

Phospholipase D inhibitors screening: Probing and evaluation of ancient and novel molecules

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Abstract:	Phospholipase D (PLD) is a ubiquitous enzyme that cleaves the distal phosphoester bond of phospholipids generating phosphatidic acid (PA). In plants, PA is involved in numerous cell responses triggered by stress. Similarly, in mammals, PA is also a second messenger involved in tumorigenesis. PLD is nowadays considered as a therapeutic target and blocking its activity with specific inhibitors constitutes a promising strategy to treat cancers. Starting from already described PLD inhibitors, this study aims to investigate the effect of their structural modifications on the enzyme's activity, as well as identifying new potent inhibitors of eukaryotic PLDs. Being able to purify the plant PLD from Vigna unguiculata (VuPLD), we obtained a SAXS model of its structure. We then used a fluorescence-based test suitable for high-throughput screening to review the effect of eukaryotic PLD inhibitors described in the literature. In this regard, we found that only few molecules were in fact able to inhibit VuPLD and we confirmed that vanadate is the most potent of all with an IC50 around 58 µM. Moreover, the small-scale screening of a chemical library of 3,120 compounds allowed us to optimize the different screening's steps and paved the way towards the discovery of new potent inhibitors.
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Phospholipase D inhibitors screening: probing and evaluation of ancient and novel molecules

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Abstract

Phospholipase D (PLD) is a ubiquitous enzyme that cleaves the distal phosphoester bond of phospholipids generating phosphatidic acid (PA). In plants, PA is involved in numerous cell responses triggered by stress. Similarly, in mammals, PA is also a second messenger involved in tumorigenesis. PLD is nowadays considered as a therapeutic target and blocking its activity with specific inhibitors constitutes a promising strategy to treat cancers. Starting from already described PLD inhibitors, this study aims to investigate the effect of their structural modifications on the enzyme's activity, as well as identifying new potent inhibitors of eukaryotic PLDs. Being able to purify the plant PLD from *Vigna unguiculata* (VuPLD), we obtained a SAXS model of its structure. We then used a fluorescence-based test suitable for high-throughput screening to review the effect of eukaryotic PLD inhibitors described in the literature. In this regard, we found that only few molecules were in fact able to inhibit VuPLD and we confirmed that vanadate is the most potent of all with an IC_{50} around 58 µM. Moreover, the small-scale screening of a chemical library of 3,120 compounds allowed us to optimize the different screening's steps and paved the way towards the discovery of new potent inhibitors.

Introduction

Phospholipase D (PLD) (EC 3.1.4.4) is a diverse family of enzymes found in microbes, fungi, viruses, plants and animals [1]. In addition to its hydrolytic activity towards phospholipids releasing phosphatidic acid (PA) and a polar moiety, PLD catalyzes a transphosphatidylation reaction as well. This reaction occurs when a primary alcohol is used instead of water, making possible the synthesis of rarer phospholipids by exchanging the polar headgroup, as phosphatidylserine from the abundant phosphatidylcholine (PC) in the presence of serine.

Contrary to several bacterial PLDs, plant and mammalian PLDs share a common feature represented by a duplicated HKD motif that is involved in the catalytic reaction, there are then two classes of PLDs based on their primary structure: the HKD-containing and the non-HKD [2]. This latter family regroups well-used enzymes that belongs to the superfamily of PLC-like phosphodiesterases [3], such as the commercial PLD from *Streptomyces chromofuscus* [4] or the PLD from arachnids [5]. The HKD-containing PLD family belongs to the PLD superfamily and is mostly found in mammals as for example PLD1 and PLD2 in *Homo sapiens*, in plants as PLDα, in yeast with SPO14 [2,6,7], and in several microbes as the crystallized PLD from *Streptomyces sp*. strain PMF[8]. The hallmark of the PLD superfamily is the presence of the so-called HKD motif that is formed by a conserved His, Lys and Asp in a typical $HxKx_4D$ sequence that was recently refined [9]. In plants and mammals, the HKD motif is duplicated in the primary structure, and both motifs are thought to be closed in the tertiary structure to form the catalytic site at the interface of the bi-lobed enzyme [10–13].

Furthermore, one common mechanism of action defined as a two-step “ping-pong” reaction mechanism is conserved between plant and mammalian PLDs [2]. The interest in PLD as a drug target is due to the fact that PLDs are essential to intracellular and extracellular signaling. PLD

have been described to be involved in settings ranging from platelet activation to response to cardiac ischemia, viral infections, neurodegenerative diseases, and in cancers. Therefore, human PLD are a valid target for a variety of drug therapies [14].

Our work focuses on identifying potential new inhibitor of eukaryotic PLDs, especially the human isoforms, by using a plant PLD as a model. Indeed, VuPLD shows an overall 19 % peptidic sequence identity with human PLD1, which reaches 42 % and 69 % around the first and second HKD motif, respectively. Thus, based on these elements, we hypothesized that an inhibitor directed against the catalytic site of plant PLD would somehow be able to inhibit mammalian PLD and *vice versa*. Previous works to screen inhibitors on mammalian PLD were weakened by the difficulty to obtain sufficient amounts of enzyme for HTS of PLD inhibitors. This difficulty was partially circumvented, either by the use of the atypical prokaryotic PLD enzyme from *Streptomyces* for preliminary screenings [15], by using *in silico* methods [16], by virtual screening [17], or other undisclosed conditions [18]. Recombinant plant PLDα is however easy to produce and could be purified in one step [19]. Therefore, considering their similarities at multiple levels, we assumed that a molecule capable of inhibiting plant PLDs could as well affect human PLDs. This strategy could thus constitute an interesting and original approach to identify novel inhibitors than using non-HKD PLDs as templates for the screening.

Only a few inhibitors of PLDs were described in the literature. N-acylethanolamines (NAEs) are a class of potent lipids released from the membrane phospholipid N-acylphosphatidylethanolamine (NAPE), and are found as endogenous constituents of desiccated seeds. The hydrolysis of NAPE is catalyzed by a NAPE-specific PLD or PLD-like enzyme [20], but not by canonical PLD such as plant PLDα. However, NAE is able to inhibit PLDα activity with an IC_{50} estimated at 0.15 µM as for NAE (12:0) [21]. Applying NAE (12:0) improved seed desiccation tolerance via the inhibition

of PLDα, that led to the reduction of PA formation, PA being a key modulator of desiccation sensitivity [22]. Likewise, lysophosphatidylethanolamine (LPE) was shown to be a specific inhibitor of plant PLDs, that could act as a lipid-derived senescence retardant via the inhibition of PLD [23]. As an example adding 10 µM of LPE (18:1) reduced by half the activity of PLD partially purified from cabbage [23].

Similarly, the immunosuppressant ciclosporin at 5 µM was also shown to completely inhibit the cabbage PLD activity [24], and so were alkylphosphate esters [25], octadecylphosphocholine being the most efficient inhibitor of this class of molecules with an IC_{50} of 6.4 µM. However, these molecules having a classical phosphoester bond they are substrate as well of PLD enzymes [26]. In fact, compared to PC, the canonical substrate tested in the same conditions, around 3% of the specific activity was found when hexadecylphosphocholine was used [25]. Hexanal and its reduced form hexanol have been shown to be effective PLD inhibitors in different subcellular fractions from corn kernels, and PLD activity was inhibited from 50 to 70 % using 0.1 % of hexanal in *in vitro* assays [27,28]. Volatile hexanal being a natural product of the catabolism of linoleic and linolenic acid during senescence, it was used as a check-point for arresting membrane lipid degradation in plants, and consequently enhances shelf-life of fruits as described in raspberry [29] and in litchi [30]. PLD inhibition is used in banana when treated with hexanal, to thick the cell wall and imped penetration of pathogenic spores [31]. Moreover, PLD in rice causes rancidity and 'stale flavor' in bran oil, and thus limits the rice bran usage [32], demonstrating the need for specific plant PLD inhibitors.

As far as mammalian PLDs are concerned, several compounds were reported in literature. For instance, fodrin [33] indirectly inhibits the PLD activity by decreasing the amount of phosphatidylinositol (4,5)-bisphosphate (PIP2), a cofactor necessary for mammalian PLD activity.

However, this compound is not considered useful for research or clinical trials. Halopemide was identified by HTS in 2007 as a modest inhibitor of PLD2 [18]. Several variants of halopemide were then developed to exhibit a preferential selectivity towards either PLD1 or PLD2 [34]. *In vitro*, halopemide has an IC_{50} of 220 and 310 nM for PLD1 and PLD2 respectively. Conversely, VU0155069 is more selective towards PLD1, with an IC_{50} of 46 nM towards PLD1 compared to 933 nM towards PLD2 *in vitro*, and 11 nM and 1800 nM in cells respectively, whereas a VU0364739 precursor is more selective towards PLD2 (IC_{50} of 140 nM *in vitro* and of 110 nM in cells, compared to 5.1 µM *in vitro* and 1 µM in cells towards PLD1) [35]. Interestingly 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) which at approximately 25 nM induced a 50% loss of activity independently on PLD1 and PLD2 [36] is thought to act on the catalytic site of both isoforms PLD1 and PLD2. In the opposite VU0364739 which is selective of PLD2 [37] was previously shown by computational modelling to use an allosteric site overlapping with the PH domain to target its activity [38], but surface plasmon resonance experiments have demonstrated that this family of inhibitors binds directly to the catalytic domain [11]. FIPI's IC_{50} on PLD from *Streptomyces* strain PMF was reported to be around 100 nM, which is 4 to 6 times higher than the values found on PLD1 and PLD2, demonstrating that some inhibitors directly block enzyme and phospholipid vesicles substrate binding [15]. Last but not least, resveratrol, an antioxidant found in red wine, was described to inhibit plant PLD [39], and human isoforms as well with an IC_{50} around 61 µM [40].

In order to find the optimal conditions for the screening and to understand the best features of a PLD inhibitor, we first characterized the purified enzyme VuPLD and described its physical shape and biochemical structural properties. Then, we characterized the effect of already-known inhibitors of eukaryotic PLD on VuPLD. Several types of inhibitors have been described in the

literature and could be basically divided into three classes. First, phosphate mimicking ions such as vanadate [41] or tungstate that compete with the phosphate moiety of the phospholipid substrate. Secondly natural products such as resveratrol [39] and ciclosporine [24], that are small organic compounds supposedly protecting enterocyte mitochondria from oxidative stress. Finally, halopemide and its derivatives that were specially designed for the two isoforms of mammalian PLD with catalytic activity (PLD1 and PLD2). We then screened a chemical library of roughly 3,120 molecules of diverse origins. The optimization of a 6-step screen could now be used for larger libraries.

Material and Methods

Reagents and material

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), choline oxidase from *Arthrobacter globiformis*, horseradish peroxidase (type VI), bovine serum albumin (BSA), 8-hydroxyquinoline (8HQ), resveratrol, ciclosporin, VU0285655, alcohols, hexanal, N-acylethanolamine, and mineral salts: sodium vanadate, vanadate derivatives (oxytriethoxide, oxytripropoxide, oxytriisopropoxide, tris(triphenylsiloxy)), tungstate and phosphate came from Sigma Aldrich-Fluka Chimie. Halopemide, VU0359595, and FIPI were from Cayman Chemical. Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA) in chloroform were purchased from Avanti Polar Lipids (Alabama, USA). Thin layer chromatography (TLC) plates were purchased from Macherey-Nagel Gmbh & Co. Kg (Germany). Mammalian-PLDs inhibitors halopemide and derivatives were from Cayman chemicals. Mineral salts: sodium vanadate and its derivatives, tungstate and phosphate were dissolved in water. All PLD inhibitors and compounds from the ICBMS chemical library were prepared in DMSO (DMSO levels used

in this study remained below 5%). Flat-bottom black 96-well microplates (Greiner bio-one) were used for fluorescence measurements.

Purification of VuPLD

Recombinant VuPLD was produced in *Pichia pastoris* and purified as described previously [19]. Small aliquots were thawed and diluted before each experiment. A single batch was used for the enzymatic characterization, and another for the screening of the chemical library. Protein concentrations were determined using the Bradford's procedure [42], with Dye Reagent and BSA as the standard.

Small angle X-ray scattering experiment

Data were collected at the ESRF beamline BM29 (Grenoble, France). VuPLD was concentrated at 2.55 mg/mL in PIPES buffer 10 mM, pH 6.2, containing EDTA 0.1 mM and NaCl 200 mM. Reconstruction of the overall shape of the VuPLD from X-ray scattering data was first achieved by the *ab initio* modeling programs DAMMIN (20 models calculated) [43] or GASBOR (50 models calculated) [44], then models were averaged using DAMAVER [45]. Comparison of the SAXS data and protein structure (PDB code : 6KZ9) was done using FoXS [46]. Protein molecular mass was estimated from scattering intensities $I(0)$ using SAXSMoW2 software [47].

PLD activity measurement

Chromogenic assay

A detailed protocol of this assay has been recently published [48]. Briefly, 0.4 mM of DMPC in SDS/Triton-X100 mixed micelles were incubated with pure PLD (0.7 to 0.9 µg/mL, final concentration) in the presence or absence of inhibitors for 10 min at 30°C. The reaction was started by the addition of 20 mM of $CaCl_2$, then blocked after 10 min by addition of 83.3 mM of EDTA, and revealed by addition of peroxidase, choline oxidase, 2-hydroxy-3,5-dichlorobenzenesulfonate and 4-aminoantipyrine. The PLD-generated choline was quantified with end point measurements by recording the absorbance at 500 nm, based on a standard curve obtained with pure choline.

Fluorescence-based assay

The preparation of 8HQ / phospholipid/ detergent mixtures was adapted from [49] with minor modifications to control the activation of the enzymes. To prepare the mixtures, 40 nmol of POPC (266 µM, final concentration) were first dissolved in chloroform, then evaporated under a stream of nitrogen. Subsequently, dried phospholipids were dispersed in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM Triton X-100, 0.7 mM SDS, 66 µM 8HQ, and 50 µM EDTA. The micellar solution was vortexed for 30 s, sonicated for 10 min using a bath sonicator (Deltasonic type O11C, Fécamp, France), then vortexed for an additional 30 s. One pmol of enzyme (VuPLD) was finally added as well as the compounds to be tested. After a 10-minute incubation period at 30°C, the reaction was triggered by adding 20 mM of $CaCl_2$. Fluorescence measurements were recorded at 30°C using a microtiter plate fluorescence reader (Tecan Infinite M200, Austria) at wavelengths 366 nm and 490 nm of excitation and emission, respectively. To monitor PLD activity, a baseline of 4 kinetic cycles of 30 s was first recorded, after which 20 mM $CaCl_2$ were injected in each well

to trigger the PLD hydrolysis. Fluorescence intensities were then measured during a 1h-kinetic cycle with interval shaking before each read. The PLD-generated PA was quantified by recording the fluorescence in the same conditions as described above, based on a standard curve obtained with pure POPA [49].

TLC analysis

The phospholipids were extracted with chloroform/methanol (2:1, v/v). The lower organic phase was collected, dried, and subjected to TLC separation together with proper phospholipid standards. TLC plates were developed in a solvent system using a mixture of chloroform/methanol/water (65:25:5, v/v/v) as the eluent. Phospholipid spots were revealed by charring the plate after spraying a mixture of 10 % copper sulfate and 10 % phosphoric acid in water and heating at 150°C for 5 min.

Statistical analysis

Statistically significant differences were determined by Brown-Forsyth and Welsh ANOVA test using the computer program Prism (GraphPad Software). Results were considered significant when $p < 0.05$ (*), highly significant when $p < 0.01$ (**), and extremely significant when $p < 0.001$ (***).

Results

Modelization of VuPLD by SAXS experiment

VuPLD was produced according to Arhab *et al.*[48]. The amount of homogenous VuPLD obtained after purification was 1 mg per liter of yeast culture. The specific activity toward DMPC was 20 U/mg. Scattering measurement with synchrotron radiation were performed at pH 6.2 with 2.55 mg/mL of VuPLD. A typical SAXS profile of VuPLD is shown in Fig. 1A and a Kratky curve displays a symmetric peak indicating that VuPLD is globular and correctly folded (Fig. 1B). From scattering intensities $I(0)$ a molecular mass of 89.1 kDa was calculated, which is in accordance with a monomer of VuPLD as the theoretical molecular mass deduced from the amino acid sequence is 91.5 kDa. The radius of gyration is 2.81 +/- 0.05 nm and D_{max} (maximum dimension) is calculated to be 9.89 nm (Fig. 1C). The calculated model by DAMMIN shows a globular and symmetric molecule with extended extremities and an ovoid shape (Fig. 1D). The program GASBOR calculated a very similar model however this one is less symmetrical and displays a larger base and a fine apical extremity (Fig. S1). The comparison of the collected SAXS data and calculated scattering curve from the published structure of the PLD show a good correlation (Fig. S2A) indicating that the protein adopts a similar conformation in the crystals and in solution. The small differences observed could be due to small motion of the C2 domain.

Characterization of the salts effect on PLD activity

Ions such phosphate, tungstate and vanadate salts were already reported as potent inhibitors of partially purified plant PLDs. We were able to confirm these results by measuring an IC_{50} for these three ions on a pure VuPLD. Vanadate was the most potent of them with an IC_{50} of 58 µM and being mostly able to totally inhibit the enzymatic activity at a concentration of 260 µM (Fig. 2A) and was consequently used as a reference for HTS of PLD inhibitors. Likewise, tungstate was also able to inhibit totally the PLD activity at a concentration of 2 mM, and harbored an IC_{50} value of

375 µM (Fig. 2C). Phosphate behaved in between, with an IC_{50} estimated to 125 µM but not being able to totally abolish the activity, 25% of residual activity was quantified at 1 mM of phosphate that is the maximal concentration usable in this test (Fig. 2B), as Ca^{2+} cofactor precipitates at higher concentration of phosphate.

An unusual slight synergistic and inhibitory effect was observed when phosphate and vanadate were incubated together (Fig. 2D). Indeed, when both molecules were incubated together at their respective IC_{50}, only 15 % of residual activity was measured that was lower than the theoretical 25 % expected at these particular concentrations, suggesting the combined salts potentiate the PLD inhibition.

Vanadate derived compounds inhibitory effect on PLD activity

Vanadate being the PLD inhibitor with the more potent inhibitory impacts, we decided to examine the effects of the environment around this particular ion by testing diverse vanadate derivatives such as vanadium oxytriethoxide, vanadium oxytripropoxide, vanadium oxytriisopropoxide and tris(triphenylsiloxy) vanadium oxide, as all these molecules harbor different hydrocarbon chain lengths and steric environment (Fig. S3). If vanadate took the place of the phosphate moiety of phospholipid substrate in the active site, changes of alkyl chain length and steric environment around vanadate would have been expected to modify its IC_{50}. However, even if the IC_{50} of vanadate derivatives were lower than the one of vanadate (Fig. S4), no dramatic variation could be observed amongst these four derivatives (Fig. 3). IC_{50} were ranging from 7 µM (vanadate oxytriethoxide) to 15 µM (vanadate oxytriisopropoxide).

Alcohols inhibitory effect on PLD hydrolysis activity

Historically, primary alcohols have been used as inhibitors of PLD hydrolysis activity. However, one must state that it is not a proper inhibitory effect as the use of alcohol derives PLD activity from hydrolysis *stricto sensu* toward the transphosphatidylation activity. In fact, alcohols can be used as secondary substrate and therefore PLD can transfer the phosphatidyl group to a primary alcohol to generate new phospholipids. The use of butan-1-ol is quite common to follow mammalian PLD activity by detecting the production of phosphatidylbutanol. We consequently tried to define which primary alcohol would be the best to avoid PLD-generated PA by testing the primary alcohols from methanol to hexan-1-ol (Fig. 4).

As expected, methanol and ethanol were not efficient secondary substrates of VuPLD. For short-chain alcohol the best candidate was butan-1-ol with an IC_{50} of 150 mM compared to propan-1-ol which was more than twice less efficient with 320 mM. Pentan-1-ol had an IC_{50} of 60 mM which is the best among all the alcohols tested. Finally, hexan-1-ol seemed not to be a proper secondary substrate as the activity quickly falls at 20 mM probably indicating enzyme denaturation (Fig. 4A). All secondary alcohols tested appeared not to be proper substrates as they displayed high IC_{50}, 160 mM for pentan-2-ol, 360 mM for butan-2-ol and propan-2-ol had no effect on PLD activity (Fig. 4A).

However, these values are based on the fluorimetric detection of PA (see Material & methods section). A more convenient way to detect phosphatidylalcohols issued from the PLD-catalyzed transphophatidylation reaction is to use TLC for phospholipids separation. As can be seen from Fig. 4B, from all alcohols tested, the transphophatidylation products were qualitatively detected only with methanol, ethanol, propan-1-ol and butan-1-ol.

Effect of known mammalian PLD inhibitors on VuPLD

Halopemide was shown previously to inhibit both human PLD1 and PLD2 isoforms, VU0359595 targets the PLD1 isoform with an IC_{50} calculated to be 3.7 nM, VU0285655 targets the PLD2 isoform with an IC_{50} calculated to be 635 nM. When tested on VuPLD, no inhibition could be observed up to 10 µM (Fig. 5). The PLD activity was reduced down to 50 % when 100 µM of each these three inhibitors were used independently.

Test of alleged plant PLD inhibitors

Amongst the molecules reported in literature to inhibit plant PLD activity we tested both ciclosporin A and resveratrol on VuPLD using our fluorescence-based assay. In our hand, neither ciclosporin A (Fig. 6A) nor resveratrol (Fig. 6B) were able to significantly affect VuPLD activity. A slight 20% PLD inhibition could be observed using 2 mM of resveratrol, which is far from the value reported by others where more than 90% of inhibition was measured with 130 µM of resveratrol [39]. As far as ciclosporin A is concerned, no inhibition was detected until 100 µM even if a quasi-total inhibition was previously reported for the PLD from cabbage using 5 µM of this compound [24,39].

Similarly, neither NAE (Fig. 6C) nor hexanal (Fig. 6D) which have been demonstrated as efficient inhibitors of castor bean [21] or raspberry [29] PLDs, respectively, displayed any inhibitory effect on VuPLD.

Screening of new PLD inhibitors

Because of its sensitivity and ease of use, we aimed to validate the 8HQ-based assay suitability for HTS of novel PLD inhibitors as a start. In order to do so, we calculated the Z'-factor, a statistical parameter used to evaluate the quality and performance of an assay. This parameter is reflective

of both the assay signal dynamic range and the data variation associated with signal measurements [50]. In light of former results showing sodium vanadate as the most potent inhibitor of VuPLD (Fig. 2B), we consequently decided to use it as a reference and a positive control for the screening. Briefly, confronting the variability of 30 values of PLD activity measured with either 100 µM of vanadate or just 3 % DMSO, we obtained a calculated value of 0.803 for the Z'-score (Fig. 7A). This value being comprised in the in the interval between 0.5 and 1.0 thus qualifies the 8HQ-based test as "excellent" [50] for HTS of PLD inhibitors.

Moreover, as most of chemical library are DMSO solubilized, DMSO was shown only to have a slight effect on PLD activity up to 5 % DMSO final concentration, but no statistical difference compared from the control was observed when 5% DMSO final concentration was used (data not shown).

We built a six-step protocol for the HTS of a PLD inhibitor (Fig. 7B). We consequently performed a small-scale screening on the chemical library of our institute that is composed of 3,120 compounds from diverse origins. These molecules include quinolones, indoles, indolinones, benzofurans, coumarins and flavonoids from chemistry and pharmaceutical laboratories.

A hit was considered every molecule capable to activate or inhibit PLD activity *in vitro* by more than 50%. Out of 3,120 initial compounds, 227 molecules were found to reduce the PLD activity and 53 to activate this activity (the others were found to have little or no effect at all). Because some of the hits could be fluorescent molecules, or molecules with putative screen effect on the 8HQ probe, or molecules that could chelate the calcium ions for example, *id est* molecules that could interfere with the fluorescent assay, we screened the putative 277 inhibitory molecules using chromogenic assay which quantifies the free choline released by the PLD. After this step, 11 molecules were retained then further checked for their absence of effect on a PA standard curve

fluorescence. Finally, the 2 remaining compounds were tested on crude extract of *Pichia pastoris* expressing VuPLD to avoid unspecific protein interaction. The last hit was confirmed against other HKD-type PLDs like cabbage PLD [51] or peanut PLD [52]. Then, it was excluded after the last step of this protocol as *de novo* synthesis of the molecule showed no effect at all and as several contaminants were present in the initial batch of molecule used to make up the library.

Discussion

Using two different *ab initio* modelization programs SAXS data indicate that VuPLD is a globular enzyme with elongated tops, thus the enzyme seems overall symmetrical with an ovoid shape. These models do not allow the positioning of the regulatory C2 domain. The determination of the overall shape of VuPLD is in accordance with the results obtained with cabbage PLDα2 in 2012 by others [53]. Both models of nearly the same length (809 residues for VuPLD compare to 812 for cabbage PLDα2) share 81.3% amino acid sequence identity and have a similar gyration radius, but the D_{max} is lower in cabbage PLD (2.81 +/- 0.05 nm (this study) *vs* 2.89 +/- 0.01 nm [53]) indicating a slightly smaller enzyme. Also, the porod volume of VuPLD is more important indicating a larger enzyme of 140.8 nm^3 *vs* 126.7 nm^3 for cabbage PLD [53]. These very slight differences for such similar enzymes in terms of sequence might be explained by the fact that, even if they are purified the same way, they are not recombinantly expressed in the same organism. Specifically, cabbage PLD was expressed in *Escherichia coli* BL21 (DE3), contrary to VuPLD that was expressed in yeast *Pichia pastoris* X33 cells which are known to glycosylate proteins. The structure of PLDα1 from Arabidopsis that was recently solved [12] positioned the C2 domain as a small protuberance interacting with the second HKD domain which is, however, not in accordance with the ovoid shape with extended extremities of the VuPLD obtained in this work.

Nevertheless, the SAXS model of VuPLD (this work) and of PLD from cabbage [53], or crystal structure of *Arabidopsis* PLD [12] are very close (Fig. S2B) and interchangeable, which makes of VuPLD another PLD model, and specially a convenient tool to study PLD inhibition as this latest protein could be recombinantly expressed and easily purified in one step [54].

To prove that VuPLD is a relevant model to study plant PLD inhibition, we started with the use of substrate analogs, in particular, analogs of the phosphate head of phospholipid. Therefore, we tested phosphate salt at first sight and structurally close chemicals like vanadate or tungstate. These salts are known to modulate PLD activity and we show here that vanadate is the best of them with the finest IC_{50}. Consequently, we tested vanadate derivatives that differ by their hydrophobic properties. Indeed, vanadate and then vanadate oxytriethoxide, vanadate oxytripropoxide, vanadate oxytriisopropoxide and tris(triphenylsiloxy) vanadium oxide are increasing hydrophobic molecules. Among them, vanadate oxytriethoxide that only display ethane moieties around vanadate salt is the best inhibitor. These results may suggest that the binding site of vanadate on PLD was not influenced by the alkyl chain length and/or steric environment and has enough room to allow the fixation of the different derivatives tested.

Another way to inhibit the hydrolase activity of PLDs is the use of alcohols, keeping in mind that alcohols are not proper inhibitors but are just another secondary substrate competing with water to induce a transphosphatidylation reaction. Here, we show that short-chained alcohols like methanol, ethanol and to a lesser extent propan-1-ol do not affect the hydrolysis activity. Secondary alcohols are also not substrates. The commonly used butan-1-ol is definitely the best primary alcohol susceptible to generate a phosphatidylbutanol at a weak concentration. With other alcohols, no transphosphatidylation products were visible on TLC plates, meaning that they are not proper

secondary substrates of PLD. But one has to notice the fact that they indeed reduce PLD activity. As stated above for hexan-1-ol, propan-2-ol and pentan-1-ol probably induce enzyme denaturation. Human PLD inhibitors do not show any inhibitory effect on plant PLD, even if these PLD [11] [12] share a common and superposable active site (Fig. S5). These results ask questions about the mechanism by which they inhibit human PLDs. We demonstrated recently that all PLDs share common sequence motifs [9]. Among them a pocket constituted by two HKD motifs form the catalytic site. But also, several similar sequences are found in all eukaryotic PLDs. Indeed, PLDs are mostly differing by their regulatory motifs [9]. For example, human PLD bears a PX and PH domain [2] whereas plant PLD bears a C2-domain responsible for the fixation of Ca^{2+} ions, a mandatory activator of plant PLD [55]. However, the catalytic sites of *Arabidopsis* PLD [12] and human PLD1 [13] share a common feature as globular fold with a funnel-shaped cavity leading to the active site. Considering the localized relative sequence similarity between human PLD and plant PLD, and considering that if human PLD inhibitors were targeting the catalytic site they would have a similar effect on plant PLD, it is possible that human PLD inhibitors are targeting (i) aminoacid sequences that do not exist in plant PLD or (ii) the regulatory domains and therefore do not inhibit the catalytic activity of the enzyme but its capacity to bind its partners and cofactors. The structure of the catalytic domains of human PLD1 and PLD2 that was recently published [11] would surely help to explain the selectivity of several molecules thought or used as generic inhibitors on plant and/or mammalian PLDs. As it was not been possible to solve the structure of PLDα1 with FIPI [12], this compound is assumed to localized in the substrate-binding pocket of the enzyme. However, our direct and continuous fluorescence based assay failed to display an inhibitory effect on VuPLD, as no inhibition could be observed up to 100 µM (Fig. 5) and only an apparent 29% reduction of activity was observed when 1 mM of FIPI was used (data not shown),

noting that such a high concentration would probably interfere with the fluorescence of the 8HQ probe. These differences of FIPI effects could be explained by the nature of the plant PLDs used, as some residues are dissimilar in this pocket among the plant PLDs, or by the nature of the test, the activity being assayed in a direct and continuous manner in this work.

At the exception of vanadate that is still the more potent plant PLD inhibitor to our knowledge, almost of all plant PLD inhibitors described in the literature failed to inhibit VuPLD, consequently there is still a need for a specific plant PLD inhibitor or a generalist eukaryotic PLD inhibitors acting both on plant and mammalian PLDs. Looking closely, it appears that the use of a purified enzyme in this study is the major difference compared to all other papers published on this topic. In this regard, it is possible that these compounds interfere with others enzymes used in the cascade of choline quantification. On the other hand, in our case the use of an assay to directly measure the PA released directly from PLD-catalysed hydrolysis, constitutes a significant advantage to quantify PLD activity [49].

Indeed, this 8HQ-based assay was statistically validated with a Z' score of 0.803 indicating an excellent test for HTS and demonstrating that vanadate should be used as a positive control for forthcoming screenings. We consequently screened as a proof of concept a chemical library of ~3,120 compounds. After six runs of enzymatic tests combining successively the advantages of both fluorescent and chromogenic assays to avoid false positives, no hit could unfortunately be retained as the unique potential candidate appeared to be a contaminant. In the future, combining the two PLD enzymatic assays with their specific strengths and weaknesses, their distinct and alternative properties, larger libraries could be screened to identify and validate new PLD inhibitors.

In conclusion, this work highlights (i) the necessity for a novel universal inhibitor potentially targeting the catalytic site of PLDs, (ii) the potential of a purified plant PLD to screen PLD inhibitors and (iii) the need to screen larger chemical libraries to find new inhibitory molecules.

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Author Contributions

YA and MVC performed SAXS and modeling experiments. AC and FP contributed to compound design and synthesis. YA, KB, HA, MA, RR performed biochemical assays. LB, SM, AA and AN devised the project and the main conceptual ideas. AN was in charge of funding acquisition. All authors contributed to data interpretation. The manuscript was written from contributions by all the authors. All the authors have given approval to the final version of the manuscript.

Competing interests

The authors have no competing interests to declare.

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Abbreviations

8-Hydroxyquinoline (8HQ), high-throughput screening (HTS), lysophosphatidylethanolamine (LPE), N-acylethanolamines (NAE), N-acylphosphatidylethanolamine (NAPE), phosphatidic acid (PA), phospholipase D (PLD), palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).

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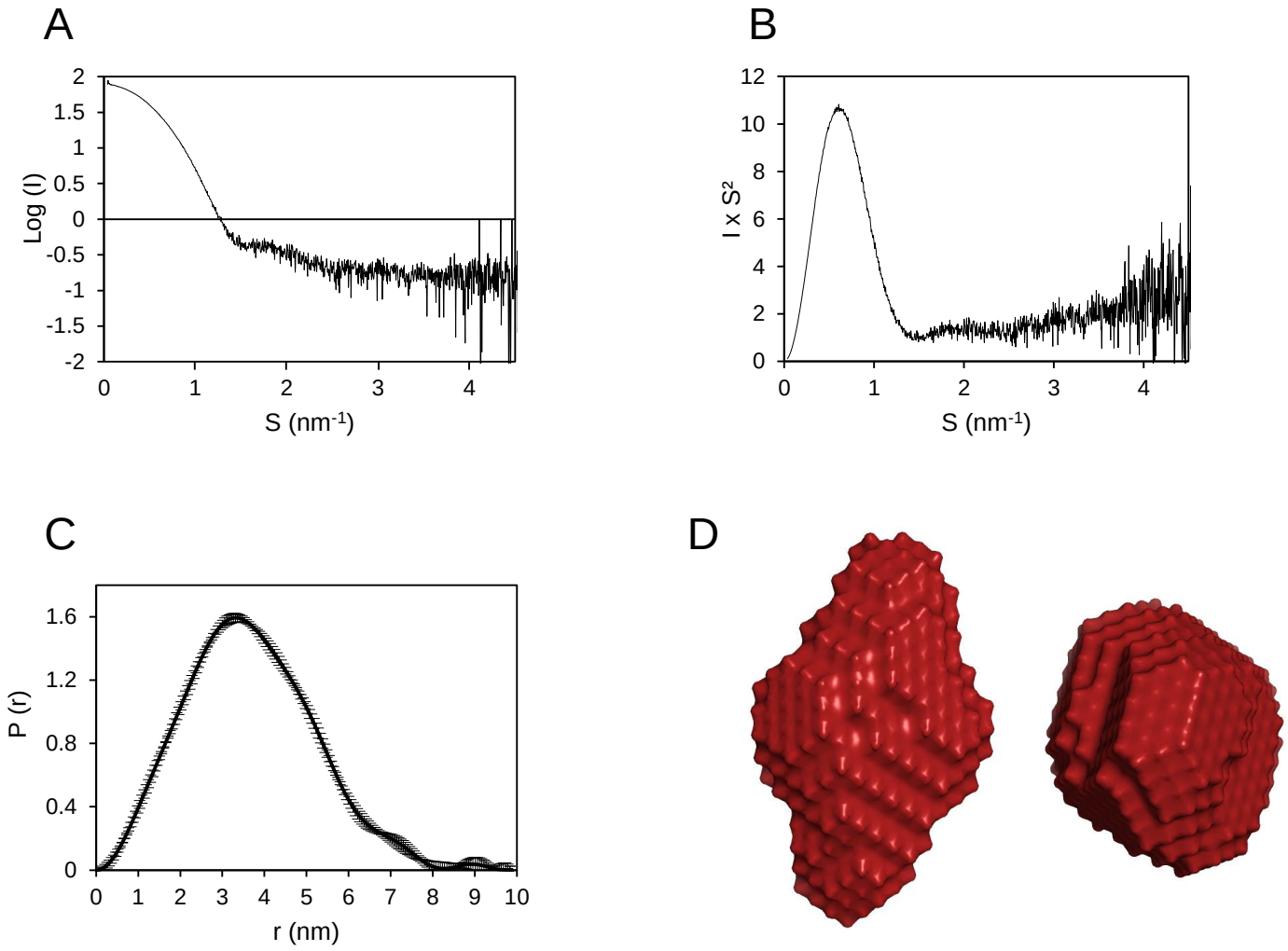


Fig. 1: SAXS modelization of VuPLD.
A) Diffusion curve. B) Kratky curve. C) Distance distribution function P(r) calculated from X-ray scattering data. D) *Ab initio* low-resolution structure model of VuPLD calculated from the SAXS pattern and the program DAMMIN. Model is shown in two different views obtained by 90° rotation around the y-axis.

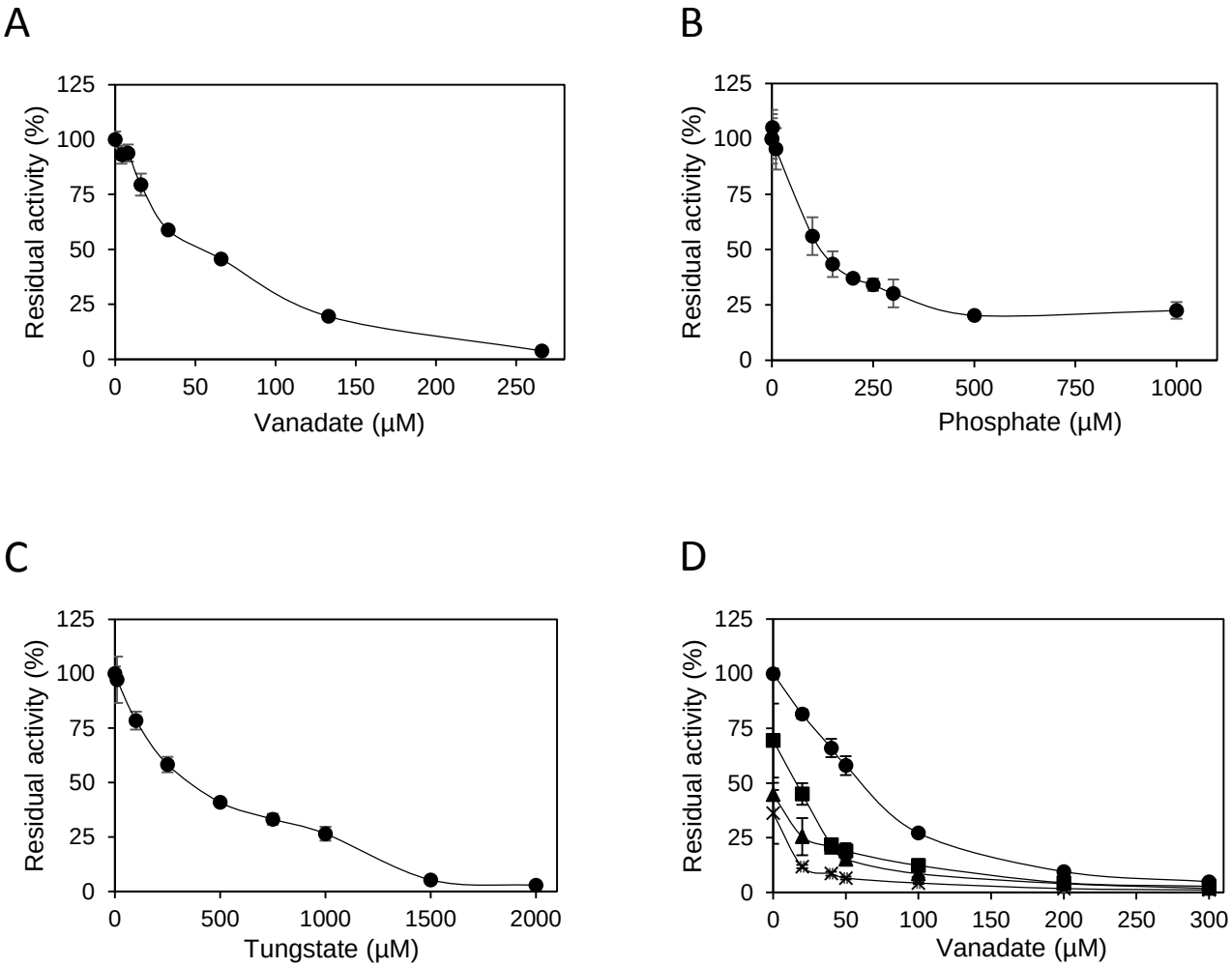


Fig. 2: Inhibitory effect of different salts on purified VuPLD.

The PLD activity was measured with the fluorimetric assay in triplicates and with increasing concentrations of sodium vanadate (A), sodium tungstate (B) or sodium phosphate (C). D) PLD relative activity incubated with 0 µM (circle ●), 25 µM (square ■), 50 µM (triangle ▲) or 100 µM (cross ⋆) of phosphate at different concentrations of vanadate. Relative PLD activities were calculated relatively to the enzyme incubated in the absence of effector set at 100 %. Values are the mean ± SD obtained from three independent assays.

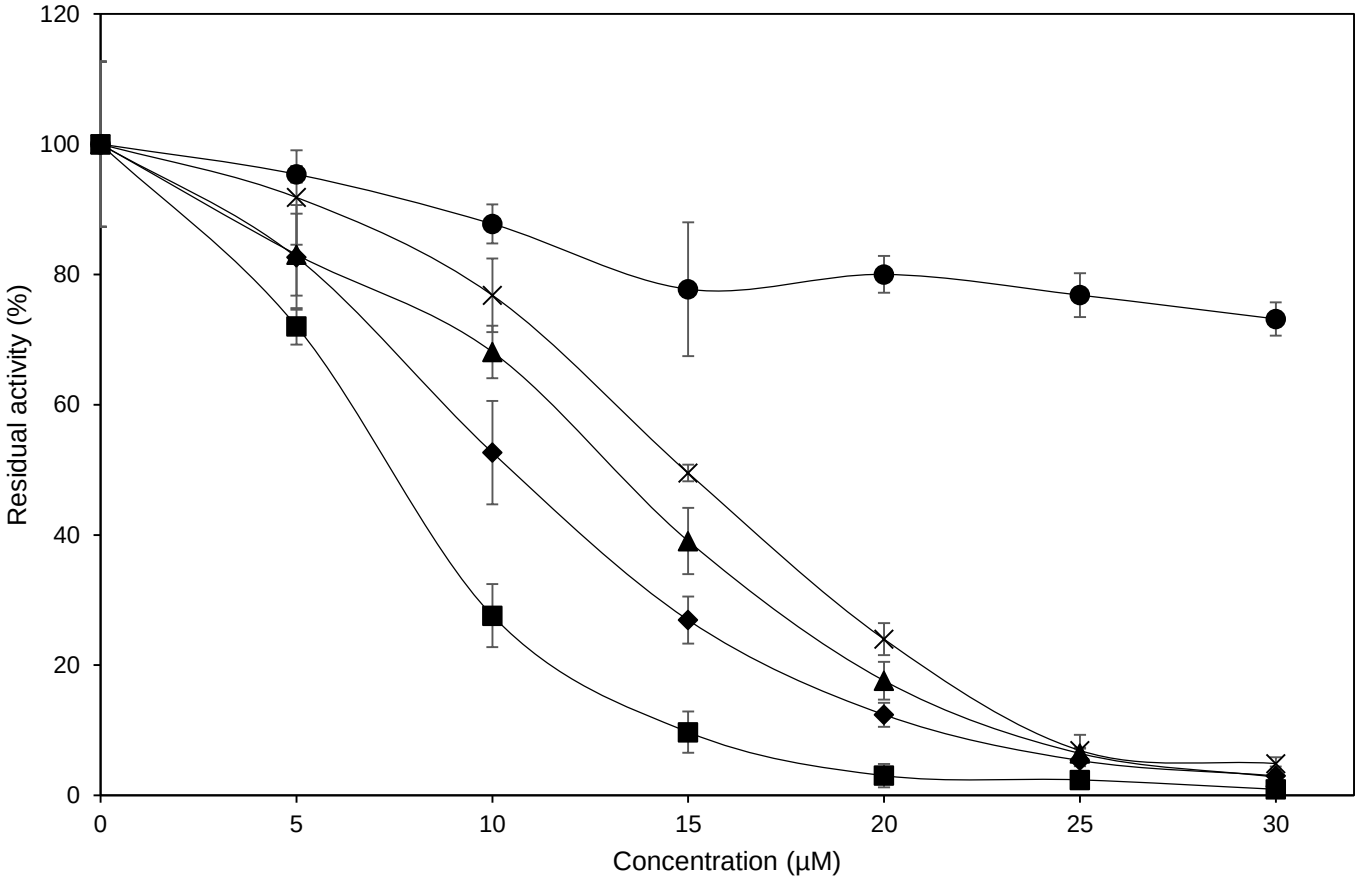


Fig. 3: Inhibitory effect of different vanadate derivatives on purified VuPLD.
The activity was measured with the chromogenic assay in triplicates with increasing concentrations of vanadate (circle ●), vanadate oxytriethoxide (square ■), vanadate oxytripropoxide (triangle ▲), vanadate oxytriisopropoxide (cross ⁕) or vanadate tris(triphenylsiloxy) (diamond◆). Values are the mean ± SD obtained from three independent chromogenic assays.

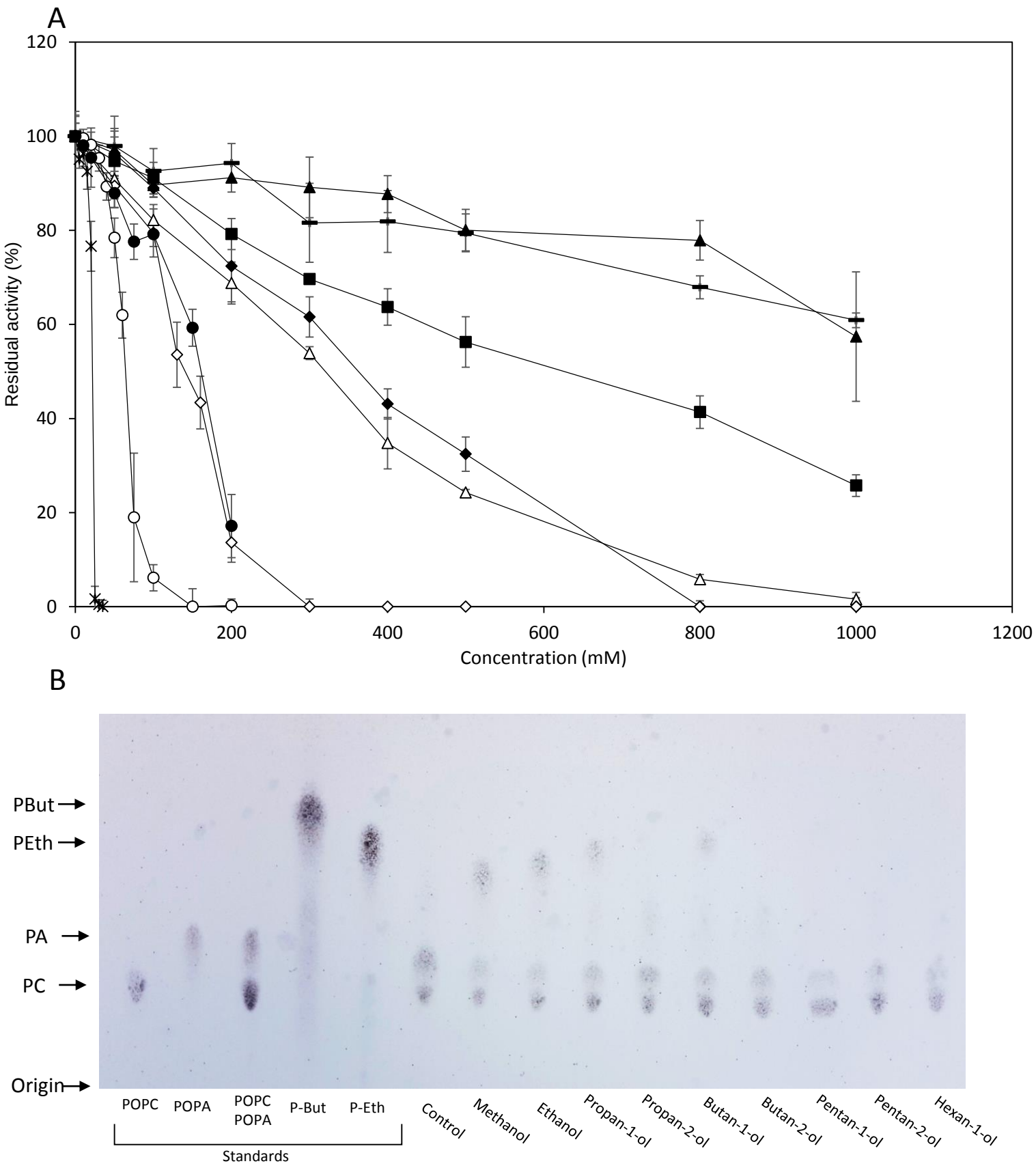


Fig. 4: Effect of different alcohols on purified VuPLD.
A) The activity was measured in triplicates with increasing concentrations of methanol (black dash−), ethanol (square■), propan-1-ol (white triangle Δ), propan-2-ol (black triangle ▲), butan-1-ol (white diamond ◇), butan-2-ol (black diamond ◆), pentan-1-ol (white circle○), pentan-2-ol (black circle●) and hexanol (cross ⋆). Relative activities were calculated relatively to the enzyme incubated in the absence of effector (100%). Values are the mean ± SD obtained from three independent fluorimetric assays. B) TLC analysis of the VuPLD catalyzed transphosphatidylation reaction products obtained from (A). Lipids were extracted, developed, and revealed as described in the experimental section. POPA, phosphatidylbutanol (P-But) and phosphatidylethanol (P-Eth) were run as standard reaction products,

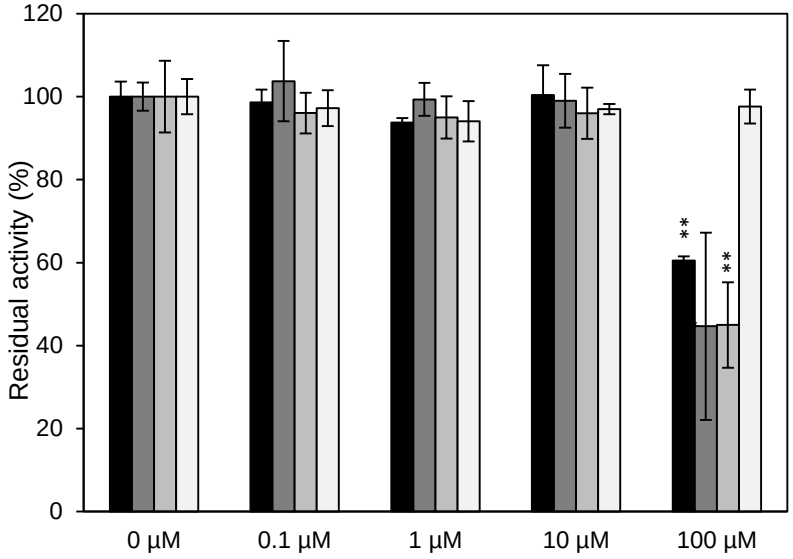


Fig. 5: Inhibitory effect of known inhibitors of human PLD on purified plant VuPLD.

The activity was measured in triplicates with increasing concentrations of halopemide (black), VU0359595 (dark grey), VU0285655 (light grey), FIPI (white). Relative activities are calculated relatively to the enzyme incubated with 0 µM of effector (100%). Values are the mean ± SD obtained from three independent fluorimetric assays. ** $P<0.01$, *** $P<0.001$ (*vs* untreated).

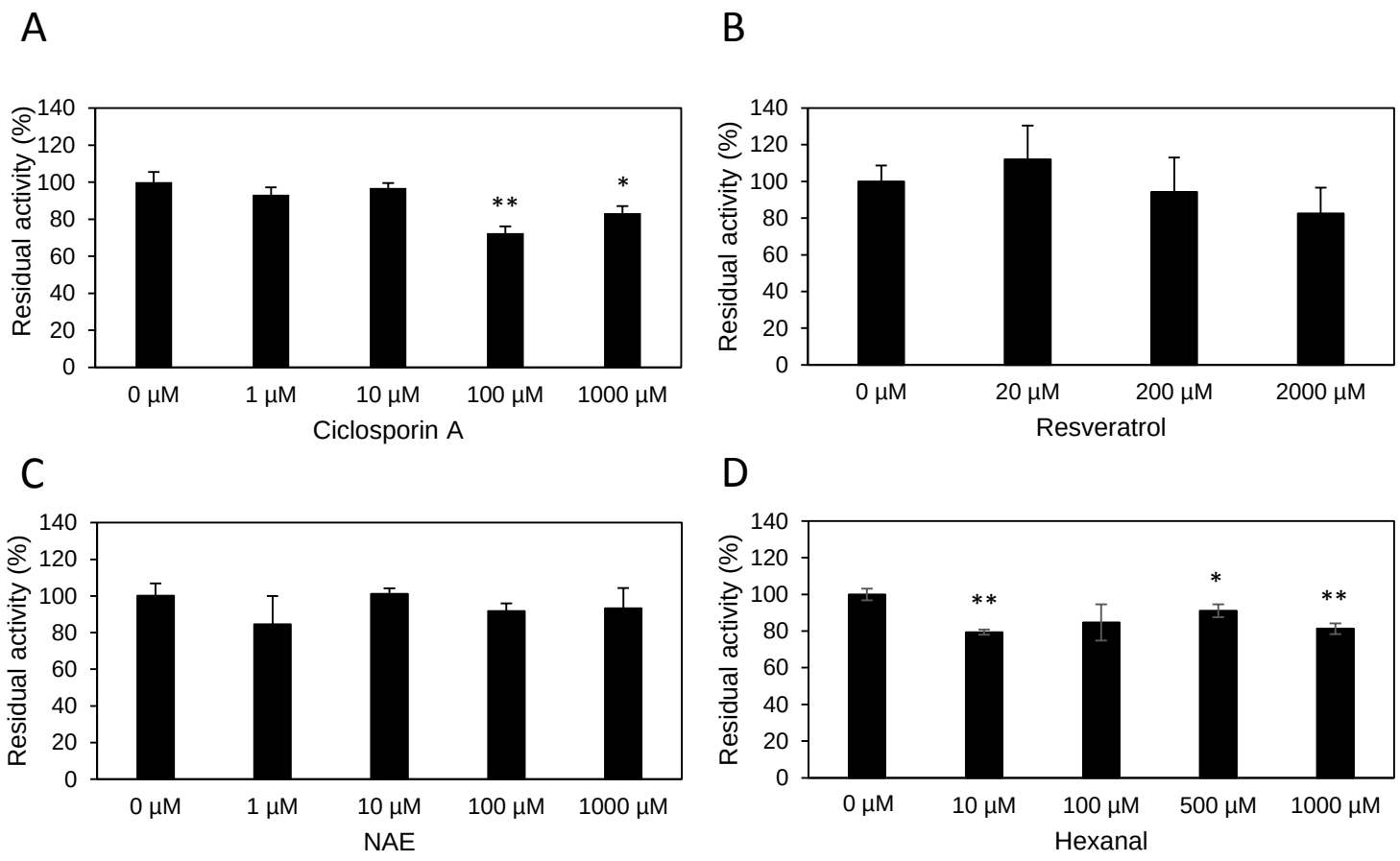


Fig. 6: Inhibitory effect of alleged inhibitors of plant PLD.
PLD described inhibitors, ciclosporin A (A), resveratrol (B), NAE C12:0 (C) and hexanal (D) were tested at different concentrations from 1 µM to 1 mM. Relative activities are then calculated relatively to the enzyme incubated in the absence of effector (100%). Values are the mean ± SD obtained from three independent fluorimetric assays. *P<0.05, **P<0.01 (*vs* untreated).

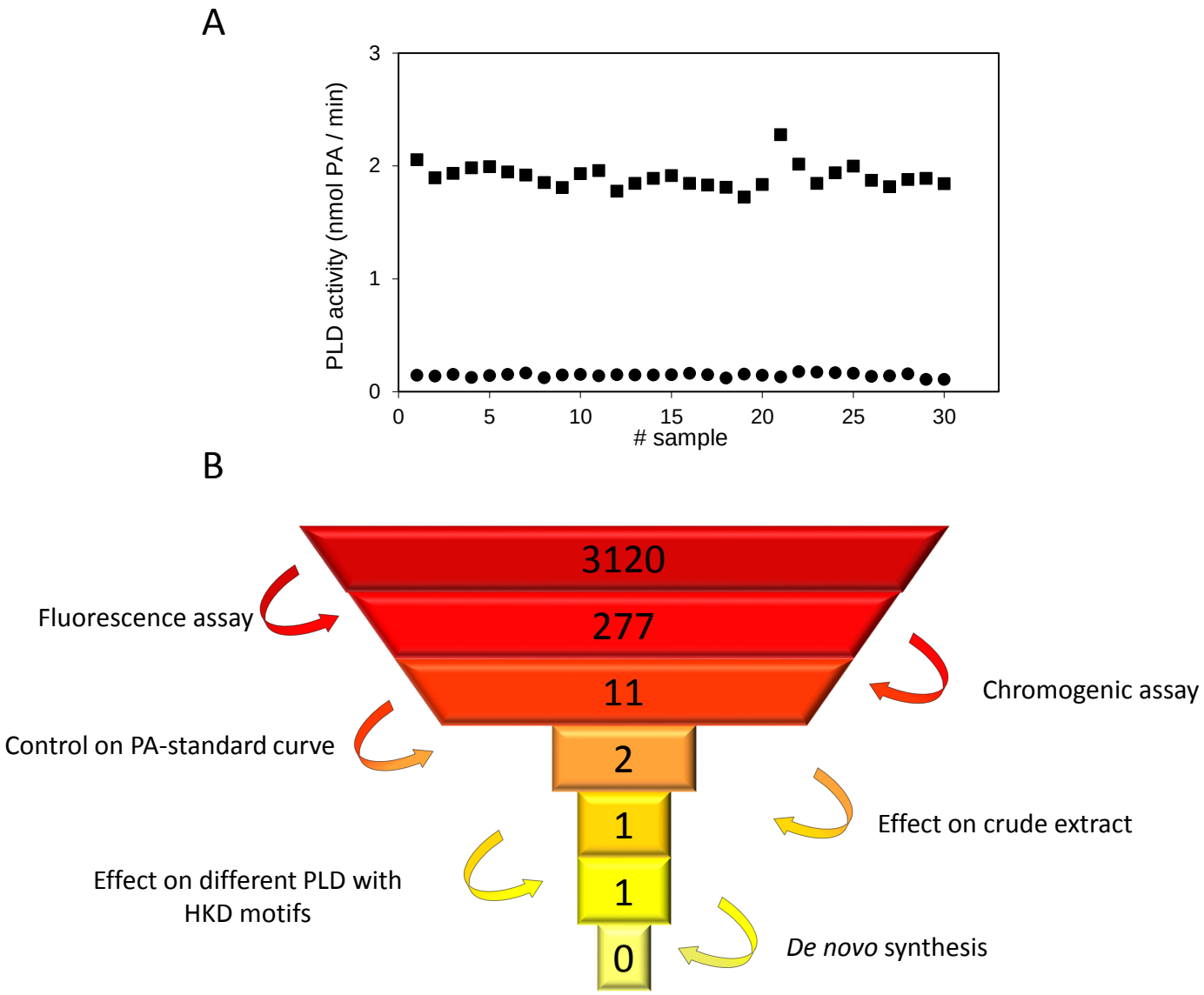


Fig. 7: HTS screening of a new PLD inhibitor.
PLD activity was measured in the presence or absence of 100 µM of vanadate in order to establish the variation of the assay and the calculation of the Z' score. Multiple repeats (n=30) were realized with (circles) or without effector (squares). B) Seven-step workflow for the identification of novel PLD inhibitors.

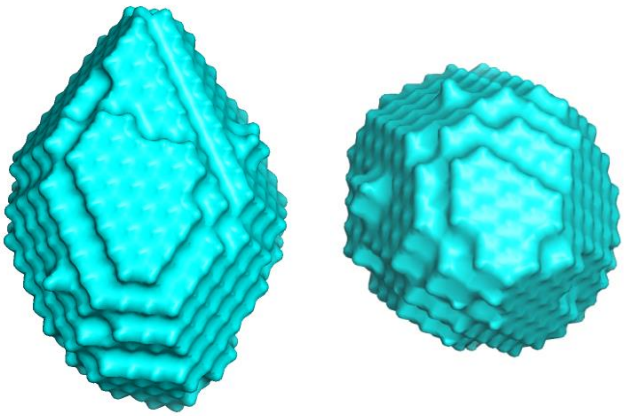
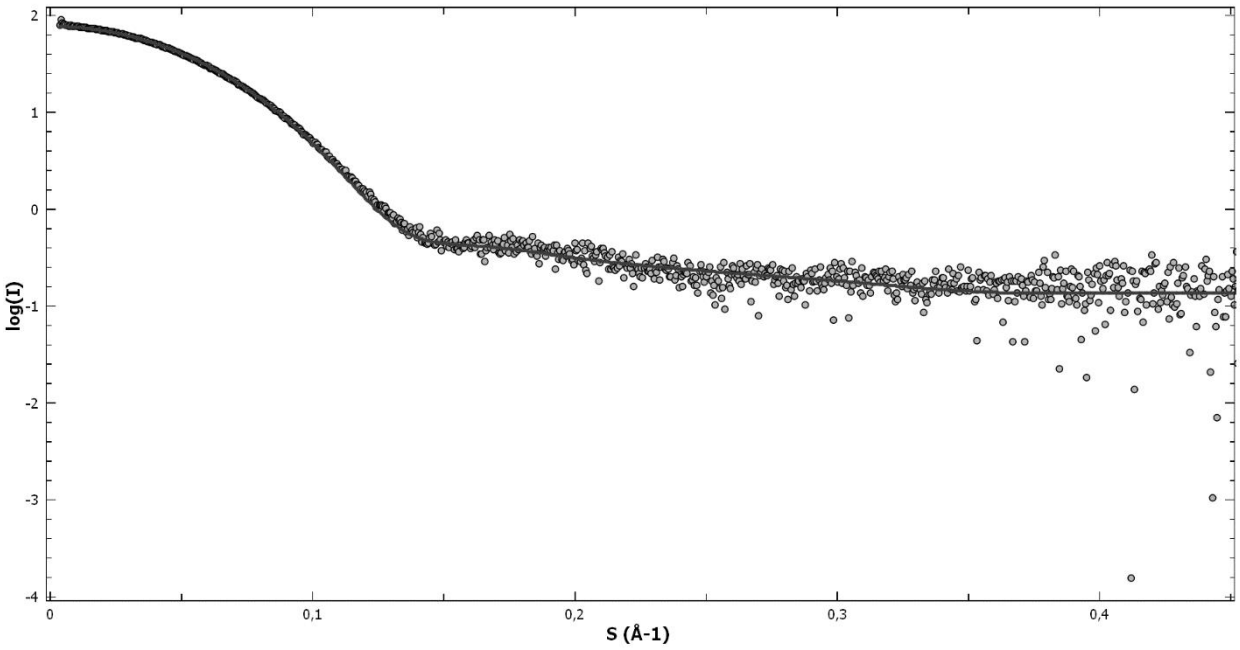


Fig. S1: SAXS modelization of VuPLD.
Ab initio low-resolution structure model of VuPLD calculated from the SAXS pattern and the program GASBOR. Model is shown in two different views obtained by 90° rotation around the y-axis

A



B

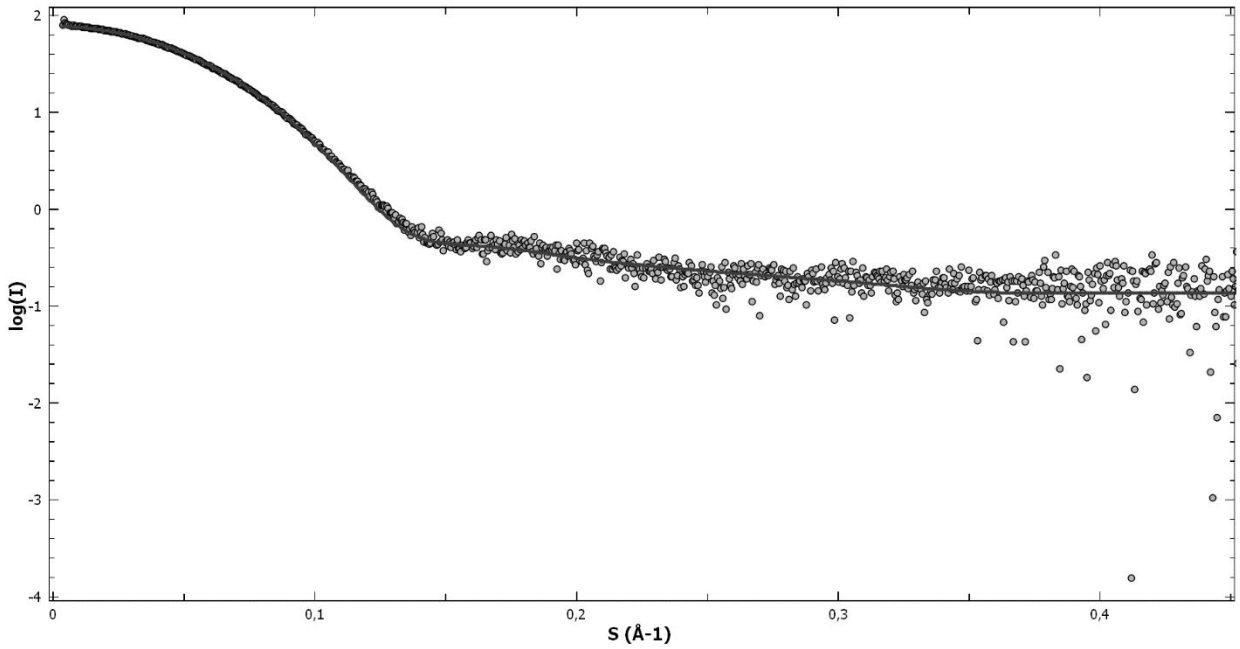


Fig. S2: Analysis of SAXS data of VuPLD.
A) Comparison of the experimental scattering data (gray circles) with the calculated scattering profile from PLD structure.
B) Experimental SAXS curve of VuPLD (grey dots) compared to the theoretical SAXS curve of PLDα1 (black line) from Arabidopsis (PDB code 6KZ9).

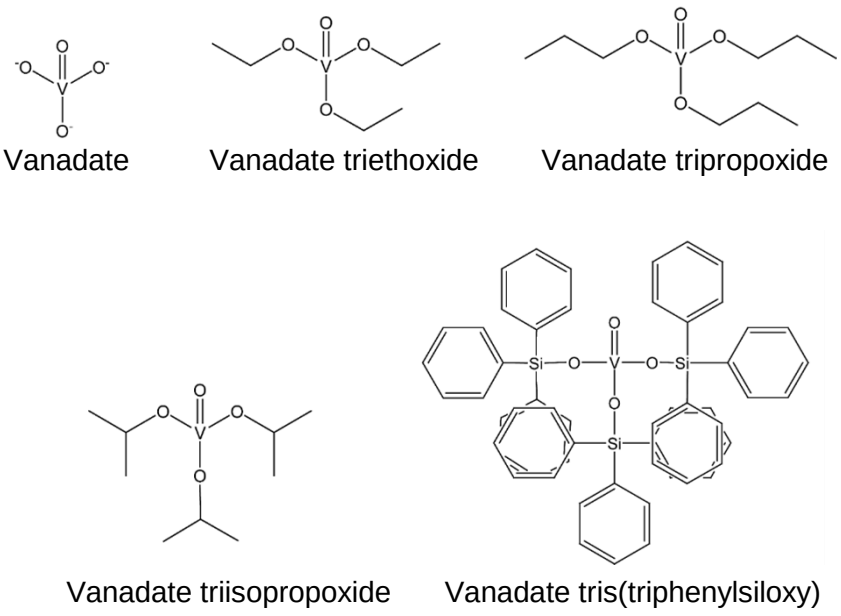


Fig. S3: Chemical structures of vanadate and its derivatives

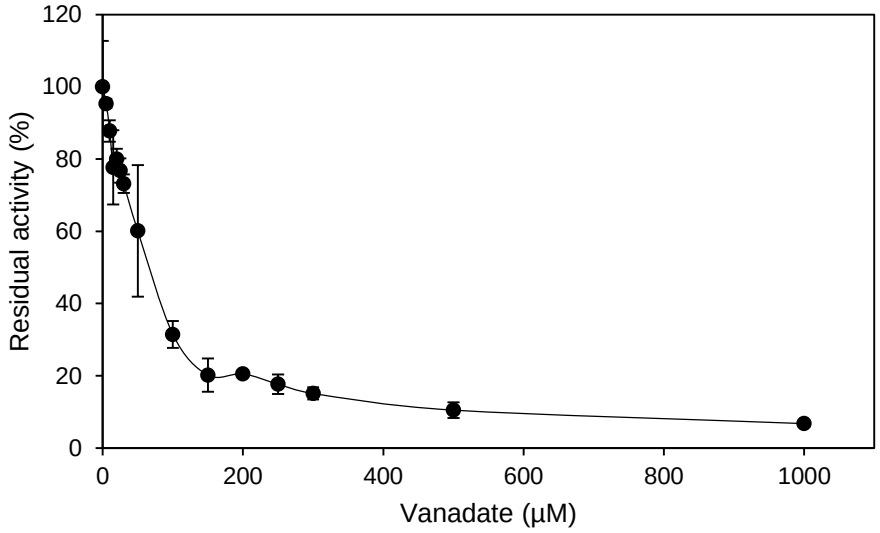


Fig. S4: Inhibition of purified plant PLD by vanadate.
The activity was measured in triplicates with increasing concentrations of vanadate. Values are the mean ± SD obtained from three independent chromogenic assays.

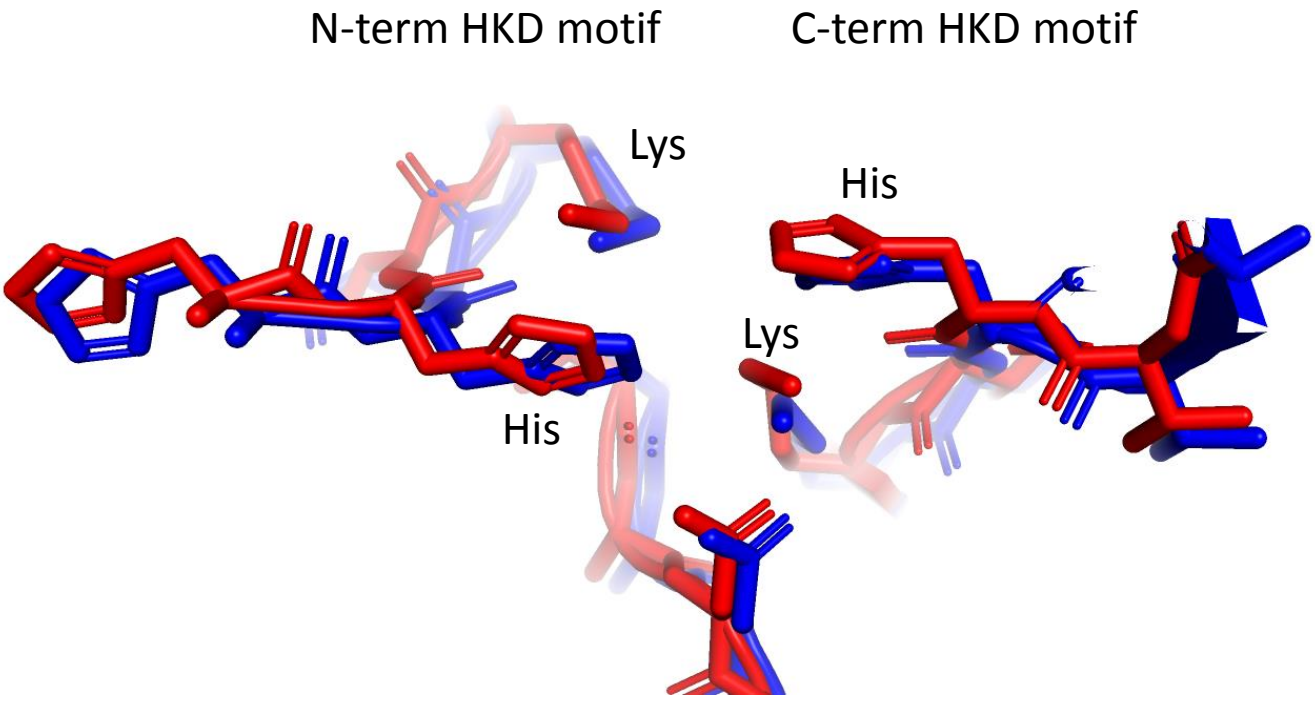


Fig. S5: Comparison of plant and mammalian PLD catalytic pockets.
Both HKD motifs are displayed in blue (human PLD, 6OHR) and red (plant PLD, 6KZ9) according to [9]. Histidyl and lysyl catalytic residues are represented (sticks) and annotated. Structure superposition was done using Pymol 2.3.2..

Author Statement

YA and MVC performed SAXS and modeling experiments. AC and FP contributed to compound design and synthesis. YA, KB, HA, MA, RR performed biochemical assays. LB, SM, AA and AN devised the project and the main conceptual ideas. AN was in charge of funding acquisition. All authors contributed to data interpretation. The manuscript was written from contributions by all the authors. All the authors have given approval to the final version of the manuscript.