

# **Bulky Barbiturates as Non-toxic Ionic Dye Insulators for Enhanced Emission in Polymeric Nanoparticles**

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## **Materials and Methods**

All starting materials for synthesis were purchased from Alfa Aesar, Sigma Aldrich or TCI Europe and used as received. NMR spectra were recorded on Bruker Avance III 400 MHz and 500 MHz spectrometers. High resolution mass spectra were obtained using an Agilent Q-TOF 6520 mass spectrometer. MilliQ-water (Millipore) was used in all experiments. 1,3-Dicyclohexyl-1,3-diazinane-2,4,6-trione was prepared as described before.<sup>[1]</sup> pKa of the newly synthesized barbiturates were calculated using chemicalize.com software tools.

### **Preparation of fluorescent NPs**

The stock solution of PLGA in acetonitrile was prepared at a concentration of 10 mg/ml. This solution was then diluted with acetonitrile containing an appropriate amount of the dye salt, to the final concentration of 2 mg/ml. 50  $\mu$ l of this solution containing the dye salt and the polymer was then added rapidly, using a micropipette and under shaking (Thermomixer comfort, Eppendorf, 1000 rpm), to 450  $\mu$ l of 20 mM phosphate buffer (pH 7.4) at room temperature. The particle solution was then quickly diluted 5-fold with the same buffer and used as is for the following measurements.

### **Characterization of NPs**

Absorption spectra were recorded using a Cary 4000-HP Scan ultraviolet–visible spectrophotometer (Varian), emission spectra using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon) equipped with a thermostated cell compartment.

DLS and zeta potential measurements were performed on a Zetasizer Nano ZSP. The data are shown based on a triplicate. Analysis based on volume statistics was used for estimation of hydrodynamic diameter.

Electron microscopy was performed on a Philips CM120 transmission electron microscope. The particle size was obtained as mean value based on analysis of >200 particles per conditions. The experimental error was estimated based on the half-width at the half maximum of the size distribution.

Encapsulation of ion pairs inside nanoparticles was measured as the ratio of dye quantity (in moles) in the sample after and before dialysis (MEMBRA-CEL MD34 14V100 membrane, MWCO 14000) versus 1 mM beta-cyclodextrin solution in water as recipient medium according to the following protocol, developed previously.<sup>[2]</sup> Absorption spectra of freshly-prepared NPs was measured, then 450 mL of NP solution were mixed with 550 mL of 1 mM beta-cyclodextrin solution. The resulting mixture was dialyzed for 24 hours with 1 mM beta-cyclodextrin solution as a recipient medium. After the dialysis, the solution inside the membrane was recovered, and its volume and absorption spectra were measured. The dye encapsulation efficiency was estimated as  $A/A_0 \times 100\%$ , where  $A_0$  is initial value of absorption and  $A$  is the absorbance after the dialysis corrected by the initial dilution and further volume change after dialysis.

Relative fluorescence quantum yields were calculated using rhodamine B in methanol (QY = 0.7) as a reference dye.<sup>[3]</sup> Absorption of NP solutions never exceeded 0.2, and the same excitation wavelength and slits width was used to measure fluorescence spectra of both the reference dye and the NPs. Absorption spectra were corrected for scattering using Origin software.

Single-particle fluorescence measurements were performed in the wide-field epi-fluorescence mode on a Nikon Ti-E inverted microscope using a CFI Plan Apo × 60 oil (NA = 1.4) objective and a Hamamatsu Orca Flash 4 sCMOS camera. The excitation was provided by light-emitting diodes (SpectraX, Lumencor) at 550 nm at a power density of 4.4 W/cm<sup>2</sup> (44 W/cm<sup>2</sup> in case of quantum dots). Single particle brightness was analyzed using ImageJ software as follows. First, the stack of images (microscopy video) was subjected to

background subtraction and despeckling. Then, to obtain all the nanoparticles coordinates, a Z-projection of maximum intensity of all images in a stack was created and a Find Maxima function was run on it to generate a set of regions of interest (ROIs), containing all the nanoparticles in all the images. This set of ROIs was then applied to the stack of images and Multi Measure function was run on the stack, measuring integrated density of the pixels brightness inside ROIs. Averaging this brightness of the nanoparticles in each image allowed us to obtain relative averaged nanoparticle brightness per image. Nanoparticle brightness presented was calculated as the average brightness of the first 10 images in a stack (>400 total number of NPs of each type).

### **Cellular studies**

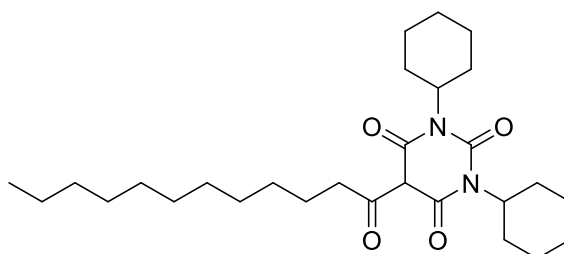
KB cells were grown in DMEM (Gibco–Invitrogen), supplemented with 10% fetal bovine serum (Dominique Dutscher) and 1% L-glutamine, 1% MEM vitamins, 1% MEM non-essential aminoacids and 0,4% antibiotic gentamicin in humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. Cells were seeded onto 8-chambered microscope slides (LabTek) at a density of 30000 cells per well 24 h before measurements. Before microscopy, the culture medium was discarded and the attached cells were rinsed with Opti-MEM (Gibco–Invitrogen) three times. Next, freshly prepared solutions of fluorescent NPs, loaded at 5 and 50 mM of ion pairs, were diluted 20 times in Opti-MEM, added to the cells and incubated for 3 hours. Cell membrane staining was done with wheat-germ agglutinin-Alexa488 at room temperature 5 minutes before the measurements.

### **Cytotoxicity evaluation**

Hela cells (ATCC® CCL-2) were grown in Dulbecco's modified Eagle's medium with low (1 g/l) glucose (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Dutscher), 1% L-glutamine (Lonza) and 1% penicillin-streptomycin (Lonza) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. For counterion cytotoxicity studies, HeLa cells were seeded in 96-well plates at a concentration of 2500 cells per well in 100 µL of the DMEM growth medium and then incubated overnight. Before addition of the counterion salts, the wells were rinsed twice with PBS. F5-TPB lithium salt and acidic forms of barbiturate counterions with 1.2 equivalents of triethylamine were dissolved in acetonitrile to create counterion salts solutions at concentrations ranging from 1 µM to 3 mM. Immediately before

addition the counterion salt solutions were diluted 1:100 in OptiMEM, and 100  $\mu$ L of those solutions were added into each well after removal of the PBS. After 3h of incubation, 100  $\mu$ L of DMEM containing 20% FBS were added and the plates were further incubated for 21 h. Then the medium was removed followed by washing cells twice with PBS, and addition of 100  $\mu$ L of cell culture medium containing 0.5 g/L of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Following incubation for 4 h at 37°C, the formed crystals were dissolved in methanol. The absorbance was then measured at 540 and 570 nm with a microplate reader. Experiments were carried out in quadruplicate and repeated at least twice. Results are expressed as the percentage of viable cells compared with the control groups treated with OptiMEM or OptiMEM containing 1% of acetonitrile.

### Chemical synthesis



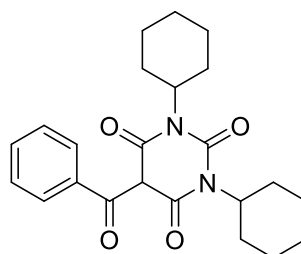
#### **1,3-Dicyclohexyl-5-dodecanoyl-1,3-diazinane-2,4,6-trione (1)**

1,3-Dicyclohexyl-1,3-diazinane-2,4,6-trione (1 eq., 500 mg, 1.71 mmol), dodecanoic acid (1.3 eq., 445 mg, 2.22 mmol) and DMAP (1.2 eq., 250 mg, 2.05 mmol) were mixed in 10 ml of dry DCM. Then EDC (1.5 eq., 491 mg, 2.57 mmol) was added to the mixture and reaction was left to stir overnight. The next day the reaction mixture was diluted with 30 ml of ethyl acetate and washed with 1M HCl (2x10 ml) and water (2x10 ml). Organic layer was dried with magnesium sulphate, filtered and evaporated. Obtained oil was purified by column chromatography with silica gel using DCM/MeOH 99/1 as eluent. 1,3-Dicyclohexyl-5-dodecanoyl-1,3-diazinane-2,4,6-trione (**1**) (583 mg, 1.23 mmol, 72 %) was obtained as a colourless viscous oil.

$^1\text{H}$  NMR (400 MHz, chloroform-*d*)  $\delta$  ppm 4.73 (tt,  $J_1 = 12.3$  Hz,  $J_2 = 3.6$  Hz, 1H), 4.70 (tt,  $J_1 = 12.3$  Hz,  $J_2 = 3.6$  Hz, 1H), 3.09 (t,  $J = 7.6$  Hz, 2H), 2.27 - 2.39 (m, 4H), 1.82 (m, 4 H), 1.59 - 1.71 (m, 8 H), 1.20 - 1.42 (m, 22H) 0.87 (t,  $J = 6.9$  Hz, 3H).

$^{13}\text{C}$  NMR (101 MHz, chloroform-*d*)  $\delta$  ppm 199.99, 170.24, 161.12, 149.61, 95.61, 54.97, 54.16, 37.27, 31.87, 29.57, 29.47, 29.45, 29.33, 29.29, 29.10, 28.98, 26.43, 26.39, 25.61, 25.27, 25.19, 22.64, 14.05.

MS (ESI)  $m/z$ :  $[\text{M}+\text{H}]$  calcd. for  $\text{C}_{28}\text{H}_{47}\text{N}_2\text{O}_4^+$ , 475.3530; found 475.3300.



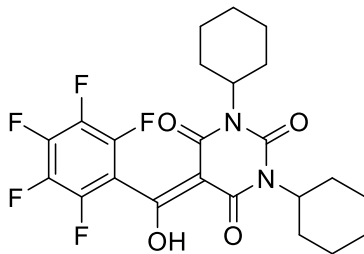
### **5-Benzoyl-1,3-dicyclohexyl-1,3-diazinane-2,4,6-trione (2)**

1,3-Dicyclohexyl-1,3-diazinane-2,4,6-trione (1 eq., 500 mg, 1.71 mmol), benzoic acid (1.3 eq., 271 mg, 2.22 mmol) and DMAP (1.2 eq., 250 mg, 2.05 mmol) were mixed in 10 ml of dry DCM. Afterwards EDC (1.5 eq., 491 mg, 2.57 mmol) was added to the mixture and reaction was left to stir overnight. The next day the reaction mixture was diluted with 30 mL of ethyl acetate and washed with 1M HCl (2x10 ml) and water (2x10 ml). Organic layer was dried with magnesium sulphate, filtered and evaporated *in vacuo*. Obtained oil was purified by column chromatography with silica gel using DCM/MeOH 99/1 as eluent. 5-Benzoyl-1,3-dicyclohexyl-1,3-diazinane-2,4,6-trione (**2**) (501 mg, 1.27 mmol, 78 %) was obtained as a white solid.

$^1\text{H}$  NMR (400 MHz, chloroform-*d*)  $\delta$  ppm 7.51-7.56 (m, 3H), 7.43-7.48 (m, 2H), 4.76 (tt,  $J_1 = 3.7$  Hz,  $J_2 = 12.2$  Hz, 1H), 4.68 (tt,  $J_1 = 3.7$  Hz,  $J_2 = 12.2$  Hz, 1H), 2.40 (dq,  $J_1 = 3.5$  Hz,  $J_2 = 12.4$  Hz, 4H), 2.29 (dq,  $J_1 = 3.4$  Hz,  $J_2 = 12.4$  Hz, 4H), 1.89 (m, 2H), 1.75 (m, 5H), 1.60 (m, 4H), 1.11-1.46 (m, 6H)

$^{13}\text{C}$  NMR (126 MHz, chloroform-*d*)  $\delta$  ppm 191.75, 170.60, 160.31, 149.72, 135.62, 131.53, 128.12, 127.75, 95.73, 55.36, 54.24, 29.20, 28.91, 26.44, 26.32, 25.21, 25.18.

HRMS (ESI)  $m/z$ :  $[\text{M}+\text{H}]$  calcd. for  $\text{C}_{23}\text{H}_{29}\text{N}_2\text{O}_4^+$ , 397.2122; found 397.2126.



**1,3-Dicyclohexyl-5-(pentafluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (BarC6)**

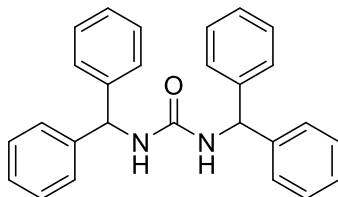
1,3-Dicyclohexyl-1,3-diazinane-2,4,6-trione (1 eq., 500 mg, 1.71 mmol), pentafluorobenzoic acid (1.1 eq., 398 mg, 1.88 mmol) and DMAP (1.2 eq., 250 mg, 2.05 mmol) were mixed in 5 ml of dry DCM. Afterwards EDC (1.5 eq., 491 mg, 2.57 mmol) was added to the mixture and reaction was left to stir overnight. The next day the reaction mixture was diluted with 30 ml of ethyl acetate and washed with 1M HCl (2x10 ml) and water (2x10 ml). Organic layer was dried with magnesium sulphate, filtered and evaporated. Obtained oil was purified by column chromatography with silica gel using DCM/MeOH 99/1 as eluent. 1,3-Dicyclohexyl-5-(pentafluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (582 mg, 1.2 mmol, 70 %) (**BarC6**) was obtained as a white-yellowish solid.

$^1\text{H}$  NMR (400 MHz, chloroform-*d*)  $\delta$  ppm 4.77 (tt,  $J_1 = 12.3$  Hz,  $J_2 = 3.8$  Hz, 1H), 4.61 (tt,  $J_1 = 12.2$  Hz,  $J_2 = 3.7$  Hz, 1H), 2.37 (dq,  $J_1 = 3.5$  Hz,  $J_2 = 12.4$  Hz, 2H), 2.24 (dq,  $J_1 = 3.5$  Hz,  $J_2 = 12.4$  Hz, 2H), 1.55-1.92 (m, 10H), 1.13-1.46 (m, 6H)

$^{13}\text{C}$  NMR (101 MHz, chloroform-*d*)  $\delta$  ppm 178.42, 169.99, 159.43, 149.04, 98.69, 56.06, 54.82, 29.13, 28.79, 26.36, 26.28, 25.12.

$^{19}\text{F}$  NMR (376 MHz, chloroform-*d*)  $\delta$  ppm -140.65 (m, 2F), -150.75 (m, 1F), -160.90 (m, 2F).

HRMS (ESI)  $m/z$ : [M+H] calcd. for  $\text{C}_{23}\text{H}_{24}\text{F}_5\text{N}_2\text{O}_4^+$ , 487.1651; found 487.1654.



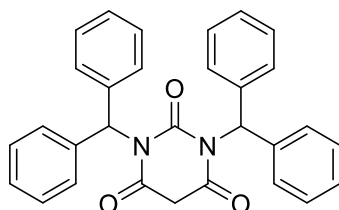
**1,3-Bis(diphenylmethyl)urea**

Aminodiphenylmethane (1 eq., 10 g, 9.43 ml, 54.6 mmol) was dissolved in 50 ml of dry THF and CDI (0.7 eq., 6.19 g, 38.2 mmol) was added. Reaction was left to reflux under

stirring for 1h, TLC after acidic micro-workup has shown the presence of both urea product and amine reagent, therefore another portion of CDI (0.7 eq., 6.19 g, 38.2 mmol) was added and reflux was continued for another 2 hrs. After cooling of the mixture 10 ml of 1M aqueous HCl was added, left to stir for another 10 mins, white precipitate of urea was filtered off, washed with 1M HCl and cold ethanol. The solid was further recrystallized from ethanol to obtain 1,3-bis(diphenylmethyl)urea (6.5 g, 16.6 mmol, 43 %) as a white powder.

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ): 7.30-7.34 (m, 8 H), 7.20-7.27 (m, 12 H), 6.95 (d,  $J$  = 8.4 Hz, 2H), 5.88 (d,  $J$  = 8.4 Hz, 2H).

$^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ ): 156.32, 143.57, 128.39, 126.78, 56.95.



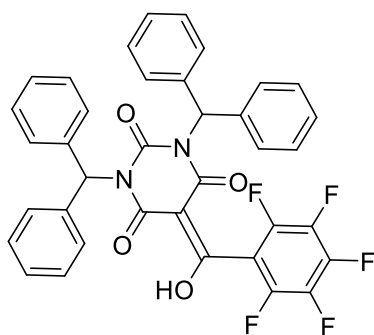
**1,3-Bis(diphenylmethyl)-1,3-diazinane-2,4,6-trione**

To a boiling dispersion of 1,3-bis(diphenylmethyl)urea (1 eq., 1000 mg, 2.55 mmol) in DCM (70 ml) malonyl chloride (1.3 eq., 466 mg, 0.322 ml, 3.31 mmol) was added as one portion. Reaction mixture was refluxed for 4hrs and followed by NMR in DMSO - peak around 7.06 belongs to the barbituric acid. Its growth continued long after the starting urea protons disappeared. On TLC the barbituric acid gave  $R_f$  around 0.5 in DCM/MeOH 99/1. With time the concentration of black resin that didn't move on TLC increased, therefore the reaction was stopped after 4hrs. The volatiles were evaporated, and the product was purified by column chromatography on silica using pure DCM as eluent to obtain 1,3-bis(diphenylmethyl)-1,3-diazinane-2,4,6-trione (384 mg, 0.834 mmol, 33 %) as a white solid.

$^1\text{H}$  NMR (400 MHz, chloroform- $d$ )  $\delta$  ppm 7.22-7.30 (m, 20H), 7.17 (s, 2H), 3.65 (s, 2H).

$^{13}\text{C}$  NMR (126 MHz, chloroform- $d$ )  $\delta$  ppm 164.34, 150.75, 137.46, 128.52, 128.22, 127.57, 60.44, 40.82.

HRMS (ESI)  $m/z$ :  $[M+H]^+$  calcd. for  $\text{C}_{30}\text{H}_{25}\text{N}_2\text{O}_3^+$ , 461.1860; found 461.1854.



**1,3-Bis(diphenylmethyl)-5-(penta-fluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (BarPh2)**

1,3-Bis(diphenylmethyl)-1,3-diazinane-2,4,6-trione (1 eq., 50 mg, 0.109 mmol), pentafluorobenzoic acid (1.1 eq., 25.3 mg, 0.119 mmol) and DMAP (1.2 eq., 15.9 mg, 0.13 mmol) were mixed in 0.5 ml of dry DCM. Afterwards EDC (1.5 eq., 31.2 mg, 0.163 mmol) was added to the mixture and the reaction was left to stir overnight. The next day the reaction mixture was diluted with 3 ml of ethyl acetate and washed with 1M HCl (2x1 ml) and water (2x1 ml). Organic layer was dried with magnesium sulphate, filtered and evaporated *in vacuo*. Obtained oil was purified by gradient column chromatography with silica gel using DCM/MeOH (from 99/1 to 9/1) as eluent. 1,3-Bis(diphenylmethyl)-5-(penta-fluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (52.6 mg, 0.0803 mmol, 74 %) (**BarPh2**) was obtained as a yellowish-white solid.

$^1\text{H}$  NMR (400 MHz, chloroform-*d*)  $\delta$  ppm 7.14 – 7.40 (22H, m)

$^{13}\text{C}$  NMR (126 MHz, chloroform-*d*)  $\delta$  ppm 181.73, 166.37, 162.37, 150.41, 138.45, 138.39, 128.51, 128.45, 128.04, 127.96, 127.21, 127.18, 98.96, 60.00, 59.26.

$^{19}\text{F}$  NMR (376 MHz, chloroform-*d*)  $\delta$  ppm -144.09 (d,  $J$  = 16.4 Hz, 2F), -154.76 (t,  $J$  = 20.4 Hz, 1F), -162.08 (t,  $J$  = 16.4 Hz, 1F), 162.09 (t,  $J$  = 20.4 Hz, 1F).

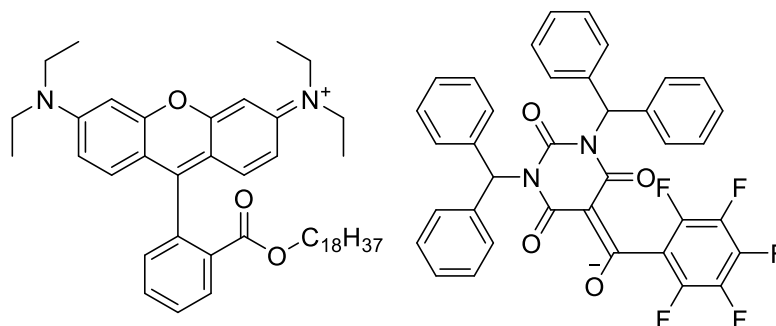
HRMS (ESI)  $m/z$ :  $[M+H]^+$  calcd. for  $\text{C}_{37}\text{H}_{24}\text{F}_5\text{N}_2\text{O}_4^+$ , 653.1505; found 653.1496.

**General procedure for ion exchange of R18/ $\text{ClO}_4^-$  with barbiturate counterions.**

A counterion in acidic form (0.05 mmol) was dissolved in absolute toluene (1 ml) in a flame-dried Schlenk flask followed by potassium tert-butoxide (7.5 mg, 0.065 mmol). The mixture was stirred at RT for 30 min in argon atmosphere. To this solution octadecyl rhodamine B perchlorate (R18/ $\text{ClO}_4^-$ ) (40 mg, 0.05 mmol) in absolute toluene (400  $\mu\text{L}$ ) was injected and kept with stirring for 5 mins. The afforded solution was filtered through a PTFE



0.2 micron filter, which was additionally rinsed with abs. toluene (0.5 ml), and concentrated *in vacuo* to obtain R18 salt with quantitative yield as a red solid bulk.



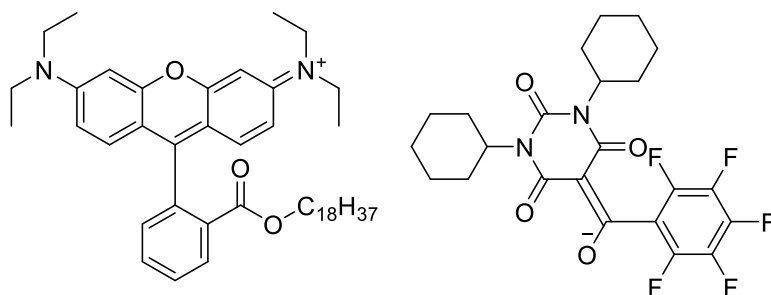
*N*-(6-(diethylamino)-9-(2-((octadecyloxy)carbonyl)phenyl)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium(1,3-dibenzhydryl-2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)(perfluorophenyl)methanolate (**R18/BarPh2**)

Yield: 65 mg (97%) as red solid bulk.

<sup>1</sup>H NMR (400 MHz, chloroform-*d*) δ ppm 8.29 (dd,  $J_1 = 7.8$  Hz,  $J_2 = 1.1$  Hz, 1H), 7.78 (td,  $J_1 = 7.5$  Hz,  $J_2 = 1.4$  Hz, 1H), 7.73 (td,  $J_1 = 7.5$  Hz,  $J_2 = 1.3$  Hz, 1H), 7.15-7.31 (m, 15H), 7.07 (d,  $J = 9.4$  Hz, 2H), 7.06 (m, 3H), 6.86 (m, 5H), 6.82 (dd,  $J_1 = 9.4$  Hz,  $J_2 = 2.5$  Hz, 2H), 6.81 (s, 2H), 4.02 (t,  $J = 6.7$  Hz, 2H), 3.59 (q,  $J = 7.2$  Hz, 8H), 1.44 (m, 2H), 1.31 (t,  $J = 7.1$  Hz, 12H), 1.14-1.29 (m, 30H), 0.89 (m, 3H).

<sup>13</sup>C NMR (101 MHz, chloroform-*d*) δ ppm 165.12, 159.08, 157.75, 155.48, 139.07, 133.31, 132.90, 131.27, 131.23, 130.36, 130.18, 130.11, 128.59, 127.70, 127.44, 126.59, 114.00, 113.52, 96.30, 65.77, 45.99, 31.87, 29.64, 29.60, 29.53, 29.40, 29.30, 29.14, 28.29, 25.77, 22.63, 14.06, 12.50.

<sup>19</sup>F NMR (376 MHz, chloroform-*d*) δ ppm -143.58 (m, 2H), -157.97 (m, 1H), -163.72 (m, 2H).



*N*-(6-(diethylamino)-9-(2-((octadecyloxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium(1,3-dicyclohexyl-2,4,6-trioxotetrahydropyrimidin-5(2H)-ylidene)(perfluorophenyl)methanolate (**R18/BarC6**)

Yield: 58 mg (99%) as red solid bulk.

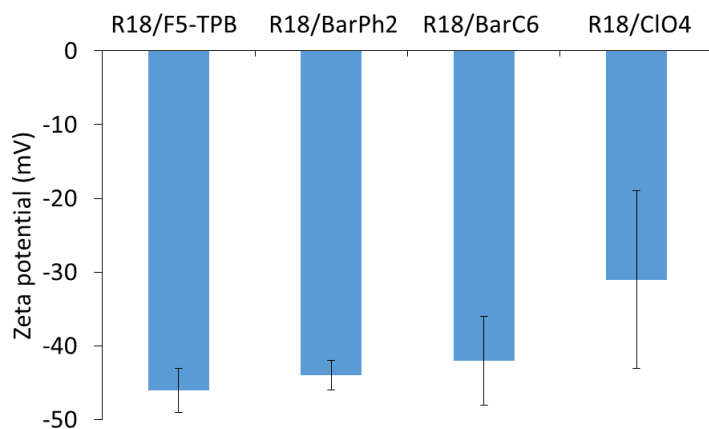
<sup>1</sup>H NMR (400 MHz, chloroform-*d*) δ ppm 8.28 (dd,  $J_1 = 6.7$  Hz,  $J_2 = 1.1$  Hz, 1H), 7.76 (td,  $J_1 = 7.5$  Hz,  $J_2 = 1.4$  Hz, 1H), 7.73 (td,  $J_1 = 7.5$  Hz,  $J_2 = 1.4$  Hz, 1H), 7.29 (dd,  $J_1 = 7.5$  Hz,  $J_2 = 1.1$  Hz, 1H) 7.07 (d,  $J = 9.5$  Hz, 2H), 6.83 (dd,  $J_1 = 9.5$  Hz,  $J_2 = 2.4$  Hz, 2H), 6.79 (d,  $J = 2.4$  Hz, 2H), 4.73 (m, 2H), 4.00 (t,  $J = 6.6$  Hz, 2H), 3.60 (q,  $J = 7.4$  Hz, 8H), 2.38 (m, 4H), 1.49-1.72 (m, 10H), 1.42 (quint,  $J = 6.9$  Hz, 2H), 1.31 (t,  $J = 7.1$  Hz, 12H), 1.12-1.31 (m, 36H), 0.87 (t,  $J = 6.9$  Hz, 3H).

<sup>13</sup>C NMR (101 MHz, chloroform-*d*) δ ppm 177.78, 165.09, 159.11, 157.72, 155.47, 133.28, 132.90, 131.25, 131.23, 130.35, 130.09, 113.98, 113.50, 97.66, 96.27, 65.75, 52.14, 45.99, 31.84, 29.61, 29.57, 29.56, 29.50, 29.37, 29.27, 29.11, 28.26, 26.60, 25.74, 25.51, 22.60, 14.03, 12.48.

<sup>19</sup>F NMR (376 MHz, chloroform-*d*) δ ppm -145.62 (m, 2H), -161.51 (m, 1H), -164.29 (m, 2H).



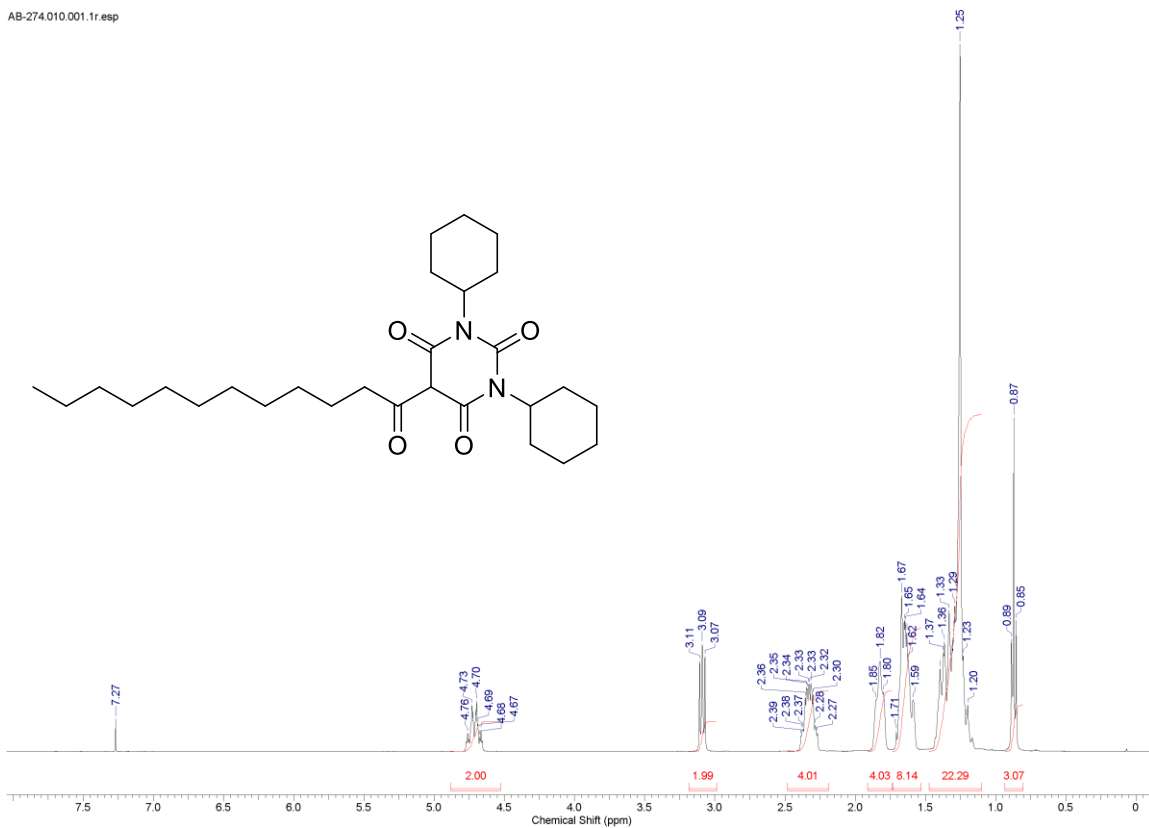
**Figure S1.** Precoated aluminum TLC plate with SiO<sub>2</sub> eluted with pure DCM. R18 perchlorate (1) and mixtures with barbiturates (2-5) were applied from reaction mixtures (1eq R18 perchlorate/0 or 10 eq barbiturate derivatives/30 eq triethylamine). R18/F5-TPB salt (6) was applied as pure individual compound. Rf: (1) – 0.06, (2) – 0.06, (3) – 0.06, (4) – 0.33, (5) – 0.40, (6) – 0.83.



**Figure S2.** Zeta potential of PLGA NPs loaded at 50 mM with ion pairs of R18 dye with different counterions. Error is standard deviation (n = 3).

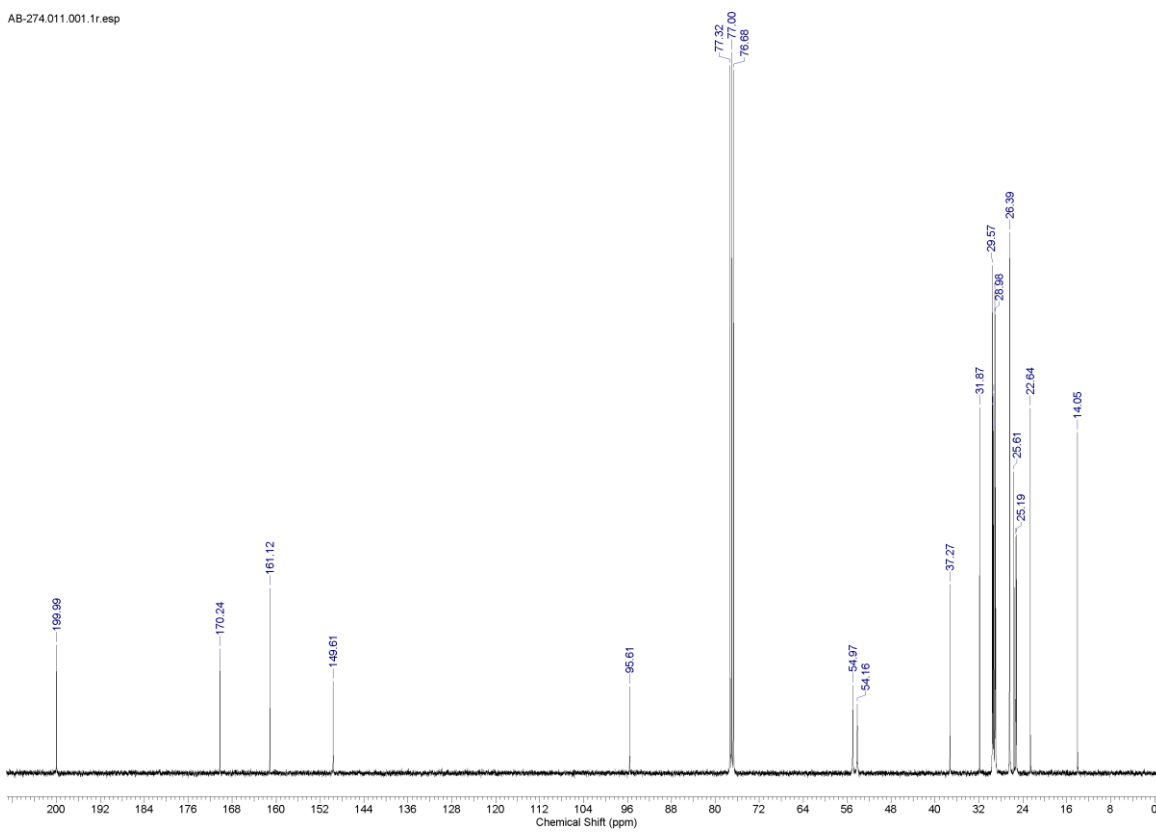
# <sup>1</sup>H NMR for 1,3-dicyclohexyl-5-dodecanoyl-1,3-diazinane-2,4,6-trione (1)

AB-274.010.001.1r.esp



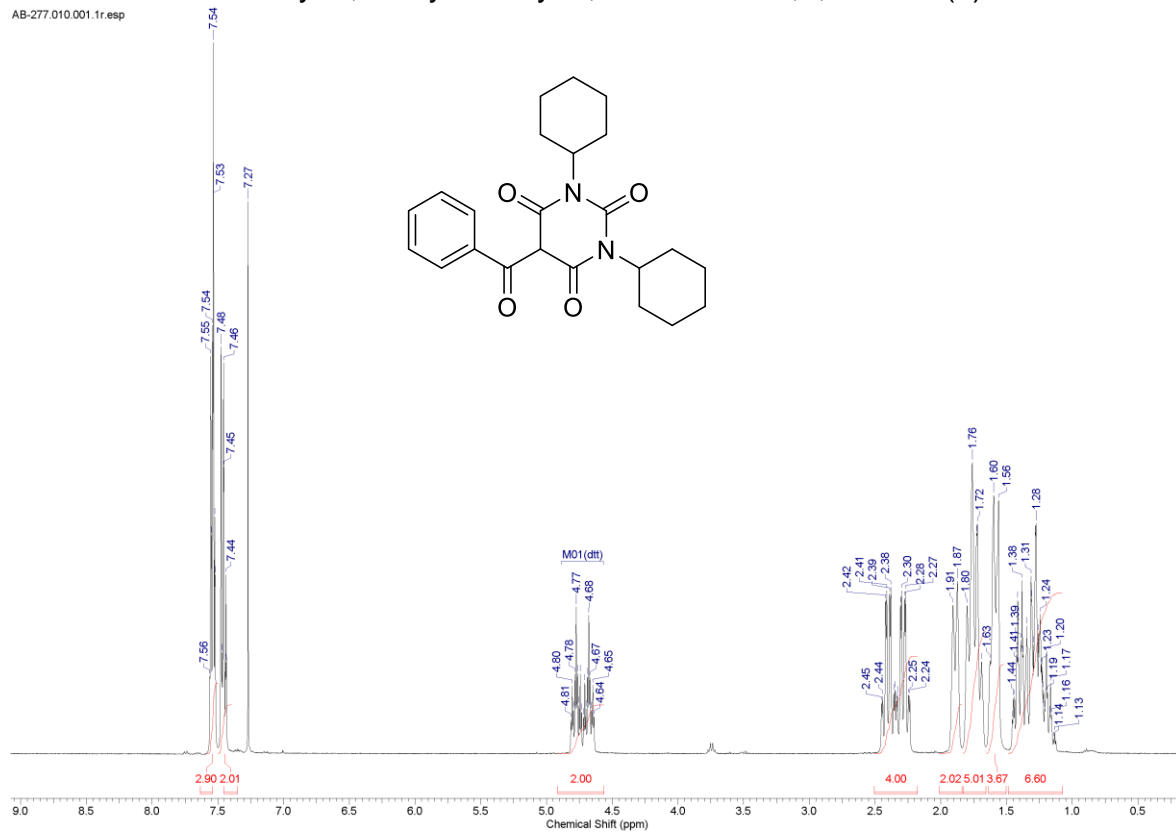
# <sup>13</sup>C NMR for 1,3-dicyclohexyl-5-dodecanoyl-1,3-diazinane-2,4,6-trione (1)

AB-274.011.001.1r.esp



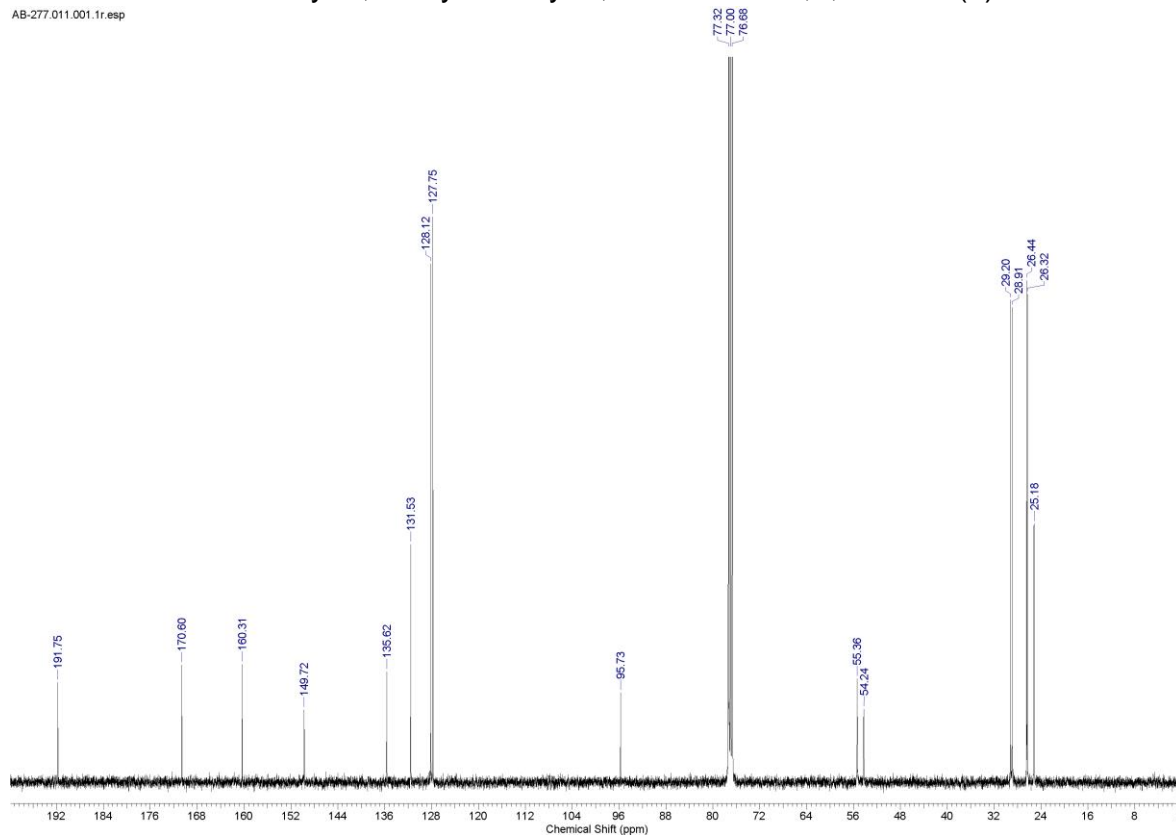
# <sup>1</sup>H NMR for 5-benzoyl-1,3-dicyclohexyl-1,3-diazinane-2,4,6-trione (2)

AB-277.010.001.1r.esp



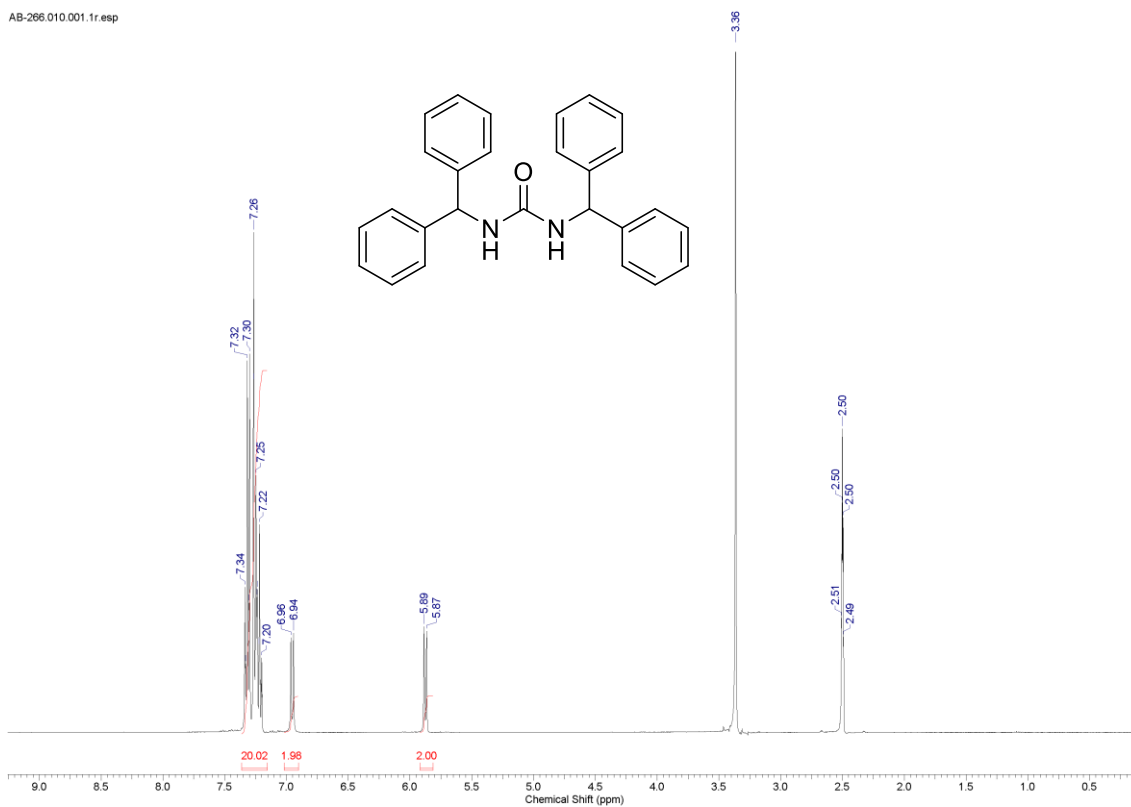
# <sup>13</sup>C NMR for 5-benzoyl-1,3-dicyclohexyl-1,3-diazinane-2,4,6-trione (2)

AB-277.011.001.1r.esp



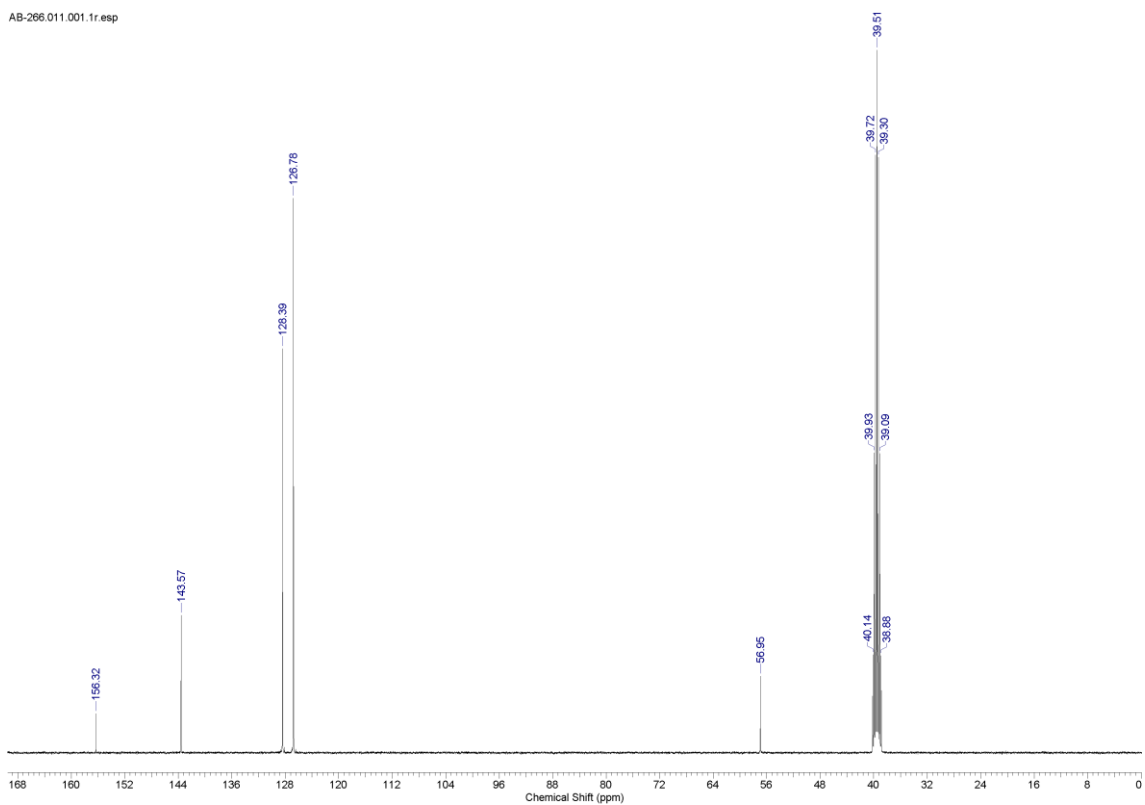
# <sup>1</sup>H NMR for 1,3-bis(diphenylmethyl)urea

AB-266.010.001.1r.esp

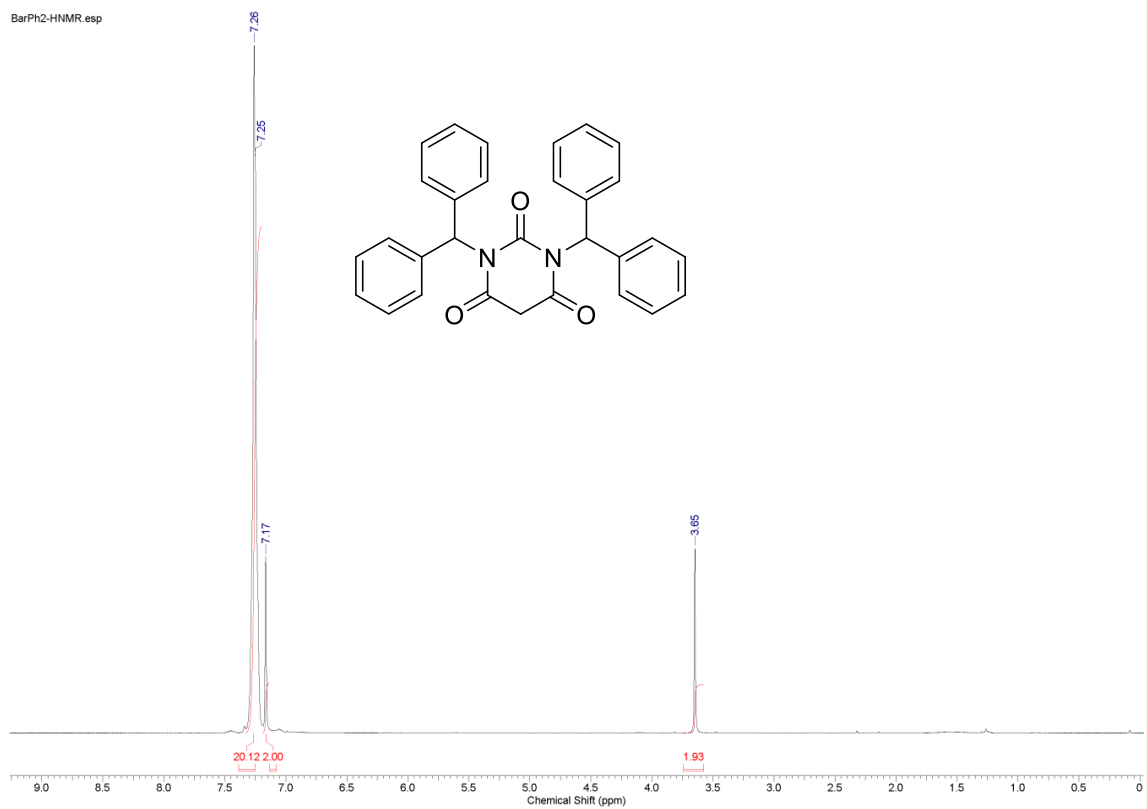


# <sup>13</sup>C NMR for 1,3-bis(diphenylmethyl)urea

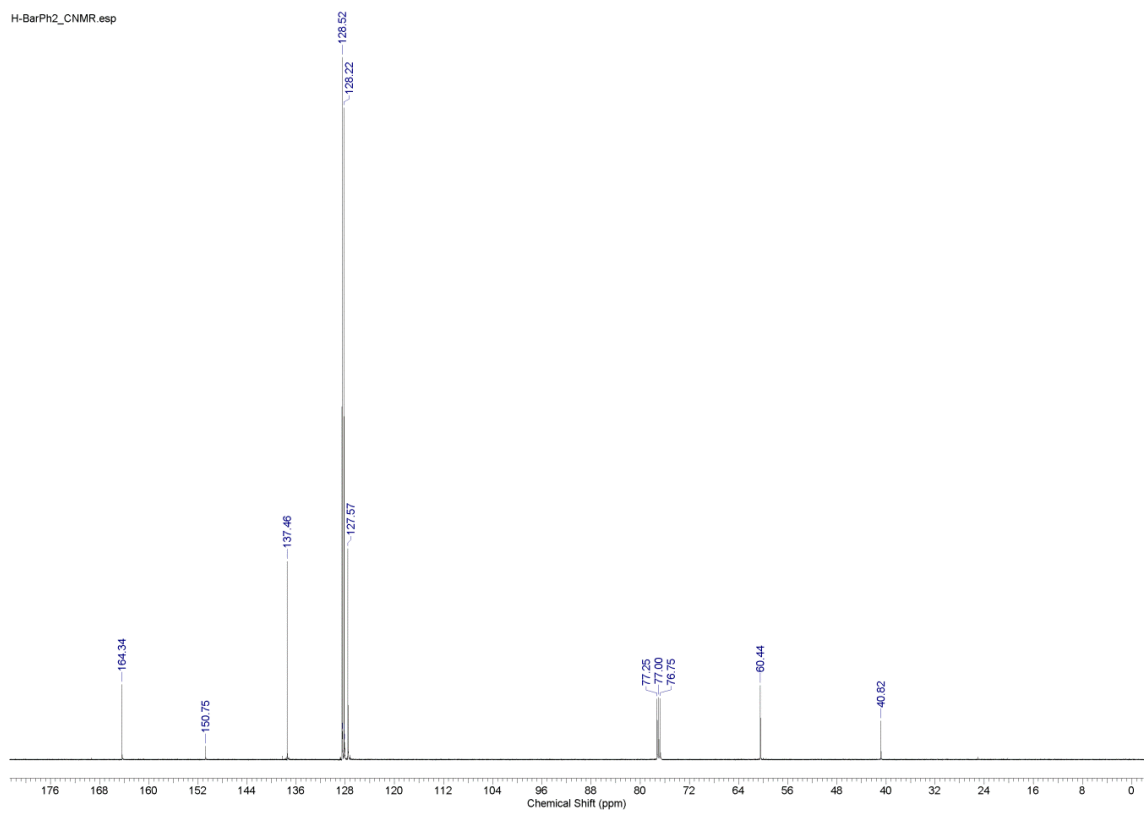
AB-266.011.001.1r.esp



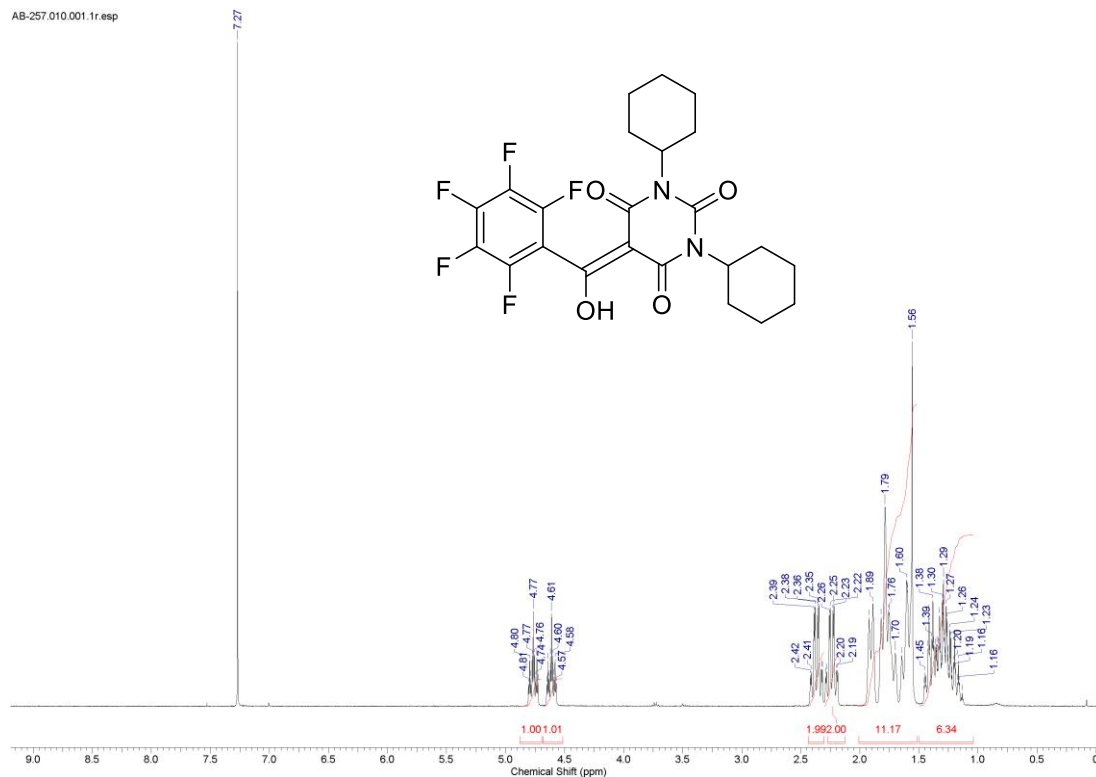
# <sup>1</sup>H NMR for 1,3-bis(diphenylmethyl)-1,3-diazinane-2,4,6-trione



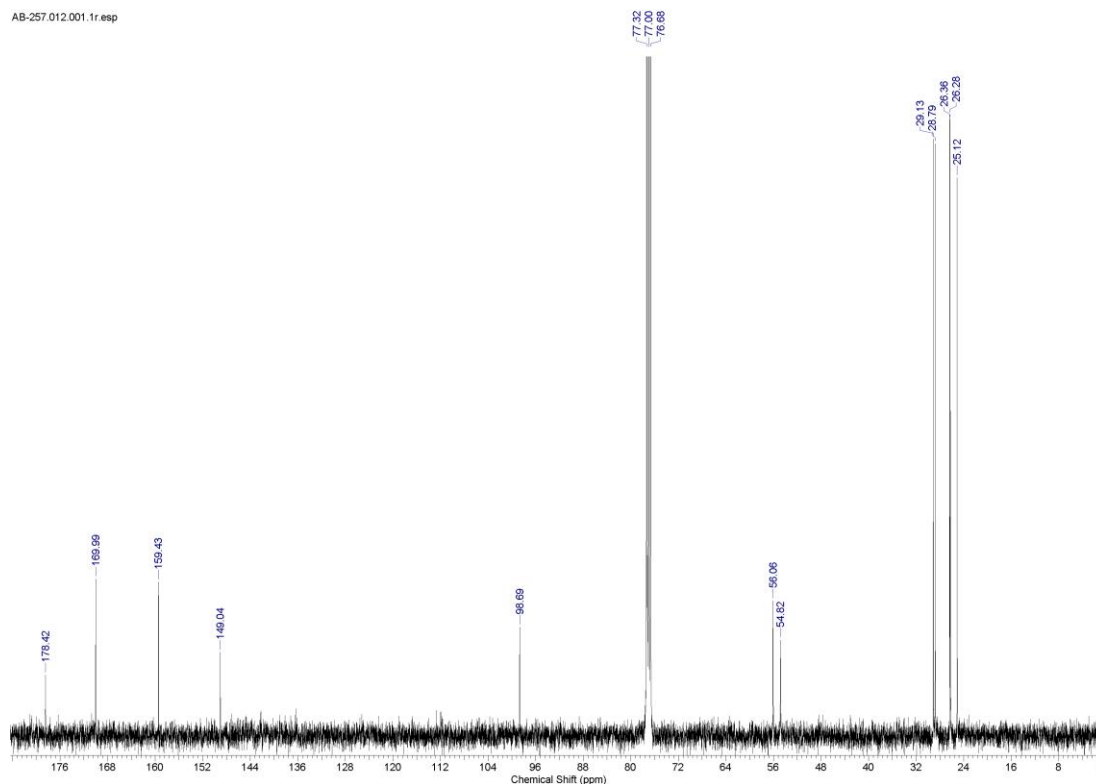
# <sup>13</sup>C NMR for 1,3-bis(diphenylmethyl)-1,3-diazinane-2,4,6-trione



<sup>1</sup>H NMR for 1,3-dicyclohexyl-5-(pentafluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (BarC6)

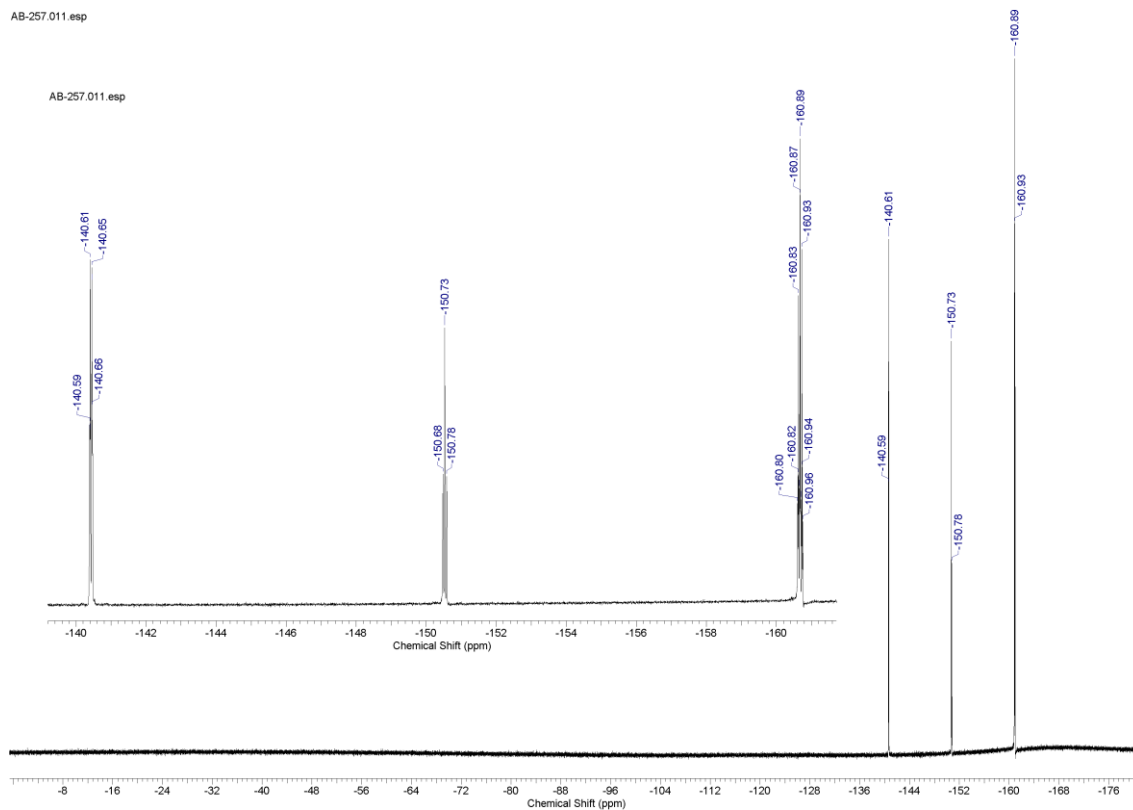


<sup>13</sup>C NMR for 1,3-dicyclohexyl-5-(pentafluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (BarC6)

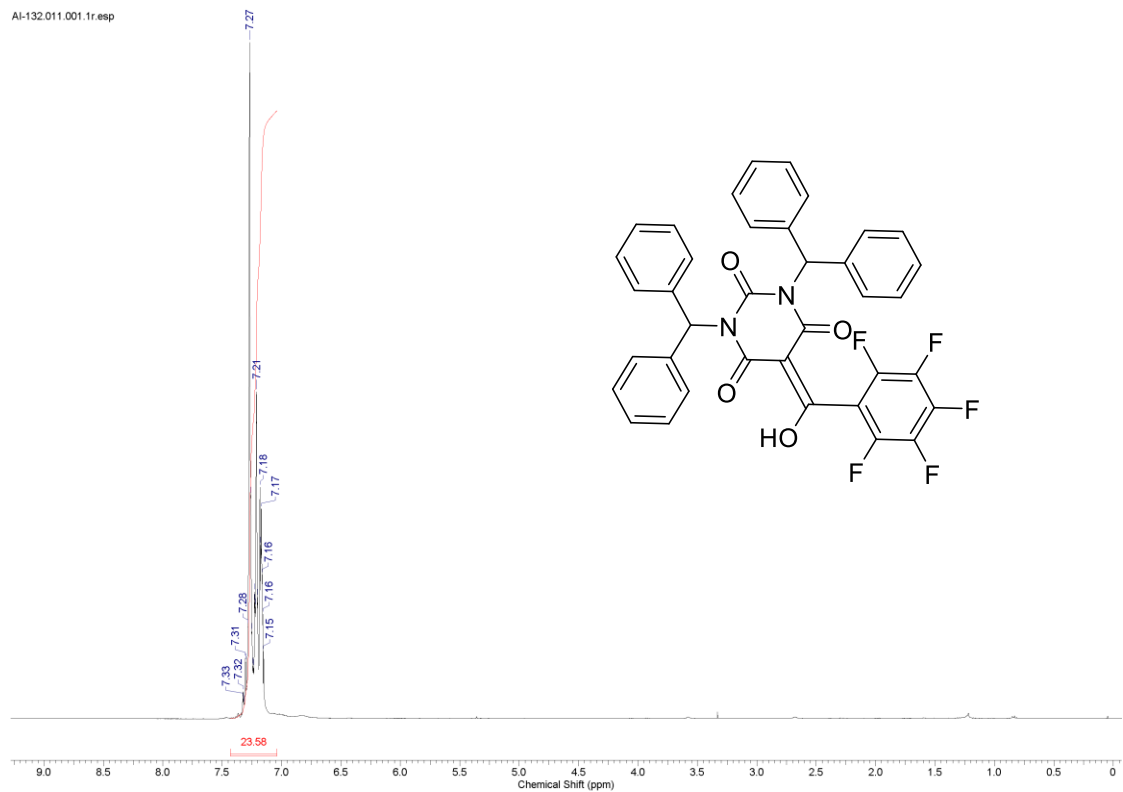




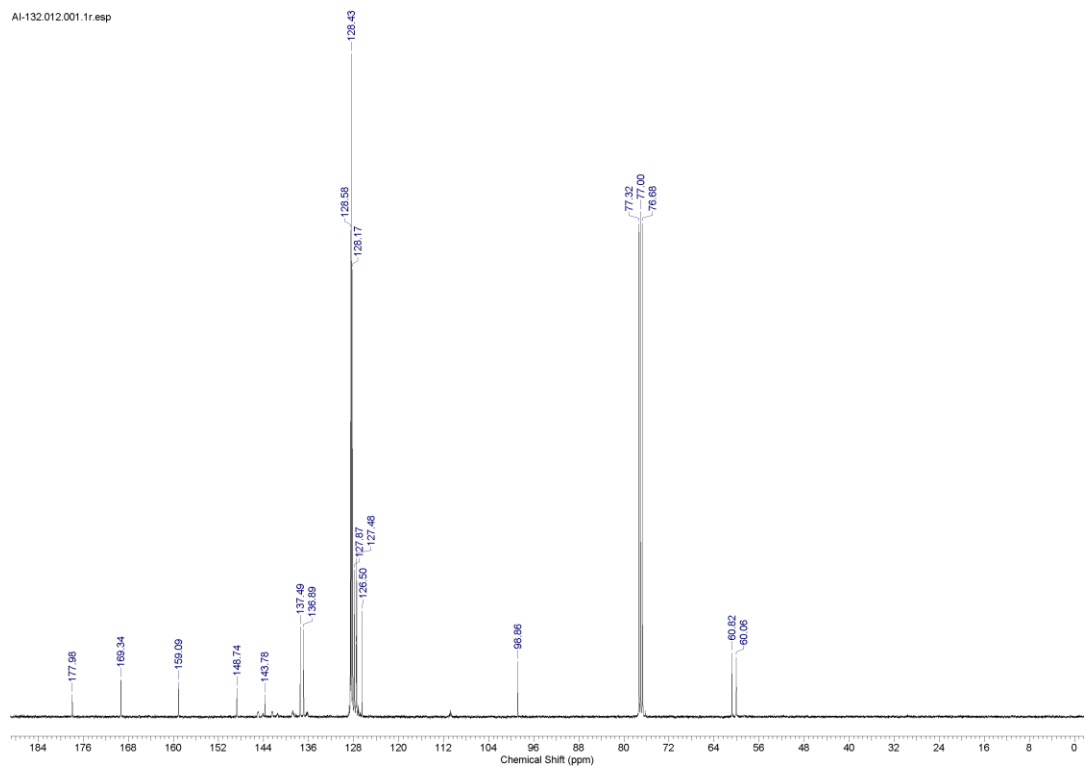
<sup>19</sup>F NMR for 1,3-dicyclohexyl-5-(pentafluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (BarC6)



<sup>1</sup>H NMR for 1,3-bis(diphenylmethyl)-5-(penta-fluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (BarPh2)

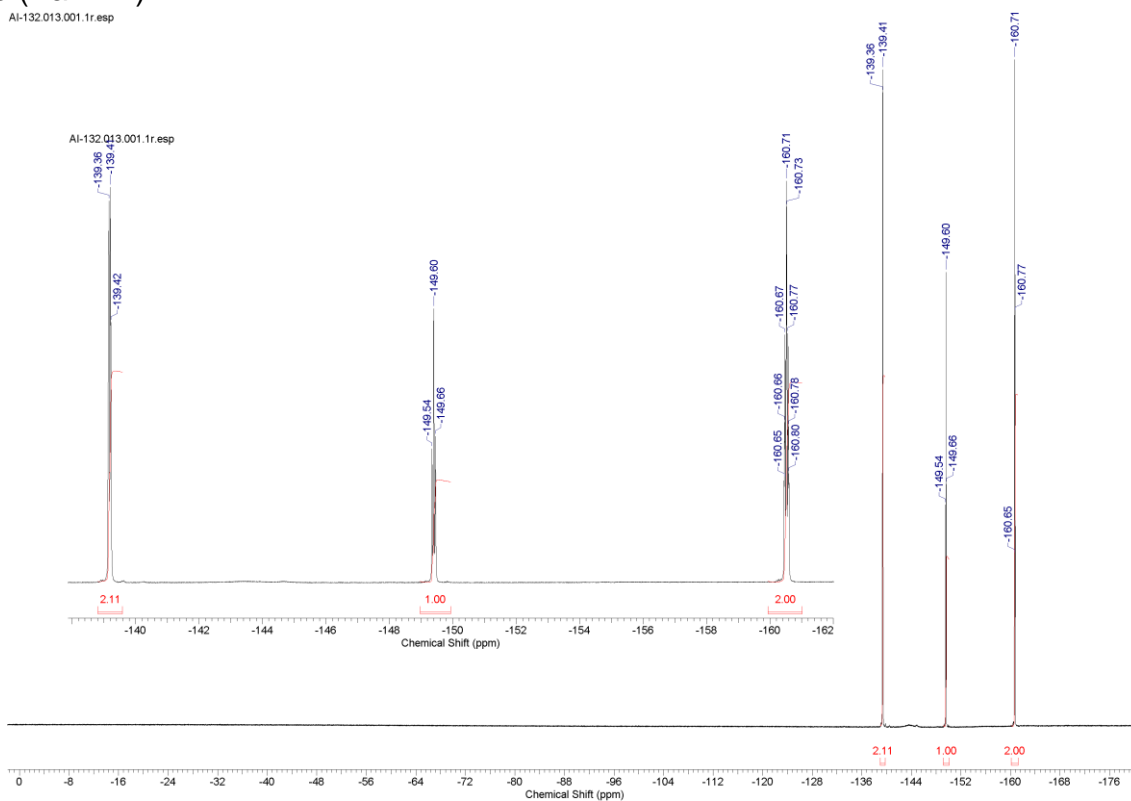


<sup>13</sup>C NMR for 1,3-bis(diphenylmethyl)-5-(penta-fluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (BarPh2)

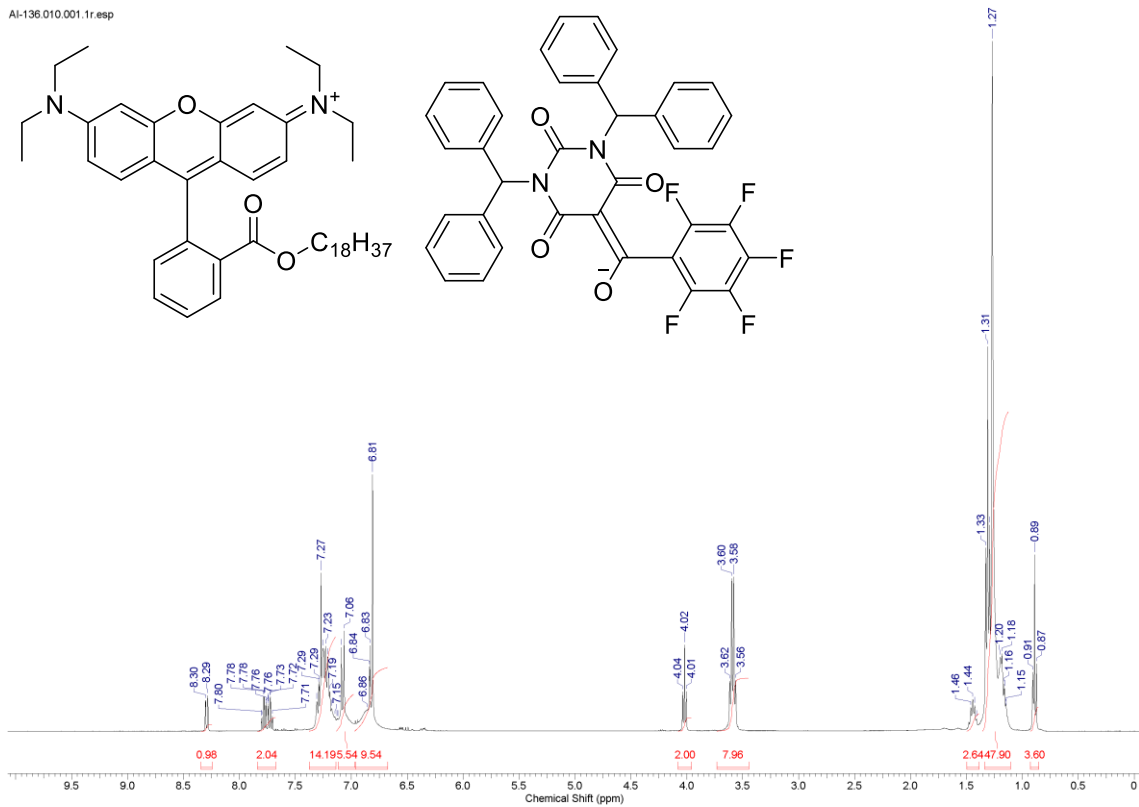


# 19F NMR for 1,3-bis(diphenylmethyl)-5-(penta-fluorophenyl)carbonyl-1,3-diazinane-2,4,6-trione (BarPh2)

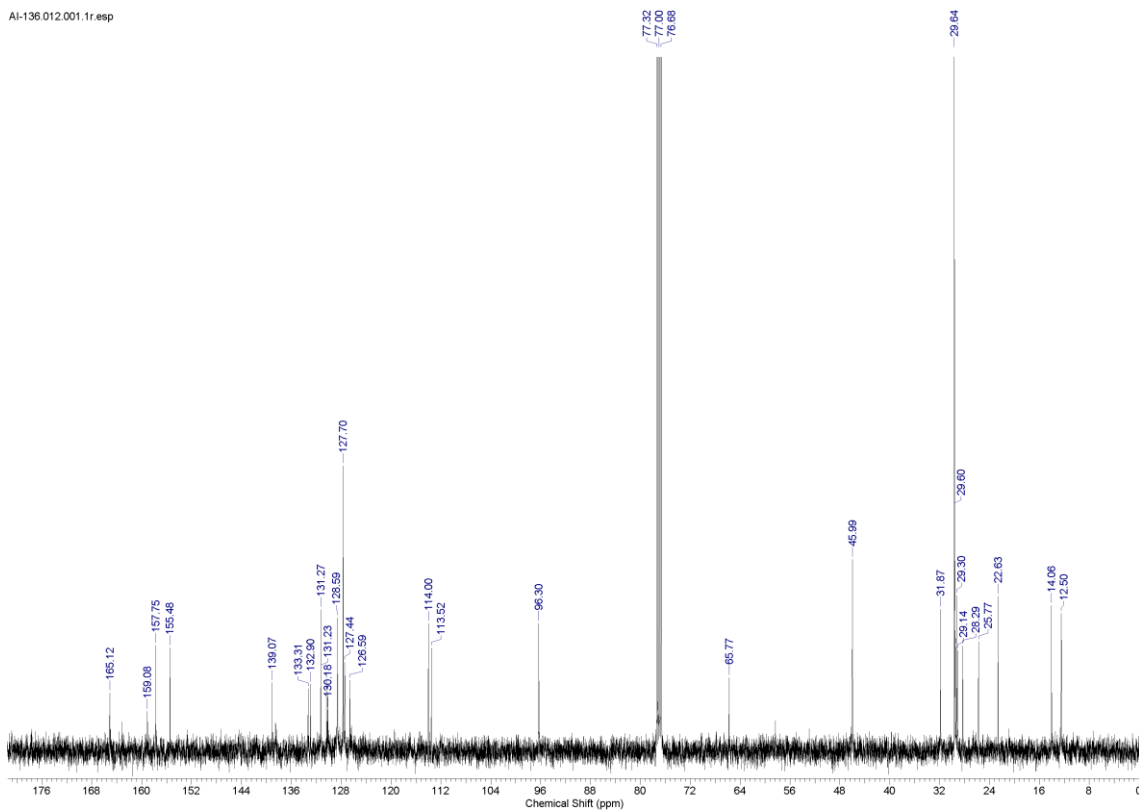
AI-132.013.001.1r.esp



# <sup>1</sup>H NMR for R18/BarPh2 ion pair

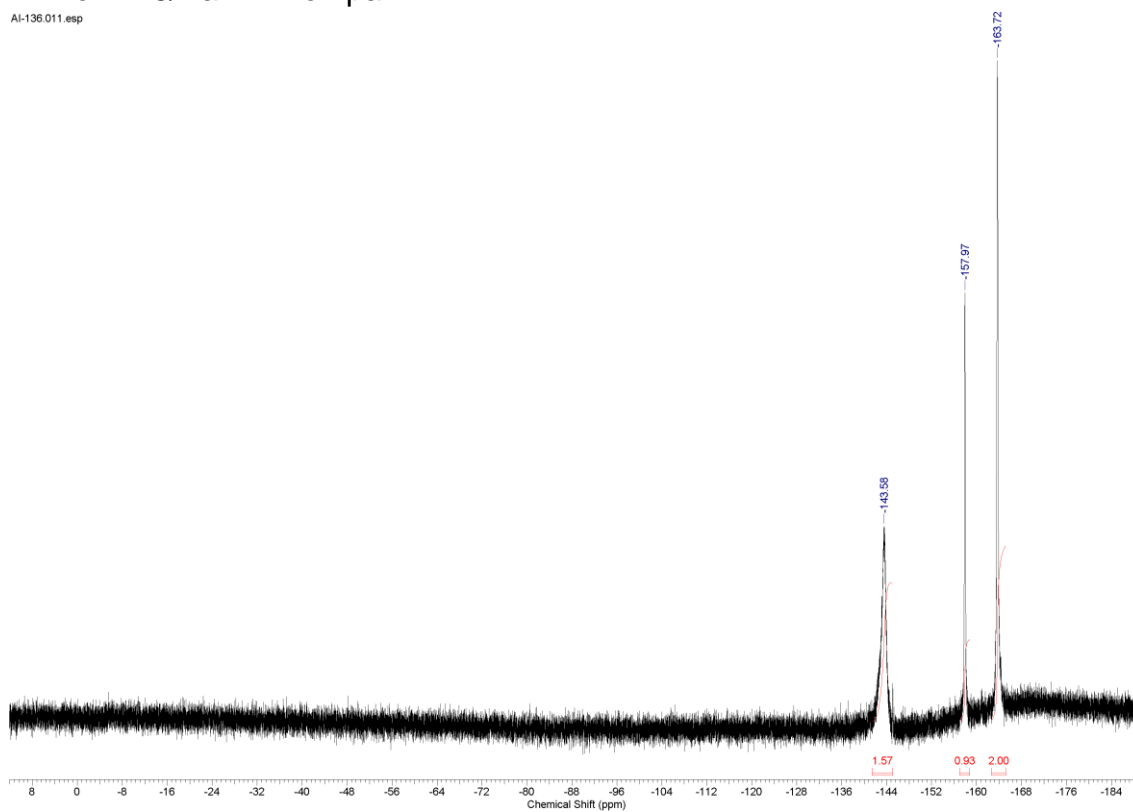


# <sup>13</sup>C NMR for R18/BarPh2 ion pair



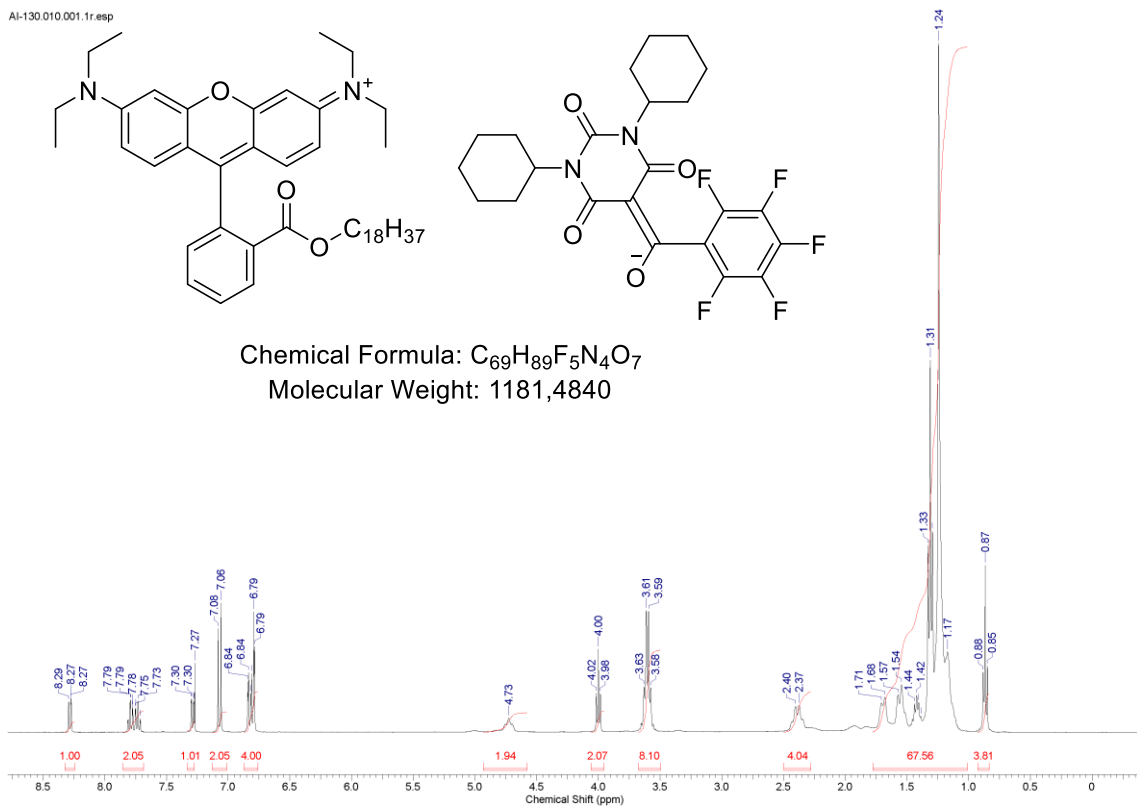
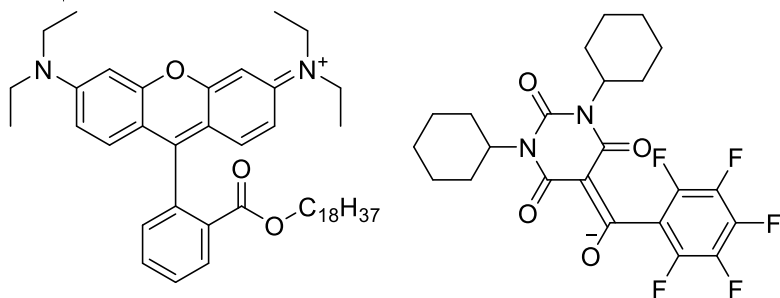
# $^{19}\text{F}$ NMR for R18/BarPh2 ion pair

Al-136.011.esp



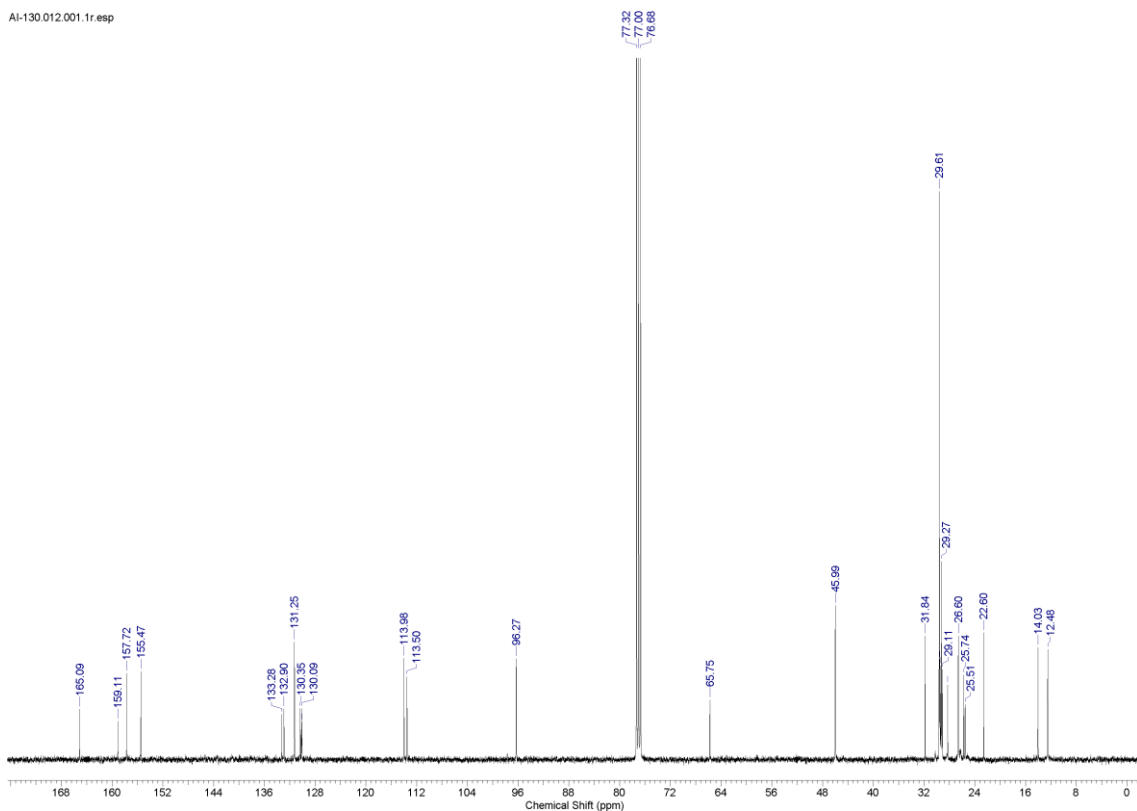
# <sup>1</sup>H NMR for R18/BarC6 ion pair

AI-130.010.001.1r.esp



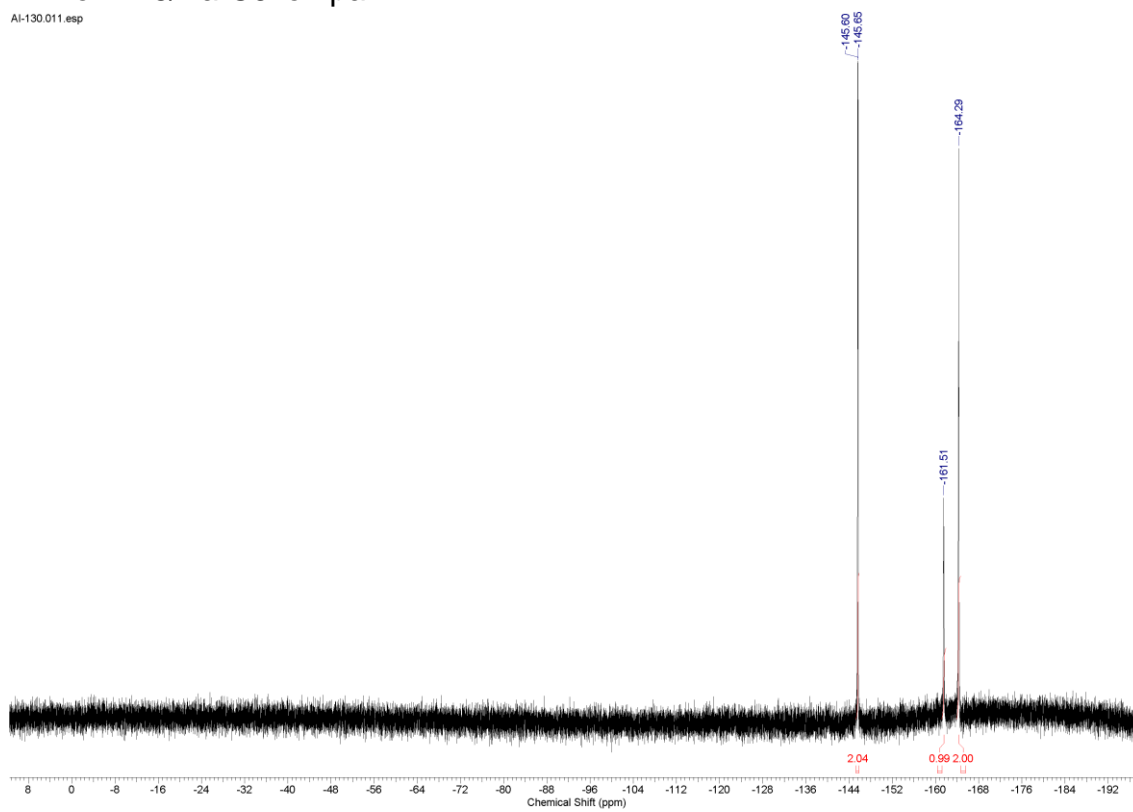
# <sup>13</sup>C NMR for R18/BarC6 ion pair

AI-130.012.001.1r.esp



# $^{19}\text{F}$ NMR for R18/BarC6 ion pair

Al-130.011.esp



## References

- [1] A. K. Bose, S. Garratt, *J. Am. Chem. Soc.* **1962**, *84*, 1310.
- [2] B. Andreiuk, A. Reisch, E. Bernhardt, A. S. Klymchenko, *Chem. Asian J.* **2019**, *14*, 836.
- [3] R. A. Velapoldi, H. H. Tonnesen, *J. Fluoresc.* **2004**, *14*, 465.