

# Alkyl vs Aryl Modifications: A Comparative Study on Modular Modifications of Triphenylphosphonium Mitochondrial Vectors

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**Abstract:** Triphenylphosphonium (TPP<sup>+</sup>) moieties are commonly conjugated to drug molecules to confer mitochondrial selectivity due to their positive charge and high lipophilicity. Although optimisation of lipophilicity can be achieved by modifying the length of the alkyl linkers between the TPP<sup>+</sup> moiety and the drug molecule, it is not always possible. While methylation of the TPP<sup>+</sup> moiety is a viable alternative to increase lipophilicity and mitochondrial accumulation, there are no studies comparing these two separate modular approaches. Thus, we have systematically designed, synthesised and tested a range of TPP<sup>+</sup> molecules with varying alkyl chain lengths and degree of aryl methylation to compare the two modular methodologies for modulating lipophilicity. The ability of aryl/alkyl modified TPP<sup>+</sup> to deliver cargo to the mitochondria was also evaluated by confocal imaging with a TPP<sup>+</sup>-conjugated fluorescein-based fluorophore. Furthermore, we have employed molecular dynamics simulations to understand the translocation of these molecules through biological membrane model systems. These results provides further insights into the thermodynamics of this process and the effect of alkyl and aryl modular modifications.

## Introduction

The triphenylphosphonium (TPP<sup>+</sup>) moiety has drawn widespread interest as a molecular vector for selective mitochondrial delivery owing to its high lipophilicity, cationic nature, high stability in physiological conditions and its ease of conjugation to molecular cargo.<sup>1</sup> The positive charge on the TPP<sup>+</sup> moiety allows for the accumulation within the mitochondrial matrix (100-1000x for a monocation) due to the high mitochondrial membrane potential ( $\Delta\psi$ , *ca.* 180 mV) in accordance with the Nernst equation.<sup>2-4</sup> The TPP<sup>+</sup> platform has been demonstrated to be highly versatile, and has been employed for the delivery of a wide range of cargo, such as spin-traps, antioxidants, prodrugs, protonophores, fluorophores, photodynamic therapy sensitisers as well as positron emitters into the mitochondria.<sup>1, 5-14</sup> With mitochondria dysfunction being inextricably linked to a myriad of diseases from neurodegenerative and cardiovascular diseases to cancer,<sup>15-19</sup> the ability to deliver compounds to mitochondria is vital for the development of novel therapeutics. Thus, this work focuses on improving the efficacy of the TPP<sup>+</sup> vector for enhanced mitochondrial delivery.

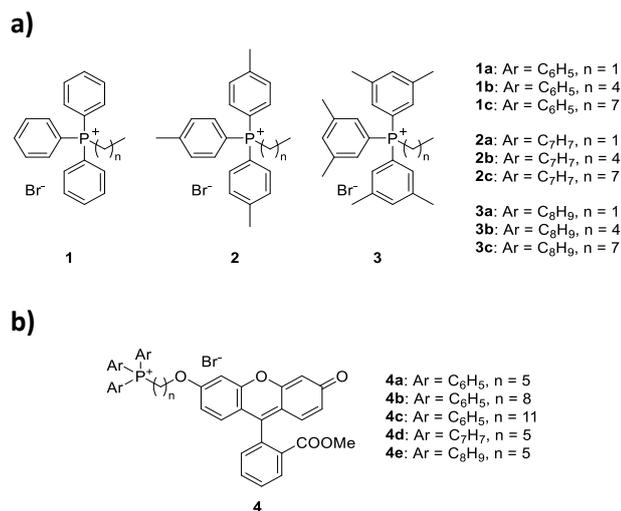
An effective strategy for enhancing mitochondrial uptake has been focused around tuning the molecule's lipophilicity, which has been demonstrated to be highly relevant for TPP<sup>+</sup>-

conjugated systems.<sup>20-24</sup> The typical strategy for enhancing lipophilicity for TPP<sup>+</sup>-conjugated compounds is by selecting a molecular fragment (module) with a higher lipophilicity as the linker – usually a longer alkyl chain<sup>6, 23, 24</sup> – between the TPP<sup>+</sup> moiety and the cargo, which increases the rate of membrane permeation and hence the mitochondrial accumulation.<sup>25</sup> However, chain length modulation has its limitations. It may be more challenging to modify linkers with a specific function, such as cleavable linkers used for drug release purposes, where modification may lead to alteration of function.<sup>14, 26, 27</sup> Aryl methylation thus presents itself as an attractive alternative when linker modification is not synthetically viable or when it affects linker functionalities. Furthermore, it enables a modular approach for the synthesis of conjugated cargo with variable lipophilicity by varying the starting phosphine. It has also been demonstrated that aryl methylation of TPP<sup>+</sup> can improve mitochondrial uptake and lipophilicity, in a similar manner to chain length modification.<sup>21, 22, 28, 29</sup> While judicious alkylation of the phenyl rings in TPP<sup>+</sup> moieties has been shown to be an effective method to enhance lipophilicity in both mono-TPP<sup>+</sup> and bis-TPP<sup>+</sup>, no systematic comparison between these two modular approaches has been reported in the literature.<sup>21</sup> A comparative study between the two methodologies was thus needed to evaluate the mitochondrial delivery efficacies of the two series of phosphonium salts.

Herein we report the first comparative analysis of alkyl vs aryl modular modification effects on mitochondrial uptake for triphenylphosphonium moieties (**Figure 1**). For this purpose, we

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**Figure 1:** (a) T<sup>+</sup>PP<sup>+</sup> salts and (b) T<sup>+</sup>PP<sup>+</sup>-Fluorescein conjugates studied in this work.

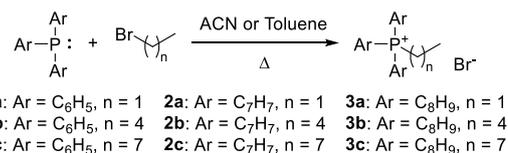
synthesised and evaluated nine alkyltriarylphosphonium salts, with permutations of three different aryl groups (phenyl, *p*-tolyl and 3,5-dimethylphenyl) as well as three alkyl groups of different lengths (ethyl, pentyl and octyl), and assessed the effects of the additional groups on lipophilicity and bioactivity. Conjugation to a fluorescein-based dye followed by confocal microscopy was employed to ascertain the mitochondrial targeting effects of these species to compare the two methodologies. To further understand the thermodynamics of these cations transport across biological membranes, we have also performed umbrella sampling molecular dynamics simulations using a 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) membrane model system.

## Results and discussion

### Synthesis and characterisation of alkyltriarylphosphonium salts.

The choice of substituted triarylphosphines was based on our previously reported work. The selected alkylated aryl groups (*p*-tolyl and 3,5-dimethylphenyl) were chosen as they exhibited the largest increase in mitochondrial uptake among mono- and dimethylated phosphonium species.<sup>21, 22</sup> The length of alkyl chain was chosen such that any increase in aryl substitution can be matched by an identical increase in alkyl chain length based on its total atom count (*i.e.*, structural isomerism). As such, an increase in aryl substitution can be matched by an equivalent increase in alkyl chain length (*i.e.* **1b/2a**, **1c/2b/3a**, **2c/3b** are isomers one another). The synthesis and characterisation for compounds **1a** – **1c** was previously reported,<sup>22</sup> whereas compounds **2a** – **3c** were obtained in good yields (75% - 99%) upon reacting triarylphosphines with the respective alkyl bromides in acetonitrile or toluene under reflux (**Scheme 1**).

The compounds were characterised by <sup>1</sup>H, <sup>31</sup>P{<sup>1</sup>H} and <sup>13</sup>C{<sup>1</sup>H} NMR spectroscopy, high-resolution mass spectrometry (HRMS), and single crystal X-ray structures were obtained for compounds **1b** – **3b**.



**Scheme 1.** Synthesis of TPP<sup>+</sup> cations **1a** – **3c**

The characterisation data was fully consistent with the proposed structures, indicating the successful synthesis of the phosphonium salts. <sup>31</sup>P{<sup>1</sup>H} NMR revealed a downfield shift for the chemical shift of the phosphorus atom (~6 ppm of triarylphosphines to ~20 ppm in the phosphonium salts), consistent with values from our previous work.<sup>21, 22</sup> All single-crystal structures for series **a** and **b** were obtained, with two previously unreported structures (**see ESI**).<sup>22, 30</sup> Unfortunately, compounds **1c** – **3c** resisted all attempts at crystallisation, presumably due to the poor packing of the long and flexible alkyl chains, forming waxy solids instead.<sup>31</sup>

In terms of structural parameters, the ranges of the average bond lengths in the six compounds were 1.794 – 1.799 Å and 1.790 – 1.798 Å for P-Aryl and P-Alkyl (*i.e.*, P-Ar and P-Ak), respectively, while the ranges of the bond angles for Ar – P – Ar and the Ar – P – Ak bond were 108.36° – 109.71° and 109.23° - 110.57°, respectively – all of them consistent with previously reported TPP<sup>+</sup> compounds.<sup>21, 22</sup> For the short alkyl chain modules in compounds **1a** – **3a**, the chains adopted a consistent anti conformation. However, for the pentyl substituted TPP<sup>+</sup>, twisting of the C-C bonds was observed for **1b** and **3b** further away from the phosphorus centre, while **2b** had an extended hydrocarbon chain. A gauche conformation was observed in **1b** for the 2<sup>nd</sup> and 3<sup>rd</sup> C-C bond, while the same was observed in **3b** for the 3<sup>rd</sup> C-C bond. The inconsistency between all three structures indicates a high degree of flexibility for the alkyl chain.

### Measurement of lipophilicity

The role of lipophilicity in drugs is well-documented and influences their biological properties greatly, and is frequently quantified by logP, where P is the partition coefficient between two immiscible phases. This is especially relevant for TPP<sup>+</sup> compounds, as there are many studies in the literature correlating the accumulation, toxicity, or drug efficacies to lipophilicity.<sup>23, 24, 32, 33</sup> Due to the large impact that lipophilicity has on these systems, we quantified the lipophilicity of our compounds in water and 1-octanol and the values are presented in **Table 1**. The measurements were done using a shake-flask method as described in the literature.<sup>34</sup> The logP values obtained correlate well with the increasing atom count, with larger molecules having larger lipophilicity. Steric parameters (molecular volume, and solvent accessible surface area) were found to have a good correlation with lipophilicity ( $r^2 = 0.96$ ), in line with our previous work (**see Table 1 and ESI, Figure S3**).<sup>21, 22</sup> An increase of the solvent accessible surface area (SASA) of the cations, has been linked with an increased (hydrophobic) surface area that is exposed to the solvents,

**Table 1.** Experimental data for compounds **1a** – **3c**

Compound	log P ( $\pm$ SD)	IC <sub>50</sub> ( $\pm$ 95% CI) / $\mu$ M	Volume ( $\pm$ SD) / nm <sup>3</sup>	SASA ( $\pm$ SD) / nm <sup>2</sup>	$\Delta G_{aq \rightarrow oct}^{vdw}$ / kJ·mol <sup>-1</sup>	logP <sub>mem</sub>
<b>1a</b>	-1.36 $\pm$ 0.09	16.15 $\pm$ 1.29	0.8912 $\pm$ 0.0264	5.381 $\pm$ 0.189	-61.77 $\pm$ 0.57	-0.73 $\pm$ 0.01
<b>1b</b>	-0.84 $\pm$ 0.01	1.64 $\pm$ 0.14	1.0481 $\pm$ 0.0313	6.255 $\pm$ 0.229	-71.87 $\pm$ 0.68	-0.47 $\pm$ 0.17
<b>1c</b>	0.22 $\pm$ 0.02	0.34 $\pm$ 0.03	1.2053 $\pm$ 0.0359	7.137 $\pm$ 0.284	-82.70 $\pm$ 0.71	0.45 $\pm$ 0.70
<b>2a</b>	-0.50 $\pm$ 0.05	3.83 $\pm$ 0.50	1.0502 $\pm$ 0.0323	6.296 $\pm$ 0.211	-73.84 $\pm$ 0.54	-0.54 $\pm$ 0.31
<b>2b</b>	0.26 $\pm$ 0.02	0.45 $\pm$ 0.05	1.2087 $\pm$ 0.0365	7.184 $\pm$ 0.244	-84.58 $\pm$ 0.53	-0.38 $\pm$ 0.20
<b>2c</b>	1.57 $\pm$ 0.02	0.30 $\pm$ 0.04	1.3646 $\pm$ 0.0420	8.056 $\pm$ 0.318	-97.36 $\pm$ 0.83	1.69 $\pm$ 0.33
<b>3a</b>	0.08 $\pm$ 0.02	0.80 $\pm$ 0.06	1.2063 $\pm$ 0.0350	7.130 $\pm$ 0.225	-83.89 $\pm$ 0.69	-0.28 $\pm$ 0.26
<b>3b</b>	0.90 $\pm$ 0.01	0.36 $\pm$ 0.04	1.3633 $\pm$ 0.0387	7.983 $\pm$ 0.269	-94.93 $\pm$ 0.81	1.10 $\pm$ 0.41
<b>3c</b>	2.25 $\pm$ 0.05	0.32 $\pm$ 0.06	1.5112 $\pm$ 0.0485	8.756 $\pm$ 0.384	-107.79 $\pm$ 0.87	2.30 $\pm$ 0.38

favouring the partition to the 1-octanol phase.<sup>21, 22</sup> The molecular volume upon normalisation with respect to charge, has also been described as an important structural parameter for the prediction of mitochondrial accumulation of a series of TPP<sup>+</sup> molecules.<sup>21</sup> Alchemical free energy calculations focused on the van der Waals (vdW) contribution to the free energy of transfer between water and octanol, also showed a good correlation ( $r^2 = 0.98$ ) with the experimental logP results (see **ESI, Figure S3**). This is also in line with our previous works.

#### IC<sub>50</sub> measurements

For a rapid screening of mitochondrial uptake, the 72-hour cell viability assay was conducted using HeLa cells by the resazurin reduction assay. The cytotoxic effects of TPP<sup>+</sup> has been noted in the literature and has been linked to increased membrane uncoupling.<sup>24, 35</sup> However, these bioenergetic effects induced by TPP<sup>+</sup> moieties are unlikely to be detrimental at relevant dosages.<sup>36</sup> Furthermore, from previously published work, mitochondrial accumulation has been found to correlate with the IC<sub>50</sub> values in TPP<sup>+</sup> species.<sup>21</sup> Thus, the dose-response curves of these compounds should reveal the mitochondrial accumulation of the compounds, as a higher accumulation of the TPP<sup>+</sup> salts should result in a greater cytotoxicity. In addition to compounds **1a** – **3c**, methyltriphenylphosphonium (TPMP) iodide was also included as a comparison. The absolute IC<sub>50</sub> values ( $\pm$  95 % confidence intervals) are presented in **Table 1**, and the dose-response curves are plotted against the experimental logP in **Figure 2**.

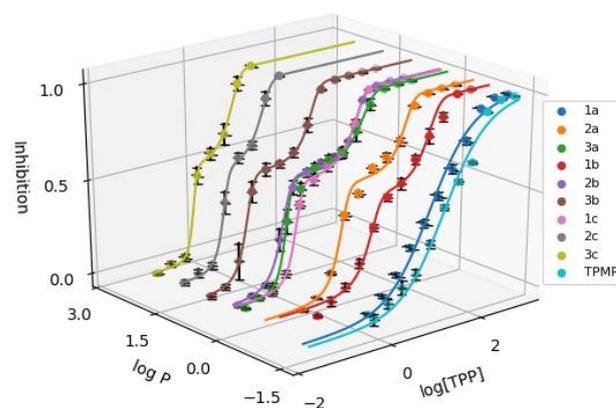
IC<sub>50</sub> was observed to decrease with both increased alkyl chain length, as well as increasing aryl methylation. With an increase of the alkyl chain length from **1a** – **1c**, the IC<sub>50</sub> decreased from 16.15 to 1.64 to 0.34. On the other hand, with an increase of the aryl methylation from **1a** – **3a**, the IC<sub>50</sub> dropped from 16.15 to 3.83 to 0.80. The two modular approaches appear to be compatible, with compound **2b** having a lower IC<sub>50</sub> compared to **1b** and **2a**. However, the effects appear to diminish with the more lipophilic compounds. For example, **3c**, with the longest alkyl chain and the most substituted aryl ring, had an IC<sub>50</sub> of 0.32  $\pm$  0.06, which was similar to both **2c** (0.30  $\pm$  0.04) and **3b** (0.32  $\pm$  0.02). This indicates that the scope of the compounds studied approaches the upper limit for the lipophilicity-linked

cytotoxicity observed in the literature, with negligible changes in IC<sub>50</sub> over an order of magnitude of [TPP<sup>+</sup>].<sup>24</sup>

Interestingly, while a sigmoidal model provided a good description for **1a** and TPMP, a double-sigmoidal model was a more accurate model for the remaining compounds, which – to the best of our knowledge – has not been previously reported for TPP<sup>+</sup> species. While there are multiple explanations in the literature for this phenomenon, ranging from cell population heterogeneity, differing mode of action at different concentrations, to cell-cycle phase specificity, detailed mechanistic studies are necessary to determine the cause of this phenomenon.<sup>37, 38</sup>

#### COMPUTATIONAL STUDIES

To understand the thermodynamics of the cations transport across biological membranes, we have also performed umbrella sampling molecular dynamics simulations. The full protocol details are provided in the supporting information accompanying this paper (see **ESI**). We explored the thermodynamics of the cations transport using a hydrated POPC bilayer system as our model membrane. This model is much closer to biological membranes than biphasic 1-octanol:water systems, accounts for ion-membrane specific interactions,<sup>39-42</sup>



**Figure 2.** Dose response curve of the methylated TPP<sup>+</sup> salts (**1a** – **3c**) with TPMP. Error bars for log P are shown for standard deviation and inhibition as standard error. [TPP] is shown in  $\mu$ M.

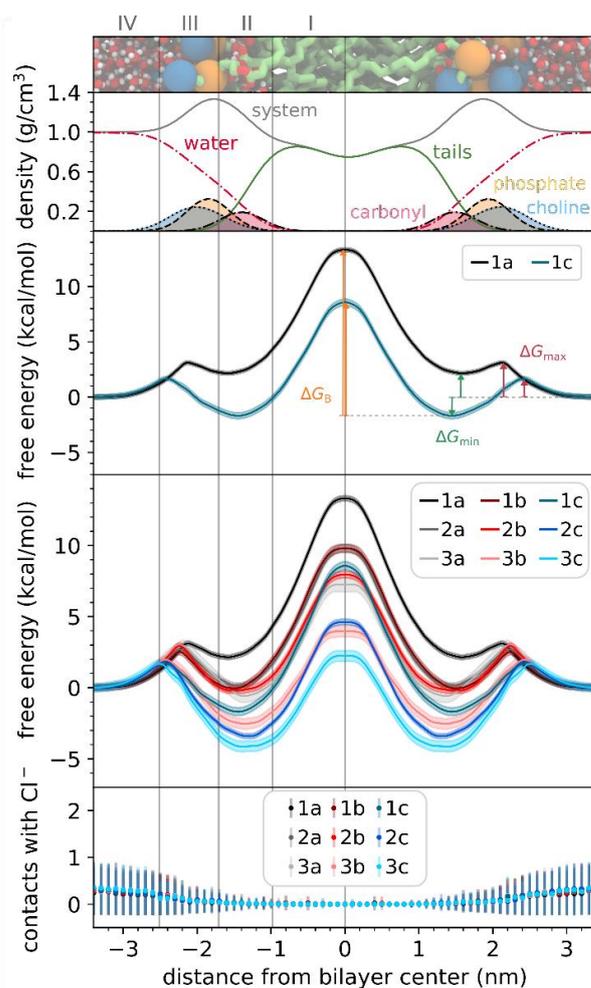
and thus can be used to examine the differences between the cation-membrane interactions as a result of structural changes in the TPP<sup>+</sup> moiety (Figure 3). In our study, the membrane model is divided into four regions based on the different density compositions, namely, bulk water (region IV); most of the charged phosphate and choline density (region III), the region that contains both hydrophobic tails and a portion of the polar headgroup density, ending where the lipid tail density intercepted the choline density (region II); and a central zone that contains only the hydrophobic lipid tails (region I). The translocation free energy profile of all nine triphenylphosphonium cations (1a – 3c) as well as detailed parameters describing local free energy maxima ( $\Delta G_{\max}$ ), minima ( $\Delta G_{\min}$ ) and free energy barrier at the centre of the bilayer ( $\Delta G_B$ ) are depicted in Figure 3 and Table S2.

The free energy profile can be qualitatively understood in 3 phases during translocation. As the TPP<sup>+</sup> ion approaches the surface of the membrane, an increase in free energy ( $\Delta G_{\max}$ , region III) is observed. There is a subsequent decrease in free energy ( $\Delta G_{\min}$ , region II), and finally, we observe a free energy maxima in region I ( $\Delta G_B$ ). The potential energy profiles of TPP<sup>+</sup> ions with lipid bilayers have been previously written down as the sum of four terms, including Born, image and dipole energy contributions, and a neutral energy term.<sup>41, 42</sup> The free energy profile diagrams obtained are highly consistent with models previously reported in the literature.<sup>43, 44</sup>

The free energy profile diagrams obtained further emphasise the importance of lipophilicity in TPP<sup>+</sup> systems; since an increase in lipophilicity can be correlated with a respective decrease in free energy at all three critical points,  $\Delta G_{\max}$ ,  $\Delta G_{\min}$  and  $\Delta G_B$ . Notably, the maximum values of the free energy profiles relative to water (defined at 0.0 kcal·mol<sup>-1</sup>) of isomeric compounds are comparable, with **1b/2a** at 9.80 - 9.85 kcal·mol<sup>-1</sup>, **1c/2b/3a** at 7.28 - 8.61 kcal·mol<sup>-1</sup>, and **2c/3b** at 3.99 - 4.63 kcal·mol<sup>-1</sup> (see ESI, Table S2). We also observed that among the four compounds with the lowest IC<sub>50</sub> (between 0.30 and 0.36  $\mu$ M), **1c**, **2c**, **3c** and **3b**, showed the smallest free energy barrier for penetrating the high-density region of the bilayer ( $\Delta G_{\max}$ ), and except for **1c**, these compounds had low  $\Delta G_B$  values (see Figure 3 and ESI, Table S2).

To allow the full translocation free energy profile to be considered, the membrane partition coefficient ( $\log P_{\text{mem}}$ ) was computed from the free energy profile diagrams by calculating the standard binding free energy of the cations to the membrane (Table 1, see ESI for more details). We observed that the four compounds with the lowest IC<sub>50</sub> (between 0.30 and 0.36  $\mu$ M) – **2c**, **3c**, **1c** and **3b**, were the only ones to show a positive  $\log P_{\text{mem}}$  (between 0.45 and 2.30). The  $\log P_{\text{mem}}$  obtained were also found to be well-correlated with experimental  $\log P$ . ( $r^2 = 0.89$ , Figure S4), with a mean unsigned difference of  $0.29 \pm 0.23$ . However, these differences were to be expected as the experimental results were obtained in water and 1-octanol.

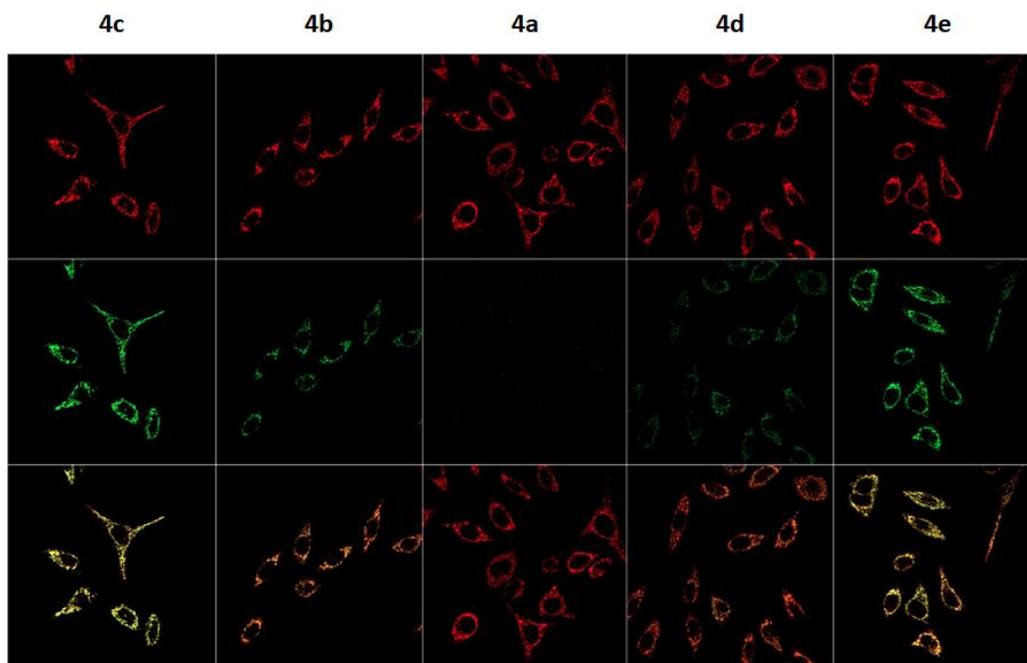
The  $\log P_{\text{mem}}$  for the different isomers were then compared in terms of aryl substitution vs alkyl chain length modules. For the **1b/2a** isomers, very similar membrane partition ( $-0.47 \pm 0.17$  and  $-0.54 \pm 0.31$ , respectively) were obtained. As for the other isomers, *i.e.*, **1c/2b/3a** and **2c/3b**, the simulations showed that



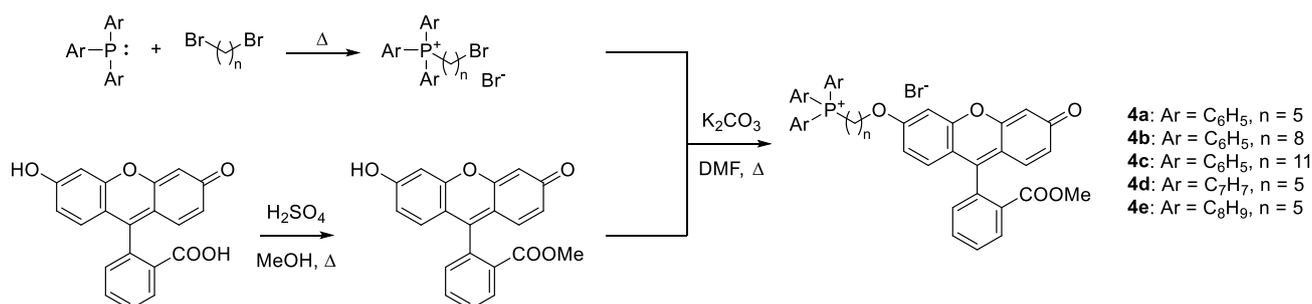
**Figure 3.** (Top 2 panels) Hydrated bilayer model system and the partial density profiles for the different functional groups or molecules in the system. (Third panel) Free energy profiles for the translocation of selected cations and illustration of  $\Delta G_{\min}$ ,  $\Delta G_{\max}$  and  $\Delta G_B$ . (Fourth panel) Free energy profiles for the translocation of all the studied cations. (Bottom panel) Average number of contacts of any of the studied cations with Cl<sup>-</sup> counter-ions in the simulation cell (considering a distance threshold of 0.6 nm). Membrane regions (from left to right): region IV (bulk water); region III (charged phosphate and choline groups); region II (hydrophobic tails and a portion of the polar headgroup); and region I (hydrophobic lipid tails).

**1c** and **2c** had a higher membrane partition when compared with their respective isomers (ranging from 0.6 to 0.8). This suggests that a longer alkyl chain length module could have an increased tendency to associate with lipid membranes despite having similar lipophilicities.

To examine the possible different tendencies to form ion-pairs for **1a – 3c**, which may affect translocation profiles, an ionic concentration of *ca.* 0.15 M of NaCl was used throughout our studies. By following the number of contacts between the cations and the Cl<sup>-</sup> ions present in the system, we did not detect the formation of ion pairs as the cations translocated the hydrated bilayer system (see Figure 3). Still, in a separate simulation, we have constrained the interatomic distance between one Cl<sup>-</sup> ion and the **1a** cation, to investigate the effect of the formation of an ion pair in the translocation free energy profile of **1a** (see ESI, Figure S5). We observed that ion pairing



**Figure 4.** Confocal fluorescence microscopy images of HeLa cells treated with **4a** – **4e** upon excitation with 488 nm and 644 nm lasers. The first row shows the fluorescence signal from MitoTracker Deep Red, the second row shows the fluorescence signal from **4a** – **4e**, and the third row shows the overlay of the two channels.



**Scheme 2:** Synthesis of TPP<sup>+</sup>-fluorescein conjugates **4a-4e**.

should not be favourable for the translocation of this cation, which agrees with what has been already proposed in the literature for TPMP and using DFT-based continuum model calculations.<sup>45</sup> Although we obtained the same partition with or without ion pairing, the free energy barrier for crossing the bilayer for **1a** as an ion pair was higher by *ca.* 2 kcal·mol<sup>-1</sup>.

#### Mitochondrial uptake and localisation of TPP<sup>+</sup>-conjugated fluorescein

To quantify the difference in mitochondrial delivery between the various TPP<sup>+</sup> vectors, a fluorescein-based dye was conjugated to the triarylphosphonium salts modules with differing chain length units. The compounds synthesised are shown in **Figure 1**, with modifications from previously reported molecule (MitoFluo) by Antonenko *et al.*<sup>46, 47</sup> To control regioselectivity, as well as to restrict the protonophoric activity observed in mitoFluo, the methyl ester of fluorescein was used. The compounds **4a/4b/4c** consist of a triphenylphosphonium linked to the fluorescent moiety with an increasing chain length,

while compounds **4a/4d/4e** consist of the fluorescent dye linked with a 5-carbon chain to different alkylated triarylphosphonium groups.

The synthesis of TPP<sup>+</sup>-conjugated fluorescein was achieved in three steps: esterification of fluorescein, synthesis of ω-bromoalkyltriarylphosphonium bromide and Williamson ether synthesis for the conjugation of TPP<sup>+</sup> to the dye (**Scheme 2**). **4a** – **4e** were characterised by <sup>1</sup>H, <sup>13</sup>C{<sup>1</sup>H} and <sup>31</sup>P{<sup>1</sup>H} NMR and HRMS. The experimental excitation/emission fluorescence spectra obtained for the five conjugated dyes were virtually identical in both intensities and wavelengths (**see ESI, Figure S2**). LogP values for the TPP<sup>+</sup>-fluorescein conjugates were also quantified experimentally and presented in **Table 2**. The relative increase in logP values were highly consistent with the differences in the unconjugated TPP<sup>+</sup> salts.

To measure the mitochondrial uptake and subcellular localisation of these conjugated dyes, HeLa cells were treated with compounds **4a** – **4e** (100 nM) together with MitoTracker DeepRed FM(25 nM) in DMEM for 30 minutes, washed with 3 x

200  $\mu$ L of PBS, and the medium was replaced with DMEM. The samples were then examined under a confocal microscope. The relative mitochondrial uptake among the cells was quantified using the ratio of fluorescence intensities between the **4a** – **4e** and the MitoTracker (Fluo/Mt), and the data is presented in **Figure 4 and Table 2**.

The images obtained revealed a colocalisation of the TPP<sup>+</sup> conjugated dyes and the MitoTracker, confirming a high selectivity of compounds **4a** – **4e** for the mitochondria. Evidently, the fluorescent intensity from the fluorescein dyes were correlated with lipophilicity. As the chain length was extended from 5 to 11 carbons in compounds **4a/4b/4c**, the Fluo/Mt ratio increased 16 times from 0.065 to 1.009. Similarly, an increase in aryl groups methylation results in an increase in mitochondrial accumulation as the Fluo/Mt ratio for compounds **4a/4d/4e** increased 12 times from 0.065 to 0.757. Both methods were found to increase mitochondrial uptake drastically, indicating the viability of both methodologies for mitochondrial delivery, consistent with other work in the literature. Fluo/Mt was found to correlate well with logP values of the TPP<sup>+</sup>-fluorescein conjugate ( $r^2 = 0.94$ ). The slightly increased performance of **4c** as compared to **4e** was hence attributed to the higher lipophilicity ( $2.72 \pm 0.07$  for **4c** and  $2.37 \pm 0.01$  for **4e**).

## Conclusions

In conclusion, we have reported the first evaluation of aryl methylation as a complementary modular strategy to the traditional methods of chain length extension for enhancing mitochondrial targeting abilities. Both strategies result in lowered membrane permeation energy and greater mitochondrial accumulation with isomeric compounds displaying analogous translocation profiles, cytotoxicity and mitochondrial accumulation. Most notably, both approaches are fully compatible, and can be simultaneously applied for the greatest increase in mitochondrial targeting. This work demonstrates that aryl methylation is both comparable and complementary with traditional methods.

Our work underscores the modular nature and complementary nature of both approaches. We hope our work will catalyse the synthesis of a wide range of fine-tuned mitochondrial delivery vectors based on a broader range of phosphonium salts and other non-conventional delivery vectors.

## Conflicts of interest

There are no conflicts to declare.

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