane structures significantly resulting in altered metabolism, localization or trafficking in cells [14–20]. In contrast, fluorescent sterols having a conjugated double bond system in the sterol rings, so-called polyene sterols (P-sterols), are particularly suitable analogs, since they have minimal impact on important lipid properties. Polyene-lipids are intrinsically fluorescent due to their conjugated double bond system in the hydrocarbon portion. For example, polyene-based analogs of fatty acids were found to mimic their natural counterparts allowing for in-depth characterization of lipid metabolism and storage [21]. Currently, mainly the natural P-sterol dehydroergosterol (**DHE**) is available and in use as an analog of **Erg** and **Chol** in spectroscopic and imaging applications [15,22–24]. **DHE** differs from **Erg** only by one additional double bound (Fig. 1), making it a suitable substitute of this sterol, especially in sterol-auxotroph organisms relying on **Erg**, as yeast cells under some conditions or the nematode *Caenorhabditis elegans* [25–27]. **DHE**'s disadvantages are however severe: It possesses low brightness, rapid bleaching and excitation in the ultraviolet (UV)

* Corresponding author. Tel.: +45 6550 8602.
** Corresponding author. Tel.: +45 6550 2304.
E-mail addresses: naabo@sdu.dk (L.J. Nåbo), kongsted@sdu.dk (J. Kongsted).

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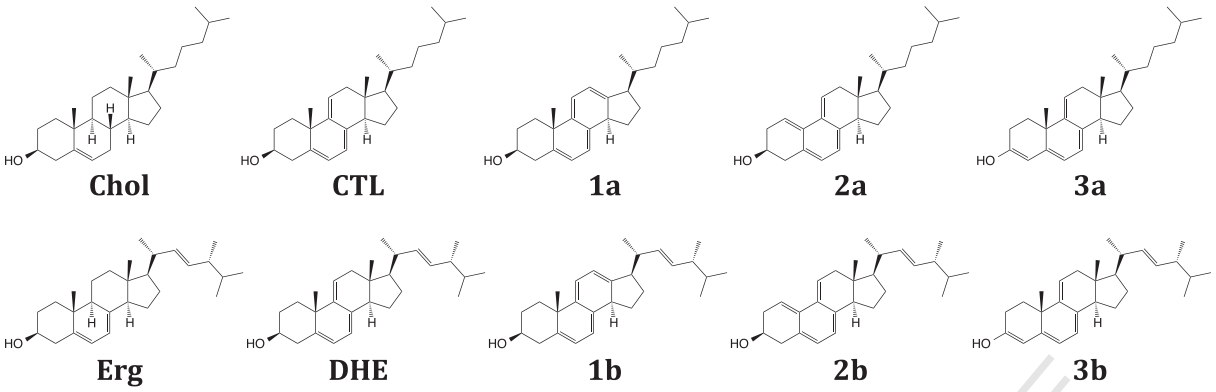

Fig. 1. Chemical structures and names of **Chol**, **Erg** and their analogs, which are investigated in this study.

region. A lower excitation energy is desirable, particularly for live cell imaging as it enables the use of less invasive exciting radiation. A related analog of **Chol** is cholestatrienol (**CTL**), which has the same side chain as **Chol** but a conjugated system in the steroid backbone identical to that of **DHE** (Fig. 1). **CTL** has been used extensively as a membrane probe in fluorescence spectroscopic investigations [28–30], but has only recently been incorporated with some success in live-cell imaging applications [31,32]. However, given the comparable and weak fluorescent properties of **DHE** and **CTL**, the quest for further improvements upon existing P-sterols for imaging applications cannot be undermined.

Electronic structure calculations and molecular dynamics (MD) simulations constitute complementary tools for obtaining guiding principles for the design of new fluorescence probes. Computations provide information about the molecular properties of the analogs and their interactions with the membrane directly on the molecular level. These computational tools are employed in this study, where we continue the search for better intrinsically fluorescent **Chol** and **Erg** analogs along the line of **CTL** and **DHE** by extending the conjugated system to four double bonds. This strategy is chosen because it is known that increasing the length of a conjugated system results in lower excitation energy, more efficient absorption and stronger fluorescence [33]. The four double bonds must be introduced in such a way that the membrane-ordering properties of **Chol** and **Erg** are retained. Herein lies the major challenge of this work, since as already stated, even small changes in the chemical structures of **Chol** and **Erg** will reduce their ability to order membranes [10,16,34–41].

A series of analogs of **Chol** and **Erg** (Fig. 1) are here examined for their suitability to serve as **Chol** or **Erg** probes, respectively. Their electronic transition properties are characterized with electronic structure calculations and the ability of each analog to mimic the ordering effects of **Chol** and **Erg** on a 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) membrane is investigated using atomistic MD simulations. A molar sterol concentration of 30% is chosen for initial simulations because 1) this concentration of **Chol** is often found in plasma membranes in the lo phase [3–5] and 2) the differences in membrane perturbations induced by the sterols will be amplified at a higher concentration, enabling us to identify the most appropriate probes. Selected sterols are also examined at 5.5 mol-% since this concentration is more relevant for imaging experiments of cell membranes [42]. Qualitatively, the effects of the probes were similar at both concentrations.

Like **CTL**, **1a**, **2a** and **3a** are analogs of **Chol**, while **DHE**, **1b**, **2b** and **3b** are **Erg** analogs. As seen in Fig. 1, analogs **1a** and **1b** do not have the C18 methyl group, while for **2a** and **2b**, the C19 methyl group is instead missing (see atom numbering in Fig. 2). Analogs **3a** and **3b** have a double bond between C3 and C4, which eliminates the asymmetric center in **Chol** and **Erg** to which the hydroxyl group is bonded. The important stereochemistry of the hydroxyl group [34] is thereby altered as well as the functionality of the group, since the chemical structures of **3a** and **3b** allow the oxygen atom to participate in conjugation. For example, the pK_a value for the hydroxyl hydrogen atom will be reduced compared to **Chol** and **Erg**. In contrast to the other four double bond analogs, all methyl groups are still present.

The structure of this paper is as follows: First, the computational methods are described. Hereafter, we present the results from the electronic structure calculations together with a discussion of the electronic properties of the different analogs. The MD simulations are then analyzed to evaluate the analogs' ability to mimic the membrane ordering effects of **Chol** and **Erg**. The outcome of the two computational methods is combined in the Conclusions section, where we point out the most promising analogs for fluorescent **Chol** and **Erg** probes.

2. Computational procedure

2.1. Electronic structure calculations

The molecular structures of the sterols were built in the Schrödinger Suite 2013 graphical interface Maestro [43]. First, a conformational search of the isolated molecule was performed based on molecular mechanics using the MM3 force field and the lowest energy conformation, with the requirement of an elongated geometry, was chosen for further optimization. An elongated geometry is likely the most relevant when the sterol is incorporated in a bilayer. All quantum mechanically (QM) based geometry optimizations as well as electronic transition properties were conducted in gas phase using Gaussian09 [44]. Ground state properties were calculated using density functional theory (DFT) employing the B3LYP [45] exchange-correlation functional while excitation energies, excited state properties and emission energies were calculated with the range-separated CAM-B3LYP [46] functional within the time-

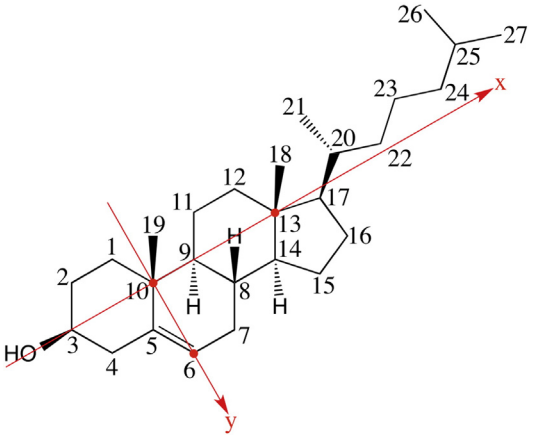

Fig. 2. Numbering of **Chol** carbons and illustration of internal coordinate system.

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