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Synthesis of tocopheryl succinate phospholipid conjugates and monitoring of phospholipase A_2 activity

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ABSTRACT

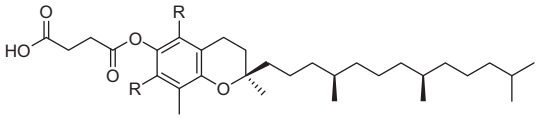
Tocopheryl succinates (TOSs) are, in contrast to tocopherols, highly cytotoxic against many cancer cells. In this study the enzyme activity of secretory phospholipase A_2 towards various succinate-phospholipid conjugates has been investigated. The synthesis of six novel phospholipids is described, including two TOS phospholipids conjugates. The studies revealed that the TOS conjugates are poor substrates for the enzyme whereas the phospholipids with alkyl and phenyl succinate moieties were hydrolyzed by the enzyme to a high extent.

1. Introduction

The lack of effective administration routes can be a bottleneck in the development of pharmaceuticals from potent lead compounds. Many promising lead molecules suffer from low water solubility and low stability during transport in the body due to labile chemical functionalities or fast metabolic clearance, whereby the use of intravenous or oral administration routes is precluded. Therefore it is of great interest to develop drug delivery systems that can enhance the drug stability through transport in the body and hence increase bioavailability.

α-Tocopheryl succinate (α-TOS, Fig. 1) is a potent growth inhibitor towards various cancer cells while the toxicity towards healthy cells is low.[1–3] Structure–activity relationship (SAR) studies have revealed that the succinate moiety is crucial for the activity and neither esters of α-TOS, α-tocopherol itself nor other members of the vitamin E family display cytotoxicity.[1,3] In addition SAR studies have demonstrated that succinates and malonates of TOS are more active than the conjugates of longer dicarboxylic acids, like the glutarate analog.[3] However the use of tocopheryl derivatives in cancer treatment is restricted due to their low water solubility, which hampers an intravenous administration route, and oral administration of α-TOS is not effective due to hydrolysis of the succinate by esterases during the transport through the gastrointestinal tract.[4,5] For in vivo studies in mice intravenous or intraperitoneal administration of α-TOS in ethanol, DMSO or vegetable oil emulsions have been applied[4,6] but these formulations are largely restricted to mouse tumor models and have little clinical relevance. Non-covalent incorporation of α-TOS into liposomal drug delivery systems has so far provided the most promising administration route with regard to clinical applicability.[7] Nevertheless, the non-covalent incorporation strategy suffers from low drug content in liposomes together with an uncontrolled release mechanism and thereby also problems with leakage from the carrier system. An improved drug delivery system is therefore needed in order to make α-TOS clinically applicable for humans.

In the present study incorporation of α-TOS into a recently developed liposomal drug delivery system[8,9] has been investigated. The drug delivery system consists of secretory phospholipase A_2 ($sPLA_2$) IIA degradable liposomes, in which α-TOS is attached



α-TOS: R = CH_3
δ-TOS: R = H

Figure 1. Structure of the potent antiproliferative compounds α-tocopheryl succinate (α-TOS) and δ-tocopheryl succinate (δ-TOS).

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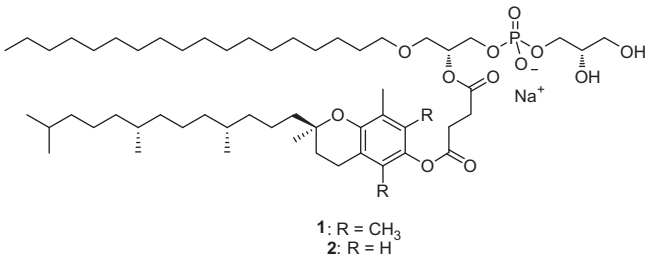


Figure 2. Structure of the targeted TOS phospholipid conjugates.

covalently to the *sn*-2 position of the phospholipid backbone, see Figure 2. $sPLA_2$ IIA is part of the PLA_2 superfamily, which consists of a broad range of enzymes defined by their ability to catalyze the hydrolysis of the ester bond at the *sn*-2 position in phospholipids yielding the carboxylic acid and lysolipids.[10,11] Overexpression of $sPLA_2$ IIA in human tumors[12,13] have, together with the enhanced permeability and retention (EPR) effect,[14,15] made selective delivery of anticancer agents to cancer tissue possible by the use of $sPLA_2$ sensitive liposomes.[16,17] Advantageously, this liposomal formulation will scavenge the biologically important carboxylate moiety in α-TOS by formation of an ester bond whereby only tissue with an over-expression of $sPLA_2$ will be affected by the circulating liposomes.

In addition to α-TOS, the less potent δ-tocopheryl succinate.[3] (δ-TOS, Fig. 1) was included in the investigation in order to provide additional information concerning enzyme activity and cytotoxocity. The δ-TOS phospholipid conjugate (**2**) is shown in Figure 2.

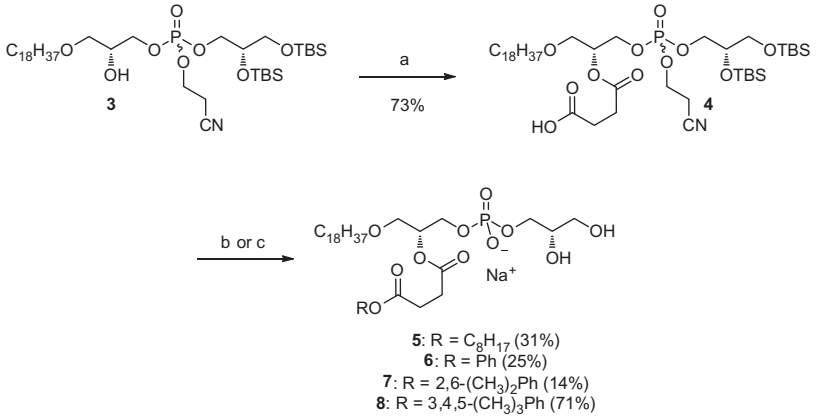
Previous studies have shown that $sPLA_2$ is less reactive towards substrates with substituents in close proximity of the *sn*-2 position,[18–21] and therefore we decided to investigate the $sPLA_2$ activity towards phospholipids with a succinate moiety in the *sn*-2 position prior to studying the TOS phospholipid conjugates. In addition to the alkyl succinate phospholipid conjugate **5** (Scheme 1) we also opted to study the enzyme activity on the phenyl succinate phospholipid conjugates **6**–**8** (Scheme 1). This was done in order to investigate the importance of the methyl-substitution on the aromatic ring for the enzymatic activity. The extensively substituted phenyl-moiety in α-TOS provides a bulky environment close to the active site, which may lead to an imperfect positioning of the phospholipid in the active site of $sPLA_2$ and/or block the entrance of water via the lipophilic channel in the enzyme.[20,22]

2. Results and discussion

2.1. Synthesis of alkyl and phenyl succinate phospholipid conjugates and $sPLA_2$ activity studies

The synthesis of the phospholipids having a succinate moiety in the *sn*-2 position was initiated from the known phospholipid precursor **3**.[23] Acylation of **3** with benzyl succinate using dicyclohexylcarbodiimide (DCC) and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) in CH_2Cl_2 gave the desired succinate. Hydrogenolysis of the benzyl ester using Pd/C in a 1:1 mixture of MeOH and EtOAc afforded the carboxylic acid **4** in 73% overall yield from **3** (Scheme 1). Precursor **4** was diversified into the desired phospholipids **5–8** by first a carbodiimide mediated coupling with the corresponding alcohol or phenol, followed by removal of the cyanoethyl and *tert*-butyl dimethylsilyl (TBS) protection groups on the glycerol head group using the reaction conditions previously described (Scheme 1).[8,9] Phospholipid **8** was made applying a Yamaguchi coupling[24] in the esterification of **4** with 3,4,5-trimethylphenol.

The phospholipids **5–8** were formulated as liposomes in 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer by extrusion[25] and dynamic light scattering (DLS) analysis revealed that particles with diameters close to 100 nm were formed, indicating formation of liposomes. The formulated solutions of **5–8** were subjected to snake (*Naja mossambica mossambica*) venom $sPLA_2$ and incubated at 37 °C for 48 h. Samples were taken after 2, 24 and 48 h and the enzyme activity was monitored by matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS) using a 2,5-dihydroxy benzoic acid (DHB) matrix.[8,26] As evident from Figure 3 the octyl succinate phospholipid **5** was completely degraded by the enzyme within 24 h, demonstrating that the succinate moiety itself is tolerated by $sPLA_2$. The enzyme activity on the phenyl succinate phospholipids **6**, **7** and **8** was almost as good as on **5**, although small peaks for the starting phospholipids remain in the MS spectra (Fig. 3), but the degree of hydrolysis is still >70% for **6–8**. The MS spectra also confirmed that the desired lysolipid was released in the enzyme catalyzed hydrolysis (see Supplementary data). In the absence of $sPLA_2$, no degradation of the phospholipids was detected (Fig. 3). From the activity studies on **5–8** it can therefore be concluded that the incorporation of a succinate moiety into the *sn*-2 position does not obstruct the ability of $sPLA_2$ to liberate molecules from the *sn*-2 position. However, alkyl succinates are slightly better substrates for the enzyme than phenyl succinates, but as the extent of degradation for the phenyl succinate phospholipids were >70%



Scheme 1. Synthesis of the succinate phospholipids **5**, **6**, **7** and **8**. Reagents: (a) (i) Benzyl succinate, DCC, DMAP, CH_2Cl_2; (ii) H_2, Pd/C, EtOAc, MeOH; (b) (i) $C_8H_{17}OH$, PhOH, or 2,6-dimethylphenol, DCC, DMAP, CH_2Cl_2; (ii) DBU, CH_2Cl_2; (iii) HF, H_2O, CH_2Cl_2, MeCN; (c) (i) 3,4,5-trimethylphenol, 2,4,6-$Cl_3C_6H_2COCl$, DMAP, THF; (ii) DBU, CH_2Cl_2; (iii) HF, H_2O, CH_2Cl_2, MeCN.

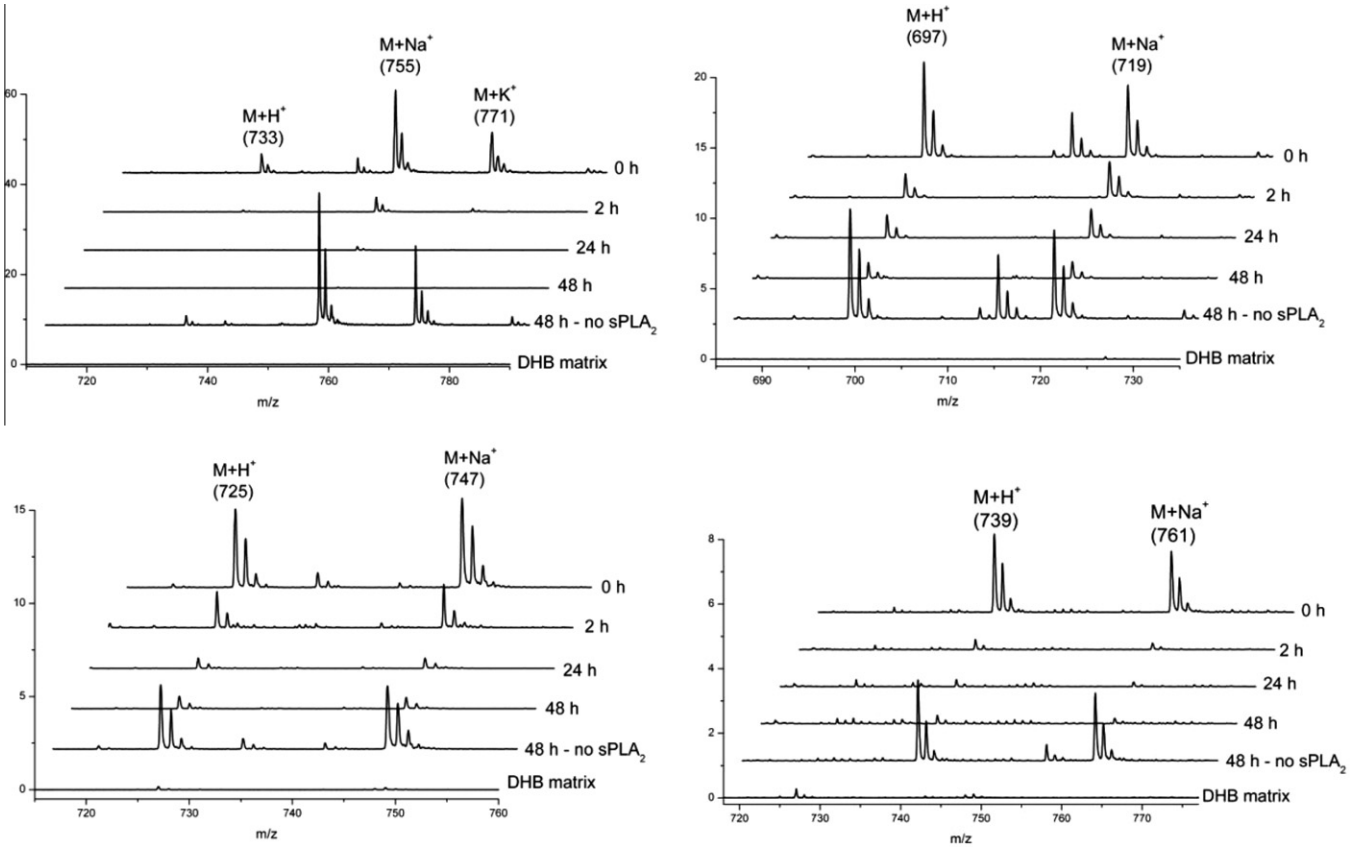


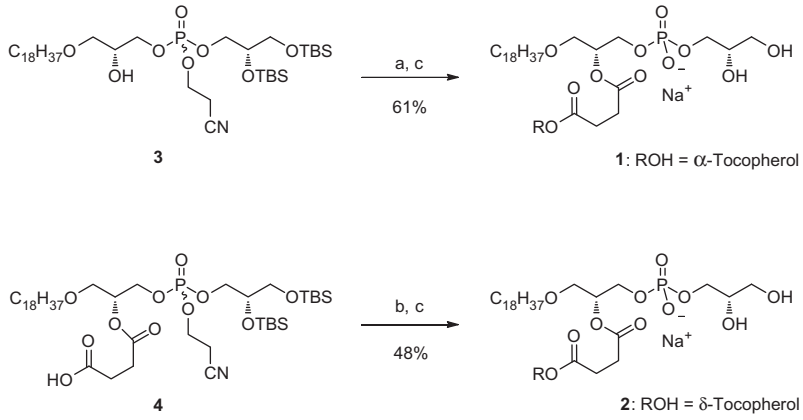
Figure 3. MALDI-TOF MS monitoring of snake (*Naja mossambica mossambica*) venom $sPLA_2$ activity on the succinate phospholipid **5** (top left), **6** (top right), **7** (bottom left) and **8** (bottom right).

we found it relevant to continue with the incorporation of α-TOS and δ-TOS.

2.2. Synthesis, enzyme activity and cytotoxicity of tocopheryl succinate phospholipid conjugates

The α-TOS phospholipid conjugate **1** was accessible in three synthetic steps from the secondary alcohol **3** (Scheme 2). Acylation of **3** with α-TOS gave the desired ester bond and the following deprotections of the cyanoethyl and the TBS groups were achieved using the same conditions as in synthesis of **5–8** (Scheme 1), giving the phospholipid conjugate **1** in 61% overall yield. In a similar fashion the synthesis of the δ-TOS phospholipid conjugate **2** was achieved from succinate **4** in 48% yield over the three steps (Scheme 2).

The TOS phospholipid conjugates **1** and **2** were hydrated in HEPES buffer yielding milky solutions and differential scanning calorimetry (DSC) scans (20–65 °C) were obtained. However, no main phase transition temperature was observed in the tested range, suggesting that **1** and **2** could be in a fluid state at 20 °C. These findings were further supported during the formulation where it was possible to extrude the solutions of **1** and **2** through a 100 nm filter at 20 °C, yielding clear and transparent solutions. DLS analysis of the formulated phospholipids revealed that particles with an average diameter of 115 nm were formed indicating formation of unilamellar vesicles.



Scheme 2. Synthesis of the phospholipid conjugate **1** and **2**. Reagents: (a) (i) α-TOS, EDCI, DMAP, EtOAc, CH_2Cl_2; (b) (i) δ-Tocopherol, 2,4,6-$Cl_3C_6H_2COCl$, DMAP, THF; (c) (i) DBU, CH_2Cl_2; (ii) HF, H_2O, CH_2Cl_2, MeCN.

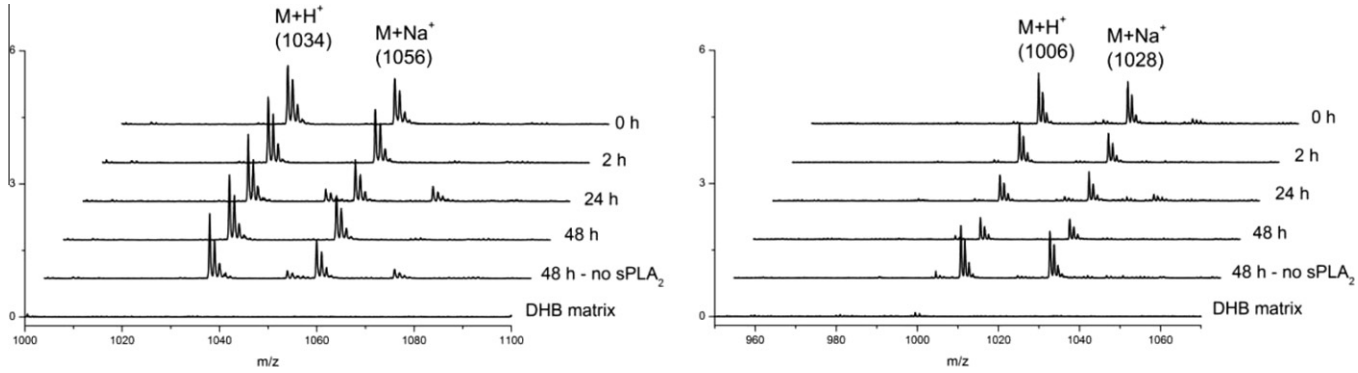


Figure 4. MALDI-TOF MS monitoring of snake (*Naja mossambica mossambica*) venom $sPLA_2$ activity on the TOS conjugates **1** (left) and **2** (right).

The $sPLA_2$ activity was investigated with snake (*Naja mossambica mossambica*) venom $sPLA_2$ and human $sPLA_2$. Upon addition of the enzymes the phospholipid solutions were incubated at 37 °C for 48 h and the activity was monitored by MALDI-TOF MS. However, as evident from the MS spectra in Figure 4 (see also Supplementary data), the α-TOS conjugate **1** is a very poor substrate for the enzyme and based on the MS spectra for **1** no significant degradation occurred within 48 h, neither by using the snake venom nor the human $sPLA_2$. Based on the MS spectra the δ-TOS conjugate **2** is a slightly better substrate for the enzyme and significant hydrolysis is observed after 24 and 48 h (see Fig. 4), which is confirmed by detection of the lysolipid and δ-TOS in the MS spectra (see Supplementary data).

The poor activity of $sPLA_2$ on **1** and **2** were confirmed by evaluation of the cytotoxicity in U2OS human osteosarcoma cells. U2OS cells are free of $sPLA_2$, which allowed us to explore the cytotoxicity both in the presence and absence of the enzyme. As evident from Figure 5 (and Supplementary data) no significant cell death was observed when **1** or **2** were subjected to the cells neither with nor without $sPLA_2$. In contrast, the TOS's and the lysolipid alone induced significantly higher cell death than **1** and **2** verifying the low extent of hydrolysis.

In order to rule out the possibility that small amounts of liberated TOS is acting as an inhibitor of the enzyme and preventing further degradation, we determined the IC_{50} of **1** to 40 μM, significantly higher than an all-*trans* retinoic acid derivative (IC_{50}: 8 μM), which has previously been successfully formulated and released by $sPLA_2$ (see Supplementary data). [9] To further ensure that the lack of hydrolysis observed was not due to inhibition of the enzyme, we co-formulated (1:1) **1** and **2** with 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol (DSPG), a known substrate for $sPLA_2$. The mixed liposomes were subjected to snake and human $sPLA_2$ (see Supplementary data) and the experiments showed that DSPG was degraded efficiently by the enzymes, whereas neither **1** nor **2** were hydrolyzed, similar to the monocomponent liposomes described above. Taken together, these experiments demonstrate that the inertness to $sPLA_2$ degradation displayed by **1** and **2** is inherent to their structure and not an effect stemming from the properties of the formulated liposomes or inhibition of the enzyme.

Based on the enzyme activity data for the succinate conjugates **5**–**8** and the TOS's conjugates **1** and **2** the difference in enzyme activity cannot directly be explained by the substituted phenyl moiety in tocopherols. Rather, it is apparent that the long lipophilic tail in tocopherols either blocks the access of water or prevents the phospholipids **1** and **2** from obtaining a productive positioning in the active site. That structural motifs so far away from the active site can influence the enzyme activity in such a strong manner

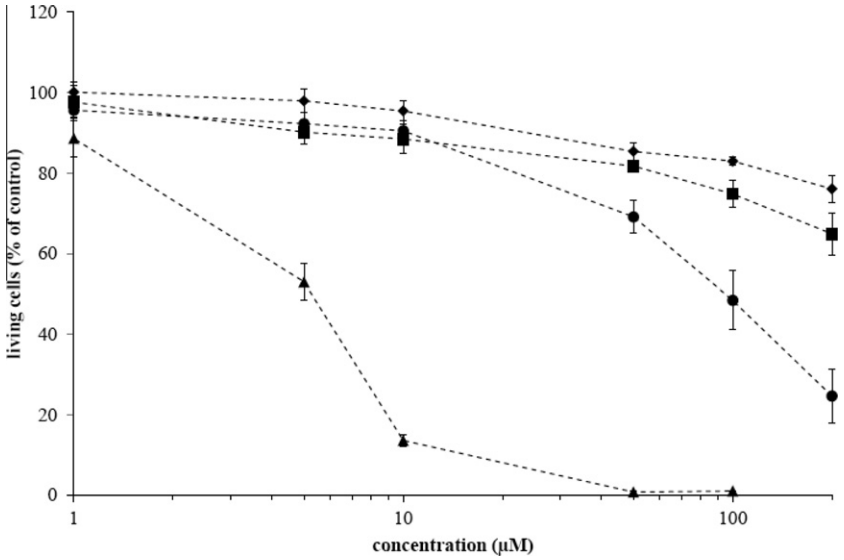


Figure 5. Dose-response curves for the treatment of U2OS cells with **1** (♦), 1 + $sPLA_2$ (■), lysolipid (●) and α-TOS (▲). The cytotoxicity was measured using the MTT assay as cell viability 48 h after incubation with the indicated substances for 24 h and shown by mean + SD (n = 3). Snake (*Agkistrodon piscivorus piscivorus*) venom $sPLA_2$ was added to a final concentration of 5 nM in the studies.

has never been observed for $sPLA_2$ and the current findings are valuable in the efforts to better understand the ability of $sPLA_2$ to bind and hydrolyze a broad range of phospholipid substrates.

3. Conclusion

In conclusion we have shown that *sn*-2 succinate phospholipid conjugates can form liposomes that are good substrates for $sPLA_2$ when they are either alkyl or phenyl substituted. However, low enzyme activity was observed for the α-TOS and δ-TOS conjugates **1** and **2**, and taking the results from the enzyme studies on phospholipid **5–8** into account, the slow hydrolysis of **1** and **2** cannot directly be associated with neither the phenyl ring nor methyl substituents in tocopherols, and therefore we hypothesize that the long lipophilic tail in combination with the ring systems in TOS's influences the enzyme activity. This means that liposomal formulation of tocopherol succinates for $sPLA_2$-triggered release is not viable and future efforts would have to consider other approaches, such as non-covalent incorporation in liposomes[7,27] or the use of alternative enzymes for triggered release, for example matrix metalloproteinases.[28] Nevertheless, the overall conclusion from the present work is that succinate conjugation provides a promising opportunity for liposomal delivery of lipophilic alcohols, which may otherwise lack administration routes due to low water solubility.

4. Experimental procedures

4.1. Organic synthesis

Starting materials, reagents, solvents and $sPLA_2$ from *Naja mossambica mossambica* were purchased from Sigma–Aldrich Chemical Co. and used without further purification. CH_2Cl_2 was dried over 4 Å molecular sieves and THF was dried over sodium/benzophenone and distilled before use. Evaporation of solvents was done under reduced pressure (in vacuo). Thin layer chromatography (TLC) was performed on Merck aluminum sheets precoated with silica gel 60 F_{254}. Column chromatography was performed using Matrex 60 Å silica gel. The purity of all tested compounds was found to be >95% by HPLC. HPLC was performed on a Waters Alliance HPLC equipped with a diode array detector, using a LiChrospher Si 60 column and eluting with water/isopropanol/hexane mixtures.[29,30] NMR spectra were recorded using a Bruker AC 200 MHz spectrometer, a Varian Mercury 300 MHz spectrometer or a Varian Unity Inova 500 MHz spectrometer. Chemical shifts were measured in ppm and coupling constants in Hz, and the field is indicated in each case. IR analysis was carried out on a Bruker Alpha FT-IR spectrometer. HRMS was recorded on an Ionspec Ultima Fourier transform mass spectrometer.

4.1.1. 1-*O*-Octadecyl-2-(4-(α-tocopheroxy)-4-oxobutanoyl)-sn-glycero-3-phospho-(*S*)-glycerol (1)

Alcohol **3** (48 mg, 0.062 mmol), α-tocopheryl succinate (0.032 M in EtOAc, 1.5 mL, 0.048 mmol) and DMAP (18 mg, 0.14 mmol) were dissolved in CH_2Cl_2 (4 mL). EDCI (28 mg, 0.14 mmol) was added and the reaction mixture was stirred for 1 h at 20 °C, then concentrated in vacuo and purified by column chromatography (heptane/EtOAc 2:1 then heptane/EtOAc 1:2) to give 61 mg of the desired ester, which was dissolved in CH_2Cl_2 (1.3 mL) along with DBU (8 μL, 0.050 mmol). The resulting mixture was stirred at 20 °C for 40 min and then purified directly by column chromatography (CH_2Cl_2/MeOH 10:1) to afford 41 mg that was dissolved in MeCN (2.3 mL) and CH_2Cl_2 (0.8 mL) and cooled to 0 °C. Aqueous HF (40%, 0.14 mL) was added dropwise and the reaction mixture was allowed to reach 20 °C. After 3 h the reaction was quenched by dropwise addition of $MeOSiMe_3$ (0.6 mL) and the mixture was stirred for 30 min, after which solid $NaHCO_3$ (5 mg, 0.059 mmol) was added and the mixture was concentrated in vacuo and purified by column chromatography (CH_2Cl_2/MeOH 20:1 then CH_2Cl_2/MeOH 4:1) to give 30 mg (61%) of **1** as a colorless amorphous solid. R_f = 0.09 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$ 4:1): δ 5.22–5.14 (m, 1H), 4.05–3.98 (m, 2H), 3.98–3.90 (m, 2H), 3.86–3.79 (m, 1H), 3.67–3.56 (m, 4H), 3.47–3.40 (m, 2H), 2.98–2.90 (m, 2H), 2.83–2.76 (m, 2H), 2.60 (t, *J* = 6.4 Hz, 2H), 2.09 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), 1.84–1.74 (m, 2H), 1.59–1.48 (m, 8H), 1.47–1.01 (m, 48H), 0.91–0.83 (m, 15H). ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ 4:1): δ 172.4, 171.8, 149.7, 140.7, 126.9, 125.2, 123.3, 117.8, 75.4, 72.6, 72.1, 71.1, 69.1, 66.7, 64.4, 62.4, 39.7 (2C), 37.7 (3C), 37.6, 33.1, 33.0, 32.2, 31.4, 30.0 (13C), 29.3, 29.0, 28.2, 26.3, 25.1, 24.7, 24.2/23.7[31], 23.0, 22.9, 22.8, 21.3, 20.9, 20.0, 19.9, 14.3, 13.1, 12.2, 12.0. ^{31}P NMR (202 MHz, $CDCl_3/CD_3OD$ 4:1): δ 3.43. IR (neat) 3423, 2923, 2853, 1722, 1696, 1660, 1397, 1264, 1062, 804 cm^{-1}. HRMS (ESI+) calcd for $C_{57}H_{103}O_{12}PNa$ $[M+H]^+$ 1033.7085, found 1033.7094.

4.1.2. 1-*O*-Octadecyl-2-(4-(δ-tocopheroxy)-4-oxobutanoyl)-sn-glycero-3-phospho-(*S*)-glycerol (2)

Carboxylic acid **4** (70 mg, 0.084 mmol) and δ-tocopherol (50 mg, 0.12 mmol) were dissolved in THF (1.1 mL). 2,4,6-Trichlorobenzoyl chloride (17 μL, 0.11 mmol) and then DMAP (15 mg, 0.12 mmol) were added and the reaction mixture was stirred at 20 °C for 17 h, after which the mixture was concentrated in vacuo and then purified by column chromatography (heptane/EtOAc 4:1 then heptane/EtOAc 2:1) to give 65 mg of the desired ester that was dissolved in CH_2Cl_2 (4.8 mL) along with DBU (8 μL, 0.053 mmol). The resulting mixture was stirred for 1 h and then purified directly by column chromatography (heptane/EtOAc 1:1 then CH_2Cl_2/MeOH 9:1) to give 59 mg of a colorless oil, which was dissolved in a mixture of MeCN (4.3 mL) and CH_2Cl_2 (1.5 mL) and cooled to 0 °C. Aqueous HF (40%, 255 μL) was added dropwise and the reaction mixture was allowed to reach 20 °C. After 3.5 h, excess reagent was quenched by dropwise addition of $MeOSiMe_3$ (1.0 mL) and the mixture was stirred for 30 min after which solid $NaHCO_3$ (13 mg) was added and the mixture was concentrated in vacuo and purified by column chromatography (CH_2Cl_2/MeOH 10:1 then CH_2Cl_2/MeOH 4:1) to give 40 mg (48%) of **2** as a colorless amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$ 4:1): δ 6.66 (d, *J* = 2.7 Hz, 1H), 6.62 (d, *J* = 2.7 Hz, 1H), 5.24–5.15 (m, 1H), 4.07–3.91 (m, 4H), 3.89–3.81 (m, 1H), 3.67–3.57 (m, 4H), 3.47–3.38 (m, 2H), 2.88–2.71 (m, 6H), 2.14 (s, 3H), 1.86–1.72 (m, 2H), 1.62–1.02 (m, 56H), 0.91–0.84 (m, 15H). ^{13}C NMR (50 MHz, $CDCl_3/CD_3OD$ 4:1): δ 170.6, 170.3, 148.2, 140.7, 125.6, 119.4 (2C), 117.3, 75.0, 70.6, 70.2, 69.2, 67.1, 64.9, 62.7, 60.5, 38.5, 37.7, 35.7 (3C), 31.1 (2C), 30.3, 30.0, 29.3 (19C), 28.0, 26.3, 24.3, 23.1, 22.8, 22.3, 20.9, 19.3, 18.0, 14.3, 12.3. ^{31}P NMR (202 MHz, $CDCl_3/CD_3OD$ 4:1): δ −1.93. IR (neat) 3368, 2922, 2852, 1738, 1466, 1222, 1148, 1046 cm^{-1}. HRMS (ESI+) calcd for $C_{55}H_{99}O_{12}PNa$ $[M+H]^+$ 1005.6766, found 1005.6736.

4.1.3. 1-*O*-Octadecyl-2-(4-hydroxy-4-oxobutanoyl)-*sn*-glycero-3-(2-cyanoethylphospho)-(*S*)-2,3-di-*O*-*tert*-butyldimethylsilyl-glycerol (4)

Alcohol **3** (96 mg, 0.12 mmol) and benzyl succinate (51 mg, 0.25 mmol) were dissolved in CH_2Cl_2 (2.5 mL). DCC (76 mg, 0.37 mmol) and DMAP (15 mg, 0.12 mmol) were added and the reaction mixture was stirred at 20 °C for 1 h, then concentrated in vacuo and purified by column chromatography (heptane then heptane/EtOAc 2:1) to afford 81 mg of the desired ester, which was dissolved in a mixture of EtOAc (0.7 mL) and MeOH (0.7 mL). Pd/C (10 wt%, 12 mg, 0.012 mmol) was added and the mixture was stirred under an atmosphere of H_2 for 2 h at 20 °C, after which filtration through celite gave 74 mg (73%) of **4** (two diastereoisomers 1:1) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$, two diastereoisomers): δ

5.23–5.16 (m, 1H), 4.32–4.13 (m, 5H), 4.06–3.97 (m, 1H), 3.92–3.84 (m, 1H), 3.62–3.53 (m, 4H), 3.48–3.39 (m, 2H), 2.77 (t, J = 6.3 Hz, 2H), 2.70–2.64 (m, 4H), 1.59–1.49 (m, 2H), 1.32–1.23 (m, 30H), 0.92–0.86 (m, 21H), 0.10 (s, 3H), 0.09 (s, 3H), 0.07 (s, 6H). ^{13}C NMR (50 MHz, $CDCl_3$, two diastereoisomers): δ 175.1, 171.5, 116.5, 72.1 (2C), 71.2, 69.7, 68.2, 66.7, 64.1, 62.2, 32.1, 29.8 (13C), 29.0 (2C), 26.0 (7C), 22.8, 19.6, 18.4 (2C), 14.2, −4.6 (2C), −5.3 (2C). ^{31}P NMR (202 MHz, $CDCl_3$, two diastereoisomers): δ −0.94, −1.13. IR (neat): 2924, 2854, 1737, 1463, 1252, 1152, 1102. HRMS (ESI+) calcd for $C_{43}H_{86}NO_{11}PSi_2$ $[M+H]^+$ 880.5793, found 880.5822.

4.1.4. 1-*O*-Octadecyl-2-(4-octanyloxy-4-oxobutanoyl)-sn-glycero-3-phospho-(*S*)-glycerol (5)

Carboxylic acid **4** (26 mg, 0.031 mmol) and octanol (7.2 μL, 0.045 mmol) were dissolved in CH_2Cl_2 (0.5 mL). DCC (8 mg, 0.04 mmol) and DMAP (2 mg, 0.016 mmol) were added and the reaction mixture was stirred at 20 °C for 1 h, after which the mixture was purified directly by column chromatography (heptane/EtOAc 4:1 then heptane/EtOAc 2:1) to give 12 mg of the desired ester, that was dissolved in CH_2Cl_2 (1.1 mL) along with DBU (1.9 μL, 0.013 mmol). The resulting mixture was stirred for 1 h and then purified directly by column chromatography (heptane/EtOAc 1:1 then CH_2Cl_2/MeOH 9:1) to give 11 mg of a colorless oil, that was dissolved in a mixture of MeCN (1.0 mL) and CH_2Cl_2 (0.33 mL) and cooled to 0 °C. Aqueous HF (40%, 60 μL) was added dropwise and the reaction mixture was allowed to reach 20 °C. After 3.5 h excess reagent was quenched by dropwise addition of $MeOSiMe_3$ (225 μL) and the mixture was stirred for 30 min, after which solid $NaHCO_3$ (3 mg, 0.035 mmol) was added and the mixture was concentrated in vacuo and purified by column chromatography (CH_2Cl_2/MeOH 10:1 then CH_2Cl_2/MeOH 4:1) to give 7 mg (31%) of **5** as a colorless amorphous solid. R_f = 0.38 (CH_2Cl_2/MeOH 4:1). ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD 4:1): δ 5.18–5.11 (m, 1H), 4.08 (t, J = 6.8 Hz, 2H), 3.97–3.90 (m, 4H), 3.85–3.79 (m, 1H), 3.66–3.62 (m, 2H), 3.60–3.55 (m, 2H), 3.47–3.40 (m, 2H), 2.68–2.61 (m, 4H), 1.67–1.58 (m, 2H), 1.58–1.50 (m, 2H), 1.35–1.23 (m, 40H), 0.89 (t, J = 6.0 Hz, 3H), 0.88 (t, J = 5.8 Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$/CD_3OD 4:1): δ 172.3, 172.0, 71.8 (2C), 70.4, 68.1, 67.3, 65.1 (2C), 62.2, 31.7 (2C), 29.6 (18C), 25.8, 24.0, 22.5 (2C), 13.8 (2C). ^{31}P NMR (202 MHz, $CDCl_3$/CD_3OD 4:1): δ −1.61. IR (neat): 3352, 2922, 2853, 1736, 1220, 1162, 1118, 1053. HRMS (ESI−) calcd for $C_{36}H_{70}O_{11}P$ $[M-Na]^-$ 709.4660, found 709.4687.

4.1.5. 1-*O*-Octadecyl-2-(4-phenyloxy-4-oxobutanoyl)-sn-glycero-3-phospho-(*S*)-glycerol (6)

The synthesis was performed as for **5**, starting from carboxylic acid **4** (30 mg, 0.034 mmol) and phenol (10 mg, 0.10 mmol) and gave 6 mg (25%) of **6** as a colorless amorphous solid. R_f = 0.14 (CH_2Cl_2/MeOH 4:1). ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD 4:1): δ 7.38 (t, J = 7.8 Hz, 2H), 7.24 (t, J = 7.8 Hz, 1H), 7.09 (d, J = 7.8 Hz, 2H), 5.22–5.17 (m, 1H), 4.05–3.96 (m, 2H), 3.93–3.89 (m, 2H), 3.79–3.76 (m, 1H), 3.63–3.57 (m, 4H), 3.47–3.38 (m, 2H), 2.90 (t, J = 6.7 Hz, 2H), 2.79 (t, J = 6.7 Hz, 2H), 1.55–1.49 (m, 2H), 1.31–1.22 (m, 30H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$/CD_3OD 4:1): δ 172.5, 171.7, 150.9, 129.8 (2C), 126.3, 121.8 (2C), 72.6, 72.2, 71.2, 69.2, 66.8, 64.5, 62.5, 32.2, 30.0 (15C), 26.3, 23.0, 14.3. ^{31}P NMR (202 MHz, $CDCl_3$/CD_3OD 4:1): δ −0.64. IR (neat): 3333, 2922, 2852, 1763, 1737, 1251, 1198, 1131, 1068. HRMS (ESI+) calcd for $C_{34}H_{58}O_{11}P$ $[M-Na]^-$ 673.3721, found 673.3702.

4.1.6. 1-*O*-Octadecyl-2-(4-(2,6-dimethylphenyloxy)-4-oxobutanoyl)-sn-glycero-3-phospho-(*S*)-glycerol (7)

The synthesis was performed as for **5**, starting from carboxylic acid **4** (71 mg, 0.086 mmol) and 2,6-dimethylphenol (32 mg, 0.26 mmol) and gave 9 mg (14%) of **7** as a colorless amorphous solid. R_f = 0.09 (CH_2Cl_2/MeOH 4:1). ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD 4:1): δ 7.06–7.05 (m, 3H), 5.20–5.15 (m, 1H), 4.05–3.97 (m, 2H), 3.95–3.89 (m, 2H), 3.82–3.77 (m, 1H), 3.63–3.56 (m, 4H), 3.47–3.38 (m, 2H), 2.95 (t, J = 6.7 Hz, 2H), 2.80 (t, J = 6.7 Hz, 2H), 2.14 (s, 6H), 1.55–1.49 (m, 2H), 1.30–1.22 (m, 30H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$/CD_3OD 4:1): δ 172.4, 171.1, 148.3, 130.4 (2C), 129.0 (2C), 126.3, 72.6, 72.2, 71.2, 69.2, 66.8, 64.5, 62.5, 32.2, 30.0, 29.3 (13C), 28.9, 26.3, 23.0, 16.4 (2C), 14.3. ^{31}P NMR (202 MHz, $CDCl_3$/CD_3OD 4:1): δ −2.62. IR (neat): 3331, 2922, 2853, 1753, 1738, 1233, 1139, 1058. HRMS (ESI−) calcd for $C_{36}H_{62}O_{11}P$ $[M-Na]^-$ 701.4035, found 701.4017.

4.1.7. 1-*O*-Octadecyl-2-(4-(3,4,5-trimethylphenyloxy)-4-oxobutanoyl)-sn-glycero-3-phospho-(*S*)-glycerol (8)

Carboxylic acid **4** (70 mg, 0.084 mmol) and 3,4,5-trimethylphenol (16 mg, 0.12 mmol) were dissolved in THF (1.1 mL). 2,4,6-Trichlorobenzoyl chloride (17 μL, 0.11 mmol) and then DMAP (15 mg, 0.12 mmol) were added and the reaction mixture was stirred at 20 °C for 17 h, after which the mixture was concentrated in vacuo and purified by column chromatography (heptane/EtOAc 4:1 then heptane/EtOAc 2:1) to give 65 mg of the desired ester, that was dissolved in CH_2Cl_2 (6.0 mL) along with DBU (10 μL, 0.068 mmol). The reaction mixture was stirred for 1 h and then purified directly by column chromatography (heptane/EtOAc 1:1 then CH_2Cl_2/MeOH 9:1) to give 54 mg of a colorless oil that was dissolved in a mixture of MeCN (5.5 mL) and CH_2Cl_2 (1.9 mL) and cooled to 0 °C. Aqueous HF (40%, 325 μL) was added dropwise and the reaction mixture was allowed to reach 20 °C. After 3.5 h excess reagent was quenched by dropwise addition of $MeOSiMe_3$ (1.2 mL) and the mixture was stirred for 30 min, after which solid $NaHCO_3$ (16 mg) was added, the mixture was concentrated in vacuo and purified by column chromatography (CH_2Cl_2/MeOH 10:1 then CH_2Cl_2/MeOH 4:1) to give 44 mg (71%) of **8** as a colorless amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD 4:1): δ 6.71 (s, 2H), 5.22–5.15 (m, 1H), 4.06–3.90 (m, 4H), 3.88–3.80 (m, 1H), 3.67–3.56 (m, 4H), 3.47–3.37 (m, 2H), 2.89–2.84 (m, 2H), 2.79–2.73 (m, 2H), 2.27 (s, 6H), 2.14 (s, 3H), 1.58–1.48 (m, 2H), 1.35–1.20 (m, 30H), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR (50 MHz, $CDCl_3$/CD_3OD 4:1): δ 171.6, 171.2, 147.3, 137.2 (2C), 132.3, 119.6 (2C), 71.4 (2C), 70.2, 68.0, 66.8, 64.5, 61.7, 31.4, 29.2 (14C), 28.6, 25.5, 22.2, 19.9 (2C), 14.3, 13.5. ^{31}P NMR (202 MHz, $CDCl_3$/CD_3OD 4:1): δ −1.56. IR (neat): 3362, 2922, 2852, 1738, 1465, 1147, 1045 cm^{-1}. HRMS (ESI+) calcd for $C_{37}H_{65}NaO_{11}P$ $[M+H]^+$ 739.4157, found 739.4144.

4.1.8. δ-Tocopheryl succinate (δ-TOS)

δ-Tocopherol (30 mg, 0.075 mmol), succinic anhydride (19 mg, 0.19 mmol) and DMAP (9 mg, 0.075 mmol) were dissolved in CH_2Cl_2 (0.3 mL). The reaction mixture was stirred at 20 °C for 14 h, concentrated in vacuo and purified by column chromatography (heptane/EtOAc 3:1 then CH_2Cl_2/MeOH 9:1) to give 26 mg (68%) of δ-TOS as a colorless amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.68 (d, J = 2.6 Hz, 1H), 6.63 (d, J = 2.6 Hz, 1H), 2.88–2.68 (m, 6H), 2.14 (s, 3H), 1.85–1.68 (m, 2H), 1.61–1.02 (m, 24H), 0.87 (d, J = 6.8 Hz, 6H), 0.86 (d, J = 6.5 Hz, 3H), 0.85 (d, J = 6.8 Hz, 3H).[3]

4.2. Procedures for biophysical and biological characterization

4.2.1. Liposome preparation and particle size determination

The phospholipid prodrugs were dissolved in $CHCl_3$ in a test tube and dried under vacuum for 15 h to form a thin film. The phospholipid prodrugs (2 mM) were solubilized by addition of aqueous buffer (0.15 M KCl, 30 μM $CaCl_2$, 10 μM EDTA, 10 mM HEPES, pH 7.5) and vortexed periodically over 1 h at 20 °C. Subsequently, the solutions were extruded through a 100 nm polycarbonate cutoff membrane (20–30 repetitions) using a Hamilton syringe extruder (Avanti Polar Lipids, Birmingham, AL). The

particle size distribution of the formulated lipids was measured by DLS. The DLS measurements were obtained using a Zetasizer Nano Particle Analyzer (ZS ZEN3600, Malvern Instrument, Westborough, MA). DSC was performed using a MicroCal MC-2 (Northhampton, MA) on samples at a scan rate of 10 °C/h. A DSC scan was also performed on the HEPES buffer solution and was used to subtract a baseline from the thermograms of **1** and **2**.

4.2.2. $sPLA_2$ activity measurements monitored by MALDI-TOF MS

The formulated phospholipid prodrugs (0.40 mL, 1–2 mM) were diluted in an aqueous buffer (0.15 M KCl, 30 μM CaCl2, 10 μM EDTA, 10 mM HEPES, pH 7.5) to a final concentration of 0.32 mM and the mixture was stirred at 37 °C in a test tube protected from light. The catalytic reaction was initiated by addition of snake (*Naja mossambica mossambica* (12 μL, 71 μM)) venom $sPLA_2$ or human $sPLA_2$ IIA (40 μL tear fluid). Sampling was done after 0, 2, 24 and 48 h by collecting 100 μL of the reaction mixture and rapidly mixing it with a solution of $CHCl_3$/MeOH/H_2O/AcOH 4:8:1:1 (0.5 mL) in order to stop the reaction. The mixture was washed with water (0.5 mL) and the organic phase (80 μL) was isolated by extraction and then concentrated in vacuo. For the MALDI-TOF MS analysis the extract was mixed with 9 μL of DHB matrix (0.5 M DHB, 2 mM CF_3COONa, 0.1 mg/mL DSPG or DPPG in MeOH as internal standard), and 0.5 μL of this mixture was used for the MS analysis. Tear fluid was used as the source for human $sPLA_2$ IIA. Tear fluid was collected from healthy adults exposed to tiger balm fumes or freshly cut onions. Tear fluid has a high concentration of $sPLA_2$ IIA and this is the only prevalent $sPLA_2$ species found in tears. The $sPLA_2$ IIA content in tears in healthy subjects (55 μg/ml) is one of the highest amounts of $sPLA_2$ IIA content reported in human secretions.[32–34]

4.2.3. Cytotoxicity

The osteosarcoma cell line U2OS was cultured in DMEM medium supplemented with 10% fetal calf serum and 1% Pen-Strep in a humidified atmosphere containing 5% CO_2. Cells were plated on 96-well plates at a density of 1×10^4 cells per well 24 h prior to addition of the tested compound. α-TOS and δ-TOS were solubilized in DMSO and water (final DMSO concentration $\leqslant$0.5%). Liposomes were diluted in PBS, and initial phospholipid concentrations in the liposome solutions were determined by phosphorus analysis.[35] After 24 h of incubation, the substances were removed and the cells were washed and incubated in complete medium for another 48 h. Cytotoxic activity was assessed using a standard 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Proliferation Kit I, Roche, Germany).[36] Cell viability is expressed as percentage reduction of incorporated MTT.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.024.

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