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ORIGINAL ARTICLE

Optimized synthesis of phosphatidylserine

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Abstract The synthesis of phosphatidyl serine containing saturated fatty acids was thoroughly studied and optimized in order to establish a protocol amenable to large-scale synthesis. The key step was a one-pot multicomponent reaction involving an *O*-benzyl phosphorodiamidite, protected serine and diacylglycerol, followed by in situ oxidation of the resulting phosphite. In order to replace expensive and poorly stable tetrazole, a screening of substitutes was carried out and imidazolium chloride was selected as the best suited one.

Keywords Phospholipids · Multicomponent reactions · Phosphorodiamidites

Introduction

Phosphatidyl serine (PS) **1** is one of the most important natural phospholipids present in the cellular membrane. It occurs quite widely in nature but usually in concentrations less than 10% of the cell phospholipid pool. The highest amount is present in brain tissue, particularly in myelin (white matter). PS plays an important role in functioning of the membranes in neurons. Apart from maintenance of the internal cell's environment, it has been proved to be implicated in cell-to-cell communication, cell growth regulation, secretory vesicle release, and signal transduction. Thus, treatment with PS was demonstrated to attenuate the age-related loss of neuronal functions and thus to improve cognitive functions (Pepeu et al. 1996). Phosphatidyl serine is also implicated in the blood coagulation process. For this last effect, it has been shown that both the PS headgroup per se and unsaturation of the 1,2 fatty acid components are important (Smirnov et al. 1999).

The main source of PS has been, to date, extraction from calf brain: a 92% pure phosphatidyl serine can be isolated from brain "cephalin" by means of solvent fractionation.

This method has currently become problematic due to increased consumer concern over bovine spongiform encephalopathy (BSE) (mad cow disease). Although a partial solution to this problem may be the extraction from eggs or plant sources (soybean), a fully synthetic procedure may be advantageous not only from an economical point of view, but also because of the possibility to obtain compounds containing a single fatty acid type, thus allowing a better assessment of the differences in biological activity related to the different fatty acid composition. It should be noted that the fatty acid content varies greatly among the various natural sources of PS, and that it is thought to have a great importance for biological activity (Blokland et al. 1999). Particularly interesting are, from this point of view, the completely saturated phosphatidylserines, containing palmitic or stearic acid, because PS extracted from natural sources is typically characterized by a high percentage of monounsaturated (soybean) or polyunsaturated (calf brain) acids.

Although synthetic methods for the synthesis of phospholipids in general and PS in particular were already known (Baer and Maurukas 1955; Eibl 1984; Hermetter et al. 1982; Lindh and Stawinski 1989; Martin et al. 1994), all of them present some practical problems that make them not well suited for a commercial production. So we

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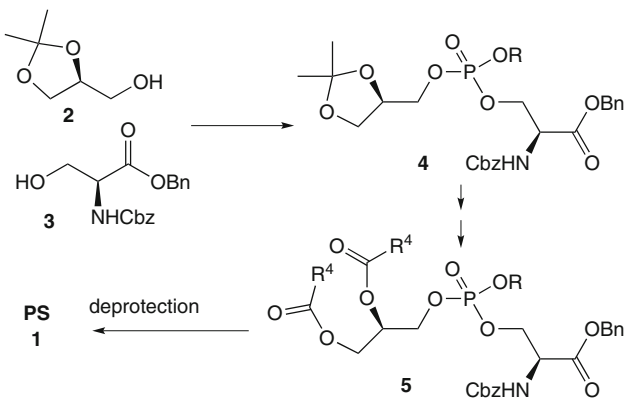
wanted to re-evaluate PS synthesis, in order to develop a robust procedure for a potential large-scale production.

Results and discussion

The most obvious methodology would be a multicomponent reaction of protected glycerol **2**, protected serine **3** and a suitable phosphorus reagent, to give phosphate **4**, which contains a further protecting group R (Scheme 1). Removal of the acetal, followed by acylation would then furnish triprotected PS **5**. Finally, deblocking of the masking groups would give PS **1**.

The crucial step is undoubtedly the multicomponent reaction leading to the phosphate **4**, which contains three different alcoholic residues, but also the choice of the protecting groups is important. In previous works, the phosphate protecting group R was often Ph (Martin and Josey 1988; Martin et al. 1994). However this option requires the use of platinum or palladium black for the final deprotection. Since the use of the less expensive Pd–C was preferable, we decided to use three protections that may be cleaved simultaneously by hydrogenation with this catalyst. Therefore we selected Bn and Cbz for the serine and Bn as R protecting group for the phosphate. While usually *N*-Cbz-L-serine benzyl ester 3 had been prepared introducing first the benzyl ester and then the urethane (Itaya et al. 1993), we found the benzylation of *N*-Cbz-L-serine with benzyl bromide more suitable for large-scale synthesis (Baer and Maurukas 1955). For this step we employed a phase-transfer methodology (see the "Experimental" section).

In order to couple **2**, **3** and benzyl alcohol to give phosphate **4**, we explored various alternative multicomponent reactions. A particularly straightforward possibility is the sequential addition of these three alcohols to $POCl_3$ in the presence of a base. We tried to optimize this protocol by changing the order of addition of the three components



Scheme 1 General plan for the synthesis of phosphatidylserine

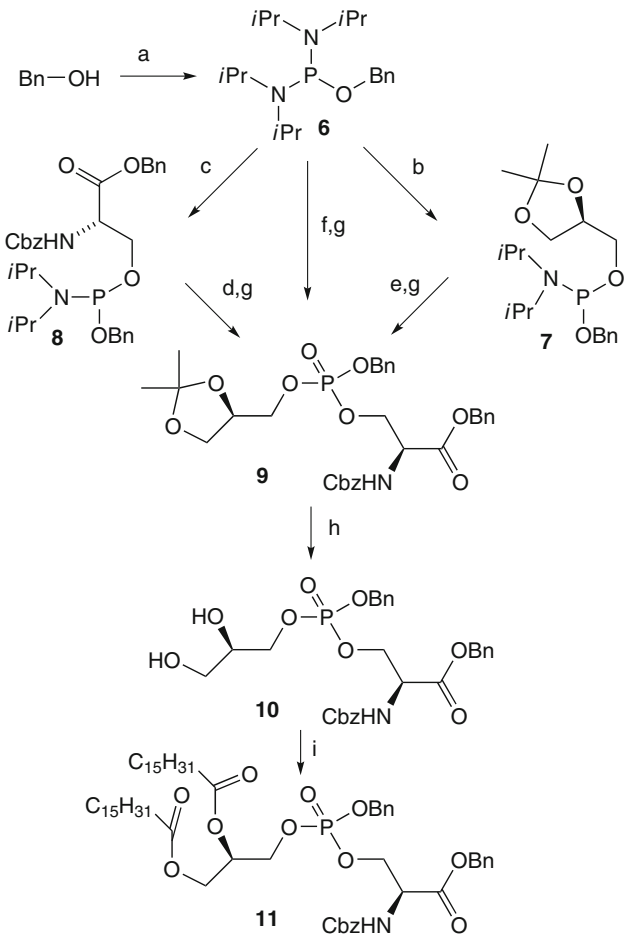
and the base. The best results were obtained at 0°C, in the presence of 2,6-lutidine, by sequential addition of **2**, **3**, and finally excess BnOH. The overall yield of **9** was however only 25%; moreover, the isolation of **9** from the various phosphates arising from inclusion of 2 equal alcohols required a complex chromatography. These and other preliminary results indicated that a synthesis employing $POCl_3$, benzyl phosphorodichloridate (Misiura et al. 1983), or other reagents at the phosphoric oxidation state (Burgos et al. 1987; van Boeckel et al. 1981, 1985; Xia and Hui 1997a, b; Yamauchi et al. 1986) was not suited for our purposes.

An alternative is represented by the use of derivatives of phosphorous acid, followed by in situ oxidation of the resulting phosphite. For example, Martin (Martin and Josey 1988; Martin et al. 1994) has reported a synthesis of protected PS by using phenyl dichlorophosphite. This method was not optimal for our purposes for two main reasons: it involved a phenyl R protection (whereas we preferred a benzyl group, easier to deprotect), and it required very low temperatures (–78°C) for the coupling step in order to achieve selectivity. Although the first problem may be overcome by using benzyl dichlorophosphite (Bannwarth and Trzeciak 1987; Claesen et al. 1985; Ogilvie et al. 1980) instead of phenyl dichlorophosphite, the former was reported to be rather unstable and even to explode during distillation (Miller et al. 1971), and thus it was not advisable to employ it in a large-scale production.

Other coupling reactions that lead to phosphites employ *O*-alkyl chlorophosphoroamidites (Bruzik et al. 1986; Inami et al. 1990; Prestwich et al. 1991) or *O*-alkyl phosphorodiamidites (Bannwarth and Trzeciak 1987; Chen et al. 1998; Mayer et al. 1999; Reddy et al. 1995; Dreef et al. 1988) instead of dichlorophosphites as reagents. These methodologies were originally developed in the realm of oligonucleotide synthesis, but later they have proved to be useful also for phospholipid synthesis. Among the two, the second method seemed to us particularly appealing, since it involves mild conditions and the same type of catalyst for the two subsequent steps (typically tetrazole), therefore allowing in principle an easier one-pot procedure. The selectivity is ensured by the strong difference in the rate of substitution of the two amino groups by alcohol molecules.

In order to exploit this strategy, we prepared the *O*-benzyl phosphorodiamidite **6** following the literature procedures (Scheme 2). There are two alternative reported methods for preparing **6**: (a) by reaction of diisopropylamine with benzyl dichlorophosphite (Bannwarth and Trzeciak 1987); (b) by reaction of PCl_3 with diisopropylamine to give chloro bis(diisopropylamino)phosphoramidite (King and Sundaram 1984) followed by coupling with benzyl alcohol. This second method can be carried out

stepwise (Dreef et al. 1988; Dreef-Tromp et al. 1992) or one-pot (Wakamiya et al. 1996). In our hands all methods worked well, but we eventually selected the second one because of the already cited potential problems associated with benzyl dichlorophosphite. In particular, we preferred the one-pot method developed by Wakamiya and coworkers (Wakamiya et al. 1996). Crude **6** obtained in this way is not pure, being contaminated by substantial amounts of dibenzyl *N*,*N*-diisopropylphosphoramidate and benzyl *N*,*N*-diisopropylphosphoramidic acid, easily detected by GC–MS. Although we tried to optimize the reaction, we were not able to completely suppress these side products, whose presence was deleterious for the yield of the ensuing multicomponent reaction. However, an extractive workup with *n*-hexane/acetonitrile (see "Experimental") allowed us to obtain 98% pure **6** (GC) in 56% yield. Although some **6** was lost in the acetonitrile washing, we



Scheme 2 Reagens and conditions: *a* PCl_3, $(iPr)_2NH$, *n*-hexane; then add BnOH/Et_3N, hexane/CH_3CN extraction, 56%. *b* **2** + tetrazole, 84% or **2** + PPTS, 67%. *c* **3** + tetrazole, 91% or **3** + PPTS, 87% or **3** + imidazolium chloride, 88%. *d* **2** + tetrazole or **2** + PPTS, or **2** + imidazolium chloride. *e* **3** + tetrazole or **3** + PPTS. *f* **3** + imidazolium chloride, then **2**, 49%. *g* MCPBA, 0°C. *h* BF_3, MeOH. *i* Palmitic acid, DCC, DMAP, CH_2Cl_2, 37%

preferred to have a higher purity, paying the fee for a lower yield.

Starting from **6** we first studied the stepwise process to **9**, reacting it with one of the two alcohols **2** (protected glycerol) and **3** (protected serine), in the presence of tetrazole and isolating, through silica gel chromatography, the intermediate phosphoramidites **7** and **8** in 84 and 91% yield, respectively. Since phosphites are sensitive to acids, we always added 0.5–1% Et_3N in the eluent. It should be noted that the synthesis of **7** by this method was already described by Prestwich et al. (Chen et al. 1998). A second acid-catalyzed substitution, followed by in situ oxidation with *m*CPBA (Chen et al. 1998), was then used for converting both **7** (by reaction with protected serine **3**) and **8** (by reaction with protected glycerol **3**) into **9**. Once again the overall yields were fully satisfactory (83 and 88%).

However, tetrazole was not well suited for our purposes: it is a rather expensive compound, and furthermore, it is not very stable. We therefore carefully studied the first step (conversion of **6** into **7** or **8**), trying to replace tetrazole with a cheaper and more stable catalyst, reasoning that any acid with a similar pKa could work similarly well. Unfortunately this was not true. We tried several alternative weak acids experiencing yields remarkably lower than those achieved with tetrazole itself (see Scheme 2). It is clear that in this reaction tetrazole does not act merely as a specific acid catalyst, but is involved in a more complex catalytic process. After a thorough screening we could obtain acceptable results with pyridinium *p*-toluenesulfonate (PPTS). However, while the synthesis of **8** worked nearly as well as with tetrazole, the related preparation of **7** was less satisfactory. Moreover, while transformation of **7** into **9** proceeded with 72% yield, conversion of **8** into **9** afforded an unsatisfactory 51% yield.

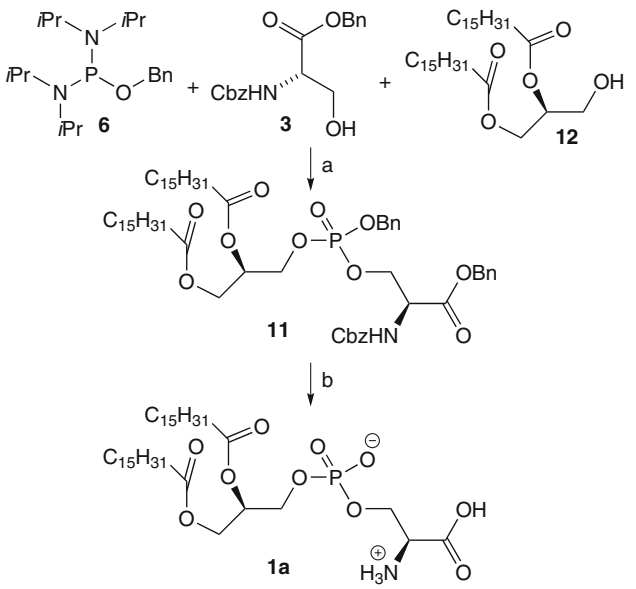
These results indicate that the most critical step appears to be the introduction of the protected glycerol. We therefore carried out a second screening of acid catalysts for the conversion of **8** to **9**. The best results were obtained with imidazolium chloride (78%). It is interesting to note that imidazolium chloride, as tetrazole, has an additional pyridine-type nitrogen. Although the overall yield with imidazolium chloride (78%) was still slightly lower than that achieved with tetrazole (88%), the low cost and high stability of imidazolium chloride makes it the catalyst of choice for this reaction.

Moreover, imidazolium chloride was also found to be efficient for the transformation of **6** into **8**, affording a yield similar to that achieved with PPTS and tetrazole. We then repeated the whole process with this catalyst, this time without isolation of the intermediates, obtaining compound **9** in 49% (unoptimized) yield after chromatography.

The next step involved deprotection of the isopropylidene group followed by acylation with a fatty acid.

Throughout this study we always used palmitic acid. Unfortunately the deblocking step turned out to be rather troublesome, maybe because of the partial lability of benzyl phosphates. We tried a lot of alternative acid conditions, but the yield was invariably low. The best result that we could achieve was a 37% yield for the two steps from **9** to **11** [deblocking followed by acylation of crude **10** with fatty acid and DCC/DMAP (Chen et al. 1996)].

This disappointing result prompted us to explore an alternative route, inserting the two fatty acids before the assembly of the phosphate (Scheme 3). In the meantime, we had developed an efficient chemoenzymatic synthesis of 1,2-diacyl-*sn*-glycerols (DAG) in high e.e., that is amenable to up-scaling, since it does not need chromatographic purifications (Guanti et al. 2004). So we had an easy access to 1,2-dipalmitoyl-*sn*-glycerol **12** as input for our multicomponent phosphate assembly (Scheme 3). Taking advantage of the experience gained in the synthesis of **9**, we performed the coupling of **6**, **3** and **12** in a one-pot fashion, using imidazolium chloride as catalyst. This reaction was carefully optimized in order to improve the yield and to obtain a crude product amenable to crystallization. During this optimization we realized that, for various reasons, MCPBA was not the best oxidant: it is not particularly cheap, nor safe, it requires low temperatures, and the resulting side product (*meta*-chlorobenzoic acid) complicates purification of the product. We investigated several other oxidants (Hébert and Just 1990; Martin and Josey 1988) and found that *tert*-butyl hydroperoxide (Kozikowski et al. 1994; Martin and Wagman 1996) was best suited for this task. After all these optimizations, the



Scheme 3 Reagents and conditions: *a* **6** + **3**, imidazolium chloride, 0°C, then **12**, imidazolium chloride, 0°C, then *t*BuOOH, 0°C, 78%. *b* H_2, Pd–C, AcOH, 95%

one-pot procedure eventually produced **11** in 78% yield from **12**. Most importantly the crude product could be conveniently purified by crystallization, notwithstanding the obvious presence of two diastereoisomers due to the chiral phosphorus atom.

Finally, hydrogenation over Pd–C afforded pure dipalmitoyl PS **1a** in nearly quantitative yield. This compound is poorly soluble in most solvents. Therefore, in order to separate it from the catalyst we had to carry out the filtration using hot $CHCl_3$/MeOH 9:1.

Conclusions

In conclusion, we have reported a highly convergent and efficient synthesis of PS **1a** in 74% yield from DAG **12** through a two-step procedure involving only two crystallizations for the purification of intermediates (no chromatography). This procedure was used in our laboratory for the synthesis of multigram quantities of PS.

The main improvements compared to the previously reported syntheses of PS are: (a) the use of the phosphorodiamidite method, that allows a multicomponent, one-pot procedure at 0°C–r.t.; (b) the use of cheap imidazolium chloride as catalyst; (c) the use of benzyl protection for the phosphate, that allows final hydrogenation under milder conditions than the phenyl group; (d) a more convergent route, since the fatty acids are already bound to one of the building blocks.

Coupled with the already reported (Guanti et al. 2004) efficient enantioselective preparation of **12** via a chemoenzymatic route, this process should be very likely amenable to scale-up. Moreover, the introduction of other saturated fatty acids (e.g., stearic acid) is expected to be feasible by the same procedure.

Experimental

NMR spectra were taken in $CDCl_3$ at 200 MHz (^{1}H), and 50 MHz (^{13}C), using TMS as internal standard for ^{1}H NMR and the central peak of $CDCl_3$ (at 77.02 ppm) for ^{13}C NMR. Chemical shifts are reported in ppm (δ scale), coupling constants are reported in hertz. Peak assignment in ^{13}C spectra was made with the aid of DEPT experiments. GC–MS was carried out on a HP-5971A instrument, using an HP-1 column (12 m long, 0.2 mm wide), electron impact at 70 eV, and a mass temperature of about 170°C. Only $m/z > 33$ were detected. All analyses were performed with a constant He flow of 0.9 ml/min with initial temperature of 100°C, initial time 2 min, rate 20°C/min, final temperature 260°C, final time 4 min, injector temperature 250°C, detector temperature 280°C. R_t are in min. Melting

points were measured on a Büchi 535 apparatus and are uncorrected. TLC analyses were carried out on silica gel plates and developed at UV or with "molybdic" reagent (21 g $(NH_4)MoO_4{\cdot}4H_2O$, 1 g $Ce(SO_4)_2$, 469 ml H_2O, 31 ml H_2SO_4), with Dittmer–Lester reagent (Dittmer and Lester 1964), or with ninhydrin. R_f was measured after an elution of 7–9 cm. Chromatographies were carried out on 220- to 400-mesh silica gel using the "flash" methodology. Petroleum ether (40–60°C) is abbreviated as PE. Dry solvents were purchased from Fluka. All reactions employing dry solvents were carried out under a nitrogen atmosphere.

O-Benzyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphorodiamidite 6 (Dreef-Tromp et al. 1992; Wakamiya et al. 1996)

Diisopropylamine (49 ml, 346 mmol) and *n*-hexane (140 ml) were placed in a two-necked flask equipped with a dropping funnel and kept under nitrogen atmosphere. The flask was cooled to 0°C and treated dropwise (during 15 min) through the dropping funnel, with a solution of PCl_3 (7.87 g, 57.0 mmol) in *n*-hexane (10 ml). After washing the funnel with few milliliters of *n*-hexane, the temperature was allowed to rise to r.t. After 3 h the milky suspension was refluxed for 19 h. After cooling again to 0°C, Et_3N (24.0 ml, 172.4 mmol) was added followed by slow addition (15 min) of a solution of Et_3N (8.0 ml, 57.4 mmol) and BnOH (6.0 ml, 57.9 mmol) in Et_2O (20 ml). After stirring for 25 min at 0°C and 35 min at r.t., the mixture was filtered through a sintered funnel and evaporated to dryness. The resulting oil was taken up in *n*-hexane (100 ml) and washed three times with CH_3CN (80 + 60 + 60 ml). The hexane phase was checked at GC–MS (purity = 98%) and evaporated to dryness to give **6** (10.92 g, 56%) as a slightly yellow liquid. $R_f = 0.38$ (PE/AcOEt 1:1). GC–MS: $R_t = 7.85$. *m*/*z* 338 (M^+, 0.6), 247 (2.2), 238 (10.3), 121 (2.6), 91 (100), 90 (3.9), 79 (6.1), 70 (3.4), 65 (3.3), 58 (5.8), 43 (4.9), 42 (5.4), 41 (4.0). ^{1}H NMR: 7.42–7.20 [5 H, m, aromatics]; 4.65 [2 H, d, CH_2Ph, $J = 7.4$]; 3.58 [4 H, d of septuplets, $CH(CH_3)_2$, $J_d = 10.8$, $J_s = 6.7$]; 1.18 [24 H, dd, CH_3, $J = 2.3$, 6.7].

N-(Benzyloxycarbonyl)-L-serine benzyl ester

N-(benzyloxycarbonyl)-L-serine (10.63 g, 44.4 mmol) was suspended in CH_3CN (50 ml), and treated with 4 M aqueous NaOH (11.175 ml, 44.7 mmol). The resulting solution was treated with *n*-Bu_4NHSO_4 (1.52 g, 4.48 mmol) and BnBr (6.40 ml, 53.9 mmol) and refluxed for 4 h. After cooling to r.t. (a white solid separates), 5% aqueous $NaHCO_3$ (55 ml) was added and the suspension stirred for 20 min. After removal of the solid through filtration, the two phases were separated and the aqueous one extracted three times with AcOEt (40 ml each). The reunited organic phases were washed with brine, well dried with Na_2SO_4 and evaporated to dryness to give an oil. It was taken up in *n*-hexane (40 ml) and stirred until a well solid separates. It was filtered on a Buchner filter and washed with *n*-hexane. Finally, the solid was crystallized from Et_2O/PE to give pure **3** (11.03 g, 75%) as a white solid, m.p. 81.9–82.6°C. $R_f = 0.34$ (PE/Et_2O 3:7). The other analytical data were identical with those reported (Itaya et al. 1993).

O-Benzyl *O*-[(*S*)-2-(benzyloxycarbonyl)-2-(benzyloxycarbonylamino)ethyl] *O*-(1,2-*O*-dipalmitoyl-*sn*-glyceryl) phosphate 11

A solution of **3** (2.195 g, 6.66 mmol) in dry CH_2Cl_2 (30 ml) was treated with a solution of diamidite **6** (2.505 g, 7.40 mmol) in dry CH_2Cl_2 (15 ml). The solution was cooled to 0°C and treated with imidazolium chloride (200 mg, 1.91 mmol). After 30 min at 0°C, the solution was stirred at r.t. for 21 h. Then dipalmitoylglycerol (4.128 g, 7.25 mmol) was added. Then imidazolium chloride (1.957 g, 18.73 mmol) was added in three equal portions, spaced out by 45 min each. After 150 min from the last addition the mixture was cooled again to 0°C, and treated with a 3 M solution of *tert*-butylhydroperoxide in toluene (3.5 ml, 10.36 mmol). After 2 h at 0°C, the reaction was quenched with 10% $NaHSO_3$ (20 ml) and 5% $NaHCO_3$ (20 ml). When the mixture was negative at iodine–starch paper, the phases were separated and the aqueous one extracted with CH_2Cl_2 three times. The united organic extracts were washed with brine, dried (Na_2SO_4) and evaporated to dryness to give a yellow foam (8.434 g). It was crystallized from methanol to give a white solid (5.431 g, 78%), corresponding to **11** as a diastereoisomeric mixture due to the chiral phosphorus (m.p., 49.8–54.6°C, $R_f = 0.5$ (PE/ACOEt 7:3). Found: C, 69.1; H, 9.2; N, 1.25. $C_{60}H_{92}NO_{12}P$ requires: C, 68.61; H, 8.83; N, 1.33%. ^{1}H NMR: 7.33 [15 H, s, aromatics]; 5.89 and 5.85 [1 H, 2d, N*H* of the two diast., $J = 8.4$]; 5.25–5.05 [2 H, m, P–O–CH_2Ph]; 5.11 [2 H, s, CH_2Ph]; 5.01 and 4.96 [2 H, 2 s, CH_2Ph of two diast.]; 4.65–4.55 [1 H, m]; 4.50–4.35 [1 H, m]; 4.35–4.18 [2 H, m]; 4.12–3.95 [4 H, m]; 2.27 [4 H, t, $CH_2C=O$, $J = 7.5$]; 1.88–1.73 [4 H, m]; 1.25 [48 H, m]; 0.88 (mc) [6 H, m, terminal CH_3].

1,2-*O*-Dipalmitoyl-*sn*-glycero-3-phosphoserine 1

Compound **11** (1.939 g, 1.835 mmol) is dissolved in AcOH (40 ml) and hydrogenated over 10% Pd–C (90 mg) for 48 h at r.t. The mixture was filter through a warm Celite cake prepared in a sintered funnel with pores = 10–20 μm. The use of a low pore size sintered funnel was necessary in order to avoid passage of the catalyst. The cake was

thoroughly washed with hot $CHCl_3$–MeOH 9:1 (160 ml overall). The filtrate was evaporated to dryness, removing completely AcOH azeotropically with the aid of *n*-heptane. The resulting white solid was transferred into a sintered funnel and washed with acetic acid, acetone, water, acetone and finally with few Et_2O. After drying, 1.29 g of **1** was obtained (95%). $R_f = 0.43$ ($CHCl_3/CH_3OH/25\%$ NH_3 50/25/6, developed with ninhydrin). M.p.: 159–160°C (dec.). Found: C, 61.1; H, 9.4; N, 1.7. $C_{38}H_{74}NO_{10}P$ requires: C, 62.01; H, 10.13; N, 1.90%. The other spectroscopic data were coincident with those reported (Eibl 1984). At ^1H or ^{13}C NMR in $CDCl_3$–MeOH the signals were rather broad.

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