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Synthesis of *sn*-1 functionalized phospholipids as substrates for secretory phospholipase A_2

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Abstract

Secretory phospholipase A_2 ($sPLA_2$) represents a family of small water-soluble enzymes that catalyze the hydrolysis of phospholipids in the *sn*-2 position liberating free fatty acids and lysophospholipids. Herein we report the synthesis of two new phospholipids (**1** and **2**) with bulky allyl-substituents attached to the *sn*-1 position of the glycerol backbone. The synthesis of phospholipids **1** and **2** is based upon the construction of a key aldehyde intermediate **3** which locks the stereochemistry in the *sn*-2 position of the final phospholipids. The aldehyde functionality serves as the site for insertion of the allyl-substituents by a zinc mediated allylation. Small unilamellar liposomes composed of phospholipids **1** and **2** were subjected to $sPLA_2$ activity measurements. Our results show that only phospholipid **1** is hydrolyzed by the enzyme. Molecular dynamics simulations revealed that the lack of hydrolysis of phospholipid **2** is due to steric hindrance caused by the bulky side chain of the substrate allowing only limited access of water molecules to the active site.

Keywords: $sPLA_2$ activity; Biomarkers; Molecular dynamics simulations; Phospholipid; Substrate specifity; Synthesis

1. Introduction

Secretory phospholipase A_2 ($sPLA_2$) constitutes a family of interfacially active mammalian enzymes. To date, 10 members (IB, IIA, IIC, IID, IIE, IIF, III, V, X and XIIA) of this family have been reported (Murakami and Kudo, 2004). Even though this group of enzymes has been studied extensively, the substrate specificity with relation to the active site architecture of the enzyme still needs to be addressed and understood, since the enzyme seems to be able to catalyze the hydrolysis of lipids with very diverse chemical structures (Andresen and Jørgensen, 2005a; Bonsen et al., 1972a,b; Slotboom et al., 1976).

This family of enzymes catalyzes the hydrolysis of phospholipids in the *sn*-2 position generating free fatty acids and lysophospholipids (Six and Dennis, 2000). Moreover, the group of enzymes shares several characteristics as they have a low molecular weight ranging from 14 to 19 kDa, require Ca^{2+} for enzymatic activa-

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tion and are secreted from cells (Laye and Gill, 2003). $sPLA_2$ catalyzes the hydrolysis of phospholipids through a highly conserved catalytic site, where His48, Asp99 and Ca^{2+} are of particular importance (Dennis, 1994). Hydrolysis proceeds through activation and specific orientation of a water molecule in the catalytic site (Scott et al., 1990). The hydrolysis is achieved by hydrogen bonding of the water molecule to His48 assisted by Asp99 where Ca^{2+} stabilizes the intermediate of the hydrolysis through coordination to the *sn*-2 carbonyl of the phospholipid substrate (Dennis, 1994; Scott et al., 1990).

An interesting aspect present in the $sPLA_2$ family of enzymes is the fact that the different subtypes of enzymes show different specificity towards lipid substrates. This is reflected in the activity profile of, e.g., the $sPLA_2$-IIA subtype, which is mainly active towards anionic phospholipids, whereas the $sPLA_2$ V and X subtypes catalyze the hydrolysis of both anionic and zwitterionic phospholipids (Murakami and Kudo, 2004). Substrate specificity has been proposed to be dependent on the ability of the enzymes to bind to the lipid-membrane interface, which is achieved by the electrostatic interactions between the enzyme and the surface of the lipid substrate (Leidy et al., 2006).

$sPLA_2$-IIA has proven to be especially interesting in therapeutic intervention, since elevated levels of $sPLA_2$-IIA is present in the microenvironment surrounding tumors, e.g., high levels of $sPLA_2$-IIA have been observed in human colorectal adenomas and in neoplastic prostatic tissue (Laye and Gill, 2003). Moreover, $sPLA_2$ plays a role in the release of arachidonic acid that is metabolized downstream by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to give, e.g., prostaglandins and leukotrienes (Andresen et al., 2005b). The elevated levels of $sPLA_2$ in tumor tissue have provided the basis of a new tumor activated drug delivery concept, which relies on the administration of $sPLA_2$ degradable liposomes (Andresen et al., 2005c; Andresen et al., 2004a). This leads to a site-specific release of the therapeutics at the tumor target site. The liposomes can entrap and transport conventional chemotherapeutics such as cisplatin and doxorubicin and release the therapeutics by using $sPLA_2$ as a tumor-specific trigger (Andresen et al., 2005d).

In this paper, we investigate the catalytic site of $sPLA_2$ by the synthesis of two new phospholipids **1** and **2** (Fig. 1), which contain allyl-substituents in the *sn*-1 position. Testing these phospholipids towards hydrolysis catalyzed by $sPLA_2$ can provide valuable insight into the structural determinants that govern substrate specificity, and reveal whether $sPLA_2$ can tolerate a bulky substituent close to the glycerol backbone. Additionally, the two new phospholipids can function as biomarker precursors, since the allyl group can serve as a functional handle for further chemical manipulation by insertion of, e.g., fluorescent molecular probes such as naphthalene. Biomarkers positioned in close proximity to the glycerol backbone are important with respect to obtaining information on the microstructure and interfacial properties of lipid membranes (Bagatolli, 2003; Bagatolli and Gratton, 2001). Most of the biomarkers known today report changes in membranes occurring along the fatty acid chains or at the end of the phosphate headgroup. It can therefore be difficult to obtain information at the membrane–water interface of phospholipid bilayers. However, incorporating a fluorescent probe in the *sn*-1 position of the glycerol backbone offers unique opportunities to investigate changes in the vicinity of the glycerol backbone of phospholipids.

The primary challenge in this work has been to design a suitable synthetic strategy that incorporates an allyl-substituent in the *sn*-1 position of the final phospholipid. As a consequence, the conventional ways of generating phospholipids (Bhatia and Hajdu, 1988; Martin et al., 1994; Paltauf and Hermetter, 1994; Roodsari et al., 1999) cannot be used, and a new and more complex synthetic route has therefore been investigated. We report the synthesis of phospholipids **1** and **2** by the use of a key aldehyde intermediate **3**, which locks the stereochemistry in the *sn*-2 position of the final phospholipid. The aldehyde functionality furthermore serves as the site for incorporating the allyl-substituents in the *sn*-1 position of the final phospholipids. This aldehyde intermediate is used for the first time in phospholipid synthesis, and as our results indicate, will be useful for future synthetic procedures towards derivatives of natural phospholipids, where new functional groups are introduced. $sPLA_2$ activity measurements in combination with molecular dynamics (MD) simulations of the two new phospho-

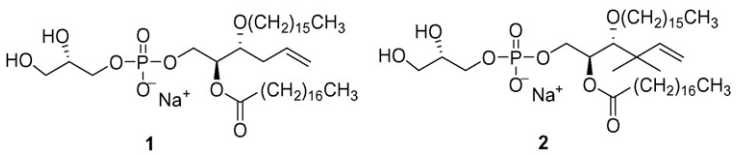


Fig. 1. Structure of the two target phospholipids.

lipids have provided useful insight on a molecular level that can explain the specificity of $sPLA_2$ towards the two phospholipids.

2. Experimental procedures

2.1. Organic synthesis

Dioxane, THF and toluene were distilled from sodium benzophenone, while CH_2Cl_2 was distilled from CaH_2. Thin layer chromatography was performed on aluminum plates coated with silica (Merck, Kieselgel 60 F_{254}). Visualization of the compounds were done by heating after dipping the plates in a solution of $Ce(SO_4)_2$ (2.5 g) and $(NH_4)_6Mo_7O_{24}$ (6.25 g) in 10% aqueous H_2SO_4 (250 mL). Flash chromatography was performed with silica gel 60. NMR spectra were recorded on a Varian Mercury 300 spectrometer with $(CH_3)_4Si$ ($\delta_H = 0.0$ ppm) as an internal reference for ^1H NMR and with $CDCl_3$ ($\delta_C = 77.0$ ppm) as an internal reference for ^{13}C NMR. Optical rotations were measured on a Perkin-Elmer 241 polarimeter and microanalyses were obtained at the Mikroanalytisches Laboratorium, Universität Wien.

2.1.1. (2S,5S,6S)-5,6-Dimethoxy-5,6-dimethyl-[1,4]dioxane-2-carbaldehyde (**3**)

Trimethyl orthoformate (62.0 mL, 567 mmol) and 2,3-butanedione (14.0 mL, 160 mmol) were added to a solution of L-ascorbic acid (25.0 g, 142 mmol) in dry CH_3OH (250 mL) under nitrogen after which the solution turned yellow. $BF_3{\cdot}OEt_2$ (1.80 mL, 14.2 mmol) was added, and the solution was stirred for 42 h at room temperature. The reaction mixture was neutralized to pH 7 with Et_3N and concentrated in vacuo to give a reddish foam. This was dissolved in CH_2Cl_2 (250 mL) and washed with brine (400 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 100 mL) and EtOAc (2 × 100 mL), and the combined organic extracts were dried over $MgSO_4$, filtered and concentrated in vacuo to afford a reddish foam (55.5 g). R_f 0.40 (10% CH_3OH/CH_2Cl_2). Crude **4** was dissolved in a solution of K_2CO_3 (39.4 g, 285 mmol) in H_2O (400 mL) and cooled to 0 °C. 35% (v/v) H_2O_2 (28.0 mL, 288 mmol) was added dropwise over 30 min to the reddish solution, and the reaction was stirred for 20 min at 0 °C. The solution was allowed to warm to room temperature and stirred overnight after which $NaHCO_3$ (53.6 g, 638 mmol) was added and the reaction was stirred for 1 h. $(CH_3)_2SO_4$ (60.0 mL, 630 mmol) was added dropwise over a period of 1 h, and the solution was heated to reflux. The mixture was refluxed for 4 h after which the yellow solution was cooled to room temperature and stirred overnight. The solution, which had turned white overnight, was filtered and the filtrate was extracted with CH_2Cl_2 (3 × 200 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated in vacuo to give white crystals (33.0 g). The mixture was used without further purification in the next step. R_f 0.77 (10% CH_3OH/CH_2Cl_2). Crude **5** was dissolved in isopropanol (350 mL) under nitrogen, and $NaBH_4$ (7.20 g, 190 mmol) was added in small portions over 6 min, which gave rise to a small increase in the temperature. The reaction was heated to 70 °C and stirred for 30 min. The solution was cooled to room temperature, and sat. aq. NH_4Cl (50 mL) was slowly added after which the solution was concentrated in vacuo to a volume of 40 mL. H_2O (200 mL) and EtOAc (100 mL) were added and the phases were separated. The aqueous phase was extracted with EtOAc (8 × 100 mL) and the combined organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuo to afford a colorless oil (32.0 g). The oil was used without further purification in the next step. R_f 0.08 (50% EtOAc/heptane). Sat. $NaHCO_3$ (50 mL) was added to a solution of crude **6** in CH_2Cl_2 (350 mL). $NaIO_4$ (40.1 g, 187 mmol) was added in small portions, and the reaction stirred overnight at room temperature. The mixture, which now contained white crystals, was dried over $MgSO_4$ and filtered. $Na_2S_2O_3$ (20.0 g, 130 mmol) was added to the filtrate and the mixture stirred at room temperature for 25 min. The mixture was filtered and concentrated in vacuo to give a yellow syrup, which was distilled at 80 °C, 3.5 mmHg using a Vigreux distillation apparatus furnishing 18.6 g (64%) of **3** as a colorless syrup. R_f 0.29 (50% EtOAc/heptane). $[\alpha]_D$ +102.8 (2.06 g/100 mL, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$): δ 9.59 (s, 1H), 4.30 (dd, J 5.6, 9.5 Hz, 1H), 3.70–3.61 (m, 2H), 3.26 (s, 3H), 3.20 (s, 3H), 1.32 (s, 3H), 1.24 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 200.3, 99.7, 98.3, 72.3, 58.3, 48.4, 48.2, 17.6, 17.5.

2.1.2. (2S,5S,6S)-2-(1′-(Hydroxy)but-3′-enyl)-5,6-dimethoxy-5,6-dimethyl-[1,4]dioxane (**7**)

Zinc (12.0 g) was activated by stirring with 1 M HCl (60 mL) for 15 min. The solution was filtered, and the filter cake was quickly washed with H_2O (2 × 25 mL) and Et_2O (2 × 20 mL), after which the zinc was transferred to a flame dried flask. The zinc was dried with a heatgun for 5 min under reduced pressure (2 mmHg) and allowed to cool under reduced pressure (2 mmHg) for at least 1 h. Aldehyde **3** (10.0 g, 49.1 mmol) was dissolved in distilled THF (100 mL) under nitrogen. The solution was cooled to 0 °C, and activated zinc (6.42 g, 98.2 mmol) and allyl bromide (8.50 mL, 98.2 mmol) were added. Sat. aq. NH_4Cl (50 mL) was added dropwise over 15 min and

the reaction was stirred for 10 min at 0 °C. The mixture was filtered and the filter cake rinsed with CH_2Cl_2 (100 mL). H_2O (100 mL) was added to the filtrate and the phases were separated. 0.5 M HCl (14 mL) was added to the aqueous phase, which turned the aqueous phase into a clear solution, and the aqueous phase was extracted with CH_2Cl_2 (2 × 100 mL). The combined organic extracts were washed with sat. $NaHCO_3$ (200 mL) and H_2O (200 mL), dried over $MgSO_4$, filtered and concentrated in vacuo to afford a colorless syrup. Purification by flash chromatography (25% EtOAc/CH_2Cl_2) gave 9.03 g (74%) of **7** as a 5:1 mixture of diastereomers (colorless oil). R_f 0.48 (50% EtOAc/heptane). Microanalysis calculated ($C_{12}H_{22}O_5$): C 58.52; H 9.00. Analysis result: C 57.87; H 8.96. Both isomers: ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.85–5.68 (m, 1H), 5.13–5.03 (m, 2H), 3.76–3.47 (m, 4H), 3.20 (s, 6H), 2.42–2.33 (m, 1H), 2.23–2.06 (m, 1H), 1.94 (br. s, OH), 1.23 (s, 3H), 1.22 (s, 3H). Major isomer: ^{13}C NMR ($CDCl_3$, 75 MHz): δ 133.9, 118.6, 99.2, 97.7, 70.7, 69.2, 60.0, 48.0, 37.2, 17.8, 17.5. Minor isomer: ^{13}C NMR ($CDCl_3$, 75 MHz): δ 133.9, 117.8, 99.2, 97.9, 70.1, 69.5, 60.3, 48.0, 37.4, 17.8, 17.5.

*2.1.3. (2S,5S,6S)-2-(1′-(Hydroxy)2′,2′-dimethyl-but-3′-enyl)-5,6-dimethoxy-5,6-dimethyl-[1,4]dioxane (**8**)*

Performed as described for **7** giving 170 mg (70%) of **8** (4:1) as a clear oil. Microanalysis calculated ($C_{14}H_{26}O_5$): C 61.29; H 9.55. Analysis result: C 60.99; H 9.59. Both isomers: ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.98–5.82 (m, 1H), 5.07–5.00 (m, 2H), 3.98 (dt, *J* 3.5, 11.0 Hz, 1H), 3.81 (t, *J* 11.3 Hz, 1H), 3.58 (dd, *J* 3.3, 11.5 Hz, 1H), 3.48 (d, *J* 3.9 Hz, 1H), 3.28 (s, 3H), 3.26 (s, 3H), 2.08 (br. s, OH), 1.28 (s, 3H), 1.27 (s, 3H), 1.10 (s, 3H), 1.09 (s, 3H). Major isomer: ^{13}C NMR ($CDCl_3$, 75 MHz): δ 144.4, 112.9, 99.4, 97.6, 79.0, 67.9, 59.9, 48.3, 48.0 40.7, 24.8, 22.8, 17.8, 17.5.

*2.1.4. (1R′,2S,5S,6S)-2-(1′-(Hexadecyloxy)but-3′-enyl)-5,6-dimethoxy-5,6-dimethyl-[1,4]dioxane (**9**)*

Alcohol **7** (7.10 g, 28.8 mmol) was dissolved in DMF (30 mL) under nitrogen. The solution was cooled to 0 °C, and cetyl iodide (36.2 mL, 115 mmol) and 50% (w/w) NaH dispersion in oil (4.15 g, 86 mmol) were added, after which gas evolved from the solution. After 10 min, the solution was heated to 50 °C and stirred for 20 h. The mixture was allowed to cool to room temperature, and CH_3OH (10 mL) was added. The solution was stirred for 10 min and Et_2O (50 mL) was added, after which the solution was washed with brine (50 mL), and the aqueous phase was extracted with Et_2O (4 × 50 mL). The combined organic extracts were washed with H_2O (2 × 70 mL), dried over $MgSO_4$ and concentrated in vacuo to give a yellow oil. The crude product was purified by dry column flash chromatography (1:0 → 3:1, heptane/EtOAc) and then by flash chromatography (5% Et_2O/heptane) to afford 8.10 g (60%) of **9** as a colorless oil and 2.38 g (18%) of its diastereomer as a colorless oil. R_f 0.12 (5% Et_2O/heptane). $[\alpha]_D$ +82.30 (2.00 g/100 mL, $CHCl_3$). Microanalysis calculated ($C_{28}H_{54}O_5$): C 71.44; H 11.56. Analysis result: C 71.09; H 11.57. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.88–5.74 (m, 1H), 5.08–4.97 (m, 2H), 3.75 (ddd, *J* 3.9, 7.9, 10.7 Hz, 1H), 3.66–3.54 (m, 2H), 3.45 (dt, *J* 6.3, 8.7 Hz, 1H), 3.32–3.23 (m, 2H), 3.21 (s, 3H), 3.19 (s, 3H), 2.49–2.40 (m, 1H), 2.23 (dt, *J* 6.9, 14.4 Hz, 1H), 1.44–1.40 (m, 2H), 1.22 (s, 3H), 1.21 (s, 3H), 1.18 (br. s, 26H), 0.81 (t, *J* 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 134.5, 117.1, 99.0, 97.9, 79.2, 70.5, 68.2, 61.7, 48.1, 48.0, 35.0, 31.9, 30.0, 29.7, 29.4, 29.3, 26.1, 22.7, 17.8, 17.6, 14.1.

*2.1.5. (1R′,2S,5S,6S)-2-(1′-(Hexadecyloxy)2′,2′-dimethyl-but-3′-enyl)-5,6-dimethoxy-5,6-dimethyl-[1,4]dioxane (**10**)*

Cetyl triflate was synthesized in the following way: trifluoromethanesulfonic anhydride (0.90 mL, 5.36 mmol) was added to a solution of dry pyridine (0.44 mL, 5.36 mmol) in dry CH_2Cl_2 (10 mL) at 0 °C under nitrogen. The ice bath was removed, and a solution of cetyl alcohol (1.0 g, 4.12 mmol) in dry CH_2Cl_2 (4 mL) was added dropwise over a period of 10 min. The reaction was stirred for 2 h at room temperature and was then quenched with H_2O (10 mL). CH_2Cl_2 (50 mL) was added, and the solution was washed with H_2O (2 × 50 mL). The combined aqueous phases were extracted with CH_2Cl_2 (50 mL), and the combined organic extracts were washed with brine (50 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was dissolved in hexane (10 mL) and filtered through a plug of silica gel with hexane and EtOAc. The solvent was removed by concentration in vacuo giving 1.41 g (92%) of cetyl triflate. R_f 0.50 (7% heptane/Et_2O). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.46 (t, *J* 6.5 Hz, 2H), 1.80–1.71 (m, 2H), 1.39–1.18 (m, 26H), 0.80 (t, *J* 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 120.7, 77.8, 31.9, 29.7, 29.6, 29.5, 29.3, 29.2, 28.8, 25.0, 22.7, 14.1. 1,8-Bis(dimethylamino)naphthalene (proton sponge, 281 mg, 1.31 mmol) was added to a solution of **8** (200 mg, 0.729 mmol) and cetyl triflate (492 mg, 1.31 mmol) in dry CH_2Cl_2 (7 mL) under nitrogen. The yellow solution was heated to reflux and stirred for 6 days. CH_2Cl_2 (50 mL) was added, and the solution was washed with brine (200 mL). The aque-

ous phase was extracted with CH_2Cl_2 (3 × 50 mL), the combined organic extracts were dried over $MgSO_4$, filtered and concentrated in vacuo. Purification by flash chromatography gave 142 mg (39%) of **10** and 63 mg (18%) of its diastereomer as colorless oils. R_f 0.10 (5% Et_2O/heptane). Microanalysis calculated ($C_{30}H_{58}O_5$): C 72.24; H 11.72. Analysis result: C 72.47; H 11.96. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.90 (dd, *J* 10.8, 17.5 Hz, 1H), 5.02–4.95 (m, 2H), 3.97 (dt, *J* 2.9, 10.9 Hz, 1H), 3.84–3.72 (m, 2H), 3.52 (dd, *J* 3.2, 11.5 Hz, 1H), 3.47–3.42 (m, 1H), 3.25 (s, 3H), 3.23 (s, 3H), 3.06 (d, *J* 2.6 Hz, 1H), 1.56–1.52 (m, 2H), 1.26 (br. s, 32H), 1.06 (s, 3H), 1.02 (s, 3H), 0.88 (t, *J* 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 145.1, 111.9, 99.2, 97.6, 88.0, 73.4, 68.4, 60.6, 48.2, 47.8, 41.2, 31.9, 30.2, 29.7, 29.6, 29.4, 26.3, 24.5, 23.3, 22.7, 17.9, 17.6, 14.1.

*2.1.6. (2S,3R)-3-Hexadecyloxy-hex-5-ene-1,2-diol (**11**)*

94% Trifluoroacetic acid in H_2O (5 mL) was added to a solution of **9** (332 mg, 0.705 mmol) in CH_2Cl_2 (5 mL), and the mixture was stirred at room temperature for 25 min. The solution was diluted with CH_2Cl_2 (20 mL), washed with H_2O (20 mL) and the aqueous phase was extracted with CH_2Cl_2 (10 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated in vacuo. Purification by flash chromatography (30% EtOAc/heptane) gave 190 mg (76%) of **11** as white crystals. R_f 0.2 (30% EtOAc/heptane). $[\alpha]_D$ − 15.28 (2.03 g/100 mL, $CHCl_3$). mp 38–39 °C (Et_2O). Microanalysis calculated ($C_{22}H_{44}O_3$): C 74.10; H 12.44. Analysis result: C 73.96; H 12.55. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.84–5.70 (m, 1H), 5.09–5.00 (m, 2H), 3.65–3.54 (m, 3H), 3.51 (dt, *J* 6.7, 9.1 Hz, 1H), 3.40–3.22 (m, 2H), 2.38–2.14 (m, 2H), 1.50–1.45 (m, 2H), 1.19 (br. s, 26H), 0.81 (t, *J* 6.5 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 134.2, 117.4, 81.1, 72.1, 70.7, 63.4, 35.0, 31.9, 30.0, 29.7, 29.6, 29.4, 29.3, 26.1, 22.6, 14.1.

*2.1.7. (2S,3R)-3-Hexadecyloxy-4,4-dimethyl-hex-5-ene-1,2-diol (**12**)*

Performed as described for **11** giving 666 mg (76%) of **12** as a clear oil. $[\alpha]_D$ − 173.8 (1.0 g/100 mL, $CHCl_3$). Microanalysis calculated ($C_{24}H_{48}O_3$): C 74.94; H 12.58. Analysis result: C 75.15; H 12.75. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.00 (dd, *J* 10.5, 17.2 Hz, 1H), 5.07–5.01 (m, 2H), 3.72–3.64 (m, 4H), 3.57–3.50 (m, 1H), 3.16–3.15 (m, 1H), 1.58–1.53 (m, 2H), 1.26 (br. s, 26H), 1.08 (s, 6H), 0.88 (t, *J* 6.6 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 145.5, 112.0, 88.1, 74.6, 72.4, 64.0, 41.5, 31.9, 30.4 29.7, 29.5, 29.3, 26.1, 25.0, 22.7 22.4, 14.1.

*2.1.8. Octadecanoic acid ((2S,3R)-1-(tert-butyldiphenylsilyloxy)-3-(hexadecyloxy)hex-5-en-2-yl)ester (**13**)*

4-(Dimethylamino)pyridine (226 mg, 1.85 mmol) and *tert*-butyl-diphenylsilylchloride (0.518 mL, 1.99 mmol) were added to a solution of **11** (0.455 g, 1.28 mmol) in dry CH_2Cl_2 (30 mL) under nitrogen, and the solution was stirred at room temperature. After 26 h, octadecanoic acid (726 mg, 2.55 mmol) and 4-(dimethylamino)pyridine (10.0 mg, 0.08 mmol) were added and the solution was cooled to 0 °C. *N*,*N*′-Dicyclohexylcarbodiimide (1 M) in CH_2Cl_2 (2.55 mL, 2.55 mmol) was added dropwise over a period of 4 min. The solution was allowed to warm to room temperature and stirred for 24 h. The mixture was filtered through a GF/A filter, and the filtrate was concentrated in vacuo. The residue was dissolved in Et_2O (50 mL) and washed with H_2O (2 × 50 mL) and brine (50 mL). The combined organic extracts were dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by flash chromatography (100:1 → 100:20 heptane/Et_2O) gave 918 mg (84%) of **13** as a colorless oil. R_f 0.15 (2.5% heptane/Et_2O). $[\alpha]_D$ +7.51 (1.97 g/100 mL, $CHCl_3$). Microanalysis calculated ($C_{56}H_{96}O_4Si$): C 78.08; H 11.23. Analysis result: C 77.93; H 11.26. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.62–7.57 (m, 4H), 7.36–7.27 (m, 6H), 5.81–5.67 (m, 1H), 4.99–4.94 (m, 3H), 3.80 (dd, *J* 5.9, 11.1 Hz, 1H), 3.70 (dd, *J* 3.8, 11.1 Hz, 1H), 3.62–3.56 (m, 1H), 3.42–3.34 (m, 2H), 2.23–2.13 (m, 4H), 1.63–1.50 (m, 4H), 1.18 (br. s, 54H), 0.97 (s, 9H), 0.81 (t, *J* 6.7 Hz, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 173.0, 135.6, 135.5, 134.6, 133.4, 133.3, 130.0, 129.7, 127.7, 117.0, 77.5, 74.6, 70.6, 62.2, 36.2, 35.2, 31.9, 30.0, 29.7, 29.6, 29.3, 26.7, 26.1, 25.1, 22.7, 19.1, 14.1.

*2.1.9. Octadecanoic acid ((2S,3R)-1-(tert-butyldiphenylsilyloxy)-3-(hexadecyloxy)-4,4-dimethyl-hex-5-en-2-yl)ester (**14**)*

The synthesis of **14** was carried out as described for the synthesis of **13** to afford 1.18 g (84%) of **14**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.69–7.64 (m, 4H), 7.43–7.35 (m, 6H), 5.80 (dd, *J* 10.5, 17.9 Hz, 1H), 5.15–5.10 (m, 1H), 4.87–4.81 (m, 2H), 3.84–3.83 (m, 2H), 3.59 (dt, *J* 6.4, 8.8 Hz, 1H), 3.35 (dt, *J* 6.6 Hz, 8.9 Hz, 1H), 3.12 (d, *J* 3.4 Hz, 1H), 2.26–2.21 (m, 2H), 1.70–1.57 (m, 2H), 1.43–1.39 (m, 2H), 1.25 (br. s, 54H), 1.10 (s, 6H), 1.02 (s, 9H), 0.88 (t, *J* 6.7 Hz, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.9, 144.8, 135.7, 135.6, 129.6, 129.5, 127.7, 127.6, 111.3, 87.1, 75.8, 73.6, 63.1, 41.2, 36.2, 34.7, 31.9, 30.1, 29.7, 29.5, 29.4, 29.3, 29.2, 26.9, 26.8, 26.1, 25.1, 24.8, 24.5, 23.5, 22.7, 19.2, 14.1.

*2.1.10. (2′S,3′R,4S)-2,2-Dimethyl-[1,3]dioxolan-4-ylmethyl 2′-octadecanoyloxy-3′-hexadecyloxy-hex-5′-enyl methyl phosphate (**17**)*

Imidazole (81.0 mg, 1.19 mmol) was added to a solution of **13** (292 mg, 0.339 mmol) in dry THF (25 mL) under nitrogen, and the solution was cooled to −15 °C. 0.1 M tetrabutylammonium fluoride in THF (10.2 mL, 1.02 mmol) was added, and the mixture was stirred for 2 h. The solution was diluted with CH_2Cl_2 (30 mL), washed with sat. $NaHCO_3$ (3 × 100 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The crude product **15** was dissolved in dry CH_2Cl_2 (7 mL) followed by addition of 2,2,6,6-tetramethylpiperidine (0.12 mL, 0.711 mmol) and methyl-*N*,*N*-diisopropylchlorophosphoramidite (0.13 mL, 0.678 mmol) under nitrogen at room temperature, after which the reaction was stirred for 45 min. The solution was diluted with CH_2Cl_2 (15 mL), washed with sat. $NaHCO_3$ (3 × 20 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was co-concentrated with toluene, kept under reduced pressure overnight and used directly in the next reaction. R_f 0.68 (20% EtOAc/heptane). Activated molecular sieves (1 g, 4 Å) and 97% 2,3-*O*-isopropylidene-*sn*-glycerol (0.09 mL, 0.678 mmol) were added to a solution of the crude compound in dry CH_2Cl_2 (7 mL) under nitrogen, and the solution was stirred at room temperature for 40 min. The reaction was cooled to 0 °C, and 5-phenyl-1*H*-tetrazole (104 mg, 0.712 mmol) was added after which the mixture was allowed to warm to room temperature and stirred for 90 min. The solution was then cooled to 0 °C. 5.5 M *t*-BuOOH in decane (0.129 mL, 0.712 mmol) was added and the solution was stirred for 1 h. 1 M Na_2SO_3 (10 mL) and sat. $NaHCO_3$ (10 mL) were added, and the mixture was allowed to warm to room temperature and stirred for 20 min. The solution was extracted with CH_2Cl_2 (4 × 20 mL), and the combined organic extracts were washed with brine (15 mL), dried over Na_2SO_4, filtered and concentrated in vacuo to give a white slurry. Purification by flash chromatography (30% EtOAc/heptane) gave 145 mg (51%) of **17** as a mixture of diastereomers (white slurry). R_f 0.2 (30% EtOAc/heptane). Microanalysis calculated ($C_{47}H_{91}O_9P$): C 67.91; H 11.04. Analysis result: C 68.28; H 11.09. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.82–5.60 (m, 1H), 5.05–4.96 (m, 3H), 4.26–4.11 (m, 3H), 4.02–3.88 (m, 3H), 3.78–3.67 (m, 4H), 3.48–3.37 (m, 3H), 2.68–2.13 (m, 4H), 1.57–1.42 (m, 4H), 1.35 (s, 3H), 1.29 (s, 3H), 1.18 (br. s, 54H), 0.80 (t, *J* 6.9 Hz, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.8, 133.6, 117.7, 109.8, 77.0, 74.0, 72.7, 70.7, 67.5, 66.0, 65.9, 54.4, 35.2, 34.3, 31.9, 29.9, 29.7, 29.6, 29.4, 29.3, 29.1, 26.7, 26.1, 25.2, 24.8, 22.7, 14.1.

*2.1.11. (2′S,3′R,4S)-2,2-Dimethyl-[1,3]dioxolan-4-ylmethyl 2′-octadecanoyloxy-3′-hexadecyloxy-4′,4′-dimethyl-hex-5′-enyl methyl phosphate (**18**)*

Performed as described for **17** giving 332 mg (58%) of **18** as a clear oil. Microanalysis calculated ($C_{49}H_{95}O_9P$): C 68.49; H 11.14. Analysis result: C 68.54; H 11.12. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.92 (dd, *J* 10.5, 17.9 Hz, 1H), 5.21–5.17 (m, 1H), 5.04–4.99 (m, 2H), 4.38–4.35 (m, 2H), 4.20–3.94 (m, 4H), 3.86–3.80 (m, 1H), 3.77 (d, *J* 3.5 Hz, 3H), 3.69–3.64 (m, 1H), 3.46–3.38 (m, 1H), 3.13 (d, *J* 2.8 Hz, 1H), 2.34–2.28 (m, 2H), 1.63–1.52 (m, 4H), 1.42 (s, 3H), 1.36 (s, 3H), 1.25 (br. s, 54H), 1.09 (s, 3H), 1.06 (s, 3H), 0.88 (t, *J* 6.7 Hz, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 172.7, 144.2, 112.1, 109.8, 87.1, 74.0, 73.9, 73.8, 67.5, 67.3, 66.1, 54.4, 41.3, 34.4, 31.9, 30.1, 29.7, 29.5, 29.3, 29.2, 29.1, 26.7, 26.2, 25.2, 24.7, 24.3, 23.6, 22.6, 14.1.

*2.1.12. (2S,2′S,3′R)-2,3-Dihydroxypropyl 2′-octadecanoyloxy-3′-hexadecyloxy-hex-5′-enyl phosphate (**1**)*

Compound **17** (210 mg, 0.244 mmol) was dissolved in a solution of CH_2Cl_2 (6 mL), CH_3CN (6 mL), isopropanol (8 mL) and 40% $(CH_3)_3N$ (7 mL), which was stirred at room temperature. After stirring overnight, EtOH (20 mL) and toluene (20 mL) were added, and the mixture was concentrated in vacuo. The residue was dissolved in a 7:1 CH_2Cl_2/CH_3OH (17.5 mL) solution and extracted with 1 M HCl (30 mL). 1 M HCl (1 mL) and additional CH_3OH (4 mL) were added to the organic extract, which gave a 65:25:4 CH_2Cl_2/CH_3OH/1 M HCl solution. The solution was stirred at room temperature overnight, and $NaHCO_3$ (286 mg, 3.4 mmol) was added followed by stirring for an additional 24 h. The neutral (pH 7) solution was dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by flash chromatography (15% CH_3OH/CH_2Cl_2) gave 122 mg (60%) of **1** as a colorless oil. R_f 0.12 (15% CH_3OH/CH_2Cl_2). ^{1}H NMR (300 MHz, 30% $CD_3OD/CDCl_3$): δ 5.92–5.79 (m, 1H), 5.18–5.04 (m, 3H), 4.07–3.87 (m, 4H), 3.80–3.76 (m, 1H), 3.62–3.53 (m, 5H), 2.38–2.29 (m, 4H), 1.65–1.48 (m, 4H), 1.27 (br. s, 54H), 0.89 (t, *J* 6.7 Hz, 6H). ^{13}C NMR (30% $CD_3OD/CDCl_3$, 75 MHz): δ 173.5, 133.9, 116.8, 77.7, 73.3, 70.6, 70.4, 66.1, 63.5, 62.0, 34.7, 34.0, 31.5, 29.6, 29.3, 29.2, 29.1, 29.0, 28.7, 25.7, 24.5, 22.3, 13.5. ^{31}P NMR (162 MHz, 30% $CD_3OD/CDCl_3$): δ 3.55. ESI HRMS calcd. for $C_{43}H_{85}O_9P$ $[M-H]^-$ *m/z* 775.5847, found: *m/z* 775.5849.

*2.1.13. (2S,2′S,3′R)-2,3-Dihydroxypropyl 2′-octadecanoyloxy-3′-hexadecyloxy-4′,4′-dimethyl-hex-5′-enyl phosphate (**2**)*

Performed as described for **1** giving 61 mg (79%) of **2** as a colorless oil. ^{1}H NMR (300 MHz, 30% $CD_3OD/CDCl_3$): δ 5.98–5.88 (m, 1H), 5.22–5.20 (m, 1H), 5.04–4.99 (m, 2H), 4.26–3.96 (m, 4H), 3.91–3.85 (m, 1H), 3.81–3.57 (m, 5H), 2.31–2.29 (m, 2H), 1.61–1.53 (m, 4H), 1.26 (br. s, 54H), 1.09 (s, 6H), 0.83 (br. s, 6H). ^{13}C NMR (30% $CD_3OD/CDCl_3$, 75 MHz): δ 173.4, 144.3, 111.7, 87.3, 74.8, 73.6, 70.7, 66.1, 65.3, 62.0, 44.5, 41.1, 34.3, 31.7, 29.9, 29.4, 29.3, 29.2, 29.0, 25.9, 24.6, 24.2, 23.1, 22.4, 13.8. ^{31}P NMR (162 MHz, 30% $CD_3OD/CDCl_3$): δ 3.83. ESI HRMS calcd. for $C_{45}H_{89}O_9P$ $[M-H]^-$ *m/z* 803.6160, found: *m/z* 803.6162.

*2.1.14. Stereochemistry of **7R***

Alcohol **7** (410 mg, 1.7 mmol) was dissolved in dry CH_2Cl_2 (15 mL) and 4-(dimethylamino)pyridine (19 mg, 0.16 mmol), followed by addition of acetic anhydride (0.32 mL, 3.4 mmol) and Et_3N (0.43 mL, 3.4 mmol) under nitrogen. The solution was stirred for 45 min and then concentrated in vacuo. The residue was purified by flash chromatography (9% EtOAc/heptane) to give 350 mg (72%) of **19** and 60 mg (12%) of its diastereomer as colorless oils. To the solution of **19** (0.31 g, 1.1 mmol) in CH_2Cl_2 (5 mL) was added 95% trifluoroacetic acid in H_2O (5 mL) and the yellow solution was stirred at room temperature for 45 min. The mixture was concentrated in vacuo, and Na (15.5 mg, 0.67 mmol) dissolved in dry CH_3OH (5 mL) was added. The flask was fitted with a $CaCl_2$-tube and after stirring for 30 min additional Na (15.3 mg, 0.67 mmol) in dry CH_3OH (5 mL) was added. The reaction was stirred for 45 min and subsequently quenched with ion exchange resin (1.4 mL, Amberlite IR-120 H^+, 1.9 meq/mL). The mixture was stirred for 35 min, filtered and concentrated in vacuo. Purification by flash chromatography gave 112 mg (79%) of **20** as white crystals. $[\alpha]_D$ +10.25 (2.00 g/100 mL, $CHCl_3$). Microanalysis calculated ($C_{14}H_{26}O_6$): C 54.53; H 9.15. Analysis result: C 54.62; H 9.19. Triol **20** (0.105 g, 0.80 mmol) was dissolved in CH_3OH (50 mL). The solution was cooled to −78 °C and treated with ozone for 30 min $(CH_3)_2S$ (0.4 mL, 5.4 mmol) was added, and the solution was stirred under nitrogen for 18 h. The mixture was concentrated in vacuo, filtered through reverse phase silica with CH_3OH and concentrated in vacuo. Purification by flash chromatography (12% CH_3OH/CH_2Cl_2) gave 55 mg (52%) of **22** and **23** and 20 mg (17%) of **21** as colorless oils. **21** (α-anomer): ^{1}H NMR (500 MHz, $(CD_3)_2SO$): δ 4.97 (dd, *J* 2.5, 5.8 Hz, 1H), 4.95 (d, *J* 4.9 Hz, 1H), 4.59 (dd, *J* 5.5 Hz, 1H), 4.08 (ddd, *J* 5.4 Hz, 1H), 3.71–3.65 (m, 1H), 3.33–3.40 (m, 2H), 3.19 (s, 3H), 1.97 (ddd, *J* 2.6, 6.4, 13.1 Hz, 1H), 1.87 (dt, *J* 5.7, 13.2 Hz, 1H). ^{13}C NMR (CD_3OD, 75 MHz): δ 105.4, 87.0, 71.6, 63.7, 54.2, 41.1. **21** (β-anomer): ^{1}H NMR (500 MHz, $(CD_3)_2SO$): δ 4.89 (dd, *J* 2.4, 5.8 Hz, 1H), 4.81 (d, *J* 5.5 Hz, 1H), 4.59 (dd, *J* 5.7 Hz, 1H), 3.91 (ddd, *J* 4.9, 5.3, 8.1 Hz, 1H), 3.71–3.65 (m, 1H), 3.49 (ddd, *J* 3.3, 5.2, 11.7 Hz, 1H), 3.33–3.40 (m, 1H), 3.22 (s, 3H), 2.25 (ddd, *J* 5.8, 8.1, 13.9 Hz, 1H), 1.63 (ddd, *J* 2.3, 4.8, 13.8 Hz, 1H). ^{13}C NMR (CD_3OD, 75 MHz): δ 105.1, 85.6, 71.1, 62.0, 54.0, 41.1. **22** (α-anomer): ^{13}C NMR ($CDCl_3$, 75 MHz): δ 98.1, 85.4, 70.9, 62.7, 41.0. **22** (β-anomer): ^{13}C NMR ($CDCl_3$, 75 MHz): δ 98.1, 85.8, 71.2, 61.5, 41.2. **23** (α-anomer): ^{13}C NMR ($CDCl_3$, 75 MHz): δ 93.9, 67.3, 66.5, 66.1, 33.7. **23** (β-anomer): ^{13}C NMR ($CDCl_3$, 75 MHz): δ 91.7, 67.5, 64.6, 62.8, 35.1.

*2.1.15. Stereochemistry of **8R***

NaH 50% (w/w) dispersion in oil (93 mg, 1.94 mmol) was added to a solution of **8** (177 mg, 0.65 mmol) in dry DMF (7 mL), and the solution was stirred at room temperature under nitrogen for 30 min. The mixture was cooled to 0 °C, and BnBr (0.25 mL, 2.06 mmol) and Bu_4NI (24 mg, 0.065 mmol) were added, and the mixture stirred at room temperature overnight. The reaction was quenched with H_2O (1 mL), toluene (20 mL) was added, and the mixture was concentrated in vacuo. Et_2O (100 mL) and H_2O (10 mL) were added, and the mixture was washed with 1 M HCl (2 × 50 mL) and H_2O (2 × 50 mL), dried over Na_2SO_4, filtered and concentrated in vacuo. Purification by flash chromatography (6% Et_2O/heptane) gave 162 mg (68%) of **24** (and 50 mg of the diastereoisomer, 21%) as a colorless oil. R_f 0.34 (10% EtOAc/heptane). Benzyl ether **24** (162 mg, 0.44 mmol) was dissolved in CH_2Cl_2 (5 mL), and 90% trifluoroacetic acid in H_2O (5 mL) was added. The yellow solution was stirred at room temperature for 20 min, where after CH_2Cl_2 (20 mL) was added and the solution was washed with H_2O (20 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 30 mL), and the combined organic extracts were dried over $MgSO_4$, filtered and concentrated in vacuo. Purification by flash chromatography (20% EtOAc/heptane) gave 60 mg (54%) of **25** as a colorless oil. R_f 0.46 (25% EtOAc/heptane). Diol **25** (60 mg, 0.24 mmol) was dissolved in *t*-BuOH (3 mL) and hydrogenated overnight at 1 atm with 10% Pd/C (6 mg). The mixture was filtered through a GF/A filter, and the filter cake was rinsed with CH_2Cl_2 (10 mL) and EtOAc (10 mL), where after the solvent was removed by concentration in vacuo. Crude **26** (38 mg, 0.23 mmol)

was dissolved in dry CH_2Cl_2 (5 mL) under nitrogen, and *p*-toluenesulfonic acid (3.5 mg, 0.02 mmol) and benzaldehyde dimethylacetal (0.04 mL, 0.23 mmol) were added to the solution. The mixture was stirred at room temperature overnight, where after the solution was washed with sat. $NaHCO_3$ (10 mL), and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic extracts were washed with brine (10 mL), dried over $MgSO_4$, filtered and concentrated in vacuo. The crude compound (**27**) was dissolved in dry CH_2Cl_2 (1 mL) under nitrogen, and 4-(dimethylamino)pyridine (337 mg, 2.76 mmol) and benzoyl chloride (0.28 mL, 2.3 mmol) were added to the solution. The mixture was stirred at room temperature overnight, where after H_2O (20 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated in vacuo. Purification by flash chromatography (6% Et_2O/heptane) gave 63 mg (77%) of **28** as a white slurry. R_f 0.2 (6% Et_2O/heptane). $[\alpha]_D$ +35.9 (1.0 g/100 mL, $CHCl_3$). Microanalysis calculated ($C_{22}H_{26}O_4$): C 74.55; H 7.39. Analysis result: C 74.30; H 7.14. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.18–8.15 (m, 2H), 7.60–7.35 (m, 8H), 5.56 (s, 1H), 5.38 (td, *J* 5.3, 9.4 Hz, 1H), 4.44 (dd, *J* 5.3, 10.7 Hz, 1H), 3.82 (d, *J* 9.5 Hz, 1H), 3.68 (dd, *J* 9.4, 10.7 Hz, 1H), 1.64–1.52 (m, 1H), 1.38–1.26 (m, 1H), 1.02 (s, 3H), 0.99 (s, 3H), 0.89 (t, *J* 7.6 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 165.1, 137.9, 133.4, 130.5, 129.7, 128.8, 128.5, 128.2, 126.0, 100.8, 83.6, 67.9, 64.8, 37.0, 31.6, 23.5, 8.1.

2.2. Preparation of liposomes

Phospholipids **1** and **2** were hydrated in an aqueous buffer (150 mM KCl, 10 mM HEPES, 30 μM $CaCl_2$, 10 μM EDTA, pH 7.5). MLVs formed spontaneously. To ensure complete hydration, the lipids were hydrated for 1 h at 60 °C. During the hydration, the lipids were vortexed every 15 min. SUVs were prepared by sonication of MLVs for 1 h, 5 °C above the lipid main phase transition temperature regime. The total concentration of lipids in the buffer solutions was 2 mM.

2.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry of 2 mM multilamellar liposomes was performed by using a Microcal MC-2 (Northhampton, MA, USA) ultrasensitive power compensating scanning calorimeter equipped with a nanovoltmeter. The scans were performed in the upscan mode at a scan rate of 10 °C/h. An appropriate baseline has been substracted from the thermograms to afford the melting enthalpies of the liposomes.

2.4. Activity measurements

The conditions used to perform the $sPLA_2$ activity measurements were as follows: 0.15 mM phospholipid as SUVs, 150 nM $sPLA_2$, 0.15 M KCl, 30 μM $CaCl_2$, 10 μM EDTA, 10 μM HEPES (pH 7.5). The catalytic reaction was initiated by addition of 8.9 μL of a 42 μM snake (*Agkistrodon piscivorus piscivorus*) venom $sPLA_2$ stock solution to a 2.5 mL liposome suspension thermostated prior to the addition of the enzyme. The time-dependent action of $sPLA_2$ was monitored from the changes in the 90° static light scattering giving information of changes in the lipid morphology as non-bilayer forming lysophopholipids and fatty acids are generated. High-performance liquid chromatography (HPLC) quantification of the products from the enzymatic reaction was performed with a 5 μm diol column. Two different mobile phases (hereafter referred to as mobile phase A and mobile phase B) were used. Mobile phase A was a chloroform/methanol/25% ammonium hydroxide (800:195:5) solution, while mobile phase B was a chloroform/methanol/water/25% ammonium hydroxide (600:340:50:5) solution. The following gradients were used at a flow rate of 1 mL/min 0–14 min: 100% A to 100% B using a linear gradient; 14–25 min: 100% B; 25–30 min: 100% B to 100% A using a linear gradient; 30–45 min: 100% A to regenerate. An evaporative light scattering detector was used for detection. The turnover of the hydrolysis was followed by HPLC. Samples were collected at different time intervals by collecting 100 μL of the reaction mixture and rapidly mixing with a 0.5 mL chloroform/methanol/acetic acid (2:4:1) solution to quench the reaction. The solution was washed with 0.5 mL of water, and 50 μL of the organic phase was used for HPLC. Fluorescence measurements were performed using an SLM DMX 1100 spectrofluorometer. Purified snake venom PLA_2 was a generous gift from Dr. R.L. Biltonen (University of Virginia, VA, USA).

2.5. Molecular dynamics simulations

The crystal structures of bee-venom (*Apis Mellifera*) phospholipase A_2 complexed with the transition-state analogue, L-1-*O*-octyl-2-heptylphosphonyl-*sn*-glycero-3-phosphoethanolamine (diC_8(2Ph)PE), resolved to 2.0 Å (White et al., 1990) and human phospholipase A_2 IIA complexed with 6-phenyl-4(*R*)-(7-phenyl-heptanoylamino)-hexanoic acid resolved to 2.1 Å (Hansford et al., 2003) were obtained from the Protein

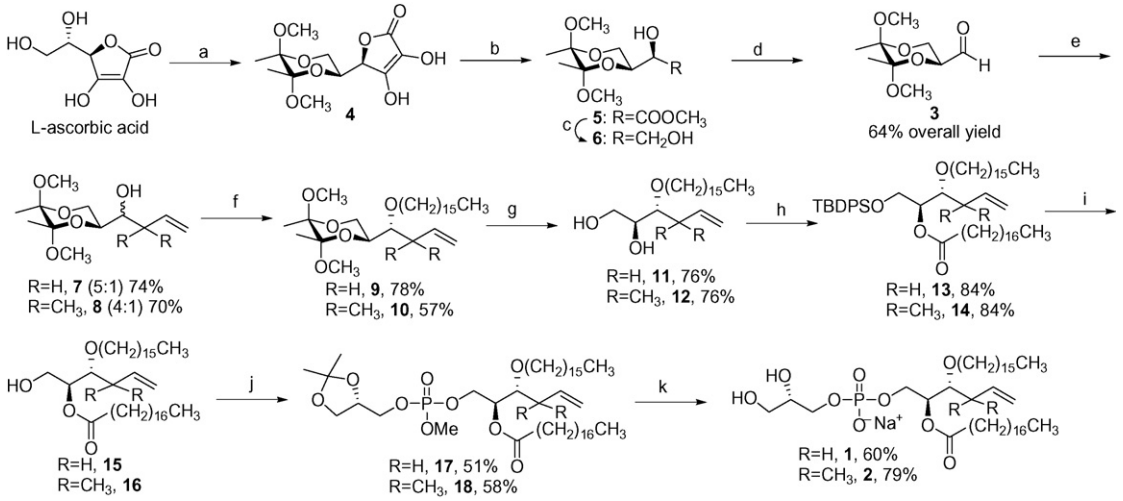
Data Bank ((Bernstein et al., 1977); entry codes: 1 poc and 1 kqu, respectively).

The initial modeling step involved placing diC_8(2Ph)PE into the binding cleft of human phospholipase A_2 IIA, which was done by (i) deleting the inhibitor in 1 kqu (keeping calcium ions and water molecules in the structure), (ii) deleting calcium ions and water molecules in 1 poc, (iii) aligning 1 poc with 1 kqu, and (iv) finally deleting the bee-venom phospholipase A_2 structure. The structures for the phospholipids **1** and **2** were built from diC_8(2Ph)PE using SPARTAN Version 1.0.2 (Wavefunction Inc., Irvine, California, USA). Missing distance, angle and torsion parameters for the allyl side chains (located at the *sn*-1 position in the phospholipids **1** and **2**) were obtained from the CHARMM27 parameter set describing similar atom types. The structures complexed with either phospholipid **1** or **2** were solvated using the program SOLVATE (by Grubmüller). Eighteen water molecules were randomly replaced with chloride ions to neutralize the systems. The final systems contained 4888 water molecules, and the simulation cell dimensions were 52.7 Å × 51.7 Å × 67.3 Å. For all simulations, NAMD (Kale et al., 1999) was used with the Charmm27 all hydrogens parameter set and with the TIP3 water model (Jorgensen et al., 1983). Initially, the energy of the systems was minimized for 5000 steps. This was followed by 100 ps of heating of the systems to $T = 300$ K. The simulations were carried out for 10 ns in the *NPT* ensemble. A time step of 1 fs was used throughout all simulations. An isotropic constant ambient pressure of 1 atm was imposed using the Langevin piston method (Feller et al., 1995) with a damping coefficient of 5 ps^{-1}, a piston period of 200 fs, and a decay of 500 fs. The Particle Mesh Ewald method was used for computation of the electrostatic forces (Essmann et al., 1995; Darden et al., 1993). The grid spacing applied was approximately 1.0 Å, and a fourth order spline was used for the interpolation. The long range part of the electrostatic forces was evaluated every fourth fs. Van der Waals interactions were cut off at 12 Å using a switching function starting at 10 Å. Periodic boundary conditions were applied in *x*, *y* and *z*-directions. The analyses of the trajectories were performed using VMD (Humphrey et al., 1996).

3. Results and discussion

3.1. Synthesis of aldehyde *3*

The synthesis of the key aldehyde **3** is based on a procedure originally reported by Michel and Ley (2002, 2003). However, we raised the overall yield from 35 to 64% by modifying the workup of the intermediates (Scheme 1). L-Ascorbic acid was butanedione protected by reaction with 2,3-butanedione, boron trifluoride ethyl



Scheme 1. Reagents and conditions: (a) 2,3-butanedione, $BF_3{\cdot}OEt_2$, $CH(OCH_3)_3$, CH_3OH; (b) H_2O_2, K_2CO_3, H_2O then $(CH_3)_2SO_4$, $NaHCO_3$, reflux; (c) $NaBH_4$, isopropanol, 70 °C; (d) $NaIO_4$, $NaHCO_3$, H_2O, CH_2Cl_2; (e) Zn, allyl bromide or 3,3-dimethylallyl bromide, sat. NH_4Cl, THF, 0 °C; (f) cetyl iodide, NaH, DMF, 50 °C or cetyl triflate, 1,8-bis(dimethylamino)naphthalene, CH_2Cl_2, reflux (g) trifluoroacetic acid, CH_2Cl_2; (h) (*i*) *tert*-butyldiphenylsilyl chloride, 4-(dimethylamino)pyridine, CH_2Cl_2; (*ii*) octadecanoic acid, *N*,*N*′-dicyclohexylcarbodiimide, 4-(dimethylamino)pyridine, CH_2Cl_2; (i) tetrabutylammonium fluoride, imidazole, THF, −15 °C; (j) (*i*) methyl-*N*,*N*-diisopropylchlorophosphoramidite, 2,2,6,6-tetramethylpiperidine, CH_2Cl_2; (*ii*) 2,3-*O*-isopropylidene-*sn*-glycerol, 5-phenyl-1*H*-tetrazole, CH_2Cl_2 then *tert*-butylhydroperoxide, 0 °C; (k) (*i*) $(CH_3)_3N/CH_2Cl_2/CH_3CN$/isopropanol; (*ii*) CH_2Cl_2/CH_3OH/HCl; (*iii*) $NaHCO_3$.

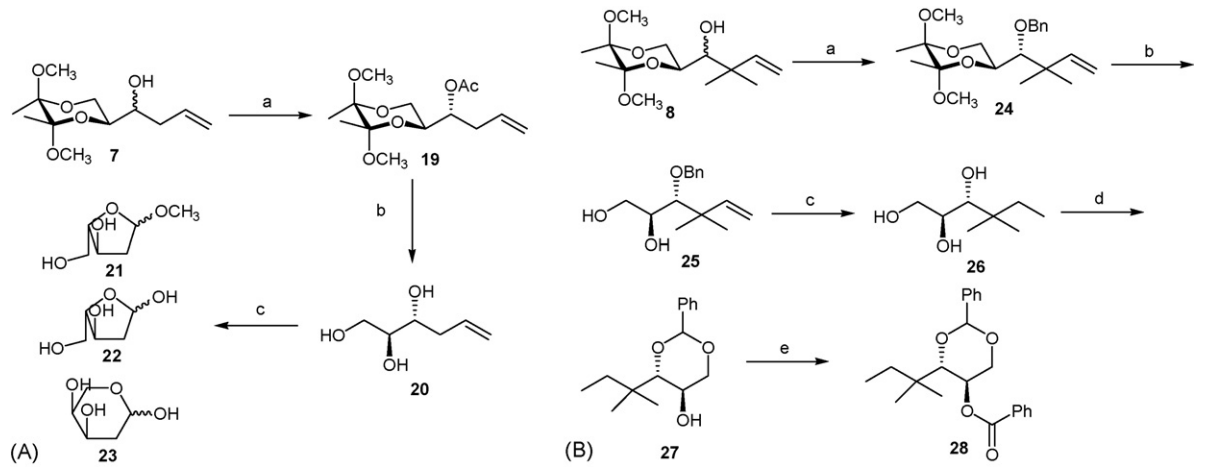
etherate and trimethyl orthoformate in methanol. The generated compound **4** was used without purification in the next reaction, where it was treated with hydrogen peroxide followed by dimethyl sulfate yielding methylester **5**. Crude **5** was reduced to diol **6** with sodium borohydride, and subsequently diol **6** was oxidatively cleaved with sodium periodate to afford aldehyde **3**. The only purification during the synthesis involved a simple distillation of the final aldehyde **3**.

3.2. Synthesis of diol *11* and *12*

The synthesis of diol **11** and **12** required the introduction of a new stereogenic center. This was done by a zinc mediated allylation of aldehyde **3** with allyl bromide giving the alcohol **7** as a 5:1 diastereomeric mixture in 74% yield (Scheme 1). Allylation with 3,3-dimethylallyl bromide using the same conditions, gave **8** as a 4:1 diastereomeric mixture in 70% yield. It was not possible to separate the diastereomeric mixtures by flash chromatography. However, it was found that the diastereomers could be separated after the next reaction. Reaction of the diastereomeric mixture of alcohol **7** with NaH and cetyl iodide gave **9*R*** and its diastereoisomer in a total yield of 78%. Under the same reaction conditions the diastereomeric mixture of **8** only gave 48% yield of **10** as a 3:2 mixture with the wrong isomer being the major compound. It was found that the reaction had not gone to completion, and 44% of the starting material could be recovered. Interestingly, NMR of the recovered starting material only showed the presence of the desired diastereomer **8*R*** which indicates that the wrong diastereomer **8*S*** is more reactive than **8*R***. Due to the low yield, the reaction conditions were changed to the procedure by Heyes et al. (2002) using cetyl triflate and 1,8-bis(dimethylamino)naphthalene (proton sponge), which improved the yield to 57%. Again, it was not possible to run the reaction to completion, and 25% of the starting material was recovered after 6 days. In contrast to the reaction with NaH and cetyl iodide, the recovered starting material consisted of **8** as a 4:1 mixture, which shows that in this case there is no difference in reactivity between the two diastereomers. Further reaction of **9** and **10** with TFA removed the butanedione protecting group in 76% yield in both cases. In order to verify the stereochemistry of **7*R***, the reactions in Scheme 2a were carried out. The stereochemistry of **7*R*** was then confirmed by comparing the NMR data of compound **21**, **22** and **23** with literature data (Birk et al., 1997; Dyatkina and Azhayev, 1984). The stereochemistry of **8*R*** was verified by the generation of compound **28** and analyzing the ^{1}H NMR data of the six-membered ring (Scheme 2b).

3.3. Synthesis of phospholipid *1* and *2*

The synthesis of phospholipids **1** and **2** relied on the introduction of a *tert*-butyldiphenylsilyl protecting group on the primary alcohol of diol **11** and **12** followed by insertion of the fatty acid chain in the secondary position with *N*,*N*′-dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine (Scheme 1). This was carried out in 84% yield for both diol **11** and **12**. The *tert*-butyldiphenylsilyl group was then removed from **13** and **14** with tetrabutylammonium fluoride at −15 °C. As the fatty acid chain easily migrates to the primary



Scheme 2. (A) Reagents and conditions: (a) acetic anhydride, 4-(dimethylamino)pyridine, Et_3N, CH_2Cl_2; (b) (*i*) trifluoroacetic acid, H_2O, CH_2Cl_2; (*ii*) Na, CH_3OH; (c) O_3, $(CH_3)_2S$, CH_3OH. (B) Reagents and conditions: (a) BnBr, NaH, Bu_4NI, DMF; (b) 90% aq. trifluoroacetic acid in CH_2Cl_2; (c) H_2, *t*-BuOH, Pd/C; (d) $PhCH(OCH_3)_2$, *p*-toluenesulfonic acid, CH_2Cl_2; (e) PhC(O)Cl, 4-(dimethylamino)pyridine, CH_2Cl_2.

position, the deprotection can be troublesome. Acyl migration was prevented by adding imidazole to the reaction mixture as described by Qin et al. (1999). After removal of the *tert*-butyldiphenylsilyl protecting group, the phosphate headgroup was inserted into the primary position by use of the phosphoramidite method (Beaucage and Iyer, 1993). Crude alcohols **15** and **16** were reacted with commercially available methyl-*N*,*N*-diisopropylchlorophosphoramidite in the presence of 2,2,6,6-tetramethylpiperidine to afford the corresponding amidites, which were not purified but reacted with 2,3-*O*-isopropylidine-*sn*-glycerol in combination with 5-phenyl-1*H*-tetrazole (Andresen et al., 2004b). The generated phosphites were oxidized to the corresponding phosphates **17** and **18** by treatment with *tert*-butylhydroperoxide in an overall yield of 51% from **13** and 58% from **14**. Treatment of **17** and **18** with $(CH_3)_3N$ followed by reaction of the crude compound with a $CH_2Cl_2/CH_3OH/HCl$ solvent system afforded the desired phospholipids **1** and **2** in 60% and 79% yield, respectively.

3.4. $sPLA_2$ activity measurements and molecular dynamics simulations

Differential scanning calorimetry (DSC) results of the two new phospholipids **1** and **2** revealed the thermodynamic properties of the liposomes. DSC of phospholipid **1** shows the main phase transition T_m at 47 °C ($T_{1/2} = 1.7$ °C, $\Delta H = 10.5$ kcal/mol) as a single peak, and DSC of phospholipid **2** shows the main phase transition T_m at 44 °C ($T_{1/2} = 0.48$ °C, $\Delta H = 9.2$ kcal/mol) as a single peak. Small unilamellar liposomes composed of phospholipids **1** and **2** were subjected to hydrolysis with $sPLA_2$. The enzymatic reaction was carried out at T_m and monitored by light scattering and HPLC. Increased intensity changes in the 90° light scattering were measured for liposomes composed of phospholipid **1** indicating morphology changes caused by the production of nonbilayer forming fatty acids and lysophospholipids and hence hydrolytic activity. This was further supported by HPLC measurements showing that there was a decrease in the substrate concentration upon addition of the enzyme (Fig. 2). Light scattering measurements of $sPLA_2$ action towards phospholipid **2** composed liposomes did not reveal any morphology changes, suggesting that $sPLA_2$ is not able to catalyze the hydrolysis of this particular phospholipid. The inability of $sPLA_2$ to hydrolyze phospholipid **2** was verified by HPLC, which showed no changes in the concentration of the phospholipid upon addition of the enzyme.

In the light of these findings, MD simulations were carried out in order to further understand the selectivity of $sPLA_2$ towards phospholipids **1** and **2**. Simulations in general can provide insight at a molecular level and might reveal the structural requirements that govern substrate specificity (Peters, 2004a,b). Figs. 3A and B show snapshots taken from the MD simulations of phospholipids **1** and **2**, respectively. The images are close-up of the active site of $sPLA_2$. The secondary protein structure is shown in ribbon mode. His48 and Asp99 involved in the hydrolysis, the substrate and a water molecule are displayed in liquorice mode. Major differences between the snapshots are observed in the orientation of His48 and the location of the water molecule. For phospholipid **1** (Fig. 3A), Asp99-His48-water are perfectly aligned for hydrolysis. In this configuration, His48 is able to abstract a proton from the water molecule that is perfectly positioned to perform a nucleophilic attack on the *sn*-2 carbonyl. Clearly, the allyl-substituent in phospholipid **1** does not seem to interfere with the incoming water molecule participating in the hydrolysis. In contrast, Fig. 3B shows that one of the two methyl substituents in phospholipid **2** prevents a water molecule from getting in place to take part in the hydrolysis. Furthermore, His48 is not optimal aligned to participate in the hydrolysis. Hence, the *sn*-1 side chain of phospholipid **2** is too bulky for allowing free access of a water molecule and perfect alignment of His48. A detailed analysis of the simulations will be provided elsewhere (unpublished material).

In summary, we have described the preparation of phospholipids **1** and **2** from L-ascorbic acid. In the synthesis, aldehyde **3** served as a key intermediate for introduction of the allyl-substituents and for controlling the stereochemistry at the *sn*-2 position of the final phospholipids. Difficulties in incorporating the ether

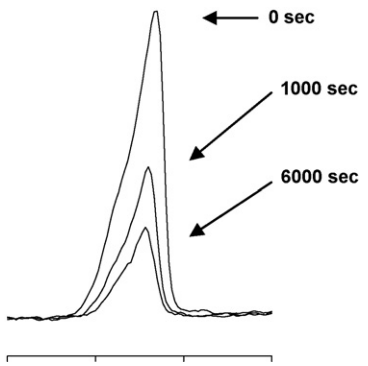


Fig. 2. $sPLA_2$-catalyzed hydrolysis at 47 °C of liposomes composed of phospholipid **1**. The HPLC chromatograms show the amount of phospholipid **1** before $sPLA_2$ was added (0 s), and the amount of unhydrolyzed lipids 1000 and 6000 s after addition of $sPLA_2$.

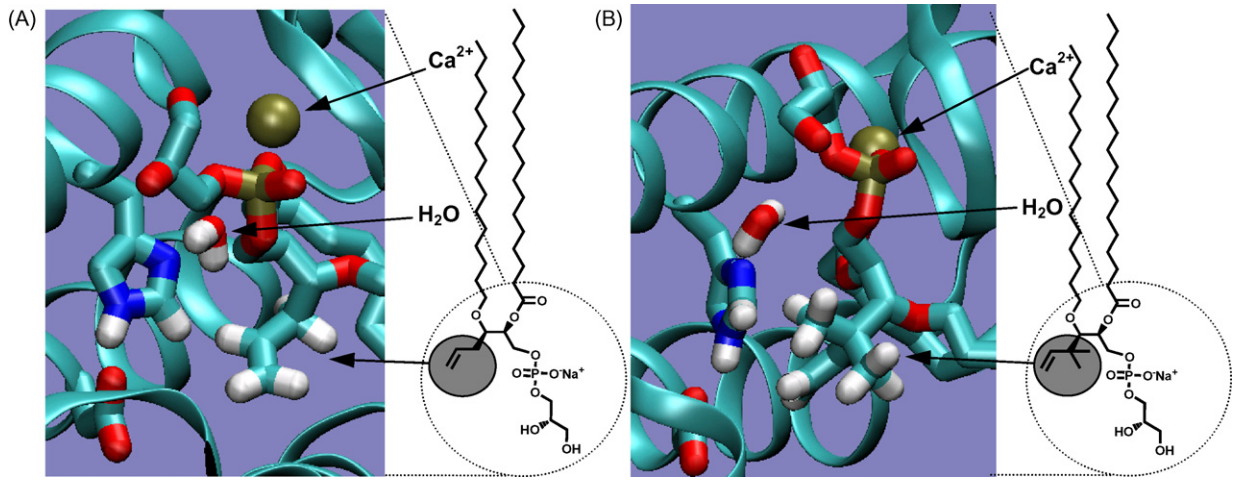


Fig. 3. Snapshots of the active site of $sPLA_2$. Images are taken from simulations of phospholipids **1** (A) and **2** (B). The secondary protein structure is shown in the ribbon mode and is colored green. His48, Asp99, the substrate and a water molecule are shown in the liquorice mode and are colored according to the atom type (H: white, N: blue, O: red, P: gold). The Ca^{2+} essential for $sPLA_2$ activity is shown in van der Waals mode and is colored gold. Lipid structures to the right of the images define the region shown in the images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

moiety in the *sn*-1 position arose in the case of alcohol **8** and consequently, the reaction conditions were changed from sodium hydride and cetyl iodide to cetyl triflate and 1,8-bis(dimethylamino)naphthalene, which improved the yield of the reaction. Additionally, $sPLA_2$ activity measurements of the phospholipids in combination with molecular dynamics simulations revealed that the limited space available in the binding cleft prohibits hydrolysis of phospholipid **2**, whereas phospholipid **1** seems to be a good substrate for $sPLA_2$.

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