Di(polyprenyl) Phosphates as Models for Primitive Membrane Constituents: Synthesis and Phase Properties

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Abstract: Identical- and mixed-chain di-(polyprenyl) phosphates, derived from 3-methyl-2-buten-1-ol, (*E*)-geraniol, (*E,E*)-farnesol, (*E,E,E*)-geranylgeraniol, (*R*)-citronellol, or (*R*)-tetrahydrogeraniol have been synthesized by phosphitylation and oxidation with iodine/water. These phosphates gave organized systems in water, in particular vesicles, the physical properties of which were studied by photon correlation spectroscopy, electron microscopy, entrapment of a fluorescent dye (calcein), ^{31}P NMR, stopped-flow/light-scattering studies, and fluorescence polarization with lipophilic probes. Interdigitation of mixed-length phosphates was not found to have any favorable effect on the stability of the vesicles. The $C_{>15}$ vesicles were formed despite the fact that they are composed of only one molecular species and are devoid of any reinforcer, but those with two geranyl chains appeared to be rather fragile. Phosphates containing C_5 chains are soluble in water. The results obtained lend weight to the hypothesis that polyprenyl phosphates might have been primitive membrane constituents, and stimulate the search for appropriate reinforcers.

Keywords
amphiphiles · cytommetic chemistry · phosphitylation · polyprenols · vesicles

Introduction

In extant organisms, except archaea, cell membranes are built of ester phospholipids with *n*-acyl chains, or often, in bacteria, with branched chains. On the other hand, polyterpenoid derivatives are apparently present in the membranes of all living organisms, either as structural constituents (polyprenyl phospholipid ethers in archaea) or as mechanical reinforcers (hopanoids and carotenoids in procaryotes; sterols in eucaryotes).[1] Possible explanations for the prebiotic formation of these terpene derivatives were independently proposed by Wächtershäuser[2a] and by us,[2b] and we have suggested that polyterpenyl phosphates could have been involved in the prebiotic formation of the most primitive membranes.[3]

The known archeal phospholipids are usually saturated, but in some examples the polyprenyl chains conserve the double bonds originating from their biosynthesis.[4] However, their polar heads, based on phosphate derivatives of glycerol or of more elaborate polyols, are of a complexity suggestive of advanced evolution, and we wondered whether "primitive" membrane-forming phospholipids could not combine the simplest lipidic chains (from a biosynthetic point of view), acyclic polyterpenyl chains, with the simplest possible head group, a simple phosphate. These phosphates, with one or with two polyprenyl chains, can in principle be obtained by a series of simple chemical reactions; they might have been the ancestors of modern phospholipids. This hypothesis implies that they should form systems such as closed vesicles, which might have been anticipated, but had never been described. We have reported briefly that the sodium salts of digeranyl, difarnesyl, and di(geranylgeranyl) phosphates form vesicles when dispersed in water.[3]

We describe here the synthesis of two series of di(polyprenoid) phosphates (Fig. 1) in which the lengths of the polyprenoid

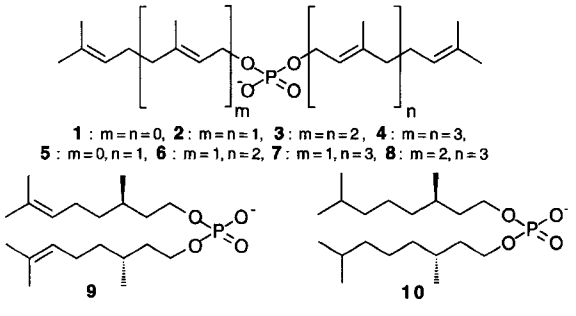

Fig. 1. Sodium di(polyprenyl) phosphates: **1**: diprenyl phosphate; **2**: digeranyl phosphate; **3**: difarnesyl phosphate; **4**: di(geranylgeranyl) phosphate; **5**: prenyl geranyl phosphate; **6**, geranyl farnesyl phosphate; **7**: geranyl geranylgeranyl phosphate; **8**: farnesyl geranylgeranyl phosphate.

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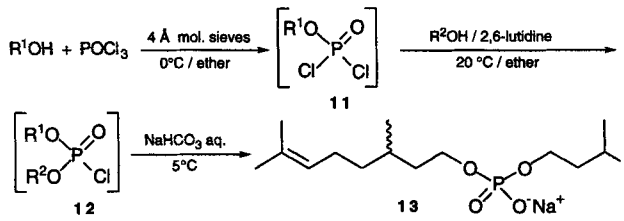
chains are identical (**1–4**, **9**, **10**) or different (**5–8**). The phase properties of these phosphates in water are then described: the formation of vesicles from the phosphates with chains of 20 or more carbon atoms was observed, in particular, by means of electron-microscopic techniques (cryo- and freeze-fracture microscopies). Their stability and water tightness were studied by ^{31}P NMR spectroscopy, probe entrapment, stopped-flow/light diffusion, and fluorescence polarization.

Results and Discussion

Synthesis of the Phosphates

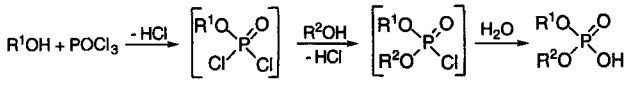
Phosphorylation of Polyprenyl Alcohols: A number of phosphorylation methods leading to identical or mixed-chain dialkyl phosphates have been described,[5] but none had been applied to the synthesis of di(polyprenyl) phosphates. Any procedure likely to be useful should take into account the high reactivity of the polyprenyl allylic alcohols, which are prone to dehydration and other side reactions during the phosphorylation step and workup. For the synthesis of mixed-chain di(polyprenyl) phosphates, a further requirement is the absence of contamination by identical-chain by-products, which can be very hard to separate from the desired product.

We first tested phosphorylation by phosphorus oxychloride, which is used for the industrial preparation of mono-, di-, and trialkyl phosphates, and even of allylic (but less substituted) derivatives.[6] It is also known that the replacement of the chlorine atoms of $POCl_3$ by alkoxy residues becomes progressively more difficult, and this allows mixed-chain dialkyl phosphates to be obtained (Scheme 1).[7] Danilov and co-workers[8] reported a failed attempt to prepare mono(polyprenyl) phosphates starting from the corresponding alcohols and $POCl_3$, using a procedure that had been successful for the phosphorylation of dolichol, a polyprenol saturated in the α,β position (i.e., not allylic).[9] The favorable outcome of phosphorylations with $POCl_3$ strongly depends on the experimental conditions,[10] in particular on the presence (or absence) and nature of bases which are able to remove the hydrogen halide generated during the reaction. In preliminary experiments (Scheme 2), we were



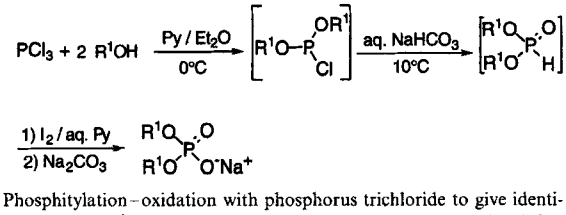
Scheme 2. Phosphorylation with phosphorus oxychloride to give a mixed-chain phosphate: R^1OH = (±)-citronellol; R^2OH = isopentanol.

able to prepare the mixed-chain (non-allylic) (±)-citronellyl isopentyl phosphate **13** in 34% yield, by adding (±)-citronellol (1 mmol) to an excess of $POCl_3$ in ether at 0 °C, in the presence of 4 Å molecular sieves as HCl scavenger;[11] isopentanol (1 mmol) was then added at 20 °C to the solution containing the phosphorodichloridate **11** and a slight excess of 2,6-lutidine. The phosphorochloridate **12** was hydrolyzed with aqueous $NaHCO_3$, and the crude product purified by column chromatography on SiO_2. Unfortunately, every attempt to synthesize digeranyl phosphate (**2**) or prenyl geranyl phosphate (**5**) by this procedure failed. Several nitrogen bases were tested as trapping agents for HCl (see Scheme 1), but we always obtained mixtures rich in non-phosphorylated substances and we were not able to recover the desired products. We also failed in our attempt to use the method of Ugi and co-workers based on a cyclic P^V reagent,[12] and had to turn to two-step methods using P^{III} derivatization and oxidation.



Scheme 1. Phosphorylation with phosphorus oxychloride: $R^1 = R^2$ or $R^1 \neq R^2$; HCl scavengers employed: triethylamine, diisopropylamine, pyridine, 2,6-lutidine, 2,3,6-collidine, quinoline, and 1,8-bis(dimethylamino)naphthalene.

Phosphitylation of Polyprenyl Alcohols: Phosphoramidites have often been used for the phosphitylation of alcohols.[13] Their application to the synthesis of phosphoric acid derivatives requires a further oxidative step, and their protecting groups must be cleaved to obtain the desired products; both oxidation and deprotection can be carried out easily and selectively under very mild conditions. However, the phosphoramidite methods described by Watanabe and co-workers,[14] Köster and co-workers,[15] and Poulter and Mautz[16] were unsatisfactory for the synthesis of **5**. This prompted us to use PCl_3 as the phosphitylating agent. Phosphorus trichloride has long been used for the synthesis of dialkyl phosphates (Scheme 3) by substitution of two chlorine atoms in the presence of an HCl scavenger, hydrolysis of the resulting dialkyl phosphochloridite, and final oxidation of the intermediate dialkyl phosphite.[5] This procedure was successfully applied to the phosphorylation of 3-methyl-2-buten-1-ol, (*E*)-geraniol, (*E,E*)-farnesol, (*E,E,E*)-geranylgeraniol, (*R*)-citronellol, and (*R*)-tetrahydrogeraniol, leading to the



Scheme 3. Phosphitylation–oxidation with phosphorus trichloride to give identical-chain phosphates: R^1OH = 3-methyl-2-buten-1-ol, (*E*)-geraniol, (*E,E*)-farnesol, (*E,E,E*)-geranylgeraniol, (*R*)-citronellol, or (*R*)-tetrahydrogeraniol.

Abstract in French: *La synthèse de phosphates de di-polyprényle, avec deux chaînes de longueurs identiques ou différentes, a été réalisée au mieux en passant par les dérivés de P^{III}, et oxydation par l'iode et l'eau. Les phosphates en $C_{>15}$ donnent des vésicules, dont les propriétés ont été étudiées par spectroscopie de corrélation photonique, cryomicroscopie électronique et cryodécapage, encapsulation d'une sonde fluorescente hydrophile (calcéine), spectroscopie de RMN de ^{31}P, flux bloqué/diffusion de la lumière et polarisation de fluorescence avec une sonde lipophile. L'interdigitation attendue des phosphates à chaînes différentes n'a pas d'effet favorable. Les vésicules contenant des chaînes géranyle sont bien formées, mais fragiles. Les phosphates contenant des chaînes en C_5 sont solubles dans l'eau. Ces résultats renforcent l'hypothèse de l'intervention de ces phosphates de polyprényle comme constituants primitifs de membranes, mais suggèrent l'importance de l'addition de renforçateurs.*

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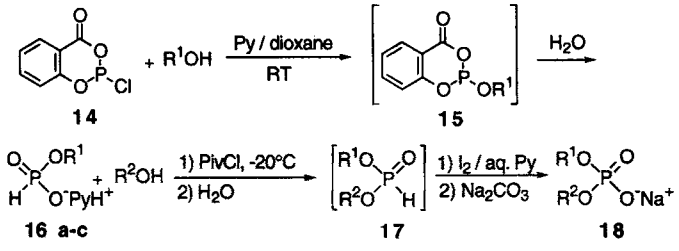
corresponding identical-chain di(polyprenyl) phosphates **1**–**4**, **9**, and **10**.[3] Phosphitylation with PCl_3 is, however, not suited for the synthesis of mixed-chain phosphates.

A general method for the preparation of nucleoside phosphonates using salicyl chlorophosphite **14** as a monophosphitylating agent was described by van Boom and co-workers.[17] This was also found to be suitable for the preparation of allylic monoesters of phosphonic acid via phosphonate intermediates.[18] Moreover, activated phosphonate monoesters can be treated with molecules containing an alcohol function to give phosphonate diesters.[19] This method led to a convenient procedure for the synthesis of mixed-chain di(polyprenyl) phosphates **5**–**8** (Scheme 4). One molar equivalent of the longer polyprenyl alcohol was added to a 1 M solution of **14** in dioxane in the presence of pyridine as an HCl trapping agent. The mono(polyprenyl) phosphonate **16**, obtained from the reaction mixture after hydrolysis of the intermediate **15**, was purified by column chromatography on silica gel. Activation of **16** with pivaloyl chloride followed by coupling with the second polyprenyl alcohol (1 equiv) afforded, after hydrolysis, the corresponding mixed-chain di(polyprenyl) phosphonate **17**, which was oxidized in situ to the phosphate **18**.



Scheme 4. Phosphitylation–oxidation with salicylchlorophosphite. R^1OH = a: (*E*)-geraniol, b: (*E,E*)-farnesol, c: (*E,E,E*)-geranylgeraniol; R^2OH = prenyl alcohol.

Oxidation of the Polyprenyl Phosphonates to Phosphates: For the oxidation of dialkyl phosphites, which exist predominantly as phosphonates in solution, permanganates, periodates, iodates, and hypochlorites have been used.[20] We found that $KMnO_4$, KIO_4, and tetra-*n*-butylammonium periodate or persulfate oxidize di(polyprenyl) phosphonates to phosphates without affecting the polyunsaturated chains; however, the isolation of the products from the reaction mixtures was very difficult. Other oxidizing agents such as [2-(phenylsulfonyl)-3-phenyl]-oxaziridine, 3-*n*-butyl-1,2-benzisothiazole 1,1-dioxide, and 80% *tert*-butylhydroperoxide in di-*tert*-butylperoxide, which do not oxidize the polyterpenic double bonds under appropriate conditions,[21] were ineffective for the oxidation of phosphonates into the desired phosphates. The mildest, most efficient and convenient reagent for this transformation was found to be iodine in aqueous pyridine 98% (v/v), as described by Garegg et al.[20b] It was successfully applied in the oxidation of all our di(polyprenyl) phosphonates.

Purification of the Polyprenyl Phosphates: Purification of the crude di(polyprenyl) phosphates is critical. For some products, such as difarnesyl phosphate, the pure product could be obtained by chromatographing only once on silica gel, followed by ion-exchange chromatography on a weak cation-exchange resin (CM Sepharose®, Na^+ form). For others, such as di(geranylgeranyl) phosphate, isolation of the desired product as the sodium salt required repeated column chromatography on silica gel before ion exchange. Our attempts to avoid silica gel chromatography, which causes much loss of product, were unsuccessful: other stationary phases, such as neutral alumina, cellulose, or Florisil®, were inefficient for the purification of the reaction mixtures. In some cases (e.g., for digeranyl phosphate) the final purification of the compound could be achieved by ion-exchange chromatography on DEAE Sepharose®,[22] but a preliminary purification of the crude product by silica gel chromatography was also required. Moreover, ion exchange could not be applied to some of the di(polyprenyl) phosphates, because of their particular amphiphilic properties. These compounds are not water-soluble and, especially those with two long polyprenyl chains, show a higher affinity for the organic mobile phase than for the anion-exchange resin, to which they were hardly bound. Final purification by HPLC on normal or reverse stationary phases was also investigated, but no satisfactory results were obtained. Attempts to isolate di(polyprenyl) phosphates from the reaction mixtures by precipitation induced by suitable inorganic or organic cations failed: the calcium salts of digeranyl and difarnesyl phosphate were obtained as microcrystalline solids, but only from the corresponding pure sodium salts and with considerable loss of product. The complexity of the purification step lowered the overall yields of sodium di(polyprenyl) phosphates, which were never higher than 30% based on the starting alcohols.

Stability of the Di(polyprenyl) Phosphates: The chemical stability of the acidic form of neat di(polyprenyl) phosphates **1**–**4** in $CHCl_3$ solution or in H_2O dispersion was checked under an inert atmosphere by ^1H NMR and TLC. The neat substances and their $CHCl_3$ solutions were found to be stable at −18 °C; when they were dispersed in water, decomposition was observed in 24 h at 20 °C. It is known that the stability of phosphate esters of allylic alcohols is improved when their phosphate head is negatively charged;[23] indeed, the sodium salts of **1**–**10** stored at −18 °C under an inert, dry atmosphere were found to be stable for at least one month, and could be used for the subsequent physical studies without previous purification.

Phase Properties of Di(polyprenyl) Phosphates: Long-chain phosphate dialkyl esters, with a charged head group and nC_{12} to nC_{16} chains or the branched C_{20} phytanyl chain, are known industrial surfactants.[24] They have been reported to give a variety of structures in water, including vesicles[25–28] and, in the case of diphytanyl phosphate (similar to **4**, but fully saturated), to stabilize vesicles made of the standard lecithin, dipalmitoylphosphatidylcholine.[29] In a preliminary communication, we have already shown that sodium digeranyl phosphate (**2**) as well as its higher homologues **3** and **4** form vesicles; no phase transition was observed by differential scanning calorimetry between 5 and 70 °C for such systems.[3b] Monolayer experiments were conducted independently and gave consistent results.[30] We have also attempted to obtain crystals of **2**–**4** suitable for X-ray crystallography, but the calcium salts, the only ones found to give crystals, gave needles that were far too small.

Electron microscopy: The sodium salts of the di(polyprenyl) phosphates with two C_5 chains (**1**) or with one C_5 and one C_{10} chains (**5**) gave clear solutions in water, as expected for molecules of such a large hydrophilicity/lipophilicity ratio. Those with two C_{10} (**2**), C_{15} (**3**), or C_{20} (**4**) chains, or with C_{10} and C_{15} (**6**), C_{10} and C_{20} (**7**), or C_{15} and C_{20} chains (**8**), as well as the partially or fully saturated compounds **9** and **10** gave opalescent suspensions by hydration of their films with a pH 7 buffer (buffer A; see Experimental Procedure, Materials) followed by

vortex mixing; this suggested that systems of larger size had been formed. We have already described the formation of vesicles in sonicated suspensions of sodium digeranyl, difarnesyl, and di(geranylgeranyl) phosphates, detected by negative-staining electron microscopy.[3] We have now studied these suspensions by cryo-electron microscopy (cryo-EM), which is the least perturbing and the most appropriate method for characterising liposome suspensions by electron microscopy.[31-33]

Suspensions of sodium digeranyl phosphate (**2**) contained vesicles, mainly unilamellar, of various shapes and sizes, and also tubules (Fig. 2) with diameters ranging from 10 to 40 nm and extending for several hundreds of nanometers. These tubular structures were multilayered (data not shown). Very small vesicles of diameter close to 10 nm (arrowheads) coexisted with larger vesicles with a diameter of 100 nm (arrows).

Fig. 2. Cryo-EM of sodium digeranyl phosphate (**2**) vesicles on a bare grid. Vesicles were prepared by vortexing and submitting a hydrated film of lipids (10 mg mL^{-1}) to several freeze-thaw cycles. Two kinds of structures are visible: unilamellar vesicles and tubules. Large unilamellar vesicles (arrows) coexist with small unilamellar vesicles (arrow heads). Fusion areas between large unilamellar vesicles and an extended vesicle are indicated by double arrow heads. Scale bar: 100 nm.

Cryo-EM showed that sonicated solutions of the higher homologue, sodium difarnesyl phosphate (**3**), contained vesicles with a rather wide size distribution (Fig. 3). The vesicles observed were all unilamellar, with sizes ranging from 10 to 150 nm. The presence of larger vesicles cannot be excluded, as they could be segregated in the thickest part of the ice film. The non-sonicated suspensions of **3** showed the same type of structures (data not shown).

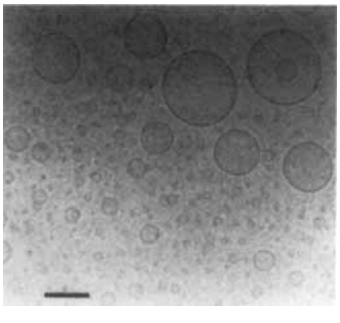


Fig. 3. Cryo-EM of sodium difarnesyl phosphate (**3**) vesicles. A homogeneous suspension of unilamellar vesicles is obtained with a wide size distribution, ranging from 10 to 150 nm.. Scale bar: 100 nm.

In the case of sodium di(geranylgeranyl) phosphate (**4**), the same solution of freeze-thawed vesicles was used for cryo-EM and for NMR studies. We observed a polydisperse solution of different types of structures (Fig. 4). Vesicular structures associated with narrow tubes were frequently observed. A large number of unusual bilamellar vesicles were present. When the solution was further sonicated, a standard population of unilamellar vesicles was observed (data not shown).

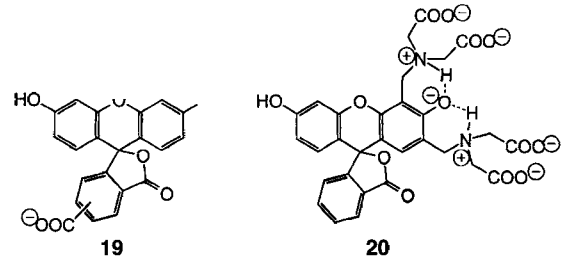
Fig. 4. Cryo-EM of sodium di(geranylgeranyl) phosphate (**4**) vesicles showing unusual bilamellar vesicles and long and narrow tubules. These tubules seem to originate from the end regions of the vesicles (or vice versa) (arrows). Scale bar 100 nm.

Sodium dicitronellyl phosphate (**9**) and ditetrahydrogeranyl phosphate (**10**) gave unilamellar vesicles, with diameters ranging from 10 to 150 nm (data not shown).

Thus, electron microscopy shows the formation of closed structures, in particular vesicles, except with phosphates containing one or two C_5 chains. A wide range of sizes and shapes are observed, depending on the method of preparation used.

Photon correlation spectroscopy (PCS): PCS was used for evaluating the size of structures formed by sonicated sodium di(geranylgeranyl) phosphate (**4**), difarnesyl phosphate (**3**), and digeranyl phosphate (**2**). Vesicle diameters of 100 ± 80 nm were thus recorded, in agreement with the cryo-EM results, which also showed that the vesicles have a rather wide size distribution. Suspensions of sodium difarnesyl phosphate (**3**) appeared by PCS to be rather more homogeneous and to essentially contain vesicles with diameters of 200 ± 50 nm; however, cryo-EM showed vesicles very heterogenous in size (from 10 to 600 nm). The discrepancy between PCS and cryo-EM values reflects the fact that PCS gives overall values for the size distribution; that is, in PCS polydispersed samples are considered as containing only spherical objects, and the presence of odd structures is not taken into account, while cryo-EM gives a view of all types of components.

Entrapment of fluorescent dyes and leakage: Closed vesicles should be capable of holding inside their cavity hydrophilic molecules unable to cross the lipidic layer. This can be tested by preparing vesicles in the presence of a water-soluble dye that does not fluoresce in concentrated solution because of self-quenching, but does upon dilution with water; this occurs once the vesicle surrounded by an aqueous environment is broken by addition of a surfactant or simply if the vesicle leaks. We have used this method with carboxyfluorescein (**19**);[3] however, the entrapment then observed was less efficient than that obtained with standard phospholipid vesicles. We have now considerably improved our results by using calcein (**20**), which carries a



19 **20**

higher net charge (-3) than carboxyfluorescein (-1) at pH 7.[34] Using a procedure derived from that of Matzuzaki et al.,[35] with 70 mM calcein, we have obtained clear-cut results for entrapment by vesicles of sodium difarnesyl, geranyl farnesyl,

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and geranyl geranylgeranyl phosphates (**3**, **6**, and **7**; Fig. 5). The procedure described by Oku et al.,[36] using 0.1 mM calcein, also gave clear-cut positive results with sodium di(geranylgeranyl) phosphate (**4**) and farnesyl geranylgeranyl phosphate (**8**). With sodium digeranyl phosphate (**2**), the fluorescence change is too weak to demonstrate the entrapment of the dye as clearly; we assume that the membrane is too thin, and that the dye can therefore leak out.

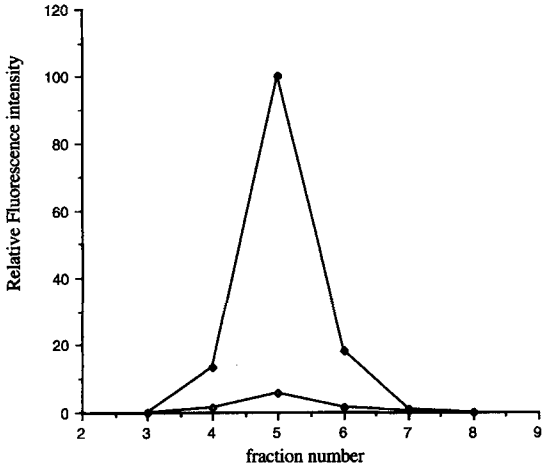

Fig. 5. Entrapment of calcein (70 mM) into vesicles of compound **3**: relative fluorescence intensity of fraction n diluted with 10% Triton X-100 [$(F_{max(n)}/F_{max(5)}) \times 100$] (♦); relative fluorescence intensity of fraction n diluted with buffer B [$(F_{(n)}/F_{max(5)}) \times 100$] without detergent (◇). Compounds **6** and **7** give comparable curves.

The failure of our attempts at entrapment with sodium di(geranylgeranyl) phosphate (**4**) and 70 mM calcein may be related to the effect of ionic strength, a factor which is known to be important for di-*n*-alkylphosphates:[37] phosphate **4** forms aggregates both in buffer B and in the isotonic 70 mM calcein. However, we have no plausible explanation for the lack of demonstrable entrapment with sodium farnesyl geranylgeranyl phosphate (**8**); it would be justified to repeat these experiments with a water-soluble non-ionic fluorescent dye.

Dye encapsulation also permits a quantitative evaluation of the volume of the total internal aqueous compartment of vesicles.[38] This can be expressed as µL per µmol of di(polyprenyl) phosphate and can be calculated from the amount of trapped dye, the concentration of the dye before encapsulation, and the molar concentration of di(polyprenyl) phosphate (see Experimental Procedure and Table 1). As expected, the freeze–thaw method gave vesicles with higher trapping efficiency than sonication, at dye concentrations of 70 or 0.1 mM.

The rate of calcein leakage was also studied by recording the fluorescence build-up in suspensions of vesicles, initially non-fluorescent because all the dye was concentrated inside the vesicles. Of course, this does not distinguish between real leakage through intact bilayers and progressive spontaneous breaking of vesicles. Figure 6 shows that, for vesicles of sodium difarnesyl phosphate (**3**), the fluorescence hardly increases with time, and therefore the suspension remains practically intact over at least 5 h. We had expected the stability of vesicles of the mixed-chain phosphates **6** and **7** to be at least as good as that of **3**. In fact, their apparent leakage rate is much higher; interdigitation is therefore an unfavorable factor with these polyprenyl chains, in contrast to the case of *n*-acyl phospholipids.[39]

Table 1. Calcein entrapment in sodium di(polyprenyl) phosphates (DPPNa) vesicles.

	V trapped (µL per µmol DPPNa)	
	70 mM sonication	0.1 mM freeze–thaw
2	[a]	[b]
3	5	10
4	[a]	39
6	3	[c]
7	<1	[c]
8	[a]	29

[a] Not tested. [b] Leakage too important. [c] No encapsulation.

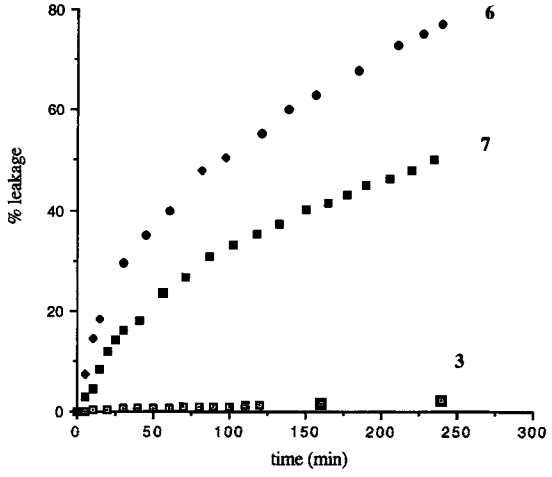

Fig. 6. Leakage of calcein (70 mM) from sonicated vesicles of compounds **3**, **6**, and **7**; % leakage evaluated by the fluorescence intensity relative to 100%, obtained after dilution with 10% Triton X-100.

^{31}P Nuclear Magnetic Resonance: ^{31}P NMR has been extensively used in the study of head-group conformations in membranes and to elucidate the nature of the phases formed by the phospholipids.[40,41] In order to interpret properly the results obtained, this technique should be used in combination with other methods;[42] we have supplemented ^{31}P NMR data by the informations obtained from cryo-EM.

The ^{31}P NMR spectra of multilamellar vesicle dispersions have a characteristic shape. The chemical anisotropy $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$ is measured as the chemical shift separation between the high-field peak ($\sigma_{\perp}$) and the low-field shoulder ($\sigma_{\parallel}$). A symmetrical signal indicates a fast and isotropic tumbling of the lamellar structures (small vesicles for instance), or a homogeneous solution, and this is where the electron microscopic evidence is useful as an additional criterion. Figure 7A shows the ^{31}P NMR spectrum of a water dispersion of sodium difarnesyl phosphate (**3**). The line shape of a powder spectrum is characteristic of a multilamellar system on which a sharp peak at around $\delta = 3.75$ is superimposed, as would be expected for single-wall vesicles or particles undergoing isotropic tumbling, but the appearance of the spectrum is not completely convincing. We therefore attempted to obtain ^{31}P NMR spectra of oriented multilayers, observed with an assembly of hundreds of parallel planar bilayers, oriented by their insertion between microscope cover glass plates; in this way, by changing the orientation of the stack of cover glasses in relation to the external magnetic field, it is possible to define the axis of motional averaging.

However, the method described in the literature,[43] which we had successfully used with phosphatidylcholines,[44] did not give satisfactory results, since sodium difarnesyl phosphate (**3**) did

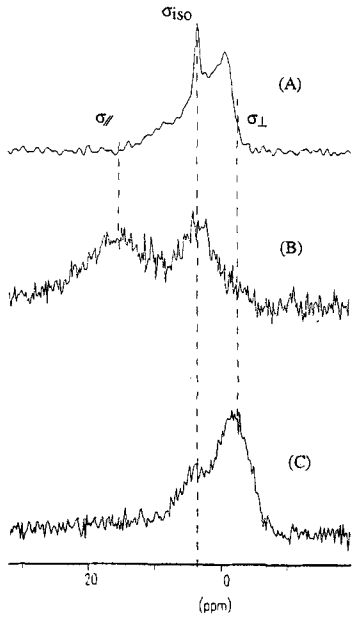

Fig. 7. A) Proton-decoupled ^{31}P NMR spectra of multilamellar dispersion of compound **3**, number of scans (NS) = 6560; B) and C) ^{31}P NMR spectra (without proton-decoupling) of partially oriented multibilayers of compound **3**: B) $\theta = 0°$, NS = 3800; C) $\theta = 90°$, NS = 4456.

not form continuous films on the glass slides, though these had been carefully washed with concentrated nitric acid, water, and acetone. We thus treated the cover glasses with concentrated ammonia, in the hope that the negative head groups of the phosphates would better wet a slightly basic surface. This was successful, but only partially. Figure 7 B and C show the resultant spectra measured in the extreme orientations $\theta = 0°$ and $\theta = 90°$: for both the experiments, the signal centered around $\delta = 3.75$, similar to the σ_{iso} of spectrum 7 A and assigned to small vesicles, is superimposed on a broad signal, centered around $\delta = +17$ for $\theta = 0°$ and at around $\delta = -5$ for $\theta = 90°$, corresponding to the $\sigma_{\parallel}$ and $\sigma_{\perp}$ signals observed for the random dispersion. These results are in agreement with what would be expected for a partially oriented system of multilayers. Of course, the quality of the spectra is not optimal (compare with those in ref. [44]), and further treatments of the glass surfaces would be required to improve their interaction with the phosphate head groups.

Water dispersions of phosphates **4** and **6–8** showed spectra similar to Figure 7 A, in agreement with the presence in suspension of a mixture of lamellar structures of various sizes and forms (Fig. 8). The chemical shift anisotropies of the bilayer spectrum obtained (Table 2) are small ($\Delta\sigma \leq 18$ ppm) relative to that of 1,2-dimyristoyl-*sn* - glycero - 3 - phosphocholine (DMPC) ($\Delta\sigma =$ 50 ppm), but are in the same range as that observed for didodecyl phosphate.[45] The chemical shift anisotropy value gives information about the motions of the phosphate head group in the bilayer; for dispersions of di(polyprenyl) phosphates it is influenced by the length of the chains. For the smallest molecules (digeranyl phosphate, **2**), only the sharp peak at around $\delta = 3.75$, due to isotropic tumbling, was observed, whereas there is some evidence of

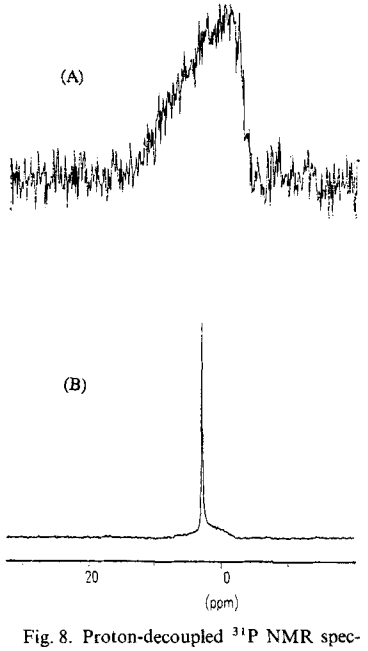

Fig. 8. Proton-decoupled ^{31}P NMR spectra of multilamellar dispersions of A) compound **4**, NS = 5200; B) compound **6**, NS = 4000.

Table 2. ^{31}P NMR of multilamellar dispersions.

	$\Delta\sigma$ [a] (ppm)	Isotropic peak
2	0	+
3	18	+
4	16	–
5	0	+
6	12	+
7	9	+
8	16	+

[a] $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$.

weak isotropic lamellar structures for the longest chains (di(geranylgeranyl) phosphate, **4**).

These ^{31}P NMR spectroscopy results confirm the decreased mobility of the polar head group with increasing length of the chain.

Water permeability—stopped-flow/light-scattering method: Bilayer-bound vesicles exhibit volume changes when subjected to an osmotic shock. This behavior is shared by multilamellar vesicles (MLV), and large and intermediate unilamellar vesicles (LUV and IUV, respectively), while it is doubtful whether small unilamellar vesicles (SUV) obtained by sonication could withstand the volume changes without breaking. Important information about the permeability of amphiphilic bilayers to solvents or solutes (ionic or neutral) can be obtained following osmotic swelling or shrinkage of vesicles.[46] Most of the studies carried out so far deal with the determination of water permeability, and they are (sometimes implicitly) based on the assumption that vesicles act as ideal osmometers (i.e., that they are permeable to the solvent but not to the solute). We have used stopped-flow measurements to evaluate not only the water permeability, but also the elasticity of bilayer vesicles, following a theoretical model that we developed.[47, 48] This theoretical model shows that the variation of scattered light intensity of a vesicle suspension should follow first-order kinetics controlled by the water permeability of the bilayer: the higher the rate constant, the higher the water permeability. The experimental procedure[47] is based on the direct measurement of the time-dependence of the scattered light intensity of a vesicle suspension following an osmotic shock. This technique[49] improves the sensitivity of the measurements in comparison with the previously used changes in absorbance (or transmittance), and allows one to use dilute vesicle suspensions (conc. lipid ca. 10^{-4}–10^{-3} M), less prone to undesired phenomena (e.g., aggregation) during the rapid mixing with the hyper- or hypotonic saline solution. In the present study we have evaluated the water permeability of vesicles of sodium di(polyprenyl) phosphates by following their shrinkage following an osmotic shock. We have not made use of the relative variation of scattered light intensity to measure the elasticity of the bilayer vesicles, because of the coexistence of different kinds of systems (MLV, LUV, IUV, SUV, tubules, etc.) in the vesicle suspensions.

We had previously shown that sonication, solvent injection, and reverse-phase evaporation are useful methods for the preparation of vesicles suitable for the osmotic shock experiments from the normal lecithins.[47–53] A concentration difference of at least 150 mM of NaCl between the inner and the outer vesicle compartments was found to be necessary, with the instrument then available, in order to obtain a good signal-to-noise ratio during the experiments. The higher instrumental sensitivity now available made it possible to detect scattered light variations even in the case of a saline concentration jump half as large, and

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our experiments were carried out by mixing vesicle dispersions in buffer E with the same volume of buffer F ($\Delta[NaCl]_{int-ext}$ = 75 mM). An intensity/time exponential recording obtained for di(geranylgeranyl) phosphate (**4**) vesicles in buffer F is shown in Figure 9.

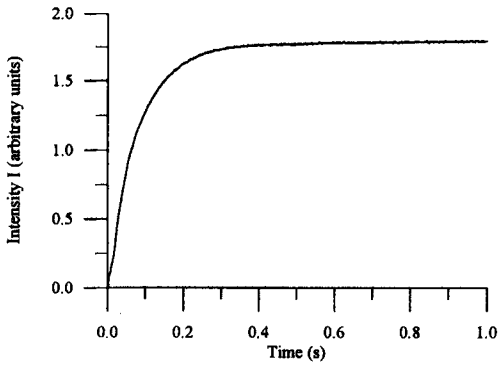

Fig. 9. Vesicle shrinkage upon osmotic shock: experimental scattered light variation vs. time: di(geranylgeranyl) phosphate (**4**) vesicles, buffer F, average diameter: 133 nm; $T = (15 \pm 1)$ °C; $\lambda = 400$ nm.

Numerical fitting of the kinetic data was achieved by means of the exponential relationship (1), in agreement with the theoretical model. This relationship holds for the initial, fast part

$$I = I_\infty + A e^{-kt} \quad (1)$$

of the signal change; it is followed by a slow drift, which we have not studied further.

In the case of vesicles of sodium digeranyl phosphate (**2**), a fast increase in the scattered light intensity was observed when the vesicles were shrinking after having been subjected to a hypertonic shock. The rate constants k calculated according to Equation (1) were in range of 150–200 s^{-1}. Due to the mixing time (≈ 3 ms) of the stopped-flow technique, more precise kinetic determinations could not be obtained. The thin sodium digeranyl phosphate (**2**) vesicles thus allow a rapid permeation of H_2O.

For the next higher homologues **3** and **4**, we were able to determine the average first-order rate constants k (Tables 3 and 4). A satisfactory statistical fitting of the exponential light-scaterring signal was observed for the shrinkage experiments on freeze–thawed samples of phosphate **4**; for **3**, the statistical fitting was poorer: the reproducibility of the kinetic experiments was good, but other processes than water permeability were most probably involved.

According to the results reported in Tables 3 and 4, the k values calculated for three samples of **4** were found to be substantially lower than those calculated for four samples of **3**. It must be pointed out that the k value of 20.5 s^{-1} for sample D, which is the smallest found in the case of **3** upon shrinkage, is still larger than the k value of 13.2 s^{-1} found for sample F, made of **4**. Thus, as expected intuitively, the permeation of water through di(geranylgeranyl) phosphate (**4**) bilayers is slower than through difarnesyl phosphate (**3**) bilayers in the case of a hyperosmotic shock.

The results found for vesicles of sodium difarnesyl phosphate (**3**) upon shrinkage and swelling were compared with those obtained for the permeability towards D_2O described by Lawaczeck:[54, 55] in this case, the variation of refractive index following the influx of D_2O into vesicles prepared in an isotonic H_2O buffer gave rise to an exponential decrease in the scattered light intensity. With difarnesyl phosphate (**3**) vesicles, the signal was small and after a fast exponential decrease of the light intensity, a slow increase could be observed; the rate constants k calculated for the exponential D_2O influx range between 13 and 16 s^{-1}, and agree well with those found for the osmotic shock treatment (Table 3), when the fact that D_2O permeates membranes more slowly than H_2O is taken into account.[54, 56]

Fluorescence polarization: Two hydrophobic fluorescent probes were incorporated in the membrane bilayers: (*E,E,E*)-diphenylhexatriene (DPH) and trimethylammonium-(*E,E,E*)-diphenylhexatriene (TMA-DPH). Fluorescence anisotropy was used to estimate the fluidity of the internal leaflet of the bilayer using TMA-DPH, or of the hydrophobic core of the membrane bilayer using DPH. Some preliminary experiments of fluorescence polarization using DPH or TMA-DPH showed that the anisotropy values r were weak compared with the values given in the literature[57] for DPH or TMA-DPH incorporated in DMPC vesicles or in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) vesicles (Table 5). These results emphasize that membranes of di(polyprenyl) phosphates **3** and **4** are much more fluid than those of DPPC or DMPC. As expected, the values of anisotropy r observed for vesicles of the longer-chain di(geranylgeranyl) phosphate (**4**) were larger than for vesicles of difarnesyl phosphate (**3**).

Table 3. Shrinkage experiments on vesicles of **3**.

Sample	d_{av} [a] (nm)	SD [b] (nm)	k [c] (s^{-1})	SD [b]
A	122	26	31	1
B	130	33	27	1
C	115	30	31	6
D	123	35	20.5	0.3

[a] Average diameter. [b] Standard deviation. [c] Average rate constant.

Table 4. Shrinkage experiments on vesicles of **4**.

Sample	d_{av} [a] (nm)	SD [b] (nm)	k [c] (s^{-1})	SD [b]
E	140	35	12.0	0.4
F	133	35	13.2	0.2
G	140	36	13.1	0.2

[a] Average diameter. [b] Standard deviation. [c] Average rate constant.

Table 5. Comparison of fluorescence anisotropy (r) of DPH and TMA-DPH in vesicles of **3**, **4**, DMPC, and DPPC.

	T (°C) [a]	Fluorescence anisotropy r TMA-DPH	DPH
3	20	0.140 (±0.004)	0.0369 (±0.004)
4	20	0.148 (±0.004)	0.0546 (±0.004)
DMPC [b]	30	0.20	0.120
DPPC [b]	45	0.19	0.076

[a] Temperature higher than the phase-transition temperature. [b] Values from ref. [57].

Conclusion

The various methods used here for the study of vesicles of sodium di(polyterpenyl) phosphates give results that are far less clear than those obtained for the usual phospholipids such as lecithins. Each technique has its own limitations; yet, their combined use shows that vesicles do form, except when one or both of the chains is as short as C_5, and that they have the expected

barrier properties. However, di(polyprenyl) phosphate vesicles are more fragile and disordered than those obtained from *n*-acyl phospholipids. Their properties vary quite abruptly from one member of the series to the next, as a consequence of the large C_5 increments in the chain lengths, and the influence of interdigitation on the bilayer stability is unexpectedly negative (this implies that the increase in fluidity due to the heterogeneity of the system is, in this case, more important than the increase in contact surfaces). The implications of these findings for our hypothesis on the possible role of such di(polyterpenyl) phosphates as primitive membrane components remain to be ascertained—it should also be taken into account that phosphates carrying only one polyterpenyl chain might be involved, too,[58] either alone or in combination. Perhaps even more importantly, it should be noted that we followed the rigorous course of working with pure components, whereas all known membranes contain complex mixtures; the next steps should involve the addition of non-phosphorylated alcohols, and of some of the reinforcers usually present in natural membranes, for which we have postulated that the most primitive ones could be the tricyclopolyprenols.[1, 2b] This is the clear direction that must now be followed in this combined study of "prebiomimetic"[2b] and "cytomimetic"[59] chemistries. We have already embarked on a study with monolayers.[30]

Experimental Procedure

General: Anhydrous pyridine, dioxane and ether were purified by distillation over calcium hydride; phosphorus trichloride was distilled before use. 2-Chloro-1,3,2-benzodioxaphosphorin-4-one (salicyl chlorophosphite, Fluka, >97%) was used as received without further purification. Carboxyfluorescein **11** and calcein **12** (Fluka) were purified before use [60]. Moisture-sensitive reactions were carried out in flame-dried glassware under an inert atmosphere. Evaporation of solvents was performed under reduced pressure without heating over 35 °C. Aqueous buffers were prepared with MilliQ water [HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA: ethylenediaminetetraacetic acid; TRIS: tris(hydroxymethyl)aminomethane]: Buffer A: NaCl 10 mM, HEPES 10 mM, EDTA 1 mM, pH = 7. Buffer B: NaCl 150 mM, TRIS·HCl 10 mM, EDTA 1 mM, pH = 7. Buffer C: HEPES 10 mM, NaCl 10 mM, pH = 7.2. Buffer D: HEPES 10 mM in D_2O, pH = 7. Buffer E: TRIS·HCl 10 mM, NaN_3 5 mM, EDTA 1 mM, pH = 7.95. Buffer F: NaCl 150 mM, TRIS·HCl 10 mM, NaN_3 5 mM, EDTA 1 mM, pH = 7.95. Calcein solution: 70 mM calcein, EDTA 1 mM, HEPES 10 mM, pH = 7; 0.1 mM calcein, HEPES 10 mM, pH = 7.

Spectra: IR: Perkin-Elmer 881 spectrometer, in $CHCl_3$, characteristic absorption bands in cm^{-1}. NMR: Bruker WP (200 MHz), Bruker AM (400 MHz), or Bruker SY (300 MHz) spectrometers; chemical shifts (δ) in ppm with respect to $CDCl_3$ (δ = 7.26) or CD_3OD (δ = 3.30) as internal standards for ^1H NMR, to $CDCl_3$ (δ = 77.0) as internal standard for ^{13}C NMR, and to H_3PO_4 (80% in $CDCl_3$, δ = 0) as external standard for ^{31}P NMR; values of coupling constants J in Hz; data annotations: superscripted a = the resolution as a multiplet was low and coupling contants were not determined; * = assignment of the peaks may be reversed; ' indicates the carbon numbers in the shorter chain of mixed-chain products; carbon peaks of the di(polyprenyl) phosphates were assigned by comparison with spectra of the corresponding alcohols. ^{31}P NMR spectra of vesicles were obtained on a Bruker ARX-500 spectrometer operating at 202.45 MHz, at 25 °C, in a 5 mm NMR tube. Multibilayer oriented phase spectra were performed on a Bruker MSL-300 spectrometer operating at 121.496 MHz using a solid ^{31}P probe without proton decoupling. Experiments were run in a 10 mm (o.d.) NMR tube. Mass spectra (MS): VG Analytical ZAB-HF spectrometer in the FAB mode, m/z relative intensities (in %) in round brackets.

Preparation of vesicles: Standard methods (freeze–thaw, vortexing or sonication) [38, 60] suitable for systems with different lamellarity, size, and trapped volume [61], chosen according to the membrane properties under investigation. All vesicles were prepared under a strictly inert atmosphere. The phosphorus content of each vesicle preparation was determined according to the method described by Morrisson [62]. Sonication in a bath (Sonorex RK 100 H Bandelin) or with a titanium tip (Sonifer cell disruptor B-30, duty cycle 50, power 4). Extrusion through polycarbonate membranes (Costar, pore size = 200 nm) in a Matesson Model 3030-580 instrument.

Photon correlation spectroscopy: Vesicle size and sample dispersity evaluated on a Coulter-Counter N4MD instrument, laser light scattering at 20 °C with a scattering angle of 30° for MLV and 90° for IUV and SUV.

Stopped flow: The variation of scattered light intensity vs. time upon osmotic shock (T = 15.0 ± 0.2 °C) was followed at the fixed wavelength of 400 nm (slit width = 4 nm) on a Biosequential DX-17MV stopped-flow ASVD spectrofluorimeter (Applied Photophysics). Analysis of data: Bio-Kine Analysis V 3.14 software (Bio-Logic).

Encapsulation: The amount of calcein (**20**) or carboxyfluorescein (**19**) in encapsulation experiments was determined fluorometrically with an Amicon SPF 500 spectrophotometer. Gel filtration for removal of non-encapsulated dye on a Sephadex® G50 (Pharmacia) column (30 × 1 cm) thermostated at 20 °C. Excitation and emission wavelengths set at 490 and 520 nm respectively (slit width 4 nm).

Microanalyses: Service Central de Microanalyse du CNRS (Vernaison).

Chromatography: Analytical TLC: precoated silica gel plates 60F-254 (Merck); elution with $CHCl_3$/MeOH (7/3 v/v); detection effected by dipping the plate in a solution of vanillin (1 $g\,L^{-1}$ in EtOH/H_2SO_4 (95/5 v/v)), and then by heating it on a hot plate; for phosphorus-containing compounds, the procedure reported by Dittmer and Lester [63] was followed. Flash chromatography (FC, P = 0.5–1.1 bar): silica gel Geduran SI 60 40–63 mm (Merck). Anion-exchange chromatography: DEAE Sepharose Fast Flow (Pharmacia); cation-exchange chromatography: CM Sepharose fast flow (Pharmacia). filter: Millex-SR 0.5 mm (Millipore).

General procedure for the synthesis of identical-chain di(polyprenyl) phosphates: Pyridine (40 mmol) was added at 0 °C to phosphorus trichloride (20 mmol) in dry ether (50 mL). The polyprenyl alcohol (40 mmol) diluted in dry ether (50 mL) was then added dropwise within 2 h and stirring was continued for 3 h at 0 °C. The precipitate was filtered through Celite under argon and washed with dry ether (100 mL). The combined ether phases at 0 °C were treated with aqueous 10% $NaHCO_3$ (30 mL). After 30 min of vigorous stirring, the organic layer was separated and dried over $MgSO_4$. The crude material could be stored several weeks at −18 °C. Its typical composition (determined by ^1H NMR) was: di(polyprenyl) phosphonate 80% and starting alcohol 20%. The crude mixture could be oxidized without preliminary purification. Iodine (1.7 mmol) dissolved in pyridine (3 mL) was added dropwise at room temperature to a stirred solution of the crude mixture (containing 1.5 mmol of di(polyprenyl) phosphonate) in pyridine/H_2O (5 mL, 98/2 v/v). The complete conversion of the phosphonate usually required 30 min (TLC). The excess iodine was then destroyed by adding saturated aqueous Na_2SO_3 (1 mL). The solution was evaporated, and the residue coevaporated with toluene (3 × 10 mL), taken up in ether (50 mL), and shaken with 1 M HCl (5 mL). The aqueous phase was washed with ether (2 × 5 mL), and solid Na_2CO_3 was added to the combined organic extracts. The mixture was vigorously stirred at 25 °C for 30 min, and the solid was then removed by filtration and carefully rinsed with ether (3 × 50 mL). Evaporation of the combined ether phases afforded the crude sodium salt di(polyprenoid) phosphate.

General procedure for the synthesis of mixed-chain di(polyprenyl) phosphates: The polyprenyl alcohol (5 mmol) in pyridine (10 mL) was added dropwise at 25 °C to a stirred 1 M solution of 2-chloro-1,3,2-benzodioxaphosphorin-4-one in dioxane (10 mL). After 10 min the alcohol was completely converted (TLC), and the excess of phosphitylating agent was decomposed by adding water to the stirred mixture. After the removal of the solvents, the residue was coevaporated with toluene (3 × 10 mL) and purified by FC on silica gel with a linear gradient of MeOH in CH_2Cl_2 (from 0 to 30% v/v) to afford the pyridinium salt of mono(polyprenyl) phosphonate in yields ranging from 50 to 80%, depending on the starting alcohol. The pyridinium salt (5 mmol) and another polyprenyl alcohol (10 mmol) were dissolved in anhydrous pyridine (7 mL) and cooled to −20 °C. Pivaloyl chloride (10 mmol) was then added to the stirred solution. After 1 h water (5 drops) was added, and the crude di(polyprenyl) phosphonate thus obtained was oxidized in situ according to the procedure reported above.

Final purification of sodium di(polyprenyl) phosphates: FC on silica gel, with a linear gradient of CH_3OH in CH_2Cl_2 (0 to 30% v/v) as eluent, allowed most impurities to be removed. Ion-exchange chromatography was successfully used for the final purification of **1**, **2**, **5**, **6**, **9**, and **10**, but a preliminary chromatography on silica gel was also necessary. 1 mmol of product was dissolved in CH_3Cl/CH_3OH (0.5 mL, 2/1 v/v) and applied to a column containing 5 mL of an anion-exchange resin equilibrated with the same solvent mixture. The product was eluted with a linear gradient of ammonium acetate (0–2 mM) in $CHCl_3/CH_3OH$ (2/1 v/v). The excess of ammonium acetate was then eliminated by gel filtration chromatography on a Sephadex LH-20 (eluent $CHCl_3/CH_3OH$ 2/1 v/v) [22] or by following a more rapid procedure: whereas ammonium salts of di(polyprenyl) phosphate are soluble in pentane, ammonium acetate is not, so the latter can be removed from a pentane solution of the product by filtration on Millex-SR filter unit (pore size = 0.5 μm). The ammonium salt of di(polyprenyl) phosphate was finally converted into the corresponding sodium salt by cation-exchange chromatography, eluting with $CHCl_3/CH_3OH$ 2/1 v/v . This procedure was inefficient for **3**, **4**, **7**, and **8**. These compounds were purified by repeated FC on silica gel and finally converted into their sodium salts by cation-exchange chromatography. After purification all the products were lyophilized and stored at −18 °C under inert atmosphere. Yields based on the starting alcohol were at most 30%.

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Sodium di((2*E*)-3,7-dimethylocta-2,6-dienyl) phosphate (digeranyl phosphate, **2**): ^{1}H NMR (400 MHz, CD_3OD): $\delta = 5.43$ (t, $J(^1H-^1H) = 6.4$ Hz, 2H; HC(2)); 5.14 (t, $J(^1H-^1H) = 7$ Hz, 2H; HC(6)); 4.40 (dd, $J(^1H-^1H) = 7$, $J(^1H-^{31}P) = 7$ Hz, 4H; $H_2C(1)$); 2.15–2.03 (brm, 8H; $H_2C(4)$ and $H_2C(5)$); 1.71 (s, 6H; CH_3); 1.70 (s, 6H; CH_3); 1.64 (s, 6H; CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 139.1$ (s, C(3)); 131.4 (s, C(7)); 124.1 (s, C(6)); 121.4 (d, $J(^{13}C-^{31}P) = 8.3$ Hz, C(2)); 62.4 (d, $J(^{13}C-^{31}P) = 4.7$ Hz, C(1)); 39.7 (s, C(4)); 26.6 (s, C(5)); 25.6 (s, C(8)); 17.6 (s, $H_3C-C(7)$); 16.3 (s, $H_3C-C(3)$). ^{31}P NMR (162 MHz, $CDCl_3$): $\delta = 2.3$. FAB-MS (neg.): *m/z*: 761.4 (14, $[2M+23]^-$); 369.2 (100, M^-); 299.1 (37); 233.1 (26). IR ($CDCl_3$): $\tilde{\nu} = 2966$ (m), 2923 (m), 2855 (m), 1446 (m), 1378 (m), 1224 (m), 1143 (w), 1096 (m), 1000 (m), 885 (w), 824 (w). All other compounds showed similar IR spectra. Analysis on the microcrystalline calcium salt prepared by addition of a calcium chloride solution and recrystallization from water: $C_{40}H_{68}O_8P_2Ca$: calcd C 61.67, H 8.80, P 7.95, Ca 5.15; found: C 61.71, H 9.1, P 7.6, Ca 5.0.

Sodium di((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyl) phosphate (difarnesyl phosphate, **3**): ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$): $\delta = 5.35$ (t, $J(^1H-^1H) = 6.4$ Hz, 2H; HC(2)); 5.05–5.08 (t, $J(^1H-^1H) = 7$ Hz, 4H; HC(6) and HC(10)); 4.38 (t, $J(^1H-^1H) = 6$, $J(^1H-^{31}P) = 6$ Hz, 4H; $H_2C(1)$); 2.10–1.90 (brm, 16H; $H_2C(4)$, $H_2C(5)$, $H_2C(8)$, and $H_2C(9)$); 1.66 (s, 6H; CH_3); 1.64 (s, 6H; CH_3); 1.58 (s, 6H; CH_3) 1.57 (s, 6H; CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 139.3$ (s, C(3)); 135.1 (s, C(7)); 131.2 (s, C(11)); 124.4* (s, C(10)); 124.0* (s, C(6)); 121.3 (d, $J(^{13}C-^{31}P) = 7.7$ Hz, C(2)); 62.4 (d, $J(^{13}C-^{31}P) = 3$ Hz, C(1)); 39.7 (s, C(4) and C(8)); 26.8 (s, C(9)); 26.7 (s, C(5)); 25.7 (s, C(12)); 17.6 (s, $CH_3-C(11)$); 16.4 (s, $CH_3-C(7)$); 15.9 (s, $CH_3-C(3)$). ^{31}P NMR (162 MHz, $CDCl_3$): $\delta = 2.44$. FAB-MS (neg.): *m/z*: 1033.7 (18, $[2M+23]^-$); 505.3 (100, M^-); 367.2 (60); 301.1 (29); 231.1 (30). Analysis on the microcrystalline calcium salt prepared by addition of a calcium chloride solution and recrystallization from water: $C_{60}H_{100}O_8P_2Ca$: calcd C 68.53, H 9.59, P 5.89, Ca 3.80; found: C 68.6, H 9.5, P 6.0, Ca 3.8.

Sodium di((2*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl) phosphate (di(geranylgeranyl) phosphate, **4**): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.36$[a] (brm, 2H; HC(2)); 5.10 [brm, 6H; HC(6), HC(10) and HC(14)]; 4.38[a] (brm, 4H; $H_2C(1)$); 2.13–1.92 (m, 24H; $H_2C(4)$, $H_2C(5)$, $H_2C(8)$, $H_2C(9)$, $H_2C(12)$, and $H_2C(13)$); 1.67 (s, 6H; CH_3); 1.64 (s, 6H; CH_3); 1.60 (s, 18H; CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 139.2$ (s, C(3)); 135.2* (s, C(7)); 134.9* (s, C(11)); 131.1 (s, C(15)); 124.4* (s, C(14)); 124.2* (s, C(10)); 123.9* (s, C(6)); 121.3[a] (brs, C(2)); 62.5[a] (brs, C(1)); 39.7 (s, C(4) and C(8)); 26.9* (s, C(12)); 26.8* (s, C(5)); 26.78* (s, C(9)); 26.72* (s, C(13)); 25.7 (s, C(16)); 17.6 (s, $CH_3-C(15)$); 16.5 (s, $CH_3-C(3)$); 16.0 (s, $CH_3-C(7)$ and $CH_3-C(11)$). ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = 2.3$. FAB-MS (neg.): *m/z*: 1305.9 (11, $[2M+23]^-$); 641.4 (100, M^-); 435.2 (41); 369.1 (22); 231 (32). Anal. $C_{40}H_{66}O_4PNa$, H_2O: Calcd C 70.35, H 10.04, P 4.54, Na 3.37; found: C 70.0, H 9.9, P 4.6, Na 3.1.

Sodium [(3-methyl-2-butenyl)((2*E*)-3,7-dimethylocta-2,6-dienyl)] phosphate (prenyl geranyl phosphate, **5**): ^{1}H NMR (200 MHz, CD_3OD): $\delta = 5.31$ (t, $J(^1H-^1H) = 7$ Hz, 2H; HC(2) and HC′(2)); 5.14 (t, $J(^1H-^1H) = 7$ Hz, HC(6)); 4.40[a] (brm, 4H; $H_2C(1)$ and $H_2C'(1)$); 2.20–2.00 (m, 4H; $H_2C(4)$ and $H_2C(5)$); 1.76 (s, 6H; CH_3); 1.71 (s, 6H; CH_3); 1.65 (s, 3H; CH_3). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 139.6$ (s, C(3) and C′(3)); 131.5 (s, C(7)); 124.0 (s, C(6)); 121.3 (d, $J(^{13}C-^{31}P) = 6.1$ Hz, C(2)); 121.0 (d, $J(^{13}C-^{31}P) = 8$ Hz, C′(2)); 62.8[a] (d, C(1) and C′(1)); 39.6 (s, C(4)); 26.5 (s, C(5)); 25.6 (s, C(8) and C′(4)); 17.9 (s, $CH_3-C'(3)$; 17.6 (s, $CH_3-C(7)$; 16.3 (s, $CH_3-C(3)$. ^{31}P NMR (121 MHz, CD_3OD): $\delta = 1.9$. FAB-MS (neg.): *m/z*: 625.2 (27, $[2M+23]^-$); 301.1 (100, M^-); 233.1 (54); 165 (60). Anal. $C_{15}H_{26}O_4PNa$: calcd C 55.55, H 8.08; found: C 54.4, H 8.3.

Sodium [((2*E*)-3,7-dimethylocta-2,6-dienyl)((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyl)] phosphate (geranyl farnesyl phosphate, **6**): ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$): $\delta = 5.35–5.30$[a] (brm, 2H; HC(2) and HC′(2)); 5.10–5.00[a] (brm, 4H; HC(6), HC′(6) and HC(10)); 4.40–4.31 (dd, $J(^1H-^1H) = 6.3$ Hz, $J(^1H-^{31}P) = 6.3$ Hz, 4H; $H_2C(1)$ and $H_2C'(1)$); 2.11–1.88 (brm, 12H; $H_2C(4)$, $H_2C(5)$, $H_2C'(4)$, $H_2C'(5)$, $H_2C(8)$ and $H_2C(9)$); 1.65 (s, 6H; CH_3); 1.63 (s, 6H; CH_3); 1.58 (s, 6H; CH_3); 1.57 (s, 3H; CH_3). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 139.3$ (s, C(3) and C′(3)); 135.1 (s, C(7) and C′(7)); 124.3* (s, C(11) and C(10)); 124.00* (s, C(6)); 123.8* (s, C′(6)); 121.1[a] (brs, C(2) and C′(2)); 62.4[a] (brs, C(1) and C′(1)); 39.7 (s, C(4), C′(4) and C(8)); 26.7* (s, C(9)); 26.5* (s, C(5) and C′(5)); 25.6 (s, C(12) and C′(8)); 17.6 (s, $CH_3-C'(7)$, $CH_3-C(11)$); 16.3 (s, $CH_3-C(7)$, $CH_3-C'(3)$); 15.8 (s, $CH_3-C(3)$). ^{31}P NMR (162 MHz, $CDCl_3/CD_3OD$): $\delta = 1.76$. FAB-MS (neg.): *m/z*: 897.4 (12, $[2M+23]^-$); 437.2 (82, M^-); 299.1 (37); 233.1 (26). Anal. $C_{25}H_{42}O_4PNa$, H_2O: Calcd. C 62.74, H 9.26; found: C 62.2, H 9.1.

Sodium [((2*E*)-3,7-dimethylocta-2,6-dienyl)((2*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl)] phosphate (geranyl geranylgeranyl phosphate, **7**): ^{1}H NMR (200 MHz, CD_3OD): $\delta = 5.45–5.39$[a] (brm, 2H; HC(2) and HC′(2)); 5.14–5.12[a] (brm, 4H; HC(6), HC′(6), HC(10) and HC(14)); 4.45–4.41[a] (brm, 4H; $H_2C(1)$ and $H_2C'(1)$); 2.22–1.96 (brm, 16H; $H_2C(4)$, $H_2C(5)$, $H_2C'(4)$, $H_2C'(5)$, $H_2C(8)$, $H_2C(9)$, $H_2C(12)$ and H_2 C(13)); 1.71 (brs, 12H; CH_3); 1.63 (s, 12H; CH_3). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 139.2$ (brs, C(3) and C′(3)); 135.0* (s, C(7)); 134.8 (s, C(11)); 131.1* (brs, C(15) and C′(7)); 124.3–123.9 (br, C(6), C(10), C(14) and C′(6)); 121.6–120.6[a] (brs, C(2) and C′(2)); 62.5–61.9[a] ((brs, C(1) and C′(1)); 39.7 (s, C(4), C(8) and C′(4)); 26.7 (s, C(5), C(9), C(13) C(12) and C′(5)); 25.6 (s, C(16) and C′(8)); 17.6 (s, $CH_3-C(15)$ and $CH_3-C'(7)$); 16.4* (s, $CH_3-C(3)$); 16.3* (s, $CH_3-C'(3)$); 15.9* (s, $CH_3-C(7)$ and $CH_3-C(11)$). ^{31}P NMR (121 MHz, CD_3OD): $\delta = 2.0$. FAB-MS (neg.): *m/z*: 505.3 (100, M^-); 369.2 (41); 316.8 (18); 276.8 (16); 233.1 (23). Anal. $C_{30}H_{50}O_4PNa$: Calcd C 68.14, H 9.53; found: C 67.9, H 9.4.

Sodium [((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyl)((2*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl)] phosphate (farnesyl geranylgeranyl phosphate, **8**): ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 5.35$ (t, $J(^1H-^1H) = 6.2$ Hz, 2H; HC(2) and HC′(2)); 5.09[a] (brm, 5H; HC(6), HC′(6), HC(10), HC′(10) and HC(14)); 4.37 (t, $J(^1H-^1H) = 6.2$ Hz, $J(^1H-^{31}P) = 6.2$ Hz, 4H; $H_2C(1)$ and $H_2C'(1)$; 1.88–2.13 (brm, 20H; $H_2C(4)$, $H_2C'(4)$, $H_2C(5)$, $H_2C'(5)$, $H_2C(8)$, $H_2C'(8)$, $H_2C(9)$, $H_2C'(9)$, $H_2C(12)$ and $H_2C(13)$); 1.67 (s, 6H; CH_3); 1.62 (s, 6H; CH_3); 1.59 (s, 14H; CH_3). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 139.5$ (s, C(3) and C′(3)); 135.1 (s, C(7) and C′(7)); 134.8 (s, C(11)); 131.3 (s, C′(11) and C(15)); 124.4* (brs, C(14)); 124.2* (brs, C(10) and C′(10)); 123.9* (brs, C(6) and C′(6)); 121.1 (d, $J(^{31}P-^{13}C) = 5.8$ Hz, C(2) and C′(2)); 62.6[a] (brs, C(1) and C′(1)); 39.7 (s, C(4), C(8), C′(4) and C′(8)); 26.8 (brs, C(12), C(5), C(9), C(13), C′(5) and C′(9)); 25.7 (s, C(16) and C′(12)); 17.6 (s, $CH_3-C(15)$, $CH_3-C'(11)$); 16.4 (s, $CH_3-C(3)$ and $CH_3-C'(7)$); 16 ((s, $CH_3-C(7)$,$CH_3-C(11)$ and $CH_3-C'(3)$). ^{31}P NMR (81.03 MHz, $CDCl_3$): $\delta = 2.4$. FAB-MS (neg.): *m/z*: 1169.7 (15, $[2M+23]^-$); 573.3 (100, M^-); 505.3 (13); 435.2 (18); 367.2 (17); 301.1 (20); 231 (28). Anal. $C_{35}H_{58}O_4PNa$, H_2O: Calcd C 68.37, H 9.83; found: C 68.0, H 9.2.

Sodium di((3*R*)-3,7-dimethyloct-6-enyl) phosphate (di(*R*)-citronellyl phosphate, **9**): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.09$ (t, $J = 7$ Hz, 2H; HC(6)); 3.90 (dd, $J(^1H-^1H) = 5$ Hz, $J(^1H-^{31}P) = 6$ Hz, 4H; $H_2C(1)$); 1.95 (m, 4H; $H_2C(5)$); 1.66–1.15 (m, 22H; $H_2C(2)$, $H_2C(4)$, HC(3) and CH_3); 0.91 (d, $J = 7.5$ Hz, $H_3-C(3)$). ^{13}C NMR (50 MHz, $CDCl_3/CD_3OD$): $\delta = 131.0$ (s, C(7)); 124.4 (s, C(6)); 63.8 (d, $J(^{31}P-^{13}C) = 5.8$ Hz, C(1)); 37.5 (d, $J(^{31}P-^{13}C) = 7.2$ Hz, C(2)); 37.1 (s, C(4)); 29.1(s, C(3)), 25.4* (s, C(8)); 25.2* (s, C(5)); 18.9 (s, $H_3C-C(3)$); 17.3 (s, $H_3C-C(7)$). ^{31}P NMR (121 MHz, $CDCl_3$) = 2.1. FAB-MS: 373.3 (100, M^-); 300.8 (67); 260.9 (59).

Sodium di((3*R*)-3,7-dimethyloctanyl) phosphate (di(*R*)-tetrahydrogeranyl phosphate, **10**): ^{1}H NMR (200 MHz, $CDCl_3/CD_3OD$): $\delta = 3.84$ (brm, 4H; $H_2C(1)$); 1.64–1.00 (brm, 20H; HC(3), HC(7), $H_2C(2)$, $H_2C(4)$, $H_2C(5)$, $H_2C(6)$); 0.84–0.78 (m, 18H; CH_3). ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 64.0$ (d, $J(^{31}P-^{13}C) = 5.8$ Hz, C(1)); 39.2 (s, C(6)); 37.7 (d, $J(^{31}P-^{13}C) = 7.21$ Hz, C(2)); 37.3 (s, C(4)); 29.4 (s, C(3)); 27.8 (s, C(7)); 24.6 (s, C(5)); 22.5* (s, $H_3C-C(7)$); 22.4* (s, C(8)); 19.0 (s, $H_3C-C(3)$). ^{31}P NMR: (121.5 MHz, $CD_3OD/CDCl_3$): $\delta = 2.1$.

Pyridinium ((2*E*)-3,7-dimethylocta-2,6-dienyl) phosphonate (geranyl phosphonate, **16a**): ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$): $\delta = 8.83$ (m, 2H; py); 8.4 (m, 1H; py); 7.94 (m, 2H; py); 7.67 (d, $J(^1H-^{31}P) = 675$ Hz, 1H; H–P); 5.98 (d, $J(^1H-^{31}P) = 675$ Hz, 1H; H–P); 5.39 (t, $J(^1H-^1H) = 6.5$ Hz, 1H; HC(2)); 5.08 (t, $J(^1H-^1H) = 6.7$ Hz, 1H; HC(6)); 4.54 (m, 2H; $H_2C(1)$); 2.07 (m, 4H; $H_2C(4)$ and $H_2C(5)$); 1.71 (s, 3H; CH_3); 1.68 (s, 3H; CH_3); 1.68 (s, 3H; CH_3). ^{31}P NMR (162 MHz, $CDCl_3/CD_3OD$): $\delta = 6.6$ ($J(^{31}P-^1H) = 675$ Hz).

Pyridinium ((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyl) phosphonate (farnesyl phosphonate, **16b**): ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 8.88$ (m, 2H; py); 8.61 (d, $J(^{31}P-^1H) = 660$ Hz, 1H; H–P); 8.46 (m, 1H; py); 7.98 (m, 2H; py); 5.3 (d, $J(^{31}P-^1H) = 660$ Hz, 1H; H–P); 5.42 (m, 2H; HC(2)); 5.08 (m, 2H; HC(6) and HC(10)); 4.54 (m, 2H; $H_2C(1)$); 2.04 (brm, 8H; $H_2C(4)$, $H_2C(5)$, $H_2C(8)$, and $H_2C(9)$); 1.69 (s, 3H; CH_3); 1.67 (s, 3H; CH_3); 1.60 (brs, 6H; CH_3). ^{31}P NMR: (81.03 MHz, $CDCl_3$): $\delta = 6.5$ ($J(^{31}P-^1H) = 655$ Hz).

Pyridinium ((2*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl) phosphonate (geranylgeranyl phosphonate, **16c**): ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 8.86$ (m, 2H; py); 8.54 (d, $J(^{31}P-^1H) = 669$ Hz, 1H; H–P); 8.23 (m, 1H; py); 7.81 (m, 2H; py); 5.40 (t, $J(^{31}P-^1H) = 6.7$ Hz, 2H; HC(2)); 5.19 (d, $J(^{31}P-^1H) = 669$ Hz, 1H; H–P); 5.10[a] (brm, 3H; HC(6), HC(10), and HC(14)); 4.53 (t, $J(^1H-^1H) = 7.9$ Hz, $J(^{31}P-^1H) = 7.9$ Hz, 2H; $H_2C(1)$; 2.00 (brm, 12H; HC(4), HC(5), HC(8), HC(9), HC(12), HC(13)); 1.70 (s, 6H; CH_3); 1.60 (s, 9H; CH_3). ^{31}P NMR (121.49 MHz, $CD_3OD/CDCl_3$): $\delta = 6.8$ ($J(^{31}P-^1H) = 667$ Hz).

Electron microscopy—*Vesicle preparation*: Sodium di(polyprenyl) phosphates (10 mg) were dissolved in $CHCl_3$ (1 mL). After evaporation of the solvent the film was dried under vacuum overnight. The film was hydrated by addition of buffer A (1 mL) and vortexed for 2 min at room temperature. Following this procedure, we could obtain unilamellar vesicles (LUV and IUV). For the preparation of MLV, we further applied freeze–thaw cycles to the vortexed samples. The aqueous suspension was frozen in liquid N_2 and thawed in a water bath at 35 °C. Sonicated vesicles were prepared by bath sonication for 15 min or tip sonication (15 min). Metal debris were removed by centrifugation (5 min, 9.8 g).

Preparation of samples: The preparations were observed in the frozen hydrated state by cryo-EM [64]. 700 mesh copper grids were dipped into lipid stock solution (≈ 10 mg mL^{-1}), the excess liquid was blotted with a piece of filter paper (Whatman no. 2) and the grids were plunged in liquid ethane or liquid propane cooled with

liquid nitrogen [65]. Grids were mounted under liquid nitrogen in a Gatan 626 Cryoholder, transferred into the electron microscope, and observed at ca. −170 °C. Observations were made in a CM 12 Philips microscope operating at 100 kV. The microscope was equipped with an additional anticontamination device [66]. Images were not taken under low-dose conditions, as pure lipid solutions are not as sensitive to beam irradiation as proteins (Schmutz M., Brisson A. unpublished results). Images were recorded on Kodak SO 163 films and developed under standard conditions.

Entrapment of calcein and leakage—*Vesicle preparation*: Calcein-containing vesicles were prepared by two different methods depending on the dye concentration. Tip-sonicated vesicles were prepared starting from 10 mg of compounds **2**–**8**, and 70 mM calcein (solution A). MLV of compounds **3**, **4**, and **8** were prepared starting from 10 mg of product and 0.10 mM calcein (solution B) by a slight modification of the general MLV preparation method reported above: after five freeze–thaw cycles, the suspension was sonicated in a bath for 10 min and submitted again to five freeze–thaw cycles.

Sonicated vesicles—leakage measurements and trapped volume: The vesicles and nonencapsulated dye were separated by gel-filtration eluting with buffer B; *n* fractions of about 2 mL were collected. 10 μL of each fraction was added both to 1 mL of buffer B and to 1 mL of Triton® X-100 10% v/v in buffer B; this provided samples with fluorescence intensities $F_{(n)}$ and $F_{max(n)}$ respectively. The estimated uncertainty was 5%. A difference between F and F_{max} larger than the experimental uncertainty indicates the entrapment of the dye (Fig. 5). In order to determine the leakage of the dye, suspensions of sonicated vesicles were filtered over a gel and collected in 500 μL fractions. Fractions containing vesicles were then collected together except for the first and the last fractions to avoid contamination by MLV and very small vesicles, respectivly. 10 μL were diluted in 2.5 mL of buffer B. The fluorescence of this sample was monitored continously for the first 40 min and then every 10 min, thus providing F_t values. F_0 represents the fluorescence intensity at time 0. 10 μL were diluted in 2.5 mL of Triton® X-100 10% v/v in buffer B, thus providing F_f. The % leakage of vesicles was calculated as $(F_t - F_0)/(F_f - F_0) \times 100$ (Fig. 6). The trapped volume was calculated from $(F_f - F_0)$. A calibration curve F vs. [calcein] built by fluorimetric titration of calcein solutions of known concentrations 3–30 μmol, was used to determine the molar amount N of trapped solute in 10 μL. N was converted into volume according to N (μmol)/70 (mM), where 70 mM is the initial concentration of the dye. In order to compare results, the relative trapped volume expressed as μL of dye solution/μmol sodium di(polyprenyl) phosphate was also calculated (Table 1).

MLV—leakage measurements and trapped volume: 50 μL of the vesicle suspension prepared as described above was added to 3 mL buffer C and the fluorescence intensity was measured before (F_{tot}) and after (F_{in}) addition of 10 μL of $CoCl_2$ (10 mM). F_{totq} represents the fluorescence intensity obtained after addition of 150 μL Triton® X-100 10% v/v in buffer C to the same sample. The % trapped volume was calculated from $(F_{in} - F_{totq} \cdot r)/(F_{tot} - F_{totq} \cdot r) \times 100$, where r is the dilution factor due to Triton® X-100 [36]. The relative trapped volume was also calculated (Table 1).

^{31}P NMR of hydrated DPPNa films—*Vesicle preparation*: A thin film of sodium di(polyprenyl) phosphate (10 mg) was prepared as described above, hydrated with 0.5 mL of buffer D and vortexed. TLC was used before and after the NMR experiments to check that the substances studied had not been degraded.

NMR experiments: Chemical shifts were measured from 85% H_3PO_4 as an external standard. The relaxation time T_1 of the vesicles was about 1.2 s, measured with the usual inversion–recovery pulse sequence. We used power-gated ^{1}H decoupling with the Waltz-16 sequence and phase-cycle Hahn-echo during free induction decay accumulation [67]. The phase-cycling avoids ringing effects and eliminates the FID part from echo. This last feature allows one to use pulses different from 90 and 180°. Pulse width was 18 μs (90°), acquisition time 0.16 s, defocusing/refocusing time 300 μs, and recycle time 4 s.

^{31}P NMR of oriented multibilayers: Cover glasses (Polylabo no. 0/1 20 × 20 × 0.15 mm) were first cleaned by immersion in conc. NH_3 aq (30% min.) during 2 d, then thoroughly washed with MilliQ water and pure acetone, and dried in an oven overnight. They were cut into 6–9 mm strips. Sodium difarnesyl phosphate (**3**) (10 mg) was dissolved in 2-propanol (0.5 mL). The organic solution was applied dropwise on the glass strips and dried under vacuum (0.01 atm) over P_2O_5. The strips were stacked in a 10 mm (o.d.) NMR tube (45 to 50 strips). To hydrate the sample, about 50 mL of MilliQ water was added, and the sample was kept at 35 °C for 24 h in the saturated water atmosphere. Then the tube was sealed. The bilayer normal was set either perpendicular (90°) or parallel (0°) to the magnetic field by positioning manually the NMR tube in the probe. We used Hahn-echo: pulse width was 5 μs (90°), acquisition time 0.04 s, defocusing/refocusing time 10 μs, recycle time 10 s.

Water permeability of vesicles measured by stopped-flow osmotic shock—*Vesicle preparation*: Freeze–thawed vesicles of DPPNa (10 mg) were prepared as described above using buffer E. The vesicle suspension was then extruded through two polycarbonate membrane (pore size 200 nm) under 10–15 atm N_2 pressure. The extrusion procedure was repeated ten times, and the sample was then diluted to 25 mL with buffer E. The final phosphorus concentration of all the samples was about 10^{-3} M. The average diameter measured by PCS ranged between 115 nm (sodium difarnesyl phosphate, **3**) and 155 nm (sodium di(geranylgeranyl) phosphate, **4**).

Shrinkage experiments: The vesicle dispersions were subjected to the osmotic shock 60 min after their preparation. The stability of the samples was checked by comparison of the average size of the vesicles (PCS) just after their preparation and their average size 5 h later. A sample was deemed stable if it remained monodispersed and the average size of the vesicles was constant. An aliquot of vesicle dispersions was rapidly mixed with the hypermolar buffer F in the stopped-flow instrument. Just before their introduction into the stopped-flow syringes, the sample and the hypo- or hypertonic buffer were filtered on Millipore 0.5 mm PTFE filters and deareated by passing through a gentle flow of argon for 10 min at room temperature. To achieve thermal equilibrium, they were left at 15 ± 1 °C in the drive syringes for at least 10 min before the beginning of the kinetic experiments.

Analysis of data: Each rate constant value k was calculated by averaging rate constant values k obtained in turn by computerized fitting of average curves I vs. t derived from the superimposition of several experimental curves (typically 3–6 for each average curve). Each experimental curve (1000 points) was obtained by monitoring the change in scattered light intensity following the rapid mixing ($t \lesssim 3$ ms) of equal volumes (100 μL) of sample and hypertonic buffer. Typically 3 to 6 injections provided independent experimental kinetic curves which were superimposed, averaged, and numerically treated by the Biokine software, which uses a factor analysis method and a Simplex algorithm. The results of 5 to 6 runs of experiments were then averaged; the corresponding k values and standard deviation are given in Tables 3 and 4. The values of the first-order rate constants k determined for the theoretical exponential model measure the H_2O permeability of the vesicles.

Fluorescence polarization—*Vesicle preparation*: Freeze–thawed vesicles of DPPNa (10 mg) were prepared as described above using buffer A. (1 mL). The vesicle suspension was then extruded five times through polycarbonate membranes (pore size 800 nm) under 10–15 atm N_2 pressure.

Fluorescence anisotropy: The membrane fluidity was measured by fluorescence polarization of lipid-specific probes [68]: 2 μL of a solution of DPH or TMA-DPH (Molecular Probes, Oreg., USA), both prepared in dimethylformamide at a concentration of 2.10^{-3} M, were added to a suspension of 50 μL of vesicles diluted in 2 mL buffer A. The sample with the DPH probe was incubated 30 min at 20 °C before measuring the fluorescence, whereas the fluorescence of the sample containing the TMA-DPH was measured immediately. Fluorescence polarization was measured with an Amicon SPF 500 spectrofluorimeter equipped with a polarization attachment. The excitation and emission wavelengths were 360 and 425 nm, respectively. The anisotropy parameter r is defined by $r = (F_{\parallel} - F_{\perp})/(F_{\parallel} + 2F_{\perp})$, where $F_{\parallel}$ and $F_{\perp}$ correspond to the fluorescence intensities measured with the emission polarizer respectivly parallel and perpendicular to the direction of polarization of the excitation beam [69]. Results are given with the mean ± uncertainty of 10 determinations.

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