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Dendrimers ended by non-symmetrical azadiphosphonate groups: Synthesis and immunological properties

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ABSTRACT

The synthesis and characterization of new series of phosphorus-containing dendrimers ended by non-symmetrical azamonophosphonates, or azadiphosphonates, or azadiphosphonic acid salts are reported. The sodium salts of the non-symmetrical azadiphosphonic dendrimers are soluble in water. Their influence towards human immune blood cells is assayed ex vivo.

Peripheral blood immune cells are easily accessible and widespread in the whole body, thus they are a target of choice for trying to increase their fighting efficiency against bacterial, viral or parasitic infections, and even against cancers. It is known that the surface of cells is composed of complex and dynamic micro-domains, and that the activation of cells often occurs with an increased efficiency if multivalent interactions can be induced.[1] For this purpose, multivalent and perfectly defined macromolecules such as dendrimers have received a tremendous attention for testing their biological properties since one decade.[2] Very few of these tests concern the influence of dendrimers towards immune blood cells, but we have already demonstrated that some phosphorus-containing dendrimers capped with azadiphosphonic acids are able to promote human monocytes activation,[3] to increase the efficiency of the IL2-dependent proliferation of human Natural Killer (NK) cells,[4] and possess anti-inflammatory properties for the human myeloid cell lineage.[5] In this Letter, we report the synthesis of new series of phosphorus-containing dendrimers capped with non-symmetrical azadiphosphonic acids, and their ability to activate human monocytes isolated from the peripheral blood of healthy individuals.

The azadiphosphonate derivatives can be linked to the dendrimer through the central nitrogen affording symmetrical terminal groups, or through one carbon affording non-symmetrical terminal groups. This latter anchorage produces racemic compounds, but offers the possibility to vary easily the nature of the R substituent, thus allowing a structure/activity relationship study (Fig. 1).

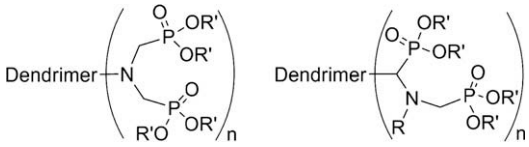

Figure 1. Types of grafting of azadiphosphonate groups to the surface of dendrimers. Left: symmetrical grafting through nitrogen; right: non-symmetrical grafting through carbon.

We have already reported the synthesis of the azadiphosphonate dendrimer where R = methyl;[3a] we report here the synthesis of new dendrimers where R has an increasing number of bonds (R = allyl (**a**), butyl (**b**), decyl (**c**)), to determine the influence of an increased hydrophobicity on the biological properties.

One of the simplest way to graft functional groups to the surface of the phosphorus-containing dendrimers consists in reacting phenols with $P(S)Cl_2$ terminal groups. Starting from 4-hydroxybenzaldehyde, azadiphosphonate groups are obtained in 3 steps. The first step is a condensation reaction of the aldehyde with primary amines bearing the R substituent, to afford compounds **1a–c**. They are generally not isolated but used directly to react with dimethylphosphite

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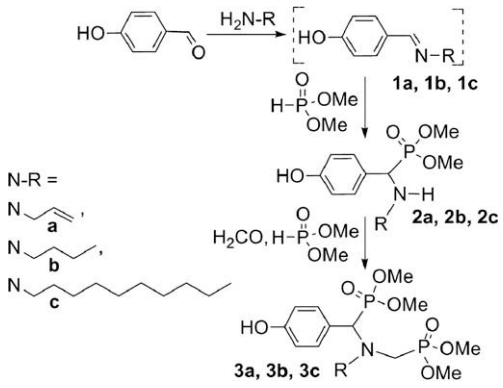


Scheme 1. Synthesis of phenol functionalized by azamonophosphonates (**2a**–**c**) and azadiphosphonates (**3a**–**c**).

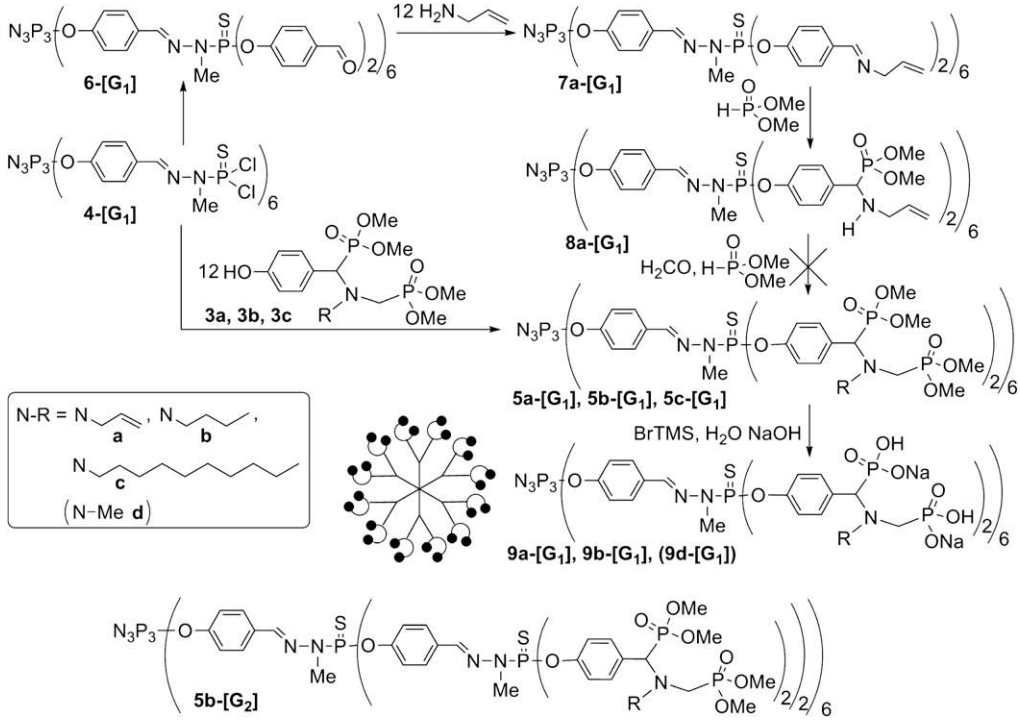
in the second step, to afford the monophosphonate derivatives **2a**–**c**, isolated in 65–92% yield. The third step is a Kabachnik–Fields procedure: formaldehyde in water is first added to compounds **2a**–**c**, then dimethylphosphite. Non-symmetrical azadiphosphonates **3a**–**c** are isolated in moderate yields (28–60%) (Scheme 1). A typical procedure of synthesis is given for compound **3a**.[6] These compounds are characterized in particular by ^{31}P NMR which displays two different signals at about 29 and 31 ppm, emphasizing the presence of two non-equivalent phosphonate groups.

The next step consists in grafting these multifunctional phenols to the surface of dendrimers ended by $P(S)Cl_2$ groups, in particular to the first generation dendrimer[7] **4-[G$_1$]**, with Cs_2CO_3 as base. The reaction affords dendrimers **5a-[G$_1$]**, **5b-[G$_1$]**, or **5c-[G$_1$]** in 76%, 65%, or 85% yield after work-up, respectively[8] (Scheme 2).

The reaction is monitored by ^{31}P NMR, which displays first the appearance of an intermediate singlet at about 70 ppm, corresponding to the substitution of one Cl on the $P(S)Cl_2$ terminal groups, together with the decrease of the signal at 65.5 ppm corresponding to $P(S)Cl_2$. When the reaction has gone to completion, both signals have totally disappeared, on behalf of a singlet at 65 ppm. Obviously, the singlets corresponding to the core (11.4 ppm) and to both types of phosphonates (28 and 31 ppm) are also observed. Such reaction can be also applied to larger generations, as shown in particular by the synthesis of the second generation **5b-[G$_2$]**.

A potentially alternative to this method for synthesizing compounds **5-[G$_1$]** consists in carrying on a dendrimer the same reactions than those carried out on 4-hydroxybenzaldehyde. In this case, dendrimers ended by benzaldehyde are needed, such as compound **6-[G$_1$]**.[7] The condensation with allylamine affords dendrimer **7a-[G$_1$]**, characterized in particular by the disappearance of the signal corresponding to the aldehydes in ^{1}H and ^{13}C NMR and IR spectra. Addition of dimethylphosphite on the imines affords dendrimer **8a-[G$_1$]** ended by monophosphonate derivatives. The completion of the reaction is shown in particular by the disappearance of the signal corresponding to the N=C(sp^2)H functions on behalf of N-C(sp^3)H functions by ^{1}H NMR, from 8.17 to 4.06 ppm. Furthermore, this signal is a doublet (coupling with phosphonate ($^2J_{HP}$ = 19.8 Hz)). The last step to obtain dendrimer **5a-[G$_1$]** from **8a-[G$_1$]** should consist in Kabachnik–Field reactions with formaldehyde in water and dimethylphosphite. However, this reaction affords numerous compounds (multiple signals in ^{31}P NMR), thus no attempt was made to isolate the expected compound, since we have a straightforward way to access to the same compound (Scheme 2).

The last step for obtaining the water-soluble dendrimers consists in the deprotection of the phosphonates, to generate the phosphonic acids, then their sodium salts, in a multi-step one-pot procedure. Addition of bromotrimethylsilane changes the O–Me groups to O–SiMe$_3$, which are highly sensitive to hydrolysis to afford O–H groups. Addition of 1 equiv of NaOH per $P(O)(OH)_2$ groups affords the P(O)(OH)(ONa) derivatives **9a-[G$_1$]**[9] and **9b-[G$_1$]** which are water-soluble (Scheme 2). This transformation is in particular characterized by ^{31}P NMR, with a shielding of the



Scheme 2. Synthesis of dendrimers ended by azamonophosphonates (**8a-[G$_1$]**), or non-symmetrical azadiphosphonates (**5a-[G$_1$]**, **5a-[G$_2$]**, **5b-[G$_1$]**, **5c-[G$_1$]**), or the sodium salt of non-symmetrical azadiphosphonic acids (**9a-[G$_1$]**, **9b-[G$_1$]**). Schematization of dendrimers **9-[G$_1$]**. Compound **9d-[G$_1$]** was previously reported.[3a]

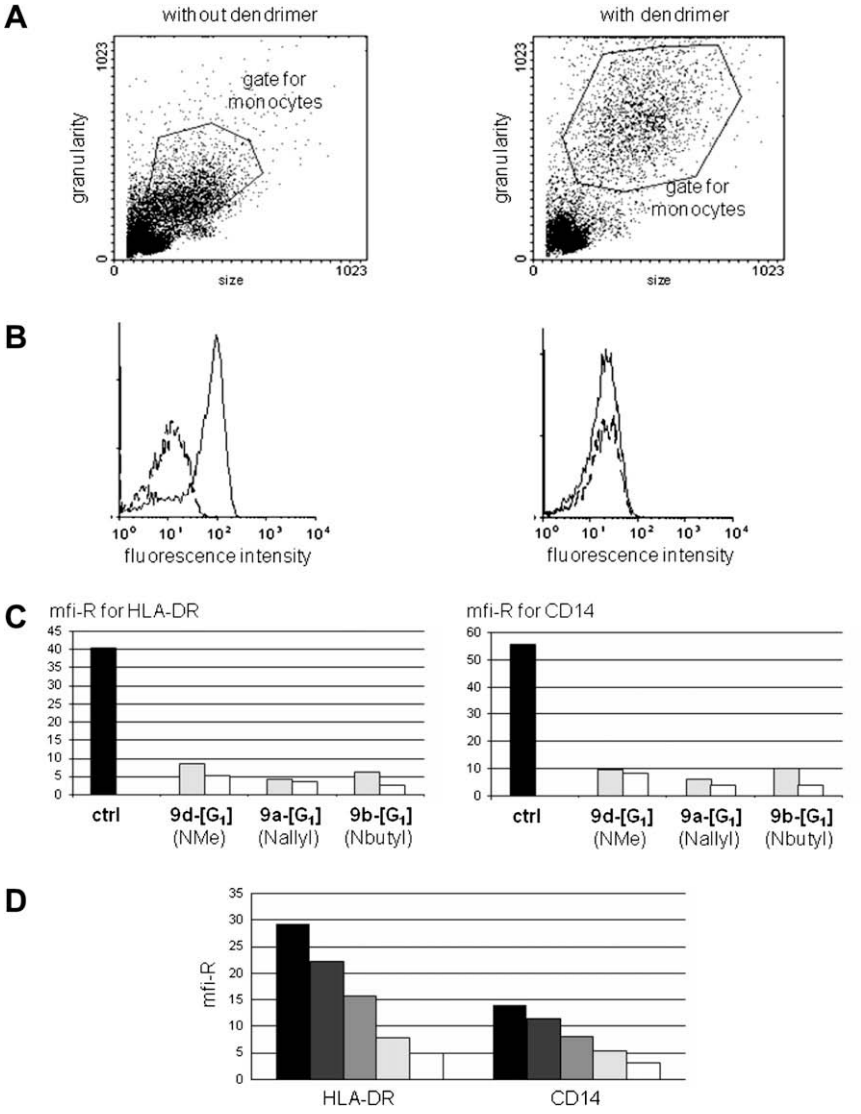


Figure 2. (A) Morphological changes induced by dendrimer on monocytes enable their selection in culture of PBMC (gating). (B) Down-regulation of the expression of CD14 on monocytes activated by dendrimer (dotted line: isotype-matched control, continuous line: staining with anti-CD14 monoclonal antibody, mAb). (C) Comparison of the bio-activity of three dendrimers (at 2 μM, gray bars, and 20 μM, white bars) on monocytes measured by the mfi-ratio (mfi-R) of two membrane markers: HLA-DR and CD14. Control corresponds to untreated monocytes. Results shown were obtained from one healthy donor and are representative of the three donors tested. (D) Dose-dependent bio-activity of dendrimer **9a-[G_1]** on human monocytes (mfi-R of HLA-DR and CD14 with 0, 0.02, 0.2, 2 and 20 μM from dark to white bars of the histograms).

signals of the P(O) groups from about 28 ppm ($P(O)(OMe)_2$) to about 11–13 ppm. Both compounds were engaged in biological tests regarding the activation of human monocytes present in peripheral blood mononuclear cells (PBMC). Upon activation by phosphorylated dendrimers, human monocytes undergo both morphological and phenotypic changes.[3] They become bigger and more granular (Fig. 2A). Expression of membrane markers such as CD14 (Fig. 2B) and HLA-DR are down regulated. We compare the bio-activity of dendrimers **9a-[G_1]** and **9b-[G_1]** to the bio-activity of a non-symmetrical azadiphosphonate dendrimer in which R = Methyl (**9d-[G_1]**).[3a] We have already reported that the bio-activity of this N-methylated dendrimer is very close to the bioactivity of symmetrical azadiphosphonate capped dendrimers.[3a] The three dendrimers were added in cultures of PBMC at 2 and 20 μM, and after 4 days the down-regulation of CD14 and HLA-DR was analyzed. This down-regulation is quantified by the mfi-R[10] a low mfi-R indicates a strong activation of monocytes, and vice versa (Fig. 2C). HLA-DR and CD14 are two independent proteins which differ in their respective levels of expression. Thus, it can be said that the 'dose-dependent' bio-activity is the same for both proteins. As dendrimer **9b-[G_1]** led to cell death in long-lasting cultures, we only test dendrimer **9a-[G_1]** to assess its dose-dependent effect between 0.02 and 20 μM (Fig. 2D).

In conclusion, we have shown that non-symmetrical azadiphosponate-capped dendrimers with various substituents on the nitrogen atom (methyl, allyl or butyl) are able to activate human monocytes in short term in vitro cultures, even at 0.02 μM. Nevertheless, the dendrimer **9b-[G_1]** with the longest hydrophobic portion of the series displays a cytotoxic effect in long term cultures. Ongoing studies will assess the particular interaction between this molecule and cell membrane

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Supplementary data

Supplementary data (Syntheses and characterization of all compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.003.

References and notes

1. Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. *Curr. Opin. Chem. Biol.* **2000**, *4*, 696.
2. For reviews about biological uses of dendrimers, see: (a) Cloninger, M. J. *Curr. Opin. Chem. Biol.* **2002**, *6*, 742; (b) Boas, U.; Heegaard, P. M. H. *Chem. Soc. Rev.* **2004**, *33*, 43; (c) Kofoed, J.; Reymond, J. L. *Curr. Opin. Chem. Biol.* **2005**, *9*, 656; (d) Lee, C. C.; MacKay, J. A.; Fréchet, J. M. J.; Szoka, F. C. *Nat. Biotechnol.* **2005**, *23*, 1517; (e) Svenson, S.; Tomalia, D. A. *Adv. Drug Delivery Rev.* **2005**, *57*, 2106; (f) Wolinsky, J. B.; Grinstaff, M. W. *Adv. Drug. Delivery Rev.* **2008**, *60*, 1037; (g) Caminade, A. M.; Turrin, C. O.; Majoral, J. P. *Chem. Eur. J.* **2008**, *14*, 7422.
3. (a) Poupot, M.; Griffe, L.; Marchand, P.; Maraval, A.; Rolland, O.; Martinet, L.; L'Faqihi-Olive, F. E.; Turrin, C. O.; Caminade, A. M.; Fournié, J. J.; Majoral, J. P.; Poupot, R. *FASEB J.* **2006**, 2339; (b) Rolland, O.; Griffe, L.; Poupot, M.; Maraval, A.; Ouali, A.; Coppel, Y.; Fournié, J. J.; Bacquet, G.; Turrin, C. O.; Caminade, A. M.; Majoral, J. P.; Poupot, R. *Chem. Eur. J.* **2008**, *14*, 4836.
4. Griffe, L.; Poupot, M.; Marchand, P.; Maraval, A.; Turrin, C. O.; Rolland, O.; Métivier, P.; Bacquet, G.; Fournié, J. J.; Caminade, A. M.; Poupot, R.; Majoral, J. P. *Angew. Chem., Int. Ed.* **2007**, *46*, 2523.
5. Fruchon, S.; Poupot, M.; Martinet, L.; Turrin, C. O.; Majoral, J. P.; Fournié, J. J.; Caminade, A. M.; Poupot, R. *J. Leukocyte Biol.* **2009**, *85*, 553.
6. *Typical sequence of reactions for the synthesis of **3a***: $MgSO_4$ (15 g) and allylamine (7.5 mL) were added to a solution of 4-hydroxy-benzaldehyde (11 g) in CH_2Cl_2 (25 mL) at 0 °C, to afford **1a** after one night at rt in situ addition of $(MeO)_2P(O)H$ (9 mL) gave a solution stirred for 3 days, then poured into water (100 mL), and extracted by CH_2Cl_2 (3 × 100 mL). The organic phases were dried ($MgSO_4$) and evaporated; the residue was washed with ether to afford **2a** as very viscous pale yellow oil in 90% yield. CH_2O at 37% in water (1.06 mL) was added to a solution of **2a** (970 mg) in THF (5 mL). $(MeO)_2P(O)H$ (492 μL) was added after 30 min. The solution was stirred for 72 h then poured into water (30 mL) and extracted with CH_2Cl_2. After drying ($MgSO_4$) and evaporating the organic phases, the residue was washed with ether to afford viscous oil purified by column chromatography (silica gel; eluent: AcOEt/MeOH, 95/5). Compound **3a** was isolated as a white powder in 28% yield.
7. (a) Launay, N.; Caminade, A. M.; Lahana, R.; Majoral, J. P. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1589; (b) Launay, N.; Caminade, A. M.; Majoral, J. P. *J. Organomet. Chem.* **1997**, *529*, 51.
8. *Typical synthesis of **5a-[G1]***: Compound **3a** (600 mg) and Cs_2CO_3 (596 mg) were added to a solution of **4-[G1]** (223 mg) in THF (3 mL). This suspension was stirred up to the full substitution of Cl. After decantation, the solution was recovered, then the solid was washed with THF and this solution was added to the previous one, then concentrated under reduced pressure then precipitated with pentane. The resulting solid was washed with THF/pentane then with THF/ether, to afford **5a-[G1]** in 76% yield as a white solid.
9. *Typical synthesis of **9a-[G1]***: Bromo-trimethylsilane (260 μL) was slowly added to a solution of **5a-[G1]** (200 mg) in CH_3CN at 0 °C. The solution was stirred for 18 h at rt then evaporated to dryness. MeOH (5 mL) was added, and the mixture was vigorously stirred for 2 h. After evaporation to dryness, the residue was washed with ether, then with water and MeOH. The residue was dried under reduced pressure. A solution of NaOH in water (0.1966 M, 3.99 mL) was slowly added to the solid, to afford a solution which was filtered then lyophilized. Compound **9a-[G1]** was isolated as a white powder in 97% yield.
10. *Blood samples, cells and cell cultures. PBMC purification and culture*: Fresh blood samples were collected from healthy adult donors, PBMC were prepared on a Ficoll-Paque density gradient (Amersham Biosciences AB, Upsalla, Sweden) by centrifugation (800 g, 30 min at room temperature). Collected PBMC were washed twice and finally diluted at 1.5 million cells per mL in complete RPMI 1640 medium, that is, supplemented with penicillin and streptomycin, both at 100 U per mL (Cambrex Bio Science, Verviers, Belgium), 1 mM sodium pyruvate and 10% heat-inactivated fetal calf serum (both from Invitrogen Corporation, Paisley, UK). PBMC (4.5 millions) were cultured in 3 mL of complete RPMI 1640 medium in 6-well plates. Sterile filtered solutions of the specified dendrimers were added to cultures at the desired final concentration. *Flow cytometry and microscopy*: Flow cytometry was performed on a LSR-II cytometer (BD Biosciences, San Jose, CA, USA). All cell stainings were done using fluorochrome-conjugated mAb from BD Biosciences, San Jose, CA, USA: clone Tü36 for anti-HLA-DR (coupled to FITC fluorochrome) and clone M5E2 for anti-CD14 (coupled to PE-Pc5 fluorochrome). Monocytes were selected upon morphological criteria. To compare the surface densities of various molecules at the surface of monocytes, we calculated the mean fluorescence intensity ratio (mfi-R), that is, the ratio between the mfi of cells stained with the selected mAb and that of cells stained with the isotype-matched control (negative control).[3]