Preparative transformation of natural phospholipids catalysed by phospholipase D from *Streptomyces*

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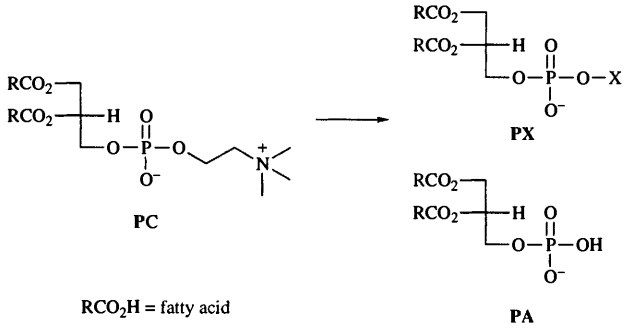
Phospholipase D from *Streptomyces sp.* catalyses the transphosphatidylation reaction of various primary and secondary alcohols with natural phosphatidylcholine in an emulsion system in a preparatively useful manner.

Introduction

Natural and synthetic phospholipids have attracted considerable interest for the multiple scientific and practical applications which follow from their amphiphilic properties. In particular their involvement in the formation of natural membranes and ability for self-organisation in water has stimulated a large body of studies on their physical properties.[1] These investigations rely on material derived from natural sources largely modified by semisynthesis or from compounds obtained by total synthesis. In this field a number of different strategies have been used starting from chiral glycerol derivatives through the extensive application of protecting/deprotecting techniques. This kind of approach has been applied mainly to the synthesis of glycerophospholipids with defined acyl chains equal or different at the two *sn*1 and *sn*2 positions. Modification of the polar head part of the molecule is usually better achieved through enzymic transesterification catalysed by phospholipase D (PLD). In fact the occurrence of rather accessible hydrolytic enzymes from vegetal[2] and microbial[3] sources has allowed the development of a useful biocatalytic approach to modified phospholipids, competing successfully with synthetic ones.[4] PLD has also been directly applied to the transformation of the most abundant natural phospholipid, phosphatidylcholine (PC), into other minor natural products, namely phosphatidylserine (PS),[5] phosphatidylethanolamine (PE)[6] and phosphatidylglycerol (PG).[7] In these transformations the peculiar catalytic ability of PLD from microbial sources to transfer a nucleophile (alcohol) moiety in the presence of water in a transesterification[3-7] has been exploited (Scheme 1).

Notwithstanding the numerous reports devoted to transphosphatidylations catalysed by PLD of different origins, clear knowledge of the catalytic capacity of these enzymes is lacking.[3-8]



Scheme 1 PLD-catalysed transphosphatidylation of PC. *Reagents:* XOH, water, PLD.

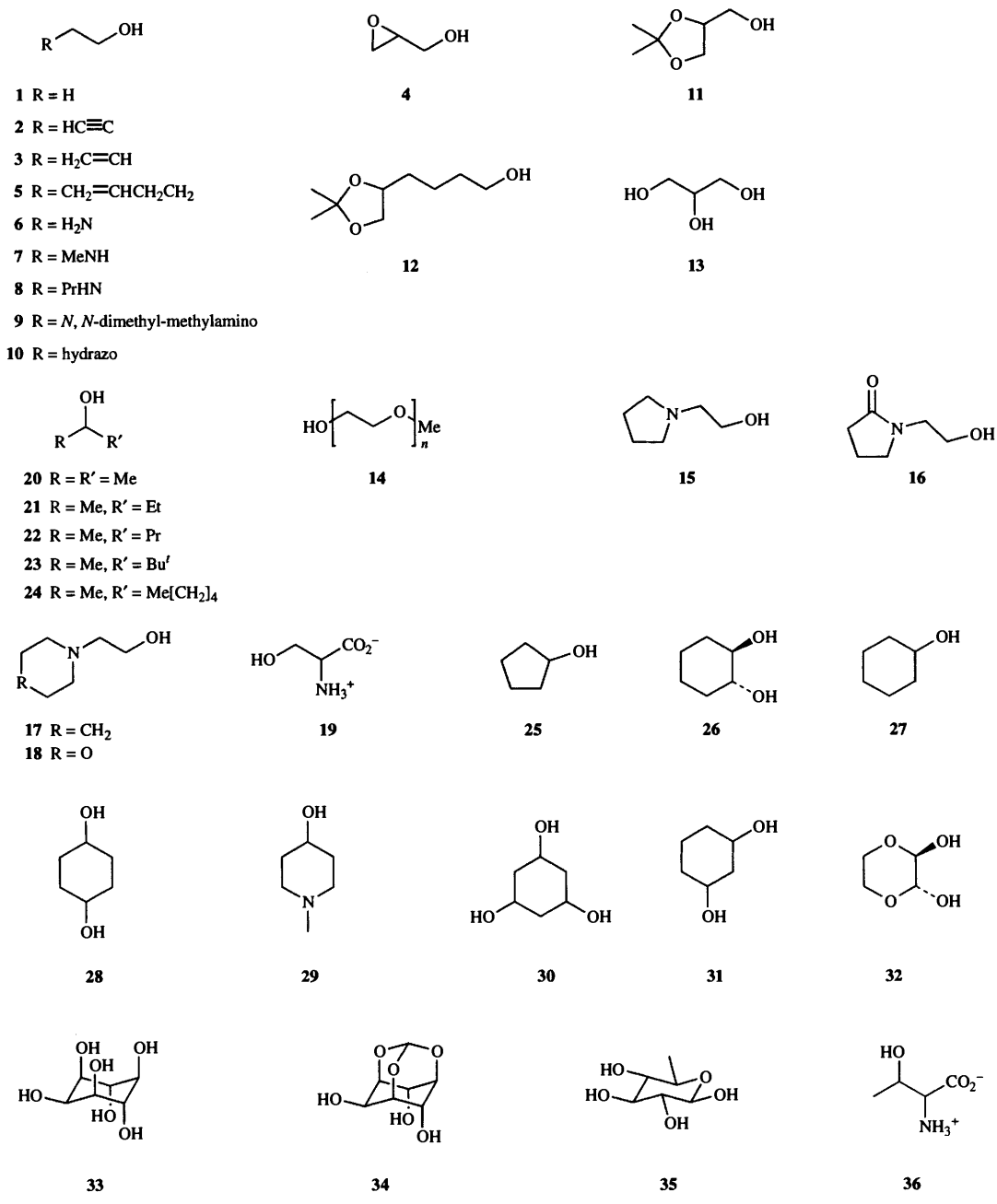
The catalytic capacities of PLD from the leaves of savoy cabbage have been studied. This enzyme is active in the transfer of numerous reactive primary alcohols, but in most cases it displays a prevalent hydrolytic activity. From a synthetic point of view the use of this biocatalyst is effective in the preparation of phosphatidic acid (PA) from other common glycerophospholipids, while the transesterification which can have a preparative significance is the preparation of PG from PC. A much larger substrate tolerance is shown by PLD from *Streptomyces*.[8-10] Enzymes catalysing the transfer of specific head-groups are widely distributed in mammalian cell membranes.[11] The preparative transphosphatidylation using as nucleophile alcohols of complex structure has recently been reported.[8,9,12]

In this article we report on our results on the preparation of modified polar head phospholipids derived from natural PC from soy beans. We also report on the reactions of PC with PLD from *Streptomyces* in the presence of two different groups of alcohols as nucleophiles.[13] First we examined a number of primary alcohols, most of which are homologues of ethanolamine in order to obtain PE analogues and to evaluate the acceptability of unnatural substrates in the PLD-catalysed transphosphatidylation. Besides the intrinsic interest in the ability of individual enzymes to accept different substrates, the preparative value of PE analogues is in relation to the preparation of phosphatidylethanolamine liposomes for gene transfer and immunodiagnostic applications[14a] and for the preparation of sterically stabilised liposomes.[14b] We then considered as possible candidates a set of secondary alcohols of different structures. Secondary alcohols have not been considered before as substrates for PLD.[15] Our investigation of the capacity for transferring secondary alcohols was prompted by the presence in Nature of phosphatidylinositol (PI) with relevant biological implication in signal transduction across membranes[16] and which is not hydrolysed by ordinary PLDs but by PI-specific enzymes. This compound is the only natural phospholipid in which the phosphoric acid function is esterified with a secondary alcoholic group. We wanted therefore to explore the possibility of transforming PC into simple inositol analogues.

Results and discussion

We deliberately used as starting material natural PC† of high purity, instead of phosphatidylcholines with defined fatty acid chains, due to their better solubility in organic solvents and to their natural character as substrates. Moreover the biocatalytic

† PC (soy beans) fatty acid chains' natural composition: palmitic 11.6, stearic 3.4, oleic 4.6, linoleic 66.4, linolenic 8.7%.



Scheme 2 Alcohols used as co-substrates in the transphosphatidylation of natural PC catalysed by PLD in a biphasic system

approach to unnatural phospholipids from readily available starting material from renewable sources adds economic significance to the transformation. Reactions were run in an emulsion system formed by the aqueous phase containing the PLD from *Streptomyces sp.* (10 U) in 1 ml of 0.1 M sodium acetate buffer and 1 M in alcohol,‡ and the organic phase formed by methylene dichloride containing 0.5 mmol of PC. The reaction conditions were dictated from the fact that PC is not water soluble, while most of the alcohols used in our experiments are. In the case of non-water-soluble nucleophiles the use of the organic phase in the presence of the enzyme in a solid or immobilised form invariably led to much longer reaction times and lower conversions. Applications of systems containing substrates and products in an organic phase, and the enzyme confined in a membrane bioreactor, or immobilised, can, however, be advantageous from the point of view of the efficient utilisation of the enzyme. Product formation was followed by HPLC and TLC on silica gel plates. The PX/PA ratio was calculated on the crude mixture from HPLC measurements. New compounds were identified by the combined examination of their ^{1}H, ^{13}C, ^{31}P NMR measurements and from MS data. Owing to the fact that products are non-homogeneous since they are derived from mixtures of natural phospholipids with mixed acyl-chain composition,† the polar head group in the new phospholipid was better characterised by comparison of the MS/MS spectra of the diagnostic fragment ions generated under ammonia or methanol chemical ionisation with those of the corresponding protonated species of the related alcohol measured under the same operating conditions. This gives a fingerprint which allows us to assign unambiguously the structures of new compounds. Moreover $[M - H]^-$ species of new phospholipids have been detected by FAB in negative-ion mode using thioglycerol or tetraethylene glycol matrices. Ions corresponding to the 18:2–16:0 $M_1 = [M - H]^-$ and 18:2–18:2 $M_2 = [M_1 + 24]^-$ combination of acyl chains were detected for all the species described.

‡ Alcohol concentration was calculated on the basis of the volume of the aqueous phase regardless of the distribution of the alcohol between the two phases.

The ease of formation of the transesterification products was evaluated by the $t_{1/2}$ of the starting PC and by the PX/PA ratio at that reaction time.

Unlike the analogous reactions observed with lipases in the interesterification with different acyl donors or with activated esters and alcohols as nucleophiles, which in the presence of water are largely shifted toward the formation of hydrolysis products, and where efficient esterification requires the use of low water activity, in the transesterification with PLD, high yields of the new ester PX can be obtained depending on the substrate used and its concentration in the water medium, if water soluble. It has been reported, for instance, that PG can be obtained without concomitant formation of PA when a 10 M solution of glycerol in the presence of cabbage PLD was used.[7] The PX/PA ratio can therefore be considered as an indicator of the relative affinity of the two competing nucleophiles for the intermediate enzyme–substrate complex.§ Table 1 shows for a number of primary and secondary alcohols the $t_{1/2}$-values, the time in minutes at which half of the starting PC has been transformed, t_f, the time in minutes at which all of the starting PC has been transformed, the PX/PA quotient at $t_{1/2}$, and the purity of the PX obtained from the crude mixture without chromatographic purification. The data show that primary alcohols are effectively transferred with very short $t_{1/2}$-values, ethanol being by far the most efficiently and selectively accepted co-substrate in the PLD-catalysed reaction.[17] In several instances, such as entries 1, 2, 5, 6, 10, 11, 12, 18, the new phospholipids are obtained in high yields with a purity higher than 90%.¶ Since purification of this class of compounds is not straightforward, the high selectivity observed in our cases allows the preparation of the compounds with this technique on a relatively large scale. The limit to reactivity of the alcohols is related in some way with water solubility, while a structure–reactivity relationship is not discernible from our observations. Compounds containing amino groups of different kinds, like **6**, **7** and **8**, are also good substrates. Primary alcohols bearing an aromatic ring in the α or β carbon are not accepted.[18] Secondary alcohols have not been considered before as substrates for PLD from any source. When glycerol is the alcohol donor for the transphosphatidylation, no product of attack with the secondary hydroxy group is observed. However, a series of compounds bearing only secondary alcoholic functions are good substrates. From the data in Table 1 one can observe that secondary alcohols of different nature can be transferred in a preparatively useful way while the efficiency of the transformation appears to be connected with several factors which at the moment have not been rationalised. The value of log *P* for the alcohols used as nucleophile has been calculated,[19] and no correlation with any parameter of the reaction outcome has been obtained. It is important to note that the results in Table 1 refer to a 1 M analytical concentration of the alcohol donor, but higher concentrations usually lead to enhanced selectivity and chemical yields, thus increasing the preparative significance of the method. When alcohol **21** is used at 3 M concentration, the PX/PA quotient raises to 3, while the final yield is 64%. Surprisingly, alcohol **24** is not accepted in the transphosphatidylation while cyclic substrates **25–29**, **31** and **32** are transformed in high rate but with different selectivity. More hydrophilic alcohols are not transferred. This is the case of *myo*-inositol **33** and its orthoester **34**, as well as L-rhamnose **35**. The cyclic triol **30** is also not accepted. This is rather surprising since solubility, hydrophilicity and steric bulk are apparently very similar to those of compounds **26**, **28** and **31**. Unlike L- and D-serine, threonine and *allo*-threonine **36** are not transformed.

The transfer reaction with secondary alcohols shows kinetic data which are comparable with those observed in the reaction with high-molecular-mass primary alcohols, but short-chain primary donors are transferred with much higher efficiency. Table 1 shows that compound **14** (mono-*o*-methylpolyethylene glycol 550) is transferred with lower efficicency than most of the secondary alcohols that we report to be substrates for PLD, while compound PX from ethanol is formed with an extremely high rate and selectivity.

Conclusions

PLD-catalysed transformation of natural phospholipids allows the preparation of a range of natural and unnatural phospholipids of wide potential applications in gram amounts using easily accessible bacterial enzymes. The method appears to be general. If coupled with known enzyme-catalysed transformations of the acyl-chain portion of the molecule,[20] phospholipids with defined polar heads and acyl chains should be available starting with low-cost, easily available natural phospholipid mixtures.

Experimental

^1H and ^{13}C NMR spectra were recorded on a Bruker AC-250 or a Bruker CPX-300 instrument with tetramethylsilane as internal standard. All spectra were recorded in $CDCl_3$/CD_3OD = 2:1. *J*-Values are given in Hz. ^{31}P NMR spectra were recorded on a Bruker AC-250. The sample (100 mg) and triphenyl phosphate (10 mg) used as internal standard were dissolved in 0.6 ml of $CDCl_3/CD_3OD$ = 2:1, stirred with 0.4 ml of a solution of CsEDTA 0.2 M in D_2O (pH 7.6) previously diluted 1:4 with CH_3OH. After 15 min, 0.2 ml of D_2O were added, the mixture was then centrifuged and poured gently into an NMR tube. The chemical ionisation (CI) and mass-analysed ion kinetic energy spectra (MIKES)[21] were performed with a VG-Micromass ZAB-2F instrument equipped with a CI ion source, heated at temperatures of 220–240 °C. The phospholipidic samples were directly introduced by a probe; the samples of alcohols were introduced with the septum inlet system, kept at 230 °C. As reactant CI gas, ammonia was used for alcohols **6–12**, **15–18** and **29**, and methanol for compounds **4**, **5**, **22**, **26–28** and **31**, as for the related phospholipidic samples. The MS/MS measurements were made by running the MIKES[21] of the protonated species of the alcohols above and of the corresponding phospholipidic fragments, upon collision activation with helium as target gas, operating at 8 or 6 eV accelerating potential for ammonia or methanol CI experiments, respectively. FAB spectra were run with a Finnigan Mat 70-70 TSQ triple-quadrupole instrument using xenon as bombarding beam, operating in negative ion mode using thioglycerol or tetraethylene glycol matrices. Ions corresponding to the 18:2–16:0 $M_1 = [M - H]^-$ and 18:2–18:2 $M_2 = [M_1 + 24]^-$ combination of acyl chains were detected.

HPLC analyses were performed on a Merck Hitachi L-6200 with a Supelcosil LC-Si 5μ column, 25 cm × 4.6 mm (Supelco) and UV (206 nm) detector using a Merck Hitachi D-2500 integrator. HPLC analysis conditions were: eluent hexane–Pr^iOH–NaOAc buffer 0.2 M, pH 4.2 = 8:8:1; flow 2 ml min^{-1}.

Silica gel 60 F254 plates (Merck) were used for analytical TLC. Developers used: $CHCl_3$–CH_3OH–NH_3 = 65:30:2.5; $CHCl_3$–CH_3OH–CH_3COCH_3–AcOH–water = 50:10:20:10:5. Phospholipids were detected by spraying with phosphomolybdic acid, followed by heating.

PC was from Lucas-Meyer AG (FRG). The microorganism was grown as previously reported.[10] The PLD activity was

§ Although it is usually assumed that the catalytic step occurs through the formation of an acyl–enzyme intermediate in analogy with other hydrolytic enzymes, this hypothesis is only partially supported by indirect evidence (see ref. 22). Also, interfacial activation in PLD catalysis has not been investigated.

¶ It should be emphasised that the PX/PA quotient (selectivity factor) is calculated at $t_{1/2}$. A rather small selectivity value is not in contrast with the final high HPLC yield in PX. In fact we have observed a rapid initial formation of PA which lowers the selectivity factor. In the progress of the reaction, however, the amount of hydrolysis product is not increasing with the same progression as the PX.

Table 1 Product ratios in the PLD-catalysed transesterification of PC to PX with various secondary and primary alcohols ROH[a]

Entry	$t_{1/2}$ (min)[b]	t_f (min)[c]	PX/PA ($t_{1/2}$)[d]	PX Purity (HPLC) at t_f
1	3	18	>100	95
2	7	90	20	94
3	5	30	45	87
4	30	180	33	64
5	5	30	31	94
6	10	120	40	98
7	42	1475	49	83
8	15	240	23	76
9	8	150	59	76
10	37	200	49	93
11-(*R*)	5	10	7	87
11-(*S*)	4	10	70	93
12	6	50	78	95
13	248	480	16	85
14	210	1310	3	75
15	200	720		70
16	21	150	23	70
17	17	120	29	83
18	13	75	5	90
19	120	360	1.5	55
20	11	120	1.3	54
21	54	430	1.1	42
22	30	420	0.9	50
23	10	300	12	73
25	7	90	8	87
26	21	300	1	70
27	17	360	4	68
28	60	500	4	69
29	51	300	6	74
31	75	360	2.8	68
32	26	1475	7	68

[a] Reaction conditions. Aqueous phase: PLD from *Streptomyces sp.* (10 U) in 1 ml of 0.1 M sodium acetate buffer pH 5.6 and 1 M in alcohol, 37 °C. Organic phase: methylene dichloride 0.5 ml. PC 0.5 mmol. Reaction in test tubes of 3 ml volume, stirred with a magnetic stirrer. Rate and selectivity are strongly influenced by the type of reaction vessel, size and stirring conditions. [b] Time in min for the transformation of half the initial amount of PC. [c] Time in min for the transformation of >98% PC. [d] Ratio of the two products at $t_{1/2}$.

measured with a colorimetric assay.[22] 1 U of activity is defined as the amount of enzyme hydrolysing one μmol of phosphatidyl-*p*-nitrophenol per min at 37 °C.

General procedure for the transphosphatidylation

3-*sn*-Phosphatidylethanol (PX1). PC (5 g) was dissolved in 50 ml of methylene dichloride and added to the aqueous phase containing 150 U of PLD from *Streptomyces sp.*, 1.03 g of NaOAc (0.1 M), 7.29 ml of ethanol (1 M), and the solution was adjusted to pH 5.6. The mixture was stirred by a mechanical stirrer at 200 rpm at 37 °C. When PC was totally transformed (20 min), the organic phase was separated and evaporated to give a residue (4 g). The product was dissolved in hexane (20 ml) and precipitated in 125 ml of chilled acetone to give compound **PX1** as a pale yellowish solid (3.8 g) of more than 95% purity as judged from HPLC analysis.

The other compounds were obtained with a similar procedure. They are designated as PX accompanied by the number identifying the alcohol from which the polar head of the PX derives. Analytical samples were purified by column chromatography if necessary to homogeneity by HPLC.

Spectral data of PXs

PX1. δ_H 0.89 (t, *J* 7, 6 H), 1.38 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H, 2.3 (t, *J* 7, 4 H), 2.75 (t, *J* 7, 4 H), 3.9 (m, 4 H), 4.2 (m, 1 H), 4.4 (dd, *J* 12.5, 1 H), 5.2 (m, 1 H) and 5.4 (m, 8 H); δ_C 14.24, 16.46, 20.88, 22.92, 23.03, 25.25, 25.95, 25.96, 27.53, 29.52, 29.63, 29.71, 29.90, 30.03, 31.69, 32.29, 34.41, 34.56, 61.96, 62.05, 63.09, 63.84, 63.90, 70.90, 71.02, 128.24, 128.25, 130.21, 130.33, 130.45, 173.91 and 174.23; MS M_1, 699.4 and M_2, 723.4.

PX2. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (q, *J* 7, 4 H), 2.55 (m, 2 H), 2.8 (m, *J* 7, 4 H), 4.0 (m, 4 H), 4.2 (m, 1 H), 4.4 (dd, *J* 12.5, 1 H) and 5.35 (m, 8 H); δ_C 14.15, 20.75, 20.87, 2.73, 22.85, 25.04, 25.79, 27.37, 29.34, 29.44, 29.52, 29.70, 29.87, 31.70, 32.10, 34.25, 34.40, 62.85, 63.84, 63.92, 70.01, 70.57, 70.70, 80.83, 128.06, 128.27, 130.13, 130.39, 173.70 and 174.05; MS M_1, 723.5 and M_2, 747.5.

PX3. δ_H 0.9 (t, *J* 7, 6 H), 1.25 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (m, 6 H), 2.75 (m, 2 H), 3.9 (m, 4 H), 4.15 (m, 1 H), 4.4 (dd, *J* 12.5, 1 H), 5.05 (m, 2 H), 5.2 (m, 1 H), 5.5 (m, 8 H) and 5.7–5.9 (m, 1 H); δ_C 14.21, 22.85, 22.95, 25.17, 25.89, 27.47, 29.44, 29.55, 29.63, 29.82, 29.85, 31.81, 32.21, 34.35, 34.51, 35.21, 35.33, 38.97, 62.99, 63.97, 65.29, 65.37, 70.78, 70.90, 117.10, 128.17, 128.37, 130.16, 130.42, 134.75, 173.78 and 174.13; MS M_1, 725.5 and M_2, 749.5.

PX5. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 11 H), 2.3 (m, 4 H), 2.8 (m, 4 H), 3.85 (m, 2 H), 3.96 (m, 2 H), 4.4 (dd, *J* 12, 1 H), 4.95 (m, 3 H), 5.15–5.45 (m, 11 H) and 5.8 (m, 2 H); δ_C 14.16, 22.82, 22.93, 25.14, 25.32, 25.86, 27.44, 29.41, 29.52, 29.60, 29.79, 29.91, 30.26, 30.38, 31.79, 32.18, 33.62, 34.32, 34.42, 62.93, 63.72, 65.90, 65.99, 70.72, 70.86, 114.86, 128.15, 128.35, 128.44, 129.91, 130.16, 130.42, 138.67, 173.73 and 174.10; MS M_1, 753.5 and M_2, 777.5.

PX6. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (t, 3 H), 3.15 (m, 1 H), 3.3 (m, 1 H), 3.95–4.1 (m, 4 H), 4.4 (dd, *J* 12.5, 1 H), 5.2 (m, 1 H) and 5.45 (m, 8 H); MS M_1, 714.3 and M_2, 738.3.

PX7. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 1.95 (m, 2 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (t, *J* 5, 2 H), 3.1 (t, *J* 5, 1 H), 3.2 (m, 1 H), 4.0 (t, *J* 5, 4 H), 4.2 (m, 1 H), 4.4 (dd, *J* 12.5, 1 H), 5.25 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.20, 22.85, 22.97, 25.19, 25.91, 27.49, 28.08, 28.14, 29.44, 29.54, 29.64, 29.82, 29.95, 31.83, 32.23, 34.37, 34.52, 37.35, 62.64, 62.73, 62.88, 63.92, 63.99, 70.63, 70.76, 128.21, 128.39, 130.23, 130.45, 173.82 and 174.24; MS M_1, 728.5 and M_2, 752.5.

PX8. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (t, *J* 7, 2 H), 2.75 (t, *J* 5, 2 H), 2.9 (t, *J* 5, 2 H), 3.9–4.0 (m, 2 H), 4.2 (m, 1 H), 4.36 (dd, *J* 12.5, 1 H), 5.25 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.16, 22.38, 22.79, 22.91, 25.11, 25.84, 26.47, 27.41, 29.37, 29.46, 29.58, 29.74, 29.86, 31.76, 32.15, 34.30, 34.46, 39.46, 62.87, 63.58, 63.66, 70.75, 128.12, 128.30, 130.16, 130.41, 173.70 and 174.12; MS M_1, 756.5 and M_2, 780.5.

PX9. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.35 (m, 4 H), 2.75 (m, 8 H), 3.2 (m, 2 H), 4.05 (m, 4 H), 4.2 (m, 1 H), 5.25 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.21, 14.41, 20.81, 20.99, 22.85, 22.96, 25.17, 25.89, 27.36, 27.47, 29.43, 29.63, 29.80, 29.95, 31.82, 32.21, 34.35, 34.49, 42.85, 43.02, 55.81, 55.97, 58.87, 62.70, 62.83, 63.13, 63.58, 63.99, 64.06, 70.55, 70.67, 70.81, 70.93, 128.18, 128.36, 130.21, 130.44, 132.17, 173.76 and 174.19; MS M_1, 756.5 and M_2, 780.5

PX10. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.7 (m, 4 H), 4.0–4.25 (m, 3 H), 4.45 (dd, *J* 12.5, 2 H), 5.2 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.23, 22.89, 22.99, 25.22, 25.94, 27.52, 29.94, 29.67, 30.00, 31.86, 32.26, 34.39, 34.53, 62.93, 64.20, 70.78, 128.12, 128.23, 128.42, 129.99, 130.24, 130.47, 173.84 and 174.22; MS M_1, 729.5 and M_2, 753.5.

PX11. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (m, 4 H), 3.8–4.5 (m, 4 H) and 5.35 (m, 8 H); δ_C 14.33, 14.53, 21.05, 23.12, 23.23, 25.51, 26.03, 26.14, 26.99, 27.73, 29.72, 29.84, 29.93, 30.11, 30.24, 32.11, 32.51, 34.58, 34.73, 54.52, 63.30, 64.23, 64.30, 66.52, 66.61, 66.91, 71.10, 71.23, 75.31, 75.43, 110.10, 127.66, 128.34, 128.45, 128.63, 130.18, 130.37, 130.48, 130.57, 132.31, 174.05 and 174.41; MS M_1, 785.5 and M_2, 809.5.

PX12. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 41 H), 1.6 (m, 11 H), 2.05 (m, 8 H), 2.3 (t, *J* 7, 4 H), 2.75 (t, *J* 7, 4 H), 3.55 (m, 2 H), 3.85 (q, *J* 6, 2 H), 3.95 (t, *J* 6, 2 H), 4.0–4.2 (m, 4 H), 4.4 (dd, *J* 12.5, 1 H), 5.25 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.21, 14.41, 20.83, 22.32, 22.43, 22.88, 22.99, 25.22, 25.78, 25.92, 26.94, 27.02, 27.50, 29.48, 29.61, 29.67, 29.87, 30.01, 30.88, 31.00, 31.85, 32.26, 32.68, 33.58, 34.37, 34.53, 62.11, 63.05, 63.84, 65.84, 65.93, 69.67, 70.79, 70.93, 76.34, 76.48, 109.13, 127.42, 128.21, 128.41, 128.56, 129.96, 130.19, 130.30, 130.43, 132.16, 173.76 and 174.15; MS M_1, 827.5 and M_2, 851.5.

PX13. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (m, 4 H), 3.7–4.45 (m, 8 H), 5.2 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.18, 22.82, 22.93, 25.12, 25.16, 25.87, 27.44, 29.44, 29.60, 29.82, 29.95, 31.79, 32.19, 34.31, 34.46, 62.57, 62.98, 63.97, 66.77, 70.64, 70.77, 71.24, 127.36, 128.14, 128.36, 128.53, 130.17, 130.43, 132.15, 173.82 and 174.18; MS M_1, 745.5 and M_2, 769.5.

PX15. δ_H 0.89 (m, 6 H), 1.3 (m, 44 H), 1.6 (m, 4 H), 2.05 (m, 4 H), 2.3 (m, 2 H), 2.8 (m, 2 H), 3.25 (s, 8 H), 3.6–4.4 (m, 6 H) and 5.35 (m, 4 H); MS M_1, 768.5 and M_2, 782.5.

PX16. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (t, *J* 7, 4 H), 2.4 (t, *J* 7, 2 H), 2.75 (t, *J* 5, 2 H), 3.55 (m, 4 H), 4.0 (m, 4 H), 4.15 (m, 1 H), 4.44 (dd, *J* 12.5, 1 H), 5.25 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.19, 14.39, 18.11, 22.82, 22.93, 25.16, 25.77, 25.87, 27.44, 29.42, 29.53, 29.60, 29.79, 29.92, 31.29, 31.79, 32.18, 34.31, 34.46, 43.72, 43.78, 62.59, 62.67, 62.95, 63.82, 70.63, 70.75, 127.36, 128.14, 128.36, 128.53, 129.91, 130.18, 130.44, 132.16, 173.68, 175.06 and 177.07; MS M_1, 782.5 and M_2, 806.5.

PX18. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (q, *J* 6, 4 H), 2.75 (t, *J* 6, 4 H), 3.3 (m, 4 H), 4 (m, 4 H), 4.2 (m, 2 H), 4.4 (dd, *J* 12.5, 1 H), 5.2 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.22, 14.41, 20.83, 22.88, 22.98, 25.21, 25.91, 27.49, 29.46, 29.58, 29.66, 29.85, 29.97, 31.84, 32.24, 34.25, 34.50, 52.91, 58.59, 59.65, 62.91, 64.17, 64.24, 64.54, 70.59, 70.72, 127.42, 128.10, 128.20, 128.40, 128.56, 129.96, 130.21, 130.45, 137.18, 173.79 and 174.15; MS M_1, 784.5 and M_2, 808.5.

PX19. δ_C 14.23, 14.43, 20.82, 22.85, 22.96, 25.18, 25.26, 25.80, 25.90, 27.47, 29.64, 30.03, 31.82, 32.22, 32.22, 34.35, 34.49, 54.39, 63.06, 70.68, 127.39, 128.16, 128.41, 130.15, 130.45, 132.16, 173.78 and 174.05; MS M_1, 758.5 and M_2, 782.5.

PX20. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (t, *J* 7, 4 H), 2.75 (t, *J* 7, 4 H), 3.95 (m, 2 H), 4.2 (m, 1 H), 4.4 (dd, *J* 12.5, 1 H) and 5.25–5.35 (m, 8 H); δ_C 14.20, 22.81, 23.98, 25.13, 25.86, 27.44, 29.41, 29.94, 31.77, 32.17, 34.32, 34.45, 62.94, 63.69, 128.12, 128.33, 130.15, 130.42, 173.74 and 174.08; MS M_1, 713.5 and M_2, 737.5.

PX21. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (t, *J* 7, 4 H), 2.75 (t, *J* 7, 4 H), 3.95 (m, 2 H), 4.2 (m, 1 H), 4.4 (dd, *J* 12.5, 1 H) and 5.35 (m, 8 H); δ_C 9.77, 14.32, 21.13, 22.97, 23.07, 25.29, 26.01, 27.59, 29.58, 29.68, 29.75, 30.11, 30.82, 31.94, 32.34, 34.47, 34.61, 54.57, 63.13, 63.90, 71.06, 74.87, 127.50, 128.27, 128.49, 130.29, 130.54, 173.90 and 174.26; MS M_1, 727.5 and M_2, 751.5.

PX22. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (t, *J* 7, 4 H), 2.8 (t, *J* 7, 4 H), 3.85–4.2 (m, 2 H), 4.2 (m, 1 H), 4.4 (dd, *J* 12.5, 1 H) and 5.2–5.35 (m, 8 H); δ_C 13.91, 14.23, 18.75, 19.2, 20.8, 21.57, 22.84, 22.94, 25.15, 25.88, 27.46, 29.44, 29.62, 29.96, 31.8, 32.21, 32.86, 32.98, 34.33, 34.47, 62.92, 63.72, 65.88, 70.7, 127.36, 128.13, 128.35, 130.16, 130.42, 132.15, 172 and 173; MS M_1, 741.5 and M_2, 765.5.

PX23. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.0 (m, 2 H), 2.3 (m, 4 H), 2.75 (t, *J* 5, 2 H), 3.6 (d, *J* 7, 2 H), 3.95 (m, 2 H), 4.35 (m, 1 H), 4.5 (m, 1 H), 5.25 (m, 1 H) and 5.35 (m, 4 H); δ_C 14.29, 22.91, 23.02, 25.21, 25.96, 27.53, 29.51, 29.62, 29.70, 29.88, 30.05, 31.88, 32.15, 32.28, 34.41, 34.55, 53.0, 63.0, 63.9, 70.8, 86.6, 128.23, 128.44, 130.26, 130.52, 173.9 and 174.3; MS M_1, 755.5 and M_2, 779.5.

PX25. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 36 H), 1.5–1.8 (m, 12 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (t, *J* 5, 4 H), 3.95–4.7 (m, 4 H) and 5.35 (m, 8 H); δ_C 14.26, 14.45, 22.95, 23.06, 23.52, 25.28, 25.99, 27.56, 29.54, 29.66, 29.74, 29.93, 30.07, 31.93, 32.30, 32.33, 34.26, 34.32, 34.44, 34.59, 54.47, 63.14, 63.75, 63.81, 70.96, 71.09, 78.90, 78.99, 127.48, 128.16, 128.27, 128.46, 128.59, 130.01, 130.23, 130.33, 130.45, 132.18, 173.8 and 174.34; MS M_1, 739.5 and M_2, 763.5.

PX26. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.65 (m, 6 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (m, 3 H), 3.3 (m, 2 H), 3.9–4.4 (m, 4 H) and 5.35 (7 H); δ_C 14.16, 14.36, 20.89, 22.88, 22.98, 24.32, 24.67, 25.30, 26.03, 27.58, 29.56, 29.89, 30.04, 31.90, 32.29, 33.15, 34.49, 34.63, 63.14, 64.22, 71.07, 74.59, 75.79, 80.40, 127.55, 128.30, 128.55, 130.26, 130.55, 132.28, 173.73 and 174.09; MS M_1, 769.5 and M_2, 793.5.

PX27. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (t, *J* 5, 4 H), 3.9–4.45 (m, 4 H) and 5.35 (m, 7 H); δ_C 14.09, 22.61, 22.71, 24.01, 24.94, 25.65, 27.26, 29.38, 29.78, 31.55, 31.96, 33.62, 34.13, 63, 64, 127.90, 128.08, 129.98, 130.23 and 173.32; MS M_1, 753.5 and M_2, 777.5.

PX28. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 35 H), 1.6 (m, 8 H), 1.9 (m, 2 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (t, *J* 5, 4 H), 3.6–3.75 (m, 2 H), 3.9 (m, 2 H), 4.2 (m, 2 H), 4.4 (m, 1 H), 5.25 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.15, 14.36, 22.39, 22.89, 25.31, 26.04, 27.59, 29.54, 29.70, 29.89, 30.04, 30.51, 31.29, 31.92, 32.29, 32.61, 33.07, 34.51, 34.66, 63.25, 63.93, 67.68, 68.02, 69.08, 69.73, 71.13, 71.23, 72.42, 74.55, 127.58, 128.35, 128.56, 130.32, 130.57, 132.31, 173.89 and 174.24; MS M_1, 769.5 and M_2, 793.5.

PX29. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (m, 4 H), 2.75 (m, 4 H), 4.0 (m, 2 H), 4.15 (m, 1 H), 4.45 (dd, *J* 12.5, 1 H) and 5.25–4.45 (m, 8 H); δ_C 14.18, 14.38, 22.82, 22.93, 25.16, 25.77, 25.87, 27.44, 29.41, 29.51, 29.60, 29.79, 29.91, 31.79, 32.18, 34.32, 34.47, 54.35, 62.96, 63.73, 63.81, 70.57, 70.69, 127.36, 128.15, 128.35, 128.44, 129.91, 130.16, 130.42, 132.14, 173.65 and 174.06; MS M_1, 768.5 and M_2, 792.5.

PX31. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (q, *J* 5, 4 H), 2.75 (t, *J* 5, 4 H), 3.6 (m, 1 H), 4.0–4.25 (m, 2 H), 4.45 (dd, *J* 3.3, 12.5, 2 H), 5.25 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.16, 20.35, 22.92, 25.33, 26.06, 27.62, 29.57, 29.73, 30.07, 31.95, 32.32, 34.28, 34.52, 34.68, 42.84, 43.78, 63.24, 64.03, 66.95, 68.62, 68.93, 71.12, 128.37, 128.58, 130.35, 130.50, 173.84 and 174.31; MS M_1, 769.5 and M_2, 793.5.

PX32. δ_H 0.89 (t, *J* 7, 6 H), 1.3 (m, 33 H), 1.6 (m, 4 H), 2.05 (m, 8 H), 2.3 (q, *J* 5, 4 H), 2.8 (t, *J* 5, 4 H), 3.75 (m, 2 H), 4.0 (m, 4 H), 4.15 (m, 1 H), 4.4 (m, 1 H), 5.25 (m, 1 H) and 5.35 (m, 8 H); δ_C 14.20, 22.84, 22.95, 25.14, 25.17, 25.77, 25.88, 27.46, 29.45, 29.57, 29.62, 29.82, 29.94, 29.98, 31.80, 32.31, 34.32, 34.47, 62.19, 62.23, 62.96, 63.54, 63.97, 67.41, 67.46, 70.70, 70.78, 128.05, 128.15, 128.36, 128.46, 128.54, 130.18, 130.28, 130.44, 173.83 and 174.18; MS M_1, 773.5 and M_2, 797.5.

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