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Synthesis of Triphenylphosphonium-Linked Derivative of 3,5-Di*tert*-butyl-4-hydroxybenzylidene-malononitrile (SF6847) via Knoevenagel Reaction Yields an Effective Mitochondria-Targeted Protonophoric Uncoupler

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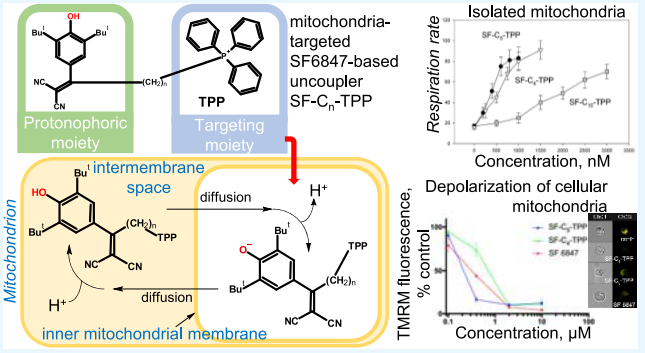
ABSTRACT: Mitochondrial uncouplers are actively sought as potential therapeutics. Here, we report the first successful synthesis of mitochondria-targeted derivatives of the highly potent uncoupler 3,5-di*tert*-butyl-4-hydroxybenzylidene-malononitrile (SF6847), bearing a cationic alkyl(triphenyl)phosphonium (TPP) group. As a key step of the synthesis, we used condensation of a ketophenol with malononitrile via the Knoevenagel reaction. SF-C_5-TPP with a pentamethylene linker between SF6847 and TPP, stimulating respiration and collapsing membrane potential of rat liver mitochondria at submicromolar concentrations, proved to be the most effective uncoupler of the series. SF-C_5-TPP showed pronounced protonophoric activity on a model planar bilayer lipid membrane. Importantly, SF-C_5-TPP exhibited rather low toxicity in fibroblast cell culture, causing mitochondrial depolarization in cells at concentrations that only slightly affected cell viability. SF-C_5-TPP was more effective in decreasing the mitochondrial membrane potential in the cell culture than SF6847, in contrast to the case of isolated mitochondria. Like other zwitterionic uncouplers, SF-C_5-TPP inhibited the growth of *Bacillus subtilis* in the micromolar concentration range.



INTRODUCTION

Compounds causing marked respiratory stimulation were described 90 years ago[1] and later called uncouplers[2] because they break the coupling between the oxidation of organic compounds and ATP synthesis; in other words, they uncouple phosphorylation from oxidation. Even in the early years, it became clear that uncoupling could be beneficial under certain conditions.[3] At present, mitochondrial uncouplers are considered promising in fighting various diseases.[4−7] Yet the medicinal application of the majority of uncouplers is precluded by rather high toxicity.[8] To this end, making their action more targeted seems to be rational. Several attempts have been made to create a mitochondria-targeted uncoupler by attaching a lipophilic cationic group, such as triphenylphosphonium (TPP), widely used for mitochondria targeting,[9−13] to conventional uncouplers, such as 2,4-dinitrophenol (DNP)[14] and carbonyl cyanide m-chlorophenyl hydrazone (CCCP).[15] Unfortunately, these derivatives appeared to be weak uncouplers. By contrast, linking decyl-TPP to fluorescein yielded a rather effective mitochondrial uncoupler coined mitoFluo, albeit showing poor protonophoric activity on model lipid membranes.[16] Of importance, mitoFluo exhibited neuro- and nephroprotective effects in rat models of traumatic brain and kidney injuries,[17] which was obviously associated with the reduction of ROS generation by mitochondria, i.e., the antioxidant action of mitoFluo. Here, we report the synthesis of one of the most potent small-molecule mitochondrial uncouplers 3,5-di*tert*-butyl-4-hydroxybenzylidene-malononitrile (SF6847)[18] appended to alkyl-TPP with varying carbon chain length, SF-C_n-TPP. Of note, SF6847, introduced in 1971[19] and extensively studied later,[20−23] now still remains a promising scaffold for the design of new mitochondrial bioenergetics' inhibitors.[24] Importantly, the pentamethylene linker-containing derivative SF-C_5-TPP not only stimulated

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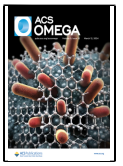
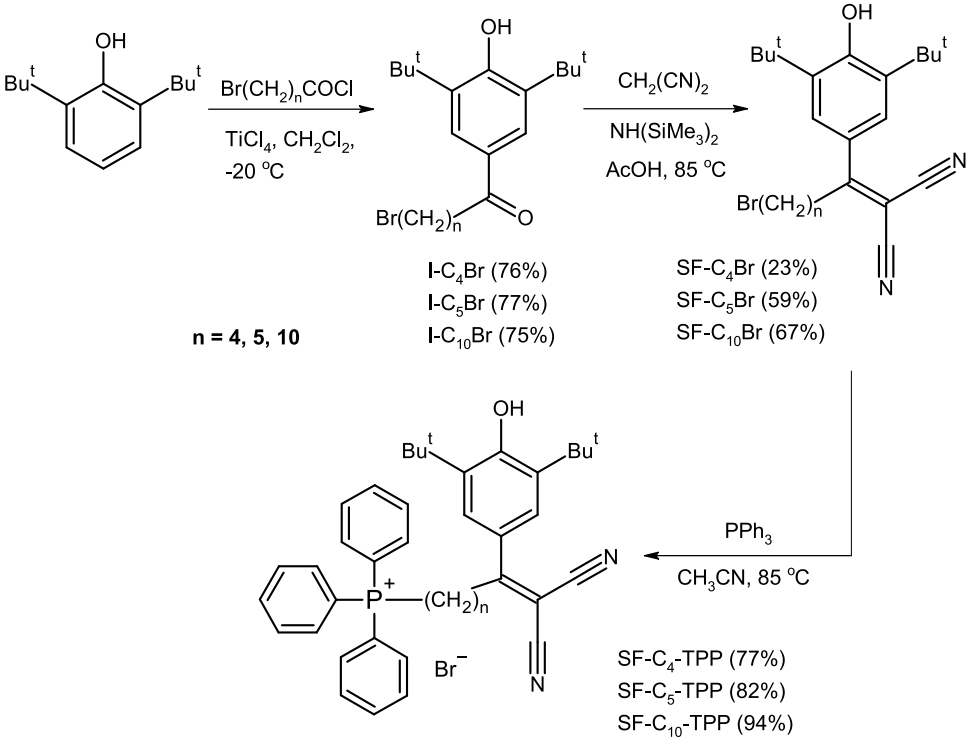
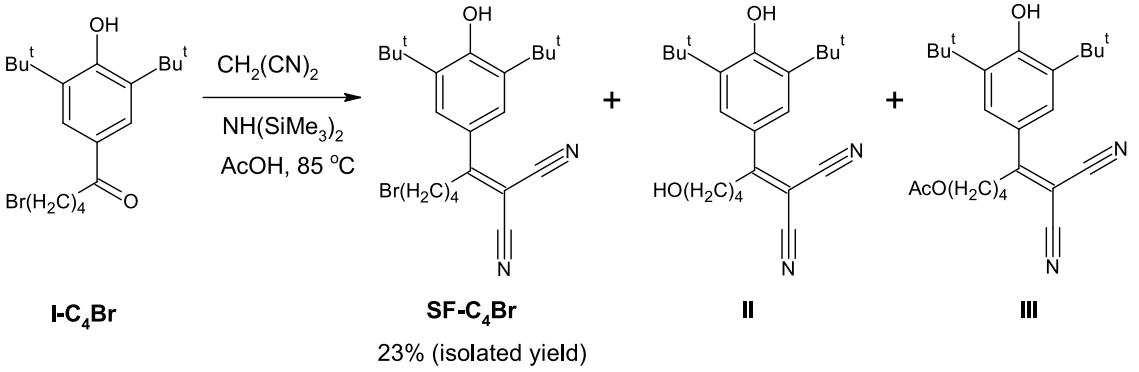


Scheme 1. Synthesis of SF-C_n-TPP Conjugates



Scheme 2. Reaction of I−C_4Br with Malononitrile



respiration and decreased membrane potential of isolated rat liver mitochondria (RLM) at submicromolar concentrations, although less effectively than SF6847, but also elicited transmembrane proton current across planar bilayer lipid membranes. In fibroblast cell culture, SF-C_5-TPP was more effective in mitochondrial depolarization than SF6847, in contrast to isolated RLM.

RESULTS AND DISCUSSION

Synthesis of SF-C_n-TPP. SF-C_n-TPP conjugates were prepared in three steps starting from 2,6-di*tert*-butylphenol according to Scheme 1.

The Friedel−Crafts acylation of 2,6-di*tert*-butylphenol with chloroanhydrides of ω-bromoalkane carboxylic acids was carried out in the presence of titanium tetrachloride at −20 °C. The low temperature seems to be crucial for the successful implementation of this reaction because the dealkylation and/or migration of *tert*-butyl substituents occurred at 0 °C or above.[25,26] The Knoevenagel condensation of 4-acyl-2,6-di*tert*-butylphenols with malononitrile appears to be a rather difficult task even in the case of the simplest representative of these ketophenols, such as 4-acetyl-2,6-di*tert*-butylphenol. In fact, the known methyl-modified SF6847 (TX-1122)[27,28] was obtained by heating in benzene in the presence of ammonium acetate as a catalyst, but the yield was poor. We found that the previously described method,[29] in which condensation occurred in hexamethyldisilazane (HMDS)/acetic acid mixture, allows the preparation of alkyl-containing derivatives of SF6847 in good yields (unpublished results). To our great surprise, the method of Knoevenagel condensation appeared to be useful also in the preparation of ω-bromoalkyl-modified SF6847 (SF-C_nBr). This procedure implies that HMDS transforms in the presence of acetic acid to $AcOSiMe_3$, which serves as a water removal agent, and $AcONH_4$, which is a promoter. There were concerns that acetate might substitute for bromide in such harsh conditions. Indeed, the reaction of 4-(5′-bromopentanoyl)−2,6-di*tert*-butylphenol, I−C_4Br (Figure S1), with malononitrile resulted in a mixture of three compounds (Scheme 2), and desirable SF-C_4Br was isolated only in a poor yield of 23%.

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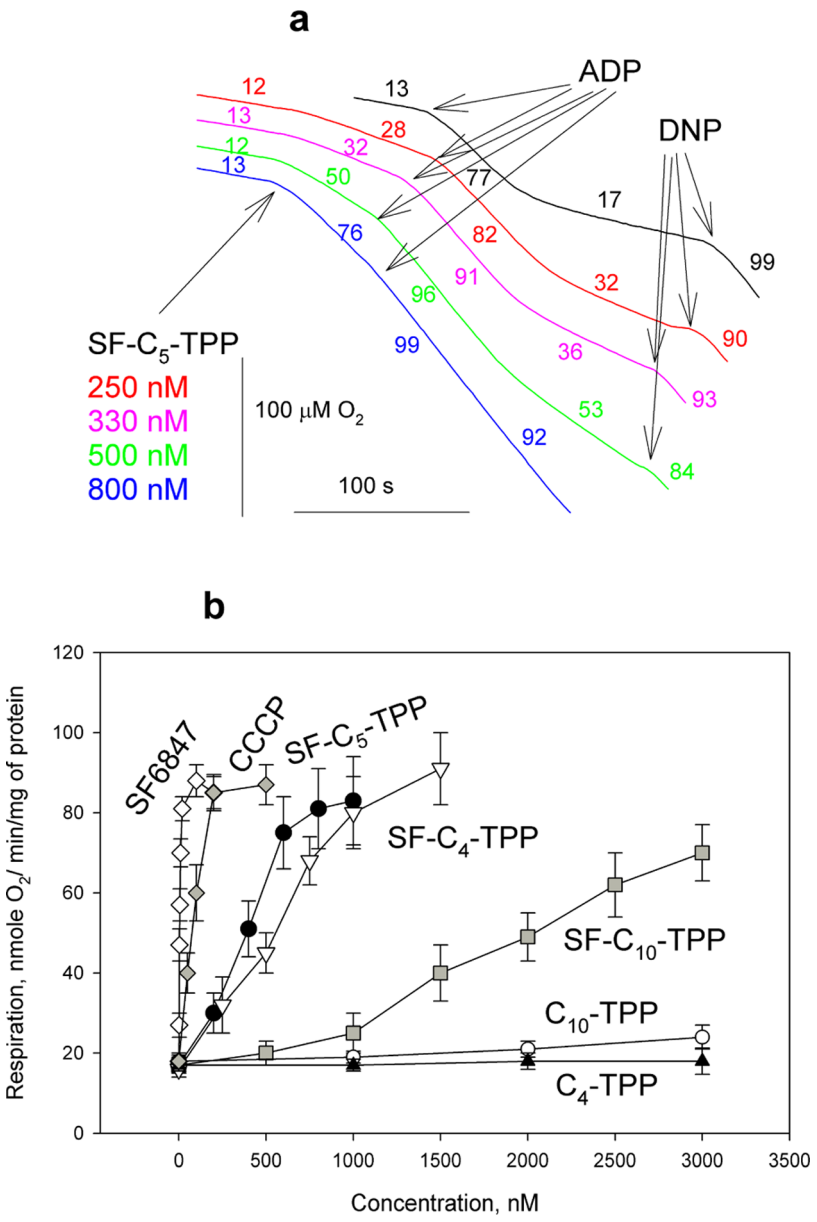

Figure 1. Effect of SF-C_5-TPP at various concentrations on the basal respiration rate and respiration rates in states 3 and 4 of the isolated RLM (a). Respiration rates are indicated as relative values in the course of each record. The maximal respiration rate for the control mitochondria could be attained by the addition of 40 μM 2,4-dinitrophenol (DNP). Incubation medium: RLM (0.5 mg protein/ml), MOPS 20 mM (pH 7.2), sucrose 250 mM, EGTA 1 mM, $MgCl_2$ 2 mM, KH_2PO_4 2 mM, succinate 5 mM, and rotenone 2 μM. The effect of SF-C_5-TPP (filled circles), SF-C_4-TPP (open triangles), SF-C_{10}-TPP (gray squares), C_{10}-TPP (open circles), C_4-TPP (filled triangles), SF6847 (open diamonds), and CCCP (filled diamonds) on RLM respiration (b). Points and error bars correspond to mean ± SD obtained from at least 3 independent experiments.

Surprisingly, if the bromoalkyl substituent contained five methylene groups or more, the reaction proceeded smoothly, and no analogous byproducts were observed. ω-Bromoalkyl-modified SF6847 (SF-C_nBr) smoothly reacted with triphenylphosphine to give desirable SF-C_n-TPP conjugates in good to excellent yields. The ^{1}H, ^{13}C, and ^{31}P NMR spectra and Liquid chromatography-mass spectrometry (LC-MS) data for the compounds are presented in Figures S1−S29.

Estimation of pK_a of SF-C_5-TPP. As expected from the literature data on the pK_a of SF6847,[21,22] the pK_a value measured spectrophotometrically from the pH dependence of absorbance at a maximum of the SF-C_5-TPP spectrum (Figure S30) was equal to 7.17 in the aqueous solution and 7.16 in the presence of DPhPC liposomes. Therefore, similar to the majority of conventional uncouplers,[30] SF-C_5-TPP is a weak acid with pK_a close to physiological pH.

SF-C_n-TPP Stimulated Respiration of the Isolated Rat Liver Mitochondria. Figure 1 displays the effect of SF-C_n-TPP on RLM respiration upon the addition of ADP (100 μM). As it is seen from the time courses of oxygen consumption by RLM (**a**), SF-C_5-TPP caused pronounced acceleration of RLM respiration at a concentration of 250 nM both before the addition of ADP and in state 4 (after ADP conversion into ATP). The comparison of the concentration dependences of the RLM respiration rate for a series of SF-C_n-TPP with n = 4, 5, 10 (**b**) shows that the pentamethylene linker-containing derivative was the most potent. Alkyl-TPP compounds lacking the 3,5-di*tert*-butyl-4-hydroxybenzylidene-malononitrile group,

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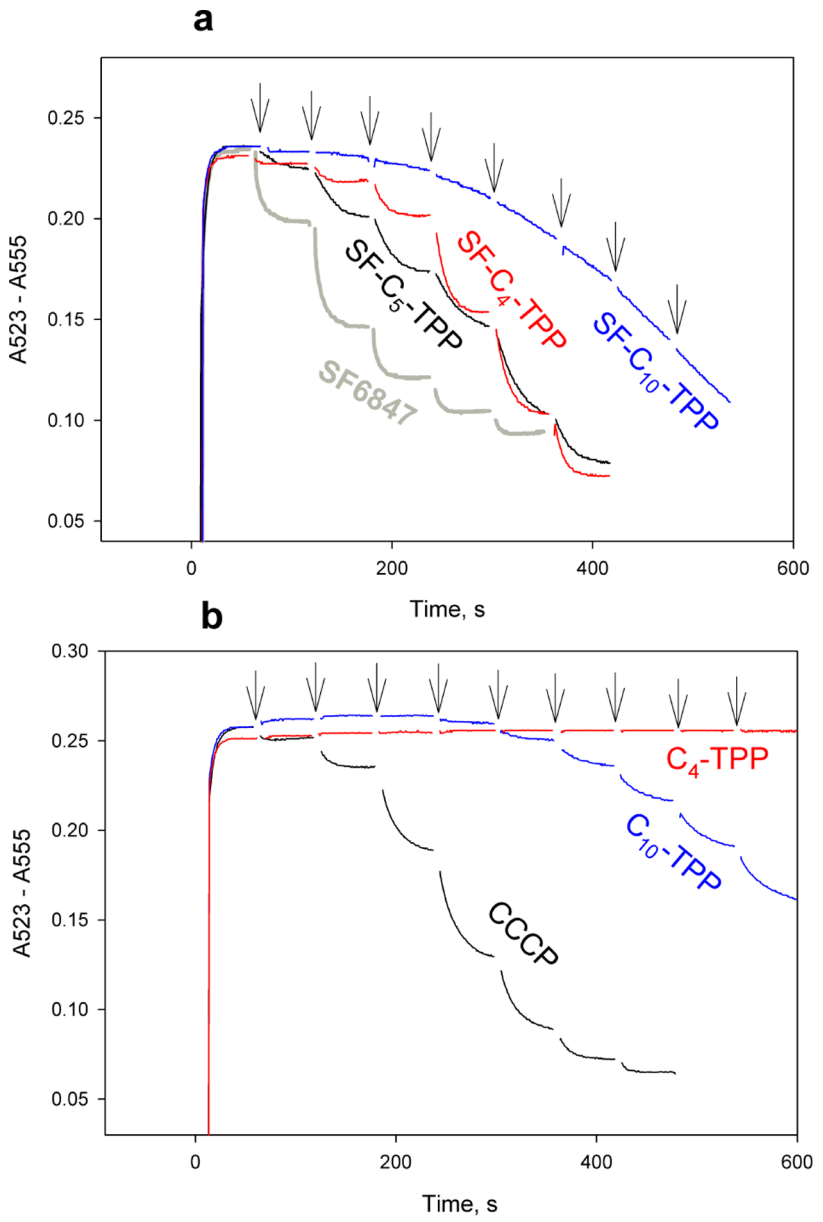

Figure 2. Effect of SF-C_5-TPP (a, black line), SF-C_4-TPP (a, red line), SF-C_{10}-TPP (a, blue line), CCCP (b, black line), C_4-TPP (b, red line), and C_{10}-TPP (b, blue line) on membrane potential in isolated RLM, as measured by changes in safranine O (15 μM) absorbance. (**a**) Three successive additions of the compounds (0.25 μM each) and subsequent additions (0.50 μM each) are marked by arrows. The gray line shows the effect of sequential additions of SF6847, 5 nM each. (**b**). Sequential additions of the compounds, 2 μM each, are marked by arrows. The black line shows the effect of sequential additions of CCCP, 50 nM each. For experimental conditions, see the Experimental Section.

C_4-TPP and C_{10}-TPP, were of no effect at the submicromolar and micromolar concentrations (open circles and filled triangles), while SF6847 (open diamonds) was effective at nanomolar concentrations.

SF-C_n-TPP Decreased Membrane Potential of the Isolated Rat Liver Mitochondria. In line with the data on mitochondrial respiration, SF-C_5-TPP also exhibited the highest efficacy of the SF-C_n-TPP series in decreasing the RLM membrane potential, as measured by changes in safranine O absorbance (Figure 2). Thus, the compounds of the SF-C_n-TPP series proved to be mitochondrial uncouplers, with n = 5 being the optimal alkyl chain length for the uncoupling activity. This kind of chain length dependence (with an optimum) is in line with previous results on the protonophores derived from fluorescein,[31] 7-nitrobenz-2-oxa-1,3-diazole,[32] rhodamine 19,[33] *p*-chlorophenol,[34,35] and 7-hydroxycoumarin,[36,37] and also corresponds to other studies of chain length impact on membrane permeability and biological activity of small molecules.[38−40]

SF-C_4-TPP Accumulated in the Isolated Rat Liver Mitochondria Upon Energization. Figure 3 depicts the effect of respiring mitochondria on the potential of a tetraphenylphosphonium (TPP^+)-selective electrode observed in the presence of SF-C_4-TPP or TPP^+. The addition of RLM to the experimental cuvette brought about the uptake of SF-C_4-TPP similar to that of TPP^+ due to membrane potential generation resulting from the oxidation of endogenous substrates, which was then suppressed by the addition of the respiratory complex I inhibitor rotenone leading to SF-C_4-TPP release. The addition of succinate as the respiratory substrate

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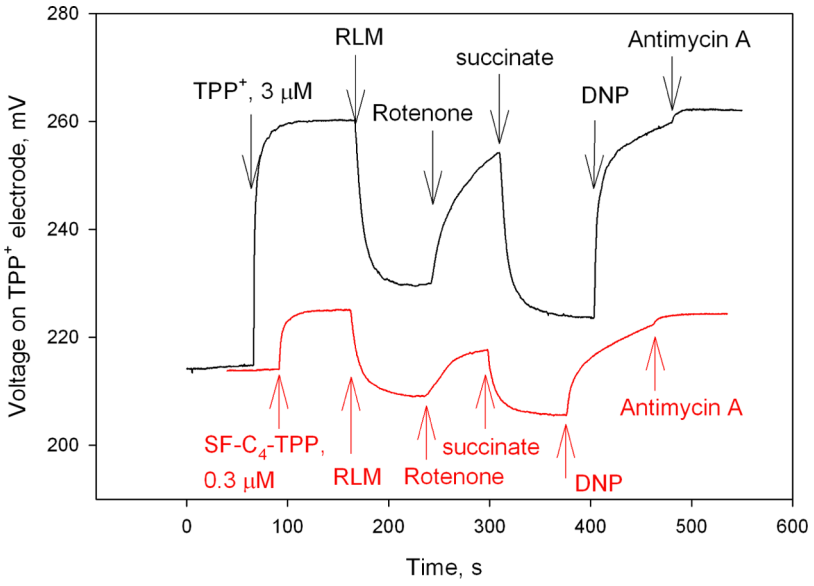

Figure 3. Effect of respiring RLM on the voltage of a tetraphenylphosphonium-selective electrode in the presence of SF-C_4-TPP (red curve) or TPP^+ (black curve). The solution was MOPS 20 mM, KH_2PO_4 10 mM, sucrose 250 mM, $MgCl_2$ 1 mM, EGTA 0.2 mM, and bovine serum albumin 0.5 mg/mL, pH 7.4. Additions: rotenone 2 μM, succinate 2 mM, DNP 40 μM, antimycin A 1 μM. T = 23 °C. Concentration of mitochondrial protein, 1 mg/mL.

again caused the uptake of SF-C_4-TPP by the mitochondria, which was followed by its release after the addition of the conventional uncoupler DNP, similar to previous observations with other zwitterionic uncouplers.[16,41,42] With SF-C_5-TPP, it was hardly possible to observe its energy-dependent accumulation in mitochondria because of its high uncoupling activity, leading to membrane potential collapse.

Direct Measurements of Proton Transport Induction by SF-C_n-TPP on the Planar Bilayer Lipid Membrane (BLM). To monitor the SF-C_5-TPP-induced transport of protons, we detected the electrical current across a BLM formed from soybean phosphatidylcholine (asolectin) under voltage-clamp conditions. The addition of 5 μM phloretin, known to cause a reduction of the membrane dipole potential, thus hindering anion translocation but promoting cation translocation,[43−45] led to a marked increase in the SF-C_5-TPP-mediated current (Figure 4a, red track) across the asolectin membrane. Therefore, translocation of the protonated cationic form of SF-C_5-TPP across the membrane contributes significantly to the total current, similar to the case of other zwitterionic uncouplers.[39] By contrast, phloretin caused a sharp drop in the current mediated by the classical anionic protonophore SF6847 (Figure 4a, black track).

To evaluate the ion selectivity of SF-C_5-TPP-mediated current, $I-V$ characteristics were determined under asymmetrical conditions of different pH values at the opposite sides of the BLM (pH_{cis} = 7.8, pH_{trans} = 6.8, Figure 4b, red open circles). The value of a zero-current voltage (V_{zero}) under these asymmetrical conditions was −51.1 mV, having a "minus" sign at the side with lower pH. The V_{zero} value showed high proton selectivity of the conductance ($V_{max} = 2.3 \cdot \frac{RT}{F} \cdot \Delta pH = 59$ mV at $\Delta pH = 1.0$), as follows from the Nernst equation. Based on three repeats, the average V_{zero} value was −43.4 ± 7.7 mV.

Depolarization of Mitochondria in Cultured Cells Incubated with SF-C_n-TPP. In further experiments, we measured the uncoupling efficacy of SF-C_n-TPP in the cell culture. Figure 5a displays concentration dependences of changes in the fluorescence intensity of the potential-sensitive dye TMRM, reflecting mitochondrial membrane depolarization in MRC5-SV40 cells incubated with SF-C_n-TPP for 60 min. It is seen that SF-C_5-TPP provoked a reduction of mitochondrial membrane potential in cells even at a concentration of 0.4 μM. The comparison of the data in Figures 5a and 2 reveals a sharp difference in the relative efficacy of SF-C_5-TPP and SF6847 in the cell culture with respect to isolated mitochondria, namely, in the cells, SF-C_5-TPP was more effective in mitochondrial depolarization than SF6847, whereas in isolated RLM, the case was vice versa, which could be associated with alleviation of hampered SF6847 penetration into cells by attaching an alkyl-TPP. The enhanced depolarizing effect of the TPP-conjugated derivatives of SF6847 on cellular mitochondria was also observed with JC-1, another potentially sensitive fluorescent dye (Figure 5b).

Influence of SF-C_n-TPP on Cell Viability. Resazurin-based measurements of cell survival in the presence of SF-C_n-TPP (Figure 6) showed rather low toxicity of these compounds toward MRC5-SV40 cells at submicromolar concentrations that caused a marked decrease in mitochondrial membrane potential (Figure 5a). This result is consistent with recent findings indicating that cell viability could not be correlated with mitochondrial bioenergetics.[46]

Antibacterial Activity of SF-C_n-TPP. Like other zwitterionic uncouplers,[35,39,47] SF-C_n-TPP (n = 4, 5, 10) exhibited antibacterial activity with Gram-positive species. In particular, the growth of *Bacillus subtilis* was inhibited at micromolar concentrations of these compounds (Table 1).

It should be noted that lipophilic TPP substituents, such as alkyl-TPP, ensure the targeting of compounds to respiring mitochondria due to their inside negative membrane potential.[9] Alkyl-TPP cations per se are not protonophoric uncouplers because they cannot bind and dissociate protons; therefore, they lack the ability to carry protons selectively by themselves across membranes. By contrast, triphenylphosphonium ylides possess high protonophoric uncoupling activity due to the acidity of a methylene group linking phosphorus to

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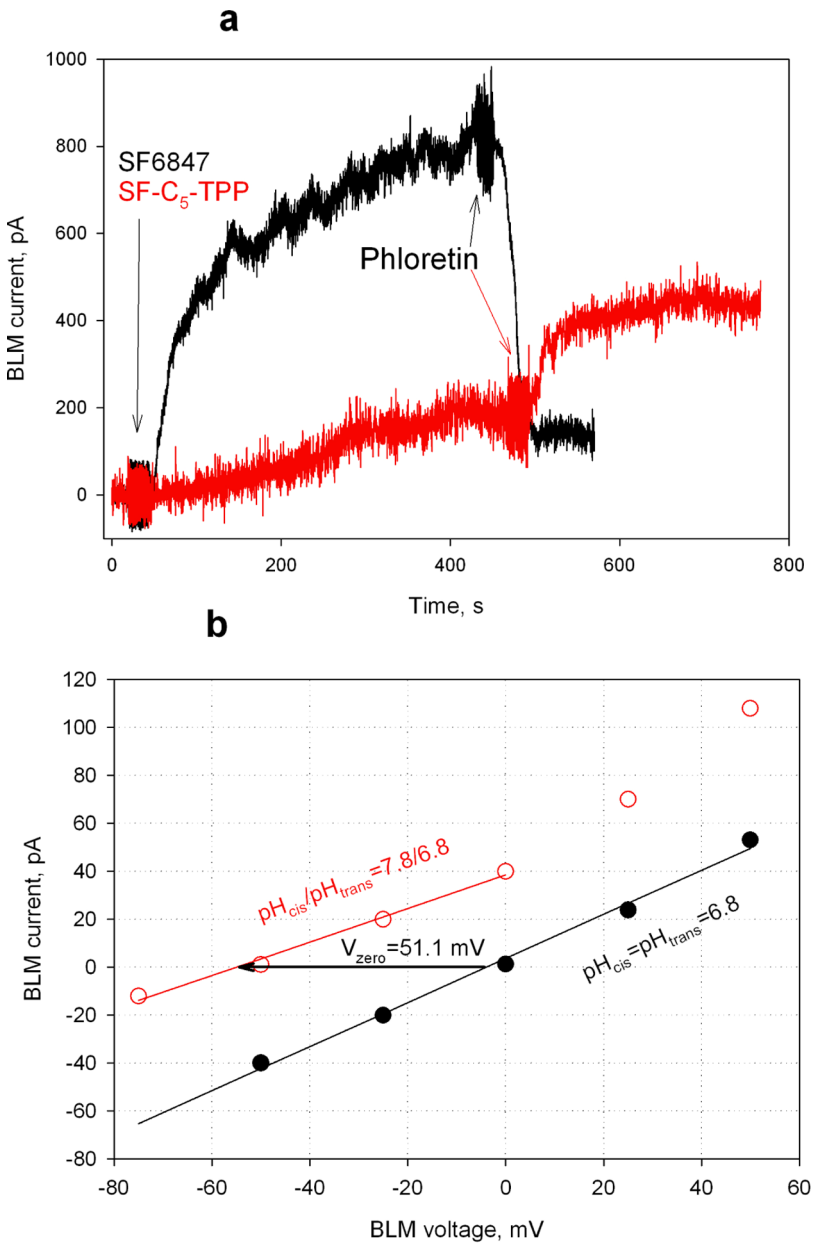

Figure 4. SF-C_5-TPP induces an electrical current through BLM that is selective for protons. (a) Typical traces of the induction of the BLM current by SF-C_5-TPP (0.5 μM, red curve) or SF6847 (0.1 μM, black curve) and the effect of phloretin (5 μM). BLM was made from a mixture of lipids from soybeans (asolectin). The solution contained Tris (10 mM), MES (10 mM), β-alanine (10 mM), and KCl (100 mM), pH 6.8. BLM voltage: 50 mV. (b) The current-voltage (I–V) curve for 0.5 μM SF-C_5-TPP under symmetrical (closed black circles, pH cis and trans were 6.8) and asymmetrical (open red circles, pH cis was 6.8, pH trans was 7.8) conditions. Straight lines were drawn to guide the eye.

an ester group in these compounds.[39] On the other hand, several research groups use the term uncoupler for lipophilic TPP cations,[48−50] because they are able to effectively depolarize mitochondria in cells. Obviously, the mechanism of their action is not the selective transport of protons through lipid membranes but can be the induction of nonspecific membrane permeability similar to the detergent action on membranes in general[51−53] and mitochondria in particular.[54−58] Another possibility is connected to the presence of fatty acids in the majority of cellular membranes. According to several studies[58,59], lipophilic TPP cations can transport protons due to the formation of complexes with fatty acids.

SF6847, which was first reported as the most powerful fungicidal and acaricidal substance of the newly synthesized 3,5-dialkyl-4-hydroxybenzylidene-malononitrile derivatives,[19] soon appeared to be the strongest mitochondrial uncoupler.[18] Later, SF6847 also turned out to be one of the tyrphostins, a group of compounds that are able to inhibit protein tyrosine kinases, playing a key role in normal cell division and abnormal cell proliferation.[60,61] Tyrphostins were proposed as useful agents for the treatment of cancer by inhibiting tumor cell proliferation.[62,63] On the other hand, a series of mitochondria-targeted compounds, including TPP derivatives, exhibited pronounced anticancer activity and were coined mitocans.[64,65] The presence of lipophilic cationic groups in the structure of mitocans ensured their selective accumulation in tumor cells due to the increased mitochondrial membrane potential compared to normal tissues.[66] Combining the advantages of

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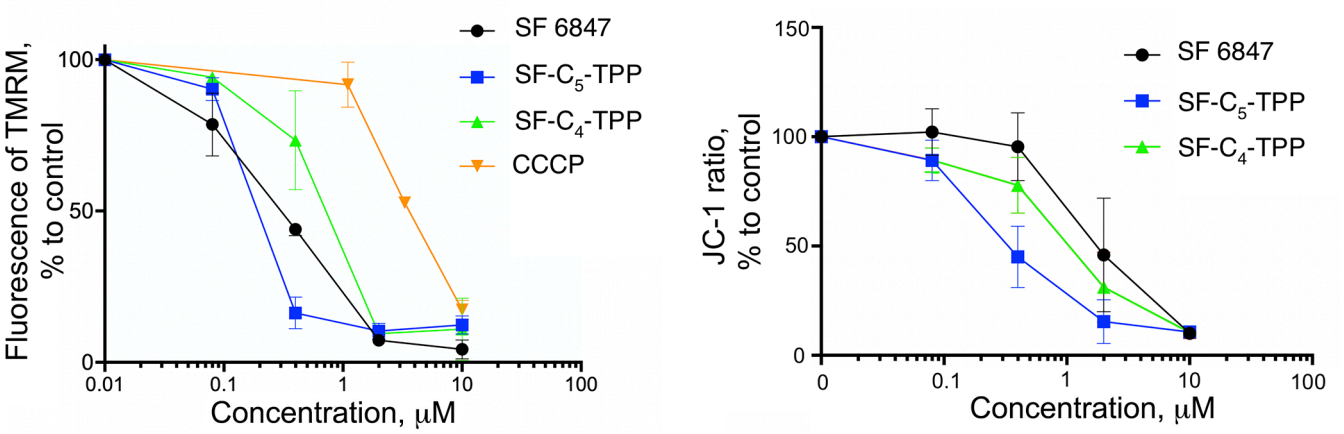

Figure 5. Effect of SF-C_5-TPP, SF-C_4-TPP, and SF6847 on mitochondrial membrane potential of MRC5-SV40 cells. (a) Changes in mitochondrial membrane potential after 60 min incubation of MRC5-SV40 cells with increasing concentrations of compounds, as assessed with the TMRM dye. (b) Changes in mitochondrial membrane potential after 60 min of incubation of MRC5-SV40 cells with increasing concentrations of the compounds, as assessed with the JC-1 dye. Results are shown as the mean of a minimum of three independent measurements with standard deviation (SD).

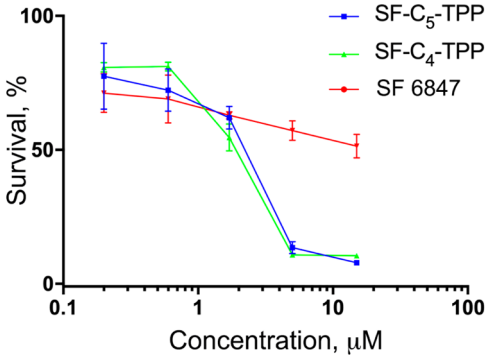

Figure 6. Effect of 24 h incubation with increasing concentrations of the compounds on the survival of MRC5-SV40 cells. The mean values of a minimum of three independent measurements with the standard deviation (SD) are shown.

Table 1. Minimal Inhibitory Concentrations (MICs) for *B. subtilis* (μM)

substance	MIC
SF6847	0.2
SF-C_4-TPP	2
SF-C_5-TPP	2
SF-C_{10}-TPP	2
CCCP	5

tyrphostins, mitocans, and protonophoric uncouplers, the TPP-linked derivatives of SF6847 may offer promising results both in treating malignant diseases and cancer chemoprevention.[67]

EXPERIMENTAL SECTION

Chemicals. Most chemicals, including diphytanoylphosphatidylcholine (DPhPC), were purchased from Sigma-Aldrich, St. Louis, MO.

NMR and LC-MS Measurements. ^{1}H, ^{13}C, and ^{31}P spectra were recorded on Bruker Avance III HD 500 operating at 500, 125, and 202 MHz, respectively. HC-HSQC and HC-HMBC were conducted on Bruker Avance III HD 500. LC-MS measurements were conducted by using an Acquity UPLC System (Waters, Milford, MA) and TQD (Waters, Milford, MA).

General Procedure for the Synthesis of Phosphonium Salts SF-C_n-TPP. A mixture of 1.3 equiv of triphenylphosphine and 1 equiv of SF-C_nBr (Supplement) in acetonitrile was heated at 85 °C for 3−4 days. Then, the solution was evaporated in vacuo, and the residue was dissolved in a minimal amount of chloroform. A 3-fold excess of hexane was added, and the dark oily precipitate was isolated by centrifugation. The dissolution−reprecipitation procedure was repeated 3 more times. Finally, the target phosphonium salt was dried in vacuo.

(6,6-Dicyano-5-(4′-hydroxy-3′,5′-di*tert*-butylphenyl)hex-5-en-1-yl)triphenylphosphonium bromide (**SF-C_4-TPP**)**:** yellow powder, 77% yield. **^{1}H NMR (500 MHz, $CDCl_3$)**: δ 7.87−7.91 (m, 6H, Ph), 7.80−7.84 (m, 3H, Ph), 7.70−7.74 (m, 6H, Ph), 7.33 (s, 2H, C*H*), 5.81 (s, 1H, OH), 3.94−4.00 (m, 2H, CH_2P), 3.05 (t, $^3J_{HH}$ = 7.2 Hz, 2H, CH_2C(=C(CN)$_2$)), 1.96−2.02 (m, 2H, CH_2), 1.66−1.74 (m, 2H, CH_2), 1.45 (s, 18H, C(CH_3)$_3$). **^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$)**: δ 179.66, 158.34, 136.67, 135.12 ($^4J_{CP}$ = 2.7), 133.90 ($^2J_{CP}$ = 10.0), 130.54 ($^3J_{CP}$ = 12.71), 125.70, 125.00, 118.13 ($^1J_{CP}$ = 86.3), 114.12, 113.69, 80.81, 35.66, 34.66 (C(CH_3)$_3$), 30.08 (C(CH_3)$_3$), 29.7 ($^2J_{CP}$ = 16.6), 22.26 ($^1J_{CP}$ = 50.6), 21.76 ($^3J_{CP}$ = 3.7). **^{31}P{^{1}H} NMR (202 MHz, $CDCl_3$)**: δ 24.75. **LC-MS (ESI+):** *m*/*z* calcd for $C_{40}H_{44}N_2OP$ ([M]$^+$), 599.32, found 599.23.

(7,7-Dicyano-6-(4′-hydroxy-3′,5′-di*tert*-butylphenyl)hept-6-en-1-yl)triphenylphosphonium bromide (**SF-C_5-TPP**)**:** yellow powder, 82% yield. **^{1}H NMR (500 MHz, $CDCl_3$)**: δ 7.85−7.89 (m, 6H, Ph), 7.79−7.82 (m, 3H, Ph), 7.69−7.73 (m, 6H, Ph), 7.38 (s, 2H, C*H*), 5.79 (s, 1H, OH), 3.89−3.95 (m, 2H, CH_2P), 2.88 (t, $^3J_{HH}$ = 7.9 Hz, 2H, CH_2C(=C(CN)$_2$)), 1.82−1.86 (m, 2H, CH_2), 1.67−1.75 (m, 2H, CH_2), 1.51−1.59 (m, 2H, CH_2), 1.46 (s, 18H, C(CH_3)$_3$). **^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$)**: δ 180.26, 158.19, 136.60, 135.01 ($^4J_{CP}$ = 3.6), 133.77 ($^2J_{CP}$ = 10.0), 130.52 ($^3J_{CP}$ = 12.7), 125.66, 125.46, 118.30 ($^1J_{CP}$ = 85.4), 114.10, 113.91, 80.66, 36.23, 34.65 (C(CH_3)$_3$), 30.07 (C(CH_3)$_3$), 29.63 ($^2J_{CP}$ = 16.4), 28.66, 22.71 ($^1J_{CP}$ = 50.5), 22.01 ($^3J_{CP}$ = 4.0). **^{31}P{^{1}H} NMR (202 MHz, $CDCl_3$)**: δ 24.46. **LC-MS (ESI+):** *m*/*z* calcd for $C_{41}H_{46}N_2OP$ ([M]$^+$), 613.33, found 613.08.

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(12,12-Dicyano-11-(4′-hydroxy-3′,5′-di*tert*-butylphenyl)-dodec-11-en-1-yl)triphenylphosphonium bromide (**SF-C_{10}-TPP**): dark-yellow powder, 94% yield. **^{1}H NMR (500 MHz, $CDCl_3$)**: δ 7.79−7.87 (m, 9H, Ph), 7.70−7.74 (m, 6H, Ph), 7.43 (s, 2H, C*H*), 5.80 (s, 1H, OH), 3.74−3.81 (m, 2H, CH_2P), 2.91 (t, $^3J_{HH}$ = 7.8 Hz, 2H, CH_2C(=C(CN)$_2$)), 1.63 (bs 4H, 2CH_2), 1.44−1.53 (m, 2H, CH_2), 1.47 (s, 18H, C(CH_3)$_3$), 1.20−1.35 (m, 10H, 5CH_2),. **^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$)**: δ 180.35, 157.99, 136.56, 134.99 ($^4J_{CP}$ = 3.6), 133.72 ($^2J_{CP}$ = 10.0), 130.50 ($^3J_{CP}$ = 12.6), 125.72, 125.60, 118.44 ($^1J_{CP}$ = 85.4), 114.28, 113.78, 80.66, 36.92, 34.67 (C(CH_3)$_3$), 30.37 ($^2J_{CP}$ = 15.8), 30.10 (C(CH_3)$_3$), 29.21, 29.17, 29.13, 29.11, 29.05, 28.84, 22.78 ($^1J_{CP}$ = 49.3), 22.68 ($^3J_{CP}$ = 4.4). **^{31}P{^{1}H} NMR (202 MHz, $CDCl_3$)**: δ 24.49. **LC-MS (ESI+)**: *m*/*z* calcd for $C_{46}H_{56}N_2OP$ ([M]$^+$), 683.41, found 682.95.

Measurements of Absorbance Spectra. Absorption spectra of SF-C_5-TPP were recorded with a SPECORD 50 ultraviolet−visible (UV−vis) spectrophotometer (Analytik Jena, Jena, Germany).

Isolation of Mitochondria from Rat Liver. Rat liver mitochondria (RLM) were isolated by differential centrifugation[68] in the medium containing 250 mM sucrose, 5 mM MOPS, 1 mM EGTA, and pH 7.4. The final washing was carried out in the medium, additionally supplemented with bovine serum albumin (0.1 mg/mL). Protein concentration was measured by using the Biuret method. Handling of animals and experimental procedures were performed according to the international guidelines for animal care and use and were adopted by the Ethics Committee of Belozersky Institute of Physico-Chemical Biology at the Lomonosov Moscow State University (protocol #3 on February 15, 2019).

Mitochondrial Respiration. To measure oxygen consumption (respiration) by RLM, we applied a standard polarographic technique based on a Clark-type oxygen electrode (Strathkelvin Instruments, U.K.) at 25 °C using 782 system software. The incubation medium contained sucrose (250 mM), MOPS (5 mM), and EGTA (1 mM) at pH 7.4. The mitochondrial protein content was 0.8 mg/mL. Oxygen consumption rates were measured in nmol/min·mg protein.

Membrane Potential ($\Delta\psi$) Measurements in Isolated Mitochondria. $\Delta\psi$ was estimated using the safranine O dye.[69] The difference in the absorbance at 555 and 523 nm (ΔA) was evaluated by using an Aminco DW-2000 spectrophotometer in dual-wavelength mode. The incubation medium contained sucrose 250 mM, MOPS 5 mM, KH_2PO_4 0.5 mM, EGTA 1 mM, rotenone 2 μM, succinate 5 mM, oligomycin 1 μg/mL, safranine O 15 μM, and pH 7.4. The mitochondrial protein concentration was about 0.6−0.9 mg of protein/ml, and the temperature was 25 °C.

Monitoring the Uptake of SF-C_4-TPP by Energized Rat Liver Mitochondria with a TPP$^+$-Selective Electrode. Accumulation of SF-C_4-TPP in RLM as compared to that of tetraphenylphosphonium (TPP$^+$) cations was monitored using a TPP$^+$-selective electrode (NIKO-ANALIT, Moscow, Russia) immersed in a 1 mL chamber with a constantly stirred buffer solution.

Electrical Current Measurements on Planar Bilayers. A bilayer lipid membrane (BLM) was formed by the brush technique[70] from a 2% decane solution of asolectin on a 0.8 mm aperture in a Teflon septum, separating the experimental chamber into two 3 mL compartments. The electrical current was measured with two Ag/AgCl electrodes placed into the solutions on the two sides of the BLM via agar bridges (ground electrode in the cis compartment) using a Keithley 428 amplifier (Cleveland, Ohio).

Flow Cytometry. Mitochondrial membrane potential changes in MRC5-SV40 cells were assessed by the fluorescence of tetramethylrhodamine methyl ester (TMRM) (Invitrogen) or JC-1 (Lumiprobe),[71] using an Amnis FlowSight Imaging Flow Cytometer (Luminex Corporation, Seattle, WA). With 100 nM TMRM, the cells were stained for 15 min. Then, the cells were incubated with SF-C_n-TPP or SF6847 at various concentrations for 1 h. After this treatment, the cells were detached with trypsin/versene and washed from the medium with phosphate-buffered saline. The median fluorescence of the cells was analyzed with a flow cytometer (excitation 548 nm, emission 574 nm). With 1 μg/mL JC-1, the cells were incubated at 37 °C for 30 min, then washed from the medium, and then analyzed with the flow cytometer. Membrane potential was assessed by the ratio of the fluorescence in the red channel (exc. 514 nm, em. 590 nm), emitted by the so-called "J-aggregates" in highly energized mitochondria, and the fluorescence in the green channel (exc. 514 nm, em. 529 nm), emitted by JC-1 in de-energized mitochondria.

Cell Viability Study. Induced cell death in MRC5-SV40 cells was measured by fluorometric analysis with the CellTiterBlue reagent (Promega) in accordance with the manufacturer's protocol. Each measurement was repeated a minimum of 3 times. The data are shown as the mean of at least 3 independent replicates with standard deviation (SD).

Growth of Bacteria. The standard laboratory strain of *B. subtilis* subs. *subtilis* Cohn 1872, specifically strain PY79, was utilized in this study. Bacterial cells from overnight cultures were diluted in a fresh Luria−Bertani broth. Subsequently, 200 μL of bacterial cell cultures containing 5 × 10^5 cells/mL were dispensed into 96-well plates (Eppendorf AG, Hamburg, Germany). The tested compounds were introduced into the wells to attain the desired concentrations. The cells were allowed to grow for 21 h at 30 °C. Optical densities at 620 nm were recorded by using a Thermo Scientific Multiskan FC plate reader equipped with an incubator (Thermo Fisher Scientific). All experiments were conducted with a minimum of three replicates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08621.

Includes the synthesis procedures, the NMR spectra, the MS spectra, and the pK_a estimation data (PDF)

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Notes

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■ ABBREVIATIONS

SF-C_5-TPP –7,7-dicyano-6-(4′-hydroxy-3′,5′-di*tert*-butylphenyl)hept-6-en-1-yltriphenylphosphonium bromide; SF-C_4-TPP –6,6-dicyano-5-((4′-hydroxy-3′,5′-di*tert*-butylphenyl)hex-5-en-1-yl)triphenylphosphonium bromide; SF-C_{10}-TPP –12,12-dicyano-11-((4′-hydroxy-3′,5′-di-*tert*-butylphenyl)dodec-11-en-1-yl)triphenylphosphonium bromide; SF6847 –3,5-di*tert*-butyl-4-hydroxybenzylidenemalononitrile; DNP –2,4-dinitrophenol; CCCP –carbonyl cyanide m-chlorophenyl hydrazone; DPhPC –diphytanoylphosphatidylcholine; RLM –rat liver mitochondria; BLM –bilayer lipid membrane; ΔΨ –mitochondrial membrane potential

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