PLA_2-mediated catalytic activation of its inhibitor 25-acetyl-petrosaspongiolide M: serendipitous identification of a new PLA_2 suicide inhibitor

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Abstract 25-Acetyl-petrosaspongiolide M (PMAc) (1), a mild non-covalent PLA_2 inhibitor, unexpectedly recovers, after incubation with *bv*PLA_2, the ability to covalently modify the enzyme target. This study demonstrates the catalytic effect of *bv*PLA_2 in converting 1 in its deacetylated congener petrosaspongiolide M (PM) (2), a strong covalent PLA_2 inhibitor whose molecular mechanism of inhibition has already been clarified. Moreover, our findings outline the potential role of PMAc as anti-inflammatory pro-drug, by virtue of its ability of delivering the active PM agent at the site of inflammation, functioning as a suicide inhibitor.

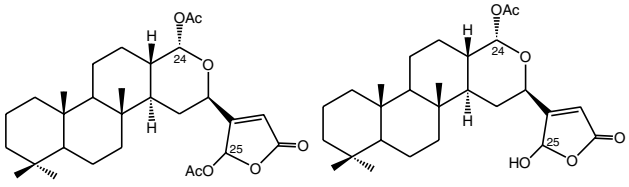
Keywords: Anti-inflammatory compound; Marine natural product; Mass spectrometry; Phospholipase A_2 inhibition

1. Introduction

Marine sesterterpenes containing a γ-hydroxybutenolide ring are well known to specifically inhibit PLA_2 enzymes [1–7]. Among them, petrosaspongiolides M-R, isolated in 1997 from the marine sponge *Petrosaspongia nigra* [8], have recently become the subject of extensive investigation in our laboratory, in the attempt to clarify their mechanism of inhibition and their interaction at the atomic level with *bv*PLA_2. The ultimate goal of our study is a deep comprehension of the principles underlying the mode of action of this family of anti-inflammatory natural agents [9,10]. In fact, armed with such knowledge, we aim at a rational design of simplified inhibitors of PLA_2 as potential new leads for the treatment of inflammation-related diseases [11–19]. In this context, *bv*PLA_2 represents a very useful model for such studies, as the pharmacological characterization of PM and its derivatives has shown an excellent correlation between inhibitory profiles obtained on this model (group III) enzyme with respect to (group IIa) human secretory counterpart (*hs*PLA_2) [8,20].

Petrosaspongiolide M (PM) (**2**), the most active among petrosaspongiolides [8,20,21], is able to covalently and specifically modify *bv*PLA_2 by means of a Schiff base formation between its C-25 masked aldehyde moiety (a cyclic hemiacetal function) and the amino group of the N-terminal Ile-1 residue of the enzyme [9]. 3D models of the complexes between *bv*PLA_2 and petrosaspongiolides M and R, respectively, were obtained by means of molecular modeling studies, leading us to a better understanding of the enzyme inactivation process at a molecular level. Our findings strongly suggest that the covalent interaction responsible for the enzymatic inhibition is mediated by a non-covalent molecular recognition event [10].

In the attempt to shed more light on the importance of such non-covalent interactions in the inactivation of *bv*PLA_2 by petrosaspongiolides, we performed a comparative study on the *bv*PLA_2 inhibition profiles of PM (**2**) and 25-acetyl-PM (PMAc) (**1**), a semi-synthetic derivative of PM, whose reactive site at C-25 was blocked by an acetyl group.



PMAc (**1**)
MW=502 Da

PM (**2**)
MW=460 Da

2. Materials and methods

2.1. Materials

All employed solvents and reagents were HPLC grade and were purchased from Baker and Fluka, respectively. *bv*PLA_2, oleic acid and DOPG were purchased from Sigma–Aldrich and the ADIFAB kit was purchased from ICN Biomedicals.

2.2. Phospholipase A_2 kinetic assay

The application of a fluorescence displacement assay to PLA_2 kinetics has been described previously [22–25]. Our experimental procedure was carried out using the fluorescent probe ADIFAB (ICN pharmaceuticals) consisting in an acrylodan derivative of rat intestinal fatty acid binding protein that exhibits a change in the fluorescence (Δ Ratio 490/440 nm)

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Abbreviations: PLA_2, Phospholipase A_2; *bv*PLA_2, bee venom PLA_2 (PDB code: 1POC); DOPG, dioleoylphosphatidylglycerol; SUV's, small unilamellar vesicles; RP-HPLC, Reverse Phase-High Performance Liquid Cromatography; ESI-MS, electrospray mass spectrometry; TFA, trifluoroacetic acid; CD, circular dichroism

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upon binding long-chain native fatty acids [23]. Measurements of the 490/440 intensity ratio (*R* values) were done on a Perkin Elmer LS55 Luminescence Spectrometer with excitation at 386 nm, excitation slit at 4 nm and emission slit at 8 nm. Calibration of fluorescence displacement was performed using oleic acid as standard. All assays were carried out at 37 °C and each measurement was repeated three times and values represented the means ± standard deviation (S.D.).

In order to obtain phospholipid substrate at a concentration of 60 μM in the form of SUV's, a stock solution of DOPG (10 mg/ml in methanol) was diluted in the assay buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $NaHPO_4$, 1 mM Glucose, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ at pH 7.4) by rapid injection [24].

Aliquots of the assay buffer containing the substrate at various concentrations, from 0.02 to 1 μM, and 20 nM ADIFAB kit were placed in the cuvette and the *R* value of the solution was registered for 1 min.

Then, *bv*PLA_2 (final concentration 1 nM) was added and the enzyme activity was monitored for 3000 s with an integration time of 2.0 s.

2.3. Kinetic analysis of inhibition

Aliquots of *bv*PLA_2 (500 fmol) were incubated with different amounts of PMAc (molar ratio *bv*PLA_2:PMAc from 1:0.5 to 1:40) for 10 min at 37 °C. Then, the mixture was placed in the cuvette with the assay buffer containing the substrate (final concentration 1 μM) and the ADIFAB kit (final concentration 20 nM) and the enzyme activity (final concentration 1 nM) was monitored.

Enzyme rates in the presence of inhibitors were then compared with those measured for enzyme only.

2.4. Circular dichroism spectroscopy

Circular dichroism measurements were performed using a Jasco J-810 spectrometer equipped with a cell holder thermostatically controlled by a circulating water bath. Measurements were recorded at 25 °C, with an 8 s time constant and 5 nm/min and averaged for eight acquisitions. The protein concentration was 15 μM and spectra were collected with rectangular quartz cells of 1 cm path length in the near-UV region (320–250 nm). Adduct formation between *bv*PLA_2 and PMAc was analyzed in a 10 mM sodium borate buffer, at pH 7.5, incubating the protein with 5:1 molar excess of ligand for 10 min at 37 °C. Spectra were recorded before and after the addition of the ligand and routinely corrected for the background signal and for dilution effects. Cotton effects are reported as molar ellipticity.

2.5. Acetylation of petrosaspongiolide M (**2**)

25-Acetyl-PM and 25-[D_3]acetyl-PM were prepared by using the procedure reported by Randazzo et al. [8] starting from PM and acetic anhydride or D_6-acetic anhydride, respectively.

2.6. *bv*PLA_2-inhibitor adducts analysis

PMAc and 25-[D_3]acetyl-petrosaspongiolide M (D_3-PMAc) were dissolved in isopropyl alcohol (1 mg/ml) and then, individually, added to a 100 μl of a solution of *bv*PLA_2 (15 μM in sodium borate 10 mM, $CaCl_2$ 10 mM at pH = 7.5) for 10 min at 37 °C with a 5:1 molar excess of inhibitor, in the presence of 20:1 molar excess of oleic acid. The final concentration of isopropyl alcohol in the reaction mixture was always kept lower than 4% v/v. It is noteworthy that in our hands the presence of oleic acid was deemed crucial for the efficacy of esterase enzyme catalysis.

When a reduction step was required, the sample was diluted with an equal volume of a solution of $NaBH_4$ in NaOH (15 mM) for 2 h at 0 °C (molar ratio $NaBH_4$:bvPLA_2 = 400:1) and the reaction was quenched adding 5 μl of 6 M HCl.

The mixture of unreacted and monomodified protein was analyzed by RP-HPLC on a Phenomenex C4 narrow-bore column by means of a linear gradient from 25% to 95% aqueous acetonitrile containing 0.05% TFA, over 45 min at 0.2 ml/min. The elution profile was monitored at 220 and 280 nm. The fractions were collected and analyzed by an electrospray ion source at 220 °C at a flow rate of 5 μl/min on a Finnigan LCQ Deca ion trap mass spectrometer (ThermoQuest, San José, California). Data were analyzed using the suite of programs Xcalibur (ThermoQuest, San José, California), while the Magtran software (Zhang and Marshall Zscore Algorithm) was used for data processing; all masses were reported as average values.

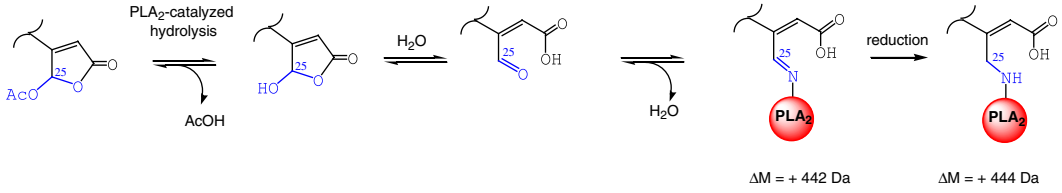
PM-oxyme (**3**) was observed upon treatment of the *bv*PLA_2-PMAc mixture with NH_2OH (with a molar excess of NH_2OH:protein of 1000:1) for 2 h at 37 °C and an LC-MS analysis was performed for the isolation and MS characterization of the various species. The mixture was analyzed by RP-HPLC on a Phenomenex C18 narrow-bore column by means of a linear gradient from 25% to 55% in 20 min and then from 55% to 95% in 50 min at a flow rate of 0.2 ml/min eluting with aqueous acetonitrile containing 0.05% TFA.

The covalent adducts between *bv*PLA_2 and PMAc and D_3-PMAc, respectively, were purified by HPLC and subjected to disulfide bond reduction by DTT (5 mM) for 2 h at 37 °C under nitrogen atmosphere. Free thiol groups were then blocked by treatment with iodoacetamide (50 mM) for 30 min at room temperature in the dark and the reaction was quenched by HPLC injection. The alkylated species were digested with endoproteinase Lys C in ammonium bicarbonate (50 mM, pH 8) at 37 °C for 4 h with a 1:50 (w/w) LysC/adduct ratio. Proteolytic fragments were analyzed by LC-MS performed on a Finnigan LCQ Deca ion trap mass spectrometer equipped with P4000 Spectra System quaternary pumps. Chromatographic separation was carried out on a Phenomenex C18 column by means of a linear gradient from 15% to 55% acetonitrile in 2% formic acid and 0.1% TFA. Mass spectra were acquired in an *m*/*z* interval of 600–1800.

3. Results and discussion

Initially, the investigation on the biological properties of PMAc was undertaken to gain insight on the nature and relevance of non-covalent interactions in the PLA_2 inhibition by PM. Indeed, PMAc, with both its reactive centers (C-24 and C-25 hemicetal functionalities) blocked as acetates, seemed to be a logical choice for highlighting the non-covalent forces shaping the PM-PLA_2 recognition process. With this background, a selective and quite efficient hydrolytic cleavage of the 25-acetate was rather unexpected, also in consideration that, to our knowledge, there are no documented examples of PLA_2 activity as generic esterase (Scheme 1).

Our study took advantage of the combined results of three different spectroscopic techniques: (a) comparative analysis of the enzyme kinetics of inhibition by means of a fluorescence displacement assay; (b) CD analysis to monitor protein tertiary structure changes associated to the inhibition process; and (c) characterization of the PMAc-*bv*PLA_2 covalent adduct by LC-ESIMS (Scheme 1).



Scheme 1. The postulated mechanism for PMAc-*bv*PLA_2 inactivation along with the expected mass increments.

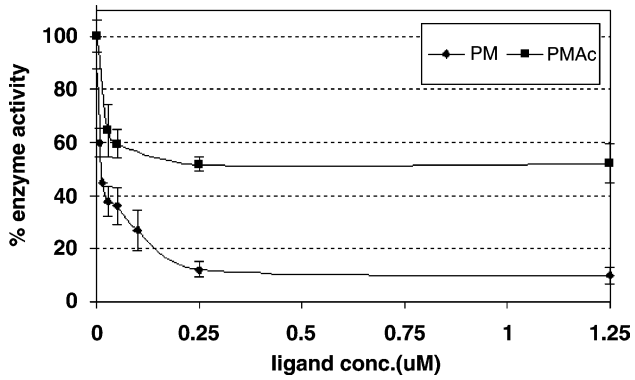


Fig. 1. *bv*PLA_2 inactivation kinetics caused by **1** and **2**. Activities were determined using the ADIFAB fluorescence displacement assay and are shown as relative percentages to enzymatic activity measured in the absence of any inhibitor (set to 100%). Data refer to means ± S.D. ($n = 3$). See Sections 3.1 and 2 for further details.

3.1. Kinetics of *bv*PLA_2 inhibition

In the aim to monitor the inhibition of *bv*PLA_2 activity by petrosaspongiolides, it was first necessary to investigate kinetic properties of *bv*PLA_2 in our experimental conditions.

Experiments were carried out using the fluorescent probe ADIFAB (see also Section 2) and anionic vesicles of DOPG as a substrate due to their high affinity to *bv*PLA_2 through an interfacial binding [25]. To set up the right conditions for subsequent assays of inhibition, the increase of enzyme activity in the presence of various concentrations of DOPG was monitored (data not shown) to allow us to optimize the concentration of DOPG for the inhibition assay.

Experiments were carried out by incubating *bv*PLA_2 with the inhibitors for 10 min and then adding the mixture to the stock assay buffer containing the substrate SUV's and the fluorescent probe ADIFAB. Enzyme rates in the presence and in the absence of the two inhibitors under investigation were then compared. In these conditions, as shown in the plots of Fig. 1, PMAc gives rise only to a moderate PLA_2 inhibition (if compared to PM), due to its yet incomplete hydrolysis to PM. Nonetheless, it is noteworthy that even after such a short incubation time, PMAc shows already an inhibitory activity of 50% at 0.25 μM (vs. a 90% inhibition by intact PM at the same concentration). In other words, the inhibitory potency of PMAc, being directly correlated to its ability to revert to PM in physiological conditions, varies with the time going, a feature typically shown by pro-drug molecules. Our results indicate that PMAc produces a steadily increasing concentration of PM, the real (covalent) PLA_2 inhibitor. After 60 min, PMAc approaches the same inhibition power of PM (see also Fig. 3).

3.2. Circular dichroism analysis

In a second experiment, the incubation solution mixture of *bv*PLA_2 with PMAc (37 °C, pH = 7, 10 min) was employed for CD measurements [26,27]. The contribution of aromatic side chains to the near-UV CD spectra of proteins is widely recognized and utilized as a sensitive probe of protein conformation and ligand binding [28–33]. We then used the near-UV CD spectra of *bv*PLA_2 for monitoring the changes in the tertiary structure occurring to the enzyme upon binding of the inhibitor. In the region between 250 and 320 nm, the CD signal arises from the aromatic side chains, namely, two Trp, eight Tyr and five Phe residues of *bv*PLA_2. The changes observed in such region of the CD curves relative to the free enzyme and the incubation mixture with **1** (Fig. 2), respectively, clearly indicated that a perturbation of the environment of the aromatic side chains of *bv*PLA_2 enzyme is induced by the inhibitor.

3.3. Characterization of *bv*PLA_2-PMAc covalent adduct

In the attempt to ascertain whether such tertiary structure perturbation could be addressed to purely non-covalent interactions between *bv*PLA_2 and PMAc or, on the contrary, to a chemical modification of the enzyme, the incubation mixture recovered after the CD measurement was submitted to overnight dialysis against 10 mM sodium borate at pH 7.5 and then analyzed again by CD and LC-MS. Unexpectedly, the CD response resulted in a curve superimposable to the one detected before dialysis. Moreover, the LC-MS analysis of the mixture led to the recovery of the free enzyme along with a large amount of a modified enzymatic species, featuring a MW increase of 442 Da. Moreover, this species resulted unstable upon acidification (pH = 3) of the medium, disappearing from the TIC trace of the LC-MS run, in agreement with the presence of a Schiff base functionality reverting back to its carbonyl and amine components in acidic media.

A full characterization of the chemical behavior of PMAc towards PLA_2 was achieved by performing a HPLC-MS analysis of aliquots, collected at different times, of the *bv*PLA_2-PMAc incubation mixture. The results provided evidence of a time-dependent formation of the covalently modified *bv*PLA_2 species (Fig. 3). A parallel experiment was performed in which the incubation mixture was treated with $NaBH_4$

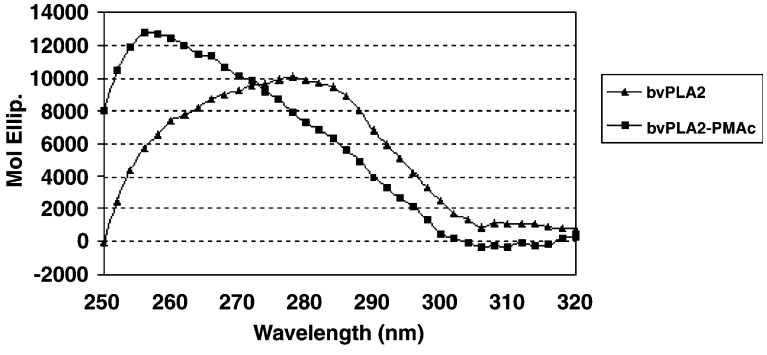


Fig. 2. CD curves of free *bv*PLA_2 and incubation mixture of *bv*PLA_2 with **1**.

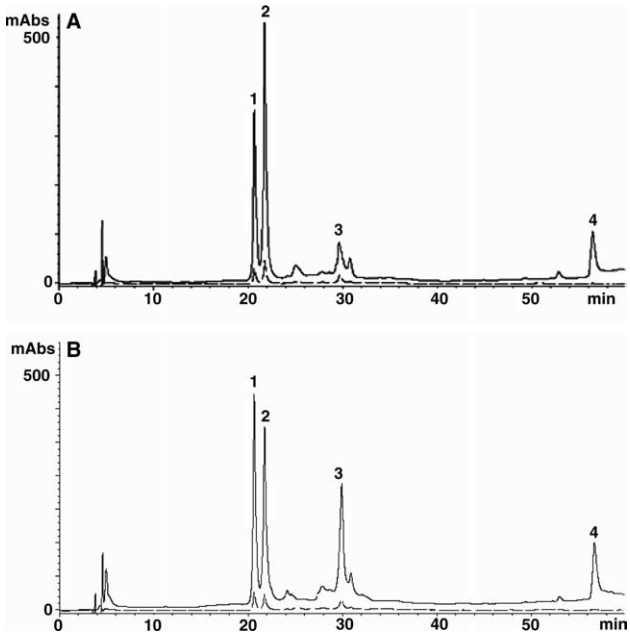
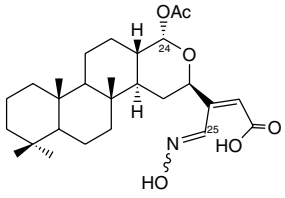


Fig. 3. Comparison between RP-HPLC traces of *bv*PLA$_2$-PMAc mixture (molar ratio 1–5) after 10 min (A) and 60 min (B) of incubation. Fractions 2 and 1 correspond to the *bv*PLA$_2$ with one (M26 or M34) and two oxy-Met residues (M26/M34 and M86), respectively; fraction 3 represents the *bv*PLA$_2$-PM adduct and fraction 4 corresponds to the unreacted excess of PMAc. It should be noted that the protein species with two oxy-Met residues is not affected by PM. M86, the second oxy-Met, lies in the proximity of the active site, protruding its side chain towards the pocket where PM binding takes place; we consider therefore likely that the extra oxygen atom may interfere with optimal positioning of PM for covalent binding (see also [9])

before LC-MS analysis. In this case, a reduced species, stable under acidification (as expected for an amine vs. an imine) and featuring a mass increment of 444 Da with respect to the free *bv*PLA$_2$, was detected.

An additional evidence for the *bv*PLA$_2$-catalyzed conversion of PMAc to PM, and its subsequent covalent reaction with the enzyme, came from isolation of PM-oxyme by LC-MS upon treatment of the PMAc-*bv*PLA$_2$ incubation mixture with excess hydroxylamine (NH_2OH) for 2 h at 37 °C. Fig. 4 displays the LC-MS run relative to the latter experiment, showing the disappearance of the covalent *bv*PLA$_2$-PM complex and the formation of the peak relative to PM-oxyme. It should be noted that PMAc itself is not reactive towards hydroxylamine, as expected for an ester. On the other hand, PM, both in its free and *bv*PLA$_2$-bound forms, should be trapped by the reaction with hydroxylamine, as previously found for other aldehyde-based anti-inflammatory compounds [34].



PM-oxyme (**3**)
MW=475.6 Da

Taken together, these evidences allowed us to confirm the plausible hypothesis that *bv*PLA$_2$, after an initial non-covalent interaction with PMAc, may work as a generic lipid esterase reactivating the C-25 aldehyde functionality. This PLA$_2$-cata-

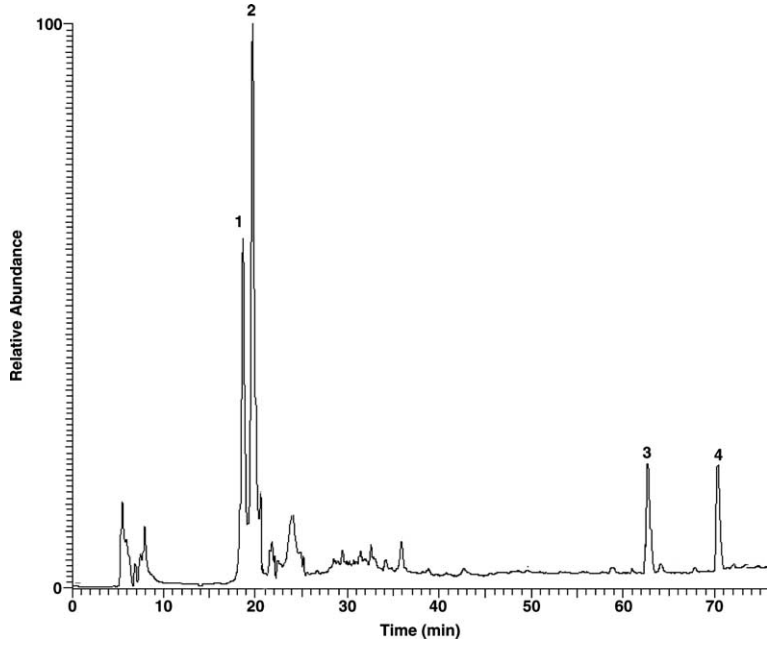
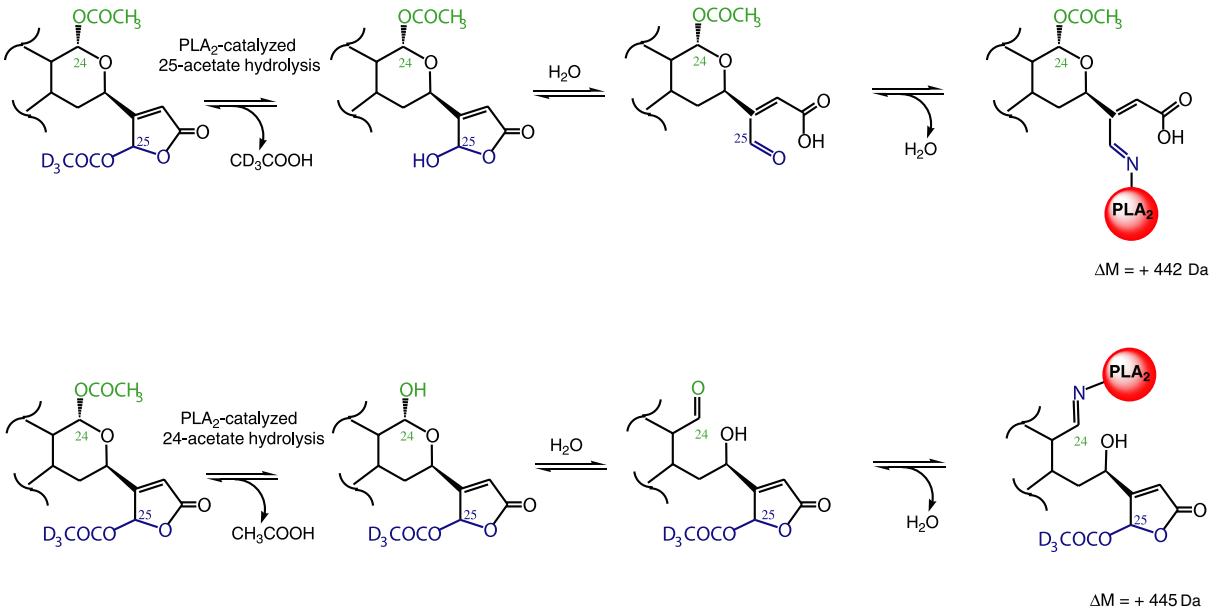


Fig. 4. LC-MS trace of the *bv*PLA$_2$-PMAc incubation mixture after hydroxylamine (NH_2OH) treatment. Fractions 2 and 1 correspond to the *bv*PLA$_2$ with one (M26 or M34) and two oxy-Met residues (M26/M34 and M86), respectively; fraction 3 corresponds to PM-oxyme (**3**) and fraction 4 contains unreacted excess of PMAc. Identification of all species has been performed by ESI-MS data.



Scheme 2. Plausible mechanisms of *bv*PLA$_2$ inactivation by D$_3$-PMAc involving PLA$_2$-mediated hydrolysis of 25-acetate and 24-acetate, respectively, along with the expected mass increments.

lyzed hydrolysis of the C-25 acetate would generate the reactive PM with the consequent covalent inhibition of the enzyme, as already shown in our previous work [9]. This suicide mechanism postulated on the basis of these considerations (Scheme 1) gives evidence of the formation of an imine intermediate, as confirmed by the mass increment of 444 Da observed for the modified PLA$_2$ species (after reduction) [9,10].

A control experiment, designed to probe PMAc selectivity towards *bv*PLA$_2$, was performed by adding to the usual incubation mixture (*bv*PLA$_2$ + PMAc) an excess of a second protein featuring a number of exposed lysine residues in its sequence. The purpose of the experiment was to monitor whether in these forcing conditions, PMAc (and hence PM) was still able to display a clear binding preference towards PLA$_2$, by virtue of a specific PM-PLA$_2$ recognition process. Alternatively, PM may be trapped by an aspecific reaction of its aldehyde function with an exposed Lys-ε-NH$_2$ group. The results (data not shown) showed that no PM-lysozyme adduct could be detected by HPLC-DAD up to 5:1 lysozyme-*bv*PLA$_2$ molar excess, confirming the strong and specific affinity of PM towards *bv*PLA$_2$.

The only issue that remained to be sorted out was whether or not the PLA$_2$ mediated de-acetylation process was carried out specifically at C-25. As a matter of fact, at least in principle, the hydrolytic process may also involve the 24-acetate, since also in this case another cyclic hemiacetal functionality (again an aldehyde in disguise) would be generated, therefore exposing another potential site for nucleophilic attack of an amino group. It has to be pointed out, however, that all the evidences gathered so far on the chemistry and biology of the whole family of petrosaspongiolides revealed that C-25 is much more prone to undergo attacks from nitrogen nucleophiles [8,10], possibly as a consequence of reduced steric hindrance at this location. On the other hand, it should be noted that an MS approach does not allow to distinguish between hydrolytic cleavages at C-24 and C-25. For a conclusive, direct proof that this was indeed the case also for PMAc, a suitable labeling of the 25-acetate group, for instance a deuterated sample, was therefore necessary for an MS discrimination among the two possible reaction pathways (Scheme 2). To this end, 25-[D$_3$]acetyl-PM (D$_3$-PMAc) was first prepared and then incubated with *bv*PLA$_2$. Application of our usual LC-MS characterization protocol yielded, upon PLA$_2$ covalent modification, a 442 Da mass increment, as typically observed for PMAc (or even intact PM). No sign at all of MS peaks with mass increments of 445 Da, as expected for molecular species undergoing 24-acetate hydrolysis, was detected. Such observation enabled us to conclude that the deuterium-containing 25-acetate was entirely lost upon exposure of the D$_3$-PMAc to *bv*PLA$_2$.

In conclusion, the serendipitous discovery of the manifestly different chemical behavior of the 25-acetate, with respect to the chemically similar 24-acetate of the same molecule, appeared to us as a relevant finding. First, the found PLA$_2$ selectivity in acting as a generic esterase raises intriguing speculations on its origin. If PLA$_2$ ability to catalyze the hydrolysis of an acetate is certainly not a remarkable finding, the observed discrimination among two chemical functionalities of the same kind and located in the same region of the molecule may be the consequence of specific three-dimensional positioning of the inhibitor within the binding pocket.

Moreover, this work may pave the way to the design of a family of new PLA$_2$ suicide inhibitors, which may be useful *leads* for the development of pro-drugs to treat inflammation-related diseases. A complication of this design, however, may be the potential metabolic instability of PMAc in vivo. If this compound happens to be also a substrate of other esterases contained in plasmatic fluids, this could prevent intact PMAc to reach the site of inflammation. On the other hand, the modulation of specific biological and pharmacological properties of PM, such as bioavailability, half-life in vivo, tissue selectivity, etc., may be achieved by a wise selection of the chemical nature of the C-25 ester. The preparation of library of different C-25 esters may be very instrumental to this particular goal and is underway in our laboratory.

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