

Biological properties of water-soluble phosphorhydrazone dendrimers

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Dendrimers are hyperbranched and perfectly defined macromolecules, constituted of branches emanating from a central core in an iterative fashion. Phosphorhydrazone dendrimers constitute a special family of dendrimers, possessing one phosphorus atom at each branching point. The internal structure of these dendrimers is hydrophobic, but hydrophilic terminal groups can induce the solubility of the whole structure in water. Indeed, the properties of these compounds are mainly driven by the type of terminal groups they bear; this is especially true for the biological properties. For instance, positively charged terminal groups are efficient for transfection experiments, as drug carriers, as anti-prion agents, and as inhibitor of the aggregation of Alzheimer's peptides, whereas negatively charged dendrimers have anti-HIV properties and can influence the human immune system, leading to anti-inflammatory properties usable against rheumatoid arthritis. This review will give the most representative examples of the biological properties of water-soluble phosphorhydrazone dendrimers, organized depending on the type of terminal groups they bear.

Uniterms: Dendrimers. Phosphorhydrazone dendrimers. Biological properties. Nanomedicine.

Dendrímeros são macromoléculas extremamente ramificadas e perfeitamente definidas constituídas de ramificações que partem de um foco central de uma forma iterativa. Dendrímeros de fosforidrazona constituem uma família especial de dendrímeros, que possuem um átomo de fósforo em cada ponto da ramificação. A estrutura interna destes dendrímeros é hidrofóbica, mas grupos hidrofílicos terminais podem induzir a solubilidade em água de toda estrutura. De fato, as propriedades destes compostos são principalmente orientadas pelos grupos terminais que apresentam, especialmente para as propriedades biológicas. Por exemplo, grupos terminais carregados positivamente são eficientes para experimentos de transfecção, como transportadores de fármacos, agentes anti-prions e como inibidores da agregação de peptídeos do Alzheimer, enquanto que dendrímeros carregados negativamente têm propriedades anti-HIV e podem influenciar o sistema imune humano, levando propriedades antiinflamatórias úteis contra artrite reumatoide. Essa revisão dará os exemplos mais representativos das propriedades biológicas de dendrímeros de fosforidrazona solúveis em água, organizados de acordo com os grupos terminais que possuem.

Unitermos: Dendrímeros. Dendrímeros de fosforidrazona. Propriedades biológicas. Nanomedicina.

INTRODUCTION

Dendrimers are at the forefront of research since almost two decades, due to the numerous properties they afford in different fields, such as catalysis, materials, and biology to name as a few (Caminade *et al.*, 2011).

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Their name has been created by D.A. Tomalia (Tomalia *et al.*, 1985) from two Greek words: *dendros* (tree) and *meros* (part), which emphasize first their shape and then their chemical structure. Indeed, dendrimers are composed of identical branches constituted of monomers, and emanating radially from a central core. Contrarily to all the other types of polymers, dendrimers are not synthesized by polymerization reactions but step-by-step. Such methodology allows a perfect control over the whole structure. The most widely used methods of synthesis of dendrimers are divergent methods. In this case, the structure is grown from the core towards the terminal groups, layer after layer. Each time a new layer of branching points is created, a new generation is obtained. The following Figure 1 displays the principle of the synthesis of dendrimers via a divergent process. Such process is compatible with different types of reactions; the only limitation is that the reactions must be quantitative. Indeed, it is impossible to purify a mixture containing a perfect dendrimer and a dendrimer in which one terminal function is missing, even if this defect can be detected (for instance by NMR).

The branching structure is widely found in the Nature at the macroscopic level, for instance in the branches and roots of trees. However, at the molecular level, there is no example to date of a natural molecule having such a branched structure. This is certainly one of the reasons of the success of dendrimers in biology and nanomedicine (Roland *et al.*, 2009b). We will focus this review on the most salient biological properties of the phosphorhydrazone dendrimers that we synthesize. These dendrimers are synthesized starting from a trifunctional core (P(S)Cl₃) (Lartigue *et al.*, 1997), or a hexafunctional core (N₃P₃Cl₆) (Launay *et al.*, 1997) as shown in Figure 2. The phosphorhydrazone linkage (P-N-N=CH) is created by the condensation reaction of a phosphorhydrazone with an aldehyde. The terminal groups are either aldehydes or P(S)Cl₂ functions. Both are particularly reactive and can be modified at will.

The internal structure of these phosphorus dendrimers is hydrophobic (Leclaire *et al.*, 2004), thus solubil-

ity in water (Caminade, Majoral, 2005), which is needed for biological purposes (Caminade *et al.*, 2010), must be afforded by the terminal groups. To achieve this goal, either positively or negatively charged compounds have been grafted as terminal groups (Caminade *et al.*, 2009). Positive charges are afforded by ammonium derivatives, and negative charges are afforded either by carboxylic acid salts or phosphonic acid salts.

PHOSPHORHYDRAZONE DENDRIMERS ENDED BY AMMONIUM GROUPS

The reaction of *N,N*-diethylethylene diamine with the P(S)Cl₂ terminations affords directly the terminal ammonium groups, as shown in Figure 3. These charged terminal groups induce the solubility in water of the whole structure. The generations 1 (12 ammonium groups) to 5 (192 ammonium groups) have been tested as transfection agents to deliver the luciferase plasmid into 3T3 cells. A clear influence of the generation of the dendrimer on the transfection efficiency, with an identical charge ratio in all cases (5:1 or 10:1 for ammonium from the dendrimer/phosphate from the plasmid). From generations 1 to 4, an increase of the transfection efficiency is observed then a plateau is reached (Loup *et al.*, 1999); the efficiency of dendrimers G₄⁺ and G₅⁺ is comparable to that of polyethyleneimine (PEI), one of the chemical standards used in transfection experiments.

These first experiments have been extended to check the ability of the G₄⁺ dendrimer to interact with the fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS), the antineoplastic drug cisplatin, and the anti-HIV siRNA siP24, and also to check its capability to deliver the green fluorescent protein gene (pGFP) into cells (Shcharbin *et al.*, 2011). The usefulness of G₄⁺ to deliver specific siRNA against HIV-1 (siNEF) in PBMCs to interfere in HIV-1 replication was also assessed. The dendriplex G₄⁺/siNEF showed a high efficiency in Nef silencing, and significantly reduced the viral replication. These results

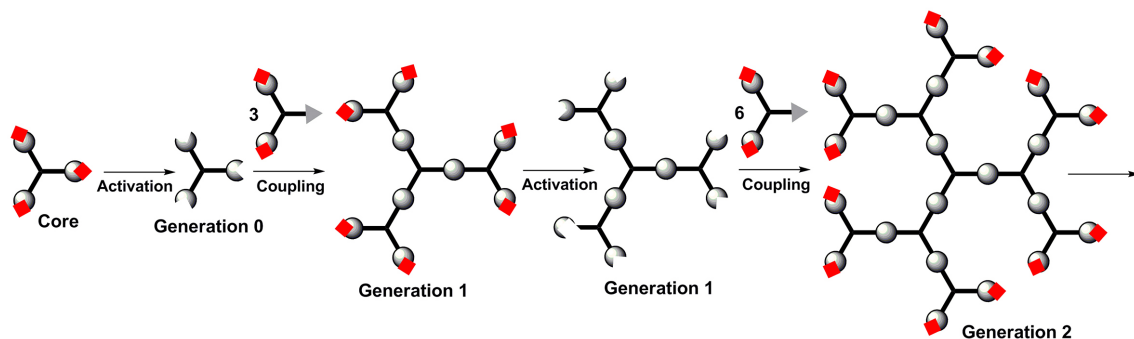


FIGURE 1 - The principle of the divergent synthesis of dendrimers.

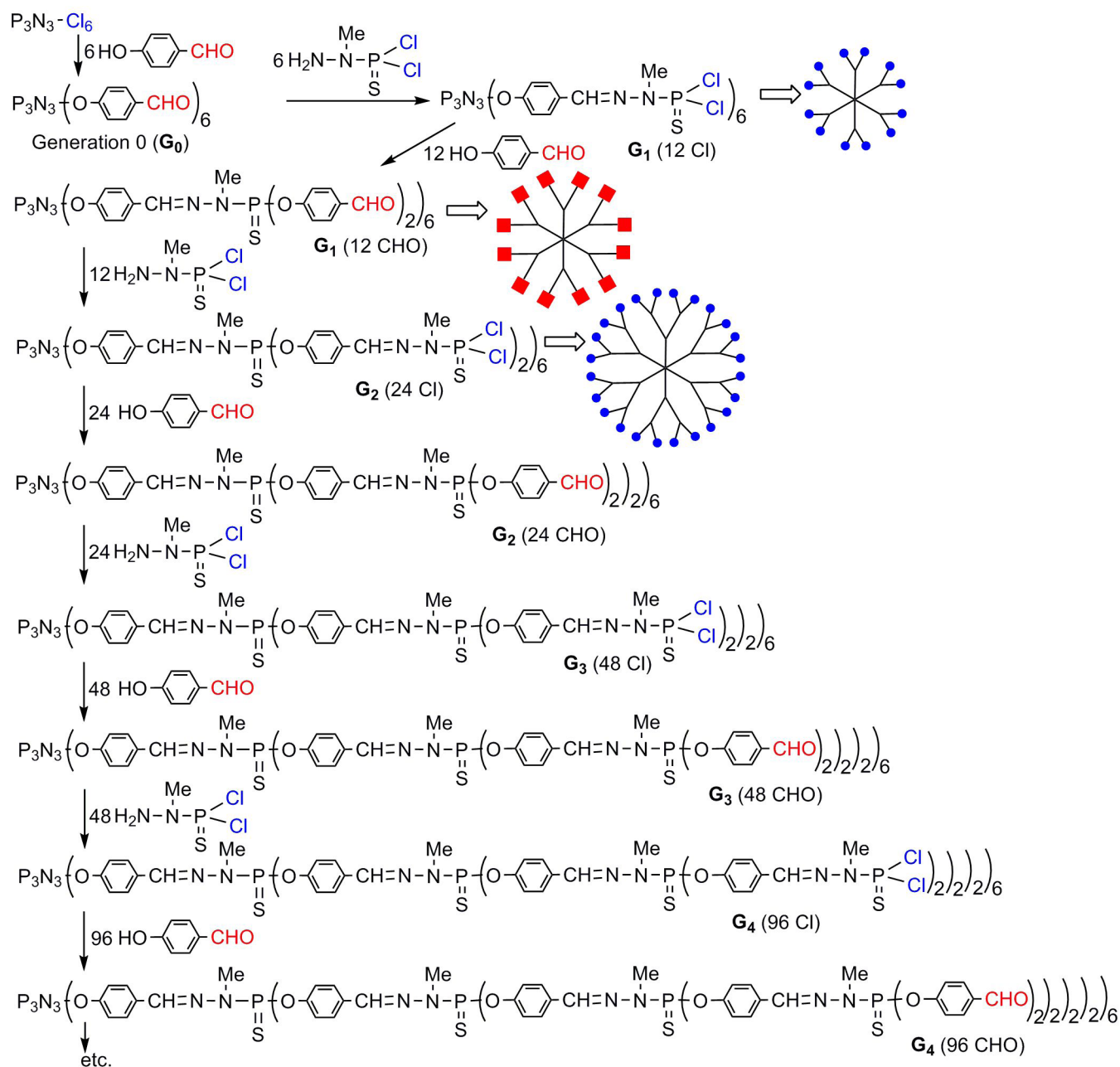


FIGURE 2 - Method of synthesis of phosphorhydrazone dendrimers, starting from the hexafunctional core.

constitute a potential alternative therapy in the HIV-1 infection (Briz *et al.*, 2012).

An analogous series of dendrimers, ended by the same ammonium groups, but built from a fluorescent phthalocyanine core (Leclaire *et al.*, 2005) instead of the cyclotriphosphazene core, was used to detect its behavior in biological media. It was shown that this dendrimer alone can penetrate in human cells, and that it goes inside the cytoplasm (Maszewska *et al.*, 2003). Grafting a maleimide fluorophore to the cyclotriphosphazene core of a second generation dendrimer allowed to studying

its interaction with DNA (Kazmierczak-Baranska *et al.*, 2010). Several other types of ammonium terminated dendrimers were synthesized using the same type of experiments from the $P(S)Cl_2$ terminal groups. Pyrrolidine, morpholine, methyl piperazine, or phenyl piperazine derivatives have been grafted (Padie *et al.*, 2009), but none of them were more efficient than the first one for transfection experiments.

The series of dendrimers displayed in Figure 3 was also shown to be efficient against various strains of the scrapie form of prions, including the one that causes the

“mad cow” disease (Bovine Spongiform Encephalopathy, BSE). The efficiency depends on the generation, but not in a linear fashion: generations 3 and 5 have a weak activity, whereas generation 4 (G_4^+) is highly active. This generation 4 (Figure 3) was so efficient on the various strains of prions tested, that it has been tested also *in vivo*. Mice were infected by cells coming from the brain of terminally-ill mice. Some of them received every two days an intrave-

nous injection (iv) of 100 μg of the G_4^+ dendrimer. After one month, all the treated mice were alive, and the quantity of the scrapie form of prion has decreased by 80% in their spleen, compared to untreated mice (Solassol *et al.*, 2004). Using spin-probe and spin-label techniques, the interaction of the same polycationic dendrimers with the PrP 106–126 peptide (prion peptide), was studied by EPR (Electron Paramagnetic Resonance) (Klajnert *et al.*, 2007a). The

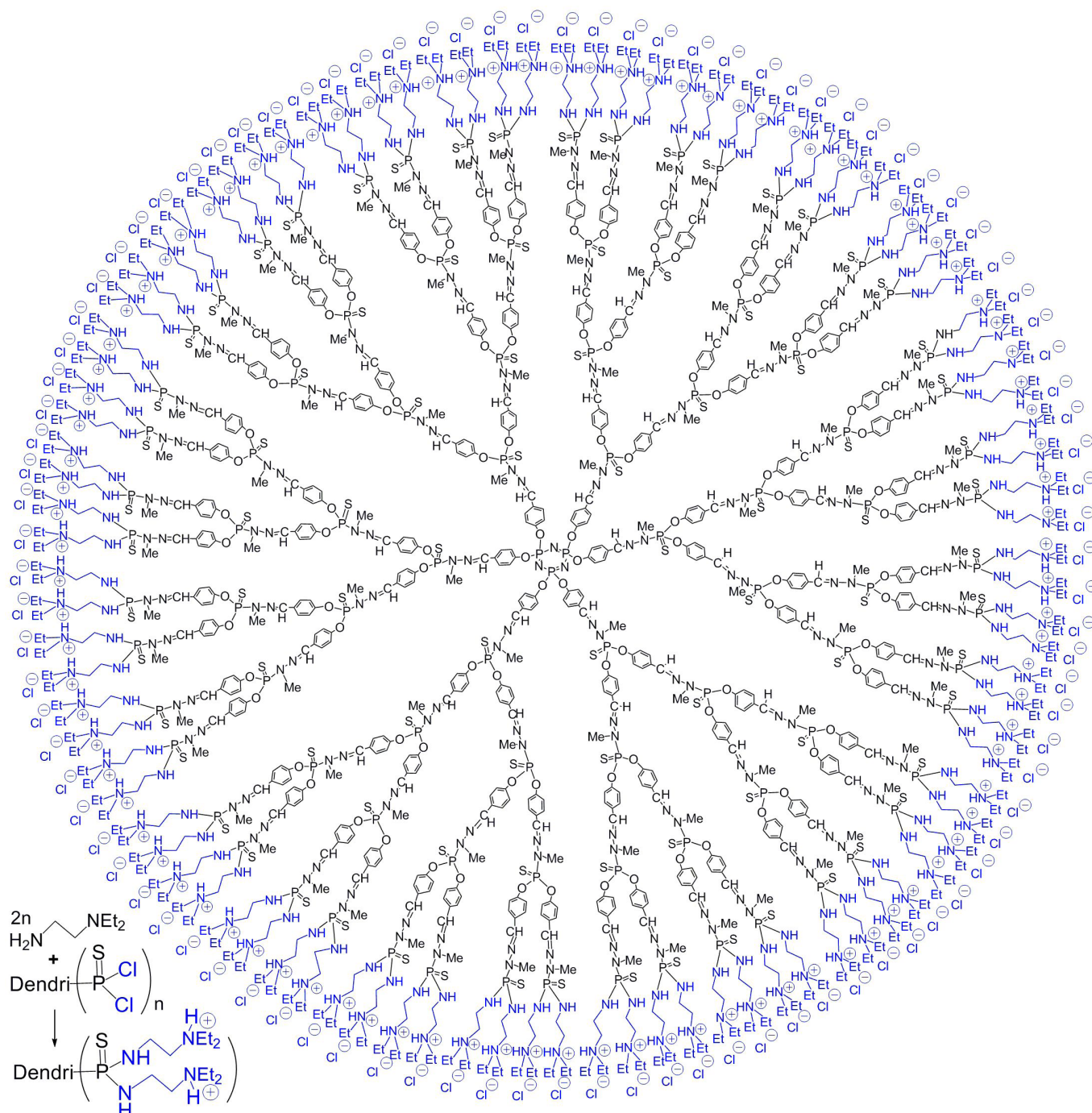


FIGURE 3 - Method of synthesis of dendrimers ended by ammonium groups, and full chemical structure of the corresponding generation 4 (G_4^+) phosphorus dendrimer.

influence of the same dendrimer on the aggregation of the prion peptide PrP 185-208 was also assessed; G_4^+ was able to clearly interfere with the PrP 185-208 aggregation process by both slowing down the formation of aggregates and by lowering the final amount of amyloid fibrils (Klajnert *et al.*, 2007b). It was shown that the polycationic dendrimers interact directly with heparin, and that this process is indirectly responsible for the inhibition of fibril formation by dendrimers (Klajnert *et al.*, 2009).

Another aspect of this work concerns the study of the influence of the polycationic dendrimers on the aggregation of the peptide involved in the Alzheimer's disease. In the case of the A β 1-28 peptide, the phosphorus dendrimers of generation 5 (G_5^+) interacts with amyloid monomers and, consequently, fibril formation (which is a hallmark of the disease) is prevented, as shown by an EPR study (Ottaviani *et al.*, 2010). A deeper insight on the process showed that it depends on the concentration in dendrimer, but not in a linear fashion. At low concentration of G_3^+ or G_4^+ dendrimers (0.01 μ M), the aggregation process is increased; at moderate concentration in dendrimers (0.1 μ M), there is practically no influence on the aggregation process; at high concentration (1 or 10 μ M), there is no trace of aggregation. These dendrimers modify also the aggregation of the MAP-Tau protein, and reduce toxicity caused by aggregated forms of A β 1-28 (Wasiak *et al.*, 2012). The effect of the same dendrimers (generations G_3^+ or G_4^+) on the fibrillation of α -synuclein (ASN) was also tested. The inhibition of fibril formation (filamentous and aggregates) is a potential therapeutic strategy for neurodegenerative disorders such as Parkinson's disease. The interaction between phosphorus-containing dendrimers and ASN was studied. The results showed that the phosphorhydrazone dendrimers inhibited fibril formation, when they were used in the ASN/dendrimer ratios 1:0.1 and 1:0.5 (Milowska *et al.*, 2012).

In view of all these biological properties of these polycationic phosphorhydrazone dendrimers, their cytotoxicity and genotoxicity were assessed in human mononuclear blood cells, A549 human cancer cells and human gingival fibroblasts (HGFs). Dendrimers G_3^+ and G_4^+ at concentrations up to 10 μ M induced a concentration dependent decrease in cell viability. They did not induce breaks in isolated DNA, but they induced DNA cross-links in the cells. The cells showed changes in their morphology, including loss of cell attachment, disruption of cell membrane and nucleus condensation (Gomulak *et al.*, 2012). These compounds alone are toxic for these cells, as expected for polycationic compounds (Fischer *et al.*, 2003), but their interaction with DNA decreases the toxicity, and the *in vivo* tests carried out in the case of the prion disease with the same dendrimer did not display any toxicity.

PHOSPHORHYDRAZONE DENDRIMERS ENDED BY CARBOXYLATE GROUPS

Negatively charged compounds are generally considered as less toxic than positive ones, thus we have also synthesized phosphorhydrazone dendrimers bearing negative charges on their terminal groups. In the first example, carboxylic acid functions have been obtained by a Doebner-like reaction, from the aldehyde terminal functions, in the presence of $\text{CH}_2(\text{CO}_2\text{H})_2$, pyridine and piperazine (Figure 4) (Boggiano *et al.*, 2000). The dendrimers ended by the carboxylic acid groups are not soluble in water, but the corresponding salts are indeed soluble. These salts can be obtained by reaction with sodium hydroxide, but also by acidobasic reactions with primary or secondary amines. These reactions have been carried out in particular with the galactosylceramide (gal β 1cer) analog hexadecylaminolactitol, as chimera for the HIV virus. These supramolecular assemblies are shown in Figure 4 for a dendrimer of second generation, built from a trifunctional core. Furthermore these assemblies spontaneously self-assemble in bilayer vesicles (Blanzat *et al.*, 2002). These compounds built from a trifunctional core, and the analogous series built from the hexafunctional cyclotriphosphazene core were synthesized with the goal of blocking HIV infection prior to the entry of the virus into human cells. Antiviral assays confirmed the crucial roles played both by multivalency effects and lipophilicity on the biological activity of Gal β 1cer analogues. Furthermore the shape of the dendrimers is also a very important criterion, since the second generation built from the trifunctional core and the first generation built from the hexafunctional core, both decorated with 12 hexadecylaminolactitol moieties, exhibit very different IC₅₀ values of 1.1 and 0.12 mM, respectively (Blanzat *et al.*, 2005).

An analogous process was applied for the ocular delivery of carteolol, which is an ocular anti-hypertensive drug used to treat glaucoma. The dendrimers were designed with the aim of increasing the residence time of carteolol in the eyes, and in order to replace benzalkonium, which is a preservative. For this purpose, an ammonium was used as core, and carboxylic acid functions were chosen as terminal groups, but they were obtained in a different way than previously. The first step is the grafting of tertibutyl benzoate on the P(S)Cl₂ functions, then the deprotection provides the carboxylic acid derivatives. Reaction with the neutral form of carteolol affords the supramolecular assembly as shown in Figure 5 for the first generation. Generations 1 and 2 are poorly soluble in water, whereas generation 0 is fairly soluble. These dendrimers have been tested *in vivo*, as vehicles for ocular drug delivery of carteolol to rabbits. No irritation

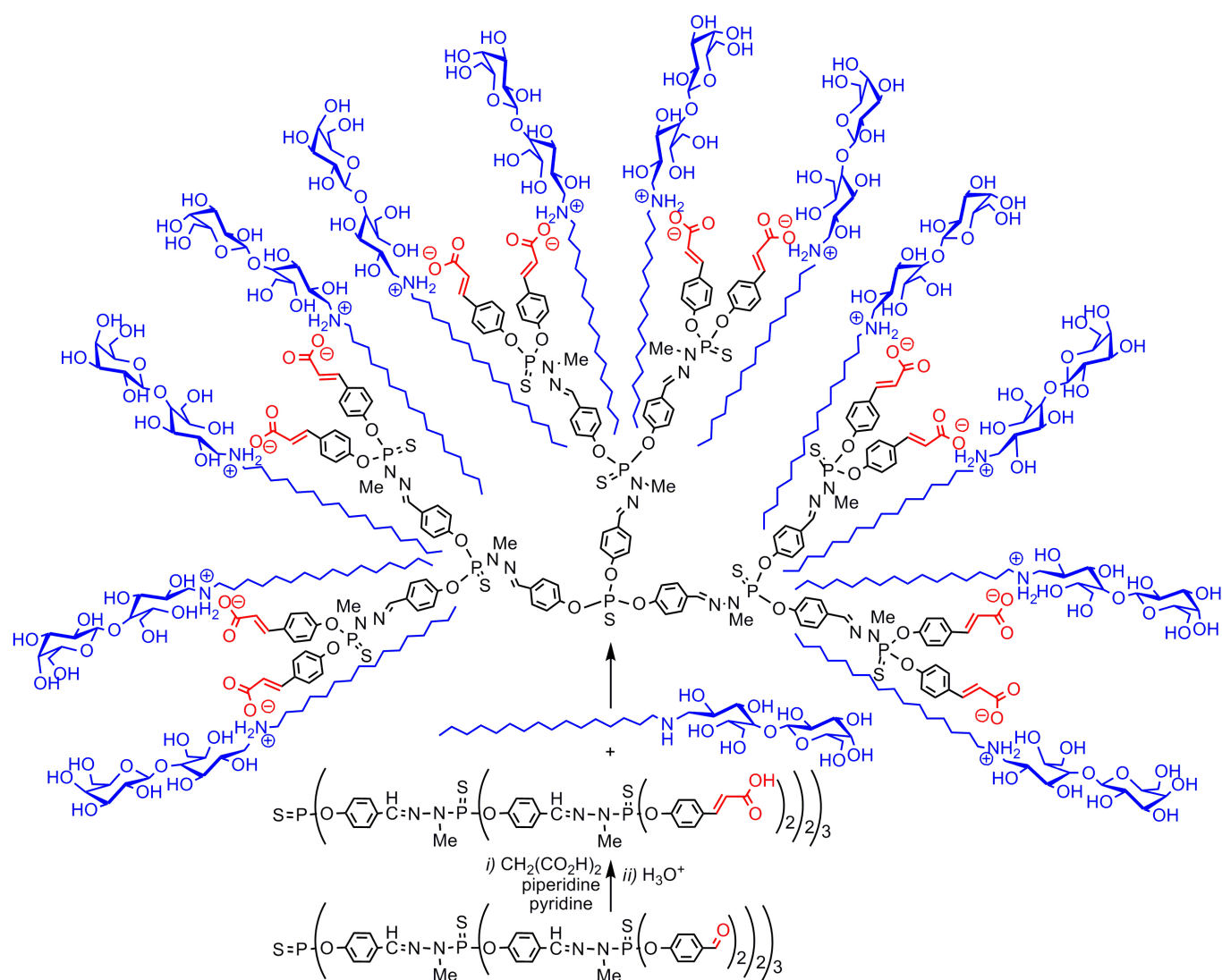


FIGURE 4 - Synthesis of a dendrimer ended by carboxylic acids, and its use for association with a Galactosylceramide analog, hexadecylaminolactitol.

of the rabbit eyes was observed, whatever the dendrimer salt used and even after several hours. Due to the very low solubility of the second generation, the quantity of carteolol instilled is low, but the quantity of carteolol that penetrates inside the eyes is larger than expected, when compared with carteolol alone (2.5 times larger). These pharmacodynamic observations highlight the biocompatibility and the potential usefulness of the phosphorhydrazone dendrimers for drug delivery (Spataro *et al.*, 2010).

PHOSPHORHYDRAZONE DENDRIMERS ENDED BY PHOSPHONATE GROUPS

The structure of the supramolecular assemblies against HIV shown in Figure 4 has been modified in order to try to increase their efficiency. In particular, the carboxylic acid functions have been replaced by various types of phosphonic acids, in which an alkyl chain of variable length (up to C_{10}) has been added to try to increase the strength of the association with the hexadecylaminolactitol (some examples are shown in Figure 6). Despite important structural differences on the terminal groups, these supramolecular assemblies have a comparable anti-HIV-1 activity. All compounds have submicromolar IC_{50} values in a cell-based HIV-infection model, but these compounds were found toxic (Perez-Anes *et al.*, 2010). On the other hand, it was shown that the sodium salts of these dendrimers (without the hexadecylaminolactitol) have also anti-HIV properties, and are non toxic at concentrations up to $1 \cdot 10^{-4} \text{ mol}\cdot\text{L}^{-1}$. Studying the influence of the length of the alkyl chain (R in Figure 6) afforded surprising results. The inhibitory activity of compounds in which $\text{R} = \text{H}$ and $\text{R} = \text{C}_{10}\text{H}_{21}$ is comparable

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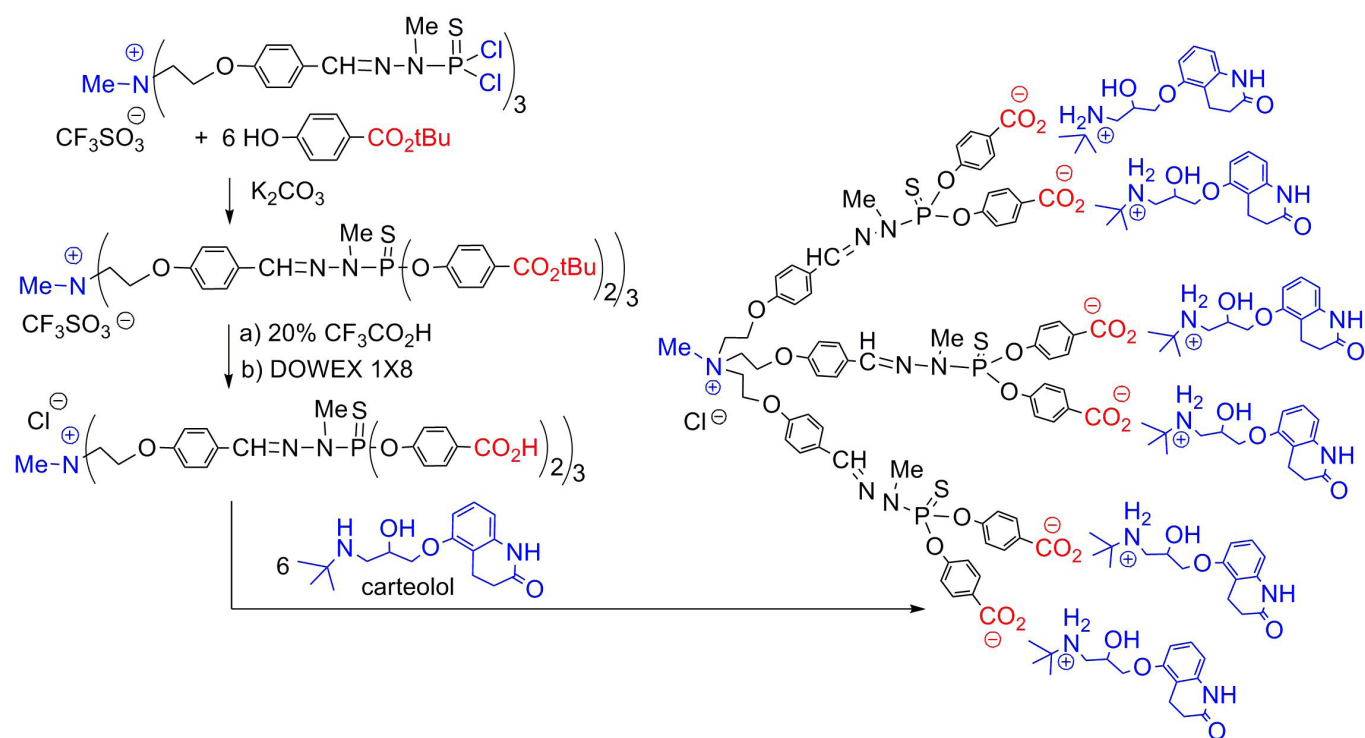


FIGURE 5 - Supramolecular assembly of a dendrimer ended by carboxylic acids and carteolol for ocular drug delivery.

(around $2 \cdot 10^{-5}$ mol.L⁻¹), whereas that of the dendrimer having a C₃ alkyl chain was found to be 10 fold better ($1.6 \cdot 10^{-6}$ mol.L⁻¹) (Perez-Anes *et al.*, 2009).

We have also synthesized as shown in Figure 7 dendrimers ended by azabisphosphonic terminal groups, called ABP (for AzaBisPhosphonic). This dendrimer displays a lot of very important and original properties towards the human immune system, and particularly the human PBMCs (peripheral blood mononuclear cells). The first property discovered concerns the amplification of a special type of PBMCs that are the Natural Killer (NK) cells. These NK cells are especially important to fight against viral and bacterial infections, and above all, against many different types of cancers. However, before our work, their multiplication was difficult and necessitated complex, expensive and poorly available biological molecules/entities. The first generation of the dendrimer ABP shown in Figure 7 is able to multiply by several hundreds the number of NK cells in the blood samples coming from several donors, after 21 days in cultures. The bioactivity of the NK cells generated in the presence of dendrimers is not modified; cultures with these dendrimers did not induce activation or inhibition of the NK cells lytic response nor compromise direct toxicity for their target cells (7 leukemia and 7 carcinoma strains tested) and preserve autologous lymphocytes (no risk of induction of auto-immune disease). Different

generations (zero, one, and two) were studied but the most efficient dendrimer is the first generation ended by the azabisphosphonic pincer (ABP) shown in Figure 7 (Griffe *et al.*, 2007).

In the search for the mechanism of action of this ABP dendrimer, it was discovered that the first interaction occurs with monocytes, which are a pivotal cell population of innate immunity. In order to have a deeper insight on the mechanism, a fluorescent analogue of the dendrimer ABP was synthesized (statistical grafting of one fluorescein isothiocyanate (FITC) among the terminal groups). It was shown by confocal videomicroscopy that this tagged dendrimer binds to isolated monocytes and gets internalized within a few seconds, following the phagolysosomal route for internalization. Here also, the dendrimer ABP was found the most efficient for the activation of monocytes (Poupot *et al.*, 2006). A large number of dendrimeric compounds ended by phosphonic acid salts have been synthesized. The modifications include the variation of the number of terminal groups, playing with the number of branches emanating from the cyclotriphosphazene core. Interestingly, it was shown that the dendrimer in which one branch is missing from the core has an analogous activity, opening the way to the possibility to graft specifically a fluorophore or a targeting entity (Rolland *et al.*, 2008). Non-symmetrical azabisphosphonic salts as terminal groups (Marchand *et al.*, 2009), but also dendrimers ended

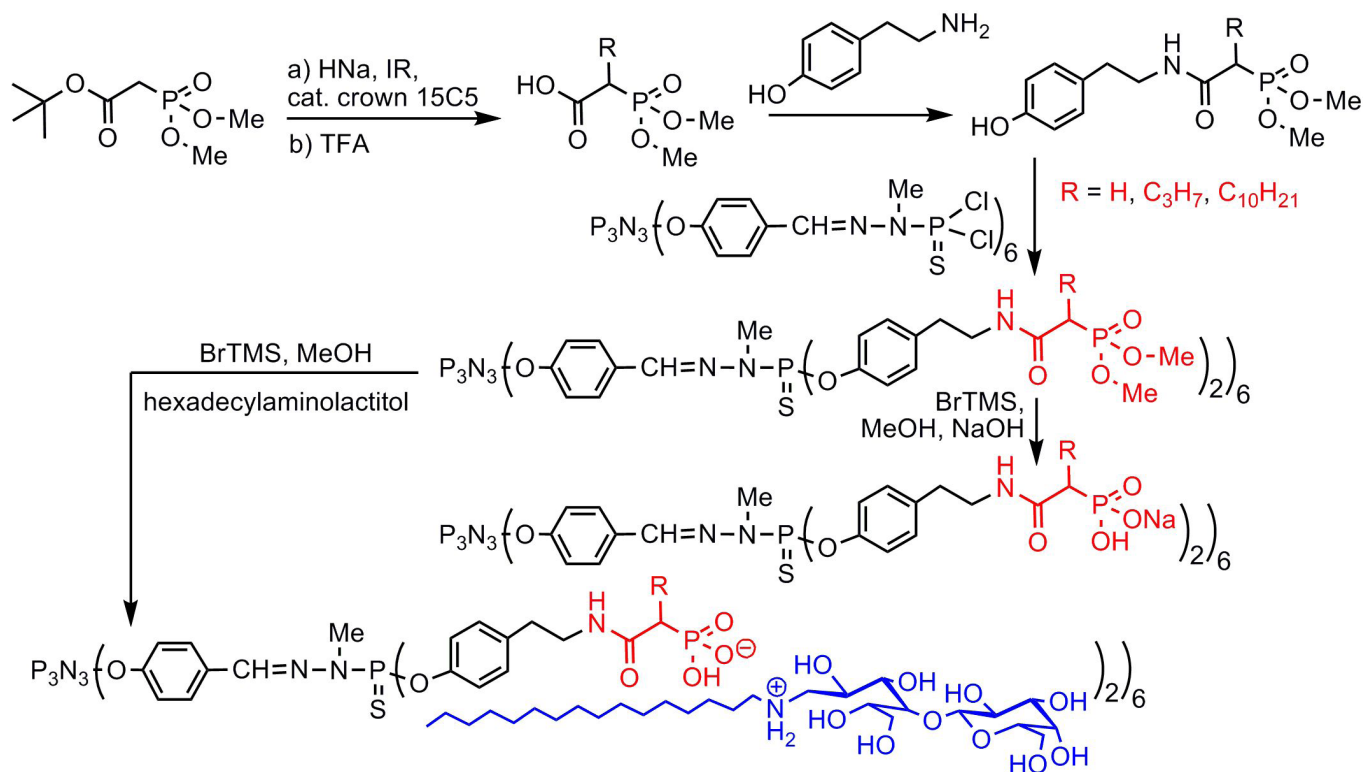


FIGURE 6 - Synthesis of dendrimers ended by phosphonic acids, and their association with the hexadecylaminolactitol.

by isosteric functions (azabis-carboxylates and azabis-sulfonates) (Rolland *et al.*, 2009a) have been synthesized. Some of the modified structures are shown in Figure 8, but none of them displayed better activities than the ABP dendrimer towards human PBMCs.

For a better understanding of the mechanism of activation of monocytes by the ABP dendrimer, the gene expression was analyzed by Affimetrix arrays. It was found that 78 genes were up-regulated, whereas 62 genes were down-regulated. Analysis of these genes indicated that the human monocytes were activated through an alternative-like, anti-inflammatory pathway. Furthermore, the ABP dendrimer induces an inhibition of the proliferation of the TCD4⁺ lymphocytes (pro-inflammatory lymphocytes) in IL2-stimulated PBMCs, without affecting their viability (Fruchon *et al.*, 2009). This inhibition is one of the factors responsible for the NK cells enrichment (Portevin *et al.*, 2009).

The ABP dendrimer also exhibited anti-osteoclastic activity (osteoclasts are giant cells responsible for bone resorption) on mouse and human cells, mediated by c-FMS (cellular-feline McDonough strain sarcoma virus oncogene homolog) inhibition. Combination of the anti-inflammatory and anti-osteoclastic properties suggests a possible use against rheumatoid arthritis (RA), which is an autoimmune inflammatory disorder characterized by

inflammation of the synovial membrane, cartilage degradation, and subsequent bone erosion by osteoclasts, leading to joint deformation and handicap. Weekly intravenous injections of dendrimer ABP at doses of 1 and 10 mg/kg inhibited the development of inflammatory arthritis in two animal models: IL-1ra^{-/-} mice and mice undergoing K/BxN serum transfer. Intravenous injections could be more inter-spaced (3 weeks, similar to anti-cytokine biotherapies). Interestingly, the same property against RA-like disease was observed when the ABP dendrimer was given orally. These preclinical assays suggest the potential use of dendrimer ABP as a nanotherapeutic for rheumatoid arthritis (Hayder *et al.*, 2012).

CONCLUSION

We have shown in this review that the phosphorhydrazone dendrimers have many different potential applications for biology and nanomedicine. Indeed, depending on the nature of their terminal functions, their properties as transfection agents, as carriers of drugs, as anti-prion and anti-HIV agents, as inhibitor of the aggregation of Alzheimer's peptides, as inhibitor or accelerator of proliferation of human immune cells, and as anti-inflammatory drugs against rheumatoid arthritis have been already demonstrated. All these tests confirm the high biocompatibility

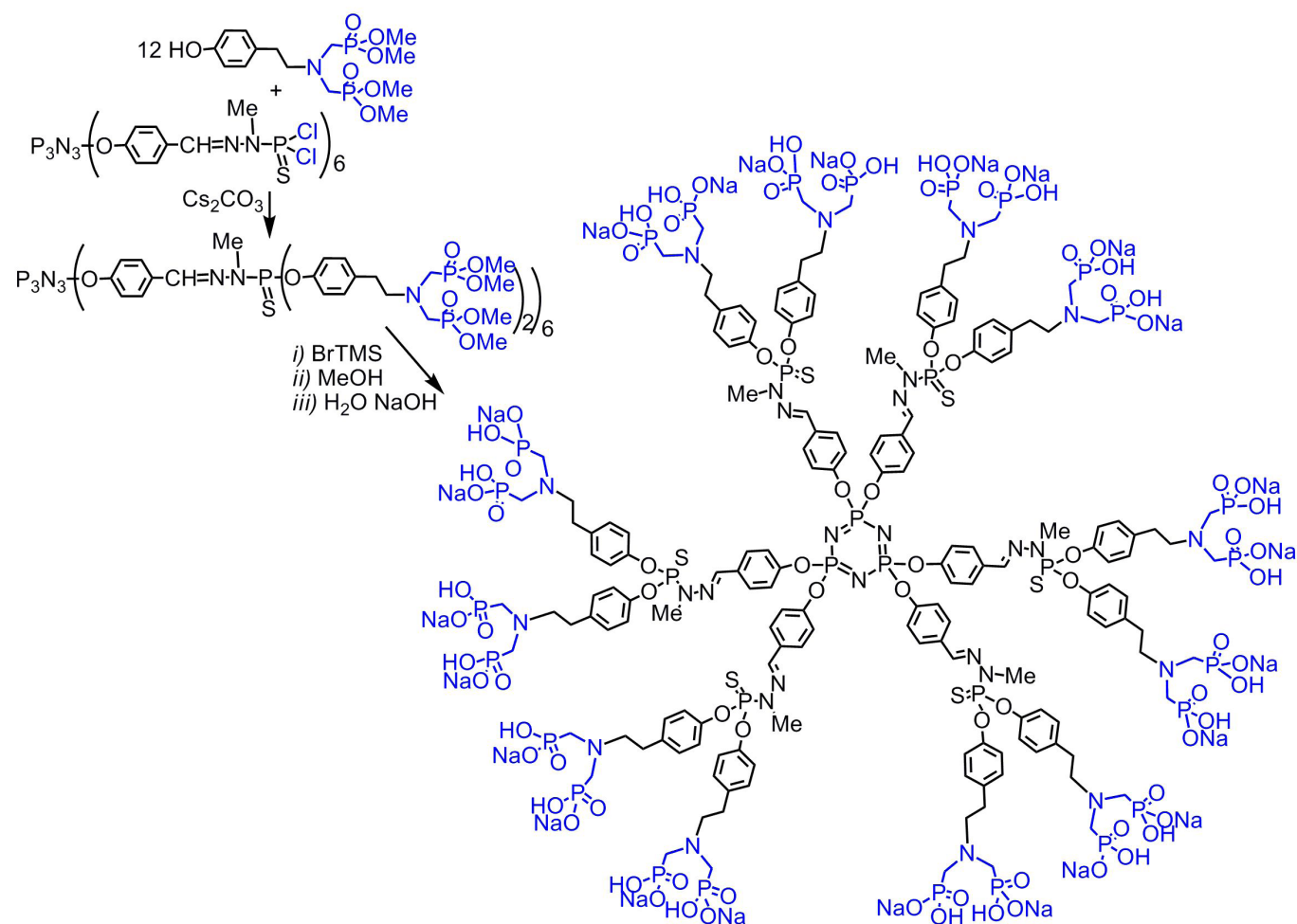


FIGURE 7 - Synthesis of the first generation dendrimer (ABP) ended by azabisphosphonic acid salts.

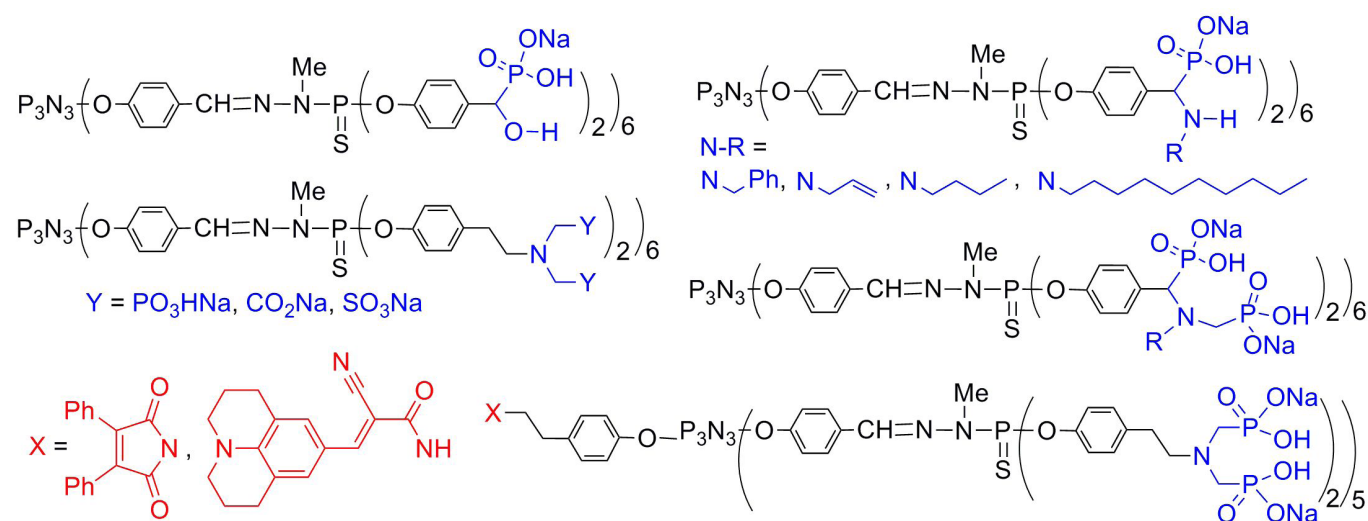


FIGURE 8 - Some examples of surface structural modifications relative to the ABP ($Y = \text{PO}_3\text{HNa}$) dendrimer.

of these dendrimers, including for long term *in vivo* tests, despite their structure totally different in shape, size, and chemical composition, from that of any natural product.

We do believe that these dendrimers are new and highly tunable nanotherapeutic candidates, and we do hope that the best is next to come.

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