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Chemical and Enzymatic Approaches to Esters of *sn*-Glycero-3-Phosphoric Acid

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Dedicated to Professor Franco Cozzi on the occasion of his 70th birthday.

Esters of *sn*-glycero-3-phosphoric acid (GPAE) are derivatives of glycerophospholipids in which both the fatty acid chains in *sn*-1 and *sn*-2 positions are removed. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) are the most abundant glycerophospholipids in Nature. Their deacylated derivatives find application in many industrial sectors; for example, *sn*-glycero-3-phosphocholine (GPC) is useful as a cognitive enhancer and as a food ingredient, whereas *sn*-glycero-3-phosphoinositol (GPI) acts as a natural anti-inflammatory agent. This minireview covers the state-of-the-art of chemical and biocatalytic methods for the obtainment of these lipid-derived components.

1. Introduction

Glycerophospholipids (GPL, Figure 1A) are the major components of cell membranes with important structural and functional properties.[1] They are a class of lipids composed of a glycerol backbone which is esterified in *sn*-1 and *sn*-2 positions with fatty acids and *sn*-3 position with a phosphate group. Although the length and degree of unsaturation of the fatty acids vary, a saturated fatty acid is typically present in *sn*-1 position, whereas an unsaturated or a poly-unsaturated fatty acid is found in *sn*-2 position.[2] The simplest GPL is phosphatidic acid (PA, Figure 1A); in other GPL the phosphate moiety of PA is further esterified with choline, *myo*-inositol, ethanolamine, or serine, thus giving rise to the most biologically important classes of GPL, *i.e.* 3-*sn*-phosphatidylcholine (phosphatidylcholine, PC), 3-*sn*-phosphatidyl-1-*myo*-inositol (phosphatidylinositol, PI), 3-*sn*-phosphatidylethanolamine (phosphatidylethanolamine, PE), and 3-*sn*-phosphatidylserine (phosphatidylserine, PS).[3]

When the two fatty acid chains of natural GPL are removed, the esters of *sn*-glycero-3-phosphoric acids are obtained (GPAE) (Figure 1B). In the last years, these latter deacylated derivatives have attracted increasing interest due to their biological and pharmacological properties. *sn*-Glycero-3-phosphoric acid (GPA, Figure 1B) is the precursor of all esters described herein. In this minireview, we aim at presenting the state-of-the-art of chemical and biocatalytic methods for the obtainment of these important lipid-derived compounds.

1.1. Nomenclature

There has been confusion in the literature about the chemical names of GPL and GPAE. The need for a novel departure in the nomenclature of lipids and, in particular, in distinguishing stereoisomers, resulted in the publication of recommended guidelines by IUPAC-IUB Commission on Biochemical Nomenclature (CBN) in 1967,[4] then revised later on.[5,6]

A stereospecific numbering (*sn*) system was, indeed, recommended by the IUPAC-IUB Commission to designate the stereochemistry of glycerol derivatives. The descriptors *sn*-1, *sn*-2, and *sn*-3 are used to identify the esterification position of carbon 1, 2, and 3 on the glycerol molecule, as depicted in Figure 2: if the secondary hydroxyl group is shown to the left of C-2 in a Fischer projection, the carbon atom above this then becomes C-1 while that below becomes C-3, and the prefix *sn* is placed before the stem name of the compound. *sn*-3-Glycerophosphoric acid (previously known as either L-α-glycerophosphoric acid or as D-glycerol-1-phosphate) is the precursor of all esters described herein.[5,6]

The system of "*sn*" numbering introduced by CBN in 1967 has been well-accepted in the field of glycerol derivatives and is still retained nowadays.[6]

3-*sn*-Phosphatidic acid (PA) can be considered a derivative of glycerophosphoric acid in which both remaining hydroxyl groups of glycerol are esterified with fatty acids. For the most common 3-*sn*-phosphatidic acids and their derivatives (see Figure 1), the locants are often omitted. This rule will be applied for the designation of the 3-*sn*-glycerophosphoric acid esters

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(GPAE) which are deacylated derivatives of GPL as follows: *sn*-glycero-3-phosphocholine (glycerophosphocholine, GPC), *sn*-glycero-3-phospho-1-*myo*-inositol (glycerophosphoinositol, GPI), *sn*-glycero-3-phosphoethanolamine (glycerophosphoethanolamine, GPE) and *sn*-glycero-3-phosphoserine (glycerophosphoserine, GPS). Glycerophospholipids in which one of the two acyl groups have been removed from either the *sn*-1 or *sn*-2 position are named lysophospholipids.[7] The old prefix 'lyso' originated from the fact that these compounds are hemolytic. It has been redefined to indicate limited hydrolysis of the phosphatidyl derivative (*i.e.*, 'deacyl').[5]

It is worth mentioning that the inositols are the nine isomeric forms of cyclohexanehexol and constitute a subgroup of a broader class of compounds known as cyclitols, *i.e.* cycloalkanes in which three or more ring atoms are each substituted with one hydroxyl group.[6] *myo*-Inositol, or *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, is the most widespread isomeric form of inositol that can be found in nature and in food.[8] In this review, later mentions of 'inositol' refer only to *myo*-inositol.

Further useful specifications about nomenclature include the term 'plasmalogen', which refers to GPL in which the glycerol moiety bears a 1-alkenyl ether group, and 'lecithin', which is a mixture of GPL, but is commonly used to indicate its main component, *i.e.* 3-*sn*-phosphatidylcholine (PC), whose systematic name is 1,2-diacyl-*sn*-glycero-3-phosphocholine.[5]

1.2. Source

Esters of *sn*-glycero-3-phosphoric acid can be produced from GPL of plant, animal, and aquatic origin (*e.g.* soy, sunflower and rapeseed oils, egg yolk, cheese whey, fish, and Antarctic krill). Vegetable lecithins are the main commercial source of GPL.[9,10]

The commercial term lecithin is referred to a complex mixture of phospholipids obtained as by-products during the vegetable oil refining process (degumming). The degumming process removes the substances containing phosphorus and glycolipids from unrefined vegetable oil, thus enhancing oil stability against sedimentation as well as facilitating further refining processes.[11] Many degumming processes are reported in the literature. Briefly, the vegetable oil is heated and stirred in presence of water which causes the hydration of polar lipids, making them insoluble and allowing their separation from oil. The obtained gum is then dried and cooled to obtain lecithin. Temperature, water amount, and mixing times as well as drying and cooling conditions may vary depending on the vegetable source.[12] As mentioned before, lecithin is also the trivial name for PC, but common usage refers to the array of GPL present in all crude vegetable oils. The most abundant GPL in lecithin are usually PC, PE, PI, and PA (Figure 1). However, slight differences can be observed in phospholipid composition depending on the natural sources and the conditions applied in the extraction and quantification process (Table 1). In most plant species, PC and PE are predominant; however, in the case of sunflower, the main GPL are PC and PI. For this reason, this source is preferred for the obtainment of the latter GPL.[13]

Due to their surface-active properties, lecithins are among the most widely used emulsifiers in a vast range of food, feed, pharmaceutical, cosmetic and technical applications.[14]

Starting from GPL, GPAE can be obtained through both chemical and enzymatic approaches. Chemical approaches generally consist of the hydrolysis of lecithin in alcohols using organic or inorganic bases. In this case, a mixture of all possible GPAE is obtained since the hydrolysis is not selective and the complexity of the mixture depends on the used lecithin and/or pre-treatment of starting material before hydrolysis. Solvent extraction and silica or ion-exchange chromatography are then applied to the mixture to purify the desired GPAE (see paragraphs 2–5).

Alternatively, GPL can be hydrolyzed by using enzymes as biocatalysts. Typically, biotransformations are carried out by incubating lecithin or pre-treated lecithin with the selected enzyme(s) (as soluble or immobilized proteins) in a fully aqueous medium or in non-conventional media such as, for instance, biphasic systems. Isolation and purification procedures are the same as for chemical hydrolysis.

Lipases and phospholipases are hydrolytic enzymes suitable for this purpose. Lipases are the most used hydrolases in the industry (*e.g.* for the formulation of laundry detergents, treatment of wastewater, and hydrolysis of triglycerides) and their applications in esterification/transesterification reactions have also been widely exploited.[15] Phospholipases can also be used to modify GPL. There are four classes of phospholipases that catalyze the cleavage of a specific ester bond, as reported in Figure 3.

Phospholipases A_1 (PLA_1) and A_2 (PLA_2) are the most suitable for the obtainment of GPAE since they hydrolyze carboxyl esters

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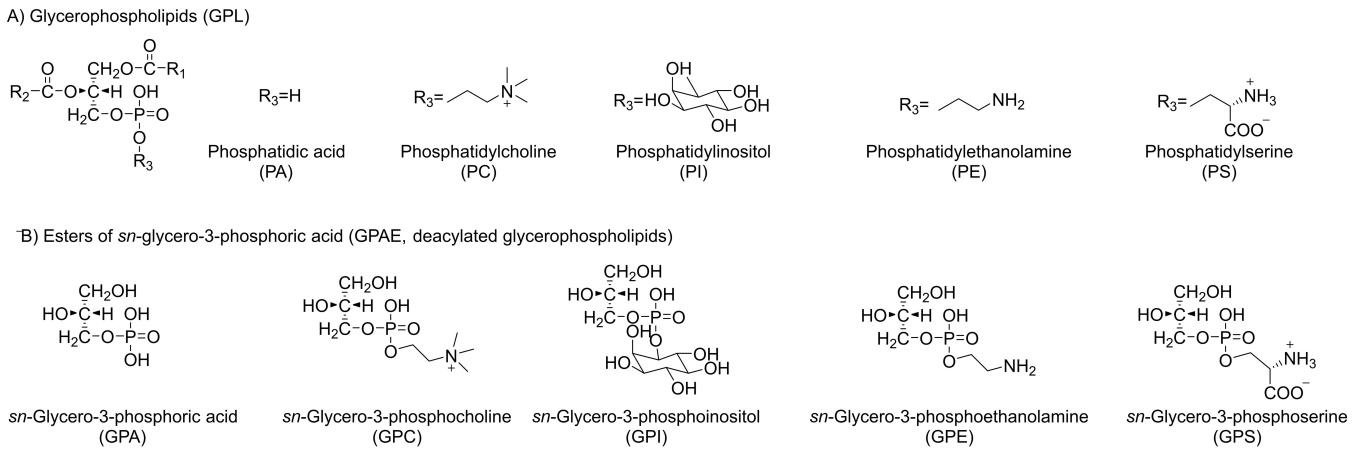


Figure 1. A) Structure of the most common glycerophospholipids (GPL); B) Structure of the most common esters of *sn*-glycero-3-phosphoric acid (GPA). R_1; R_2 = alkyl or alkenyl groups.

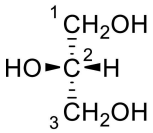


Figure 2. Glycerol "*sn*" numbering.

Table 1. Average composition of GPL in the main plant sources (adapted from [13]).

Lecithin	Composition [%]		
	PC	PE	PI
Soybean	25.5	17.6	15.6
Sunflower	28.2	12.0	20.2
Rapeseed	38.5	23.1	16.7

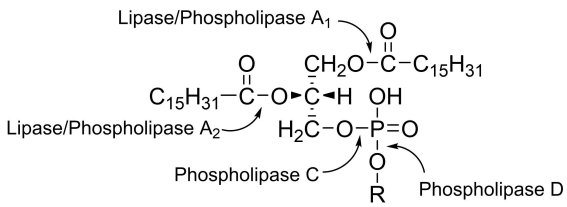


Figure 3. Enzymatic toolbox for GPL modification. R=H, choline, inositol, ethanolamine, or serine. Ester of 1,2-dipalmitoyl *sn*-glycero-3-phosphoric acid is depicted.

at the *sn*-1 and *sn*-2 position releasing free fatty acids similarly to lipases. Phospholipases C and D (PLC and PLD, respectively) have phosphodiesterase activity instead. PLC cleaves the glycerophosphate bond, thus producing a natural diacylglycerol and a phosphoric ester; PLD cleaves the terminal phosphodiester bond releasing phosphatidic acid and the polar head. As for lipases, the phospholipase-catalyzed reaction can be reversed to synthesize specific GPL and their derivatives.[2,16] Enzymatic catalysis is expected to simplify the purification procedure. Due to the intrinsic selectivity and specificity of enzymes, fewer by-products are generally formed.[2]

1.3. Biological activity

In the last years, many biological and pharmacological properties of GPAE have been reported.

Glycerophosphoinositols, which include non-phosphorylated glycerophosphoinositol (GPI) and its phosphorylated derivatives glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate, are ubiquitous, water-soluble metabolites. They constitute a class of biologically active lipid-derived mediators whose intracellular levels are modulated during physiological and pathological cellular processes. The functional roles and mechanisms of action of these different glycerophosphoinositols have been investigated in different cellular contexts and it has been observed that they can exert different functions based on the specific target/receptor to which they bind. As a result, roles for these mediators in cell proliferation, immune and inflammatory responses have been recently defined and reviewed by Corda et al.[17–20] After an inflammatory stimulus such as exposure of monocytes and macrophages to bacterial lipopolysaccharide (LPS), GPI is generated from membrane PI carrying arachidonic acid in position *sn*-2 of the glycerol backbone through two sequential deacylation reactions both catalyzed by PLA_2IVα, which has intrinsic PLA_2 and lysolipase activities.[20] It has been demonstrated that GPI can act as an anti-inflammatory agent, being part of a negative feedback loop that inhibits the *de novo* synthesis of pro-inflammatory and pro-thrombotic compounds. GPI can inhibit the signaling of Toll-like receptor 4 (TLR4) induced by LPS leading to a decrease in the nuclear translocation and binding of the transcription factor NF-kB to promoters, thus reducing the transcription of inflammatory genes,[20] and in parallel inhibiting both the expression[21] and activity of PLA_2.[22] Based on these *in vitro* results, the potential efficacy of GPI as an anti-inflammatory agent was also

demonstrated in an *in vivo* model of endotoxin shock in mice[20] and its use in sepsis syndrome has been recently patented.[23] The anti-inflammatory properties of GPI have been also demonstrated *in vitro* at blood-brain barrier (BBB) level and compared to corticosteroid dexamethasone, showing comparable effects in preventing culture-induced BBB decline after several days, as well in promoting recovery of function in compromised cells.[24]

A plethora of clinical and scientific studies have documented the central role of GPC in human health. GPC is a component of breast milk and an important source of choline required by infants for organ growth and membrane biosynthesis. Most important from an applicative viewpoint, GPC has the status of 'Generally Recognized as Safe' (GRAS) as an ingredient in beverages and foods when consumed at a level not to exceed 196.2 mg/person/day.[25,26] The use of GPC as a food additive and in health products makes its application increasingly important in numerous industries.[27] GPC was identified as the "meat factor" which is reported to enhance non-heme iron absorption, and this finding provides new opportunities for iron fortification of foods.[28]

In addition, GPC is present in brain tissue as a product of phospholipid metabolism[29–31] and has a well-established use as a therapeutic agent in the field of cognitive disorders.[32] GPC is the direct precursor of acetylcholine (ACh) in the brain and has shown significant advantages for increasing learning, memory abilities among stroke patients, and has been demonstrated to be effective in the treatment of Alzheimer's disease (AD) and dementia.[33–35] A primary trait of AD is the degeneration of basal forebrain cholinergic neurons, which causes a remarkable deficit of avenues of cortical cholinergic neurotransmission, such as ACh synthesis, release, and uptake, and choline acetyltransferase and acetylcholinesterase (AChE) activities.[36] Reduction of AChE activity is related with the degree of dementia and neuropathological hallmarks of AD, confirming a close association between cholinergic biochemical abnormalities and the disease.[37] Under conditions of reduced cholinergic synthesis and increased neuronal demand, neurons increase their ability to incorporate exogenous choline;[38] this suggests that the systemic administration of a choline precursor, such GPC, should antagonize biochemical disorders of the cholinergic system, thereby improving cognitive function. In fact, GPC is metabolized into phosphorylcholine that is able to reach cholinergic synaptic endings where it enhances ACh synthesis and release. Moreover, GPC positively affects the expression of choline uptake transporter (CHT) and vesicular Ach transporter (VAChT), thus improving synaptic efficiency.[39] Recent clinical studies indicated that the association between the AChE inhibitor donepezil (1-benzyl-4-[(5,6-dimethoxy-1-indanon-2-yl) methyl]piperidine) and GPC is accompanied by an improvement in several cognitive tests which is superior to that induced by donepezil alone.[40] It was suggested that this association may represent a therapeutic option to prolong the beneficial effects of cholinergic therapies in AD's patients with concomitant ischemic cerebrovascular disorders.[40] Among many other reported functions, being a putative ACh precursor, GPC can enhance growth hormone secretion and fat oxidation in young adults.[41] Finally, GPC is also involved in the balance of osmotic pressure in the kidney, acting as osmoprotective organic osmolyte in renal medullas.[42,43]

The tolerance of GPC is excellent and side effects are rare, never severe.[34,44] An oral NOAEL (No Observed Adverse Effect Level) of 150 mg/kg body weight/day following 26 weeks oral exposure has been reported.[25]

Although GPE and GPS do not have an application to date, some preliminary studies on their biological activities have been reported.

Tallan et al.[45] observed that GPE was present in normal liver tissue in higher levels compared to other organs, where it stimulated the growth of hepatocytes during liver regeneration.[46,47] This regeneration capacity was further confirmed in cultured hepatocytes where GPE could enhance the activity of epidermal growth factor (EGF).[47] Moreover, a recent study has demonstrated that GPE does not show any toxicity effect on human kidney-2 (HK-2) cells in concentrations up to 1000 μM, thus suggesting its possible use as a pharmaceutical candidate.[48]

In the human retina, GPE levels represent 22% of the total PE components. However, this relatively high content is not still completely understood, even if it has been demonstrated that GPE can form a direct adduct with bisretinoid called A2-GPE.[49] The formation and accumulation of these retinoid-derivatives in the retinal pigment epithelial (RPE) cells are observed in some inherited and age-related forms of macular degeneration. Investigations of potential treatments for macular degeneration include approaches that would reduce bisretinoid formation.

Although a plethora of biological activities of PS, an important membrane component, has been reported, a handful of information about GPS is available to date.

When PS is exposed on cell surface it acts as signaling triggering phagocytosis and subsequent apoptosis. Phagocytosis of apoptotic lymphocytes was inhibited in a dose-dependent manner when treated with liposomes containing PS but also by its derivatives structurally related such as GPS and phosphoserine.[50] In 1999, Tait et al.[51] have confirmed the activity of PS vesicle also on the monocytic leukemia cell lines THP-1 but no effects were observed for phosphoserine or GPS. By contrast, in recent years lyso-PS have emerged as signaling molecules in several biological processes, and deregulation of their metabolism has been linked to various human pathophysiological conditions.[52] Lyso-PS are involved in the release of histamine from Mast cells, macrophage-mediated efferocytosis, and inhibition of lymphocytes proliferation. Furthermore, lyso-PS stimulate intracellular calcium signaling and promote chemotactic migration in human cancer cells. Finally, lyso-PS have hormone-like signaling properties facilitating the transport and adsorption of glucose into muscle fibres and adipocytes.[52]

2. *sn*-Glycero-3-phosphoinositol (GPI)

Only one synthesis of GPI, specifically of '1-glyceryl-2-*myo*-inosityl phosphate', as named in the original paper, has been reported so far. It dates back to 1959 when the position of the

phosphate group in *myo*-inositol moiety (whether it was 1 or 2) had not been elucidated, yet.[53] This synthesis starts from 3,4,5,6-tetracetyl-*myo*-inositol which was converted into 1,3,4,5,6-pentacetyl-*myo*-inositol and then treated with phenylphosphodichloride followed by addition of D,L-1,2-isopropilydene glycerol, thus affording '1,2-isopropylidene-glyceryl-pentacetyl-2-*myo*-inosityl-phenylphosphate'. Hydrogenolysis in the presence of Adam's catalyst, removal of the isopropylidene group, treatment with sodium methoxide in methanol, and isolation by passing the resulting mixture through an ion exchange resin led to the free acid derivative in an estimated 30% yield. Since the product was unsuitable for characterization and handling, its sodium, potassium, and barium salts were isolated by further treating the product with an ion-exchange column in the appropriate basic form. Both final products and intermediates were characterized by elemental analysis and melting point determination, only. When D-isopropylidene glycerol was used as starting material, although various optically active intermediates were obtained, the final '1-glyceryl-2-*myo*-inosityl phosphate' was inactive, owing to racemization occurred during removal of the isopropylidene group. '1-Glyceryl-2-*myo*-inosityl phosphate' was compared with the product of the alkaline hydrolysis of liver PI as well as with the same compound prepared by an alternative synthesis based on the condensation of D,L-isopropylidene glycerol with 1,3,4,5,6-pentacetyl-*myo*-inositol-2-phosphate in the presence of dicyclohexylcarbodiimide.[53]

By contrast, numerous synthetic routes to PI and its various phosphorylated derivatives have been described.[54–59] This is not surprising, taking into account the crucial role played by inositol glycerophospholipids and phosphates in cellular signaling coupled to difficulties in isolating them from natural sources in sufficient amounts for biochemical studies. The need for chemical probes to profile PI metabolism and measure activity of PI metabolic enzymes has stimulated, indeed, the development of methods for the efficient chemical synthesis of naturally occurring phosphatidylinositols as well as unnatural derivatives such as those depicted in Figure 4.[55,60–62]

The key steps in the reported syntheses of PI are the choice of most suitable strategy for the selective protection/deprotection of the six hydroxyl groups of the inositol moiety, the preparation of *myo*-inositol derivatives in enantiomerically pure form, and the formation of the phosphodiester bonds, *i.e.*, one to the glycerolipid portion and the other to the inositol head groups.

Recently, Greco and coworkers[63] reported the synthesis of the first fluorescent GPI probe (**6**) in 37% yield through a convergent approach involving two successive coupling reactions between the three key moieties: *myo*-inositol, glycerol, and the fluorophore (NBD-aminohexanoic acid, ((7-nitrobenzo [c][1,2,5]oxadiazol-4-yl) amino)hexanoic acid) (Scheme 1).

Using a well-established resolution strategy based on the use of camphor dimethyl acetal as a chiral auxiliary,[64,65] *myo*-inositol (**7**) was converted into the enantiomerically pure and regioselectively protected derivative D-3,4,5,6-tetra-*O*-benzyl-*myo*-inositol (**8**). Regioselective allylation of **8**, followed by insertion of a *N*-protected six-carbon spacer in 2 position afforded **9**. After selective removal of the allyl group, the resulting intermediate **10** was coupled with the phosphoramidite (**11**) containing the glycerol moiety. Final steps include debenzylation and deprotection of **12** by hydrogenolysis,

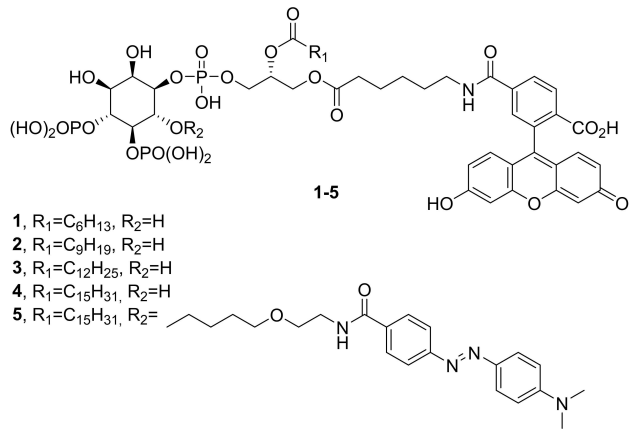
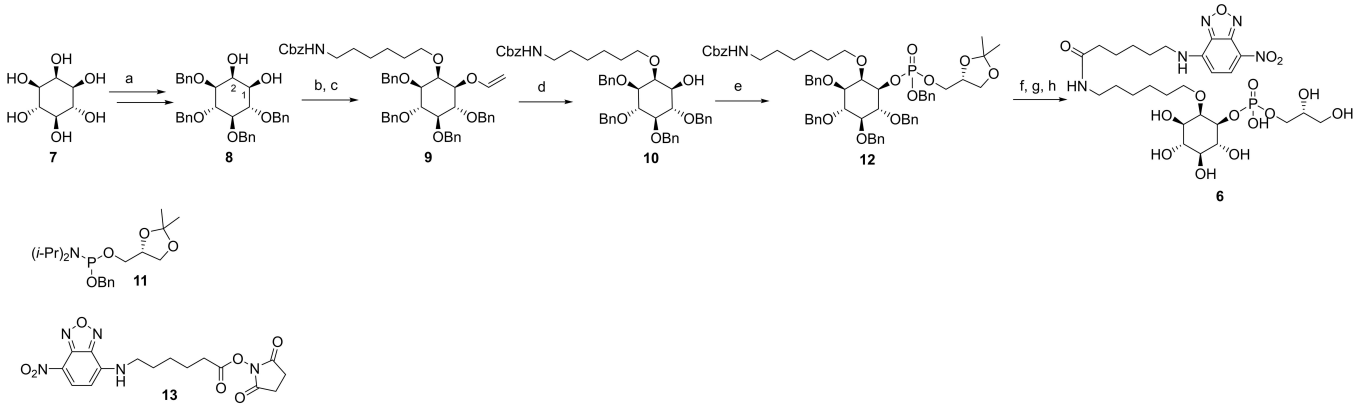


Figure 4. PI Analogues.[61,62]



Scheme 1. Synthesis of a GPI fluorescent derivative **6**.[63] Reagents and conditions: a) ref. [64,65]; b) Bu_2SnO, allyl bromide, CsF, dry MeOH, dry DMF, 24 h, 88%; c) $I(CH_2)_6NHCbz$, NaH, DMF, r.t., 16 h, 38%; d) PMHS, $ZnCl_2$, $Pd(PPh_3)_4$, THF, r.t., 4.5 h, 80%; e) **11**, 1*H*-tetrazole, *t*-BuOOH, dry MeCN, r.t., 2.5 h, 28%; f) H_2, $Pd(OH)_2$/C, EtOH, $NaHCO_3$, r.t., 24 h, 99%; g) **13**, Et_3N, DMF, r.t., 4 h, 60%; h) Dowex 50, H^+, MeOH, r.t, 12 h, 72%.

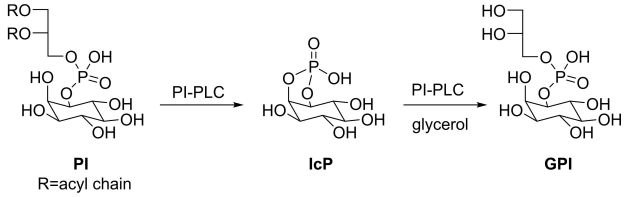
coupling between the resulting glycerol-protected derivative and the fluorophore activated as *N*-hydroxysuccinimidyl ester (NHS) (**13**), and acid-catalyzed methanolysis of the isopropylidene acetal to give **6**.

An alternative approach to the preparation of GPI and its organic (cyclohexylamine) and inorganic salts (calcium, magnesium, barium) was patented by Tronconi in 1994.[66] Deoiled soy lecithin, a low-cost commercial product, was hydrolyzed with sodium methoxide in methanol. After filtration, the residue was submitted to a number of tedious sequential steps of acidification, washing with methanol/water, elution through ion exchange resins, concentration, and precipitation by acetone and ethanol, to give GPI. Starting from 1.22 Kg of deoiled soy lecithin, 31 g of the target compound were obtained.

Corda et al.[22] have used this process to claim the preparation of a series of alkyl or acyl derivatives of GPI as modulators of activation or over-stimulation of cytosolic A_2 phospholipase.

2.1. Enzymatic synthesis

The enzymatic synthesis of GPI was reported by Bruzik et al.[67] within research focused on the transesterification of primary alcohols with inositol 1,2-cyclic phosphate (IcP), in the presence of a PI-specific phospholipase C (PI-PLC). IcP is in turn produced *via* a transesterification reaction involving the PI-PLC-catalyzed attack of the inositol 2-hydroxyl group on the phosphorus atom of PI with the release of 1,2-diacylglycerol (Scheme 2). Preparation of IcP was carried out starting either from PI-enriched soybean lecithin in a sodium deoxycholate dispersion or from crude soybean lecithin in a biphasic system water/diethyl ether. After purification, IcP was incubated in water with PI-PLC in the presence of glycerol (4.0 M) at room temperature. The resulting GPI was purified (81%) by ion-exchange chromatography by ammonium formate elution and characterized by ^{31}P NMR, ^{1}H NMR, and ES-MS. The reaction was shown to be regioselective as only the primary hydroxyl group of glycerol was phosphorylated. As for stereoselectivity, the reaction afforded two stereoisomers in 45:55 ratio arising from the phosphorylation of either the pro-*S* or pro-*R* primary hydroxyl group, as confirmed by the comparison with a single isomer of GPI obtained by deacylation of PI. To the best of our knowledge, this is the only enzymatic synthesis of GPI reported to date.



Scheme 2. Synthesis of GPI by PI-specific phospholipase C (PI-PLC)-catalyzed transesterification. IcP: inositol 1,2-cyclic phosphate.[67]

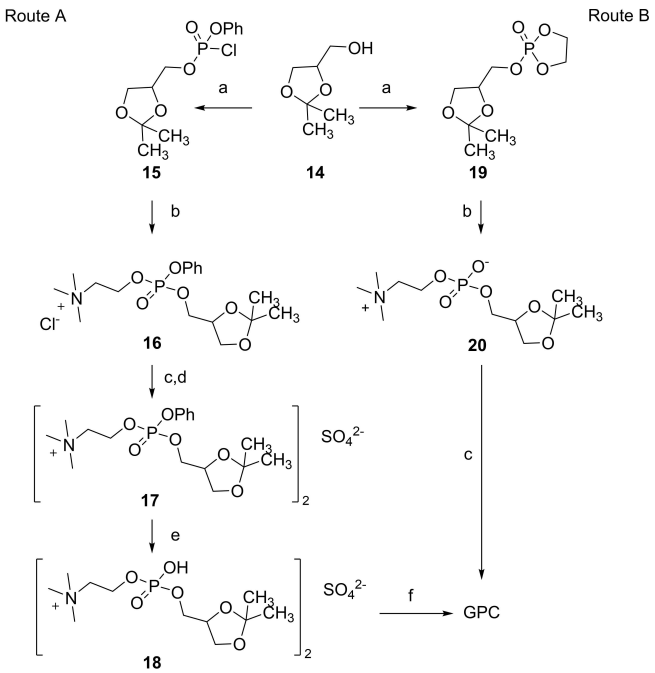
3. *sn*-Glycero-3-phosphocholine (GPC)

3.1. Chemical approaches

In 1945, Schmidt, Hershman, and Thannhauser[68] succeeded in isolating levorotatory GPC in fairly pure form from beef pancreas and elucidated its structure as the choline ester of GPA. Since isolation from natural sources hardly affords amounts exceeding a few grams, a number of chemical syntheses of GPC have been developed.

The first synthesis was reported by Baer and Kates (Scheme 3, route A).[69] Starting from 1,2-isopropylidene glycerol (**14**), GPC was obtained as a colorless, hygroscopic and viscous liquid in an overall 35–40% yield. Phenylphosphoryl chloride in the presence of quinoline was used for the phosphorylation of (**14**), followed by esterification with choline chloride and removal of the protecting groups.

Compound (**14**) was used as starting material also in the syntheses reported by other authors.[70–72] In a more straightforward preparation patented by Puricelli in 1992, the phosphorylation step was carried out by using 2-chloro-2-oxa-3,3,2-dioxophospholan to give isopropylidene 3-glyceryl-ethylenecyclic phosphate (**19**), which was treated with trimethylamine and finally deprotected to give GPC in 57% overall yield (Scheme 3, route B). It is noteworthy mentioning that GPC is obtained in optically active levorotatory, dextrorotatory or racemic form



Scheme 3. Route A: synthesis of GPC as described by Baer and Kates.[69] Reagents and conditions: a) $C_6H_5OPOCl_2$, dry quinoline, 10 min c.a., −10 °C→ r.t.; b) $HO(CH_2)_2N(CH_3)_3^+Cl^-$, dry pyridine, 4 h, r.t., c) $NH_4[Cr(NH_3)_2(NCS)_4]\cdot H_2O$, aq. Na_2CO_3, 45–55%; d) acetone-95% EtOH (2:3), Ag_2SO_4, r.t.; 79%; e) H_2, PtO_2 (Adam's catalyst), EtOH, r.t.; f) H_2O/H^+ (pH 1.5), 15 h, r.t., 65–75%. Route B: synthesis of GPC as described by Puricelli.[72] Reagents and conditions: a) $C_2H_4O_2POCl$, Et_3N, Et_2O, 0 °C→10 °C, 18 h, 97%; b) $(Me)_3N$, DCM, r.t., 5 h; c) 0.1 N HCl, r.t., 3 h, 62%.

starting from D,L or racemic 1,2-isopropylidene glycerol (**14**), respectively.

Other authors reported the use of both (*R*)- and (*S*)-glycidol (**21** and **27**, respectively) as starting material to produce GPC (Scheme 4).[27,73] (*R*)-Glycidol (**21**, Scheme 4, route A) was reacted with benzyl alcohol, and the resulting protected diol (**22**) was acetylated (**23**) and debenzylated to give **24**. Diacetate **24** was phosphorylated with phosphorous oxychloride and treated with choline tosylate affording **25**. The final deacetylation of **25** in MeOH afforded the target compound in a 69% overall yield for the last two steps.

Phosphorylation, the introduction of choline moiety, and deacetylation can be carried out in a one-pot mode, although with a lower yield (58%).[27] On the other hand, the direct reaction of (*R*)-glycidol with phosphocholine chloride allows achieving **26** in about 80% yield.

A more straightforward route to GPC involves the direct phosphorylation of (*S*)-glycidol (**27**, Scheme 4, route B) followed by treatment with choline tosylate and *in situ* ring-opening of the intermediate **28** (34% overall yield).[27]

Recently, the use of racemic or optically pure (*S*) or (*R*)-3-halo-1,2-propanediol instead of glycidol has been patented in the reaction with choline phosphate to prepare racemic or optically active GPC in high yield (97%) and gram scale.[74]

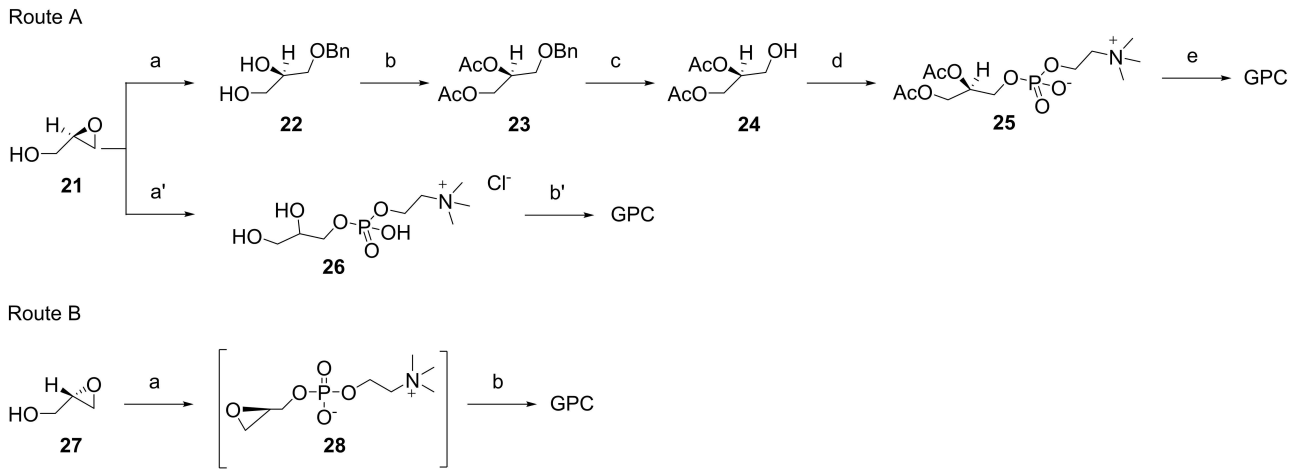
GPC can be obtained by deacylation of lecithin with mercuric chloride,[75] methanolic NaOH,[76] lithium aluminium hydride,[77] alkaline hydroxylamine,[78] or methanolic tetrabutylammonium hydroxide solution,[79,80] in 60–85% yields.

As for GPI, Tronconi disclosed a process for the preparation of GPC and GPE from crude or deoiled soy lecithin or from crude egg lecithin by deacylation with sodium methoxide in EtOH or MeOH.[81] After acidification to remove fatty acids, the methanolic solution was treated with zinc chloride or bromide and then with organic bases such as pyridine, to give a mixture of GPC and GPE that were separated by ion exchange resin: elution with water afforded GPC, followed by recovery of GPE by elution with dilute aqueous acetic acid. From 460 g of deoiled soy lecithin, 16.9 g of GPC were attained after crystallization from EtOH/Et_2O, whereas 4 g of GPE were recovered. In the following patent, isolation of GPC and GPE from mixtures obtained by deacylation of crude or deoiled soy or egg lecithin as well as from alcoholic extracts, thereof, is described.[82]

In another patent, mixtures of GPL obtained by treatment of deoiled soy lecithin with alcohols were eluted in a reactor containing a basic ion exchange resin, thus achieving deacylation and fractionation in a single step.[83] After elution from the ion exchange resins, GPC is purified from fatty acid esters and from other lipophilic impurities by extraction or treatment with apolar resins. GPC is finally crystallized from *n*-BuOH (yield: 13.5 g/100 g of ethanol-soluble soy lecithin). GPE and GPS are then recovered from the resin by eluting with solvents containing organic acids, such as acetic acid (1–10% v/v); GPE was obtained by crystallization from a 3:8 v/v H_2O/EtOH, whereas the eluted solution containing GPS is concentrated to dryness, and the residue is crystallized as calcium salt from a 1:0.4:0.7 v/v H_2O/EtOH/acetone mixture.

GPC can be also obtained by methanolysis of PC catalyzed by primary or secondary amines. The highest yields (>75%) were obtained with *iso*-propylamine, *tert*-butylamine, and 1,3-diaminopropane.[84]

Isolation and purification of GPC are often the major bottlenecks of all procedures described above. Crystallization,[85,86] resin column chromatography, and silica gel column chromatography[87] are the most frequently used techniques.



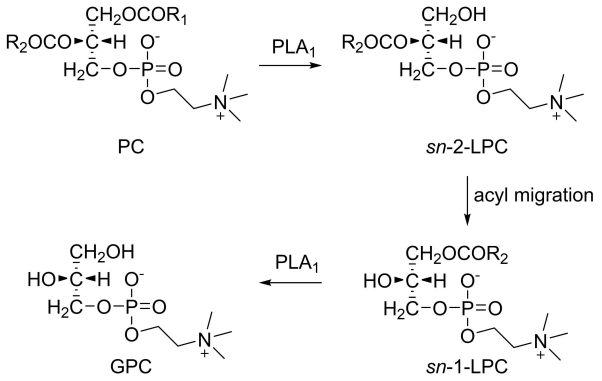
Scheme 4. Route A[73,27] synthesis of GPC starting from (*R*)-glycidol (**21**). Reagents and conditions: a) CsF, BnOH, 120 °C, 5 h, quant.; b) Et_3N, DMAP, CH_3COCl, DCM, 0 °C→r.t., 2.5 h, 92%; c) H_2, 10% Pd/C, *i*-PrOH, 40 °C, 9 h, 91%; d) i. $POCl_3$, Et_3N, DCM, 0 °C, 50 min.; ii. $HO(CH_2)_2N(CH_3)_3^+OTs^-$, pyridine, r.t., 12 h; iii. H_2O, r.t., 1 h, 76%; e) MeOH, K_2CO_3, r.t., 5 min., 91%. a') $(HO)_2(O)PO(CH_2)_2N(CH_3)_3^+Cl^-$, EtOH, 80 °C, 12 h, then *i*-$PrNH_2$ to pH 8, 3 h; b') H_2O, IONAC NM60 SG ion exchange resin, 60% c.a. (from **21**). Route B[27]: synthesis of GPC starting from (*S*)-glycidol (**27**). Reagents and conditions: a) i. $POCl_3$, Et_3N, DCM, 0 °C, 50 min.; ii. $HO(CH_2)_2N(CH_3)_3^+OTs^-$, pyridine, r.t., 12 h; iii. H_2O, r.t., 1 h, 80% (crude); b) K_2CO_3 aq., reflux, 5 h, 34% (from **27**).

3.2. Enzymatic approaches

The broad application of GPC as a cognitive enhancer and food ingredient makes its supply increasingly important in pharma and food sectors,[32,88] also considering that GPC is designated with the GRAS status by the US Food and Drug Administration.[89]

Enzymatic methods are a valuable alternative to chemical methods discussed above (see paragraph 3.1) which involve hydrolysis of PC or condensation of glycerol derivatives with phosphocholine donors. Even if chemical methods for lecithin modification are straightforward and inexpensive, enzymes can afford GPL and lysophospholipids with defined molecular structures due to their inherent selectivity.[90]

Several studies have reported the preparation of GPC through enzymatic hydrolysis of lecithin and/or PC-enriched lecithin under different reaction conditions, as summarized in Table 2. Due to the poor solubility of lecithin in water, most of the efforts have been devoted to the search of reaction media which could conjugate substrate solubility, enzyme stability, reaction yield, reaction time, and scalability. The most common strategies involved the use of biphasic systems, surfactants, or the control of substrate concentration by a fed-batch mode. Blasi et al. reported the preparation of GPC from egg yolk PC by using a combination of lipase from *Mucor miehei*, which is selective for the *sn*-1 position of PC and thus behaves like a PLA_1, and PLA_2 from hog pancreas. The highest conversion (94%) was achieved when the reaction was performed in a microemulsion system (isooctane/water) at 35 °C.[91] Lecitase Ultra is the most used PLA_1 as a biocatalyst to obtain GPC starting from PC under a wide range of experimental conditions. More in detail, Zhang et al.[92] reported the hydrolysis in aqueous medium starting from PC of food grade soy lecithin: GPC was obtained in 70–74% yield and up to 98.8% purity by silica or ion-exchange chromatography.[92,93] Hydrolysis of PC catalyzed by PLA_1 can lead to the formation of GPC as well as *sn*-1-LPC due to the migration of the acyl group from the *sn*-2 position to the *sn*-1 position in LPC.[94] Plückthun and Dennis[95] investigated acyl migration in LPC and reported that about 90% of thermodynamically stable 1-acyl LPC and 10% 2-acyl LPC were present in the mixture at equilibrium under the experimental conditions used. The presence of more *sn*-1 LPC results in an increased formation of GPC during the reaction because the acyl group at the *sn*-1 position of LPC is hydrolyzed by PLA_1 (Scheme 5).



Scheme 5. Synthesis of GPC from PC catalyzed by PLA_1. Mechanism of acyl migration.[90,92,95]

The acyl migration phenomenon can be intentionally induced to facilitate the formation of GPC, by tuning temperature, reaction time, water content, and enzyme loading.[96] As soybean accounts for more than 80% of the global supply of lecithin, it is not surprising that soy lecithin is the most widely used starting material for the enzymatic preparation of GPC.[97]

However, some challenges remain to be tackled, such as the poor water solubility of PC, which results in a low GPC productivity and could thus limit the industrial application of biotransformations. This evidence has prompted many authors to explore biphasic systems. Bang et al.[98] prepared GPC *via* Lecitase Ultra-catalyzed hydrolysis of soy PC in an *n*-hexane-water system, thus achieving enhanced productivity of GPC compared to other published enzymatic reaction systems.[91–93]

More recently, Kim et al.[26] have proposed the use of the same biphasic system but using Novozym 435 (an immobilized *Candida antarctica* lipase B) as the biocatalyst. In this case, both PC and the ethanol-soluble fraction of soy lecithin, which is very rich in PC, were used as starting material. After 6–8 hours, PC was completely converted into GPC; the target compound was recovered from the aqueous phase in 98.6% purity (ELSD-HPLC analysis). In addition, the immobilized lipase was easily removed from the reaction by filtration.

Surfactants are commonly used to enhance the solubility and dispersibility of non-polar chemicals in aqueous media. On this basis, aqueous solutions of lecithin have been supplemented with surfactants to increase the solubility of PC.[88] Among the six assayed surfactants, Tween 20 was the most

Table 2. Enzymatic synthesis of GPC by hydrolysis of lecithin.

Lecithin	Reaction system	Enzyme	Reaction time [h]	[Substrate] [$g \cdot L^{-1}$]	GPC yield [%]	GPC productivity [$g \cdot L^{-1} h^{-1}$]	References
Egg yolk[a]	Isooctane-water	Lipozyme IM and PLA_2	48	5.56	94[b]	–	[91]
Soybean	Water	Lecitase Ultra	3.5	6.67	73	0.65	[92]
Soybean	Hexane-water	Lecitase Ultra	30	240	85	15.74	[98]
Soybean	Hexane-water	Novozym 435	8	200	32	–	[26]
Egg yolk	Water-Tween 20	Lecitase Ultra	3	360	91[b]	37.52	[88]
Soybean	Soy oil-water	Lecitase Ultra	3	200	83	–	[99]

[a] PC and LPC from soybean lecithin; [b] HPLC conversion.

effective for enhancing GPC yield and productivity (see Table 2). In addition, to avoid substrate inhibition the enzymatic hydrolysis was carried out in a fed-batch mode. Under these conditions, this medium was found to be more tolerated by the enzyme (Lecitase Ultra) with respect to the biphasic system.

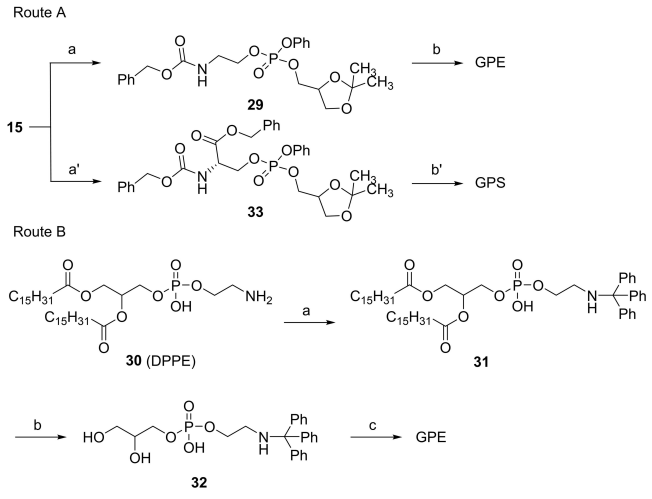
Cai et al.[99] developed a novel reaction medium consisting in solvent-free water-in-soybean oil for the hydrolysis of soy lecithin by using Lecitase Ultra as biocatalyst: 99.1% of the PC in soy lecithin was hydrolyzed with a GPC yield of 95.8% under optimized conditions (oil to PC ratio 4:1, water 30 wt%, enzyme loading 5 wt%, 55 °C, 3 h). The purity and recovery of GPC (by silica chromatography) were 94.8% and 83.0%, respectively.

4. *sn*-Glycero-3-phosphoethanolamine (GPE)

4.1. Chemical approaches

The presence of GPE in extracts of animal tissues or cellular membranes (*e.g.* pig and rabbit liver,[100] rat and ox brain,[101] and boar spermatozoa[102]) has been described. However, such procedures to extract GPE are laborious, time-consuming, and, needless to say, are not suitable for preparative purposes. Due to the interest in GPE bioactivities, efficient synthetic methods to produce GPE have been developed afterward.

The first synthesis of GPE was reported by Baer and Stancer[103] by following the same approach developed for GPC, as previously depicted in Scheme 3. Thus, GPE was obtained in about 30% yield starting from **15** in two steps (Scheme 6, route A). Specifically, treatment of **15** with Cbz-ethanolamine in pyridine afforded **29**, which was submitted to catalytic hydrogenolysis and then to acid hydrolysis. In Scheme 6 (route B) a semi-synthetic approach to GPE is sketched[48] using the commercially available 1,2-dipalmitoyl *sn*-glycero-3-phosphoethanolamine (DPPE) as the starting material. As the effort of using alkaline hydrolysis of DPPE (**30**) was unsuccessful, the amino group was protected with an acid-labile trityl group. The resulting *N*-trityl-DPPE (**31**) was subsequently deacylated to *N*-trityl-GPE (**32**) under alkaline conditions. Final deprotection with trifluoroacetic acid/DCM (1:2) at 0 °C for 5 minutes gave GPE in an overall 66% yield.



Scheme 6. Route A: total synthesis of GPE[103] and GPS[105] (see paragraph 5.1), respectively. Reagents and conditions: a) $OH(CH_2)_2NHCbz$, dry pyridine, 15–20 °C, 2 h, 81.8% (from **15**, see Scheme 3); b) i. Pd, H_2, 99% EtOH, 1 h; ii. PtO_2, 5 N H_2SO_4, H_2, 2.5 h; iii. H_2O, pH 1.7–1.9, r.t., 6–7 h, followed by Amberlite IR 120 (H^+) and recrystallization (99% EtOH), 43.5% (from **29**); a') $CbzNHCH(CH_2OH)COOCH_2Ph$, dry pyridine, 15 °C→25 °C, 3 h, 91.5% (from **15**, see Scheme 3); b') i. Pd, H_2, CH_3COOH, 99% EtOH, 75 min; ii. PtO_2, H_2, CH_3COOH, 99% EtOH, 3 h, followed by Amberlite IR 120 (H^+), 75.5% (from **15**, see Scheme 3). Route B: semi-synthesis of GPE.[48] Reagents and conditions: a) trityl bromide, Et_3N, DCM, r.t., o/n, 91%; b) MeONa, $CHCl_3$:MeOH (2:3), r.t., 3 h, 90%; c) TFA:DCM (1:2), 0 °C, 5 min., 81%.

4.2. Enzymatic approaches

Alternatively, as in the case of GPC (see paragraph 3.1), GPE can be prepared by deacylation of PE contained in lecithin.[81–83,104] To date, no enzymatic approaches have been reported for GPE synthesis.

5. *sn*-Glycero-3-phosphoserine (GPS)

5.1. Chemical and enzymatic approaches

GPS was synthesized by the same approach used for GPC and GPE as depicted in Scheme 6 (route A): the reaction of glycerylphenylphosphoryl chloride **15** with *N*-carbobenzoxy-L-serine benzyl ester in the presence of quinoline gave the intermediate **33**. Removal of the protecting groups by two subsequent catalytic hydrogenolyses and acid hydrolysis afforded the target compound in 75% overall yield.[105] More recently, the use of *tert*-butoxycarbonyl (BOC) and ethyl ester as protecting groups of L-serine was reported, without affecting the final yield (74%).[106]

When GPS was prepared by using lecithin as the starting material, mixtures enriched with PS were obtained by transphosphatidylation reaction catalyzed by PLD; this enzyme is able to transfer the phosphatidyl moiety to an alcohol acceptor.[2,16,83,106] For example, starting from 7 g of a phospholipid mixture, containing up to 41% of PS, 0.65 g of GPS were obtained as a white crystalline solid (see paragraph 3.1).

6. Conclusions and Outlook

Esters of *sn*-glycero-3-phosphoric acid (GPAE) are highly polar, water-soluble molecules endowed with important biological and pharmacological activities. Particularly, *sn*-glycero-3-phosphocholine (GPC) has a well-established use as a food ingredient and a cognitive enhancer, whereas *sn*-glycero-3-phosphoinositol (GPI) acts as a natural anti-inflammatory agent. Generally speaking, methods for GPAE manufacturing, each with its own associated advantages and disadvantages, are based either on deacylation of glycerophospholipids by lipases/phospholipases, or on a multi-step total synthesis from commercially available building blocks such as 1,2-isopropylidene glycerol, glycidol, or 3-halo-1,2-propanediol, either racemic or in optically pure form. The use of lecithin as starting material does not require protection/deprotection steps, thus

resulting in a more straightforward approach, no matter if the deacylation is carried out by either alcoholysis or enzymatic hydrolysis. The main bottleneck of both approaches is the complexity of the starting material as well as reaction work-up and product isolation. Indeed, the presence of structurally related molecules strongly affects product downstream. Solvent extraction, precipitation, ion exchange and/or silica chromatography, and recrystallization are routinely all required techniques for product isolation and purification. On the other hand, using lecithin as a raw material allows upgrading this abundant oilseed industry by-product into high-added-value molecules. The increasing interest in the study and supply of glycerophospholipids as well as their deacylated derivatives (GPAE), requires the development of versatile and efficient synthetic routes to these compounds. This minireview was conceived with the aim to provide the reader with an overview of the current manufacturing routes to all GPAE derived from glycerophospholipids as well as their intricate biological functions.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Esters · Enzyme catalysis · Phosphoric acids · Phospholipids · Synthetic methods

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