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Synthesis and Hydrolytic Stability of Cyclic Phosphatidic Acids: Implications for Synthetic- and Proto- cell Studies †

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Cyclic phosphatidic acids (cPAs) are bioactive compounds with therapuetic potential, but are in short supply. We describe a robust synthesis of cPAs employing an efficient cyclophosphorylation procedure and report on their hydrolytic properties – which should facilitate the study of their biological properties and as plausible proto- and synthetic-cell components.

Cyclic phosphatidic acids (cPAs) are naturally occurring bioactive cyclic phospholipids found in a wide range of organisms including humans (Fig. 1).[1-4] An important member of this class is the cyclic phosphatidic acid (PHYLPA), a close structural analog of lysophosphatidic acid (LPA), first isolated from Physarum polycephalum and was shown to act as an inhibitor of eukaryotic DNA polymerase.[5-7] cPAs are also widespread in mammalian serum and brain tissues, and are known to play important roles in many biological functions.[8] Even though cyclic phosphatidic acids (cPAs) are generated by intramolecular transphosphatidylation of lysophosphatidylcholine (LPC)[9] and are structural analogs of LPA binding to the same receptors as LPA, cPAs exhibit unique activity and elicit completely different behavior than LPA, intensifying the scrutiny of the role of cPAs and its analogs as therapeutic (PPARγ agonist or antagonistic properties) agents,[10, 11] as candidates for increasing the level of hyaluronic acid in the dermis,[12] and as a novel pruritic agent[13]. The cPA bioactivity potential (regulation of cell proliferation, morphology and migration) is gaining attention[10, 14] and their synthetic availability for medicinal and therapeutic investigations has become important.

Our interest in cyclic phospholipids is based on the discovery that short chain **cPA 9:0** formed vesicle-like structures that are stable and retain dyes over a period of time.[15] This was surprising since the biological cPAs are known to be disrupters of membrane-formation.[10, 14] Further, Duhan et al discovered that short chain cyclic phospholipids (**cPA 9:0** and **cPA 10:0**) enhanced the formation and the stability of synthetic vesicles.[16] Thus cPAs, apart from being biologically relevant, may provide a new build-platform for the emerging field of artificial/synthetic cells.[17] For our ongoing studies in the area of protocells,[16] we needed access to large quantities (300-500 mg) of the cyclic phospholipids with different fatty acid chain lengths.

The current methods of synthesis of cyclic phospholipids[18-21] were far from satisfactory to deliver reproducible yields and sufficient quantities of pure material. The problem is (a) the capricious nature of the (cyclo)phosphorylation reactions, and (b) the difficulty caused by the emulsions during work-up of the reaction compromising the purity and quantity of cPAs. Consequently, the commercially available cPAs are prohibitively expensive (1 mg, 164-291 US$),[22] limiting their availability for studies. Herein, we report on the development of a method that gives reliable access to a range of cyclic phospholipids in very good, isolated yields. Critical to this success are (a) the use of a novel cyclophosphorylating reagent, bis-dimethyl diamidophosphate (BDMDAP, **6**),[23] and (b) specific work-up procedures that takes advantage of the critical aggregate concentration (CAC), which delivers the pure cyclic phospholipid consistently in relatively large (300-500 mg) scales.

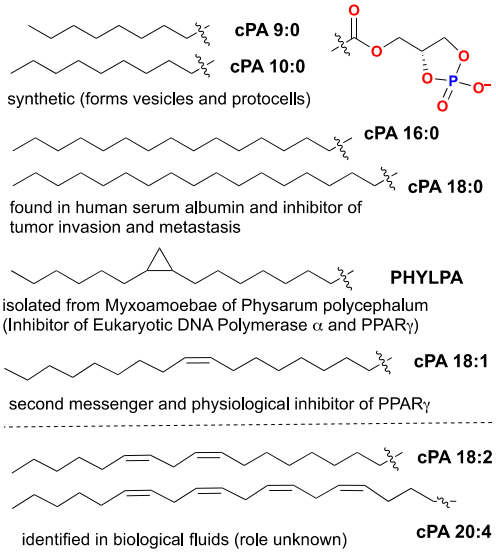

Figure 1. Cyclic phospholipids (**cPA 16:0** and higher analogues) have been implicated in a wide-ranging bioactivity.[1-14] Synthetic cyclic phospholipid **cPA 9:0** and **cPA 10:0** have been shown to be components of vesicles/protocells.[15,16]

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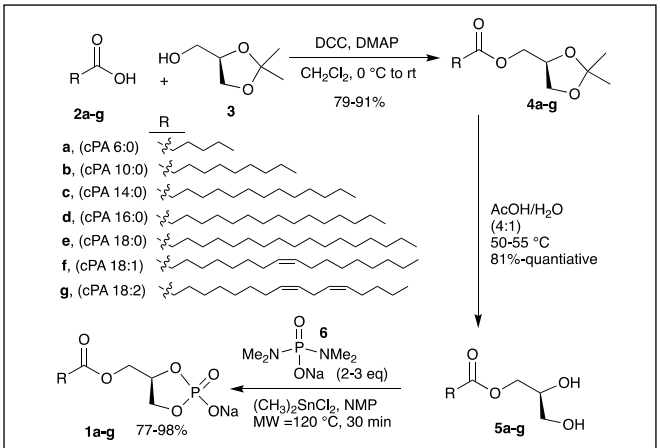

Scheme 1. Three step syntheses of cPAs starting from R-(–)-solketal and the respective fatty acids. Cyclophosphorylation was achieved using BDMDAP (**6**) under microwave reaction conditions. BDMDAP = bis(dimethylamino) phosphorodiamidate. NMP = N-methylmorpholine.

Furthermore, we report on the hydrolytic behaviour of the carboxylate- and cyclic phosphate-ester bonds of the cPAs and a surprising reversal in the trend with increasing carbon chain length in the cPA series that has implications for their roles in proto-cell and synthetic-cell formation.

The reaction of the corresponding diols **5a-g** with a cyclophosphorylating reagent would be the most straightforward way to access cPAs (Scheme 1).[15] The synthesis started with the Steglich esterification between commercially available fatty acids **2a-g** and *R*-(-)-solketal **3**. These reactions can be performed reliably on a multigram scale and the yields (79-91%) are not significantly affected by the length of the lipid chain in the fatty acids. The subsequent acid hydrolysis of the acetonide group in **4a-g** with aqueous acetic acid with mild heating (50-55 °C)[24] yielded diols **5a-g** in high yields (81%-quantitative). With diols **5a-g** in hand, we investigated various phosphorylation procedures reported for cPA synthesis using $POCl_3$ or phosphoryl tris-triazole[25] but were unsuccessful (Table S1). The capricious nature of reactions –with inconsistent yields and purity of the cPA products– led us to develop alternative methods that would address these shortcomings.

We have developed a cyclophosphorylating reagent BDMDAP (**6**) which proved to be highly efficient for the regioselective cyclophosphorylation of vicinal diols of small molecules.[23] Therefore, we evaluated **6** for the cyclophosphorylation of short, medium and long alkyl chain diols, **5a-g**. When we conducted the cyclophosphorylation reaction of **5b** and **5e** with 2 equivalents of BDMDAP **6**, catalysed by dimethyltin dichloride in NMP as solvent,[23] only 40% conversion for **5b**, and none for **5e**, to the corresponding cyclophosphates was observed by ^{13}C NMR. The divergent solubility of the two reactants was the cause: BDMDAP is a polar molecule requiring polar solvents, while long chain fatty acid ester substrates are insoluble in polar solvents. A screening of solvents including different mixtures of THF/NMP and THF/DMF and high temperature (100 °C) demonstrated some promise for cyclophosphate **1e** generation, but with low conversion (less than 10%). At this stage, we explored replacement of conventional heating by microwave (MW) irradiation (based on our previous experience).[26] We observed that under MW irradiation, BDMDAP (2 equiv.) reacted with **5e** in NMP, cleanly, to produce **1e** quantitatively. Furthermore, these conditions were found to be also efficient for the nearly quantitative formation of short (**1a**-**b**), medium chain cPAs (**1c**) and long chain, saturated and unsaturated, cPAs **1d**-**g** (Table S1). This transformation does not require strict anhydrous conditions and could be conducted in parallel MW tubes to enable large scale synthesis of the cPAs. This protocol was also amenable for the synthesis of functionalized fatty acids (e.g., 2-amino- and 2-hydroxyoctanoic acids (**2h**, **2i**) and 10-hydroxydecanoic acid **2j** and 16-hydroxyhexadecanoic acid **2k**, with acetyl protecting groups (Fig. S15-S28, S43-S50, S79-S94).

Next, it was crucial to overcome the loss of cPAs during the work up and isolation/purification procedures. In this effort, the aggregation behaviour of the cPAs offered crucial clues. We observed that cPAs having short alkyl chains such as **1a** and **1b** behave as polar entities than as amphiphilic molecules (possessing less surfactant properties) and can be purified by silica gel chromatography (see SI). However, such normal or reverse phase chromatography was unsuccessful for purification of medium, and long chain cPAs (**1c-1g**). For these, we took advantage of the observations that cPAs can form insoluble aggregates in water above 20 mM concentrations[16], while the cPAs below these concentrations are soluble in water (Fig. 2). Thus, by manipulating the cPA concentrations in water (step 1) to form aggregates or not, we were able to eliminate the polar impurities by (a) first extracting the hydrophobic cPA aggregates into n-butanol layer (step 2) and (b) then removing the non-polar impurities by reversing the aggregation by lowering their concentration to about 6 mM (step 3), so that the cPAs were now soluble in the water layer and the impurities were left behind in the *n*-butanol layer (step 4, Figure 2). Lyophilization to remove the water gave the desired cPAs in good purity (by NMR, Fig. S55-S94) and were used without further purification. This robust protocol yielded, reproducibly, good quantities (300-500 mg) of these cPAs in both the (S)- and (R)-glycidol series. We further sought to improve and streamline the two differing isolation procedures based on the varying alkyl chain lengths. We applied the same extraction by purification (Fig. 2) to crude **1b** (decanoate-cPA) but (a) increased the concentration of **1b** to 100

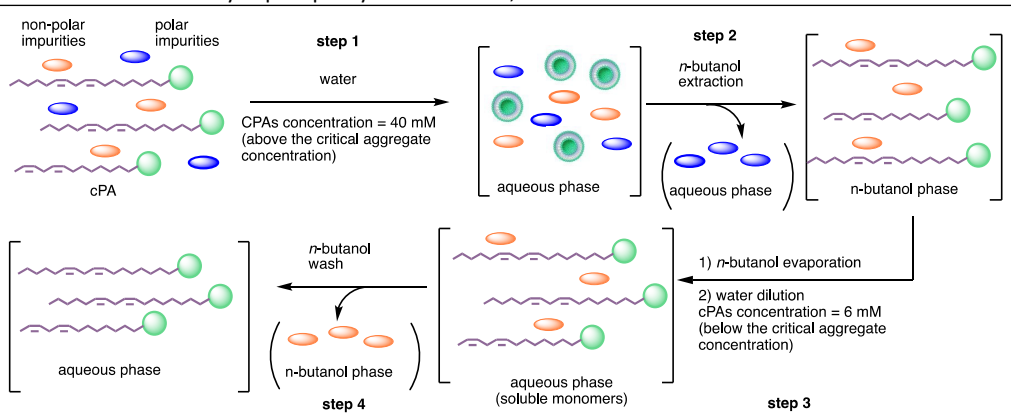

Figure 2. Isolation of medium and long chain cPAs based by manipulating the critical aggregate concentration (CAC) during workup. By keeping the concentration above the CAC, the cPAs are extracted into the organic (n-butanol) phase in steps 1 and 2. Removal of n-butanol followed by addition of water to keep the concentration well below the CAC, results in the cPAs being retained in water phase (step 3), while the non-polar impurities are extracted into the organic (n-butanol) phase (step 4).

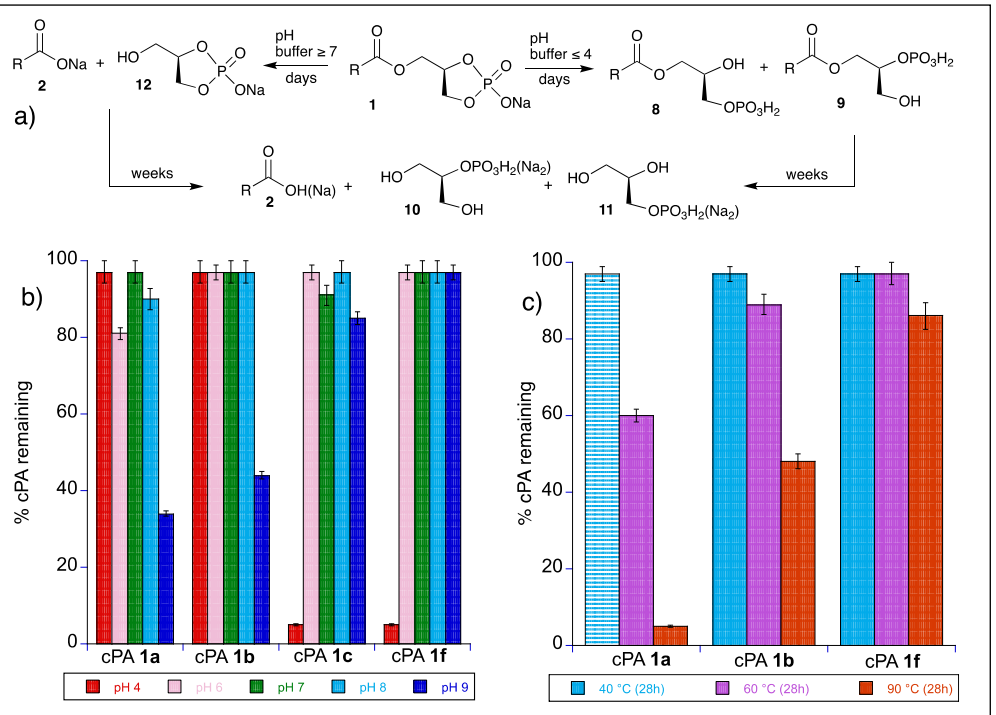

Figure 3. (Top) The two divergent hydrolytic pathways of cyclic phosphate **1** under acidic and alkaline conditions. Long term, the hydrolysis gives **2**, **10** and **11**. (Bottom) Comparison of the hydrolytic stability of the ester bonds in cPAs with varying chain length at (left) 20 mM in buffers pH 4.0, 6.0, 7.2 and 9.0 at room temperature, 7 days and (right) at 40 mM in buffer pH 7.0 at 40, 60 and 90 °C at 28 h. % hydrolytic stability as determined by ^{31}P-NMR of reaction mixture (See SI). Error bars are ±3%.

mM and (b) after step 2, removed the *n*-butanol under rotavap and washed the residue with ether to remove the impurities. This shortened procedure afforded cPA **1b** (66%). We also tested this truncated work-up method (replacing steps 3 and 4 with the ether wash after step 2 in Fig. 1) with crude oleate-cPA **1f**, **1k** and isolated them in high yields. This alternative procedure avoids the time-consuming column purification and the lyophilization steps to afford these cPAs **1b** and **1f** in good purity (>90-95%, Figs. S57 and S73). The protocol in Figure 2 was also applicable for purification of the acetamido- and acetate derivatives **1h**-**1k** (Figs. S79-S94).

Since the cPAs have two ester moieties (the cyclic phosphate-ester and the carboxylate-ester bond), understanding their hydrolytic behaviour was important (Fig. 3a) in the context of our ongoing studies with protocells and for their potential to form vesicles. First, we monitored the stability of the ester bonds of selected cPAs **1a**, **1b**, and **1c** at room temperature, based on the distinct signals in ^{1}H- and ^{31}P-NMR spectrum for the carboxylate-ester and the cyclic phosphate hydrolysis products (Figs S100-S112). Over a 24-hr period at pH 2.0, hydrolysis of the cyclic phosphate-ester occurred predominantly to furnish the regioisomers of linear phosphates **8**, and **9** with the carboxylate-ester persisting, while at pH 12 the carboxylate-ester hydrolysed first to produce the corresponding cyclic phosphate **12**, and fatty acid **2** with the cyclic phosphate-ester moiety persisting (Fig. 3a). Continuing for a prolonged time (7 days) at either pH resulted in further hydrolysis of both carboxylate- and cyclic phosphate-ester to form the linear phosphates **10**, **11**, and the fatty acids **2**. At pH 7.0, both ester bonds of cPAs were quite stable for 7 days at room temperature. The hydrolysis of the cyclic phosphate-ester moiety parallels the observations from the hydrolysis of glycerol 1,2-cyclic phosphate **12**.[27] Motivated by these results, we compared the hydrolysis behaviour of cPA **1a-c, 1f** under controlled buffer conditions (Table S2, pH 4.0, 6.0, 7.2, 8.0 and 9.0, Fig. 3b). Where possible, distributions of hydrolysed products shown in Scheme 3a were quantified by ^{31}P NMR (Tables S4-S7). At 20 mM at room temperature, between pH ranges 6-8 both the ester bonds of the cPAs were stable to hydrolysis for a week. At pH 4.0, the cyclic phosphate-ester bond along with the carboxylate-ester bond of the long chain cPAs myristate **1c** and oleate **1f** were hydrolysed in 7 days to mixture of products (Fig. S113- S132). On the contrary, in the short chain cPAs hexanoate **1a** and decanoate **1b** both ester bonds were quite stable with little sign of hydrolysis at pH 4 (Fig. S113 and S118). At pH 9.0 both ester bonds of **1c** and **1f** were stable whereas only the carboxylate-ester bond (and not the cyclic phosphate-ester bond) of **1a** and **1b**, was hydrolysed (Figs. S79, S84, S89 and S94).

Similar trends were also observed at 40 mM at pH 4 (Figs. S133 versus S154) suggesting that an aggregation-type behaviour may not be the only reason for this unexpected diverging hydrolytic behaviour at pH 4 versus pH 9. It may also be related to the pKa of the cyclic phosphate moiety (≈2)[28], and the modulation of the negative charge of the cyclic phosphate moiety (by protonation with change in pH), which could enable a hydrolytic attack on the cyclic phosphate and the neighbouring carboxylate-ester moiety. The apparent pKa of the cyclic phosphate moiety in myristic **1c** and oleate **1f** lies closer to 4, while for hexanoate **1a** and decanoate **1b** it is closer to 2 (Table S3). This increase in pKa with chain length of cPA is consistent with what is known for pKa of fatty acids with increase in chain lengths,[29] and seems to be reflective of the increase in pKa value of cyclic phosphates in an increasing non-polar environment[28]. If this interpretation is correct, then the increasing pKa would result in a protonated neutral cyclic phosphoric acid species at pH 4, to a greater extent for **1c** and **1f** (when compared **1a** and **1b**). And, therefore, **1c** and **1f** would be more prone to nucleophilic attack by water leading to hydrolysis at pH 4. Kinetic studies (Figs S155-S158) also reflected these trends with **1c** ($t_{1/2}$≈2d) and **1f** ($t_{1/2}$≈1.5d) exhibiting first-order profile of hydrolysis at pH 4, whereas at pH 9, **1a** ($t_{1/2}$≈4d) and **1b** ($t_{1/2}$≈7d) displayed first-order kinetics of hydrolysis at 20 mM.

A comparative study with the hydrolysis of the corresponding glycerides **5a**-**g** was attempted but it was limited due to their insolubility (except for **5a**) in these buffers and the NMR signals were broad, making interpretations difficult. In the one case we could compare, the carboxylate-ester bond in hexanoate-glyceride **5a** was reasonably stable at room temperature in the range of pH 4-9 for the short term (<48 hours); However, at pH 9.0, 90% hydrolysis of the carboxylate-ester bond for **5a** (versus 70% for cPA **1a**) was observed in two weeks (Fig. S164). Thus, the potential for the carboxylate ester-bond hydrolysis of these short chain fatty acid-glycerides and

phospholipids could become important especially in the context of protocell studies involving metal ions and/or high temperatures.

We also investigated the effect of temperature at 40 mM in pH 7 buffer and observed that the stability of the carboxylate-ester bond to hydrolysis increased with increasing chain length. The long chain cPA oleate **1f** was quite stable to hydrolysis (<10%) even at 90 °C for over a day, while the decanoate cPA **1b** was moderately stable (≈ 50%) and hexanoate **1a** was hydrolysed to a large (> 95%) extent (Fig. 3c). In all these cases, little or no hydrolysis of the cyclic phosphate moiety was observed (Figs. S165-S176).

The diverging hydrolytic behaviour of the long-chain cPAs described here, perhaps, may underlie some of cPA's biological behaviour such as the inhibition of cell proliferation and tumour cell invasion (when compared to the beneficial effects of phospholipids such as LPA).[8, 30] In contrast, it is known that shorter chain cPAs form vesicles[15] and those with the corresponding hybrid versions with dodecanol mixtures are more robust to a variety of conditions when compared to their fatty acid counterparts.[16] All these intriguing observations emphasize the need for further Investigations – and work is currently underway in our laboratory to study the vesicle forming properties of these cyclic phospholipids. The results will allow us to understand the scope and potential of cPAs and their ability to form and function as synthetic- and proto-cells.

Conclusions

In summary, a practical scalable synthesis for short, medium and long alkyl chain cPAs has been developed. The key step is the microwave assisted cyclophosphorylation of lipid diols using BDMDAP **6**. We also developed a simple isolation procedure (avoiding chromatography) where medium/long chain cPAs can be easily purified using extraction-steps by manipulating their capacity to form aggregates in water. Overall, this strategy allowed us to access significant amounts of, difficult to obtain, unsaturated cPAs containing one (**1f**) or more (**1g**) double bonds. The cPAs seem to be reasonably stable to hydrolysis in the pH range (6-8) relevant to prebiotic chemistry and biology. The methodology described here is expected to increase the general synthetic availability of various cPAs, thus enabling investigation of their diverse biological activities[14] while providing a venue for fashioning artificial/synthetic cells.[15-17] And the latter may lead to an understanding of what roles cyclic phospholipids could have played in chemical evolution on early Earth.

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Conflicts of interest

There are no conflicts to declare.

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