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Atomistic picture of fluorescent probes with hydrocarbon tails in lipid bilayer membranes: an investigation of selective affinities and fluorescent anisotropies in different environmental phases

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Abstract

By reverting to spectroscopy, changes in the biological environment of a fluorescent probe can be monitored and the presence of various phases of the surrounding lipid bilayer membranes can be detected. However, it is currently not always clear in which phase the probe resides. The well-known orange 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbo-cyanine perchlorate (DiI-C18(5)) fluorophore for instance as well as the new, blue BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) derivative were experimentally seen to target and highlight identical parts of giant unilamellar vesicles of various compositions, comprising mixtures of dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), sphingomyelin (SM) and cholesterol (Chol). However, it was not clear which of the coexisting membrane phases were visualized (Bacalum *et al.*, Langmuir 32 (2016), 3495). The present study addresses this issue by utilizing large-scale molecular dynamics simulations and the z-constraint method, which allows evaluating Gibbs free energy profiles. The current calculations give an indication why, at room temperature, both BODIPY and DiI-C18(5) probes prefer the gel (S_o) phase in DOPC/DPPC (2:3 molar ratio) and the liquid ordered (L_o) phase in DOPC/

SM/Chol (1:2:1 molar ratio) mixtures. This study highlights the important differences in orientation and location and therefore in efficiency between the probes when they are used in fluorescence microscopy to screen various lipid bilayer membrane phases. Dependent on the lipid composition, the angle between the transition state dipole moments of both probes and the normal to the membrane are found to deviate clearly from 90°. It is seen that the DiI-C18(5) probe is located in the headgroup region of the SM:Chol mixture, in close contact with water molecules. A fluorescence anisotropy study indicates also that DiI-C18(5) gives rise to a distinctive behavior in the SM:Chol membrane compared to the other considered membranes. The latter behavior has not been seen for the studied BODIPY probe, which is located deeper in the membrane.

Introduction

Molecular insight into the condition and properties of lipid membranes, which are fundamental components of living cells, is of utmost importance for various areas of biomedical research including drug design, drug pharmacology, or medical diagnosis and prognosis [1–4]. To give only one example of the crucial role of membranes, it has been shown that increased fluidity and polarity of cell membranes correlate with the metastasis in cancer cells [5]. Well-designed membrane-specific probes can picture biological membrane properties by means of optical imaging and suited spectroscopic techniques. Cholesterol (Chol) highly contributes to the structure of the membranes of many mammal cells [6]. For example, in hepatocellular carcinoma, which is the fifth most frequent cancer worldwide, high Chol levels were found to lead to tumor progression and malignancy [7–9]. The specific development of probe molecules which have an expressed affinity for Chol-abundant membrane regions is a particularly relevant and challenging topic. In the current work, computer modeling is used to investigate interactions of optically active probes with various membrane models and to evaluate whether they can identify the spectral fingerprints of specific biological conditions.

Natural membranes can be organized in different phases, with distinction between single-component and multicomponent membranes. For lipid systems of a single type, a gel phase (S_o) membrane is characterized by a high order of lipid packing. The liquid-crystalline or liquid disordered phase (L_d) of the membrane is characterized by a reduced lipid packing and higher diffusion coefficients. In complex lipid systems, Chol promotes phase segregation and gives access to the liquid-ordered state (L_o), a phase which is often also enriched in sphingomyelin

(SM) [10–13]. The different ratios among the lipid components of a membrane are important parameters that determine its phase. Single component membranes made of dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC) or distearoylphosphatidylcholine (DSPC) have been extensively studied [12,14,15]. The phase in a single component system depends on the lipid chemical structure and the temperature. DOPC with its transition temperature of -17 °C adopts a liquid phase at room temperature; the DPPC membrane is in the S_o phase at room temperature but adopts the L_d phase above its transition temperature of 41 °C [16]. Two and/or three components membranes have also been evaluated, *e.g.*, made of DOPC/DPPC or DOPC/SM/Chol in studies, which highlighted that different ratios between the components modulate membrane properties [10,17–21]. Considering the number of possible combinations of membrane components, as well as possible lipid segregations, mixtures of phases are expected in biological membranes. The ternary mixtures can be schematically visualized along with their relevant tie lines in temperature dependent triangular diagrams, from which the phase compositions as well as their coexistence can be read [22–24]. There is finally the need of techniques capable of distinguishing and (locally) characterizing these different phases.

One of the most popular dyes to unravel this complex membrane structure is 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiI-C18(5)). It is a dialkyl carbocyanine (see Figure 1), which is amphiphilic due to the positively-charged head chromophore consisting of two indole rings connected by 5-carbon cyanine moiety and the 18-carbon saturated alkyl chains, which are important for the phase-selective partitioning in the membrane [25,26]. DiI-C18(5) exhibits a high extinction coefficient and a high fluorescence quantum yield, and is highly fluorescent and photostable when incorporated into membranes [27]. Fluorescence spectroscopic analyses of the DiI-family have been used to investigate: membrane rotational lipid mobility [28]; membrane potential [27]; membrane fusion [29]; fluorescence resonance energy transfer [30]; phase separation [31]; lipid leaflet transmigration [32]; and the existence of lipid rafts [33]. As the precise location, orientation and lipid/phase selectivity of the dye is often unknown or only partially described, the interpretation of fluorescence lifetime, anisotropy and rotational dynamics may be complex. Gullapalli *et al.* theoretically investigated the properties of two and four DiI-C18(3) probes, which have a cyanine backbone made of 3 carbon atoms, within a DPPC lipid bilayer in its L_d phase at 323 K, safely above the transition temperature [34]. The probes were found below the head group – water interface and report well the rotational and lateral diffusion components of the lipid dynamics. The calculations showed that the dye

causes minor changes at the interface in the ordering of the water dipoles and electrostatic potential.

Recently, the *meso*-amino substituted BODIPY probe 8-[(2-sulfonatoethyl)amino]-4,4-difluoro-3,5-dioctadecyl-4-bora-3a,4a-diaza-*s*-indacene (BNP, see Figure 1) was synthesized and optically characterized [35]. This probe expresses similar behavior with respect to membranes as DiI-C18(5), but fluoresces in the blue part of the visible spectrum. The BODIPY dyes are known to combine outstanding spectroscopic and (photo)-physical properties, such as bright fluorescence with absorption and emission bands in the visible range, as well as stability toward light and chemicals. In particular, BNP was found to be excitable by either 1 or 2 photons in combination with a high fluorescence quantum yield; this probe was found to preferentially partition in the same lipid phase as DiI-C18(5) [35]. In this experimental work by Bacalum *et al.*, BNP and DiI-C18(5) were studied in a 2:3 mixture of DOPC:DPPC ($L_d:S_o$ phases) and a 1:2:1 mixture of DOPC:SM:Chol ($L_d:L_o$ phases) at room temperature. Although we could expect a tiny contribution of DOPC to the L_o phase, for simplicity it has been further omitted. Li and Cheng observed that the smaller DiI-C18(3) probe preferentially partitioned in the DPPC S_o phase of the DOPC:DPPC binary mixture [36]. With respect to the ternary mixture, Baumgart *et al.* investigated a 50:27:23 ratio (DOPC:SM:Chol), and reported that DiI-C18(3) preferentially partitions in the DOPC L_d phase [17]. Fluorescence microscopy provided insights into the DiI-C18(3) probe embedded in a dozen ternary mixtures [18]. However, neither for the larger DiI-C18(5) probe nor for BNP, the phase partitioning is known for the specific ratio of lipid systems considered in [35].

It is currently a challenge to accurately evaluate optical properties of the probe within various lipid bilayers. This task first requires a correct and comprehensive evaluation of large scale structural features of the molecular assembly made of the probe and the lipid bilayer, which can be obtained by Molecular Dynamics (MD) simulations. For the current work therefore, MD simulations were performed to gain insight into the interactions of specifically both DiI-C18(5) and BNP probes within biological membranes and to understand their phase preference. Attention is paid to their locations and motions within the lipid bilayers and how this impacts on their spectroscopic features. *In silico* membrane models have been constructed in the past and a vast development with increasing accuracy is noted [37–42]. MD calculations have been used to accurately evaluate simultaneously equilibrium positions of xenobiotics in lipid bilayers, their partition and diffusion coefficients at subpicosecond and atomic resolution [43–

⁴⁸]. Focusing on the simulation of optically active probes opens the possibility towards the development of non-invasive techniques which provide insights into the impact of surrounding environment in (non) linear and fluorescence spectroscopy [^{49,50}]. Here, MD simulations are used to assess the interaction of both DiI-C18(5) and BNP in four different lipid bilayers and lipid phases. One of them is the DPPC membrane in its Ld phase, which is considered at the same temperature as in the study of Gullapalli *et al.* [³⁴] to enable a direct comparison. The structural and physical-chemical properties of the four lipid bilayer models are discussed in terms of their areas per lipid, order parameters and non-bonding interaction energies. The Gibbs free energy profiles of DiI-C18(5) and BNP are investigated along the *z*-axis of the membrane, which is oriented perpendicular to the membrane surface. The differences between the equilibrium positions and orientations of both probes, and the variations of their transition dipole moments within the various environments are identified as being decisive for the linear and non-linear optical spectra [^{51,52}]. Finally, the fluorescence anisotropy of both probes is modelled and similarities as well as differences in the behavior of DiI-C18(5) and BNP are highlighted.

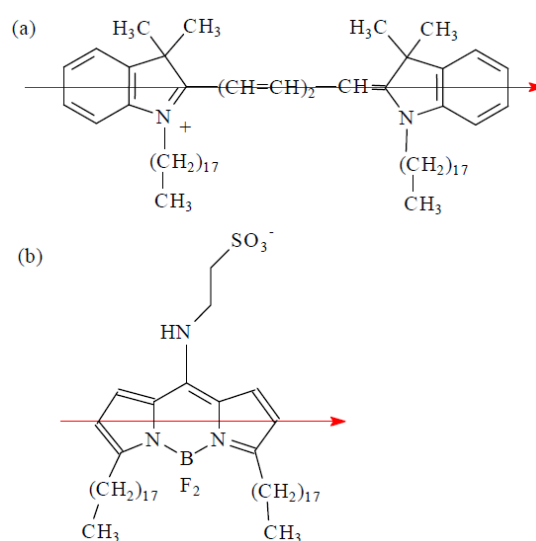


Figure 1: Molecular structures of (a) DiI-C18(5) and (b) BNP. The red arrows are the transition state dipole moments for both probes. To describe the positions of the probes, the middle carbon atom of the $-(CH=CH)_2-CH=$ bridge is considered for DiI-C18(5) and the Boron atom for BNP. Remark that the π -conjugated core in both molecules is confined to those parts of the molecules without tails and – in the case of BNP – without headgroup.

Computational details

The MD simulations were performed using the Gromacs 4.5.7 [^{53,54}] software and the Gromos 43A1-S3 force field [^{55–58}]. The lipid bilayer models consisted of 128 lipid molecules

surrounded by at least 4500 explicit water molecules, which were described by the extended single point charge (SPC/E) model. Na⁺ and Cl⁻ ions were added to bulk water at a physiological concentration (0.9%). The spatial reference frame is such that the *x*- and *y*-axes are taken in the plane of the bilayer, whereas the *z*-axis is perpendicular to the membrane surface. Periodic boundary conditions were considered in 3 dimensions. Electrostatic interactions were treated by the particle-Mesh Ewald method [59] and bonds were constrained by the LINCS algorithm [60]. Electrostatics and van der Waals short-range interaction cutoffs were set to 1.6 nm. The NPT ensemble was used, with the Nosé–Hoover thermostat [61,62], and a Parrinello–Rahman barostat [63] for a semi-isotropic pressure coupling at 1 bar and compressibility of 4.5×10^{-5} bar⁻¹. The simulation time step was set to 2 fs and the coordinates in the simulation were saved every 500 steps.

The four lipid bilayer models were built with a homemade script, they consisted out of one probe and in total 128 lipids (two leaflets of 64 lipids): pure DOPC at 298 K (L_d phase), pure DPPC at 298 K (S_o phase) and at 323 K (L_d phase), and a 2:1 SM:Chol mixture at 298 K (L_o phase). The SM acyl chains contain 17 and 15 methyl groups for the sn1 and sn2 acyl chains, respectively. Upon these systems, periodic boundary conditions in all directions have been applied. All membranes were equilibrated during 20 to 40 ns long free simulations, after which convergence of structural parameters (*i.e.*, area per lipid, lipid order parameters...) were ensured. In line with previous work [35], atom types were assigned by PRODRG [64], while partial atomic charges have been used which result from the restrained fit of electrostatic potential (RESP) [65]. They were calculated at the level of density functional theory (DFT) by means of the B3LYP functional [66,67], Dunning's correlation consistent cc-pVDZ basis set [68], and a PCM model which was chosen to describe an implicit solvent model with a dielectric constant of diethyl ether ($\epsilon = 4.24$) [69]. The Lennard-Jones parameters of the boron atom for the BNP probe, which are not by default present in the applied force field, have been taken from reference [70]. Further parametrizations for the bonded interactions of the Boron atom have been performed by means of previous DFT method.

The Gibbs free energy profiles for BNP and DiI-C18(5) were calculated by means of the *z*-constraint method [71,72], in which bulk water was put as a reference. The distance between the centers of masses of the lipid bilayer and the Boron atom for BNP, or the middle carbon atom of the $-(CH=CH)_2-CH=$ bridge for DiI, was constrained, and the required force was monitored.

The averaged force was then used to calculate the Gibbs free energy profile, also called potential of the mean force [72,73], as:

$$\Delta G(z) = - \int_{outside}^z \langle F(z') \rangle_t dz', \quad (1)$$

where $\langle F(z) \rangle_t$ is the force which is needed to keep the molecule at a given depth z . A series of windows was obtained every 0.1 nm for z -constraint simulations. The initial structures for each window were generated by merging probe and membrane coordinates, minimized to avoid steric clashes, and *g_membed* [74] was used to remove overlapping lipids when appropriate. In this process, the probes were oriented along the z -axis, with the lipid tails in the direction of membrane center. For the z -constraint process, 100 ns simulations were performed per window, ensuring convergence of Gibbs free energy profiles. The computational error was found to be ~ 1 kcal/mol. Starting from the minimum energy positions of the Gibbs free energy profiles, 300 ns long MD simulations were performed without applying additional constraints, of which the first 40 ns were discarded from the simulation window, as being the time required to equilibrate the system. The analysis of the structures of the membranes were performed on these unbiased simulations with GROMACS internal tools, area per lipid for individual lipid types was obtained by the FATS LIM script [75].

The transition state dipole moments of the BNP and DiI-C18(5) probes have been calculated using approximate second order coupled cluster theory (CC2) and the double zeta polarized (DZP) basis set.

In total, these simulations required a computational effort of more than 40 μ s. To perform these calculations, the *Lindgren* cluster at the PDC Center for High Performance Computing in Stockholm (864 000 core hours, 2013-2014), the *muk* tier-1 cluster of the Flemish Supercomputer Centre (VSC) (264 960 core hours, 2014-2015), as well as the *Beskow*, *Triolith* and *Abisko* clusters with in total 105 000 core hours/month (2015) were used.

Results and discussion

Characterization of the membranes

If the simulated DOPC (L_d), DPPC (S_o), DPPC (L_d) and SM:Chol (L_o) bilayer membranes are expected to influence the distribution and dynamic behavior of the embedded probes, their inherent properties should be accurately modelled. The structure of lipid membranes can be

well described by the density plots of various membrane components along the normal axis to the membrane plane. The density distributions of the lipid constituents and of water from the center of the membrane were constant between 2 nm and 0.8 nm for SM:Chol (Figure 2). In the other three membranes, locally higher lipid density was found with a peak at around 1.7 nm from the center, followed by a rapid decrease to the center. This effect is explained by the presence of free volumes just beneath the aqueous interface in contact with the polar head group region [34,76,77] and is manifestly seen in the SM:Chol membrane. Concomitantly, the thickness of the SM:Chol membrane was greater, as seen by a shifted point where the density of the water equals that of the lipids (*i.e.*, crossing at 2.5 nm for SM:Chol with respect to 2.3 nm for the L_d -phase DOPC and DPPC, see Figure 2). As expected, a similar increase of the thickness was observed for DPPC (S_o).

The thickness, in terms of distance of the highest density peaks, agrees well with experimental data. We observed differences in thickness between the different membranes, namely 4 and 4.5 nm for the DPPC (L_d) and the SM:Chol bilayer, respectively. The latter simulated thickness agrees with the experimental value of 4.6-4.7 nm [78]. This value mainly depends on SM, as Chol is known not to significantly modify the conformation of SM molecules [14]. The thickness of the DOPC and DPPC (S_o) bilayers is found in between 4 and 4.5 nm.

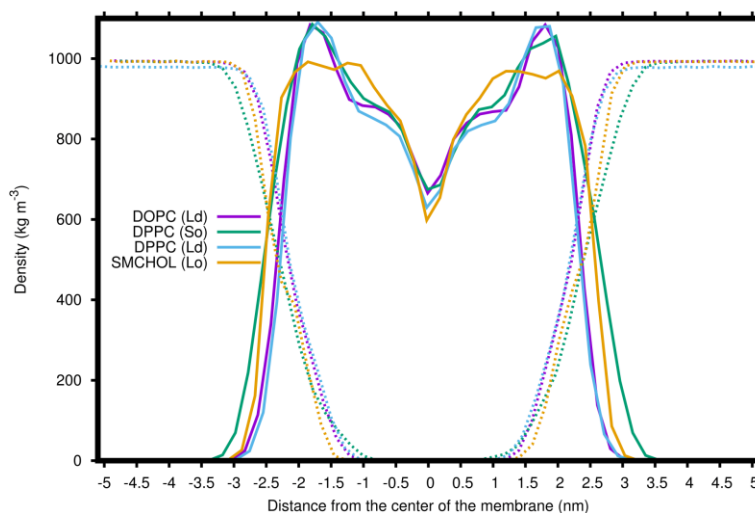


Figure 2: Density distributions of the lipid constituents (full line) and of water (dotted line) within the various membrane phases with respect to the center of the membrane

Over the 300 ns of MD simulations, the area per lipid exhibited constant values, *i.e.*, $\sim 0.45 \text{ nm}^2/\text{lipid}$ for SM:Chol (L_o , $0.40 \text{ nm}^2/\text{Chol}$ and $0.48 \text{ nm}^2/\text{SM}$), $\sim 0.51\text{-}0.52 \text{ nm}^2/\text{lipid}$ for DPPC (S_o), $0.58 \text{ nm}^2/\text{lipid}$ for DOPC and DPPC (L_d) (Figure S1). Although the calculated area per

lipid in the L_d phase is lower than some of the reported experimental data [79], the area/lipid values represent well the differences in the studied phases. A tighter packing and condensing effect were previously observed in S_o phase as well as in the presence of Chol [80].

The potential energy of interaction between the lipid tails (V_{tails}) can be derived from the average sum of Lennard-Jones and short-range Coulomb potentials between all pairs of atoms in the lipid tail region [80,81]. Concerning Chol, all atoms but the hydroxyl group were included, whereas for phospholipids, all tail atoms up to the three glycerol carbon atoms were included. The potential energy was averaged from 180 to 280 ns. The V_{tails} values per atom are very similar for all four membrane models (1.193, 1.127 and 1.110 kcal/mol for DPPC (S_o), SM:Chol and DPPC (L_d), respectively). The decreasing values from 1.193 to 1.110 kcal/mol mainly point the decrease of van der Waals contacts between lipid tails. The latter value is different from that of DOPC, which amounts to 1.182 kcal/mol, likely be due to the greater van der Waals interactions between the unsaturated bonds deep in the DOPC tails not present in the DPPC molecules. Finally, the value for DPPC (S_o) agrees with the one communicated by Wennberg *et al.* in 2012 [80].

To characterize the employed membrane models, the order parameter $|S_{CD}|$ is calculated, too. It is experimentally obtained using deuterium NMR by using the equation [34,82]

$$S_{CD} = \left\langle \frac{3}{2}(\cos^2 \theta_{CD}) - \frac{1}{2} \right\rangle, \quad (2)$$

with θ_{CD} being the angle between C-H bond of the lipid tails and the z -axis. The brackets denote time averaging and corresponds to an ensemble averaging when experiments are performed. The value of the order parameter S_{CD} can vary from -0.5 with $\theta_{CD} = 90^\circ$ (indicating full ordering of the C-H bonds perpendicular to the z -axis and to a lesser extent an orientation of the C-C bonds along the z -axis) to 1 with $\theta_{CD} = 0^\circ$ (indicating full ordering of the C-H bonds along the z -axis and the C-C bonds therefore more oriented perpendicular to the z -axis). Based on S_{CD} values, we confirmed the typical differences between the membranes in the L_d , S_o and L_o phases: as reported in Figure S2, $|S_{CD}|$ values for the sn-1 and sn-2 tails amount maximally to ~0.40 for SM:Chol, ~0.35 for DPPC (S_o), and 0.25 for both DOPC and DPPC (L_d). These maxima are obtained at carbon C8 for SM:Chol and DPPC (S_o), while for DOPC and DPPC, the maxima are reached at C6. For C3, close to the headgroup and the glycerol moiety of the lipids, S_{CD} amount to 0.27 for SM:Chol as well as for DPPC (S_o), and to 0.20 for both L_d

membranes. For SM:Chol, the quite strong increase in $|S_{CD}|$ towards the middle of the tail can be linked with the presence of Chol, which pushes the tails of SM deeper in the membrane, so as to accommodate the perpendicular orientation of the C-H bonds, diminishing hydrophobic effects. On the other hand, for DPPC (S_o), the high $|S_{CD}|$ values are related to the high packing, in agreement with V_{tails} values, and with the higher amount of water present at the level of the glycerol group of the tails (Figure 2).

Gibbs free energy profiles for DiI-C18(5)

z -Dependent Gibbs free energy profiles provide information about partition and preferred positions (free-energy minima), as well as capacity of transfer from one to the other leaflet (Gibbs free energy barriers) independently from diffusion effects. The profile for DiI-C18(5) (Figure 3, left hand side) exhibits the deepest well (-38 kcal/mol) in the DPPC (S_o) membrane. The well is energetically less favorable by 5 kcal/mol in both the DPPC (L_d) and SM:Chol (L_o) bilayers; therefore based on the Gibbs free energy alone, one cannot distinguish any preferred affinity to both DPPC (L_d) and SM:Chol (L_o) bilayers. The affinity of DiI-C18(5) to DOPC (L_d) membrane is the least favorable one (potential well of -28 kcal/mol). The here presented data seem to answer therefore the question which membrane DiI-C18(5) prefers in a DOPC:DPPC ($L_d:S_o$) and a DOPC:SM:Chol ($L_d:L_o$) mixture, like has been used by Bacalum *et al.* in ref. [35]. Namely, the simulations indicate that in the former case, after equilibration of the biological environment, confocal microscopy will allow visualizing the DPPC (S_o) regions of the unilamellar vesicle, whereas in the latter case, the L_o region of the SM:Chol mixture will be bright. For the concentrations used in the current study, DiI-C18(5) should thus be considered as a L_o marker, and contrasts therefore with the findings of Baumgart *et al.* and Kahya *et al.* for DiI-C18(3) embedded in ternary lipid mixtures with other concentration ratios [17,18].

From the analysis given in Figure 3, the position of the global minima were similar except for SM:Chol (1.3, 1.3, 1.2 and 1.9 nm for DOPC (L_d), DPPC (S_o), DPPC (L_d) and SM:Chol (L_o), respectively). Although in this latter case, the bilayer thickness is greater, this makes DiI-C18(5) closer to the polar group region in SM:Chol with respect to the other membranes.

As we applied the z -constraint method from the center of the membrane and used a window for every Ångström, the barriers of transfer from one to the other leaflet have been obtained.

Significant differences are seen: the barrier at the middle of the bilayer is ~ 8 kcal/mol with DOPC and SM:Chol, and it is lower (4-5 kcal/mol) with DPPC (L_d) and DPPC (S_o). As repeatedly seen with amphiphilic compounds, the insertion into fluid bilayers requires small or even no energetic barriers in the polar head group region. Noncovalent interactions (electrostatic and H-bonding) mainly drive insertion and positioning, with little influence of size within the μ s timescale.

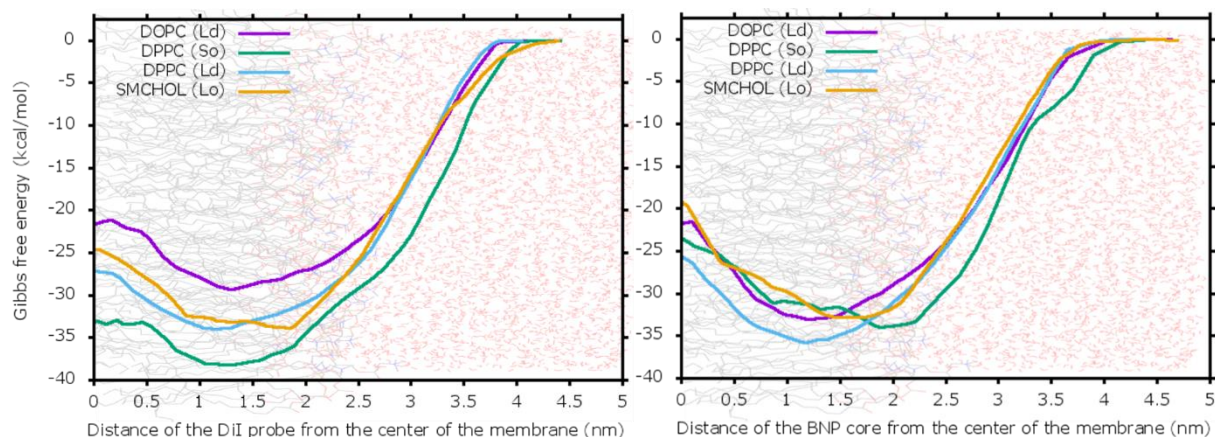


Figure 3: Gibbs free energy surfaces of (left) DiI-C18(5) and (right) BNP in function of the distance (in nm) from the center of the membrane along the z -axis, perpendicular to the membrane surface. The centers of mass of the DiI-C18(5) and BNP cores have been constrained. The error bar is contained in the thickness of the line.

Analysis of the unconstrained trajectories for DiI-C18(5) in the various membranes

It is worth noting that the Gibbs free energy profiles are generated based upon a constrained movement of the core of the probe. To discuss the equilibrated positions and orientations of DiI-C18(5) and to profoundly evaluate the influence of the finite temperature, a free production run of 300 ns was performed for each membrane in the presence of DiI-C18(5), with the minima of the Gibbs free energy profile as starting geometries. Illustrations of the DiI-C18(5) probe in the various membranes are given in Figure 4. As a measure for the position of DiI-C18(5), the middle carbon atom of the cyanine-backbone was considered with respect to the membrane center. For SM:Chol, DiI-C18(5) is situated at 1.75 ± 0.11 nm from the membrane center, in close contact to the polar head group region (Figure 5). For both DPPC bilayers, DiI-C18(5) is located deeper, at ~ 1.0 nm from the membrane center, *i.e.*, in contact with the lipid tails (the exact value for the S_o is 1.04 ± 0.09 nm, while it is 1.09 ± 0.09 nm for the L_d phase). Gullapalli *et al.* observed a value which was with its 1.26 nm a bit higher for DiI-C18(3) in DPPC (S_o) [34]. In DOPC, the location is an intermediate of the other two, however with a broad distribution ranging from 1.3 to 1.8 nm (1.47 ± 0.21). Except for SM:Chol, the mean positions in free

simulations were slightly deeper than the positions of the free energy minima, but these differences were found within errors and thermal motion. In all membranes, the probes have their light sensitive core embedded in lipid head groups and the lipophilic tails pointing towards the center of the membrane. We calculated the angles of the tails of DiI-C18(5) with the z -axis (Figure S3). These angles take the value $\sim 155^\circ$ for both L_d membrane phases, $\sim 165^\circ$ for DPPC in the S_o phase, and $\sim 170^\circ$ for SM:Chol in the L_o phase.

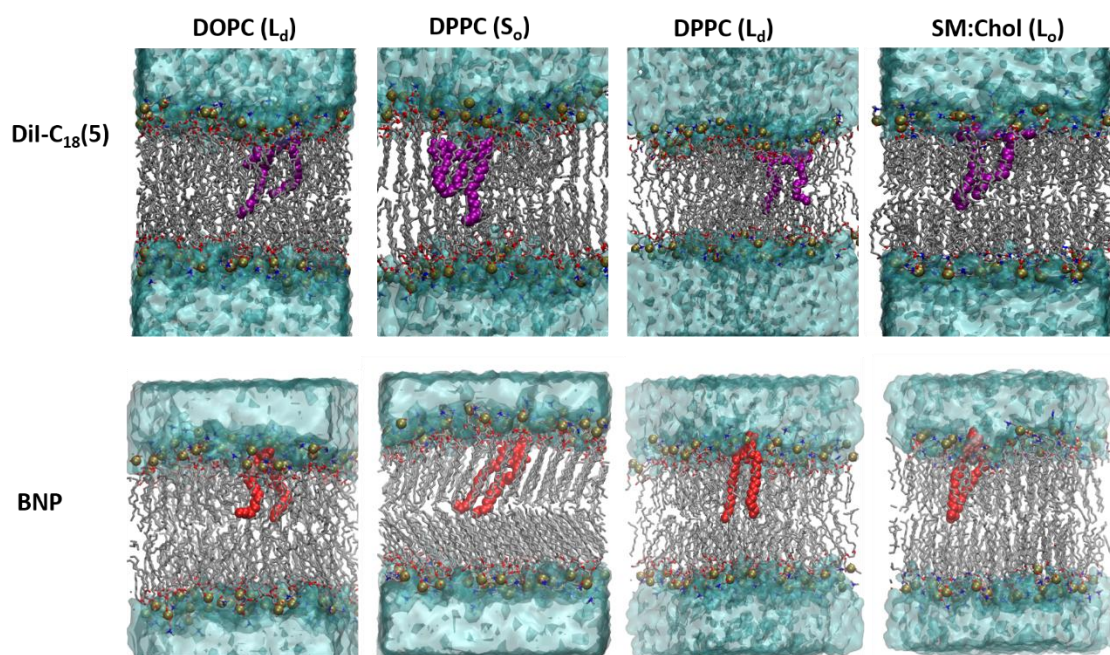


Figure 4: Illustrations of the DiI-C18(5) and BNP probes in the different environments under investigation in the current study.

While in SM:Chol the chromophore moiety of DiI-C18(5) is located at the surface of the membrane in contact with bulk water, it is located significantly more deeply in the other membranes. The DiI-C18(5) π -conjugated core is located below the level of the phosphates at a distance of 0.5 nm in SM:Chol, 0.3-0.8 nm in the L_d phase of DOPC, 1.0 nm in the L_d phase of DPPC and 1.2 nm in S_o phase (DPPC). In the SM:Chol membrane one has to consider not only the average level of membrane surface, but also a local arrangement of the membrane. The chromophore moiety of DiI-C18(5) experiences here free volumes and induces a small cavity, in which water molecules are pulled (Figure S4). Indeed, due to this surface position and such re-arrangements, DiI-C18(5) is more surrounded by water molecules in the SM:Chol membrane than *e.g.* in the S_o phase or the L_d phases of DPPC. For SM:Chol, at the DiI-C18(5) preferred position, the density of water is still 45% of that of the pure water layer, while practically no water is left with DPPC both in S_o and L_d phases (Figure 2). It can also be remarked that the maximum density of water experienced by DiI-C18(5) in the DOPC (L_d) membrane amounts

to 20%. This effect is quantified by the radial distribution functions of DiI-C18(5) and the surrounding water molecules in the various membranes (Figure S5): the first maxima (at 0.45 nm from the DiI-C18(5) core) is very low for both phases of DPPC membranes (<0.2), slightly higher in DOPC (0.3) and significantly higher in SM:Chol (0.6). The water cavity experienced in the SM:Chol membrane is then responsible for the different behavior of DiI-C18(5) in this membrane.

Being decisive for the photoselection of the probe, the distribution of the angles between the transition dipole moment and the z -axis of the membrane is given in Figure 5. For DiI-C18(5), the transition dipole moment is oriented along the cyanine backbone and is displayed in Figure 1. Knowing that a perfect photoselection in confocal microscopy requires an angle of 90° , DiI-C18(5) in DOPC appears the most efficient with a most populated angle of $\sim 85^\circ$. For SM:Chol and DPPC (S_o), the most abundant peak is seen at 72° . It can be remarked that for DPPC (S_o), the distribution of the angle is rather symmetric around its maximum, while for SM:Chol a slight asymmetry is seen together with a minor shoulder at higher values. The DPPC (L_d) lipid bilayer is characterized by a broad distribution of angles of a similar population, which are between 70° and 80° , which agrees with the angle of 77° reported for the smaller DiI compounds investigated by Gullapalli *et al.* [³⁴] or with the range of $\pm 10^\circ$ around the perpendicular position with respect to the z -axis reported by Axelrod for erythrocyte ghosts [⁸³]. The pronounced angles of the transition state dipole moments in the different membranes can be related to the differences in orientation between the sn-1 and sn-2 chains of the lipids and to the differences in position of the probe along the z -axis. To better describe the orientation of DiI-C18(5), the angle between the normal to the coplanar core and the z -axis was followed as well. A symmetric distribution was obtained centered at around 51° only for SM:Chol. In DOPC, essentially all values between 30° and 80° were observed, with only a slight preference for 35 - 40° . For DPPC (L_d), the angle increased from 30° to 80° . In DPPC (S_o), the angle distribution was ranging from 70° to 80° . Combining the analyses for both angles, S_o , and to a less extend L_o , restrain orientation to the probe.

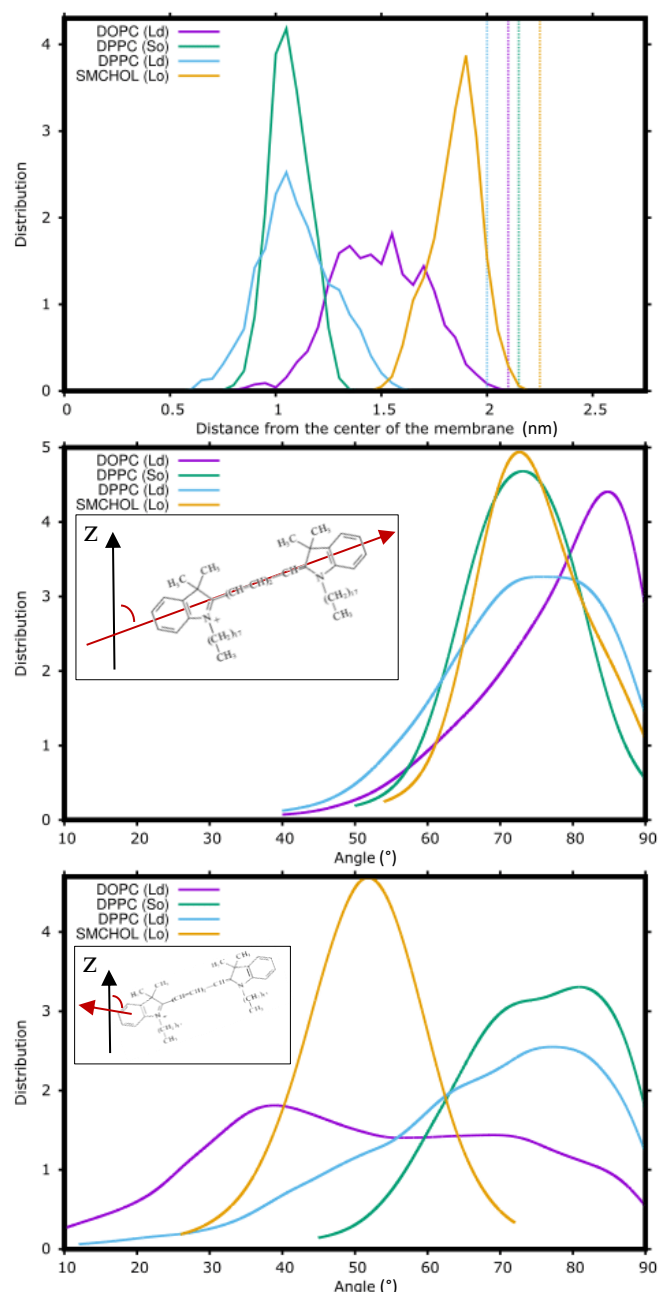


Figure 5: DiI in various membrane phases – (top) the position of the middle atom of the cyanine backbone of DiI along the z -axis expressed in terms of the distance from the center of the membrane, the dotted vertical lines denote the most abundant position of the phosphor atoms; (center) the angle between the transition dipole moment and the z -axis; (bottom) angle between the axis perpendicular to the plane of the DiI-C18(5) molecule and the z -axis. These data are taken from a free MD run and are convoluted with Gaussian profile peaks with a full width half maximum of 8° . The errors are displayed in Figure S6.

The order parameter profiles of DiI-C18(5) show the same trend in all four domains, *i.e.*, higher values close to the polar head group region which decrease when inserting deeper in the bilayer, as expected for lipid-type compounds (Figure 6). Close to the polar head, the highest $|S_{CD}|$ values (0.35-0.39) are observed in SM:Chol (L_o) and DPPC (S_o), whereas lower values (0.20-0.23) are observed in DOPC and DPPC (L_d). A further analysis can be performed making use

of the above definition of S_{CD} which relates to the angles between C-H bonds of the lipid tails and the z -axis. Due to the free space which is available at the top of the SM/Chol bilayer and the high abundance of water molecules, the mid C-C bonds of the tails of DiI-C18(5) are seen to straightly enter further down towards the center of the membrane, parallel to the z -axis. In DPPC (S_o), the DiI-C18(5) $|S_{CD}|$ value is also high for the first bonds below the nitrogen atoms, but the curve flattens down and the slope diminishes due to the high packing between the lipid tails, assuring a well-defined and orientation of the last carbon-carbon bonds of the tails. As expected from the position of the probe and the characteristics of the subsequent lipid bilayers, $|S_{CD}|$ values are lower in both L_d -phase membrane models, while the typical decrease along the tails is less steep than for the other two lipid bilayers. In the DOPC membrane, in the middle of the tails of DiI-C18(5), a slight increase of the S_{CD} value is further on observed, which even surpasses the corresponding values for DiI-C18(5) in the DPPC (S_o) environment, which is in DOPC attributed to the double bond.

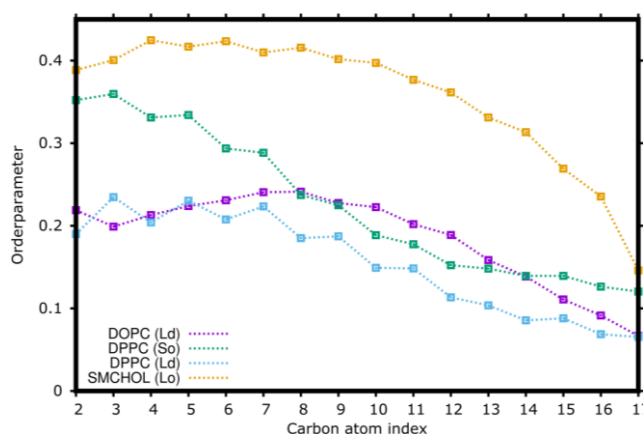


Figure 6: Order parameters for DiI-C18(5) in the various membranes. The carbon atom index points at the number of the carbon in one of the tails, starting from the carbons attached to each of the nitrogens.

Gibbs free energy profiles for BNP

The Gibbs free energy profiles of BNP given in at the right hand side in Figure 3 show a well of -33 kcal/mol in DOPC and SM:Chol; it is marginally deeper in DPPC (S_o) and significantly deeper in DPPC (L_d). Also, the most stable positions of BNP can to some extent be identified within the limits of the used z -constraint method. It mostly partitions at 1.2, 1.9, 1.1 and 1.5 nm in DOPC (L_d), DPPC (S_o), DPPC (L_d) and SM:Chol (L_o), respectively. The differences in the preferred position are however less clear than for DiI-C18(5). The markedly small Gibbs free energy differences in these profiles illustrate why within the constraints of the employed theories and simulations a comparison with DiI-C18(5) was needed to identify the lipid phases

present in the bright areas which were seen in the confocal microscopy images published in [35]. Based upon our simulations for DiI-C18(5) and the related discussion above, it is subsequently safe to assume that BNP in the employed biological environments can be found in the S_o phase when a mixture of DOPC (L_d) and DPPC (S_o) is considered and in the L_o phase when a mixture of DOPC (L_d) and SM/Chol (L_o) is involved. We would like to stress here again the importance of the ratio of the employed mixture, as we employed DOPC:SM:Chol in a 1:2:1 ratio. By means of comparison, Baumgart *et al.* reported the L_d phase as the preferred one for the DiI-C18(3) probe in a DOPC/SM/Chol mixture in basically a 2:1:1 ratio [17]. Other authors who reverted to the benchmark DiI-C18(5) probe, discussed the ternary mixtures in other ratios, too, without solving the issue for the mixture under investigation in the current study, but warning for the particular strong influence of the mixed lipid constituents when phase preferences are concerned [18–21].

The barrier for the transfer of BNP between the upper and lower leaflet amounts to ~ 10 kcal/mol for both DPPC membranes as well as for DOPC. It is calculated as the difference between the minimum of the potential energy surface and the maximum Gibbs free energy value found around the membrane center. The barrier amounts to ~ 14 kcal/mol for SM:Chol (L_o). The largest differences between both probes are therefore found for DPPC (S_o) and SM:Chol (L_o); the larger barriers are here reported for BNP and should be allocated to the influence of the Boron and Fluorine atoms.

Analysis of the unconstrained trajectories for BNP in the various membranes

As for DiI-C18(5), selecting the frames from the global minima of the Gibbs free energy profiles, a free production run was performed for 300 ns. Illustrations of the BNP probe in the various membranes are given in Figure 4. The boron atom of BNP was at $1.4\text{--}1.5 \pm 0.2$ nm from the membrane center in both the DOPC and SM:Chol membranes (Figure 7), while it was inserted deeper (at 1.2 ± 0.2 nm) in DPPC (L_d). Conversely, in DPPC (S_o), it was at $\sim 1.7 \pm 0.1$ nm, closer to the phosphorus atoms of the membrane surface, being located at 2.25 nm. It can be remarked that the boron atom is located rather close to the lipid tails, while the middle atom of the cyanine backbone of DiI-C18(5) is found higher in the molecule.

This difference in preferred position in the DPPC (S_o) and DPPC (L_d) environments is related to the difference in packing and area per lipid between both membranes. In DPPC (S_o), the packing in between lipid tails is likely to complicate insertion of BNP. Moreover, the core of

BNP has a weak zwitterionic charge distribution between the nitrogen and boron atoms, making them slightly positive and negative, respectively. This favors interactions with water molecules abundant in this region of DPPC (S_o) (up to 20% of the density of pure water). The similar position of BNP in DOPC and SM:Chol is a manifestation of the interaction with tail unsaturation and Chol.

The angle between the transition dipole moment of BNP and the z -axis amounts to 70° - 75° in DOPC (Figure 7). With an angle of 85° (and a minor distribution at 50°), the photoselection was found to be stronger in DPPC (L_d). In the DPPC (S_o) bilayer, the maximum of the distribution is found at 67° , however a shoulder can also be seen at 86° . Rather in contrast to DiI-C18(5), the angle distribution in SM:Chol is very broad with many contributions between 30° and 60° , and a major peak at 73° .

The orientation of the molecular plane of BNP with respect to the z -axis showed that this probe is rather perpendicular to the surface, with an angle of $\sim 85^\circ$ for DPPC (S_o) and SM:Chol. In DOPC, the maximum is at $\sim 71^\circ$, although a shoulder is noticed at 85° . In DPPC (L_d), the distribution is broader, with a shallow maximum at 59° .

Although it has been experimentally found that both DiI-C18(5) and BNP probes target the same membrane phases and in contradiction to the first assumptions [³⁵], it can be concluded based upon the current MD simulations that BNP behaves rather differently from the relatively known DiI-C18(5) one in terms of its orientation and equilibrium position in the membrane.

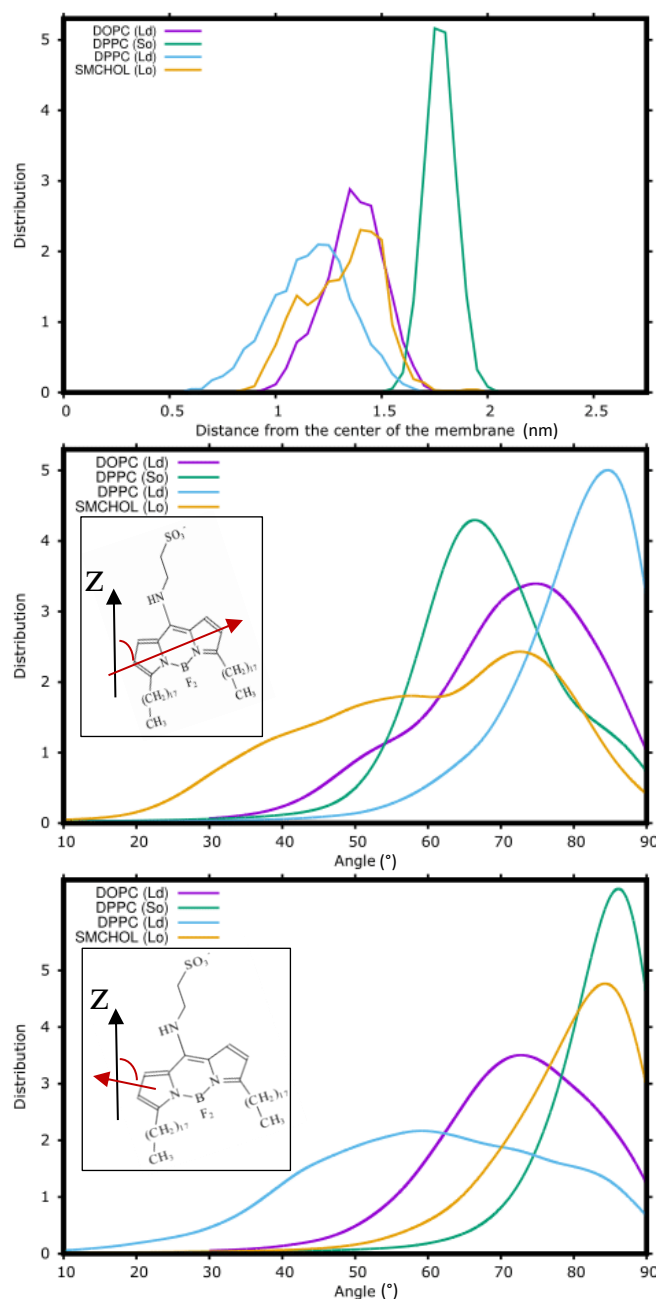


Figure 7: BNP in various membrane phases – (top) the position of the boron atom along the z -axis expressed in terms of the distance from the center of the membrane; (center) the angle between the transition dipole moment and the z -axis; (bottom) angle between the axis perpendicular to the plane and the z -axis. These data are taken from a free MD run and are convoluted with Gaussian profile peaks with a full width half maximum of 8° . The errors for the angle distributions are given in Figure S6.

Fluorescence anisotropy

When polarized light is applied to a biological environment, the probability of excitation of the probe depends on the angle between the transition state dipole moment and the electric field vector of the incoming electromagnetic radiation. A smaller angle leads to a higher excitation

probability. As a consequence, the initial emission after pulsed excitation has a defined polarization. Rotational mobility within a time span determined by the fluorescence lifetime will reduce the fluorescence polarization. The fluorescence anisotropy r is generally defined by means of the fluorescence intensities obtained parallel ($I_{//}$) and perpendicular (I_{\perp}) to the polarization of the excitation light via

$$r = \frac{I_{//} - I_{\perp}}{I_{//} + 2I_{\perp}}, \quad (3)$$

when the sample is excited with vertically excited light [84].

For DiI-C18(5) and BNP in lipid bilayers of various composition, the relaxation of $r(t)$ after a δ -pulse excitation was investigated. This relaxation depends on the rotational dynamics, the intrinsic anisotropy r_0 (corresponding to the anisotropy at $t=0$) and the conditions of the environment surrounding the light sensitive probe. In agreement with the study by Lipari and Szabo upon the effect of librational motion upon fluorescence depolarization [85] and in line with the theoretical models advocated by Heyn, Jähnig and Ameloot [86–88], the rotational correlation function $C(t)$ is an autocorrelation function and is given in terms of the second order Legendre polynomial $P_2(x)=(3x^2-1)/2$ and the orientation of the transition dipole moment at $t = 0$, $\mu(0)$, and time t after excitation, $\mu(t)$ [34,89]:

$$C(t) = \langle P_2(\mu(0)\mu(t)) \rangle, \quad (4)$$

where the brackets denote the ensemble average, or equivalently, the average over all initial times in the MD calculations, and with $C(t) = \frac{r(t)}{r_0}$ [85]. Since our quantum chemical calculations indicate that the absorption and emission dipoles of the probes under investigation are parallel to each other and as the intrinsic anisotropy r_0 or the anisotropy at time $t = 0$ for 1-photon excitation depends on the angle δ between both dipoles via [84]:

$$r_0 = \frac{2}{5} P_2(\cos \delta), \quad (5)$$

a maximum value of $r_0 = 0.4$ has been considered.

Being embedded in a lipid bilayer, the fluorophore has a limited rotational freedom. The fluorescence lifetime (ranging from hundreds of picoseconds to a few nanoseconds) sets a time window over which the rotational motions can be monitored in an experimental context. In line with previous theoretical and experimental analysis [^{34,35}], a double exponential function is used to describe the rotational correlation function:

$$C(t) = \beta_1 \exp(-t / \theta_1) + \beta_2 \exp(-t / \theta_2) + C_\infty, \quad (6)$$

where θ_1 and θ_2 are correlation times. The C_∞ constant reflects that the rotational correlation function, and therefore the fluorescence anisotropy, does not decay to zero. One can define the

mean correlation time $\langle \theta \rangle$ as: $\langle \theta \rangle = \frac{\sum_i \beta_i \theta_i^2}{\sum_i \beta_i \theta_i}$.

The results of the analysis are given in Table 1. The quality of the fit was tested by the χ^2 analysis. As our fit leads here to a deviation in the order of barely 10^{-6} , the high quality of the function used is ensured with a time window up to 25 ns. The C_∞ parameter in the S_o phase for both DiI-C18(5) and BNP are the highest ones in the range of investigated environments, pointing at a particularly confined freedom of rotation. The residual C_∞ for both compounds decreases when a more fluid-like lipid environment is considered. It can also be seen that the L_d phase of DPPC displays a slightly smaller constant than the one of DOPC in the same phase. From our analysis, it has been found that $C_\infty(S_o) > C_\infty(DOPC, L_d) > C_\infty(DPPC \text{ at } 323K, L_d) > C_\infty(L_o)$. These inequalities have to be put in relation to the nature and packing of the various membranes. For the difference between the results for the L_d and L_o phase, the particular position of DiI-C18(5) in the SM:Chol membrane and the presence of the free volumes with water can be recalled. The restricted motions of the probes are finally confirmed by the smaller (larger) relaxation time constants θ_1 (θ_2). For DPPC (L_d) and DiI-C18(3), Gullapalli *et al.* reported $\theta_1 = 0.99$ ns and $\theta_2 = 6.9$ ns for the fast and slow components [³⁴]. These values have to be compared with the ones of 0.11 ns (θ_1) and 11.57 ns (θ_2) found for DiI-C18(5) in this study. The values reported by Ariola *et al.*, who studied DiI-C12(3) in the DOPC (L_d) membrane, can be compared with the ones of Gullapalli *et al.* and amount to $\theta_1 = 1.2$ ns and $\theta_2 = 9.6$ ns [⁹⁰]. The obtained time constants for the SM:Chol membrane with not only a very low fast component but also a low slow component point at the special place of the DiI-C18(5) probe: a low steric hindrance of the chromophore is seen in the neighborhood of the top of the

lipid acyl chains, while also the collective motion of the lipids in the membrane does not stretch the decay of the rotational autocorrelation function.

A steady-state fluorescence anisotropy of ~ 0.35 has been measured for BNP in the DPPC S_o phase, while it decreased to ~ 0.15 upon transition to the L_d phase. The fluorescence lifetimes of this probe reaching up to 4.4 ± 0.2 ns were found to be independent of the phase and the temperature of the lipid system [35]. Especially for BNP, changes in fluorescence anisotropy can consequently be entirely ascribed to restricted tumbling motions of the probe, which are described by Table 1 with the two relaxation times and the limiting anisotropy at long times. From the time constants, it can be seen that the mean relaxation times are larger for BNP than for DiI-C18(5). As the carbocyanines are known to have a shorter fluorescence lifetime of ~ 1.0 ns [26], the steady state fluorescence anisotropy of BNP is thereupon more sensitive to slower rotational motions than DiI-C18(5). The presented data confirm therefore successfully the assumptions made for BNP at the time of its synthesis [35].

The profoundly low value of 0.12 for C_∞ in SM:Chol as well as the small associated average decay time of 0.43 ns found for DiI-C18(5) point at a strongly pronounced decay of the fluorescence anisotropy and might be another manifestation of the presence of free volumes and a high amount of water molecules in the top polar region of the lipid bilayer. As depicted in Figure 4, the tails of the probe are located along with the acyl tails of the lipids in the membrane. The tails of DiI in SM:Chol are almost parallel to the z -axis as can be deduced from the angle of $\sim 170^\circ$ between the z -axis and the vector described by the first and one of the last carbon atoms of the acyl tails of DiI (See Figure S3). Differences between the fitted parameters (*e.g.* $C_\infty \sim 0.62$ and 0.41 for DiI-C18(5) and BNP in DPPC(L_d) – or 0.12 for DiI-C18(5) and 0.69 for BNP) for DiI-C18(5) and BNP can finally be related to the differences in position of the probes in the lipid bilayer. It is again an indication for the fundamental differences between the two probes. The anisotropy results, together with the Gibbs energy profiles of DiI-C18(5) embedded in the various lipid bilayers, correct and supplement the image for DiI-C18(5) provided in [76] as the probe is not found to perform surface dynamics in the water phase of the membrane but rather tumbles with two relaxation time constants at different distances from the center of the bilayer.

To give an interpretation to the C_∞ parameter, Kinoshita *et al.* proposed in 1977 a so-called ‘wobbling in a cone’ model, in which the transition dipole and the symmetry axis of the probe are assumed to move without restriction in a cone fixed with respect to the membrane [91]. The model relates the C_∞ parameter to half the cone angle such that a large value of C_∞ corresponds to a small cone angle. It can be remarked that the transition state dipole moments for DiI-C18(5) and for BNP are not oriented along the lipid tails of the respective membranes, which invalidates the ‘wobbling in a cone’ model [85].

When DiI-C18(5) is approximated to a rod which is oriented along the backbone of the probe, Kinoshita’s other model of ‘wobbling outside the cone’ could be considered [91], which describes a spatial angle which is avoided by the transition state dipole moment. The analysis of the spherical coordinates (See Figure S7) gives a limited range for the angle between the transition dipole moment and the z-axis, which would be natural for any model describing a wobbling motion, *as well as* for the movement in the plane of the membrane described by the angle φ . It is this hindrance in φ which invalidates the ‘wobbling outside the cone’ model as it assumes a free movement of the emission dipole moment for this angle. In the figure, it is also seen that the restriction of the motion of DiI-C18(5) in the plane is less severe for the L_d phases than it is for the S_o and L_o phase. These plots are disentangled in Figures S8 and S9, in which the densities for the individual movements along the φ and θ angles are given. All in all, for DOPC(L_d) and DPPC (L_d), the probe can move in the plane of the membrane over angles of 1.4 and 1.2 radians ($\sim 80^\circ$ and $\sim 70^\circ$), respectively. For DPPC (S_o) and SM:Chol (L_o), the range of φ amounts to 0.3 and 0.4 radians ($\sim 17^\circ$ and $\sim 22^\circ$), respectively. Discarding small artefacts due to a limited simulation time, these plots are found to be symmetric around 0° for φ and 90° for θ . For DiI-C18(5) embedded in SM:Chol, the theta angle is however exclusively restricted to the first quadrant.

Since the tails of the DiI-C18(5) probe can be compared to e.g. the two acyl chains of a DPPC lipid and making abstract of the flexibility of the upper bonds and the out-of-plane distortions of the upper dihedral angles in the tails, the tumbling motion of the backbone and therefore transition state dipole moment of DiI-C18(5) can be related to any wobbling motion of the neighboring lipids. The 3-dimensional movement of the transition state dipole moment is given in Figure 8, showing the specific and restricted movement of the dye up to a timescale of 100 ps. For DPPC (L_d), the movement of the probe can be read and a connection can be made with

the areas of high density in the plane of the molecule, as visualized by the angle ϕ in Figure S8. The transition dipole moment of the probe describes zones in time with periods of ~ 60 ns due to a rather constrained movement in phase with the neighboring lipids and exhibits herein a motion with a smaller solid angle. For DOPC, analogous solid areas are seen. For SM:Chol (L_o), the zones are described in ~ 75 ns, while for DPPC (S_o), this period increases to almost 90 ns.

Table 1 – Pre-exponential parameters β and rotational correlation time θ for DiI-C18(5) and BNP in the four considered environments. All rotational correlation times are given in ns.^a

		β_1	θ_1	β_2	θ_2	C_∞	$\langle \theta \rangle$
DiI-C18(5)	DOPC (L_d)	0.02	0.07	0.09	2.93	0.89	2.91
	DPPC (S_o)	0.02	0.05	0.02	2.67	0.97	2.63
	DPPC (L_d)	0.04	0.11	0.34	11.57	0.62	11.55
	SM:Chol (L_o)	0.47	0.06	0.41	0.48	0.12	0.43
BNP	DOPC (L_d)	0.06	0.39	0.27	24.69	0.65	24.60
	DPPC (S_o)	0.04	0.08	0.03	7.97	0.93	7.83
	DPPC (L_d)	0.11	0.49	0.45	19.31	0.41	19.20
	SM:Chol (L_o)	0.06	0.05	0.25	15.57	0.69	15.56

^a The mean correlation time $\langle \theta \rangle$ and the C_∞ are also reported.

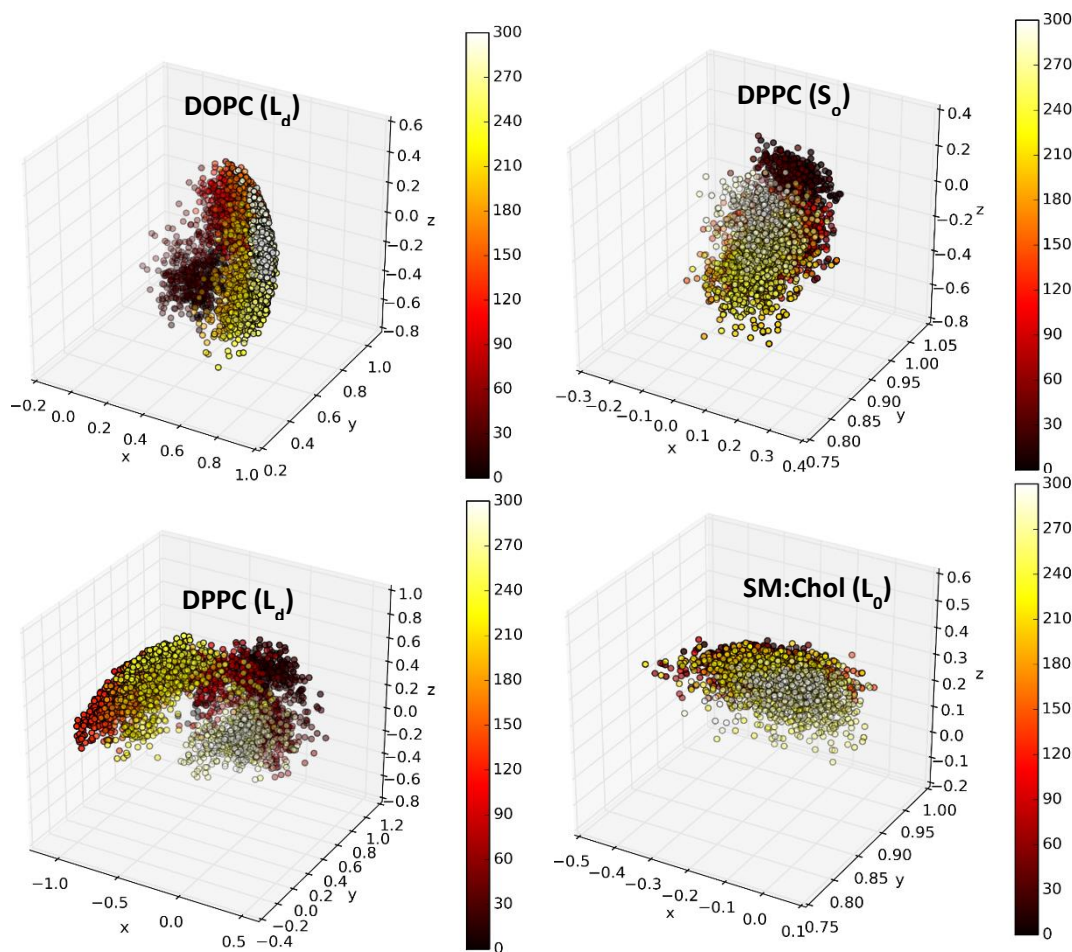


Figure 8: The movement of the transition state dipole moment vector of DiI-C18(5) along the MD trajectory. All vectors have been translated to the origin. One dot corresponds to 100 ps; the time runs from 0 ns (black) to 300 ns (white), as indicated by the color bar.

Conclusions and outlook

The behavior of BNP and DiI-C18(5) molecular probes was investigated in various lipid bilayers in three different phases. By means of demanding MD simulations, the Gibbs free energy profiles of both probes showed that they preferentially partition into the S_o phase of the DPPC bilayer rather than in the L_d phase of the DOPC bilayer. The L_o phase of a 2:1 SM:Chol mixture was also preferred with respect to the L_d phase.

The positions and orientations of the probes are primordial to anticipate their optical properties *in situ*, e.g., in biological membranes. The depths of insertion differ depending on the phase, and that relative to this, the probes in the SM:Chol mixture are stabilized more towards the polar head group region of the membrane. The orientation of the transition dipole moment is

very different with the two probes: for DiI-C18(5), the angle between the transition dipole moment and the z -axis in DOPC (L_d) is closer to a perfect 90° value than for the rather new probe BNP. A striking difference is however seen for the molecules in the DPPC (L_d) phase, for which the distribution of the angle ranges from 70° to 80° for DiI-C18(5), while for BNP it peaks at around 85° . From investigations of the membrane density and supported by simulations of the fluorescence anisotropy, it follows that in the SM:Chol (L_o) phase, a high amount of water molecules is found in the vicinity of the probes and that the embedded probes are less restricted in their movement than when they are surrounded by the other membrane phases.

Although the blue fluorescing BNP probe has been introduced as an alternative for the older yellow DiI-C18(5) one, it has been proven that they may behave differently with respect to their interaction with membranes. It is expected that the differences in position and orientation in various biological membranes will affect the linear and more the non-linear absorption spectra. The current research opens therefore a gateway towards a better investigation of the properties of biological membranes and tissues using nonlinear and fluorescent properties of selective molecular probes.

Supplementary information

Area per lipid along the simulated trajectory for the various membranes; order-parameters for the various membrane phases for the sn-1 and sn-2 tails; illustrations of the DiI-C18(5) and BNP probes in the different environments under investigation in the current study; radial distribution functions of DiI-C18(5) and surrounding water molecules for the considered membranes; angle with the z -axis of the vector described by the first and fifteenth carbon atom of the acyl tails of DiI; distribution of the vector of the transition dipole moment in spherical coordinates θ and φ ; density plots for the vector of the transition dipole moment in function of the azimuthal angle φ ; density plots for the vector of the transition dipole moment in function of the angle θ , .itp-files for DiI-C18(5) and BNP.

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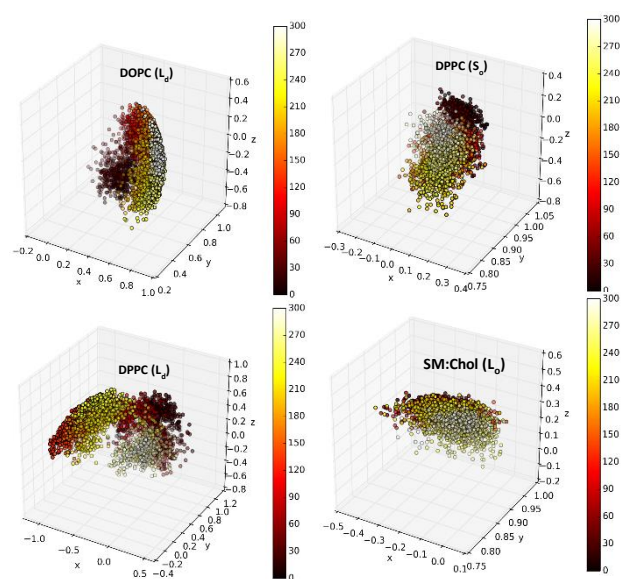
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TOC:



Movement of Dil-C18(5)

**Atomistic picture of fluorescent probes with hydrocarbon tails in lipid bilayer membranes:
an investigation of selective affinities and fluorescent anisotropies in different
environmental phases**

Supplementary information

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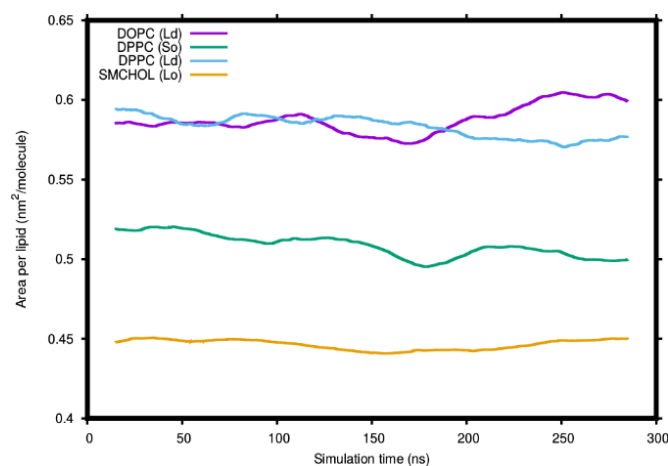


Figure S1: Area per lipid (given in nm²/molecule) along the simulated trajectory for the various membranes. The moving average is given using 30 terms.

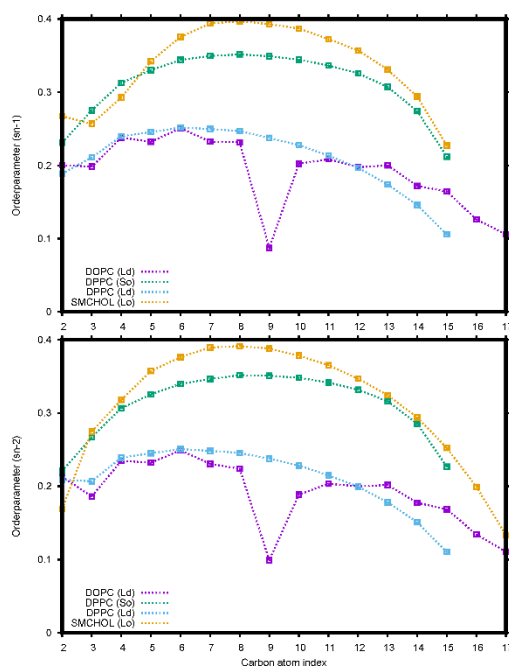


Figure S2: Order-parameters $|S_{cd}|$ for the various membrane phases for the sn-1 tail (top) and the sn-2 tail (bottom). The CH₂ groups are numbered consecutively from 2 to the end (18 for DOPC and Sphingomyelin, and 16 for DPPC). Number 1 is the carbonyl carbon. Due to the nature of $|S_{cd}|$, the value for it as well as for the terminal CH₃ group cannot be obtained. The sn-2 tail can be distinguished as it is attached to the middle carbon of the glycerol backbone.

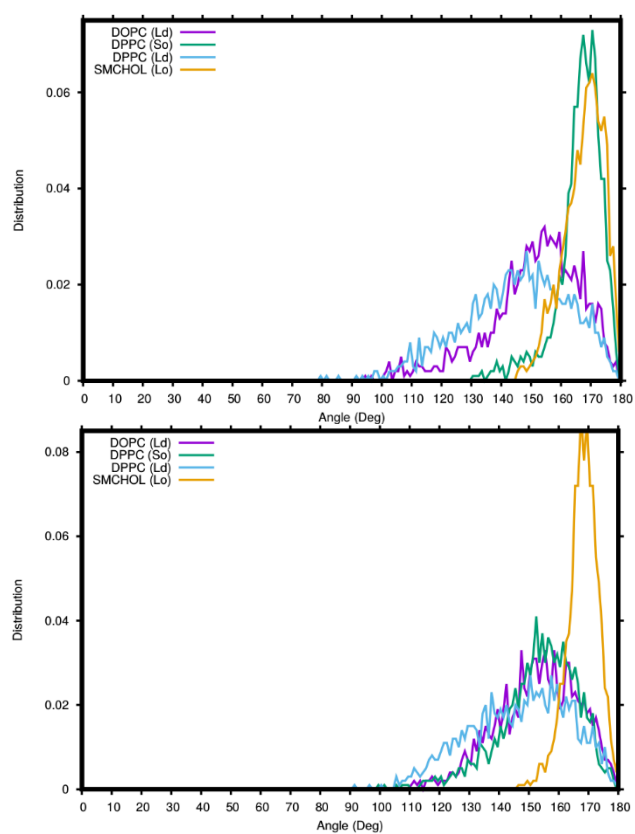


Figure S3: The angle with the z -axis of the vector described by the first and fifteenth carbon atom of the acyl tails of DiI. The two tails (top and down part of the figure) are separated out for clarity.

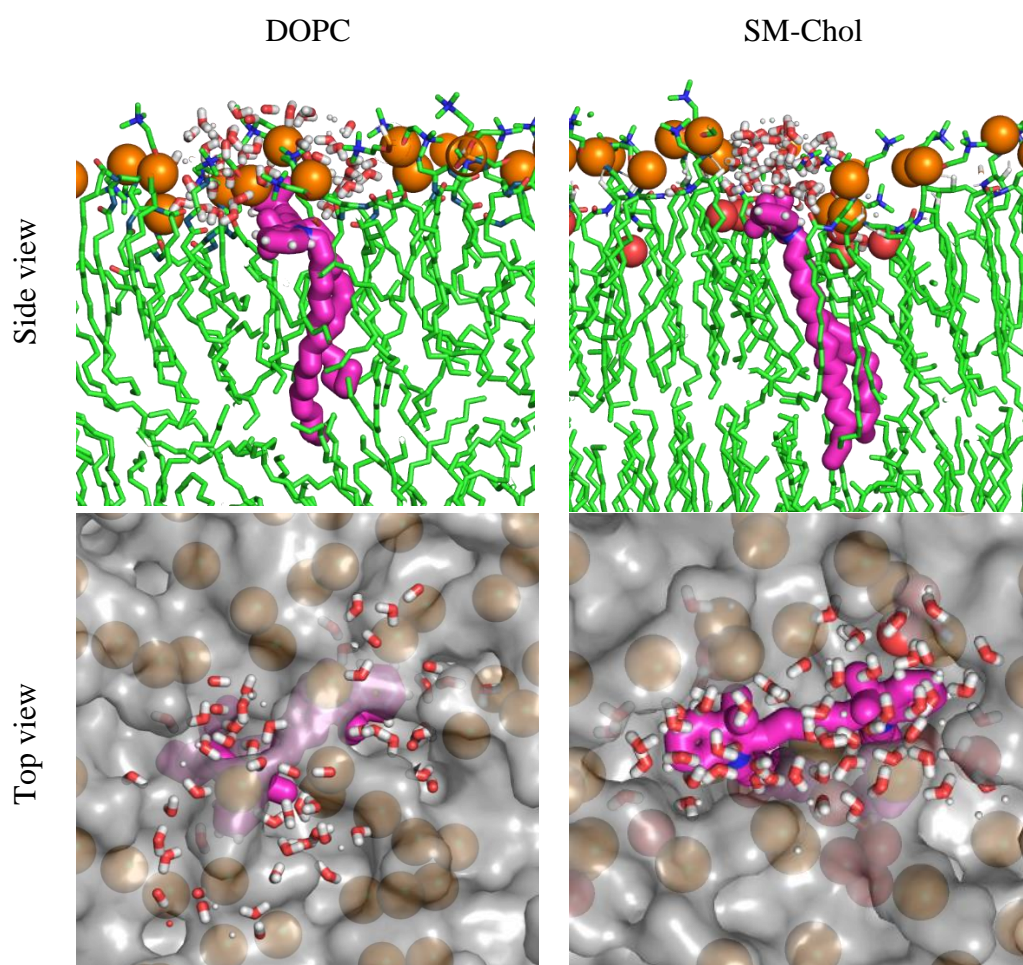


Figure S4: Side view and top view of final frames of free simulation of DiI-C18(5) in DOPC and SM/Chol membranes with highlighted surrounding water molecules. The DiI-C18(5) molecules are shown in magenta sticks, water within 8 Å are shown as red and white sticks, phosphates are displayed as orange balls, oxygen of cholesterol as red balls. In side view, lipid molecules are shown as green sticks (green – carbon, blue – nitrogen, red – oxygen), in top view lipids are displayed as semi-transparent grey surface. In both cases, DiI-C18(5) is in contact with lipid head groups, but in DOPC lipid head groups cover and hinder DiI, in SM/Chol DiI is in direct contact with bulk water and is therefore less hindered by lipid head groups.

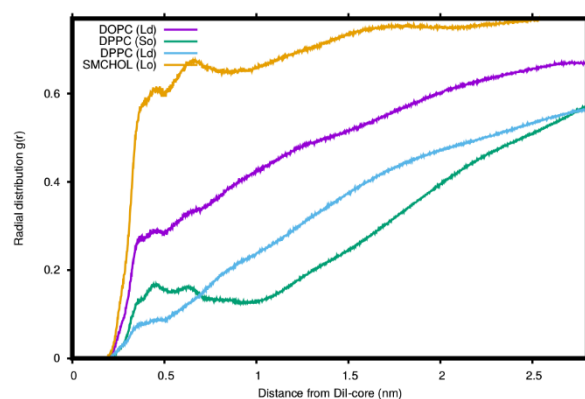


Figure S5: Radial distribution functions of the core of the DiI probe and surrounding water molecules for the various membrane phases.

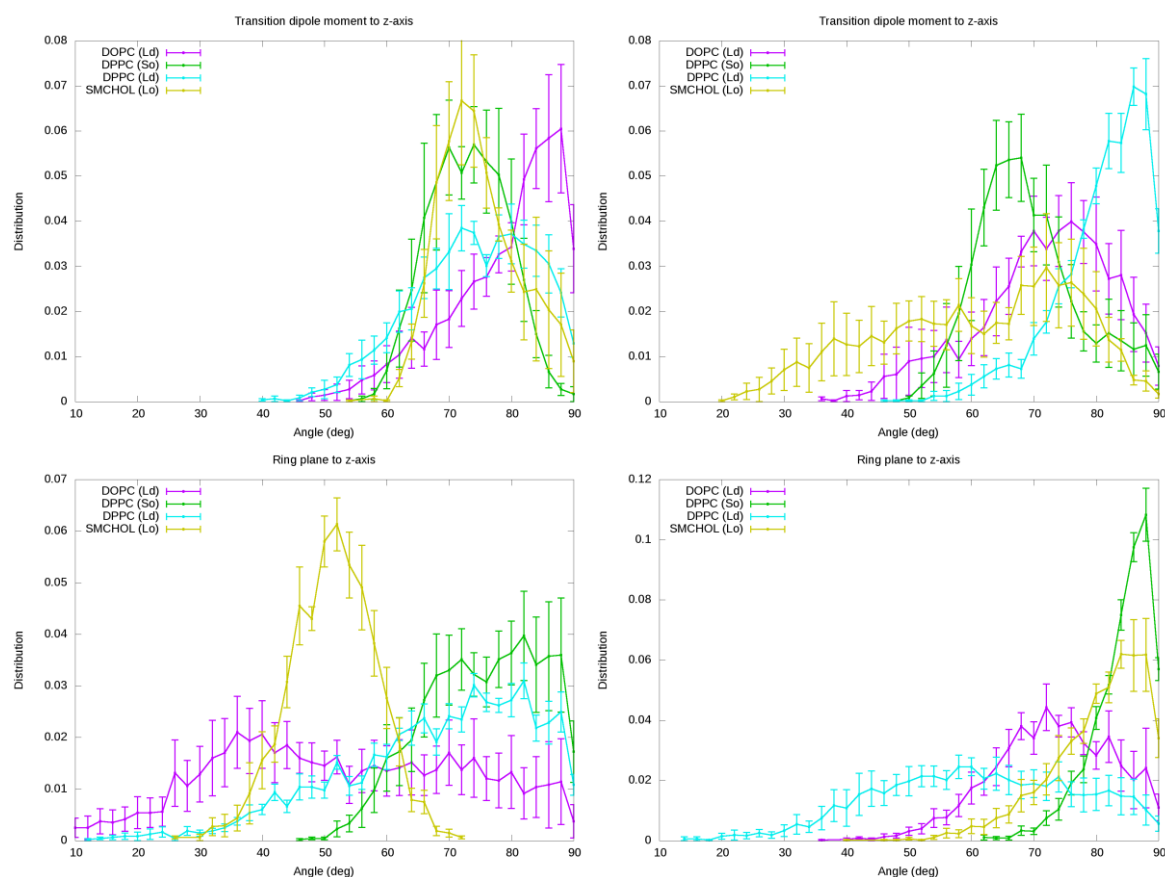


Figure S6: Top: Distribution of the angle between the transition dipole moment and the z-axis in various membrane phases; Bottom: angle between the axis perpendicular to the plane of the DiI-C18(5) (left) or BNP (right) molecule and the z-axis. Smoothed and rescaled plots are displayed in Figures 5 and 7. To obtain the error bars, the distribution plots were calculated for every 40 ns of simulations with bins of 2° (discarding the first 40 ns of the simulations). For each bin, the average and standard error were calculated.

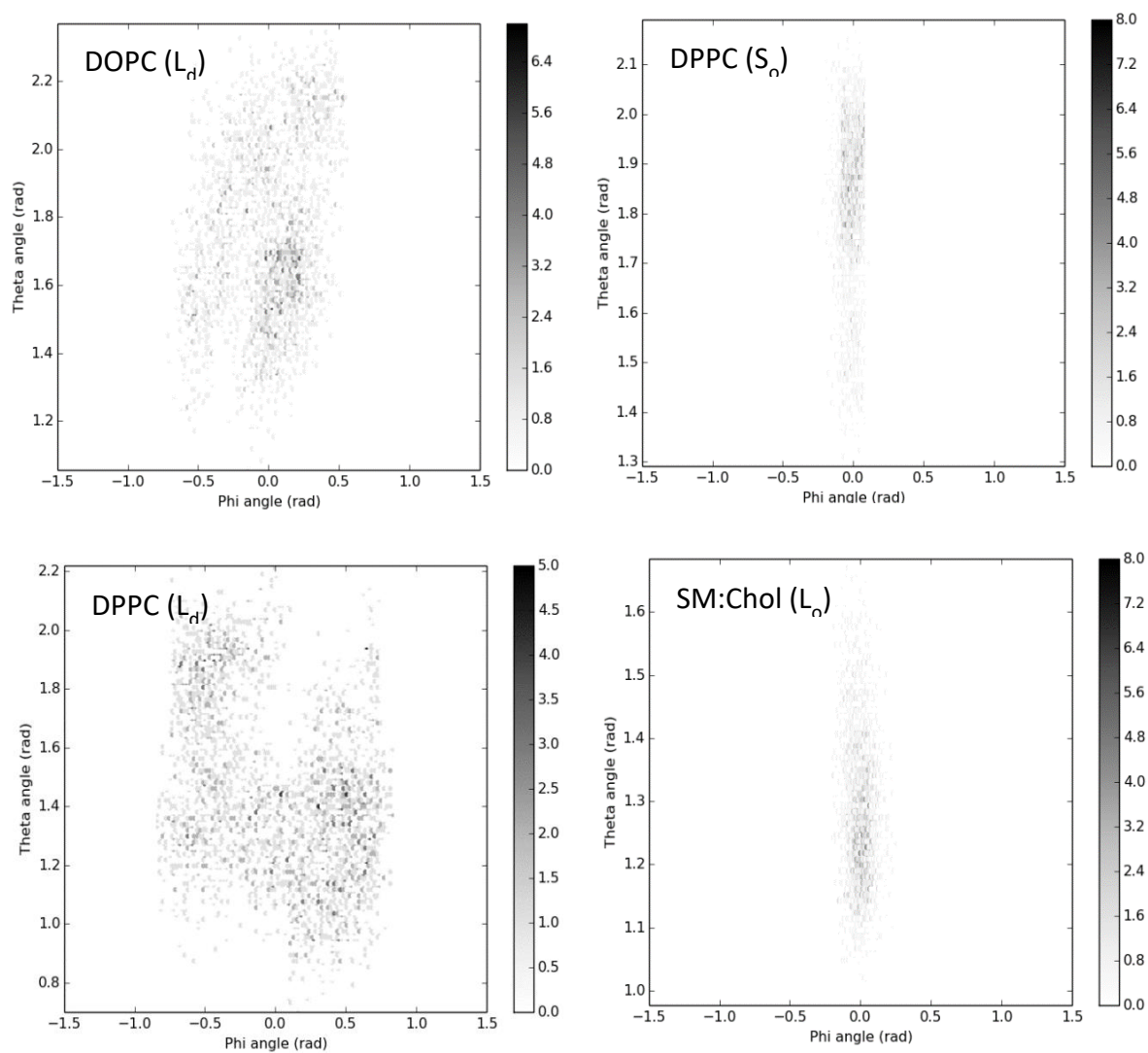


Figure S7: Distribution of the vector of the transition dipole moment in spherical coordinates θ and φ for the four membranes. θ is the angle with the z-axis of the membrane, while φ denotes the angle in the plane of the membrane.

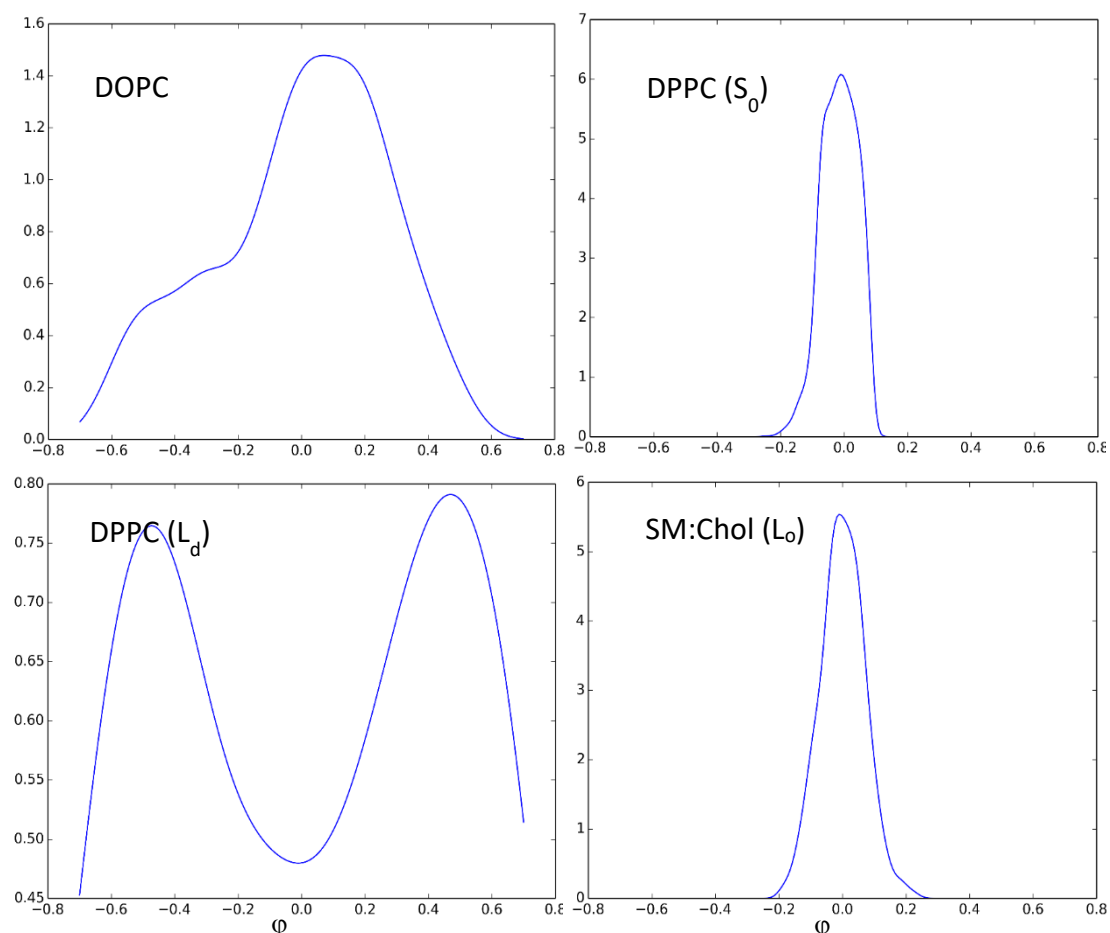


Figure S8: Density plots for the vector of the transition dipole moment in function of the azimuthal angle ϕ for the four membranes. It denotes the angle in the plane of the membrane in radians. Since the x-axis in the plane of the membrane (to which is referred by the ϕ -angle) is not uniquely defined, in these plots 0 radians is taken as the midpoint of the sampled angles. For DOPC, the two symmetric peaks are covered within the maximum at $\phi=0.0$ and 0.2 radians.

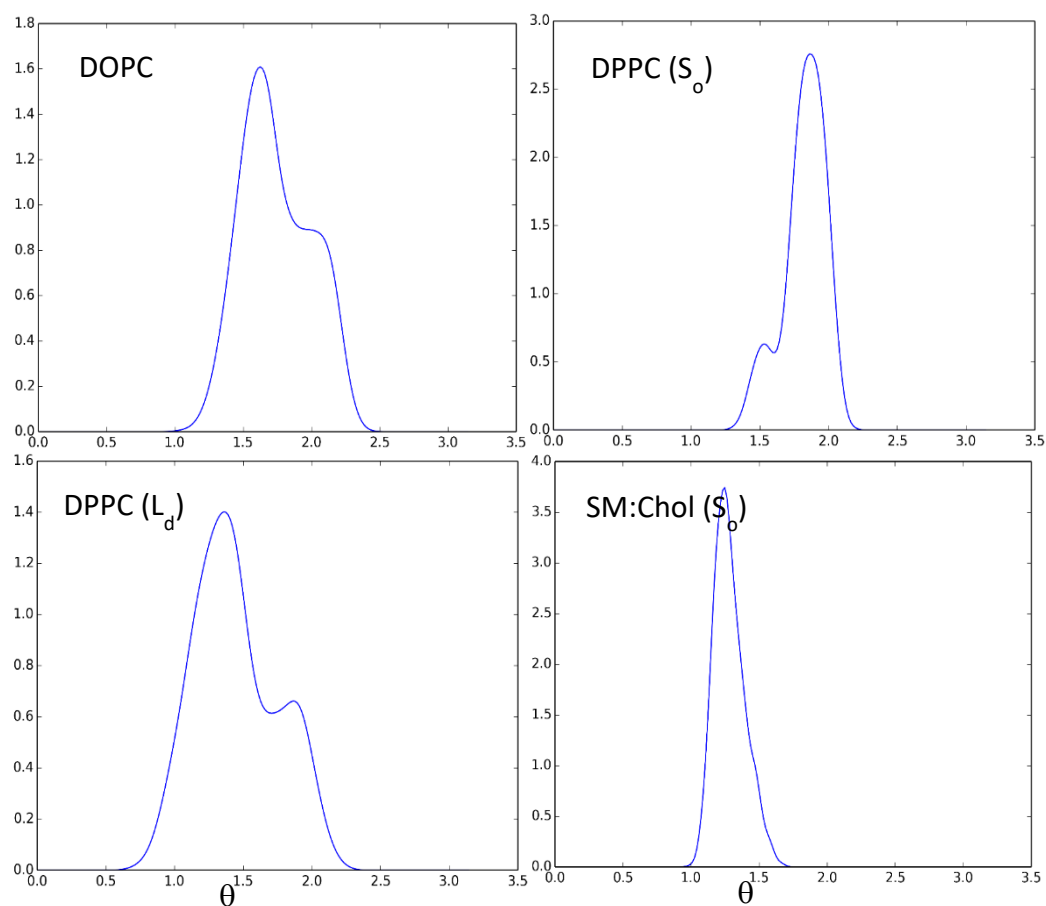


Figure S9: Density plots for the vector of the transition dipole moment in function of the angle θ for the four membranes. It denotes the angle between the transition dipole moment and the z-axis of the membrane in radians. The first peak for DPPC (S_o) (at $\theta \approx 1.4$ rad) and the second peak for DPPC (L_d) (at $\theta \approx 1.9$ rad) are less expressed due to a limited simulation time.

Table S1: dii.itp

```
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SCRF(IEFPCM,Solvent=diethylether)
;
;   This file was generated by PRODRG version AA100323.0717
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;   Acta Crystallogr. D60, 1355--1363.
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LIG      3
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2	CH2	1	LIG	CBR	1	0.0271	14.0270
3	CH2	1	LIG	CBQ	1	0.0076	14.0270
4	CH2	1	LIG	CBP	1	-0.0071	14.0270
5	CH2	1	LIG	CBO	1	-0.0019	14.0270
6	CH2	1	LIG	CBN	1	0.0056	14.0270
7	CH2	1	LIG	CBM	1	-0.0004	14.0270
8	CH2	1	LIG	CBL	2	-0.0012	14.0270
9	CH2	1	LIG	CBK	2	0.0004	14.0270
10	CH2	1	LIG	CBJ	2	0.0035	14.0270
11	CH2	1	LIG	CBI	2	-0.0004	14.0270
12	CH2	1	LIG	CBH	3	-0.0011	14.0270
13	CH2	1	LIG	CBG	3	0.0071	14.0270
14	CH2	1	LIG	CBF	3	0.0109	14.0270
15	CH2	1	LIG	CBE	3	0.0007	14.0270
16	CH2	1	LIG	CBD	3	0.0068	14.0270
17	CH2	1	LIG	CBC	4	0.0932	14.0270
18	CH2	1	LIG	CBB	4	0.1068	14.0270
19	NR	1	LIG	NBA	4	0.0572	14.0067
20	C	1	LIG	CAT	4	0.0180	12.0110
21	CR1	1	LIG	CAV	4	-0.1198	12.0110
22	HC	1	LIG	HAV	4	0.1042	1.0080
23	CR1	1	LIG	CAX	4	-0.1396	12.0110
24	HC	1	LIG	HAX	4	0.1405	1.0080
25	CR1	1	LIG	CAW	4	-0.1166	12.0110
26	HC	1	LIG	HAW	4	0.1311	1.0080
27	CR1	1	LIG	CAU	5	-0.1906	12.0110
28	HC	1	LIG	HAU	5	0.1593	1.0080
29	C	1	LIG	CAS	5	0.0317	12.0110
30	CH2	1	LIG	CAR	5	0.1061	12.0110
31	CH3	1	LIG	CAY	5	0.0298	15.0350
32	CH3	1	LIG	CAZ	5	0.0276	15.0350
33	C	1	LIG	CAQ	6	0.0321	12.0110
34	C	1	LIG	CAP	6	-0.0785	13.0190
35	C	1	LIG	CAO	6	0.0792	13.0190
36	C	1	LIG	CAN	7	-0.0032	13.0190
37	C	1	LIG	CAM	7	0.0792	13.0190
38	C	1	LIG	CAL	7	-0.0711	13.0190
39	C	1	LIG	CAH	7	0.0323	12.0110
40	CH2	1	LIG	CAG	7	0.0942	12.0110
41	CH3	1	LIG	CAJ	7	0.0284	15.0350
42	CH3	1	LIG	CAK	7	0.0316	15.0350
43	C	1	LIG	CAD	8	0.0394	12.0110

44	CR1	1	LIG	CAE	8	-0.1866	12.0110
45	HC	1	LIG	HAE	8	0.1566	1.0080
46	CR1	1	LIG	CAF	8	-0.1278	12.0110
47	HC	1	LIG	HAF	8	0.1335	1.0080
48	CR1	1	LIG	CAA	8	-0.1302	12.0110
49	HC	1	LIG	HAA	8	0.1381	1.0080
50	CR1	1	LIG	CAB	8	-0.1192	12.0110
51	HC	1	LIG	HAB	8	0.1018	1.0080
52	C	1	LIG	CAC	8	0.0206	12.0110
53	NR	1	LIG	NAI	8	0.0540	14.0067
54	CH2	1	LIG	CBT	9	0.1048	14.0270
55	CH2	1	LIG	CBU	9	0.0926	14.0270
56	CH2	1	LIG	CBV	9	0.0074	14.0270
57	CH2	1	LIG	CBW	9	0.0013	14.0270
58	CH2	1	LIG	CBX	9	0.0101	14.0270
59	CH2	1	LIG	CBY	10	0.008	14.0270
60	CH2	1	LIG	CBZ	10	-0.0018	14.0270
61	CH2	1	LIG	CCA	10	0.0006	14.0270
62	CH2	1	LIG	CCB	10	0.0026	14.0270
63	CH2	1	LIG	CCC	10	0.0013	14.0270
64	CH2	1	LIG	CCD	10	-0.0018	14.0270
65	CH2	1	LIG	CCE	11	0.0002	14.0270
66	CH2	1	LIG	CCF	11	0.0048	14.0270
67	CH2	1	LIG	CCG	11	-0.0006	14.0270
68	CH2	1	LIG	CCH	11	-0.0088	14.0270
69	CH2	1	LIG	CCI	11	0.0079	14.0270
70	CH2	1	LIG	CCJ	11	0.0259	14.0270
71	CH3	1	LIG	CCK	11	-0.0269	15.0350

[bonds]

```
; ai aj fu c0, c1, ...
 2 1 2 0.153 7150000.0 0.153 7150000.0 ; CBR CBS
 2 3 2 0.153 7150000.0 0.153 7150000.0 ; CBR CBQ
 3 4 2 0.153 7150000.0 0.153 7150000.0 ; CBQ CBP
 4 5 2 0.153 7150000.0 0.153 7150000.0 ; CBP CBO
 5 6 2 0.153 7150000.0 0.153 7150000.0 ; CBO CBN
 6 7 2 0.153 7150000.0 0.153 7150000.0 ; CBN CBM
 7 8 2 0.153 7150000.0 0.153 7150000.0 ; CBM CBL
 8 9 2 0.153 7150000.0 0.153 7150000.0 ; CBL CBK
 9 10 2 0.153 7150000.0 0.153 7150000.0 ; CBK CBJ
10 11 2 0.153 7150000.0 0.153 7150000.0 ; CBJ CBI
11 12 2 0.153 7150000.0 0.153 7150000.0 ; CBI CBH
12 13 2 0.153 7150000.0 0.153 7150000.0 ; CBH CBG
13 14 2 0.153 7150000.0 0.153 7150000.0 ; CBG CBF
14 15 2 0.153 7150000.0 0.153 7150000.0 ; CBF CBE
15 16 2 0.153 7150000.0 0.153 7150000.0 ; CBE CBD
16 17 2 0.153 7150000.0 0.153 7150000.0 ; CBD CBC
17 18 2 0.153 7150000.0 0.153 7150000.0 ; CBC CBB
19 18 2 0.148 5730000.0 0.148 5730000.0 ; NBA CBB
19 20 2 0.133 11800000.0 0.133 11800000.0 ; NBA CAT
19 33 2 0.133 11800000.0 0.133 11800000.0 ; NBA CAQ
20 21 2 0.133 11800000.0 0.133 11800000.0 ; CAT CAV
20 29 2 0.133 11800000.0 0.133 11800000.0 ; CAT CAS
21 22 2 0.109 12300000.0 0.109 12300000.0 ; CAV HAV
21 23 2 0.139 10800000.0 0.139 10800000.0 ; CAV CAX
23 24 2 0.109 12300000.0 0.109 12300000.0 ; CAX HAX
23 25 2 0.139 10800000.0 0.139 10800000.0 ; CAX CAW
25 26 2 0.109 12300000.0 0.109 12300000.0 ; CAW HAW
25 27 2 0.139 10800000.0 0.139 10800000.0 ; CAW CAU
27 28 2 0.109 12300000.0 0.109 12300000.0 ; CAU HAU
29 27 2 0.133 11800000.0 0.133 11800000.0 ; CAS CAU
30 29 2 0.139 8660000.0 0.139 8660000.0 ; CAR CAS
30 31 2 0.153 7150000.0 0.153 7150000.0 ; CAR CAY
30 32 2 0.153 7150000.0 0.153 7150000.0 ; CAR CAZ
30 33 2 0.139 8660000.0 0.139 8660000.0 ; CAR CAQ
33 34 2 0.133 11800000.0 0.133 11800000.0 ; CAQ CAP
34 35 2 0.153 7150000.0 0.153 7150000.0 ; CAP CAO
35 36 2 0.153 7150000.0 0.153 7150000.0 ; CAO CAN
36 37 2 0.153 7150000.0 0.153 7150000.0 ; CAN CAM
```

37	38	2	0.153	7150000.0	0.153	7150000.0	;	CAM	CAL
39	38	2	0.133	11800000.0	0.133	11800000.0	;	CAH	CAL
40	39	2	0.139	8660000.0	0.139	8660000.0	;	CAG	CAH
39	53	2	0.133	11800000.0	0.133	11800000.0	;	CAH	NAI
40	41	2	0.153	7150000.0	0.153	7150000.0	;	CAG	CAJ
40	42	2	0.153	7150000.0	0.153	7150000.0	;	CAG	CAK
40	43	2	0.139	8660000.0	0.139	8660000.0	;	CAG	CAD
43	44	2	0.133	11800000.0	0.133	11800000.0	;	CAD	CAE
43	52	2	0.133	11800000.0	0.133	11800000.0	;	CAD	CAC
44	45	2	0.109	12300000.0	0.109	12300000.0	;	CAE	HAE
44	46	2	0.139	10800000.0	0.139	10800000.0	;	CAE	CAF
46	47	2	0.109	12300000.0	0.109	12300000.0	;	CAF	HAF
46	48	2	0.139	10800000.0	0.139	10800000.0	;	CAF	CAA
48	49	2	0.109	12300000.0	0.109	12300000.0	;	CAA	HAA
48	50	2	0.139	10800000.0	0.139	10800000.0	;	CAA	CAB
50	51	2	0.109	12300000.0	0.109	12300000.0	;	CAB	HAB
52	50	2	0.133	11800000.0	0.133	11800000.0	;	CAC	CAB
52	53	2	0.133	11800000.0	0.133	11800000.0	;	CAC	NAI
53	54	2	0.148	5730000.0	0.148	5730000.0	;	NAI	CBT
54	55	2	0.153	7150000.0	0.153	7150000.0	;	CBT	CBU
55	56	2	0.153	7150000.0	0.153	7150000.0	;	CBU	CBV
56	57	2	0.153	7150000.0	0.153	7150000.0	;	CBV	CBW
57	58	2	0.153	7150000.0	0.153	7150000.0	;	CBW	CBX
58	59	2	0.153	7150000.0	0.153	7150000.0	;	CBX	CBY
59	60	2	0.153	7150000.0	0.153	7150000.0	;	CBY	CBZ
60	61	2	0.153	7150000.0	0.153	7150000.0	;	CBZ	CCA
61	62	2	0.153	7150000.0	0.153	7150000.0	;	CCA	CCB
62	63	2	0.153	7150000.0	0.153	7150000.0	;	CCB	CCC
63	64	2	0.153	7150000.0	0.153	7150000.0	;	CCC	CCD
64	65	2	0.153	7150000.0	0.153	7150000.0	;	CCD	CCE
65	66	2	0.153	7150000.0	0.153	7150000.0	;	CCE	CCF
66	67	2	0.153	7150000.0	0.153	7150000.0	;	CCF	CCG
67	68	2	0.153	7150000.0	0.153	7150000.0	;	CCG	CCH
68	69	2	0.153	7150000.0	0.153	7150000.0	;	CCH	CCI
69	70	2	0.153	7150000.0	0.153	7150000.0	;	CCI	CCJ
70	71	2	0.153	7150000.0	0.153	7150000.0	;	CCJ	CCK


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[ pairs ]
; ai aj fu c0, c1, ...
  1  4  1 ; CBS CBP
  2  5  1 ; CBR CBO
  3  6  1 ; CBQ CBN
  4  7  1 ; CBP CBM
  5  8  1 ; CBO CBL
  6  9  1 ; CBN CBK
  7 10  1 ; CBM CBJ
  8 11  1 ; CBL CBI
  9 12  1 ; CBK CBH
10 13  1 ; CBJ CBG
11 14  1 ; CBI CBF
12 15  1 ; CBH CBE
13 16  1 ; CBG CBD
14 17  1 ; CBF CBC
15 18  1 ; CBE CBB
16 19  1 ; CBD NBA
17 20  1 ; CBC CAT
17 33  1 ; CBC CAQ
18 21  1 ; CBB CAV
18 29  1 ; CBB CAS
18 30  1 ; CBB CAR
18 34  1 ; CBB CAP
19 22  1 ; NBA HAV
19 23  1 ; NBA CAX
19 27  1 ; NBA CAU
19 31  1 ; NBA CAY
19 32  1 ; NBA CAZ
19 35  1 ; NBA CAO
20 24  1 ; CAT HAX
20 25  1 ; CAT CAW

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20	28	1	;	CAT	HAU
20	31	1	;	CAT	CAY
20	32	1	;	CAT	CAZ
20	34	1	;	CAT	CAP
21	26	1	;	CAV	HAW
21	27	1	;	CAV	CAU
21	30	1	;	CAV	CAR
21	33	1	;	CAV	CAQ
22	24	1	;	HAV	HAX
22	25	1	;	HAV	CAW
22	29	1	;	HAV	CAS
23	28	1	;	CAX	HAU
23	29	1	;	CAX	CAS
24	26	1	;	HAX	HAW
24	27	1	;	HAX	CAU
25	30	1	;	CAW	CAR
26	28	1	;	HAW	HAU
26	29	1	;	HAW	CAS
27	31	1	;	CAU	CAY
27	32	1	;	CAU	CAZ
27	33	1	;	CAU	CAQ
28	30	1	;	HAU	CAR
29	34	1	;	CAS	CAP
30	35	1	;	CAR	CAO
31	34	1	;	CAY	CAP
32	34	1	;	CAZ	CAP
33	36	1	;	CAQ	CAN
34	37	1	;	CAP	CAM
35	38	1	;	CAO	CAL
36	39	1	;	CAN	CAH
37	40	1	;	CAM	CAG
37	53	1	;	CAM	NAI
38	41	1	;	CAL	CAJ
38	42	1	;	CAL	CAK
38	43	1	;	CAL	CAD
38	52	1	;	CAL	CAC
38	54	1	;	CAL	CBT
39	44	1	;	CAH	CAE
39	50	1	;	CAH	CAB
39	55	1	;	CAH	CBU
40	45	1	;	CAG	HAE
40	46	1	;	CAG	CAF
40	50	1	;	CAG	CAB
40	54	1	;	CAG	CBT
41	44	1	;	CAJ	CAE
41	52	1	;	CAJ	CAC
41	53	1	;	CAJ	NAI
42	44	1	;	CAK	CAE
42	52	1	;	CAK	CAC
42	53	1	;	CAK	NAI
43	47	1	;	CAD	HAF
43	48	1	;	CAD	CAA
43	51	1	;	CAD	HAB
43	54	1	;	CAD	CBT
44	49	1	;	CAE	HAA
44	50	1	;	CAE	CAB
44	53	1	;	CAE	NAI
45	47	1	;	HAE	HAF
45	48	1	;	HAE	CAA
45	52	1	;	HAE	CAC
46	51	1	;	CAF	HAB
46	52	1	;	CAF	CAC
47	49	1	;	HAF	HAA
47	50	1	;	HAF	CAB
48	53	1	;	CAA	NAI
49	51	1	;	HAA	HAB
49	52	1	;	HAA	CAC
50	54	1	;	CAB	CBT
51	53	1	;	HAB	NAI

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52 55 1
53 56 1
54 57 1
55 58 1
56 59 1
57 60 1
58 61 1
59 62 1
60 63 1
61 64 1
62 65 1
63 66 1
64 67 1
65 68 1
66 69 1
67 70 1
68 71 1

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; CAC CBU
; NAI CBV
; CBT CBW
; CBU CBX
; CBV CBY
; CBW CBZ
; CBX CCA
; CBY CCB
; CBZ CCC
; CCA CCD
; CCB CCE
; CCC CCF
; CCD CCG
; CCE CCH
; CCF CCI
; CCG CCJ
; CCH CCK

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[ angles ]
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; ai aj ak fu c0, c1, ...
1 2 3 2 109.5 520.0 109.5 520.0 ; CBS CBR CBQ
2 3 4 2 109.5 520.0 109.5 520.0 ; CBR CBQ CBP
3 4 5 2 109.5 520.0 109.5 520.0 ; CBQ CBP CBO
4 5 6 2 109.5 520.0 109.5 520.0 ; CBP CBO CBN
5 6 7 2 109.5 520.0 109.5 520.0 ; CBO CBN CBM
6 7 8 2 109.5 520.0 109.5 520.0 ; CBN CBM CBL
7 8 9 2 109.5 520.0 109.5 520.0 ; CBM CBL CBK
8 9 10 2 109.5 520.0 109.5 520.0 ; CBL CBK CBJ
9 10 11 2 109.5 520.0 109.5 520.0 ; CBK CBJ CBI
10 11 12 2 109.5 520.0 109.5 520.0 ; CBJ CBI CBH
11 12 13 2 109.5 520.0 109.5 520.0 ; CBI CBH CBG
12 13 14 2 109.5 520.0 109.5 520.0 ; CBH CBG CBF
13 14 15 2 109.5 520.0 109.5 520.0 ; CBG CBF CBE
14 15 16 2 109.5 520.0 109.5 520.0 ; CBF CBE CBD
15 16 17 2 109.5 520.0 109.5 520.0 ; CBE CBD CBC
16 17 18 2 109.5 520.0 109.5 520.0 ; CBD CBC CBB
17 18 19 2 111.0 530.0 111.0 530.0 ; CBC CBB NBA
18 19 20 2 125.0 375.0 125.0 375.0 ; CBB NBA CAT
18 19 33 2 125.0 375.0 125.0 375.0 ; CBB NBA CAQ
20 19 33 2 108.0 465.0 108.0 465.0 ; CAT NBA CAQ
19 20 21 2 132.0 760.0 132.0 760.0 ; NBA CAT CAV
19 20 29 2 108.0 465.0 108.0 465.0 ; NBA CAT CAS
21 20 29 2 120.0 560.0 120.0 560.0 ; CAV CAT CAS
20 21 22 2 120.0 505.0 120.0 505.0 ; CAT CAV HAV
20 21 23 2 120.0 505.0 120.0 505.0 ; CAT CAV CAX
22 21 23 2 120.0 505.0 120.0 505.0 ; HAV CAV CAX
21 23 24 2 120.0 505.0 120.0 505.0 ; CAV CAX HAX
21 23 25 2 120.0 505.0 120.0 505.0 ; CAV CAX CAW
24 23 25 2 120.0 505.0 120.0 505.0 ; HAX CAX CAW
23 25 26 2 120.0 505.0 120.0 505.0 ; CAX CAW HAW
23 25 27 2 120.0 505.0 120.0 505.0 ; CAX CAW CAU
26 25 27 2 120.0 505.0 120.0 505.0 ; HAW CAW CAU
25 27 28 2 120.0 505.0 120.0 505.0 ; CAW CAU HAU
25 27 29 2 120.0 505.0 120.0 505.0 ; CAW CAU CAS
28 27 29 2 120.0 505.0 120.0 505.0 ; HAU CAU CAS
20 29 27 2 120.0 560.0 120.0 560.0 ; CAT CAS CAU
20 29 30 2 108.0 465.0 108.0 465.0 ; CAT CAS CAR
27 29 30 2 132.0 760.0 132.0 760.0 ; CAU CAS CAR
29 30 31 2 109.5 520.0 109.5 520.0 ; CAS CAR CAY
29 30 32 2 109.5 520.0 109.5 520.0 ; CAS CAR CAZ
29 30 33 2 104.0 444.4 104.0 444.4 ; CAS CAR CAQ
31 30 32 2 109.5 520.0 109.5 520.0 ; CAY CAR CAZ
31 30 33 2 109.5 520.0 109.5 520.0 ; CAY CAR CAQ
32 30 33 2 109.5 520.0 109.5 520.0 ; CAZ CAR CAQ
19 33 30 2 108.0 465.0 108.0 465.0 ; NBA CAQ CAR
19 33 34 2 120.0 560.0 120.0 560.0 ; NBA CAQ CAP
30 33 34 2 120.0 560.0 120.0 560.0 ; CAR CAQ CAP
33 34 35 2 115.0 610.0 115.0 610.0 ; CAQ CAP CAO
34 35 36 2 115.0 610.0 115.0 610.0 ; CAP CAO CAN

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52	53	50	43	2	0.0	167.4	0.0	167.4	; imp	CAC	NAI	CAB	CAD
53	54	52	39	2	0.0	167.4	0.0	167.4	; imp	NAI	CBT	CAC	CAH
43	44	46	48	2	0.0	209.3	0.0	209.3	; imp	CAD	CAE	CAF	CAA
44	46	48	50	2	0.0	209.3	0.0	209.3	; imp	CAE	CAF	CAA	CAB
46	48	50	52	2	0.0	209.3	0.0	209.3	; imp	CAF	CAA	CAB	CAC
48	50	52	43	2	0.0	209.3	0.0	209.3	; imp	CAA	CAB	CAC	CAD
50	52	43	44	2	0.0	209.3	0.0	209.3	; imp	CAB	CAC	CAD	CAE
52	43	44	46	2	0.0	209.3	0.0	209.3	; imp	CAC	CAD	CAE	CAF
20	21	23	25	2	0.0	209.3	0.0	209.3	; imp	CAT	CAV	CAX	CAW
21	23	25	27	2	0.0	209.3	0.0	209.3	; imp	CAV	CAX	CAW	CAU
23	25	27	29	2	0.0	209.3	0.0	209.3	; imp	CAX	CAW	CAU	CAS
25	27	29	20	2	0.0	209.3	0.0	209.3	; imp	CAW	CAU	CAS	CAT
27	29	20	21	2	0.0	209.3	0.0	209.3	; imp	CAU	CAS	CAT	CAV
29	20	21	23	2	0.0	209.3	0.0	209.3	; imp	CAS	CAT	CAV	CAX
4	3	2	1	1	0.0	5.9 3	0.0	5.9 3	; dih	CBP	CBQ	CBR	CBS
5	4	3	2	1	0.0	5.9 3	0.0	5.9 3	; dih	CBO	CBP	CBQ	CBR
6	5	4	3	1	0.0	5.9 3	0.0	5.9 3	; dih	CBN	CBO	CBP	CBQ
7	6	5	4	1	0.0	5.9 3	0.0	5.9 3	; dih	CBM	CBN	CBO	CBP
8	7	6	5	1	0.0	5.9 3	0.0	5.9 3	; dih	CBL	CBM	CBN	CBO
9	8	7	6	1	0.0	5.9 3	0.0	5.9 3	; dih	CBK	CBL	CBM	CBN
10	9	8	7	1	0.0	5.9 3	0.0	5.9 3	; dih	CBJ	CBK	CBL	CBM
11	10	9	8	1	0.0	5.9 3	0.0	5.9 3	; dih	CBI	CBJ	CBK	CBL
12	11	10	9	1	0.0	5.9 3	0.0	5.9 3	; dih	CBH	CBI	CBJ	CBK
13	12	11	10	1	0.0	5.9 3	0.0	5.9 3	; dih	CBG	CBH	CBI	CBJ
14	13	12	11	1	0.0	5.9 3	0.0	5.9 3	; dih	CBF	CBG	CBH	CBI
15	14	13	12	1	0.0	5.9 3	0.0	5.9 3	; dih	CBE	CBF	CBG	CBH
16	15	14	13	1	0.0	5.9 3	0.0	5.9 3	; dih	CBD	CBE	CBF	CBG
17	16	15	14	1	0.0	5.9 3	0.0	5.9 3	; dih	CBC	CBD	CBE	CBF
18	17	16	15	1	0.0	5.9 3	0.0	5.9 3	; dih	CBB	CBC	CBD	CBE
19	18	17	16	1	0.0	5.9 3	0.0	5.9 3	; dih	NBA	CBB	CBC	CBD
17	18	19	33	1	0.0	1.0 6	0.0	1.0 6	; dih	CBC	CBB	NBA	CAQ
29	20	19	18	1	180.0	33.5 2	180.0	33.5 2	; dih	CAS	CAT	NBA	CBB
34	33	19	18	1	180.0	33.5 2	180.0	33.5 2	; dih	CAP	CAQ	NBA	CBB
33	30	29	20	1	180.0	33.5 2	180.0	33.5 2	; dih	CAQ	CAR	CAS	CAT
set as double bond													
29	30	33	34	1	180.0	33.5 2	180.0	33.5 2	; dih	CAS	CAR	CAQ	CAP
set as double bond													
35	34	33	19	1	180.0	16.7 2	180.0	16.7 2	; dih	CAO	CAP	CAQ	NBA
gd_12 from RTOL													
36	35	34	33	1	180.0	33.5 2	180.0	33.5 2	; dih	CAN	CAO	CAP	CAQ
gd_14 from RTOL													
37	36	35	34	1	180.0	16.7 2	180.0	16.7 2	; dih	CAM	CAN	CAO	CAP
gd_12 from RTOL													
38	37	36	35	1	180.0	33.5 2	180.0	33.5 2	; dih	CAL	CAM	CAN	CAO
gd_14 from RTOL													
39	38	37	36	1	180.0	16.7 2	180.0	16.7 2	; dih	CAH	CAL	CAM	CAN
gd_12 from RTOL													
53	39	38	37	1	180.0	33.5 2	180.0	33.5 2	; dih	NAI	CAH	CAL	CAM
gd_14 from RTOL													
43	40	39	38	1	180.0	33.5 2	180.0	33.5 2	; dih	CAD	CAG	CAH	CAL
set as double bond													
38	39	53	54	1	180.0	33.5 2	180.0	33.5 2	; dih	CAL	CAH	NAI	CBT
set as double bond													
39	40	43	52	1	180.0	33.5 2	180.0	33.5 2	; dih	CAH	CAG	CAD	CAC
43	52	53	54	1	180.0	33.5 2	180.0	33.5 2	; dih	CAD	CAC	NAI	CBT
55	54	53	39	1	0.0	1.0 6	0.0	1.0 6	; dih	CBU	CBT	NAI	CAH
56	55	54	53	1	0.0	5.9 3	0.0	5.9 3	; dih	CBV	CBU	CBT	NAI
57	56	55	54	1	0.0	5.9 3	0.0	5.9 3	; dih	CBW	CBV	CBU	CBT
58	57	56	55	1	0.0	5.9 3	0.0	5.9 3	; dih	CBX	CBW	CBV	CBU
59	58	57	56	1	0.0	5.9 3	0.0	5.9 3	; dih	CBY	CBX	CBW	CBV
60	59	58	57	1	0.0	5.9 3	0.0	5.9 3	; dih	CBZ	CBY	CBX	CBW
61	60	59	58	1	0.0	5.9 3	0.0	5.9 3	; dih	CCA	CBZ	CBY	CBX
62	61	60	59	1	0.0	5.9 3	0.0	5.9 3	; dih	CCB	CCA	CBZ	CBY
63	62	61	60	1	0.0	5.9 3	0.0	5.9 3	; dih	CCC	CCB	CCA	CBZ
64	63	62	61	1	0.0	5.9 3	0.0	5.9 3	; dih	CCD	CCC	CCB	CCA
65	64	63	62	1	0.0	5.9 3	0.0	5.9 3	; dih	CCE	CCD	CCC	CCB
66	65	64	63	1	0.0	5.9 3	0.0	5.9 3	; dih	CCF	CCE	CCD	CCC
67	66	65	64	1	0.0	5.9 3	0.0	5.9 3	; dih	CCG	CCF	CCE	CCD
68	67	66	65	1	0.0	5.9 3	0.0	5.9 3	; dih	CCH	CCG	CCF	CCE

69	68	67	66	1	0.0	5.9	3	0.0	5.9	3 ; dih	CCI	CCH	CCG	CCF
70	69	68	67	1	0.0	5.9	3	0.0	5.9	3 ; dih	CCJ	CCI	CCH	CCG
71	70	69	68	1	0.0	5.9	3	0.0	5.9	3 ; dih	CCK	CCJ	CCI	CCH

Table S2: bodipy.itp

```
[ moleculetype ]
; Name nrexcl
_2      3

[ atoms ]
;  nr      type  resnr resid  atom  cgnr  charge  mass
  1         CH3    1    _2    CBM    1    0.000  15.0350
  2         CH2    1    _2    CBL    2    0.000  14.0270
  3         CH2    1    _2    CBK    3    0.000  14.0270
  4         CH2    1    _2    CBJ    4    0.000  14.0270
  5         CH2    1    _2    CBI    5    0.000  14.0270
  6         CH2    1    _2    CBH    6    0.000  14.0270
  7         CH2    1    _2    CBG    7    0.000  14.0270
  8         CH2    1    _2    CBF    8    0.000  14.0270
  9         CH2    1    _2    CBE    9    0.000  14.0270
 10         CH2    1    _2    CBD   10    0.000  14.0270
 11         CH2    1    _2    CBC   11    0.000  14.0270
 12         CH2    1    _2    CBB   12    0.000  14.0270
 13         CH2    1    _2    CBA   13    0.000  14.0270
 14         CH2    1    _2    CAZ   14    0.000  14.0270
 15         CH2    1    _2    CAY   15    0.000  14.0270
 16         CH2    1    _2    CAX   16    0.000  14.0270
 17         CH2    1    _2    CAW   17    0.000  14.0270
 18         CH2    1    _2    CAV   18    0.000  14.0270
 19          C     1    _2    CAH   19    0.280  12.0110
 20         CR1    1    _2    CAG   19   -0.311  12.0110
 21          HC     1    _2    HAG   19    0.170   1.0080
 22         CR1    1    _2    CAF   19   -0.242  12.0110
 23          HC     1    _2    HAF   19    0.182   1.0080
 24          C     1    _2    CAJ   19    0.044  12.0110
 25          C     1    _2    CAK   20    0.134  12.0110
 26          N     1    _2    NAO   20   -0.413  14.0067
 27          H     1    _2    HAO   20    0.287   1.0080
 28         CH2    1    _2    CAP   20    0.369  14.0270
 29         CH2    1    _2    CAQ   21   -0.106  14.0270
 30        SDMSO    1    _2    SAR   21    0.945  32.0600
 31          OM     1    _2    OAT   21   -0.613  15.9994 ; ODmso ?
 32          OM     1    _2    OAU   21   -0.613  15.9994 ; ODmso ?
 33          OM     1    _2    OAS   21   -0.613  15.9994 ; ODmso ?
 34          NR     1    _2    NAI   22   -0.179  14.0067
 35          B     1    _2    BAL   22    0.494  10.8110 ; boron
 36          F     1    _2    FAM   22   -0.354  18.9984
 37          F     1    _2    FAN   22   -0.354  18.9984
 38          NR     1    _2    NAE   22   -0.179  14.0067
 39          C     1    _2    CAC   23    0.044  12.0110
 40         CR1    1    _2    CAB   23   -0.242  12.0110
 41          HC     1    _2    HAB   23    0.182   1.0080
 42         CR1    1    _2    CAA   23   -0.311  12.0110
 43          HC     1    _2    HAA   23    0.170   1.0080
 44          C     1    _2    CAD   23    0.229  12.0110
 45         CH2    1    _2    CBN   24    0.000  14.0270
 46         CH2    1    _2    CBO   25    0.000  14.0270
 47         CH2    1    _2    CBP   26    0.000  14.0270
 48         CH2    1    _2    CBQ   27    0.000  14.0270
 49         CH2    1    _2    CBR   28    0.000  14.0270
 50         CH2    1    _2    CBS   29    0.000  14.0270
 51         CH2    1    _2    CBT   30    0.000  14.0270
```

52	CH2	1	2	CBU	31	0.000	14.0270
53	CH2	1	2	CBV	32	0.000	14.0270
54	CH2	1	2	CBW	33	0.000	14.0270
55	CH2	1	2	CBX	34	0.000	14.0270
56	CH2	1	2	CBY	35	0.000	14.0270
57	CH2	1	2	CBZ	36	0.000	14.0270
58	CH2	1	2	CCA	37	0.000	14.0270
59	CH2	1	2	CCB	38	0.000	14.0270
60	CH2	1	2	CCC	39	0.000	14.0270
61	CH2	1	2	CCD	40	0.000	14.0270
62	CH3	1	2	CCE	41	0.000	15.0350

[bonds]

```
; ai aj fu c0, c1, ...
  2  1  2  0.153  7150000.0  0.153  7150000.0 ; CBL CBM
  2  3  2  0.153  7150000.0  0.153  7150000.0 ; CBL CBK
  3  4  2  0.153  7150000.0  0.153  7150000.0 ; CBK CBJ
  4  5  2  0.153  7150000.0  0.153  7150000.0 ; CBJ CBI
  5  6  2  0.153  7150000.0  0.153  7150000.0 ; CBI CBH
  6  7  2  0.153  7150000.0  0.153  7150000.0 ; CBH CBG
  7  8  2  0.153  7150000.0  0.153  7150000.0 ; CBG CBF
  8  9  2  0.153  7150000.0  0.153  7150000.0 ; CBF CBE
  9 10  2  0.153  7150000.0  0.153  7150000.0 ; CBE CBD
10 11  2  0.153  7150000.0  0.153  7150000.0 ; CBD CBC
11 12  2  0.153  7150000.0  0.153  7150000.0 ; CBC CBB
12 13  2  0.153  7150000.0  0.153  7150000.0 ; CBB CBA
13 14  2  0.153  7150000.0  0.153  7150000.0 ; CBA CAZ
14 15  2  0.153  7150000.0  0.153  7150000.0 ; CAZ CAY
15 16  2  0.153  7150000.0  0.153  7150000.0 ; CAY CAX
16 17  2  0.153  7150000.0  0.153  7150000.0 ; CAX CAW
17 18  2  0.153  7150000.0  0.153  7150000.0 ; CAW CAV
19 18  2  0.153  7150000.0  0.153  7150000.0 ; CAH CAV
19 20  2  0.133 11800000.0  0.133 11800000.0 ; CAH CAG
19 34  2  0.133 11800000.0  0.133 11800000.0 ; CAH NAI
20 21  2  0.109 12300000.0  0.109 12300000.0 ; CAG HAG
20 22  2  0.133 11800000.0  0.133 11800000.0 ; CAG CAF
22 23  2  0.109 12300000.0  0.109 12300000.0 ; CAF HAF
24 22  2  0.133 11800000.0  0.133 11800000.0 ; CAJ CAF
24 25  2  0.133 11800000.0  0.133 11800000.0 ; CAJ CAK
24 34  2  0.133 11800000.0  0.133 11800000.0 ; CAJ NAI
25 26  2  0.133 10600000.0  0.133 10600000.0 ; CAK NAO
26 27  2  0.100 18700000.0  0.100 18700000.0 ; NAO HAO
25 39  2  0.133 11800000.0  0.133 11800000.0 ; CAK CAC
28 26  2  0.147  8710000.0  0.147  8710000.0 ; CAP NAO
28 29  2  0.153  7150000.0  0.153  7150000.0 ; CAP CAQ
30 29  2  0.183  5620000.0  0.183  5620000.0 ; SAR CAQ
30 31  2  0.153  8040000.0  0.153  8040000.0 ; SAR OAT
30 32  2  0.153  8040000.0  0.153  8040000.0 ; SAR OAU
30 33  2  0.153  8040000.0  0.153  8040000.0 ; SAR OAS
35 34  2  0.155  291912.0  0.155  291912.0 ; BAL NAI B-N OK
35 36  2  0.139  453246.0  0.139  453246.0 ; BAL FAM B-F OK
35 37  2  0.139  453246.0  0.153  453246.0 ; BAL FAN B-F OK
35 38  2  0.155  291912.0  0.155  291912.0 ; BAL NAE B-N OK
38 39  2  0.133 11800000.0  0.133 11800000.0 ; NAE CAC
38 44  2  0.133 11800000.0  0.133 11800000.0 ; NAE CAD
39 40  2  0.133 11800000.0  0.133 11800000.0 ; CAC CAB
40 41  2  0.109 12300000.0  0.109 12300000.0 ; CAB HAB
40 42  2  0.133 11800000.0  0.133 11800000.0 ; CAB CAA
42 43  2  0.109 12300000.0  0.109 12300000.0 ; CAA HAA
44 42  2  0.133 11800000.0  0.133 11800000.0 ; CAD CAA
44 45  2  0.153  7150000.0  0.153  7150000.0 ; CAD CBN
45 46  2  0.153  7150000.0  0.153  7150000.0 ; CBN CBO
46 47  2  0.153  7150000.0  0.153  7150000.0 ; CBO CBP
47 48  2  0.153  7150000.0  0.153  7150000.0 ; CBP CBQ
48 49  2  0.153  7150000.0  0.153  7150000.0 ; CBQ CBR
49 50  2  0.153  7150000.0  0.153  7150000.0 ; CBR CBS
50 51  2  0.153  7150000.0  0.153  7150000.0 ; CBS CBT
51 52  2  0.153  7150000.0  0.153  7150000.0 ; CBT CBU
52 53  2  0.153  7150000.0  0.153  7150000.0 ; CBU CBV
```

53	54	2	0.153	7150000.0	0.153	7150000.0	;	CBV	CBW
54	55	2	0.153	7150000.0	0.153	7150000.0	;	CBW	CBX
55	56	2	0.153	7150000.0	0.153	7150000.0	;	CBX	CBY
56	57	2	0.153	7150000.0	0.153	7150000.0	;	CBY	CBZ
57	58	2	0.153	7150000.0	0.153	7150000.0	;	CBZ	CCA
58	59	2	0.153	7150000.0	0.153	7150000.0	;	CCA	CCB
59	60	2	0.153	7150000.0	0.153	7150000.0	;	CCB	CCC
60	61	2	0.153	7150000.0	0.153	7150000.0	;	CCC	CCD
61	62	2	0.153	7150000.0	0.153	7150000.0	;	CCD	CCE

[pairs]									
;	ai	aj	fu	c0, c1, ...					
	1	4	1		;	CBM	CBJ		
	2	5	1		;	CBL	CBI		
	3	6	1		;	CBK	CBH		
	4	7	1		;	CBJ	CBG		
	5	8	1		;	CBI	CBF		
	6	9	1		;	CBH	CBE		
	7	10	1		;	CBG	CBD		
	8	11	1		;	CBF	CBC		
	9	12	1		;	CBE	CBB		
10	13	1			;	CBD	CBA		
11	14	1			;	CBC	CAZ		
12	15	1			;	CBB	CAY		
13	16	1			;	CBA	CAX		
14	17	1			;	CAZ	CAW		
15	18	1			;	CAY	CAV		
16	19	1			;	CAX	CAH		
17	20	1			;	CAW	CAG		
17	34	1			;	CAW	NAI		
18	21	1			;	CAV	HAG		
18	22	1			;	CAV	CAF		
18	24	1			;	CAV	CAJ		
18	35	1			;	CAV	BAL		
19	23	1			;	CAH	HAF		
19	25	1			;	CAH	CAK		
19	36	1			;	CAH	FAM		
19	37	1			;	CAH	FAN		
19	38	1			;	CAH	NAE		
20	25	1			;	CAG	CAK		
20	35	1			;	CAG	BAL		
21	23	1			;	HAG	HAF		
21	24	1			;	HAG	CAJ		
21	34	1			;	HAG	NAI		
22	26	1			;	CAF	NAO		
22	35	1			;	CAF	BAL		
22	39	1			;	CAF	CAC		
23	25	1			;	HAF	CAK		
23	34	1			;	HAF	NAI		
24	28	1			;	CAJ	CAP		
24	36	1			;	CAJ	FAM		
24	37	1			;	CAJ	FAN		
24	38	1			;	CAJ	NAE		
24	40	1			;	CAJ	CAB		
25	29	1			;	CAK	CAQ		
25	35	1			;	CAK	BAL		
25	41	1			;	CAK	HAB		
25	42	1			;	CAK	CAA		
25	44	1			;	CAK	CAD		
26	30	1			;	NAO	SAR		
26	34	1			;	NAO	NAI		
26	38	1			;	NAO	NAE		
26	40	1			;	NAO	CAB		
27	29	1			;	HAO	CAQ	added manually	
27	24	1			;	HAO	CAJ	added manually	
27	39	1			;	HAO	CAC	added manually	
28	31	1			;	CAP	OAT		
28	32	1			;	CAP	OAU		
28	34	1			;	CAP	OAS		

28 39 1
 34 39 1
 34 44 1
 35 40 1
 35 42 1
 35 45 1
 36 39 1
 36 44 1
 37 39 1
 37 44 1
 38 41 1
 38 43 1
 38 46 1
 39 43 1
 39 45 1
 40 45 1
 41 43 1
 41 44 1
 42 46 1
 43 45 1
 44 47 1
 45 48 1
 46 49 1
 47 50 1
 48 51 1
 49 52 1
 50 53 1
 51 54 1
 52 55 1
 53 56 1
 54 57 1
 55 58 1
 56 59 1
 57 60 1
 58 61 1
 59 62 1

; CAP CAC
 ; NAI CAC
 ; NAI CAD
 ; BAL CAB
 ; BAL CAA
 ; BAL CBN
 ; FAM CAC
 ; FAM CAD
 ; FAN CAC
 ; FAN CAD
 ; NAE HAB
 ; NAE HAA
 ; NAE CBO
 ; CAC HAA
 ; CAC CBN
 ; CAB CBN
 ; HAB HAA
 ; HAB CAD
 ; CAA CBO
 ; HAA CBN
 ; CAD CBP
 ; CBN CBQ
 ; CBO CBR
 ; CBP CBS
 ; CBQ CBT
 ; CBR CBU
 ; CBS CBV
 ; CBT CBW
 ; CBU CBX
 ; CBV CBY
 ; CBW CBZ
 ; CBX CCA
 ; CBY CCB
 ; CBZ CCC
 ; CCA CCD
 ; CCB CCE

[angles]

; ai aj ak fu c0, c1, ...

1	2	3	2	109.5	520.0	109.5	520.0 ;	CBM	CBL	CBK
2	3	4	2	109.5	520.0	109.5	520.0 ;	CBL	CBK	CBJ
3	4	5	2	109.5	520.0	109.5	520.0 ;	CBK	CBJ	CBI
4	5	6	2	109.5	520.0	109.5	520.0 ;	CBJ	CBI	CBH
5	6	7	2	109.5	520.0	109.5	520.0 ;	CBI	CBH	CBG
6	7	8	2	109.5	520.0	109.5	520.0 ;	CBH	CBG	CBF
7	8	9	2	109.5	520.0	109.5	520.0 ;	CBG	CBF	CBE
8	9	10	2	109.5	520.0	109.5	520.0 ;	CBF	CBE	CBD
9	10	11	2	109.5	520.0	109.5	520.0 ;	CBE	CBD	CBC
10	11	12	2	109.5	520.0	109.5	520.0 ;	CBD	CBC	CBB
11	12	13	2	109.5	520.0	109.5	520.0 ;	CBC	CBB	CBA
12	13	14	2	109.5	520.0	109.5	520.0 ;	CBB	CBA	CAZ
13	14	15	2	109.5	520.0	109.5	520.0 ;	CBA	CAZ	CAY
14	15	16	2	109.5	520.0	109.5	520.0 ;	CAZ	CAY	CAX
15	16	17	2	109.5	520.0	109.5	520.0 ;	CAY	CAX	CAW
16	17	18	2	109.5	520.0	109.5	520.0 ;	CAX	CAW	CAV
17	18	19	2	109.5	520.0	109.5	520.0 ;	CAW	CAV	CAH
18	19	20	2	120.0	560.0	120.0	560.0 ;	CAV	CAH	CAG
18	19	34	2	120.0	560.0	120.0	560.0 ;	CAV	CAH	NAI
20	19	34	2	108.0	465.0	108.0	465.0 ;	CAG	CAH	NAI
19	20	21	2	126.0	575.0	126.0	575.0 ;	CAH	CAG	HAG
19	20	22	2	108.0	465.0	108.0	465.0 ;	CAH	CAG	CAF
21	20	22	2	126.0	575.0	126.0	575.0 ;	HAG	CAG	CAF
20	22	23	2	126.0	575.0	126.0	575.0 ;	CAG	CAF	HAF
20	22	24	2	108.0	465.0	108.0	465.0 ;	CAG	CAF	CAJ
23	22	24	2	126.0	575.0	126.0	575.0 ;	HAF	CAF	CAJ
22	24	25	2	132.0	760.0	132.0	760.0 ;	CAF	CAJ	CAK
22	24	34	2	108.0	465.0	108.0	465.0 ;	CAF	CAJ	NAI
25	24	34	2	120.0	560.0	120.0	560.0 ;	CAK	CAJ	NAI
24	25	26	2	115.0	610.0	115.0	610.0 ;	CAJ	CAK	NAO

24	25	39	2	120.0	560.0	120.0	560.0 ;	CAJ	CAK	CAC	
26	25	39	2	115.0	610.0	115.0	610.0 ;	NAO	CAK	CAC	
25	26	28	2	128.9	700.0	128.9	700.0 ;	CAK	NAO	CAP	ga_30
modified to 128.9Å°											
26	28	29	2	109.5	520.0	109.5	520.0 ;	NAO	CAP	CAQ	
27	26	25	2	115.8	415.0	115.8	415.0 ;	HAO	NAO	CAK	ga_31
modified to 115.8Å°											
27	26	28	2	115.3	460.0	115.3	460.0 ;	HAO	NAO	CAP	ga_17
modified to 115.3Å°											
28	29	30	2	109.5	520.0	109.5	520.0 ;	CAP	CAQ	SAR	
29	30	31	2	109.5	518.0	109.5	518.0 ;	CAQ	SAR	OAT	
29	30	32	2	109.5	518.0	109.5	518.0 ;	CAQ	SAR	OAU	
29	30	33	2	109.5	518.0	109.5	518.0 ;	CAQ	SAR	OAS	
31	30	32	2	109.5	518.0	109.5	518.0 ;	OAT	SAR	OAU	
31	30	33	2	109.5	518.0	109.5	518.0 ;	OAT	SAR	OAS	
32	30	33	2	109.5	518.0	109.5	518.0 ;	OAU	SAR	OAS	
19	34	24	2	108.0	465.0	108.0	465.0 ;	CAH	NAI	CAJ	
19	34	35	2	125.0	375.0	125.0	375.0 ;	CAH	NAI	BAL	C-N-B C
24	34	35	2	125.0	375.0	125.0	375.0 ;	CAJ	NAI	BAL	C-N-B C
34	35	36	2	110.1	696.4	110.1	696.4 ;	NAI	BAL	FAM	N-B-F
OK											
34	35	37	2	110.1	696.4	110.1	696.4 ;	NAI	BAL	FAN	N-B-F
OK											
34	35	38	2	109.5	447.3	109.5	447.3 ;	NAI	BAL	NAE	N-B-N C
36	35	37	2	110.7	788.2	110.7	788.2 ;	FAM	BAL	FAN	F-B-F
OK											
36	35	38	2	110.1	696.4	110.1	696.4 ;	FAM	BAL	NAE	F-B-N
OK											
37	35	38	2	110.1	696.4	110.1	696.4 ;	FAN	BAL	NAE	F-B-N
OK											
35	38	39	2	125.0	375.0	125.0	375.0 ;	BAL	NAE	CAC	B-N-C C
35	38	44	2	125.0	375.0	125.0	375.0 ;	BAL	NAE	CAD	B-N-C C
39	38	44	2	108.0	465.0	108.0	465.0 ;	CAC	NAE	CAD	
25	39	38	2	120.0	560.0	120.0	560.0 ;	CAK	CAC	NAE	
25	39	40	2	132.0	760.0	132.0	760.0 ;	CAK	CAC	CAB	
38	39	40	2	108.0	465.0	108.0	465.0 ;	NAE	CAC	CAB	
39	40	41	2	126.0	575.0	126.0	575.0 ;	CAC	CAB	HAB	
39	40	42	2	108.0	465.0	108.0	465.0 ;	CAC	CAB	CAA	
41	40	42	2	126.0	575.0	126.0	575.0 ;	HAB	CAB	CAA	
40	42	43	2	126.0	575.0	126.0	575.0 ;	CAB	CAA	HAA	
40	42	44	2	108.0	465.0	108.0	465.0 ;	CAB	CAA	CAD	
43	42	44	2	126.0	575.0	126.0	575.0 ;	HAA	CAA	CAD	
38	44	42	2	108.0	465.0	108.0	465.0 ;	NAE	CAD	CAA	
38	44	45	2	120.0	560.0	120.0	560.0 ;	NAE	CAD	CBN	
42	44	45	2	120.0	560.0	120.0	560.0 ;	CAA	CAD	CBN	
44	45	46	2	109.5	520.0	109.5	520.0 ;	CAD	CBN	CBO	
45	46	47	2	109.5	520.0	109.5	520.0 ;	CBN	CBO	CBP	
46	47	48	2	109.5	520.0	109.5	520.0 ;	CBO	CBP	CBQ	
47	48	49	2	109.5	520.0	109.5	520.0 ;	CBP	CBQ	CBR	
48	49	50	2	109.5	520.0	109.5	520.0 ;	CBQ	CBR	CBS	
49	50	51	2	109.5	520.0	109.5	520.0 ;	CBR	CBS	CBT	
50	51	52	2	109.5	520.0	109.5	520.0 ;	CBS	CBT	CBU	
51	52	53	2	109.5	520.0	109.5	520.0 ;	CBT	CBU	CBV	
52	53	54	2	109.5	520.0	109.5	520.0 ;	CBU	CBV	CBW	
53	54	55	2	109.5	520.0	109.5	520.0 ;	CBV	CBW	CBX	
54	55	56	2	109.5	520.0	109.5	520.0 ;	CBW	CBX	CBY	
55	56	57	2	109.5	520.0	109.5	520.0 ;	CBX	CBY	CBZ	
56	57	58	2	109.5	520.0	109.5	520.0 ;	CBY	CBZ	CCA	
57	58	59	2	109.5	520.0	109.5	520.0 ;	CBZ	CCA	CCB	
58	59	60	2	109.5	520.0	109.5	520.0 ;	CCA	CCB	CCC	
59	60	61	2	109.5	520.0	109.5	520.0 ;	CCB	CCC	CCD	
60	61	62	2	109.5	520.0	109.5	520.0 ;	CCC	CCD	CCE	
[dihedrals]											
; ai aj ak al fu c0, c1, m, ...											
19	18	34	20	2	0.0	167.4	0.0 167.4 ;	imp	CAH	CAV	NAI CAG
20	19	22	21	2	0.0	167.4	0.0 167.4 ;	imp	CAG	CAH	CAF HAG
22	20	24	23	2	0.0	167.4	0.0 167.4 ;	imp	CAF	CAG	CAJ HAF
24	34	25	22	2	0.0	167.4	0.0 167.4 ;	imp	CAJ	NAI	CAK CAF

25	39	26	24	2	0.0	167.4	0.0	167.4	; imp	CAK	CAC	NAO	CAJ
30	29	32	31	2	35.3	334.8	35.3	334.8	; imp	SAR	CAQ	OAU	OAT
34	35	24	19	2	0.0	167.4	0.0	167.4	; imp	NAI	BAL	CAJ	CAH
24	34	38	35	2	180.0	46.2	180.0	46.2	; imp	CAJ	NAI	NAE	BAL
custom for boron													
39	34	38	35	2	180.0	46.2	180.0	46.2	; imp	CAJ	NAI	NAE	BAL
custom for boron													
35	34	36	37	2	35.3	334.8	35.3	334.8	; imp	BAL	NAI	FAM	FAN
38	35	44	39	2	0.0	167.4	0.0	167.4	; imp	NAE	BAL	CAD	CAC
39	25	38	40	2	0.0	167.4	0.0	167.4	; imp	CAC	CAK	NAE	CAB
40	39	42	41	2	0.0	167.4	0.0	167.4	; imp	CAB	CAC	CAA	HAB
42	40	44	43	2	0.0	167.4	0.0	167.4	; imp	CAA	CAB	CAD	HAA
44	38	45	42	2	0.0	167.4	0.0	167.4	; imp	CAD	NAE	CBN	CAA
19	20	22	24	2	0.0	209.3	0.0	209.3	; imp	CAH	CAG	CAF	CAJ
20	22	24	34	2	0.0	209.3	0.0	209.3	; imp	CAG	CAF	CAJ	NAI
22	24	34	19	2	0.0	209.3	0.0	209.3	; imp	CAF	CAJ	NAI	CAH
24	34	19	20	2	0.0	209.3	0.0	209.3	; imp	CAJ	NAI	CAH	CAG
34	19	20	22	2	0.0	209.3	0.0	209.3	; imp	NAI	CAH	CAG	CAF
38	39	40	42	2	0.0	209.3	0.0	209.3	; imp	NAE	CAC	CAB	CAA
39	40	42	44	2	0.0	209.3	0.0	209.3	; imp	CAC	CAB	CAA	CAD
40	42	44	38	2	0.0	209.3	0.0	209.3	; imp	CAB	CAA	CAD	NAE
42	44	38	39	2	0.0	209.3	0.0	209.3	; imp	CAA	CAD	NAE	CAC
44	38	39	40	2	0.0	209.3	0.0	209.3	; imp	CAD	NAE	CAC	CAB
4	3	2	1	1	0.0	5.9 3	0.0	5.9 3	; dih	CBJ	CBK	CBL	CBM
5	4	3	2	1	0.0	5.9 3	0.0	5.9 3	; dih	CBI	CBJ	CBK	CBL
6	5	4	3	1	0.0	5.9 3	0.0	5.9 3	; dih	CBH	CBI	CBJ	CBK
7	6	5	4	1	0.0	5.9 3	0.0	5.9 3	; dih	CBG	CBH	CBI	CBJ
8	7	6	5	1	0.0	5.9 3	0.0	5.9 3	; dih	CBF	CBG	CBH	CBI
9	8	7	6	1	0.0	5.9 3	0.0	5.9 3	; dih	CBE	CBF	CBG	CBH
10	9	8	7	1	0.0	5.9 3	0.0	5.9 3	; dih	CBD	CBE	CBF	CBG
11	10	9	8	1	0.0	5.9 3	0.0	5.9 3	; dih	CBC	CBD	CBE	CBF
12	11	10	9	1	0.0	5.9 3	0.0	5.9 3	; dih	CBB	CBC	CBD	CBE
13	12	11	10	1	0.0	5.9 3	0.0	5.9 3	; dih	CBA	CBB	CBC	CBD
14	13	12	11	1	0.0	5.9 3	0.0	5.9 3	; dih	CAZ	CBA	CBB	CBC
15	14	13	12	1	0.0	5.9 3	0.0	5.9 3	; dih	CAY	CAZ	CBA	CBB
16	15	14	13	1	0.0	5.9 3	0.0	5.9 3	; dih	CAX	CAY	CAZ	CBA
17	16	15	14	1	0.0	5.9 3	0.0	5.9 3	; dih	CAW	CAX	CAY	CAZ
18	17	16	15	1	0.0	5.9 3	0.0	5.9 3	; dih	CAV	CAW	CAX	CAY
19	18	17	16	1	0.0	5.9 3	0.0	5.9 3	; dih	CAH	CAV	CAW	CAX
17	18	19	34	1	0.0	1.0 6	0.0	1.0 6	; dih	CAW	CAV	CAH	NAI
39	25	24	22	1	180.0	33.5 2	180.0	33.5 2	; dih	CAC	CAK	CAJ	CAF
set as double bond to be planar													
26	25	24	22	1	180.0	33.5 2	180.0	33.5 2	; dih	CAC	CAK	CAJ	CAF
set as double bond to be planar													
24	25	26	28	1	180.0	33.5 2	180.0	33.5 2	; dih	CAJ	CAK	NAO	CAP
180Å° angle between NHR and aromatic cycle.													
24	25	26	27	1	180.0	33.5 2	180.0	33.5 2	: dih	CAJ	CAK	NAO	HAO
180Å° angle between NHR and aromatic cycle.													
40	39	25	26	1	180.0	33.5 2	180.0	33.5 2	; dih	CAB	CAC	CAK	NAO
set as double bond to be planar													
29	28	26	25	1	180.0	1.0 6	180.0	1.0 6	; dih	CAQ	CAP	NAO	CAK
30	29	28	26	1	0.0	5.9 3	0.0	5.9 3	; dih	SAR	CAQ	CAP	NAO
28	29	30	33	1	0.0	2.9 3	0.0	2.9 3	; dih	CAP	CAQ	SAR	OAS
38	35	34	19	1	0.0	1.0 6	0.0	1.0 6	; dih	NAE	BAL	NAI	CAH
34	35	38	44	1	0.0	1.0 6	0.0	1.0 6	; dih	NAI	BAL	NAE	CAD
46	45	44	38	1	0.0	1.0 6	0.0	1.0 6	; dih	CBO	CBN	CAD	NAE
47	46	45	44	1	0.0	5.9 3	0.0	5.9 3	; dih	CBP	CBO	CBN	CAD
48	47	46	45	1	0.0	5.9 3	0.0	5.9 3	; dih	CBQ	CBP	CBO	CBN
49	48	47	46	1	0.0	5.9 3	0.0	5.9 3	; dih	CBR	CBQ	CBP	CBO
50	49	48	47	1	0.0	5.9 3	0.0	5.9 3	; dih	CBS	CBR	CBQ	CBP
51	50	49	48	1	0.0	5.9 3	0.0	5.9 3	; dih	CBT	CBS	CBR	CBQ
52	51	50	49	1	0.0	5.9 3	0.0	5.9 3	; dih	CBU	CBT	CBS	CBR
53	52	51	50	1	0.0	5.9 3	0.0	5.9 3	; dih	CBV	CBU	CBT	CBS
54	53	52	51	1	0.0	5.9 3	0.0	5.9 3	; dih	CBW	CBV	CBU	CBT
55	54	53	52	1	0.0	5.9 3	0.0	5.9 3	; dih	CBX	CBW	CBV	CBU
56	55	54	53	1	0.0	5.9 3	0.0	5.9 3	; dih	CBY	CBX	CBW	CBV
57	56	55	54	1	0.0	5.9 3	0.0	5.9 3	; dih	CBZ	CBY	CBX	CBW
58	57	56	55	1	0.0	5.9 3	0.0	5.9 3	; dih	CCA	CBZ	CBY	CBX
59	58	57	56	1	0.0	5.9 3	0.0	5.9 3	; dih	CCB	CCA	CBZ	CBY

60	59	58	57	1	0.0	5.9 3	0.0	5.9 3 ; dih	CCC	CCB	CCA	CBZ
61	60	59	58	1	0.0	5.9 3	0.0	5.9 3 ; dih	CCD	CCC	CCB	CCA
62	61	60	59	1	0.0	5.9 3	0.0	5.9 3 ; dih	CCE	CCD	CCC	CCB