Supplementary Data for

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Stereo Specific Platelet Inhibition by the Natural LXR Agonist 22(R)-OH-Cholesterol and its Fluorescence Labelling with Preserved Bioactivity and Chiral Handling in Macrophages

by

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1. General procedure for the esterification reaction

The fluorescence labelled 22OH-cholesterol esters were synthesized using a modified Steglich acylation (S-1).

The glassware is dried for at least 16 h in an oven (120 °C). The reactants 22-hydroxycholesterol, BODIPY-N1C12-fatty acid and 4-pyrrolidinopyrridine (PPY) were dried under high vacuum for three hours. CH_2Cl_2 was freshly distilled over CaH_2 prior to use. Stock solutions of 22(R)- and 22(S)-hydroxycholesterol (1 mg / 110 µl), BODIPY-N1C12-fatty acid (1 mg / 40 µl), PPY (1 mg / 10 µl) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (10 µl / 50 µl (v/v)) were prepared with distilled CH_2Cl_2 .

The stock solutions containing all reactants (see above) were combined in the reaction vessel (GC-vial). The BODIPY-N1C12-fatty acid (1 eq., 2.484 µmol) followed by PPY (1.6 eq., 3.974 µmol), EDC (2 eq., 4.968 µmol) and 22-hydroxycholesterol (1 eq., 2.484 µmol) were added under inert gas atmosphere with a Hamilton syringe (25 µl). After wrapping the

microreactor and the enclosed GC-vial with parafilm the reaction was stirred at room temperature for 6 days. The solvent was then evaporated and the residue redissolved in 100 µl CH_2Cl_2. Aliquots were checked by SiO_2 TLC run with hexane : ethylacetate 80 : 20 (v/v) and localized by UV light with a 480 nm filter. The mixture was separated by chromatography on a SiO_2-cartridge eluted with a n-hexane : diethylether gradient 10.: 0 to 5 : 5 (v/v) and fractions were checked by TLC.

2) Description of the microreactor

The microreactor consists of an outer and an inner part. The outer part is a 120 mm long glass tube with NS29 joint and a diameter of 30 mm that narrows after 70 mm to a diameter of 15 mm. On the bottom of the narrowed part a screw thread is attached that fits for a GL18 cap. On the upper part of the glass tube after 50 mm (from the top) a valve is attached in order to connect to a vacuum and/or inert gas Schlenk-line. The inner part consists of a commercially available GC-vial (32x11.6 mm) with a screw cap (8 mm) and a septum. It is placed in the centre of the narrowed part of the glass tube above the GL18 screw and contains a micro stir bar. The whole micro reactor can be sealed with a NS29 rubber septum and wrapped with parafilm to avoid intrusion of air and moisture.

3. Assembly of the microreactor

The glassware of the micro reactor (disassembled) is dried for at least 16 h in an oven (120 °C). After that the micro reactor is assembled quickly while still hot: the GL18 cap is screwed onto the bottom of the glass tube and the GC-vial (without cap but containing a micro stir bar) is inserted in the lower part of the glass-tube. The glass tube is then sealed with a NS29 rubber septum and the valve is connected to a Schlenk-line. After flame drying and nitrogen purge of the whole micro reactor, the rubber septum is removed while keeping the nitrogen stream flowing. Now the cap of the GC-vial is screwed with tweezers. After closing the reactor with the NS29 Septum again, the same procedure (flame drying and nitrogen purge) is repeated. After that the reactor can be used for reactions of 0.5 to 1 mg under inert gas atmosphere. The components are inserted in form of stock solutions with Hamilton syringes and long iron needles by piercing through both septa. After the addition has been finished, the rubber septum is removed again and the GC-vial is wrapped in parafilm, placed in the narrowed glass tube cavity again and the glass tube is sealed with the rubber septum and evacuated again. Finally the micro reactor is flushed with nitrogen, wrapped in parafilm and placed above a stirrer.

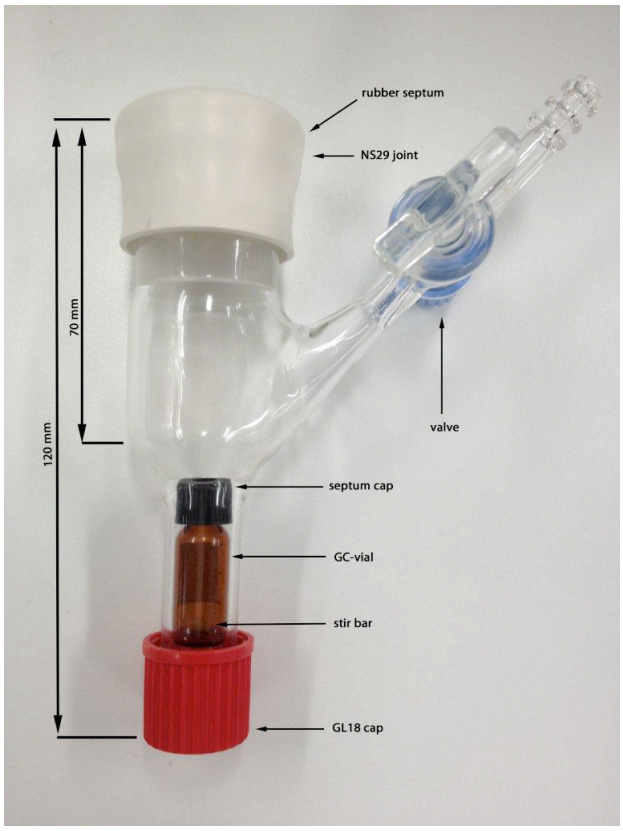


Figure S1: Assembled microreactor

4. Product analysis

The products were identified by NMR spectroscopy using a Varian 600 MHz spectrometer.

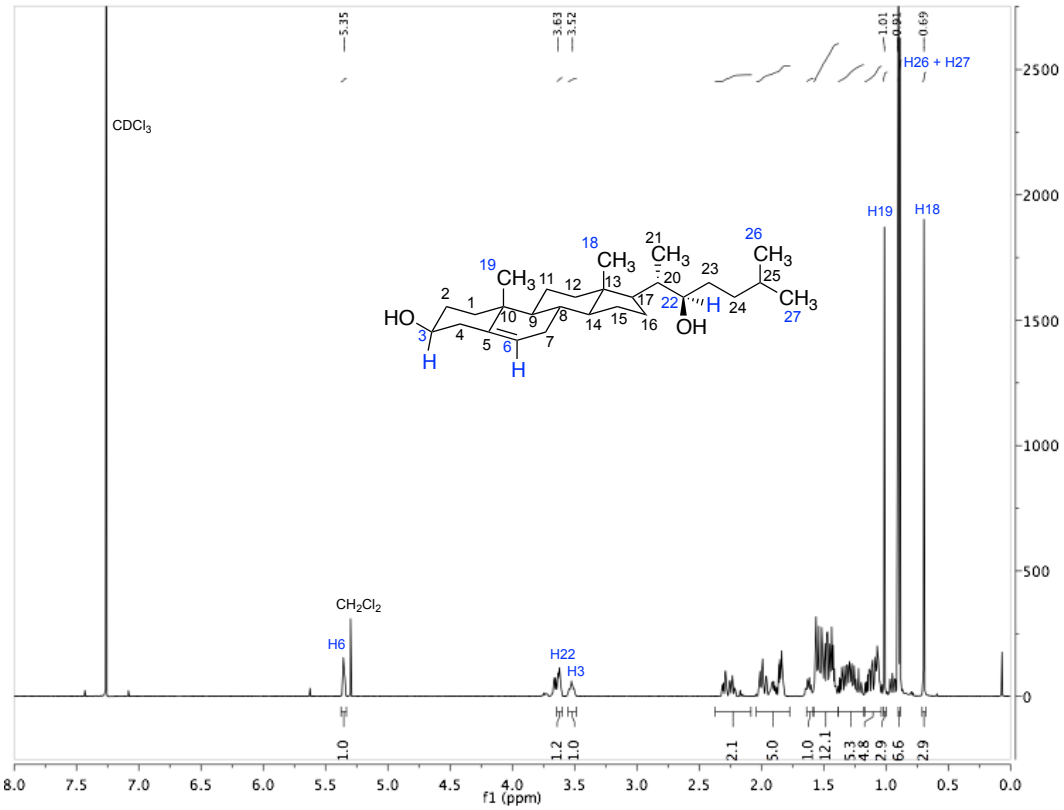


Figure S2. 600 MHz ^{1}H NMR spectrum of starting material 22(*R*)-OHC.

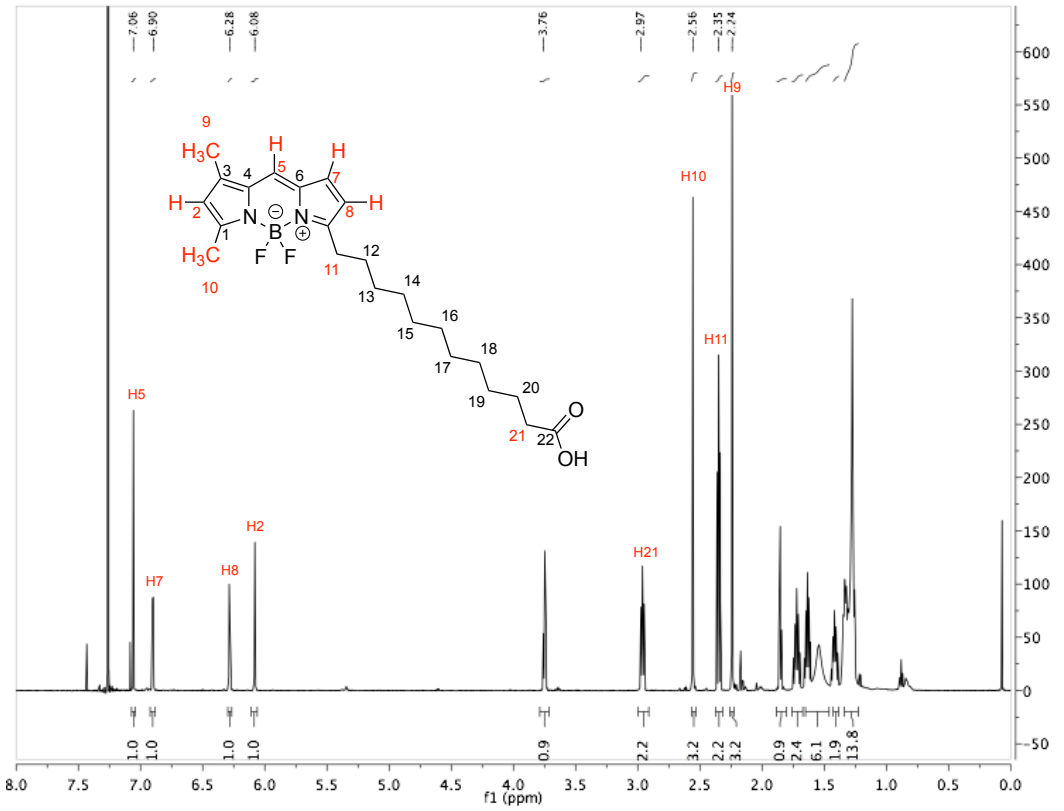


Figure S3. 600 MHz ^{1}H NMR spectrum of starting material BODIPY.

The unit of the x-axis in the NMR-spectra is ppm. The signals are reported relative to the signal of tetramethylsilane (TMS) which is used as the reference and thus set to 0 ppm. The unit of the y-axis is arbitrarily chosen and usually aligned to the highest signal (in this case $CDCl_3$).

The characteristic protons of the starting material 22(*R*)-hydroxycholesterol (blue) and that of the BODIPY fl C12 (red) were aligned and both sets of signals were found in the obtained product in the right intensity relation (see Fig. 1). A proof for the regioselective acylation at the hydroxyl group in 3 position in the cholesterol moiety is that the product still contains the H22 signal whereas the signal of the H3 proton broadened up and is thus not clearly to align.

According to the general procedure the substrates were employed on a reaction scale of 10 mg. Purification of the crude mixture gave the product in 96 % yield.

5. Characterization of byproducts

The side products of the esterification reaction were isolated and analysed by NMR and MS-ESI/HRMS spectroscopy using a Thermo Finnigan LTQ FT instrument.

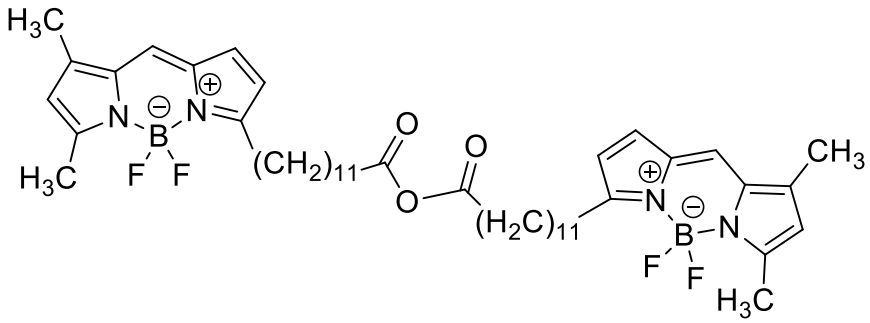


Figure S5: BODIPY-anhydride.

This byproduct was isolated from the reaction of (S)-OHC and BODIPY FL C12 according to the general procedure in chloroform. The chloroform was in that case not distilled, but used as purchased.

HRMS (ESI) $[M+Na^+ + Et_2O]^+$ calc. for $C_{50}H_{74}B_2F_4N_4NaO_4^+$: requires 915.5725, found: 915.5739.

MS (ESI): m/e 915 ($M+Na^+ + Et_2O$), 910, 764, 469, 464,427, 425, 399.

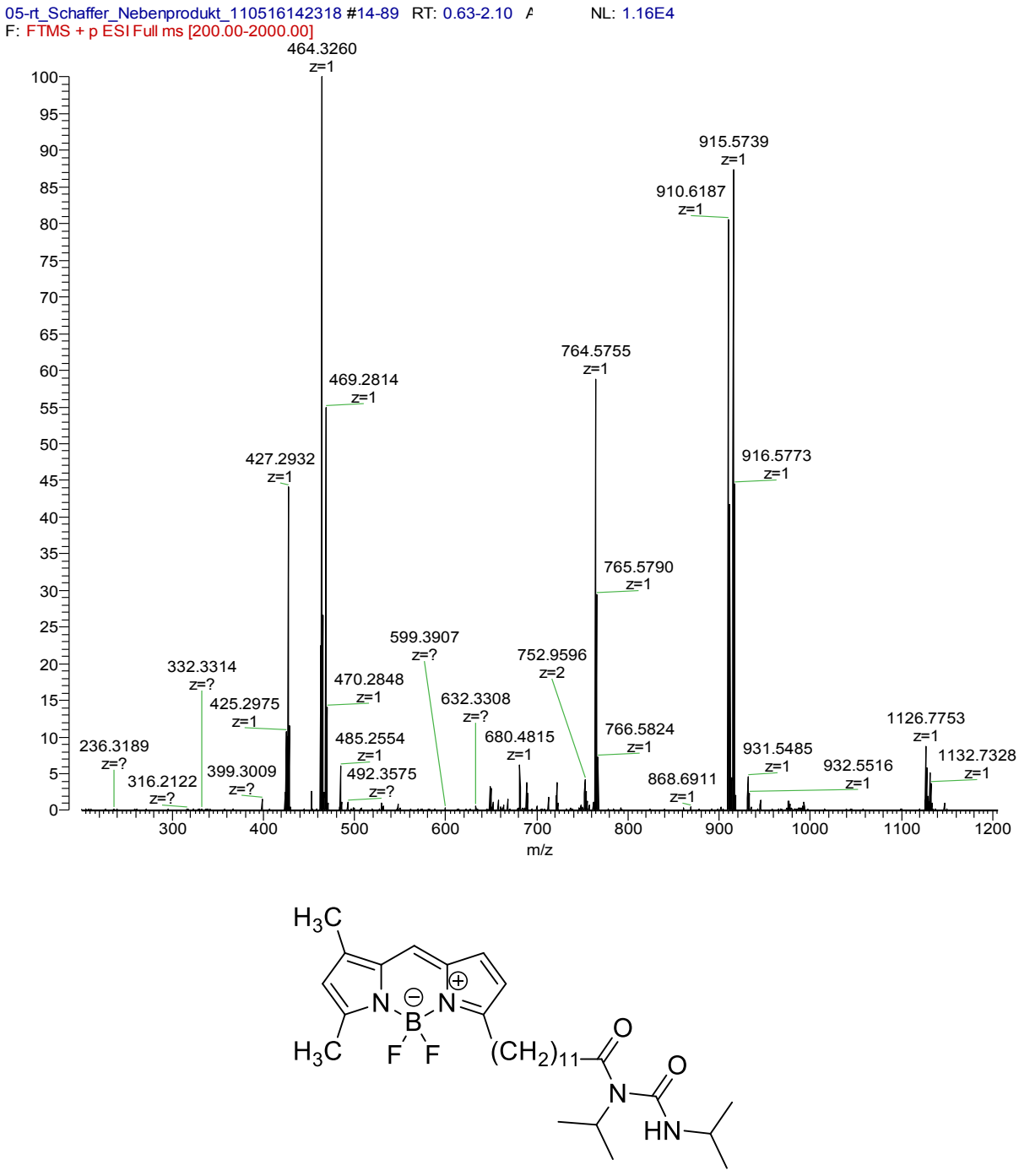
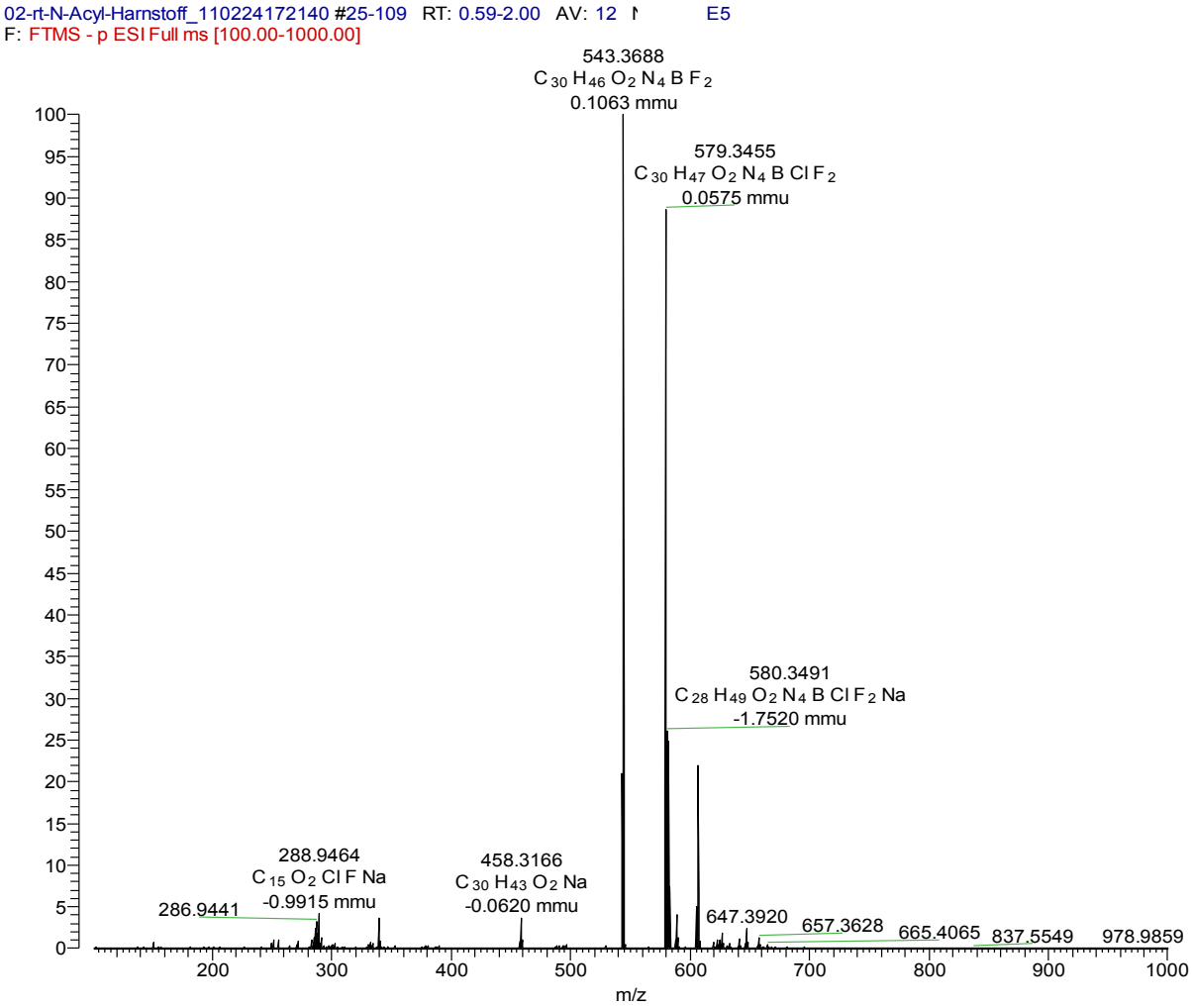


Figure S6: BODIPY N-acylurea.

This byproduct was isolated from the reaction of Sitosterol and BODIPY FL C12 according to the general procedure with the use of DIC instead of EDC.

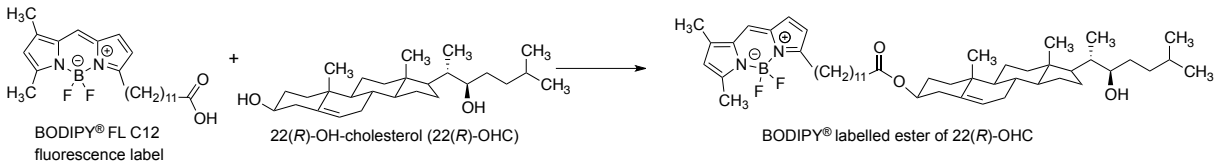
HRMS (ESI) $[M-H]^-$ calc. for $C_{30}H_{46}BF_2N_4O_2^-$: requires 543.3687, found: 543.3688.

MS (ESI): m/e 543 $(M-H)^-$, 542, 458, 339, 288, 285.

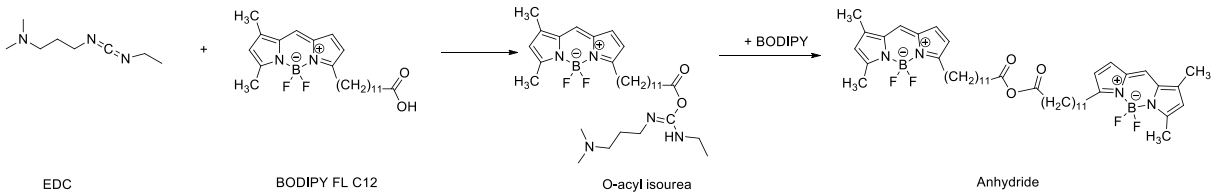


6. Reaction schemes for the formation of products and byproducts

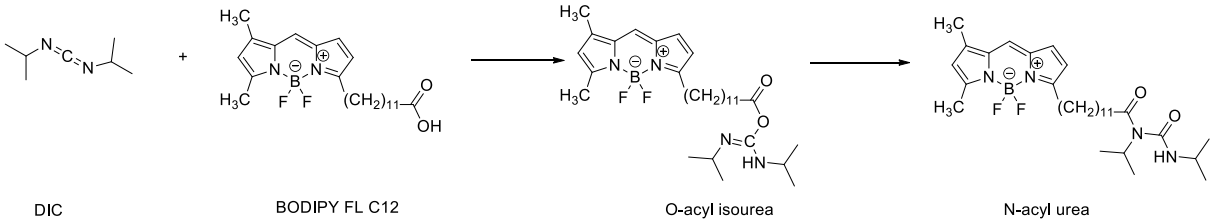
Formation of the product BODIPY-22(R)-OH-cholesteryl ester:



Formation oft the anhydride:



Formation of the N-acyl urea:



7. Metabolic stability of 22(R)- and 22(S)-OHC-BP in macrophages

Figure S-7: Aliquots if lipid extracts from macrophages incubated for 24 h with 10 µM (from left to right) 22(R)-, 22(S)-OHC-BP, cholesterol-BP or free BP-duodecanoic acid (dd-BP) and standards as added to cells plus free BODIPY chromophore standard (BP) were separated by SiO_2-TLC and visualized by UV-fluorescence detection.

extracts from cells **standards as added to cells**

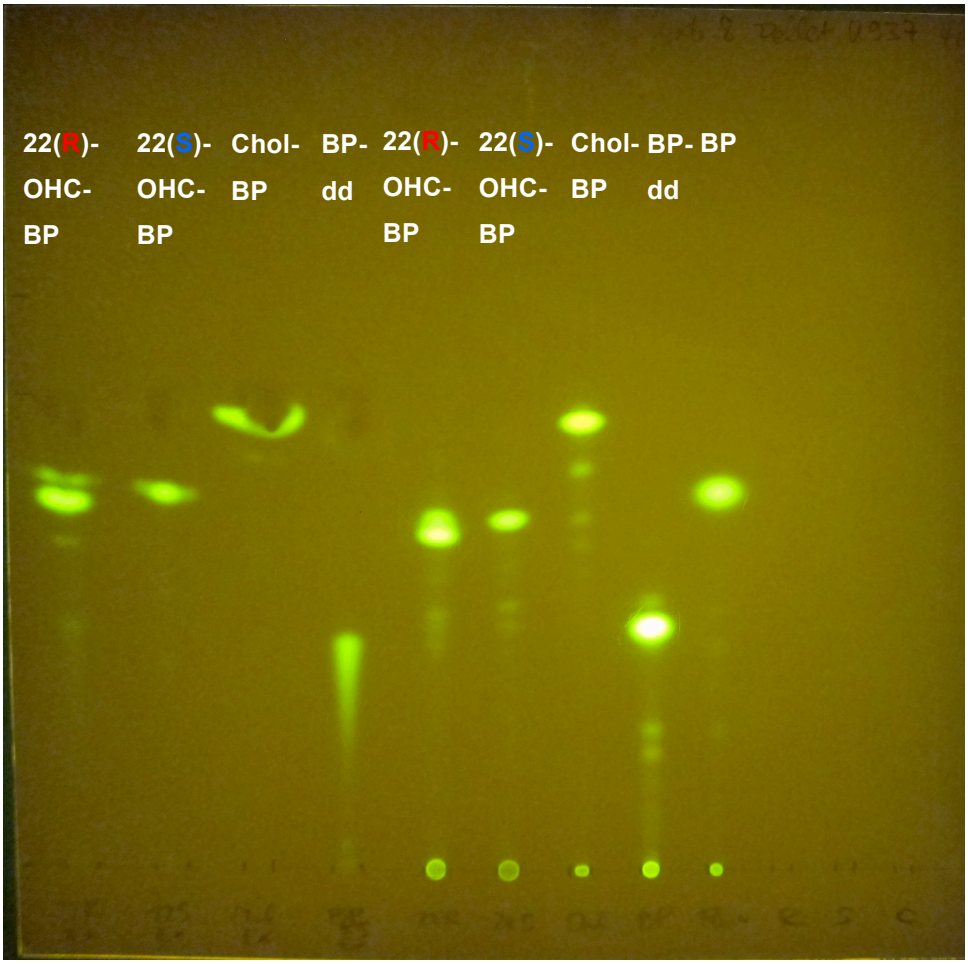
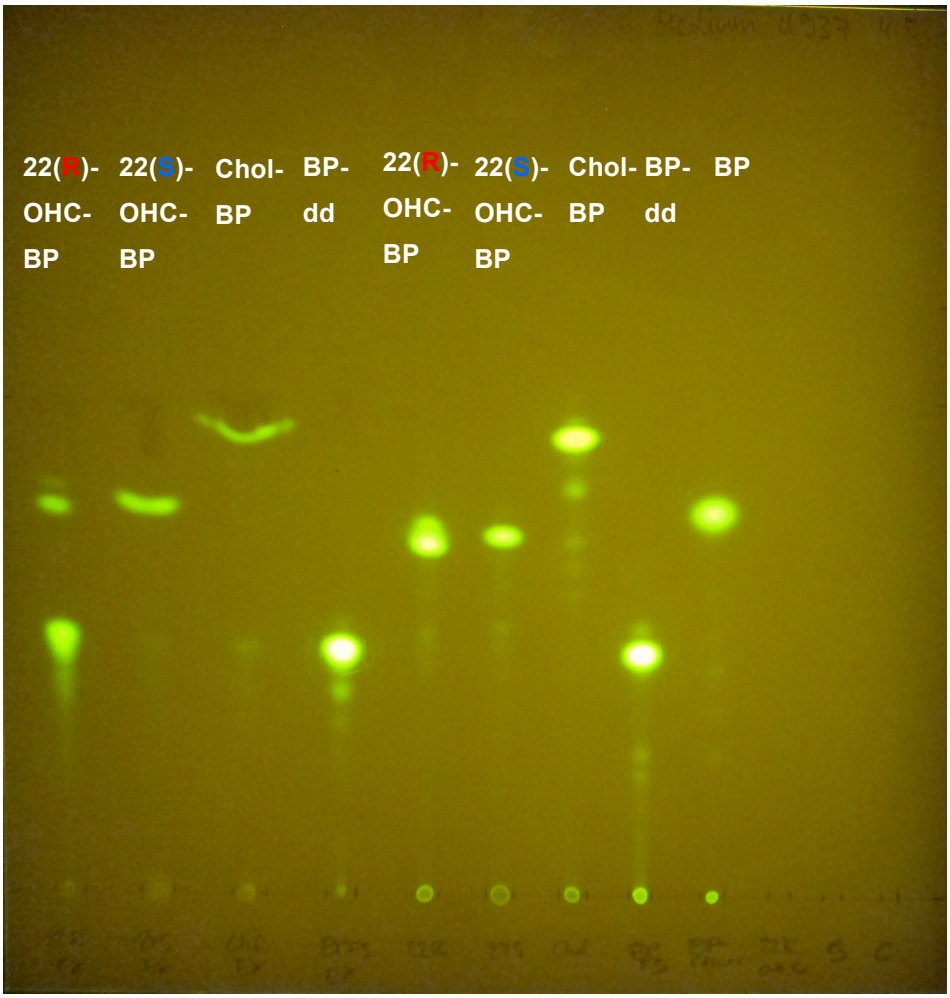


Fig. S-8: Concentrated lipid extracts from supernatant medium of macrophages incubated for 24 h with 10 µM (from left to right) 22(R)-, 22(S)-OHC-BP, cholesterol-BP or free BP-duodecanoic acid (dd-BP) and standards as added to cells plus free BODIPY chromophore standard (BP) were separated by SiO_2-TLC and visualized by UV-fluorescence detection.

extracts from supernatants standards as added to cells



Reference:

(S1) Neises B, Steglich W. Simple Method for the Esterification of Carboxylic Acids. Angew. *Chem. Int. Ed. Engl.* **1978**, *17*, 522 – 524.