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Supporting Information



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# Phase Behavior of a Designed Cyclopropyl Analogue of Monoolein: Implications for Low-Temperature Membrane Protein Crystallization**

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## Materials and Methods

**General Information**

Glycerol anhydrous was purchased from Fluka, diethyl zinc was purchased from Acros Organics, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) was purchased from TCI, and all the other reagents and solvents were purchased from Sigma Aldrich or VWR. All chemicals and solvents were used as received, unless otherwise stated. Reactions were carried out under an inert atmosphere of argon in dry solvents. Dichloromethane was degassed with argon and purified by passage through activated alumina solvent column (MC Brown solvent system) prior to use. Column chromatography was performed using silica gel Merck 60 (particle size 0.040–0.063 mm). Analytical thin-layer chromatography (TLC) was performed using Merck pre-coated silica gel plates 60 $F_{254}$; visualization by UV absorption and/or by dipping in a solution of $KMnO_4$ (1 g), $K_2CO_3$ (2 g) in $H_2O$ (100 mL) and subsequent heating. $^1$H-NMR spectra were recorded on a Bruker AV2-500 (500MHz) spectrometer. Chemical shifts are given in parts per million (ppm) relative to the solvent residual peak: $CDCl_3$ = 7.26 ppm. Coupling constants *J* are expressed in Hz and multiplicities are abbreviated as follows: s (singlet), br (broad), d (doublet), t (triplet), q (quadruplet), quint (quintet), m (multiplet). $^{13}$C-NMR chemical shifts are reported relative to the solvent residual peaks: $CDCl_3$ = 77.16 ppm. High-resolution electrospray mass spectra were performed on a Bruker maXis QTOF-MS instrument. A mass accuracy ≤2 ppm was obtained in the peak-matching acquisition mode.

**Synthesis of 1-(*cis*-9,10-methylene-octadecanoyl)-*rac*-glycerol (compound 3 (MDS)**

**8-(2-octylcyclopropyl)octanoic acid** (**2**)

To a stirred solution of 2,4,6-trichlorophenol (8.60 g, 43.5 mmol) in 200 mL of dry $CH_2Cl_2$ at -40 °C under argon was added a 1 M hexane solution of diethyl zinc (44 mL, 44 mmol). The solution was stirred for 15 min at -40 °C and then diiodomethane (11.66 g, 3.50 mL, 43.5 mmol) was slowly added over a period of 10 min. After stirring for 15 min at the same temperature oleic acid **1** (3.07 g, 3.47 mL, 10.88 mmol) was added. The reaction mixture was stirred at -40 $^o$C for an additional 1 h and then allowed to warm to rt and stirred overnight. The mixture was washed with 300 mL of 10 % HCl. The organic phase was separated and the aqueous phase was extracted with $CH_2Cl_2$ (3 x 150 mL). The combined organic phases were washed with a saturated solution of NaCl and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel eluted with a mixture $CH_2Cl_2$/MeOH 95:5. The solvent was evaporated to afford a yellow oil (2.4 g), which was precipitated with cold acetonitrile to afford compound **2** as a white solid (2.1 g, 65%). HRMS (ESI $[M + Na]^+$ ) m/z: calcd for ($C_{19}H_{36}NaO_2$) 319.26074, found 319.26075.

**1-(*cis*-9,10-methylene-octadecanoyl)-*rac*-glycerol (3)**

To a stirred solution of acid **2** (660 mg, 2.23 mmol) in 50 mL of dry $CH_2Cl_2$ was added EDC (556 mg, 2.90 mmol) followed by DMAP (27 mg, 0.223 mmol) at 0 °C under argon. The solution was stirred for 1 h at 0 °C and then was added dropwise over a period of 30 min to a solution of glycerol (1.23 g, 13.38 mmol in 50 mL of dry $CH_2Cl_2$ at 0 °C. The mixture was stirred for 12 h at room temperature and then washed with a saturated solution of $NaHCO_3$ The organic phase was separated and the aqueous phase was extracted with $CH_2Cl_2$. The combined organic phases were washed with brine and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel and eluted with a mixture $CH_2Cl_2$/MeOH 98:2 to 90:10. The solvent was evaporated to afford a yellow oil (550 mg), which was precipitated with cold acetonitrile to afford a yellow oil which was then purified by low-temperature crystallization in acetonitrile (-20 °C to 4 °C) to yield 370 mg of 1-MDS **3** as a white solid (45%), mp: 27 °C.

**NMR of 1-MDS and 2-MDS (6-8%) mixture**

$^1$H NMR (500 MHz, $CDCl_3$) δ 4.93 (quint, *J* = 5.0 Hz, 1H of 2-MDS), 4.21 (dd, *J* = 11.6, 4.6 Hz, 1H), 4.15 (dd, *J* = 11.6, 6.2 Hz, 1H), 3.91 - 3.95 (m, 1H), 3.82 – 3.84 (m, 4H of 2-MDS), 3.70 (dd, *J* = 11.4, 4.0 Hz, 1H), 3.60 (dd, *J* = 11.4, 5.8 Hz, 1H), 2.32 – 2.40 (m, 2H), 1.96 (br s, 2H, OH) 1.59 – 1.69 (m, 2H), 1.22 – 1.44 (m, 22H), 1.07 – 1.20 (m, 2H), 0.88 (t, *J* = 7.0 Hz, 3H), 0.53 – 0.69 (m, 3H), -0.31- -0.33 (m, 1H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ 174.48, 70.35, 65.15, 63.49, 34.26, 32.03, 30.31, 30.24, 29.79 (2 C), 29.56, 29.45 (2 C), 29.27, 28.82, 28.77, 25.00, 22.79, 15.86, 15.81, 14.22, 11.02; HRMS (ESI $[M + Na]^+$ ) m/z: calcd for ($C_{22}H_{42}NaO_4$) 393.29749, found 393.29753.

**Purification of bR from purple membranes**

Lyophilized *Halobacterium salinarum* purple membranes (PM) were purchased from Sigma-Aldrich (product No. B0184). Bacteriorhodopsin (bR) was purified from PM according to a published procedure.[1]

Briefly, 2 mg of lyophilized PM were resuspended in 2 ml of an aqueous solution of 150 mM KCl and collected by centrifugation (30 min, 20,000 *g*, 4 °C). The pellet was resuspended in 6 ml of 25 mM sodium phosphate buffer, pH 6.9. Subsequently, 2 ml of an aqueous 10% (w/v) n-octyl-β-D-glucopyranoside (OG, Anatrace) were added, the sample was sonicated for 1 min and incubated overnight in the dark. Prior to ultra-centrifugation (45 min, 120,000 g, 4 °C), the pH was adjusted to 5.5 with 0.1 N HCl. The supernatant was concentrated to 500 µl with an Amicon Ultra centrifugal concentrator (30 kDa cutoff, Millipore) and subjected to size-exclusion chromatography on a Superdex 200 Increase 10/300GL column (GE Healthcare) pre-equilibrated with a 25 mM sodium phosphate buffer, pH 5.5, 1.2% OG at 4 °C. Pooled peak fractions were concentrated with an Amicon Ultra centrifugal concentrator (30 kDa cutoff) to 10.2 mg/ml as judged by the absorption at 560 nm. The final absorbance ratio $A_{280}/A_{560}$ was 1.51.

**Lipidic cubic phase crystallization of bR**

Detergent-solubilized bR was reconstituted into the LCP by mixing with molten MO or MDS in a 2:3 (v/v) ratio (protein solution:lipid) using a syringe lipid mixer as previously described.[2] Following formation of a transparent, uniformly colored purple LCP, 50-70 nl LCP boli were dispensed onto 96-well plastic plates (Swissci) and overlaid with 800 nl precipitant solution using the Gryphon LCP robot (Art Robbins Instruments). An apposite screening of precipitant conditions was made, varying systematically the pH (from 5.2 to 7.7) and salt concentration (from 1.5 to 3 M of Na/K-Pi). Crystallization plates were incubated either at 20 °C (MO, MDS) or at 4 °C (MDS).

Plate-like hexagonal crystals with an average size of ~18x18x7 µm were observed after 3 days in MDS at 20 °C, and grew to the full size (~40x40x20 µm) in 10 days. In contradistinction, crystallization in MO was slower, yielding fewer initial crystals (~20x20x7 µm) under identical conditions (2.1 M Na/K-Pi, pH 5.3) after 10 days. These crystals grew to the full size in 28 days but were smaller (~30x30x12 µm). Moreover, using the same crystallization screen, crystals appeared in many more conditions in MDS than in MO. The best crystals (~15x15x5 µm) obtained at 4 °C could be observed in MDS after 28 days with the following conditions: saturated Na/K-Pi, pH 6.9.

A Zeiss Axioskop 2 MOT optical microscope and a Nikon Eclipse TE300 inverted microscope were used for observations with brightfield and cross polarized light. Images were captured by a Hamamatsu C5810 CCD camera.

**Data collection and structure determination**

Crystals were grown from MO and MDS LCPs as described previously,[3] and appeared as thin hexagonal plates. The crystals were mounted on the Dual-Thickness MicroMounts (MiTeGen) directly from the lipidic mesophase and were flash-frozen in liquid nitrogen.

*Crystals grown from MDS LCP at 20 °C*

Crystals belonged to space group $P6_3$, with one protein molecule in the asymmetric unit. X-ray diffraction data were collected from several crystals cryo-cooled to 100 K at beamline X06DA (Swiss Light Source, Villigen, Switzerland) with the PILATUS 2M high-resolution diffractometer at a wavelength λ = 1 Å. Data were processed and scaled with XDS.[4] Resolution of the data crossed I/σ(I) = 2.0 at ~2.1 Å and the data were finally cut at 1.83 Å with $CC_{1/2}$= 10.2%.[5] As previously reported,[6, 7] the crystals exhibited merohedral twinning with a twin fraction of 30% - 40%.

The structure was determined by molecular replacement in Phaser [8] using the highest-resolution bR structure (Protein Data Bank accession code 1M0L, 1.47 Å, crystals grown in the LCP) as a search model. The search model was prepared by removing all non-protein atoms except the retinal. Model building was done in Coot [9] and refinement was performed with REFMAC5 [10] and phenix.refine.[11] The final $R/R_{free}$ of the model is 0.1824 / 0.2120, with Ramachandran favored

99% and Ramachandran outliers 0%. Data collection and refinement statistics are shown in Table 1 and were generated with Phenix Graphical Tools.[12]

*Crystals grown from MDS LCP at 4 °C*

Crystals belonged to space group $P6_3$, with one protein molecule in the asymmetric unit and with identical unit cell parameters as those of the crystals grown from MDS LCP at 20 °C. X-ray diffraction data were collected from a single crystal cryo-cooled to 100 K at beamline X06SA (Swiss Light Source, Villigen, Switzerland) with the PILATUS 6M high-resolution diffractometer at a wavelength λ = 1 Å. Data were processed and scaled with XDS.[4] Resolution of the data crossed I/σ(I) = 2.0 at ~7.1 Å and the data were finally cut at 4.2 Å with $CC_{1/2}$ = 66.7% [5] and with completeness = 92.5% in the highest resolution shell.

The structure was determined by 5 cycles of rigid-body refinement in REFMAC5[10] with the high-resolution 20 °C-generated bR structure as a starting model, and due to the relatively low resolution of the data was not refined further. Data collection and merging statistics are shown in Table 1.

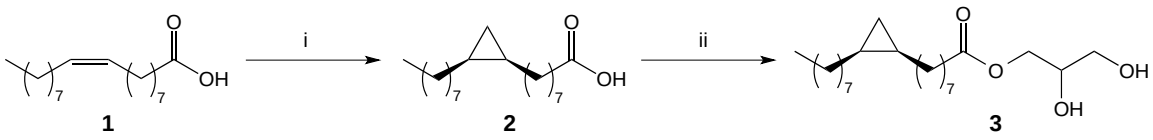



**Figure S1**. Synthesis of compound **3**: i) 2,4,6-trichlorophenol, 1 M $Et_2Zn$ soln in hexane, $CH_2I_2$, $CH_2Cl_2$, 12 h, -40 °C to r.t., 65%; ii) glycerol, EDC, DMAP, $CH_2Cl_2$, 12 h, 0 °C to r.t., 45%.

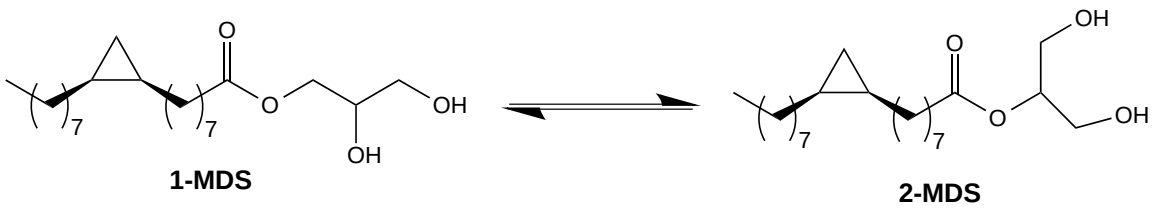



**Figure S2.** Transesterification of 1-MDS to 2-MDS under the experimental conditions used. The natural equilibrium between the 1- and 2-isomers is 9:1

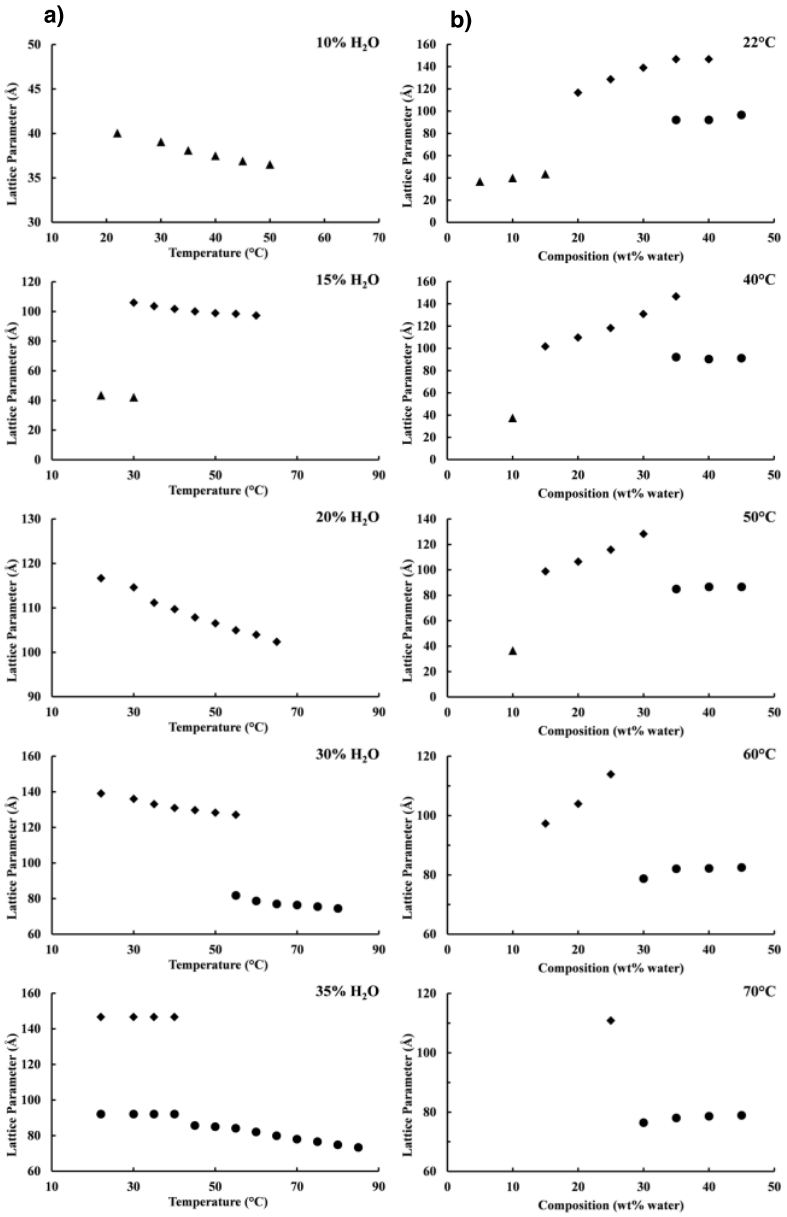



**Figure S3.** (a) Temperature dependence of the lattice parameters for the different phases observed in the MDS:water system at the indicated sample compositions (% (w/w) water). (b) Composition dependence of the different MDS:water mesophase lattice parameters at the indicated temperatures.

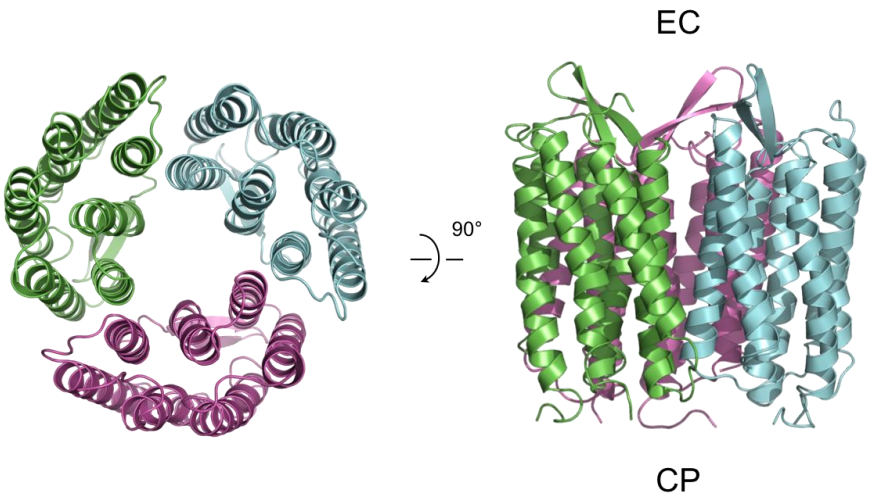



**Figure S4.** Crystal structure of bR in MDS at 20 °C, shown as a physiological trimer. Left: view perpendicular to the plane of the membrane from the cytoplasmic side. Right: view along the plane of the membrane depicting the extracellular (EC) and cytoplasmic (CP) sides.

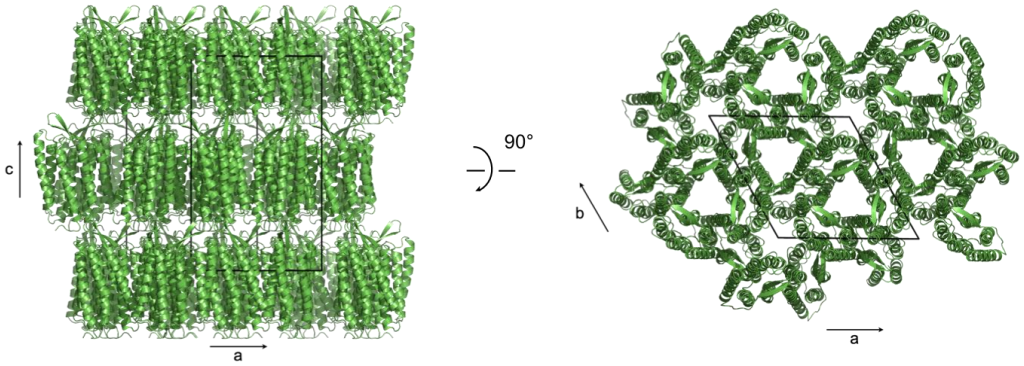



**Figure S5.** Crystal packing of bR crystallized from MDS LCP at 20 °C. Unit cell axes are indicated.

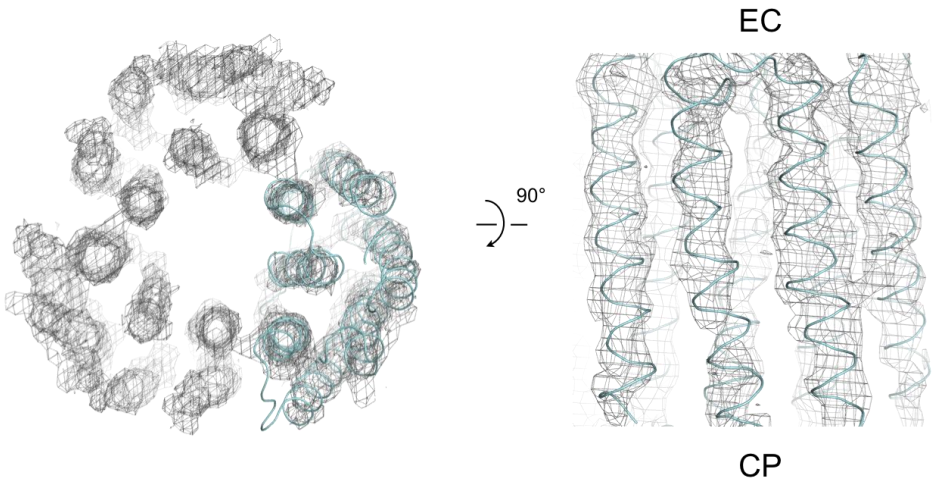
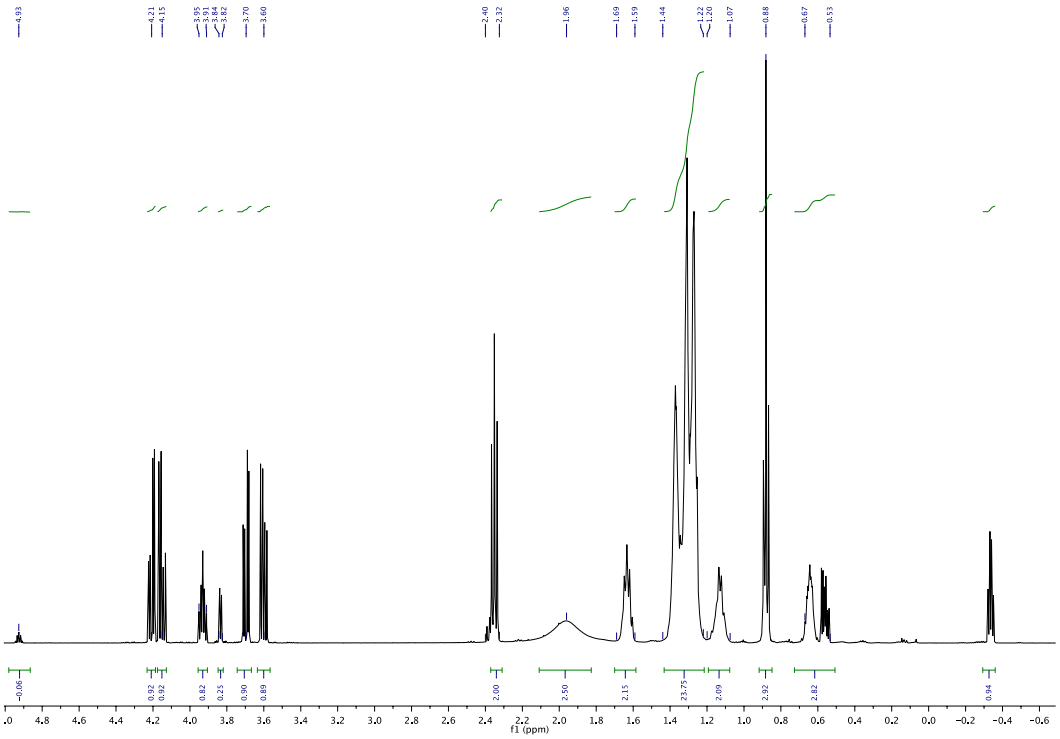



**Figure S6.** $\sigma_A$-weighted $2mF_o$-$DF_c$ electron density map of bR crystallized from MDS LCP at 4 °C, contoured at 1.5 σ. Left: view perpendicular to the plane of the membrane from the cytoplasmic side. Right: view along the plane of the membrane depicting the extracellular (EC) and cytoplasmic (CP) sides.

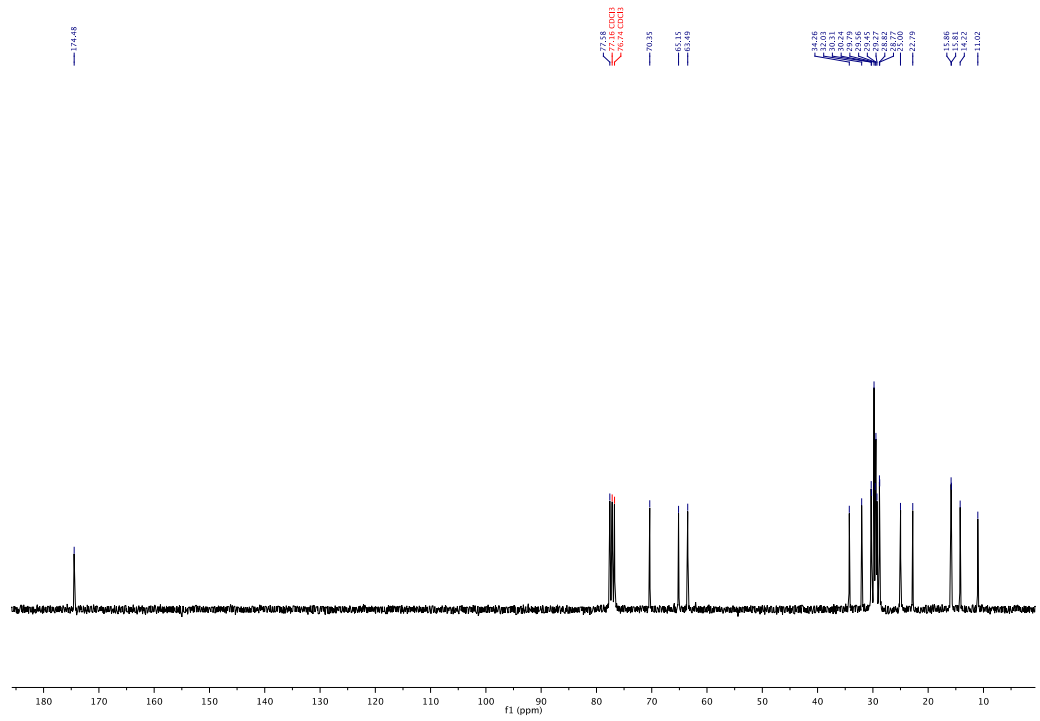
**Table S1.** Data collection and refinement statistics

| | bR 20 °C | bR 4 °C |
|---|---|---|
| **Wavelength (Å)** | 1 | 1 |
| **Resolution range (Å)** | 46.69 - 1.83 (1.895 - 1.83) | 36.36 - 4.04 (4.318 - 4.174) |
| **Space group** | $P6_3$ | $P6_3$ |
| **Unit cell (Å)** | 60.94 60.94 100.13 | 61.05 61.05 100.15 |
| | 90° 90° 120° | 90° 90° 120° |
| **Total reflections** | 238704 (24408) | 7304 (722) |
| **Unique reflections** | 18616 (1862) | 1296 (144) |
| **Multiplicity** | 12.8 (13.1) | 4.9 (5.0) |
| **Completeness (%)** | 99.99 (100.00) | 80.80 (92.31) |
| **Mean I/sigma(I)** | 9.22 (0.59) | 1.06 (0.98) |
| **R-merge** | 0.2502 (4.378) | 1.068 (1.697) |
| **R-meas.** | 0.2607 | 1.198 |
| **$CC_{1/2}$** | 0.998 (0.102) | 0.909 (0.667) |
| **CC*** | 0.999 (0.372) | 0.976 (0.786) |
| **R-work** | 0.1824 | N/A |
| **R-free** | 0.212 | N/A |
| **Number of non-hydrogen atoms** | 1988 | N/A |
| **macromolecules** | 1760 | N/A |
| **ligands** | 217 | N/A |
| **water** | 11 | N/A |
| **Protein residues** | 226 | N/A |
| **RMS(bonds) (Å)** | 0.002 | N/A |
| **RMS(angles) (°)** | 1.1 | N/A |
| **Ramachandran favored (%)** | 99 | N/A |
| **Ramachandran outliers (%)** | 0 | N/A |
| **Clashscore** | 6.03 | N/A |
| **Average B-factor** | 48.1 | N/A |
| **macromolecules** | 45.4 | N/A |
| **ligands** | 69.4 | N/A |
| **solvent** | 51.9 | N/A |

Statistics for the highest-resolution shell are shown in parentheses.




**Spectrum 1**. $^{1}H$ NMR (500 MHz, $CDCl_3$) of 1-MDS and 2-MDS (6-8%) mixture




**Spectrum 2** . $^{13}C$ NMR (125 MHz, $CDCl_3$) of 1-MDS and 2-MDS (6-8%) mixture

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