

Synthesis and NMR characterization of dendrimers based on 2, 2-bis-(hydroxymethyl)-propanoic acid (*bis*-HMPA) containing peripheral amino acid residues for gene transfection

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Abstract: Dendrimers, the emerging man made, highly branched, star-shaped macromolecules with nanometer-scale dimensions are well known for their well defined and high controlled architecture, their versatility and high functionality and are of eminent interest in nanomedical applications such as drug delivery, gene transfection, and imaging. In this paper, versatile protocols for the synthesis of polyester-based, hydrolysable, polycationic dendrimers have been setup. A fourth generation dendrimer equipped with 48 peripheral hydroxyl groups was prepared from 2,2-bis(hydroxymethyl)propanoic acid and was used for grafting BOC-amino acids or as “hypercores” on which dendrons functionalized with BOC-amino acids were attached. A library of 15 polycationic *homo*- and *hetero*-dendrimers in the form of hydrochloride was obtained. Their structures and composition were confirmed by NMR analysis and by experimental molecular weight computed by volumetric titration. Their buffer capacity and results obtained from cytotoxicity assays and tests of binding with both *p*DNA and *si*RNA were very satisfactory.

Keywords: Polyester-based polycationic dendrimers; 2, 2-bis(hydroxymethyl)propanoic acid; amino acids; *p*DNA and *si*RNA binding; buffer capacity. ©2017 ACG Publication. All right reserved.

1. Introduction

Dendrimers^{1,2} are a class of nano-sized, radially symmetric synthetic polymers with well-defined, homogeneous and monodisperse structure, characterized by globular shape, interior cavities and a large number of functions at the periphery which favor the interaction with solvents and reagents, thus facilitating their functionalization. A variety of dendrimers exists, and each has biological properties such as self-assembling, electrostatic interactions, chemical stability, low cytotoxicity. These varied characteristics make dendrimers “nonpareil” materials and to date, they represent a good choice in the medical field and for biomedical applications such as drug delivery nanocarriers,³ biosensors,^{4,5} bio imaging agents⁶ and theranostics.⁷

Dendrimers containing nitrogen atoms, which can be protonated at physiological pH, are deeply investigated as non-viral polymeric vectors⁸⁻¹⁷ for delivering nucleic acids into specific cell of patients to replace defective genes (gene therapy). Protonated dendrimers, like other cationic polymeric systems, can establish in fact electrostatic interactions with phosphate groups of genetic

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materials, promoting the formation of nanoparticles known as “polyplexes” where the polymeric component can protect the therapeutic genes from degradation by nucleases during trafficking to the nucleus¹⁰ and successively release them, if the binding strength of the two components of the polyplex are not too strong.

As polyethyleneimine (PEI) between polymeric systems, polyamidoamines (PAMAM)^{18,19} are among the most investigated dendrimeric vectors and are considered as good reference in the field of gene delivery. Though characterized by efficient gene transfer, *in vivo*, PAMAM do not find real applications owing to their cytotoxicity, mainly deriving from the high density of protonated amino groups in the polymeric framework. It is necessary to interact with negatively charged DNA and with membrane sphingolipids thus facilitating the entry into the cell but, if excessive, could, on the one hand, cause irreversible damage to the cell membranes and, could oppose the release of the genetic material once in the nucleus.

To decrease toxicity and improve biodegradability of PAMAM, research efforts focused on their chemical modification such as acetylation,^{20,21} PEGylation,²² introduction of pyrrolidone,²³ carbohydrate,^{24,25} amino acid²⁶⁻²⁸ and peptide²⁹ residues.

A different approach to reduce cytotoxicity in polycationic vectors was to introduce hydrolyzable linkages, as the ester groups, as applied both to PAMAM³⁰ itself and to non dendrimeric vectors such as poly(β -amino esters).³¹⁻³³

Moreover, dendrimers containing amino acid residues^{26,34-36} have aroused great interest since the diversity of amino acid moiety can meet the recognized requirement for modulating the buffer capacity of the vector and facilitate its escape from endosomal acidic compartments through membrane disruption of endosomes.¹⁰

On this ground, with the aim of preparing hydrolyzable dendrimer structures which were, at the same time, adequately protonated but also well tolerated by the cells and which could harmonize transfection capacity with a low level of toxicity, we report in this work versatile chemical protocols for the synthesis of a library of 15 dendrimers of fourth, fifth and sixth generation derived from 2,2-*bis*(hydroxymethyl)propanoic acid (*bis*-HMPA), a monomer of the AB₂ type used to prepare a variety of dendritic architectures.³⁷

The prepared dendrimers have an internal polyester structure known for its biocompatibility³⁸ and contain at the surface a set of amino acid residues, natural or not, alone or in combination which can be protonated at physiological pH thus making dendrimers able to bind DNA, to enter into the cells and to escape the endosomal compartment as soon as possible to avoid degradation.

A selection of samples, elected on the basis of molecular weight and of the N value (N = number of protonated residues), as representatives of the entire library have been investigated in cytotoxicity tests, proofs of adhesion to genetic materials (*p*DNA and *si*RNA) and transfection.

2. Experimental

2.1. Chemical Material And Apparatus

4-Dimethylaminopyridinium *p*-toluenesulfonate (DPTS),³⁹ isopropylidene-2,2-*bis*(methoxy)-propanoic acid **D1(A)**, (**1**)⁴⁰ (mp 124-126 °C), benzyl 2,2-*bis*(hydroxymethyl)propanoate **D1(Bn)**, (**2**)⁴⁰ (mp 88-90 °C), dendrons **D2(BnA)** (**3**),⁴⁰ **D2(A)** (**4**),⁴⁰ **D2(Bn)** (**5**)⁴⁰ (mp 74-76 °C), **D4(BnA)** (**6**),⁴⁰ and **D4(A)** (**7**)⁴⁰ were prepared according to known procedures. Branched polyethyleneimine (PEI-b, 25 kDa) and all the reagents and solvents were purchased from Aldrich or Merck. *N*-BOC-amino acids **16a-f** were prepared according to literature procedure and synthetic and analytical data are reported and available in Supporting Information (pages 3-5). The solvents were dried and distilled according to standard procedures. Petroleum ether refers to the fraction with boiling point 40-60 °C. Melting points, determined on Leica Galen III hot stage apparatus or Mettler Toledo MP50 Melting Point System, are uncorrected.

FTIR spectra were recorded as films or KBr pellets on a Perkin Elmer System 2000 spectrophotometer. ¹H and ¹³C NMR spectra were acquired on a Bruker Avance DPX 300 Spectrometer at 300 and 75.5 MHz respectively and assigned through DEPT-135 and decoupling experiments. Coupling constant values were given in Hertz. Fully decoupled ¹³C NMR spectra were reported. Chemical shifts were reported in δ (parts per million) units relative to the internal standard

tetramethylsilane ($\delta = 0.00$ ppm) and the splitting patterns were described as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad signal).

Freeze-drying was performed on an EDWARDS Super Modulyo Freeze Dryer, Ice capacity 8 kg, 8kg/24hrs, refrigeration down to -55 °C with 24-place Drum Manifold. Centrifugations were performed on an ALC 4236-V1D Centrifuge at 3400-3500 rpm. Thin layer chromatography (TLC) system for routine monitoring the course of reactions and confirming the purity of analytical samples employed aluminium-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F254) and detection of spots was made by UV light and/or by ninhydrin solution 0.2% in ethanol and heating in stove at 100 °C. Chromatographic columns and filtrations on silica gel were performed on Merck Silica gel (0.040-0.063 mm). Elemental analyses were performed with an EA1110 Elemental Analyser (Fison-Instruments). Organic solutions were dried over anhydrous sodium sulphate and were evaporated using a rotatory evaporator operating at reduced pressure of about 10-20 Torr.

2.2. Biological Materials and Apparatus

Tris borate EDTA (TBA), ethidium bromide, 7-aminoactinomycine D (7-AAD), 4', 6-diammidino-2-phenylindole (DAPI), propidium iodide, annexin, CHO (Chinese Hamster Ovary) and Hela (by Henrietta Lacks, whose cancer cells of the uterine cervix were the first to be immortalized) cell lines were purchased by Termofischer Scientific, while *Jet*PEI from PolyPlus. KMS-12-BM and LP1 cell lines were taken from a patient suffering from multiple myeloma (MM).

The factors necessary to cell growth [Dulbecco's Minimal Essential Medium (DMEM), fetal bovine serum (FBS), amino acids, sodium pyruvate, antibiotics, Reduced Serum Medium (OptiMEM), trypsin], *p*DNA and *si*RNA were purchased from Invitrogen™ (Termofischer Scientific).

Electrophoresis was performed in Agarose Gels, 4% TBA (Agarose 4g in 100 mL TBA buffer) and Agarose was purchased from Sigma-Aldrich. Cytofluorometric analysis were performed on Beckman Coulter's Gallios 10/3 Citofluorimeter. Fluorescence emissions were evaluated and quantified by a double fluorescence FM YG100 microscope produced by Suzhou Flyingman Precision Instruments Co. Ltd.

2.3. Chemistry

2.3.1. Determination of Molecular Weights of Dendrimers

Molecular weights of dendrimers in the form of hydrochlorides were obtained by volumetric titrations with HClO_4 in AcOH.⁴¹ A sample of the dendrimer (10-30 mg) was dissolved in AcOH (5 mL), treated with 2-4 mL of a solution of mercury acetate (1.5 g) in AcOH (25 mL), added with a few drops of a solution of quinaldine red (100 mg) in AcOH (25 mL) and titrated with a standardized 0.17-0.18 N solution of HClO_4 in AcOH. The very sharp end points were detected by observing the disappearance of the red color or its change to yellow and in some cases the appearance of a fine white precipitate.

2.3.2. Potentiometric Titrations of Dendrimers

Potentiometric titrations to determine the buffer capacity $[\beta = dC_{(\text{HCl})}/d(\text{pH})]^{42}$ and then the average buffer capacity $[\bar{\beta} = dV_{(\text{HCl})}/d\text{pH}(I)]^{43}$ of dendrimers in the form of hydrochlorides were performed at room temperature with a Hanna Microprocessor Bench pH Meter. The dendrimer (20-30 mg) was dissolved in Milli-Q water (30 mL) then was treated with standard 0.1 N NaOH (1-1.5 mL, pH = 10-12). The solution was potentiometrically titrated by adding 0.2 mL samples of standard 0.1 N HCl up to total 3.0 mL and measuring the corresponding pH values.⁴⁴

2.3.3. Synthesis of Dendrimer G4(OH) (10):

A mixture of D4(A) (**7**)⁴⁰ (3.52 g, 1.69 mmol), **8** (0.062 g, 0.52 mmol), DPTS (0.347 g, 1.18 mmol) in CH_2Cl_2 (26 mL) was treated with dicyclohexylcarbodiimide (DCC) (0.38 g, 1.84 mmol) at room temperature under N_2 and magnetic stirring for 24 h. The precipitated dicyclohexylurea (DCU) was removed by filtration and washed with CH_2Cl_2 . Filtrate and washings were combined,

concentrated at reduced pressure and taken with ethyl acetate (EtOAc) (25 mL) to make precipitate DPTS which was filtered and washed with EtOAc. The solvent was removed at reduced pressure to give dendrimer G4(A) (**9**) which was submitted to a careful column chromatography to eliminate traces of unreacted DCC and *N*-acylureic adduct of **7** performed as follows. Dendrimer **9** was dissolved in the minimum quantity of a mixture petroleum ether/EtOAc = 1:1 and passed through a short silica gel column (h = 20 cm, ϕ = 2 cm) using the same mixture of solvents (100 mL) collecting 1 mL fractions up to disappearance of IR bands at 2118 cm^{-1} (DCC) and at 1648, 1527 cm^{-1} (*N*-acylureic adduct of **7**). The chromatography was completed with petroleum ether/EtOAc = 2:3 (100 mL) and EtOAc 100% (200 mL) collected as a single fraction. The removal of the solvent at reduced pressure afforded **9** which was brought to constant weight under vacuum.

Compound 9: Glassy white solid (2.67 g, 82% isolated yield). FTIR (KBr, cm^{-1}): 1739 (C=O). ^1H NMR (CDCl_3 , 300 MHz): δ = 0.89 (s, 3H, CH_3 of core), 1.14 (s, 72H, CH_3 of fourth generation), 1.28 (s, 36H, CH_3 of third generation), 1.32 (s, 18H, CH_3 of second generation), 1.35 (s, 72H, CH_3 acetonide), 1.42 (s, 72H, CH_3 acetonide), 1.45 (s, 9H, CH_3 of first generation), 3.63 (d, 48H J = 11.3 Hz, CH_2O acetonide), 4.15 (d, 48H, J = 11.8 Hz, CH_2O acetonide), 4.17-4.36 (m, 90H, CH_2O dendrimer + core). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 16.88 (CH_3 of core), 17.52, 17.55, 17.68, 18.49 (CH_3 dendrimer generations), 21.91-22.10 (CH_3 acetonide), 25.17- 25.32 (CH_3 acetonide), 42.06 and 42.09 (quaternary C), 46.07 (quaternary C of core), 46.72 (quaternary C), 46.78 (quaternary C), 46.88 (quaternary C), 64.98, 65.72, 65.93 and 65.96 (CH_2O of dendrimer generations), CH_2O core not detected, 98.10, 98.12 and 98.19 (quaternary C acetonide), 171.49, 171.71, 171.80 and 173.48-173.60 (C=O). Anal. Cald. for $\text{C}_{302}\text{H}_{468}\text{O}_{138}$: C, 57.51; H, 7.48%. Found: C, 57.38; H, 7.35.

A solution of **9** (1.37 g, 0.22 mmol) in MeOH (34 mL) was treated with four spatula tips of acid resin Dowex 50 WX2-200 at room temperature with magnetic stirring for 24 h. The resins were removed by filtration and washed with fresh MeOH. Filtrate and washings were combined, concentrated at reduced pressure to give a pink glassy solid which was left under magnetic stirring overnight in excess of dry Et_2O , filtered to give **10** as a pink hygroscopic solid (1.20 g) which was further purified by dissolution in H_2O (80 mL), centrifuged to remove insoluble residues and freeze-dried to obtain **10** which was stored in a dryer on P_2O_5 .

Compound 10: Fluffy white solid (1.14 g, 98% isolated yield), m.p. 77 °C. FTIR (KBr, cm^{-1}): 3424 (OH), 1739 (C=O). ^1H NMR ($\text{DMSO}-d_6$, 300 MHz): δ = 0.80 (s, 3H, CH_3 of core), 1.01 (s 72H, CH_3 of fourth generation), 1.16 (s, 36H, CH_3 of third generation), 1.18 (s, 18H, CH_3 of second generation), 1.22 (s, 9H, CH_3 of first generation), 3.29-3.49 (m, 96H, CH_2OH); 4.08-4.30 (m, 90H, CH_2O of dendrimer), 4.55 (br q, 48H, OH). ^{13}C NMR ($\text{DMSO}-d_6$, 75.5 MHz): δ = 16.67, 16.84, 16.88, and 17.12 (CH_3), 46.16, 46.19, 46.23 and 50.20 (quaternary C), 63.63 (CH_2OH), 64.33, 64.86 and 65.29 (CH_2O), 171.42 (two signals overlapped), 171.79 and 174.00 (C=O), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{230}\text{H}_{372}\text{O}_{138}$: C, 51.68; H, 7.01%. Found: C, 51.86; H 7.18.

2.3.4. General Procedure for Synthesis of G4-Boc Dendrimers 17-22:

A solution of **10** in dry DMF (50 mg/mL) was added with *N*-BOC-amino acid (1.2 equiv./OH of **10**), 4-dimethylaminopyridine (DMAP) (0.6 equiv./OH of **10**) and *N*-ethyl-*N*-(3-dimethylamino)propyl carbodiimide hydrochloride (EDC) (1.2 equiv./OH of **10**). The solution was kept under magnetic stirring at rt for 24 h then added with EtOAc (15 mL) to produce a suspension which was washed with 10% aq. KHSO_4 (3x15 mL). The aqueous washings were extracted with EtOAc and the combined organic phases washed with aq. 15% NaOH followed by water then dried (Na_2SO_4). The removal of the solvent at reduced pressure afforded the functionalized dendrimer.

Dendrimer G4(16a) (17): Off white glassy solid, Yield 88%. FTIR (KBr, cm^{-1}): 3407 (NH), 1744 (C=O ester), 1721 (C=O urethane), 1528 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.18-1.28 (m, 135 H, CH_3 of G1, G2, G3, G4), 1.44 (s, 432 H, CH_3 of BOC), 3.88 (d, 96 H, J = 5.3 Hz, CH_2NH), 4.26 (m, 186 H, CH_2O of dendrimer), 5.46 (br s, 48 H, NH). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.32-17.87 (CH_3 of G1, G2, G3, G4), 28.35 (CH_3 of BOC), 42.26 (CH_2NH),

46.44-46.72 (quaternary C of G1, G2, G3, G4), 65.34-65.85 (CH₂O of G1, G2, G3, G4), 79.93 (quaternary C of BOC), 155.98 (C=O urethane), 170.15 (C=O amino acid), 171.52-171.93 (C=O ester of G1, G2, G3, G4), CH₃, quaternary C and CH₂O of core were no detectable. Anal. Cald. for C₅₆₆H₉₀₀N₄₈O₂₈₂: C, 52.74; H, 7.04; N, 5.22%. Found: C, 52.58; H, 7.34; N, 5.27.

Dendrimer G4(16b) (18): Colorless oil, Yield, 89%. FTIR (film, cm⁻¹): 1747 (C=O ester), 1698 (C=O urethane). ¹H NMR (CDCl₃, 300 MHz): δ = < 1.00 (CH₃ of core was no detectable), 1.11-1.21 (m, 135 H, CH₃ of G1, G2, G3, G4), 1.35 and 1.38 (two s, 432 H, CH₃ of BOC), 2.83 (s, 144 H, CH₃N), 3.86 and 3.91 (two s, 96 H, CH₂N), 4.19 (m, 186 H, CH₂O of dendrimer). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 18.56-19.67 (CH₃ of G1, G2, G3, G4), 28.75 (CH₃ of BOC), 35.85 and 36.00 (CH₃N), 46.93-47.13 (quaternary C of G1, G2, G3, G4), 50.52 and 51.12 (CH₂N), 65.65 (CH₂O), 80.64 (quaternary C of BOC), 155.73 and 156.41 (C=O urethane), 169.85 (C=O amino acid), 171.88-172.16 (C=O ester of G1, G2, G3, G4), CH₃, quaternary C and CH₂O of core were no detectable. Anal. Cald. for C₆₁₄H₉₉₆N₄₈O₂₈₂: C, 54.37; H, 7.40; N, 4.96%. Found: C, 54.19; H, 7.47; N, 5.10.

Dendrimer G4(16c) (19): Pale yellow oil, Yield, 78%. FTIR (film, cm⁻¹): 3383 (NH), 1741 (C=O ester), 1709 (C=O urethane), 1517 (NH). ¹H NMR (CDCl₃, 300 MHz): δ = < 1.00 (CH₃ of core was no detectable), 1.21-1.26 (m, 135 H, CH₃ of G1, G2, G3, G4), 1.43 (s, 432 H, CH₃ of BOC), 1.78 (m, 96 H, CH₂), 2.36 (t, 96 H, J = 7.3 Hz, CH₂), 3.12 (m, 96 H, CH₂NH), 4.20 (m, 186 H, CH₂O of dendrimer), 5.05 (br s, 48 H, NH). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 17.46-17.83 (CH₃ of G1, G2, G3, G4), 25.18 (CH₂), 28.45 (CH₃ of BOC), 31.19 (CH₂), 39.80 (CH₂NH), 46.43-46.67 (quaternary C of G1, G2, G3, G4), 64.97-65.11 (CH₂O of G1, G2, G3, G4), 79.08 (quaternary C of BOC), 156.08 (C=O urethane), 171.42 (C=O ester), 171.54 (C=O ester), 172.07 (C=O ester), 172.19 (C=O ester), 172.68 (C=O amino acid), CH₃, quaternary C and CH₂O of core were no detectable. Anal. Cald. for C₆₆₂H₁₀₉₂N₄₈O₂₈₂: C, 55.85; H, 7.73; N, 4.72%. Found: C, 55.97; H, 8.05; N, 5.08.

Dendrimer G4(16d) (20): Colorless oil, Yield, 97%. FTIR (film, cm⁻¹): 3383 (NH), 1743 (C=O ester), 1695 (C=O urethane). ¹H NMR (CDCl₃, 300 MHz): δ = < 1.00 (CH₃ of core was no detectable), 1.23-1.26 (m, 135 H, CH₃ of G1, G2, G3, G4), 1.44 (s, 432 H, CH₃ of BOC), 1.81 (m, 96 H, CH₂), 2.31 (t, 96 H, J = 7.4 Hz, CH₂), 2.84 (s, 144 H, CH₃N), 3.23 (t, 96 H, J = 7.1 Hz, CH₂N), 4.21 (m, 186 H, CH₂O of dendrimer). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 17.90-18.25 (CH₃ of G1, G2, G3, G4), 23.46 (CH₂), 28.88 (CH₃ of BOC), 31.46 (CH₂), 34.64 (CH₃N), 46.82-47.09 (quaternary C of G1, G2, G3, G4), 48.20-48.64 (CH₂N), 65.24 (CH₂O), 65.43 (CH₂O), 79.79 (quaternary C of BOC), 156.16 (C=O urethane), 171.87 (C=O ester), 172.35 (C=O ester), 172.91 (C=O amino acid), CH₃, quaternary C and CH₂O of core were no detectable. Anal. Cald. for C₇₁₀H₁₁₈₈N₄₈O₂₈₂: C, 57.20; H, 8.03; N, 4.51%. Found: C, 57.30; H, 8.33; N, 4.90.

Dendrimer G4(16e) (21): Viscous resin, Yield, 90%. FTIR (KBr, cm⁻¹): 3431 (NH), 1739 (C=O ester), 1694 (C=O urethane), 1528 (NH). ¹H NMR (CDCl₃, 300 MHz): δ = < 1.00 (CH₃ of core was no detectable), 1.00-1.70 (m, 423 H, CH₃ of G1, G2, G3, G4 + CH₂CH₂CH₂ of Lys), 1.36 (s, 432 H, CH₃ of BOC), 1.37 (s, 432 H, CH₃ of BOC), 2.87 (m, 96 H, CH₂NH), 3.44-4.22 (m, 234 H, CH₂O of dendrimer + CHNH of Lys), 6.65, 6.75 and 7.05 (br signals, 48H, ⁶NH), 6.95 (d, J = 7.9 Hz, 48 H, ^αNH). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 17.00-18.00 (CH₃ of G1, G2, G3, G4), 22.61 (CH₂), 28.39 (CH₃ of BOC), 28.49 (CH₃ of BOC), 29.58 (CH₂), 31.81 (CH₂), 40.06 (CH₂NH), 46.00-49.00 (quaternary C G1, G2, G3, G4), 53.41 (CHNH), 65.31-65.37 (CH₂O of G1, G2, G3, G4), 78.99 (quaternary C of BOC), 79.77 (quaternary C of BOC), 155.66 (C=O urethane), 156.23 (C=O urethane), 171.80-172.45 (C=O amino acid + C=O ester of G1, G2, G3, G4), CH₃, quaternary C and CH₂O of core were no detectable. Anal. Cald. for C₉₉₈H₁₇₁₆N₉₆O₃₇₈: C, 56.79; H, 8.19; N, 6.37%. Found: C, 56.98; H, 8.49; N, 6.06.

Dendrimer G4(16f) (22): Viscous resin, Yield, 87%. FTIR (KBr, cm⁻¹): 3383 (NH), 1748 (C=O ester), 1716 (C=O urethane). ¹H NMR (CDCl₃, 300 MHz): δ = < 1.00 (CH₃ of core was no detectable), 1.18-1.28 (m, 135 H, CH₃ of G1, G2, G3, G4), 1.39 (s, 432 H, CH₃ of BOC), 1.58 (s, 432 H, CH₃ of BOC), 2.96 (m, 96 H, CH₂ of His), 4.30 (m, 186 H, CH₂O of dendrimer), 4.55 (m, 48 H,

CHNH of His), 5.50 and 5.93 (m, 48 H, NH), 7.19 (m, 48 H, CH of imidazole), 7.95 (m, 48 H, CH of imidazole). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.52-17.83 (CH_3 of G1, G2, G3, G4), 27.88 (CH_3 of BOC), 28.34 (CH_3 of BOC), 29.97 (CH_2), 46.47-46.69 (quaternary C of G1, G2, G3, G4), 53.07 (CHNH), 65.45-65.68 (CH_2O of G1, G2, G3, G4), 79.49 (quaternary C of BOC), 85.38 (quaternary C of BOC), 114.86 (CH of imidazole), 136.86 (CH of imidazole), 138.65 (quaternary C of imidazole), 146.90 (C=O urethane), 155.43 (C=O urethane), 171.25-171.79 (C=O amino acid + C=O ester of G1, G2, G3, G4), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{998}\text{H}_{1476}\text{N}_{144}\text{O}_{378}$: C, 55.65; H, 6.91; N, 9.36%. Found: C, 61.32; H, 6.75; N, 9.24.

2.3.5. General Procedure for Removing the Boc Groups from 17-22

A solution of the BOC-protected dendrimer in ethanol (320 mg/mL) was cooled to 0 °C and treated with acetyl chloride (2 equiv./BOC to be removed). The solution was kept at rt under magnetic stirring for 24 h then was concentrated at reduced pressure, taken with MeOH and precipitated into acetone. The dendrimer in the form of hydrochloride was directly filtered or recovered as oil after centrifugation, washed repeatedly with fresh acetone, filtered or recovered by centrifugation, dried at reduced pressure and stored under vacuum over P_2O_5 .

Dendrimer G4(15aHCl) (23): Hygroscopic off-white solid, Yield, 97%. FTIR (KBr, cm^{-1}): 3433 (NH_3^+), 1744 (C=O), 1629 (NH). ^1H NMR ($\text{DMSO-}d_6$, 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.03-1.24 (m, 135 H, CH_3 of G1, G2, G3, G4), 3.81 (m, 96 H, CH_2NH_3^+), 4.00-4.45 (m, 186 H, CH_2O of dendrimer), 8.69 (br s, 144 H, NH_3^+). ^{13}C NMR ($\text{DMSO-}d_6$, 75.5 MHz): δ = 19.05-19.47 (CH_3 of G1, G2, G3, G4), 40.16 (CH_2NH_3^+), 47.84-48.99 (quaternary C of G1, G2, G3, G4), 67.69 (CH_2O), 169.15 (C=O amino acid), 173.52 (C=O ester), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G4(15bHCl) (24): Hygroscopic off-white solid, Yield, 98%. FTIR (KBr, cm^{-1}): 3425 (NH_3^+), 1747 (C=O). ^1H NMR ($\text{DMSO-}d_6$, 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.16-1.25 (m, 135 H, CH_3 of G1, G2, G3, G4), 2.58 (s, 144 H, CH_3NH_2^+), 3.99 (m, 96 H, CH_2NH_2^+), 4.10-4.50 (m, 186 H, CH_2O of dendrimer), 9.59 (br s, 96 H, NH_2^+). ^{13}C NMR ($\text{DMSO-}d_6$, 75.5 MHz): δ = 16.74 (CH_3 of G4), 16.86 (CH_3 of G3), 16.95 (CH_3 of G1), 17.11 (CH_3 of G2), 32.39 (CH_3NH_2^+), 46.23, 47.87, 47.91 (quaternary C of G1, G2, G3), 48.37 (CH_2NH_2^+), 50.12 (quaternary C of G4), 63.37 (CH_2O), 167.96 (C=O amino acid), 172.67-174.13 (C=O ester of G1, G2, G3, G4), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G4(15cHCl) (25): Hygroscopic glassy solid, Yield, 85%. FTIR (KBr, cm^{-1}): 3419 (NH_3^+), 1738 (C=O). ^1H NMR (CD_3OD , 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.16-1.32 (m, 135 H, CH_3 of G1, G2, G3, G4), 1.99 (m, 96 H, CH_2), 2.54 (t, 96 H, J = 7.0 Hz, CH_2), 3.03 (m, 96 H, CH_2NH_3^+), 4.29 (m, 186 H, CH_2O of dendrimer). ^{13}C NMR (CD_3OD , 75.5 MHz): δ = 18.00-18.34 (CH_3 of G1, G2, G3, G4), 23.80 (CH_2), 31.69 (CH_2), 40.17 (CH_2NH_3^+), quaternary C of dendrimer hidden under solvent, 65.52-66.71 (CH_2O of G1, G2, G3, G4), 173.65-173.90 (C=O amino acid + C=O ester of G1, G2, G3, G4), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G4(15dHCl) (26): Very hygroscopic pale yellow solid, Yield, 99%. FTIR (KBr, cm^{-1}): 3418 (NH_3^+), 1739 (C=O). ^1H NMR (CD_3OD , 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.10-1.50 (m, 135 H, CH_3 of G1, G2, G3, G4), 1.87-2.02 (m, 96 H, CH_2), 2.46-2.55 (m, 96 H, CH_2), 2.73 (m, 144 H, CH_3NH_2^+), 3.02-3.20 (m, 96 H, CH_2NH_2^+), 4.11-4.44 (m, 186 H, CH_2O of dendrimer). ^{13}C NMR (CD_3OD , 75.5 MHz): δ = 17.61-18.41 (CH_3 of G1, G2, G3, G4), 22.49 (CH_2), 31.69 (CH_2), 33.71 and 33.90 (CH_3NH_2^+), 47.81-48.10 (quaternary C of G1, G2, G3, G4), 49.80 (CH_2NH_2^+), 66.56-68.90 (CH_2O of G1, G2, G3, G4), 173.54 (C=O amino acid + C=O ester of G1, G2, G3, G4), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G4(15e2HCl) (27): Very hygroscopic glassy solid, Yield, 92%. FTIR (KBr, cm^{-1}): 3431 (NH_3^+), 1742 ($\text{C}=\text{O}$), 1629 (NH). ^1H NMR ($\text{DMSO-}d_6$, 300 MHz): $\delta = < 1.00$ (CH_3 of core was no detectable), 1.03-1.99 (m, 423 H, CH_3 of G1, G2, G3, G4 + $\text{CH}_2\text{CH}_2\text{CH}_2$ of Lys), 2.76 (m, 96 H, CH_2NH_3^+ of Lys), 3.99 (m, 48 H, CHNH_3^+ of Lys), 4.10-4.50 (m, 186 H, CH_2O of dendrimer), 8.20 (br s, 144 H, NH_3^+), 8.82 (br s, 144 H, NH_3^+). ^{13}C NMR ($\text{DMSO-}d_6$, 75.5 MHz): $\delta = 19.33$ (CH_3), 23.14 (CH_2), 28.01 (CH_2), 31.01 (CH_2), 40.02 (CH_2NH_3^+), 47.70 (quaternary C), 53.55 (CHNH_3^+), 67.65-67.82 (CH_2O and of G1, G2, G3, G4), 170.68-173.33 ($\text{C}=\text{O}$ of amino acid + ester of G1, G2, G3, G4), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G4(15f2HCl) (28): Hygroscopic glassy solid, Yield 99%. FTIR (KBr, cm^{-1}): 3435 (NH), 1747 ($\text{C}=\text{O}$), 1626 (NH). ^1H NMR ($\text{DMSO-}d_6$, 300 MHz): $\delta = < 1.00$ (CH_3 of core was no detectable), 1.00-1.35 (m, 135 H, CH_3 of G1, G2, G3, G4), 3.35 (m, 96 H, CH_2 of His), 4.24 (m, 186 H, CH_2O of dendrimer), 4.57 (m, 48 H, CHNH_3^+), 7.55 (br s, 48 H, CH of imidazole), 8.00-10.00 (very broad s, 144 H, $^n\text{NH}_3^+$), 9.12 (br s, 48 H, CH of imidazole), 12.80-16.00 (very br s, 96 H, $^m\text{NH}^+ + ^{im}\text{NH}$). ^{13}C NMR ($\text{DMSO-}d_6$, 75.5 MHz): $\delta = 17.47$ -19.03 (CH_3 of G1, G2, G3, G4), 25.11 and 25.54 (CH_2 His), 45.52-47.53 (quaternary C of dendrimer), 51.49 and 51.70 (CHNH_3^+), 65.87-66.80 (CH_2O of G1, G2, G3, G4), 118.49 (CH of imidazole), 127.21 (quaternary C of imidazole), 134.56 (CH of imidazole), 167.97-173.87 ($\text{C}=\text{O}$ amino acid + $\text{C}=\text{O}$ ester of G1, G2, G3, G4), CH_3 , quaternary C and CH_2O of core were no detectable.

2.3.6. General Procedure for Functionalization of **2** or **5** with *N*-Boc Amino Acids:

A solution of the dendron (**2** or **5**) in dry DMF (65 mg/mL) was treated with *N*-BOC amino acid (1.2 equiv./OH of dendron), DMAP (0.6 equiv./OH of dendron) and EDC (1.2 equiv./OH of dendron) for 24 h at rt under N_2 and magnetic stirring. For the isolation of the products in the case of amino acids **16a-d** the final solution was added with Et_2O (15 mL) to produce a suspension which was washed with 10% aq. KHSO_4 (3x15 mL). The aqueous washings were extracted with Et_2O and the combined organic phases washed with aq. 15% NaOH followed by water then dried (Na_2SO_4). In the case of amino acids **16e** and **16f**, EtOAc was used as extraction solvent instead of Et_2O . The removal of the solvent at reduced pressure afforded the functionalized dendron.

Dendron D1(Bn16a) (29): Colorless viscous oil, Yield, 83%. FTIR (KBr, cm^{-1}): 3384 (NH), 1721 ($\text{C}=\text{O}$ ester + $\text{C}=\text{O}$ urethane), 1513 (NH). ^1H NMR (CDCl_3 , 300 MHz): $\delta = 1.26$ (s, 3 H), 1.44 (s, 18 H), 3.79 (d, 4 H, $J = 4.4$ Hz), 4.31 (m, 4 H, $J = 11.1$ Hz), 4.99 (br s, 2 H, NH), 5.16 (s, 2 H), 7.36 (m, 5 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): $\delta = 17.99$ (CH_3), 28.40 (CH_3 of BOC), 42.31 (CH_2NH), 46.44 (quaternary C), 65.91 (CH_2O), 67.10 (benzyl CH_2O), 80.16 (quaternary C of BOC), 128.43 (phenyl CH), 128.61 (phenyl CH), 128.74 (phenyl CH), 135.56 (quaternary phenyl C), 155.78 ($\text{C}=\text{O}$ urethane), 170.02 ($\text{C}=\text{O}$ amino acid), 172.26 ($\text{C}=\text{O}$ ester). Anal. Cald. for $\text{C}_{26}\text{H}_{38}\text{N}_2\text{O}_{10}$: C, 57.98; H, 7.11; N, 5.20%. Found: C, 57.78; H, 7.49; N, 5.57.

Dendron D1(Bn16b) (30): Colorless oil, Yield, 84%. Mixture of rotamers. FTIR (KBr, cm^{-1}): 1743 ($\text{C}=\text{O}$ ester), 1702 ($\text{C}=\text{O}$ urethane). ^1H NMR (CDCl_3 , 300 MHz): $\delta = 1.26$ (s, 3 H), 1.41 and 1.46 (s, 18 H), 2.86 and 2.87 (s, 3 H), 3.84 and 3.91 (s, 4 H), 4.30 (m, 4 H), 5.16 (s, 2 H), 7.34 (m, 5 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): $\delta = 17.71$ (CH_3), 28.26 and 28.31 (CH_3 of BOC), 35.44 and 35.54 (CH_3N), 46.46 (quaternary C), 50.14 and 50.73 (CH_2N), 65.67 (CH_2O), 66.98 (benzyl CH_2O), 80.22 and 80.27 (quaternary C of BOC), 128.22 (phenyl CH), 128.44 (phenyl CH), 128.64 (phenyl CH), 135.49 (quaternary phenyl C), 155.27 and 155.97 ($\text{C}=\text{O}$ urethane), 169.40 ($\text{C}=\text{O}$ amino acid), 172.21 ($\text{C}=\text{O}$ ester). Anal. Cald. for $\text{C}_{28}\text{H}_{42}\text{N}_2\text{O}_{10}$: C, 59.35; H, 7.47; N, 4.94%. Found: C, 59.21; H, 7.78; N, 4.67.

Dendron D1(Bn16c) (31): Colorless viscous oil, Yield, 97%. FTIR (KBr, cm^{-1}): 1736 ($\text{C}=\text{O}$ ester), 1716 ($\text{C}=\text{O}$ urethane), 1515 (NH). ^1H NMR (CDCl_3 , 300 MHz): $\delta = 1.26$ (s, 3 H), 1.44 (s, 18 H), 1.74 (m, 4 H), 2.27 (t, 4 H, $J = 7.4$ Hz), 3.12 (m, 4 H), 4.24 (m, 4 H, $J = 11.1$ Hz), 4.67 (br s, 2 H, NH),

5.16 (s, 2 H), 7.34 (m, 5 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.85 (CH_3), 25.16 (CH_2), 28.42 (CH_3 of BOC), 31.21 (CH_2), 39.78 (CH_2NH), 46.42 (quaternary C), 65.36 (CH_2O), 66.86 (benzyl CH_2O), 79.24 (quaternary C of BOC); 128.23 (phenyl CH), 128.42 (phenyl CH), 128.61 (phenyl CH), 135.60 (quaternary phenyl C), 155.96 (C=O urethane), 172.56 (C=O ester), 172.62 (C=O amino acid). Anal. Cald. for $\text{C}_{30}\text{H}_{46}\text{N}_2\text{O}_{10}$: C, 60.59; H, 7.80; N 4.96%. Found: C, 60.80; H, 7.80; N, 5.06.

Dendron DI(Bn16d) (32): Colorless oil, Yield, 76%. FTIR (KBr, cm^{-1}): 1741 (C=O ester), 1694 (C=O urethane). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.26 (s, 3 H), 1.45 (s, 18 H), 1.76 (m, 4 H), 2.24 (t, 4 H, J = 7.4 Hz), 2.82 (s, 6 H), 3.20 (t, 4 H, J = 6.9 Hz), 4.24 (m, 4 H, J = 11.1 Hz), 5.16 (s, 2 H), 7.34 (m, 5 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.79 (CH_3), 22.99 (CH_2), 28.45 (CH_3 of BOC), 31.05 (CH_2), 34.17 (CH_3N), 46.43 (quaternary C), 47.96 (CH_2N), 65.42 (CH_2O), 66.83 (benzyl CH_2O), 79.24 (quaternary C of BOC), 128.14 (phenyl CH), 128.24 (phenyl CH), 128.60 (phenyl CH); 135.59 (quaternary phenyl C); 155.75 (C=O urethane); 172.50 (C=O amino acid); 172.56 (C=O ester). Anal. Cald. for $\text{C}_{32}\text{H}_{50}\text{N}_2\text{O}_{10}$: C, 61.72; H, 8.09; N 4.50%. Found: C, 61.76; H, 8.41; N, 4.33.

Dendron DI(Bn16e) (33): Colorless viscous oil, Yield, 98%. FTIR (KBr, cm^{-1}): 3358 (NH), 1740 (C=O ester), 1713 (C=O urethane), 1520 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.27 (s, 3 H), 1.33-1.80 (m, 12 H), 1.44 (s, 36 H), 3.01 (m, 4 H), 4.24 (m, 4 H), 4.34 (m, 2 H), 4.73 (m, 2 H, ^6NH), 5.16 (s, 4 H, $\text{PhCH}_2+^6\text{NH}$), 7.35 (m, 5 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.51 (CH_3), 22.11 (CH_2), 28.00 (CH_3 of BOC), 28.11 (CH_3 of BOC), 29.21 (CH_2), 31.59 (CH_2), 39.69 (CH_2NH), 46.07 (quaternary C), 52.95 (CHNH), 65.41 (CH_2O), 66.68 (benzyl CH_2O), 78.74 (quaternary C of BOC), 79.52 (quaternary C of BOC), 127.81 (phenyl CH), 128.13 (phenyl CH), 128.31 (phenyl CH), 135.08 (quaternary phenyl C), 155.13 (C=O urethane), 155.77 (C=O urethane), 171.86 (C=O amino acid), 180.05 (C=O ester). Anal. Cald. for $\text{C}_{44}\text{H}_{72}\text{N}_4\text{O}_{14}$: C, 59.98; H, 8.24; N, 6.36%. Found: C, 59.86; H, 8.54; N, 6.56.

Dendron DI(Bn16f) (34): Glassy solid, Yield, 86%. FTIR (KBr, cm^{-1}): 3387 (NH), 1753 (C=O ester), 1717 (C=O urethane). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.23 (s, 3 H), 1.42 (s, 18 H), 1.59 (s, 18 H), 2.96 (m, 4 H), 4.19-4.34 (m, 4 H), 4.54 (m, 2 H), 5.15 (m, 2 H), 5.75 (m, 2 H, NH), 7.15 (s, 2 H), 7.34 (m, 5 H), 7.91 (s, 2 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.67 (CH_3), 27.88 (CH_3 of BOC), 28.33 (CH_3 of BOC), 29.89 (CH_2 His), 46.46 (quaternary C), 53.09 and 53.12 (CHNH His), 66.07 (CH_2O), 66.93 (benzyl CH_2O), 79.67 (quaternary C of BOC), 85.49 (quaternary C of BOC), 114.77 and 114.82 (CH of imidazole), 128.18 (phenyl CH), 128.38 (phenyl CH), 128.62 (phenyl CH), 135.55 (quaternary phenyl C), 136.88 (CH of imidazole), 138.48 (quaternary C of imidazole), 146.87 (C=O urethane), 155.42 (C=O urethane), 171.27 (C=O amino acid), 172.27 (C=O ester). Anal. Cald. for $\text{C}_{44}\text{H}_{62}\text{N}_6\text{O}_{14}$: C, 58.44; H, 6.64; N, 12.03%. Found: C, 58.06; H, 7.00; N, 12.21.

Dendron D2(Bn16a) (41): Glassy solid, Yield, 81%. FTIR (KBr, cm^{-1}): 3410 (NH), 1747 (C=O ester), 1720 (C=O urethane), 1523 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.17 (s, 6 H), 1.27 (s, 3 H), 1.44 (s, 36 H), 3.88 (d, 8 H, J = 5.6 Hz), 4.26 (m, 12 H), 5.16 (s, 2 H), 5.22 (m, 4 H, NH), 7.36 (m, 5 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.66 (CH_3), 17.80 (CH_3), 28.33 (CH_3 of BOC), 42.28 (CH_2NH), 46.39 (quaternary C), 46.68 (quaternary C), 65.69 (benzyl CH_2O), 65.81 (CH_2O), 67.29 (CH_2O), 80.03 (quaternary C of BOC), 128.48 (phenyl CH), 128.62 (phenyl CH), 128.74 (phenyl CH), 135.27 (quaternary phenyl C), 155.82 (C=O urethane), 170.03 (C=O amino acid), 171.85 (C=O ester). Anal. Cald. for $\text{C}_{50}\text{H}_{76}\text{N}_4\text{O}_{22}$: C, 55.34; H, 7.06; N, 5.16%. Found: C, 55.46; H, 7.42; N, 4.80.

Dendron D2(Bn16c) (42): Colorless viscous oil, Yield, 64%. FTIR (KBr, cm^{-1}): 3386 (NH), 1740 (C=O ester), 1712 (C=O urethane), 1516 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.16 (s, 6 H), 1.28 (s, 3 H), 1.43 (s, 36 H), 1.77 (m, 8 H), 2.34 (t, 8 H, J = 7.4 Hz), 3.13 (m, 8 H), 4.20 (m, 12 H, J = 11.1 Hz), 4.85 (br s, 4 H, NH), 5.16 (s, 2 H), 7.36 (m, 5 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 18.18 (CH_3), 18.29 (CH_3), 25.70 (CH_2), 28.98 (CH_3 of BOC), 31.72 (CH_2), 40.32 (CH_2NH), 46.97 (quaternary C), 47.23 (quaternary C), 65.61 (CH_2O), 66.20 (CH_2O), 67.74 (benzyl CH_2O), 79.71 (quaternary C of BOC), 128.94 (phenyl CH), 129.12 (phenyl CH), 129.26 (phenyl CH), 135.86 (quaternary phenyl C),

156.56 (C=O urethane), 172.60 (C=O ester), 172.64 (C=O ester), 173.19 (C=O amino acid). Anal. Cald. for C₅₈H₉₂N₄O₂₂: C, 58.18; H, 7.74; N, 4.68%. Found: C, 58.47; H, 7.45; N, 4.96.

Dendron D2(Bn16e) (43): Off-white solid, Yield, 94%. FTIR (KBr, cm⁻¹): 3394 (NH), 1742 (C=O ester), 1715 (C=O urethane), 1525 (NH). ¹H NMR (CDCl₃, 300 MHz): δ = 1.26 (s, 6 H), 1.30 (s, 3 H), 1.30-1.90 (m, 24 H), 1.43 (s, 36 H), 1.44 (s, 36 H), 3.09 (m, 8 H), 4.00-4.40 (m, 16 H), 4.80 (m, 4 H, ^sNH), 5.17 (s, 2 H, PhCH₂), 5.30 (m, 4 H, ^aNH), 7.36 (m, 5 H). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 17.62 (CH₃), 17.75 (CH₃), 22.56 (CH₂), 28.36 (CH₃ of BOC), 28.47 (CH₃ of BOC), 29.59 (CH₂), 31.90 (CH₂), 40.08 (CH₂NH), 46.41 (quaternary C), 46.56 (quaternary C), 53.38 (CHNH), 65.27 (CH₂O), 65.42 (CH₂O), 65.93 (benzyl CH₂O), 79.82 (quaternary C of BOC), 79.99 (quaternary C of BOC), 128.50 (phenyl CH), 128.59 (phenyl CH), 128.51 (phenyl CH), 135.33 (quaternary phenyl C), 155.56 (C=O urethane), 156.16 (C=O urethane), 171.76, 172.07, 172.27 (C=O). Anal. Cald. for C₈₆H₁₄₄N₈O₃₀: C, 58.35; H, 8.20; N, 6.33%. Found: C, 58.12; H, 8.19; N, 6.18.

2.3.7. General Procedure for Debonylation of Dendrons 29-34 and 41-43

A slurry of the dendron in EtOAc (100 mg/1.5 mL) and 10% Pd/C (10% w/w) was subjected to hydrogenation in a 100 mL flask connected to a glass burette at rt and atmospheric pressure until ceased absorption of the gas. The slurry was then filtered through a silica plug (h = 10 cm, ø = 2 cm) which was washed with fresh EtOAc (25-50 mL). Filtrate and washings were combined and evaporated at reduced pressure to remove the solvent and gave pure debonylated dendrons.

Dendron D1(16a) (35): Glassy solid, Yield, 99%. Mixture of rotamers. FTIR (KBr, cm⁻¹): 3500-2400 (OH), 3397 (NH), 1747 (C=O ester + carboxyl), 1721 (C=O urethane), 1529 (NH). ¹H NMR (CDCl₃, 300 MHz): δ = 1.21 (s, 3 H), 1.37 (s, 18 H), 3.83 (d, 4 H, *J* = 5.1 Hz), 4.25 (m, 4 H, *J* = 11.2 Hz), 5.29 and 6.23 (br s, 2 H, NH), 8.30 (br s, 1 H, OH). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 16.89 (CH₃), 27.29 (CH₃ of BOC), 41.28 (CH₂NH), 44.98 (quaternary C), 64.84 (CH₂O), 79.21 (quaternary C of BOC), 155.06 (C=O urethane), 169.18 (C=O amino acid), 175.08 (C=O carboxyl). Anal. Cald. for C₁₉H₃₂N₂O₁₀: C, 50.89; H, 7.19; N, 6.25%. Found: C, 50.81; H, 7.03; N, 6.15.

Dendron D1(16b) (36): Viscous resin, Yield, 99%. Mixture of rotamers. FTIR (KBr, cm⁻¹): 3500-2400 (OH), 1748 (C=O ester + carboxyl), 1705 (C=O urethane). ¹H NMR (CDCl₃, 300 MHz): δ = 1.28 (s, 3 H), 1.42 and 1.46 (s, 18 H), 2.92 and 2.99 (s, 6 H), 3.93 and 4.00 (s, 4 H), 4.30 (m, 4 H), 9.30 (br s, 1 H, OH). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 16.68 (CH₃), 27.24 and 27.29 (CH₃ of BOC), 34.51 and 34.57 (CH₃N), 45.08 (quaternary C), 49.19 and 49.89 (CH₂N), 64.53 (CH₂O), 79.42 and 79.55 (quaternary C of BOC), 154.44 and 155.11 (C=O urethane), 168.39 (C=O amino acid), 175.47 (C=O carboxyl). Anal. Cald. for C₂₁H₃₆N₂O₁₀: C, 52.93; H, 7.61; N, 5.88%. Found: C, 52.81; H, 7.91; N, 5.83.

Dendron D1(16c) (37): Viscous oil, Yield, 91%. Mixture of rotamers. FTIR (KBr, cm⁻¹): 3500-2400 (OH), 3355 (NH), 1738 (C=O ester + carboxyl), 1711 (C=O urethane), 1521 (NH). ¹H NMR (CDCl₃, 300 MHz): δ = 1.27 (s, 3 H), 1.44 (s, 18 H), 1.81 (m, 4 H), 2.37 (t, 4 H, *J* = 7.3 Hz), 3.14 (m, 4 H), 4.25 (m, 4 H, *J* = 11.1 Hz), 4.92 and 6.03 (br s, 2 H, NH), 9.80 (br s, 1 H, OH). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 17.79 (CH₃), 25.21 (CH₂), 28.40 (CH₃ of BOC), 31.36 (CH₂), 39.79 (CH₂NH), 46.10 (quaternary C), 65.49 (CH₂O), 79.46 (quaternary C of BOC), 156.26 (C=O urethane), 172.75 (C=O carboxyl), 175.84 (C=O amino acid). Anal. Cald. for C₂₃H₄₀N₂O₁₀: C, 54.75; H, 7.99; N, 5.55%. Found: C, 54.78; H, 7.76; N, 5.84.

Dendron D1(16d) (38): Viscous oil, Yield, 99%. FTIR (KBr, cm⁻¹): 3500-2400 (OH), 1740 (C=O ester + carboxyl), 1695 (C=O urethane). ¹H NMR (CDCl₃, 300 MHz): δ = 1.28 (s, 3 H), 1.45 (s, 18 H), 1.80 (m, 4 H), 2.32 (t, 4 H, *J* = 7.3 Hz), 2.84 (s, 6 H), 3.25 (m, 4 H), 4.28 (m, 4 H, *J* = 11.1 Hz). ¹³C NMR (CDCl₃, 75.5 MHz): δ = 17.72 (CH₃), 22.98 (CH₃ of BOC), 23.02 (CH₂), 28.44 (CH₃ of BOC), 30.91 (CH₂), 34.19 (CH₃N), 46.14 (quaternary C), 47.66 (CH₂N), 65.52 (CH₂O), 79.86 (quaternary C), 155.99 (C=O urethane), 172.57 (C=O amino acid), 175.84 (C=O carboxyl). Anal. Cald. for C₂₅H₄₄N₂O₁₀: C, 56.38; H, 8.33; N, 5.26%. Found: C, 56.54; H, 8.14; N, 5.06.

Dendron D1(16e) (39): Glassy solid, Yield, 99%. Mixture of rotamers. FTIR (KBr, cm^{-1}): 3500-2400 (OH), 3406 (NH), 1740 (C=O ester + carboxyl), 1705 (C=O urethane), 1526 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.22 (s, 3 H), 1.10-1.80 (m, 12 H), 1.36 (s, 36 H), 2.97 (m, 4 H), 3.90-4.50 (m, 6 H), 4.60-6.60 (m, 4 H, $^6\text{NH} + ^9\text{NH}$). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 16.63 (CH_3), 21.39 (CH_2), 27.33 (CH_3 of BOC), 27.43 (CH_3 of BOC), 28.54 (CH_2), 31.13 (CH_2), 39.11 and 39.16 (CH_2NH), 44.96 (quaternary C), 52.37 (CHNH), 65.64 (CH_2O), 78.90 (quaternary C), 154.47 and 154.53 (C=O urethane), 155.36 and 155.47 (C=O urethane), 171.26 (C=O amino acid), 171.34 (C=O carboxyl). Anal. Cald. for $\text{C}_{37}\text{H}_{66}\text{N}_4\text{O}_{14}$: C, 56.19; H, 8.41; N, 7.08%. Found: C, 56.18; H, 8.18; N, 7.08.

Dendron D1(16f) (40): Glassy solid, Yield, 99%. Mixture of regioisomers. FTIR (KBr, cm^{-1}): 3500-2400 (OH), 1752 (C=O ester + carboxyl), 1716 (C=O urethane). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.19 (s, 3 H), 1.34 (s, 18 H), 1.52 (s, 18 H), 2.98 (m, 4 H), 4.17-4.37 (m, 4 H), 4.48 (m, 2 H), 5.63 and 5.69 (two d, 2 H, J = 8.3 Hz, *NH*), 7.11 (br s, 2 H), 7.96 (s, 2 H). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 16.71 (CH_3), 26.83 (CH_3 of BOC), 27.28 (CH_3 of BOC), 29.01 (CH_2 His), 45.12 (quaternary C), 53.09 (CHNH), 65.34 (CH_2O), 78.66 (quaternary C of BOC), 84.84 (quaternary C of BOC), 113.88 and 113.99 (CH of imidazole), 135.94 (CH of imidazole), 137.03 and 137.14 (quaternary C of imidazole), 145.67 (C=O urethane), 154.43 (C=O urethane), 170.21 and 170.28 (C=O amino acid), 174.01 (C=O carboxyl). Anal. Cald. for $\text{C}_{37}\text{H}_{56}\text{N}_6\text{O}_{14}$: C, 54.94 ; H, 6.98 ; N, 10.39 %. Found: C, 54.98; H, 6.81; N, 10.44.

Dendron D2(16a) (44): Glassy solid, Yield, 99%. FTIR (KBr, cm^{-1}): 3500-2400 (OH), 3406 (NH), 1747 (C=O ester + carboxyl), 1721 (C=O urethane), 1527 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.26 (s, 6 H), 1.30 (s, 3 H), 1.45 (s, 36 H), 3.89 (d, 8 H, J = 5.3 Hz), 4.29 (m, 12 H), 5.24 (m, 4 H, *NH*). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.81 (CH_3), 17.92 (CH_3), 28.31 (CH_3 of BOC), 42.20 (CH_2NH), 46.34 (quaternary C), 46.49 (quaternary C), 66.08 (CH_2O), 66.67 (CH_2O), 80.47 (quaternary C of BOC), 156.17 (C=O urethane), 169.96 (C=O amino acid), 171.91 (C=O ester). Anal. Cald. for $\text{C}_{43}\text{H}_{70}\text{N}_4\text{O}_{22}$: C, 51.90; H, 7.09; N, 5.63%. Found: C, 52.13; H, 7.04; N, 5.40.

Dendron D2(16c) (45): Viscous oil, Yield, 99%. FTIR (KBr, cm^{-1}): 3500-2400 (OH), 3382 (NH), 1740 (C=O ester + carboxyl), 1711 (C=O urethane), 1523 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.24 (s, 6 H), 1.29 (s, 3 H), 1.44 (s, 36 H), 1.79 (m, 8 H), 2.36 (t, 8 H, J = 7.2 Hz), 3.14 (m, 8 H), 4.23 (m, 12 H), 4.87 (br s, 4 H, *NH*). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.67 (CH_3), 17.81 (CH_3), 25.16 (CH_2), 28.41 (CH_3 of BOC), 31.23 (CH_2), 39.81 (CH_2NH), 46.12 (quaternary C), 46.49 (quaternary C), 65.29 (CH_2O), 79.29 (quaternary C of BOC), 156.21 (C=O urethane), 172.29 (C=O ester), 172.68 (C=O amino acid). Anal. Cald. for $\text{C}_{51}\text{H}_{86}\text{N}_4\text{O}_{22}$: C, 55.32; H, 7.83; N, 5.06%. Found: C, 55.29; H, 7.60; N, 4.80.

Dendron D2(16e) (46): White solid, Yield, 99%. FTIR (KBr, cm^{-1}): 3500-2400 (OH), 3397(NH), 1746 (C=O ester + carboxyl), 1712 (C=O urethane), 1528 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = 1.26 (s, 6 H), 1.30 (s, 3 H), 1.30-1.90 (m, 24 H), 1.44 (s, 72 H), 3.10 (m, 8 H), 4.10-4.40 (m, 16 H), 4.80 (m, 4 H, ^6NH), 5.30 (m, 4 H, ^9NH). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.74 (CH_3), 17.89 (CH_3), 22.57 (CH_2), 28.36 (CH_3 of BOC), 28.47 (CH_3 of BOC), 29.57 (CH_2), 31.85 (CH_2), 40.00 (CH_2NH), 46.31-46.41 (quaternary C), 53.37 (CHNH), 64.00-68.00 (CH_2O), 79.13 (quaternary C of BOC), 80.07 (quaternary C of BOC), 155.74 (C=O urethane), 156.18 (C=O urethane), 171.00-173.00 (C=O ester + amino acid). Anal. Cald. for $\text{C}_{79}\text{H}_{138}\text{N}_8\text{O}_{30}$: C, 56.48; H, 8.28; N, 6.67%. Found: C, 56.68; H, 8.56; N, 6.40.

2.3.8. General Procedure for the Synthesis of G5 and G6 BOC-Dendrimers 47-52 and 53-55

A solution of **10** in CH_2Cl_2 (7-10 mg/mL) was added with the proper *N*-Boc-amino acid dendron (1.1 equiv./OH of **10**), DPTS (0.75 equiv./OH of **10**) and DCC (1.25 equiv./OH of **10**) and kept under magnetic stirring at rt for 24 h. The precipitated DCU was removed by filtration and washed with fresh CH_2Cl_2 (30 mL). Filtrate and washings were combined, concentrated at reduced pressure and added with EtOAc (30 mL) to precipitate DPTS which was removed by filtration. The

solvent was removed by evaporation at reduced pressure to give a creamy solid which was purified by a slow careful column chromatography performed as the following. The dendrimer was dissolved in the minimum quantity of a mixture petroleum ether /EtOAc = 2:3 and passed through a short silica gel column (h = 20 cm, ϕ = 2 cm) using the same mixture above (50 mL) followed by a mixture petroleum ether /EtOAc = 1:4 (30 mL), collecting 1 mL fractions up to disappearance of the last traces of unreacted DCC and *N*-acylureic adduct identified through real-time FTIR spectra. The chromatography was completed with EtOAc 100% (50 mL) and acetone 100% (50 mL) which were pooled. The pooled fractions were concentrated at reduced pressure, taken with acetone and filtered to remove small quantities of DCU and brought to constant weight to give the dendrimer.

Dendrimer G5(16a) (47): Viscous resin, Yield, 85%. FTIR (KBr, cm^{-1}): 3410 (NH), 1745 (C=O ester), 1719 (C=O urethane), 1528 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.17-1.28 (m, 279 H, CH_3 of G1, G2, G3, G4, G5), 1.44 (s, 864 H, CH_3 of BOC), 3.89 (m, 192 H, CH_2NH), 4.27 (m, 378 H, CH_2O of dendrimer), 5.00-5.80 (m, 96 H, NH). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.32-17.88 (CH_3 of G1, G2, G3, G4, G5), 28.34 (CH_3 of BOC), 42.27 (CH_2NH), 46.00-46.72 (quaternary C of G1, G2, G3, G4, G5), 65.60-66.16 (CH_2O of G1, G2, G3, G4, G5), 79.96 and 80.01 (quaternary C of BOC), 156.01 (C=O urethane), 170.06-172.20 (C=O amino acid + C=O ester of G1, G2, G3, G4, G5), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{1142}\text{H}_{1812}\text{N}_{96}\text{O}_{570}$: C, 52.74; H, 7.02; N, 5.17%. Found: C, 52.74; H, 7.05; N, 5.09.

Dendrimer G5(16b) (48): Viscous resin, Yield, 78%. Mixture of rotamers. FTIR (KBr, cm^{-1}): 3432 (NH), 1747 (C=O ester), 1702 (C=O urethane). ^1H NMR (CDCl_3 , 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.16-1.28 (m, 279 H, CH_3 of G1, G2, G3, G4, G5), 1.42 and 1.45 (two br s, 864 H, CH_3 of BOC), 2.90 (br s, 288 H, CH_3N), 3.94 and 3.98 (two s, 192 H, CH_2N), 4.26 (m, 378 H, CH_2O of dendrimer). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.31-17.74 (CH_3 of G1, G2, G3, G4, G5), 28.32 (CH_3 of BOC), 35.43 and 35.58 (CH_3N), 46.45-46.69 (quaternary C of G1, G2, G3, G4, G5), 50.09-50.68 (CH_2N), 65.19-65.70 (CH_2O of G1, G2, G3, G4, G5), 80.22 and 80.35 (quaternary C of BOC), 155.31 and 155.99 (C=O urethane), 169.44 (C=O amino acid), 171.72-172.00 (C=O ester of G1, G2, G3, G4, G5), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{1238}\text{H}_{2004}\text{N}_{96}\text{O}_{570}$: C, 54.36; H, 7.38; N, 4.92%. Found: C, 54.06; H, 6.98; N, 4.81.

Dendrimer G5(16c) (49): Glassy solid, Yield, 92%. FTIR (KBr, cm^{-1}): 3375 (NH), 1740 (C=O ester), 1698 (C=O urethane), 1522 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.17-1.32 (m, 279 H, CH_3 of G1, G2, G3, G4, G5), 1.43 (s, 864 H, CH_3 of BOC), 1.79 (m, 192 H, CH_2), 2.37 (q, 192 H, J = 6.9 Hz, CH_2), 3.13 (m, 192 H, CH_2NH), 4.25 (m, 378 H, CH_2O of dendrimer), 4.88 (m, 96 H, NH). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 17.29-17.82 (CH_3 of G1, G2, G3, G4, G5), 25.15 (CH_2), 28.43 (CH_3 of BOC), 31.13 (CH_2), 39.77 (CH_2NH), 46.50-48.01 (quaternary C of G1, G2, G3, G4, G5), 64.72-65.19 (CH_2O of G1, G2, G3, G4, G5), 79.21 (quaternary C of BOC), 156.13 (C=O urethane), 167.66, 172.56, 172.71 and 172.76 (C=O amino acid + C=O ester of G1, G2, G3, G4, G5), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{1334}\text{H}_{2196}\text{N}_{96}\text{O}_{570}$: C, 55.83; H, 7.71; N, 4.96%. Found: C, 55.54; H, 8.04; N, 5.05.

Dendrimer G5(16e) (50): Glassy solid, Yield, 76%. FTIR (KBr, cm^{-1}): 3380 (NH), 1747 (C=O ester), 1710 (C=O urethane), 1527 (NH). ^1H NMR (CDCl_3 , 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.09-1.90 (m, 855 H, CH_3 of G1, G2, G3, G4, G5) + $\text{CH}_2\text{CH}_2\text{CH}_2$ of Lys), 1.43 (s, 864 H, CH_3 of BOC), 1.44 (s, 864 H, CH_3 of BOC), 3.10 (m, 192 H, CH_2NH), 4.25 (m, 474 H, CH_2O of dendrimer + CHNH of Lys), 4.70-5.50 (m, 192 H, ^uNH + ^eNH). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 14.20-17.90 (CH_3 of G1, G2, G3, G4, G5), 22.57 (CH_2), 28.36 (CH_3 of BOC), 28.47 (CH_3 of BOC), 29.57 (CH_2), 31.84 (CH_2), 40.04 (CH_2NH), 46.42 (quaternary C), 53.37 (CHNH), 65.41-65.60 (CH_2O of G1, G2, G3, G4, G5), 79.02 (quaternary C of BOC), 79.80 (quaternary C of BOC), 155.63 (C=O urethane), 156.17 (C=O urethane), 172.32 (C=O amino acid + C=O ester of G1, G2, G3, G4, G5), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{2006}\text{H}_{3444}\text{N}_{192}\text{O}_{762}$: C, 56.76; H, 8.18; N, 6.34%. Found: C, 56.41; H, 8.48; N, 6.33.

Dendrimer G5(16f) (51): Glassy solid, Yield, 66%. Mixture of regioisomers. FTIR (KBr, cm^{-1}): 3399 (NH), 1755 (C=O ester), 1717 (C=O urethane). ^1H NMR (CDCl_3 , 300 MHz): $\delta = < 1.00$ (CH_3 of core was no detectable), 1.17-1.32 (m, 279 H, CH_3 of G1, G2, G3, G4, G5), 1.41 and 1.42 (two s, 864 H, CH_3 of BOC), 1.59 and 1.60 (two s, 864 H, CH_3 of BOC), 3.02 (m, 192 H, CH_2 of His), 4.05-4.60 (m, 474 H, CH_2O of dendrimer + CHNH of His), 5.67, 5.77 and 5.92 (three d, 96 H, $J = 8.0, 8.1, 7.9$ Hz, NH), 7.19 (broad s, 96 H, CH of imidazole), 7.80-8.00 (m, 96 H, CH of imidazole). ^{13}C NMR (CDCl_3 , 75.5 MHz): $\delta = 17.18$ -17.87 (CH_3 of G1, G2, G3, G4, G5), 27.87 (CH_3 of BOC), 28.32 (CH_3 of BOC), 29.76 and 29.95 (CH_2 His), 46.44 (quaternary C), 53.12 (CHNH), 65.61-66.62 (CH_2O of G1, G2, G3, G4, G5), 79.66 and 79.76 (quaternary C of BOC), 85.56 and 85.97 (quaternary C of BOC), 114.90 and 114.96 (CH of imidazole), 136.92 (CH of imidazole), 138.40 (quaternary C of imidazole), 146.83 (C=O urethane), 155.43 (C=O urethane), 171.12-171.24 (C=O amino acid + C=O ester of G1, G2, G3, G4, G5), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{2006}\text{H}_{2964}\text{N}_{288}\text{O}_{762}$: C, 55.63; H, 6.90; N, 9.31%. Found: C, 50.43; H, 6.58; N, 9.58.

Dendrimer G5[16e(50)16f(46)] (52): Glassy solid, Yield, 69%. FTIR (KBr, cm^{-1}): 3384 (NH), 1744 (C=O ester), 1714 (C=O urethane), 1513 (NH). ^1H NMR (CDCl_3 , 300 MHz): $\delta = < 1.00$ (CH_3 of core was no detectable), 1.10-2.17 [m, 279 H (CH_3 of G1, G2, G3, G4, G5) + 300 H ($\text{CH}_2\text{CH}_2\text{CH}_2$ of Lys)], 1.41, 1.43, 1.44 (three s, 1314 H, CH_3 of BOC Lys and His), 1.60 (s, 414 H, CH_3 of BOC His), 3.09 (m, 192 H, CH_2 His and CH_2NH Lys), 4.00-4.60 (m, 474 H, CH_2O of dendrimer + CHNH Lys + CHNH His), 4.70-5.55 (three m, 100H, NH Lys), 5.60-6.10 (m, 46 H, ^nNH His), 7.19 (s, 46 H, CH of imidazole), 8.00 (m, 46 H, CH of imidazole). ^{13}C NMR (CDCl_3 , 75.5 MHz): $\delta = 17.29$ -17.89 (CH_3 of G1, G2, G3, G4, G5), 22.59 (CH_2 Lys), 27.88, 28.06, 28.34 and 28.48 (CH_3 BOC), 29.96, 32.15 and 33.97 (CH_2 Lys and His), 39.97 (CH_2NH Lys), 46.50 (quaternary C), 53.16-53.42 (CHNH Lys and CHNH His), 64.96-66.54 (CH_2O of G1, G2, G3, G4, G5), 79.81, 79.91, 85.58 and 85.69 (quaternary C of BOC), 114.94 (CH of imidazole), 136.82 (CH of imidazole), 138.29 (quaternary C of imidazole), 146.81 (C=O urethane), 155.34-156.27 (C=O urethane), 171.15-174.06 (C=O), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{2006}\text{H}_{3214}\text{N}_{238}\text{O}_{762}$: C, 56.21; H, 7.56; N, 7.78%. Found: C, 56.10; H, 7.75; N, 8.04.

Dendrimer G6(16a) (53): Viscous resin, Yield, 76%. FTIR (KBr, cm^{-1}): 3376 (NH), 1752 (C=O ester), 1710 (C=O urethane), 1527 (NH). ^1H NMR (CDCl_3 , 300 MHz): $\delta = < 1.00$ (CH_3 of core was no detectable), 1.24-1.29 (m, 567 H, CH_3 of G1, G2, G3, G4, G5, G6), 1.44 (s, 1728 H, CH_3 of BOC), 3.88 (d, 384 H, $J = 5.3$ Hz, CH_2NH Gly), 4.27 (m, 762 H, CH_2O of dendrimer), 5.47 (m, 192 H, NH). ^{13}C NMR (CDCl_3 , 75.5 MHz): $\delta = 17.73$ -18.04 (CH_3 of G1, G2, G3, G4, G5, G6), 28.31 (CH_3 of BOC), 42.29 (CH_2NH), 45.90-50.75 (quaternary C of G1, G2, G3, G4, G5, G6), 65.89, 65.96 and 67.43-67.65 (CH_2O of G1, G2, G3, G4, G5, G6), 80.05 and 80.12 (quaternary C of BOC), 155.93 (C=O urethane), 169.73-170.46 (C=O amino acid + C=O ester of G1, G2, G3, G4, G5, G6), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{2294}\text{H}_{3636}\text{N}_{192}\text{O}_{1146}$: C, 52.74; H, 7.01; N, 5.15%. Found: C, 53.01; H, 6.84; N, 5.16.

Dendrimer G6(16c) (54): Viscous resin, Yield, 80%. FTIR (KBr, cm^{-1}): 3448 (NH), 1740 (C=O ester), 1711 and 1695 (C=O urethane), 1526 (NH). ^1H NMR (CDCl_3 , 300 MHz): $\delta = < 1.00$ (CH_3 of core was no detectable), 1.24-1.35 (m, 567 H, CH_3 of G1, G2, G3, G4, G5, G6), 1.43 (s, 1728 H, CH_3 of BOC), 1.78 (m, 384 H, CH_2), 2.36 (t, 384 H, $J = 7.2$ Hz, CH_2), 3.13 (m, 384 H, CH_2NH), 4.24 (m, 762 H, CH_2O of dendrimer), 4.96 (m, 192 H, NH). ^{13}C NMR (CDCl_3 , 75.5 MHz): $\delta = 17.12$ -17.96 (CH_3 of G1, G2, G3, G4, G5, G6), 25.12 and 25.20 (CH_2), 28.41 (CH_3 of BOC), 31.10 (CH_2), 39.77 (CH_2NH), 46.03-50.56 (quaternary C of G1, G2, G3, G4, G5, G6), 64.70-65.52 (CH_2O of G1, G2, G3, G4, G5, G6), 79.22 and 79.37 (quaternary C of BOC), 156.10 and 156.17 (C=O urethane), 167.65, 172.57, 172.75 and 173.43 (C=O amino acid + C=O ester), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Cald. for $\text{C}_{2678}\text{H}_{4404}\text{N}_{192}\text{O}_{1146}$: C, 58.43; H, 7.63; N, 4.34%. Found: C, 58.06; H, 7.22; N, 3.98.

Dendrimer G6(16e) (55): Glassy solid, Yield, 89%. FTIR (KBr, cm^{-1}): 3380 (NH), 1747 (C=O ester), 1710 (C=O urethane), 1527 (NH). ^1H NMR (CDCl_3 , 300 MHz): $\delta = < 1.00$ (CH_3 of core was

no detectable), 1.10-2.17 (m, 1719 H, $\text{CH}_2\text{CH}_2\text{CH}_2$ Lys + CH_3 of G1, G2, G3, G4, G5, G6), 1.43 (s, 3456 H, CH_3 of BOC), 3.09 (m, 384 H, CH_2NH), 4.00-4.50 (m, 954 H, CHNH + CH_2O of dendrimer), 4.85 and 5.37 (two br s, 384 H, NH). ^{13}C NMR (CDCl_3 , 75.5 MHz): δ = 14.20-17.90 (CH_3 of G1, G2, G3, G4, G5, G6), 22.57 (CH_2), 28.36 (CH_3 of BOC), 28.47 (CH_3 of BOC), 29.57 (CH_2), 31.85 (CH_2), 40.04 (CH_2NH), 46.42 (quaternary C), 53.37 (CHNH), 65.41-65.60 (CH_2O of G1, G2, G3, G4, G5, G6), 79.02 (quaternary C of BOC), 79.80 (quaternary C of BOC), 155.63 ($\text{C}=\text{O}$ urethane), 156.18 ($\text{C}=\text{O}$ urethane), 172.33 ($\text{C}=\text{O}$), CH_3 , quaternary C and CH_2O of core were no detectable. Anal. Calcd. for $\text{C}_{4022}\text{H}_{6900}\text{N}_{384}\text{O}_{1530}$: C, 56.75; H, 8.17; N, 6.32%. Found: C, 57.06; H, 8.00; N, 5.96.

2.3.9. Removal of BOC Groups from Dendrimers 47-55

The removal of BOC groups was carried out as described above for 17-22.

Dendrimer G5(15aHCl) (56): Hygroscopic glassy solid, Yield, 97%. FTIR (KBr, cm^{-1}): 3424 (NH_3^+), 1744 ($\text{C}=\text{O}$), 1624 (NH). ^1H NMR ($\text{DMSO-}d_6$, 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.02-1.34 (m, 279 H, CH_3 of G1, G2, G3, G4, G5), 3.81 (m, 192 H, CH_2NH_3^+), 4.10-4.40 (m, 378 H, CH_2O of dendrimer), 8.56 and 8.69 (br s, 288 H, NH_3^+). ^{13}C NMR ($\text{DMSO-}d_6$, 75.5 MHz): δ = 17.15-17.79 (CH_3 of G1, G2, G3, G4, G5), 41.00 (CH_2NH_3^+), 45.44-46.37 (quaternary C of G1, G2, G3, G4, G5), 65.53-66.21 (CH_2O of G1, G2, G3, G4, G5), 167.27 ($\text{C}=\text{O}$ amino acid), 167.46 ($\text{C}=\text{O}$ ester of G5), 171.65-173.81 ($\text{C}=\text{O}$ ester of G1, G2, G3, G4), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G5(15bHCl) (57): Hygroscopic glassy solid, Yield, 96%. FTIR (KBr, cm^{-1}): 3433 (NH_3^+), 1748 ($\text{C}=\text{O}$). ^1H NMR ($\text{DMSO-}d_6$, 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.16-1.25 (m, 279 H, CH_3 of G1, G2, G3, G4, G5), 2.58 (m, 288 H, CH_3NH_2^+), 3.99 (m, 192 H, CH_2NH_2^+), 4.10-4.50 (m, 378 H, CH_2O of dendrimer), 9.59 (br s, 192 H, NH_2^+). ^{13}C NMR ($\text{DMSO-}d_6$, 75.5 MHz): δ = 16.37-17.11 (CH_3 of G1, G2, G3, G4, G5), 32.44 (CH_3NH_2^+), 46.17-47.91 (quaternary C of G1, G2, G3, G4), 48.37 (CH_2NH_2^+), 50.11 (quaternary C of G5), 65.56-66.37 (CH_2O of G1, G2, G3, G4, G5), 166.37 ($\text{C}=\text{O}$), 167.72 ($\text{C}=\text{O}$ amino acid), 172.67-174.13 ($\text{C}=\text{O}$), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G5(15cHCl) (58): Hygroscopic glassy solid, Yield, 83%. FTIR (KBr, cm^{-1}): 3432 (NH_3^+), 1736 ($\text{C}=\text{O}$), 1629 (NH). ^1H NMR (CD_3OD , 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.16-1.32 (m, 279 H, CH_3 of G1, G2, G3, G4, G5), 1.99 (m, 192 H, CH_2), 2.55 (m, 192 H, CH_2), 3.03 (m, 192 H, CH_2NH_3^+), 4.20-4.40 (m, 378 H, CH_2O of dendrimer). ^{13}C NMR (CD_3OD , 75.5 MHz): δ = 18.00-18.34 (CH_3 of G1, G2, G3, G4, G5), 23.80 (CH_2), 31.69 (CH_2), 40.17 (CH_2NH_3^+), quaternary C of dendrimer hidden under solvent, 65.24-66.71 (CH_2O of G1, G2, G3, G4, G5), 173.75-173.90 ($\text{C}=\text{O}$ ester of G1, G2, G3, G4, G5), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G5(15e2HCl) (59): Hygroscopic glassy solid, Yield, 97%. FTIR (KBr, cm^{-1}): 3431 (NH_3^+), 1744 ($\text{C}=\text{O}$), 1635 (NH). ^1H NMR ($\text{DMSO-}d_6$, 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.03-1.99 (m, 855 H, CH_3 of G1, G2, G3, G4, G5 + $\text{CH}_2\text{CH}_2\text{CH}_2$ of Lys), 2.76 (m, 192 H, CH_2NH_3^+ of Lys), 3.99 (m, 96 H, CHNH_3^+ of Lys), 4.10-4.50 (m, 378 H, CH_2O of dendrimer), 8.20 (br s, 288 H, NH_3^+), 8.82 (br s, 288 H, NH_3^+). ^{13}C NMR ($\text{DMSO-}d_6$, 75.5 MHz): δ = 19.33 (CH_3), 23.14 (CH_2), 28.01 (CH_2), 31.01 (CH_2), 40.02 (CH_2NH_3^+), 47.70 (quaternary C), 53.55 (CHNH_3^+), 67.65-67.82 (CH_2O and of G1, G2, G3, G4), 170.68-173.33 ($\text{C}=\text{O}$ of amino acid + ester of G1, G2, G3, G4), CH_3 , quaternary C and CH_2O of core were no detectable.

Dendrimer G5(15f2HCl) (60): Hygroscopic glassy solid, Yield, 99%. FTIR (KBr, cm^{-1}): 3420 (NH_3^+), 1751 ($\text{C}=\text{O}$), 1625 (NH). ^1H NMR ($\text{DMSO-}d_6$, 300 MHz): δ = < 1.00 (CH_3 of core was no detectable), 1.15-1.35 (m, 279 H, CH_3 of G1, G2, G3, G4, G5), 3.34 (m, 192 H, CH_2), 4.04-4.31 (m, 378 H, CH_2O of dendrimer), 4.55 (m, 96 H, CHNH_3^+), 7.55 (br s, 96 H, CH of imidazole), 9.10 and 9.14 (m, 96 H, CH of imidazole), 8.00-10.00 (br s, 288 H, NH_3^+), 13.00-16.00 (br s, 192 H, $^{\text{im}}\text{NH} + ^{\text{im}}\text{NH}^+$). ^{13}C NMR ($\text{DMSO-}d_6$, 75.5 MHz): δ = 17.47-18.04 (CH_3 of G1, G2, G3, G4, G5), 25.54

(CH₂), 45.45-46.02 (quaternary C of G1, G2, G3, G4, G5), 51.48 (CHNH₃⁺), 66.61-66.80 (CH₂O of G1, G2, G3, G4, G5), 118.49 (CH of imidazole), 127.21 (quaternary C of imidazole), 134.56 (CH of imidazole), 167.97 (C=O amino acid), 168.08 (C=O ester of G5), 168.13-173.88 (C=O ester of G1, G2, G3, G4), CH₃, quaternary C and CH₂O of *core* were no detectable.

Dendrimer G5[15e2HCl(50)15f2HCl(46)] (61): Hygroscopic glassy solid, Yield, 97%. FTIR (KBr, cm⁻¹): 3424 (NH₃⁺), 1747 (C=O ester), 1623 (NH). ¹H NMR (DMSO-*d*₆, 300 MHz): δ = < 1.00 (CH₃ of *core* was no detectable), 1.00-1.99 [m, 279 H (CH₃ of G1, G2, G3, G4, G5) + 300 H (CH₂CH₂CH₂ Lys)], 2.76 (m, 100 H, CH₂NH₃⁺ Lys), 3.34 (m, 92 H, CH₂ His), 3.99 (m, 50 H, CHNH₃⁺ Lys), 4.10-4.41 (m, 378 H, CH₂O of dendrimer), 4.56 (m, 46 H, CHNH₃⁺ His), 7.56 (br s, 46 H, CH of imidazole), 8.22 (br s, 150 H, ^αNH₃⁺ Lys), 8.81 (br s, 288 H, ^αNH₃⁺ His), 9.11 (m, 46 H, CH of imidazole), 12.00-16.00 (br s, 92 H, ^{im}NH + ^{im}NH⁺). ¹³C NMR (DMSO-*d*₆, 75.5 MHz): δ = 17.39-18.11 (CH₃ of G1, G2, G3, G4, G5), 21.78 (CH₂), 25.58 (CH₂), 26.71 (CH₂), 29.74 (CH₂), 38.67 (CH₂NH₃⁺ Lys), 45.51-46.77 (quaternary C of G1, G2, G3, G4, G5), 51.53 (CHNH₃⁺ Lys or CHNH₃⁺ His), 52.19 (CHNH₃⁺ Lys or CHNH₃⁺ His), 65.53-66.86 (CH₂O of G1, G2, G3, G4, G5), 118.54 (CH of imidazole), 127.21 and 127.27 (quaternary C of imidazole), 134.61 (CH of imidazole), 168.22 (C=O amino acid), 169.48 (C=O amino acid), 169.52 (C=O ester of G5), 172.09-172.48 (C=O ester of G1, G2, G3, G4), CH₃, quaternary C and CH₂O of *core* were no detectable.

Dendrimer G6(15aHCl) (62): Hygroscopic glassy solid, Yield, 87%. FTIR (KBr, cm⁻¹): 3422 (NH₃⁺), 1747 (C=O), 1625 (NH). ¹H NMR (DMSO-*d*₆, 300 MHz): δ = < 1.00 (CH₃ of *core* was no detectable), 1.05-1.23 (m, 567 H, CH₃ of G1, G2, G3, G4, G5, G6), 3.81 (m, 384 H, CH₂NH₃⁺), 4.10-4.40 (m, 762 H, CH₂O of dendrimer), 8.64 (m, 576 H, NH₃⁺). ¹³C NMR (DMSO-*d*₆, 75.5 MHz): δ = 17.31-17.69 (CH₃ of G1, G2, G3, G4, G5, G6), 40.96 and 41.16 (CH₂NH₃⁺), 46.29-48.50 (quaternary C of G1, G2, G3, G4, G5, G6), 65.51-66.42 (CH₂O of G1, G2, G3, G4, G5, G6), 167.48 (C=O amino acid), 167.54 (C=O ester of G6), 171.85 (C=O ester of G5), 174.15 (C=O ester), CH₃, quaternary C and CH₂O of *core* were no detectable.

Dendrimer G6(15cHCl) (63): Hygroscopic glassy solid, Yield, 81%. FTIR (KBr, cm⁻¹): 3413 (NH₃⁺), 1733 (C=O), 1630 (NH). ¹H NMR (CD₃OD, 300 MHz): δ = < 1.00 (CH₃ of *core* was no detectable), 1.16-1.32 (m, 567 H, CH₃ of G1, G2, G3, G4, G5, G6), 1.99 (m, 384 H, CH₂), 2.55 (m, 384 H, CH₂), 3.03 (m, 384 H, CH₂NH₃⁺), 4.20-4.40 (m, 762 H, CH₂O of dendrimer). ¹³C NMR (CD₃OD, 75.5 MHz): δ = 18.00-18.34 (CH₃ of G1, G2, G3, G4, G5, G6), 23.80 (CH₂), 31.69 (CH₂), 40.17 (CH₂NH₃⁺), quaternary C of dendrimer hidden under solvent, 65.24-66.71 (CH₂O of G1, G2, G3, G4, G5), 173.75-173.90 (C=O ester of G1, G2, G3, G4, G5), CH₃, quaternary C and CH₂O of *core* were no detectable.

Dendrimer G6(15e2HCl) (64): Very hygroscopic white solid, Yield, 88%. FTIR (KBr, cm⁻¹): 3434 (NH₃⁺), 1747 (C=O ester). ¹H NMR (DMSO-*d*₆, 300 MHz): δ = < 1.00 (CH₃ of *core* was no detectable), 1.03-1.99 (m, 1719 H, CH₂CH₂CH₂ Lys + CH₃ of G1, G2, G3, G4, G5, G6), 2.76 (m, 384 H, CH₂NH₃⁺), 3.99 (m, 192 H, CHNH₃⁺), 4.33 (m, 762 H, CH₂O of dendrimer), 8.20 (br s, 576 H, NH₃⁺), 8.82 (br s, 576 H, NH₃⁺). ¹³C NMR (DMSO-*d*₆, 75.5 MHz): δ = 19.33 (CH₃), 23.14 (CH₂), 28.01 (CH₂), 31.02 (CH₂), 40.02 (CH₂NH₃⁺), 47.70 (quaternary C), 53.55 (CHNH₃⁺), 67.74-67.82 (CH₂O of G1, G2, G3, G4, G5, G6), 170.68-173.33 (C=O), CH₃, quaternary C and CH₂O of *core* were no detectable.

2.4. Biological Assay

2.4.1. pDNA and siRNA Dendrimers Adhesion Assay

pDNA or siRNA (2 μL of a 20 μM buffer solution consisting of 100 mM potassium acetate (K⁺AcO⁻), 30 mM 4,2-hydroxyethyl-1-piperazinyl ethanesulfonate (HEPES-KOH) and 2 mM magnesium acetate (Mg⁺AcO⁻) at pH 7.4) was treated with dendrimer hydrochloride solutions (200 μg/ml in H₂O mQ) to obtain N/P ratios of 20, 40, 60, 80 (pDNA) and 2.5, 5.0, 10 (siRNA). After 30 minutes, necessary to allow complexation, the samples are diluted with sterile and bidistilled water to

15 μL of total volume and loaded into agarose gel wells (4% solution in TBE buffer in the presence of ethidium bromide). The electrophoretic run is carried out at room temperature for 30 minutes at 90V.

2.4.2. Cytotoxicity Assay

KMS-12-BM, LP1, CHO or HeLa cells were increased in DMEM enriched with FBS (10%), non-essential amino acids (1%) and antibiotics (1%, penicillin and streptomycin) and maintained in atmosphere containing 5% CO_2 at 37 °C.

The cells were sown at the density of 2×10^4 cells per well in a 24-well plate and in 4-wells slides in 500 μL of medium and incubated at 37 °C for 72 h. For each dendrimer were prepared complex with N/P = 20 ratio at increasing concentrations of *pDNA* and N/P = 10 ratio at increasing concentrations of *siRNA* (250 ng, 500 ng, 1 μg) diluted to 500 μL both with complete medium and with serum free medium. The complexes were added to the cells in replicates of two and incubated at 37 °C.

For serum free samples, after 4 hours the medium was replaced by the full one and incubated cells for 24 or 48 h at 37 °C. Then the cells were detached from the plate by trypsin treatment and incubated with annexine and propidium iodide (Hela cells/*siRNA*) or 7-amino-actinomycin D (7-AAD) (other cells/*pDNA*) and cytotoxicity was evaluated by cytofluorometry.

2.4.3. Transfection Experiments with Dendrimers

HeLa cells were increased in DMEM enriched with FBS (10%), non-essential amino acids (1%) and antibiotics (1%, penicillin and streptomycin) and maintained in atmosphere containing 5% CO_2 at 37 °C.

The cells were sown at the density of 2×10^4 cells per well in a 24-well plate and in 4-wells slides in 500 μL of medium and incubated at 37 °C for 72 h. For each dendrimer were prepared complexes in ratio N/P = 10 at increasing concentrations of *siRNA* (250 ng, 500 ng, 1 μg) diluted to 500 μL both with complete medium and with serum free medium. The complexes were added to the cells in replicates of two and incubated at 37 °C. For serum free samples, after 4 hours the medium was replaced by the full one and cells incubated for 24 or 48 h at 37 °C. The transfection was then evaluated by microscopic and cytofluorometric analysis. Microscopically analyzed cells were fixed with 4% formaldehyde, permeabilized with 0.1% triton and incubated with DAPI. Cytofluorometric analyzed cells were detached from the plate by trypsin treatment and incubated with annexine and propidium iodide.

3. Results and Discussion

3.1. Chemistry

The goal of this work was setting up synthetic protocols for the obtainment of dendrimeric vectors for gene delivery endowed with hydrolyzable inner matrix of the ester type and basic outer periphery whose buffer capacity could be tuned by changing the type of basic residues. The ester matrix should have ensured low cytotoxicity levels of the prepared materials while a variety of basic residues with different pK_a should have helped in selecting the polyplex more suitable to bind genetic material, to enter the cells and to escape from the endosomal acidic compartments thus resisting lysosomal degradation.

As monomer of AB_2 type to build the hydrolyzable dendrimeric architecture we chose the 2,2-bis(hydroxymethyl)propanoic acid (*bis*-HMPA), a known building block used to prepare an ample variety of dendrimeric scaffolds³⁷ and we adopted the synthetic strategy known as double-stage convergent approach^{37,40} as previously described in another our paper accepted for publication on Macromolecular Research and currently in print. This strategy required the synthesis of a fourth generation acetonide-protected dendron starting from *bis*-HMPA, its reaction with a *tris*-hydroxyl core molecule and final removal of the acetonide protection to obtain the fourth generation dendrimer having 48 peripheral hydroxyl groups (**G4OH**, **10**).

The dendron growth was easily monitored through FTIR and NMR spectroscopy that allowed us to verify the absence of defective intermediates dendrons, minimizing the risk of structural imperfections of the final dendrimer. Careful column chromatography was a necessary step for purifying the completely protected forms of dendrons and dendrimer from secondary products such as anhydrides and *N*-acylureic derivatives caused by side reactions of the DCC-activated acid reagents.

Figure 1 shows the fourth generation dendron **7**, its precursors, and *core* molecule **8** used to prepare the polyester scaffold. Dendrons (D) and dendrimers (G) have been associated with both an identification number and a code made by a letter (D or G), indicating the molecule type, followed by a number to specify the generation and finally, within parenthesis, an identifier of the protected functional part (A for acetonide and Bn for benzyl).

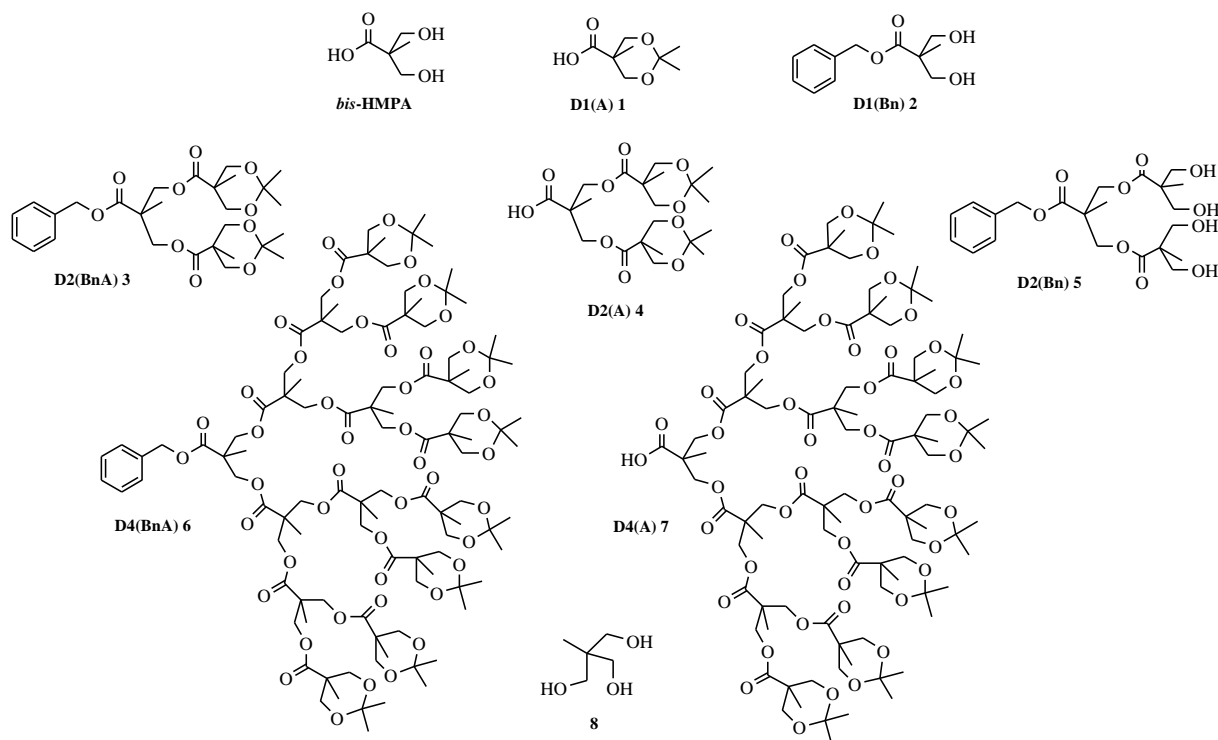
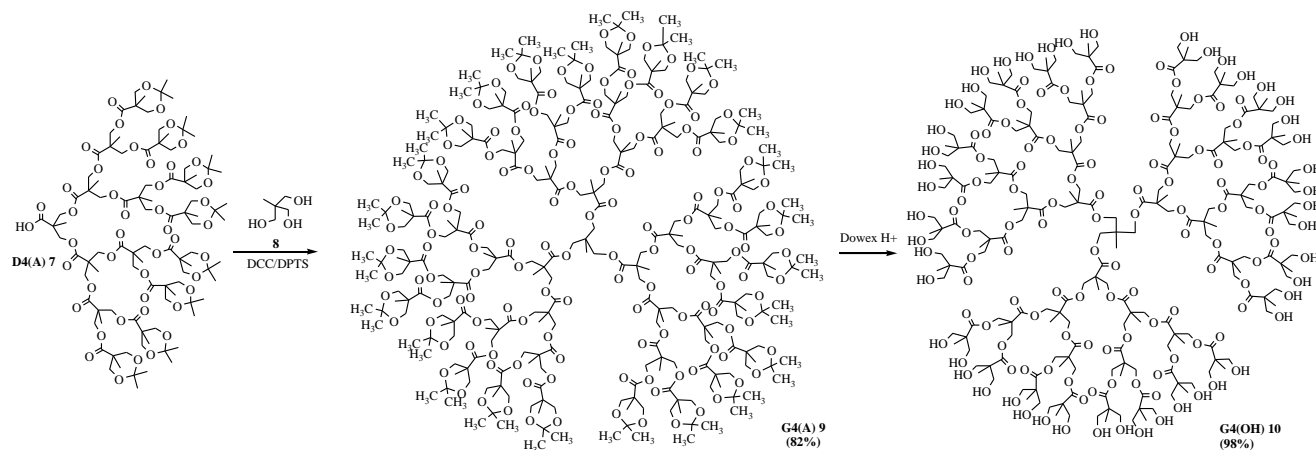


Figure 1. Structures of dendrons **1-7** and *core* molecule **8** used to prepare dendrimer **G4(OH)** (**10**)

Dendron **7**⁴⁰ was used to synthesize the new acetonide dendrimer **G4(A)** (**9**) by reaction with 2,2-bis(hydroxymethyl)propan-3-ol (**8**) as the *core* molecule (¹H and ¹³C NMR spectra of **9** are available in Supporting Information, Figure S1, 2, pages 6, 7), the successive removal of the acetal protection with acid resin Dowex 50 WX2-200 afforded **G4(OH)** (**10**) having 48 hydroxyl groups at the periphery susceptible of further functionalization (Scheme 1). We remember that attempts to bind **7** to other *core* molecules such as 2,2-bis(hydroxymethyl)-1,3-propanediol (pentaerythritol, **11**) to obtain a four-dendron dendrimer failed for steric buttressing, presumably.

The reaction of the carboxylic function of **7** with DCC brought only to the isolation of the side products which in this case were significant quantities of anhydride **12** (60%) and *N*-acylureic adduct **13** (20%) both derived from DCC-activated **7**. The reaction of **7** with 2-[[3-hydroxy-2,2-bis(hydroxymethyl)propoxy]methyl]-2-(hydroxymethyl)propane-1,3-diol (dipentaerythritol, **14**) having less buttressed OH groups was successful but the low yields recorded (16%) and the lack of reactivity of the hydroxyl-free dendrimer towards esterification in preliminary test reactions made us not to consider the molecule further.



Scheme 1. Synthesis of dendrimer **G4(OH) (10)**

Figure 2 shows *core* molecules **11** and **14**, anhydride **12** and adduct **13**.

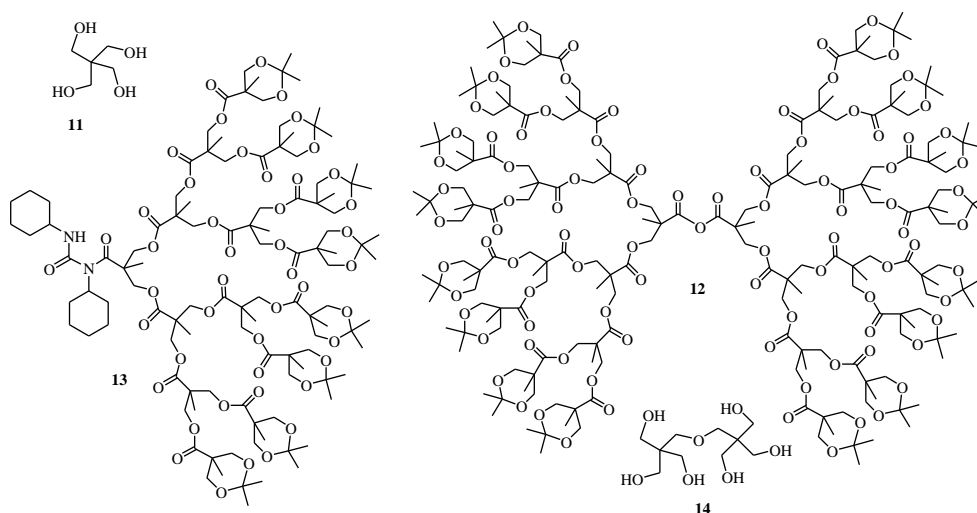


Figure 2. Structures of *core* molecules **11** and **14**, anhydride **12** and adducts **13**

Dendrimer **10** was soluble in DMF, DMSO, H₂O and insoluble in organic solvents. The ¹H NMR spectrum of **10** was very diagnostic of the dendrimeric structure (Supporting Information, Figure S3, page 8) and was characterized by the broad singlet of 48 OH at 4.44 ppm, by the multiplets of CH₂ of first, second, third generation and of the *core* in the interval 3.92-4.18 ppm and by the multiplet corresponding to 96 protons of methylene groups in CH₂OH at 3.43 ppm.

The signals of decreasing intensities corresponding to the CH₃ of *bis*-HMPA in the fourth generation and to the CH₃ of the *core* were also visible at 1.01, 1.16, 1.18, 1.21 and 0.80 ppm.

The structure of **10** was further confirmed by ¹³C NMR spectrum (Supporting Information, Figure S4, page 9) where the signals relative to C=O of esters of the fourth, third, second and first generation were observed at 175.02, 172.80, 172.44, and 172.38 ppm respectively.

The signals of inner CH₂O residues of the three generations were found between 65.39 and 66.30 ppm while those of peripheral CH₂OH at 64.64 ppm.

The signals for the quaternary carbons were visible at 51.22 ppm (fourth generation) and between 47.10 and 47.30 ppm (first, second and third generation). Methyl groups of all the generations and of the *core* were observed at 17.68, 18.12, 17.88, 17.85 and 17.37 ppm.

To functionalize the surface of **10** with residues endowed with different basic properties and structures we chose amino acids **15a-f**.

Their structural features are: different methylene sequence between amino and carbonyl groups, presence or absence of a methyl group on nitrogen atoms, presence or absence of a second protonable function atom in side chain.

Figure 3 shows the structures of the free amino acids **15a-f** and protected amino acids **16a-f**.

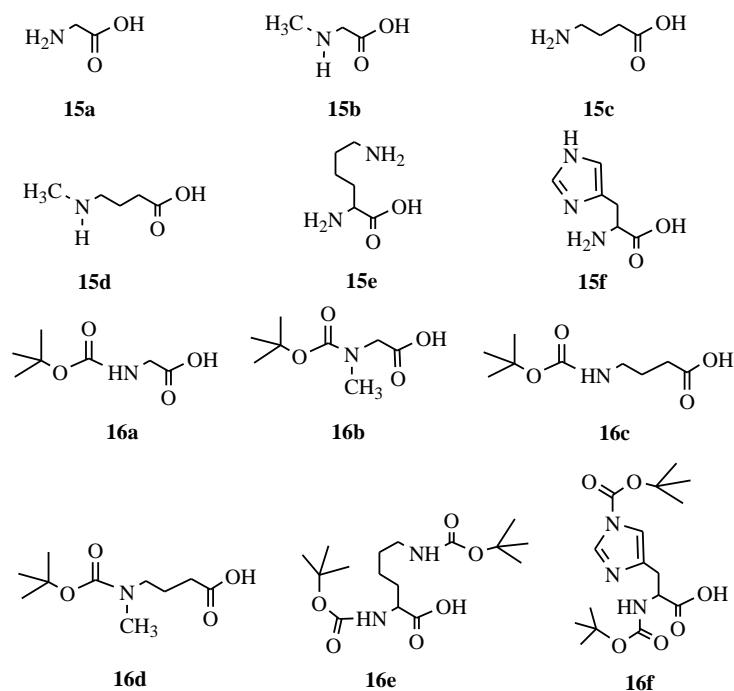


Figure 3. Free and protected amino acids used to functionalize dendrimer **10**

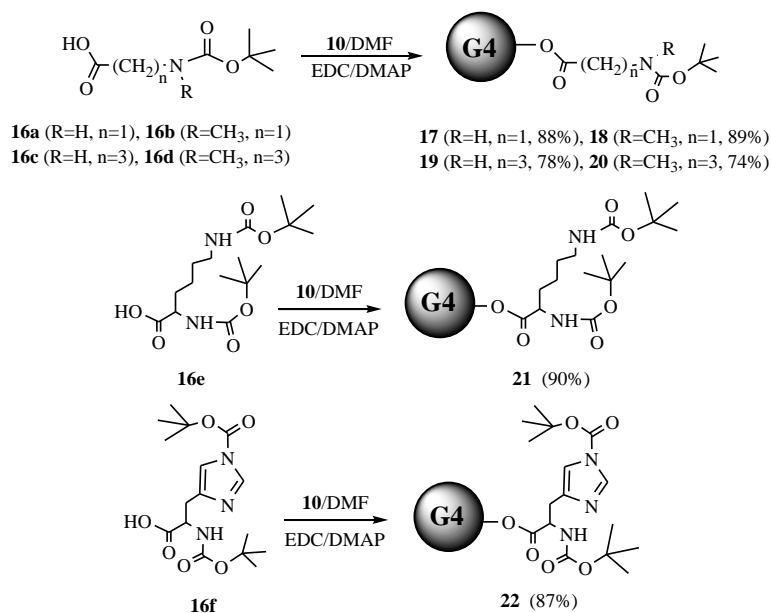
The synthetic protocol to obtain dendrimers from amino acids **15a-f** was based on the following three steps: BOC-protection of amino acids, esterification of the dendrimer **10** with BOC-protected amino acids through carbodiimide activation and final removal of the BOC-group to afford dendrimer in the form of hydrochlorides.

Amino acids **15a-f** were converted into their *N*-BOC-derivatives **16a-f** (Figure 3) following standard procedures. Their structure were confirmed by ^1H and ^{13}C NMR spectroscopy. Chemical and analytical data are available in Supporting Information (pages 3-5).

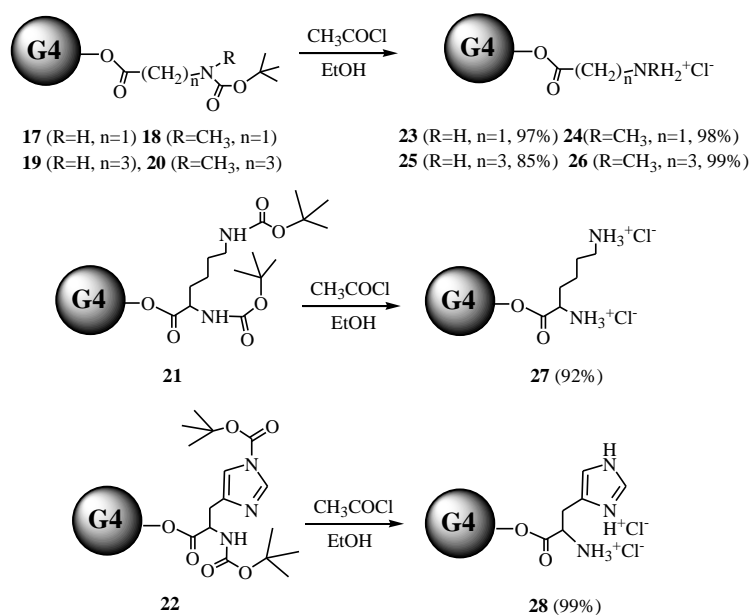
It is interesting to note the occurrence of rotamers due to restricted rotation of the urethane bond⁴⁵ in the case of **16a**, **16b**, **16c** and **16e**, particularly evident in **16b** (Supporting Information, Figures S5 and S6, pages 10, 11).

In addition, the NMR analysis allowed us to ascertain the presence of regioisomers where BOC group was found both on τ -nitrogen and on π -nitrogen of **16f**.⁴⁶

The grafting of **16a-f** onto **10** was carried out through esterification of the hydroxyl groups of **10** promoted by *N*-ethyl-*N*-(3-*N,N*-dimethylamino)propyl carbodiimide (EDC) in the presence of 4-(dimethylamino)pyridine (DMAP) (Scheme 2). The use of EDC required only a final extractive work-up after hydrolysis to obtain analytically pure products being the ureic by-product and acylureic adduct derived from EDC easily removable by acid washings.


Scheme 2. Synthesis of G4 BOC-protected dendrimers **17-22**

Dendrimers **17-22** were glassy solids, oils, or viscous resins soluble in almost all organic solvents except for pentane, hexane, cyclohexane, petroleum ether and diethyl ether. Their FTIR spectra were characterized by two strong bands near 1700 cm⁻¹ and 1750 cm⁻¹ indicating the presence of *tert*-butoxycarbonyl groups and the ester groups respectively. The successive removal of BOC groups was performed with anhydrous HCl produced by reacting acetyl chloride with ethanol, conditions that proved compatible with the ester matrix of the dendrimers (Scheme 3).


Scheme 3. Synthesis of G4 dendrimers **23-28**

^1H NMR spectra and signals integration were very helpful in following the chemical modifications performed on dendrimers.

As an example the Figure S7 in Supporting Information (page 12) shows the typical changes of the ^1H NMR spectra when **10** was transformed into the BOC-protected dendrimer **17**, containing glycine residues and then into hydrochloride **23**.

The broad signal of the 48 hydroxyl groups at 4.24 ppm and the complex signal of the 96 protons of methylene of the CH_2OH group at 3.48 ppm, typical of **10**, disappeared in the spectrum of **17** while a new signal at 3.87 ppm as a doublet corresponding to 96 protons of the CH_2NH group of glycine and a new broad signal at 5.46 ppm corresponding to 48 protons of the NHBOC groups were quite evident.

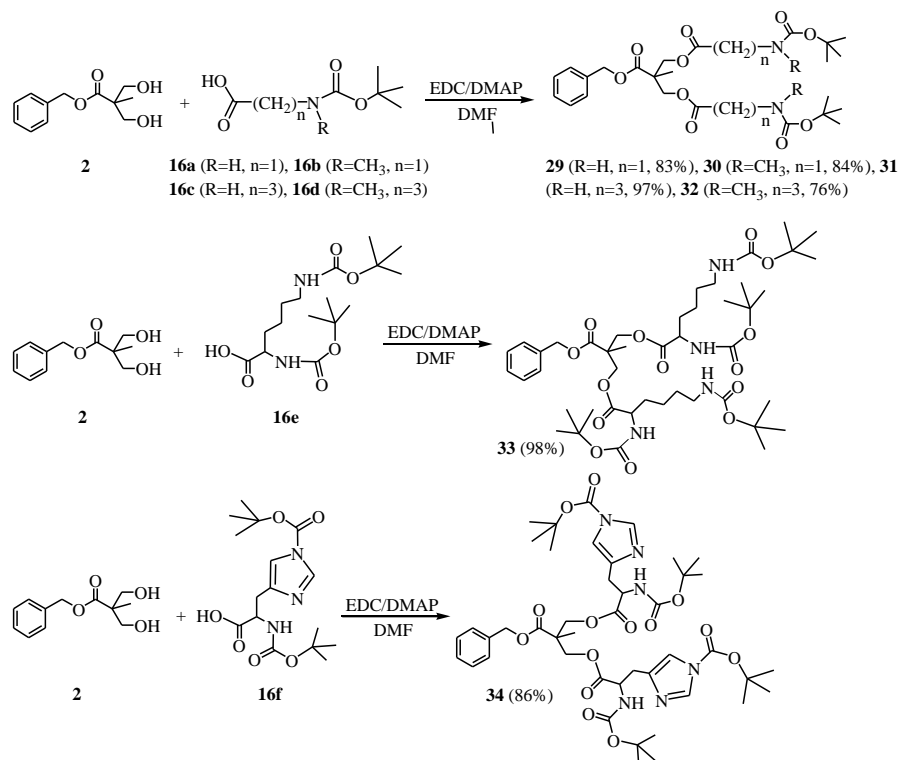
This last signal disappeared in the spectrum of dendrimer **23** after the removal of the protective groups and the signal of NH_3^+ groups corresponding to 144 protons popped up at 8.69 ppm. This pattern of changes was a common feature to all the series of dendrimers.

Since it is quite difficult to establish *a priori* which are the optimal dimensions of a vector to efficaciously bind genetic material, compact, protect and bring it to the nucleus, we included in our research the achievement of higher generations dendrimers.

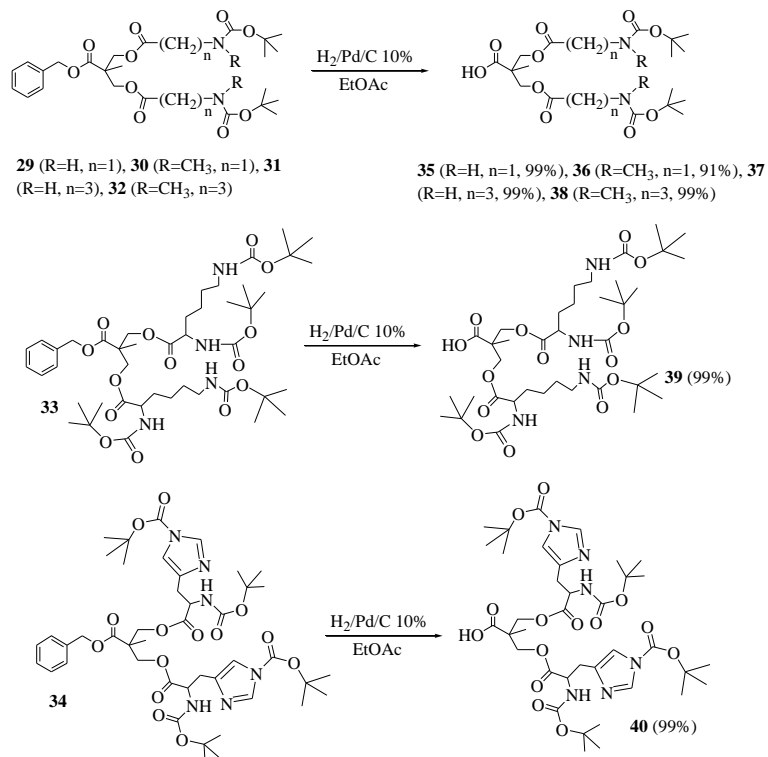
To this end we considered the fourth generation dendrimer **10** as a “hypercore”² to which graft dendrons of the D1-type (**2**) and D2-type (**5**) (Figure 1) functionalized with amino acids.

The reaction of dendron **2** with *N*-BOC-amino acids **16a-f** afforded dendrons **29-34** (Scheme 4) which after debenzoylation with H_2 , Pd/C 10% gave dendrons **35-40** as carboxylic acids (Scheme 5) to use in esterifications reaction of **10**.

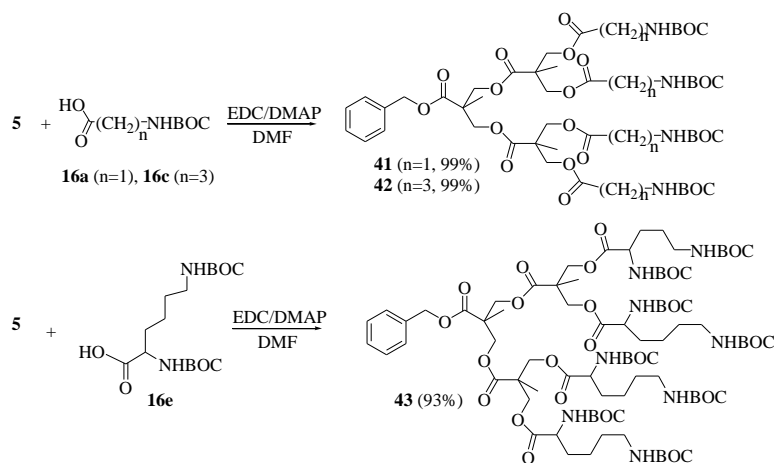
The reactions of second generation dendron **5**, was instead limited to explore *N*-BOC-amino acids **16a, c** and **16e** achieving compounds **41-43** (Scheme 6) which after removal of benzyl group provided dendrons **44-46** (Scheme 7).

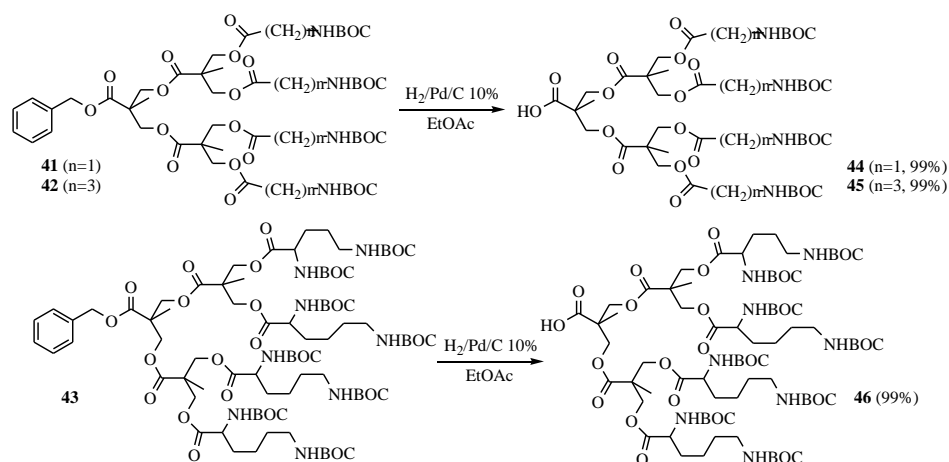


Scheme 4. Synthesis of BOC-protected dendrons **29-34** from **2**


Scheme 5. Synthesis of debenzylated dendrons **35-40**

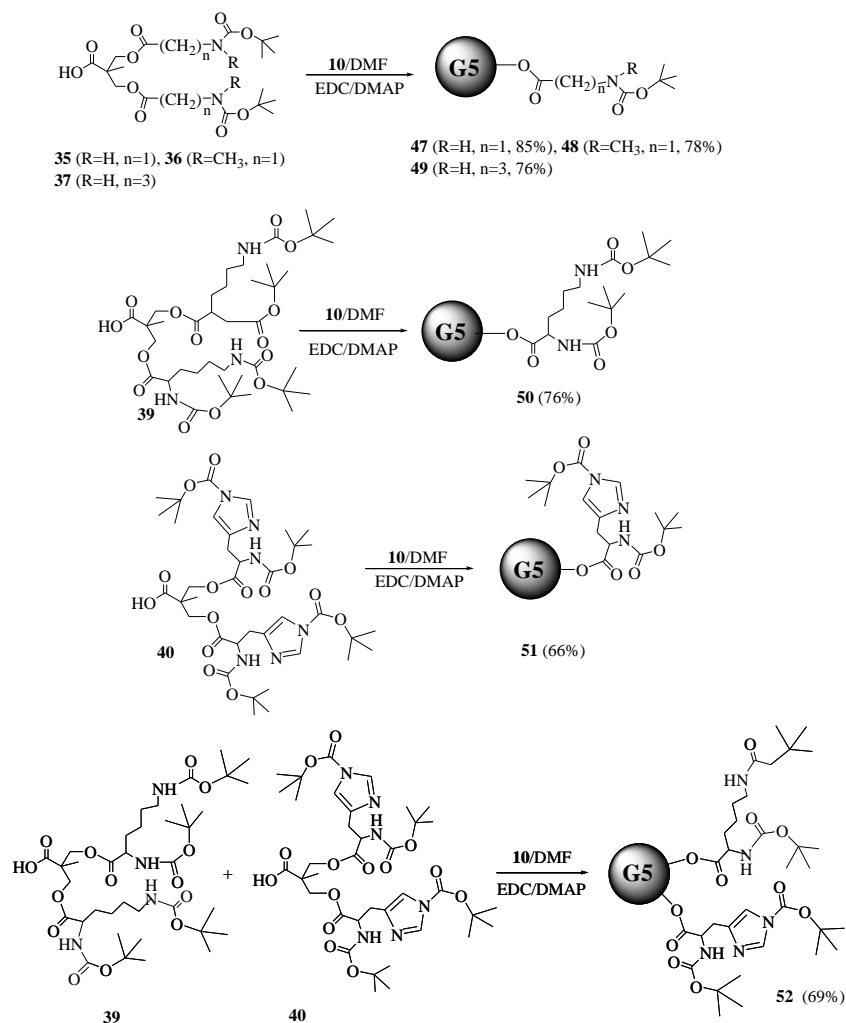
FTIR and NMR spectra confirmed the proposed structures and the assignment of signals was possible also for dendrons **34** and **40** even if difficult to interpret for the presence of regioisomers where Boc groups were found both on τ -nitrogen and on π -nitrogen of imidazole ring.


Scheme 6. Synthesis of BOC-protected dendrons **41-43** from **5**



Scheme 7. Synthesis of debenzylated dendrons **44-46**

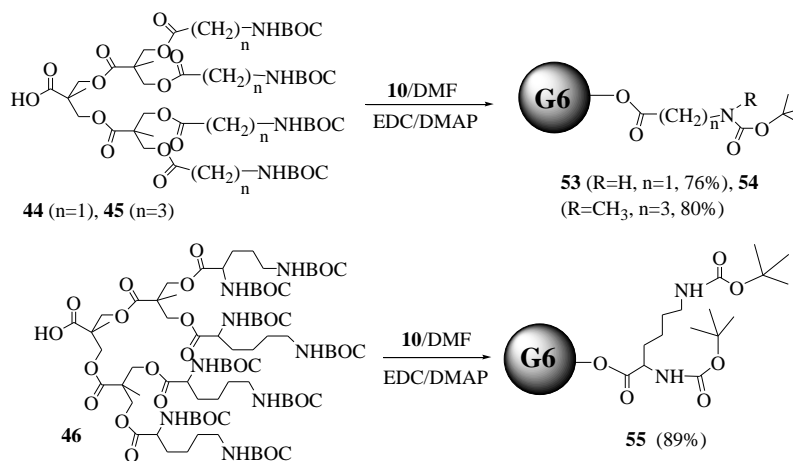
The BOC-protected dendrons **35-37**, **39** and **40** were then grafted successfully onto **10** to afford fifth generation dendrimers **47-51** while, unexpectedly, dendron **38** containing *N*-methyl GABA residues failed the grafting. It was also possible to synthesize the fifth generation *hetero*-dendrimer containing lysine and histidine residues by treating **10** with a 1:1 feed molar ratio mixture of dendrons **39** and **40** to give the protected dendrimer **52** (Scheme 8).



Scheme 8. Synthesis of G5 BOC-dendrimers **47-52**

The integration of the broad proton signal containing $\text{CH}_2^{\alpha}\text{N}$ group of lysine and $\text{CH}_2^{\beta}\text{N}$ of histidine (2.9-3.20 ppm) with respect to the signal of the imidazolic C(5)H (7.19 ppm) allowed to establish that lysine/histidine ratio was 1.08 corresponding to 25 lysine and 23 histidine residues at the periphery of dendrimer respectively.

The BOC-protected dendrons **44-46** were analogously grafted to **10** to afford sixth generation dendrimers **53-55** (Scheme 9).



Scheme 9. Synthesis of G6 BOC-dendrimers **53-55**

The successive removal of BOC groups following the tested procedures described above afforded dendrimers of the fifth (**56-61**) and sixth generation (**62-64**) in the form of hydrochlorides (Schemes 10 and 11 respectively).

The NMR analysis based on integration of selected signals allowed to confirm the growth of **10** to fifth and sixth generation.

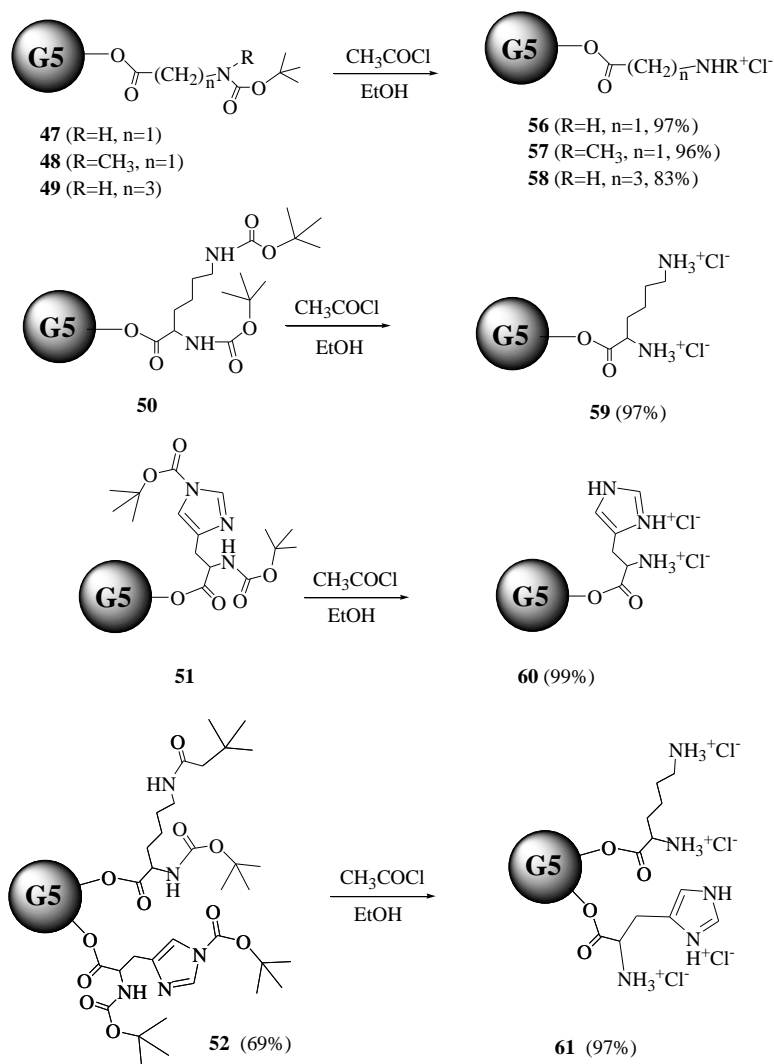
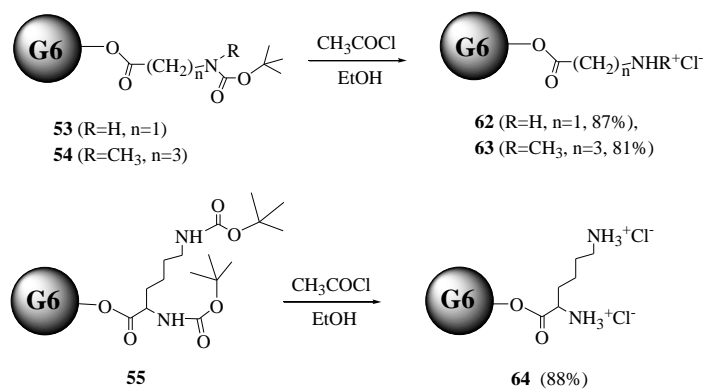
Figure S8 in Supporting Information (page 13) shows, as an example, the ^1H NMR spectra of **10** (a), BOC-protected glycine G5 dendrimer **47** (b) and deprotected **56** in the form of hydrochloride (c). In the spectrum of **47** the broad signal of 48 hydroxyl groups at 4.24 ppm and the complex signal of 96 protons of methylene of the CH_2OH group at 3.48 ppm, typical of **10**, disappeared while a new signal at 3.87 ppm corresponding to 192 protons of the CH_2NH group of glycine and a new broad split signal between 5.10 and 5.60 ppm corresponding to 96 protons of the NHBOC groups appeared.

The removal of the protective groups caused the disappearance of protons of the NHBOC residues between 5.10 and 5.60 ppm and the appearance of 288 protons of NH_3^+ groups between 8.40 and 8.90 ppm in the spectrum of **56**.

This variation pattern was quite similar for the series **48-55** and **57-64**.

Figure 4 shows simplified structures of all the prepared dendrimers in the form of hydrochlorides. The generally high tendency of these materials to absorb water precluded the obtainment of reliable microanalytical data such as Elemental Analysis.

Since the total number of synthesized compounds was quite high, in order to not overload the Supporting Information file, the ^1H and ^{13}C NMR spectra of dendrons **29-46** were not inserted and the spectra both of BOC-protected dendrimers and of the hydrochlorides of fourth, fifth and sixth generation were reported only for compounds containing glycine (**17**, **47**, **53**, **23**, **56**, **62**) (Figures S 9-20 pages 14-25). While, since the appearance of the spectrum plot does not change with the change of generation, for the dendrimers containing the amino acids **b-f** were reported the spectra of the protected and deprotected forms only of one generation as example (Figures 21-24, pages 26-29; Figures 27-30, pages 32-35; Figures 35-38, pages 40-43; Figures 43-46, pages 48-51 and Figures 49-56, pages 54-61). Regarding the remaining dendrimers, only the spectra of final hydrochlorides were reported. (Figures S25, 26, 31-34, 39-42, 47, 48, pages 30, 31, 36-39, 44-47 and 52, 53).

Scheme 10. Synthesis of G5 dendrimers **56-61**Scheme 11. Synthesis of G6 dendrimers **62-64**

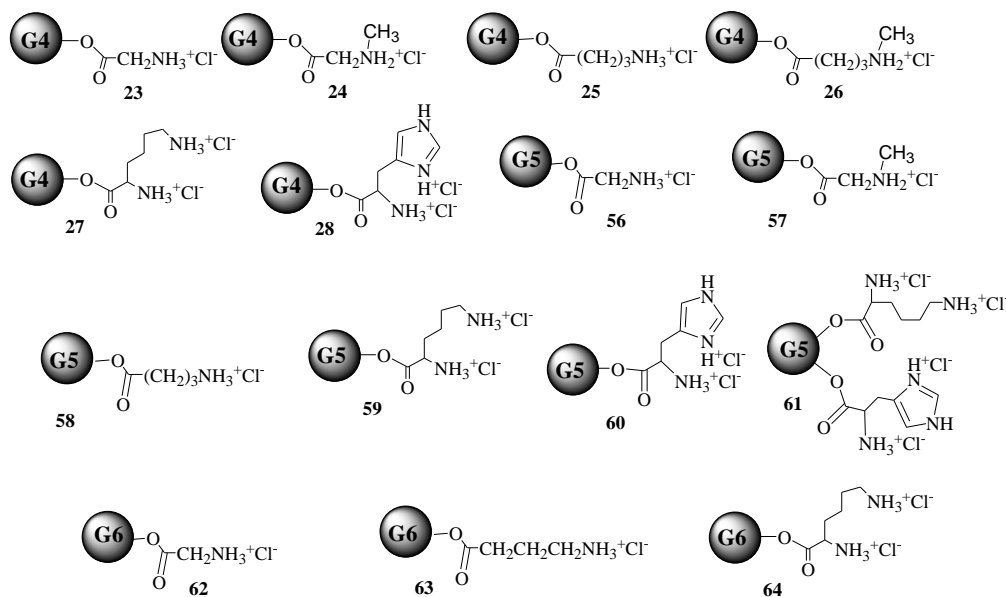


Figure 4. Synopsis of the simplified structures of the prepared dendrimers in the form of hydrochlorides

To determine the molecular weight of the 15 dendrimers reported in Figure 4 and have additional evidence of the prepared structures and composition at the periphery, without having to resort to routine well known but very expensive techniques like MALDI-TOF, the titration of amine hydrochlorides with HClO_4 solutions in AcOH in the presence of mercuric acetate and quinaldine red as indicator⁴¹ proved simple and affordable. We have been the pioneers of using this innovative, cheap and fast method for dendrimers as reported in our recent work currently in print on *Macromolecular Research* and its accuracy has been secured by a sharp endpoint of titration, while its reliability have been demonstrated by the reproducibility of results. Table 1 collects the comparison between calculated and observed molecular weights as obtained by titration. A histogram plot that displays the comparison between experimental molecular weight values and calculated ones on the bases of ^1H NMR spectra is available in Supporting Information (Figure S57, page 62).

The generally good or very good agreement of observed data with the calculated one confirmed the molecular structures of the prepared dendrimers and the goodness of the method. The content of the brackets indicate the dendrimer compositions at the periphery.

It is generally accepted that non viral gene carriers improve their efficacy of transfection if endowed with proper buffer capacity [$\beta = dc_{(\text{HCl})}/dp\text{H}$]⁴² and with an average buffer capacity [$\bar{\beta} = dV_{(\text{HCl})}/dp\text{H}(1)$]⁴³ in the pH range 4.5-7.5 suitable to make them capable to escape from endosomes/lysosomes compartments where pH is in the 5-6 range¹⁰ thus shirking the lysosomal attack.

To have an estimate of the buffer capacity of the prepared dendrimers, potentiometric titrations of **23-28** and **56-64** were performed according to Bennis et al.⁴⁴

Commercial branched PEI-b (25 kDa), a recognized reference standard for non-viral polymeric vectors, was also assayed in the same titration conditions.

Furthermore, since, between dendrimers, PAMAMs are considered as good reference for efficacy in *in vitro* gene transfection, literature data for three G4-PAMAM derivatives⁴⁷ potentiometrically titrated with the same protocol, were used to obtain their titration curve.

β and $\bar{\beta}$ of each synthesized sample, together with β and $\bar{\beta}$ of commercial PEI-b (25 kDa) and G4-PAMAM derivatives were calculated from the titration data. Table 2 collects the β values recorded at pH around 6 and the $\bar{\beta}$ values calculated in the pH range 4.5-7.5 for three degree of

freedom. Figures of all the titration and β values curves and of the histogram plot of $\overline{\beta}$ values are available in Supporting Information (Figures S58-S60, pages 63-65).

Table 1. Molecular Weights of dendrimers **23-28** and **56-64** from titration with HClO₄.⁴¹

Dendrimer	N*	MW (Calc.)	MW (obs.)
G4(15aHCl) 23	48	9834	9887
G4(15bHCl) 24	48	10507	10920
G4(15cHCl) 25	48	11181	11351
G4(15dHCl) 26	48	11854	11639
G4(15e2HCl) 27	96	14998	15503
G4(15f2HCl) 28	96	15428	17225
G5(15aHCl) 56	96	19896	19455
G5(15bHCl) 57	96	21243	23941
G5(15cHCl) 58	96	22589	27962
G5(15e2HCl) 59	192	30849	28966
G5(15f2HCl) 60	192	31085	29141
G5[15e2HCl(50)15f2HCl(46)] 61	192	30637	30592
G6(15aHCl) 62	192	40021	39129
G6(15cHCl) 63	192	45407	42082
G6(15e2HCl) 64	192	60725	62367

*number of peripheral basic groups as determined by NMR

Table 2. β (pH around 6) and $\overline{\beta}$ (pH = 4.5-7.5) of dendrimers **23-28** and **56-64**, PEI-b and G4-PAMAMs from potentiometric titrations.

Dendrimer	N*	β	$\overline{\beta}$ [§]
G4(15aHCl) 23	48	0.0206	0.219
G4(15bHCl) 24	48	0.0426	0.314
G4(15cHCl) 25	48	0.0500	0.256
G4(15dHCl) 26	48	0.0455	0.317
G4(15e2HCl) 27	96	0.0596	0.387
G4(15f2HCl) 28	96	0.0674	0.370
G5(15aHCl) 56	96	0.0390	0.280
G5(15bHCl) 57	96	0.0408	0.303
G5(15cHCl) 58	96	0.0362	0.242
G5(15e2HCl) 59	192	0.0472	0.287
G5(15f2HCl) 60	192	0.0653	0.510
G5[15e2HCl(50)15f2HCl(46)] 61	192	0.0870	0.430
G6(15aHCl) 62	192	0.0344	0.260
G6(15cHCl) 63	192	0.0250	0.220
G6(15e2HCl) 64	192	0.0244	0.250
PEI-b [#]	-	0.0760	0,517
G4-PAMAM ^a	-	0.0014	0,017
G4-PAMAM-Arg ^b	-	0.0015	0,018
G4-PAMAM-HisHisArg ^c	-	0.0038	0,041

*number of peripheral basic groups as determined by NMR; [§]calculated for three degree of freedom; [#] non-dendrimeric branched structure; ^afourth generation PAMAM; ^bG4-PAMAM containing arginine; ^cG4-PAMAM containing the His-His-Arg sequence.

Though no clear, typical patterns came out from data in Table 2 correlating buffer capacity (β) with number of generation and nature of amino acid, it must be noted the importance on this respect of lysine and histidine.

The fourth generation dendrimers containing lysine **27** or histidine **28** and fifth generation dendrimer containing histidine **60** showed in fact buffer capacity and average buffer capacity values very close to PEI and fifth generation dendrimer containing the mixture lysine-histidine **61** showed β value even higher than PEI itself. Finally all the prepared dendrimers showed β values from ten to twenty times higher than those of G4-PAMAM derivatives taken as a reference.

3.2. Biological Assay

3.2.1 Genetic Material Adhesion Assay

The binding assays with genetic material, in a first set of experiments, were performed using plasmid DNA (*pDNA*) (1 μ g) and dendrimers **56-58**, **62**, and **63** with a fixed ratio N/P = 20, obtaining results that associated to the ones obtained in cytotoxicity and transfection assays were considered unsatisfactory. Then we chose to use the fourth and fifth generation dendrimers containing lysine **27** and **59** and the fifth generation ones containing histidine and the mixture histidine-lysine **60** and **61**.

We prepared dendriplexes with *pDNA* in N/P ratios of 10, 20, 40 and 80 which were charged on 4% agarose gel in TBE buffer (Tris-Borate-EDTA) containing ethidium bromide (Figure 1), an interlayer of DNA bases that emits fluorescence after UV irradiation.

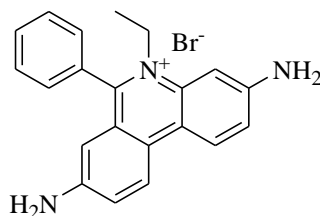


Figure 5. Ethidium bromide structure

As a reference, pure *pDNA* was loaded too and was subjected to electrophoresis together with the dendriplexes. If the *pDNA* does not adhere to the dendrimer, it is not retained and then advances freely in the electrophoretic stroke. An image that shows the obtained results is available in Supporting Information (Figure S61, page 66). Only in the case of fifth generation dendrimer containing only histidine, there was no vector/*pDNA* interaction, whereas all other samples were capable to bind the *pDNA* to any N/P ratio value.

In the light of these satisfactory results, we also evaluated the ability of these samples to bind *siRNA* that adheres to polycationic vectors with lesser affinity because of its very small size and lower density of negative charges.⁴⁸

The small/short interfering RNA (*siRNA*) identifies double stranded oligonucleotides consisting of about 21 pairs of bases of about 6-7 nm in size and molecular weight around 13500 g/mol. The *siRNA* is used to enter the cells in order to silence the expression of target genes by specific matching with the *mRNA* perfectly complementary to one of its filaments.

This time were prepared dendriplexes at N/P ratio of 2.5, 5.0, 10.0, were charged on 4% agarose gel in TBE buffer (Tris-Borate-EDTA) containing ethidium bromide, *siRNA* was also charged as reference but this time was not subjected to electrophoretic stroke. After about 30 minutes we could observe the very satisfactory results displayed in Figure S62 (Supporting Information, page 66).

The image clearly shows a framework similar to that obtained using *pDNA* as genetic material. Briefly, *siRNA* is not bound by **60** which contain only histidine (*siRNA*, not retained by the dendrimer, has run freely in the gel), while remaining well attached to the dendrimers **27** (b) **61** (c) and **59** (d) the last two being successfully also at N/P ratio = 2.5.

3.2.2 Cytotoxicity Assay

A first set of experiments to evaluate the cytotoxicity of some of the prepared materials was performed using KMS-12-BM and LP1 cell taken from a patient suffering from Multiple Myeloma (MM), a bone marrow cancer due to uncontrolled plasma cell replication. Despite of some difficulties in cultivating and maintaining cells that do not easily penetrate surfaces the results obtained with a selection of dendrimers containing amino acids with only one amino group (**56-58** and **62,63**) are reported in Table 3.

Table 3. Citotoxicity [IC_{50} 24h ($\mu\text{g/ml}$)] of compounds **56-58** and **62, 63**

Dendrimer	MW obs.	Number NH_2	IC_{50} 24h ($\mu\text{g/ml}$)	
			KMS-12-BM	LP-1
G5(15aHCl) 56	19455	96	1329	455
G5(15bHCl) 57	23941	96	940	1200
G5(15cHCl) 58	27962	96	176	62
G6(15aHCl) 62	39129	192	650	207
G6(15cHCl) 63	42082	192	133	24

Samples containing GABA, both of fifth and of sixth generation, were the most toxic to both cell lines, while glycine and sarcosine dendrimers were well tolerated by cells, and in particular the samples **56** and **57**.

Subsequent biological investigations were conducted using more easily cultivable cell lines, such as the CHO (Chinese Hamster Ovary) line and HeLa (by Henrietta Lacks, whose cancer cells of the uterine cervix were the first to be immortalized) line. The tables below collect results obtained from cytotoxicity tests using plasmid DNA (*pDNA*) as genetic material complexed with the fourth generation dendrimer **27**, the fifth generation dendrimers **56-61** and the sixth generation ones **62** and **63** on CHO cells (Table 4) and using *siRNA* as genetic material complexed with the fourth generation dendrimer **27** and the fifth generation dendrimers **59** and **61** (Table 5).

Table 4. Citotoxicity (% of CHO dead cells) of compounds **27, 56-61** and **62, 63**.

Dendrimer	MW obs.	Number NH_2	Mortality* %
G4(15e2HCl) 27	15503	96	2.1
G5(15aHCl) 56	19455	96	>30
G5(15bHCl) 57	23941	96	<1
G5(15cHCl) 58	27962	96	>30
G5(15e2HCl) 59	28966	192	2.6
G5(15f2HCl) 60	29141	192	2.5
G5[15e2HCl(50)15f2HCl(46)] 61	30592	192	2.5
G6(15aHCl) 62	39129	192	28
G6(15cHCl) 63	42082	192	28
JetPEI	22000	-	28.6

*% dead cells

Table 5. Citotoxicity (% of Hela dead cells) of compounds **27, 59** and **61**.

Dendrimer	MW obs.	Number NH_2	Mortality* %
G4(15e2HCl) 27	15503	96	3.9
G5(15e2HCl) 59	28966	192	3.9
G5[15e2HCl(50)15f2HCl(46)] 61	30592	192	3.9
JetPEI	22000	-	28.6

*% dead cells

Cytotoxicity was evaluated by cytofluorometry after incubation of cell and dendrimers together with 7-amino-actinomycin D (7-AAD, Figure 4), a DNA intercalating fluorescent substance, selective for the pair of cytosine-guanine nitrogen bases. 7-AAD is excluded from live cells, but penetrates the dead and dying cell membrane, and once intercalated into genetic material it emits fluorescence at a wavelength of 674 nm.

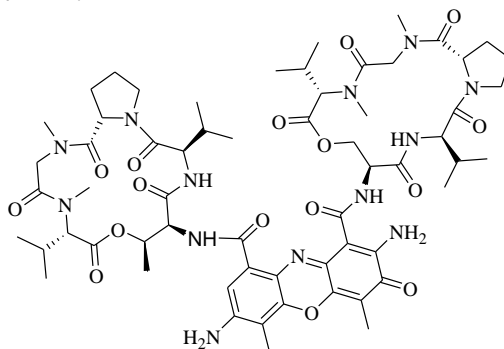


Figure 6. Structure of 7-amino-actinomycin D (7-AAD).

The results of the cytotoxic assays were compared to those obtained with the same dendrimers in the presence of *Jet*PEI (PolyPlus), a linear polyethyleneimine highly effective as vector of genetic material but also very cytotoxic. The fifth generation dendrimers containing glycine (**56**) and GABA (**58**) were very toxic (more than 30% mortality), the sixth generation dendrimers containing glycine (**62**) and GABA (**63**) resulted as toxic as *Jet*PEI, but dendrimers containing lysine, histidine or the mixture histidine-lysine showed very low cytotoxicity (2-2.6%) and finally the fifth generation dendrimer containing sarcosine (**57**) was very well tolerated by cells, demonstrating a cytotoxicity less than 1%.

3.2.3 Transfection Assays

Dendrimers **56-58** and **62, 63** were evaluated in transfection tests after binding with *p*DNA (N/P = 20) on CHO cell line and after 24 hours of incubation. The results were compared with that obtained with *Jet*PEI as reference. The transfection was measured by the intensity of the fluorescent light emitted by the Green Fluorescent Protein (GFP) encoded by the gene contained in the *p*DNA used to prepare the dendriplexes.

The transfection capacity observed with the examined dendrimers and the *p*DNA used was considered too low for further development. Thinking that a reduction in the size of the dendriplexes could facilitate their internalization, *si*RNA was considered as a genetic material because of its significantly smaller dimensions than *p*DNA.

Subsequent transfection tests were conducted only with dendrimers **27, 61** and **59** which showed good adhesion to *si*RNA, maintaining N/P = 10 at *si*RNA concentrations of 250 ng, 500 ng and 1 μ g.

Experiments were conducted on the HeLa cells line in parallel using both a fluorescence-labeled *si*RNA whose transfection would be well visible at the nuclear level by means of a double fluorescence microscope, and a *si*RNA capable of inhibiting the expression of the p53 gene responsible for cell proliferation by resulting in apoptosis by means of cytofluorimetric analysis.

The complexes were prepared both in complete medium with fetal bovine serum, amino acids and antibiotics, and in serum-free medium which, being rich in polyanionic proteins, especially albumin, is often the cause of formation and precipitation of aggregates resulting in a negative outcome of transfection.

After 24 hours of incubation, the results of the experiments conducted with fluorescent *si*RNA were verified by using the double fluorescence microscope after marking nuclei with coloring 4', 6-diaminodino-2-phenylindole (DAPI, Figure 5).

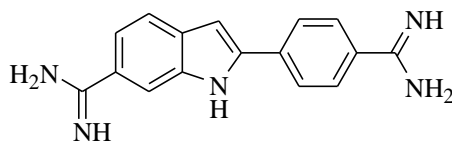


Figure 7. Structure of 4',6-diamidino-2-phenylindole (DAPI).

Unfortunately, no fluorescence was observed at nuclear level as expected in case of transfection (Figure S63a, page 67 in Supporting Information), but fluorescence was observed for precipitated aggregates on cells incubated with complete medium as shown in Figure S63b, page 67 in Supporting Information, deliberately magnified to better highlight the fluorescence only on the outer surface of the cells and to verify its absence inside. This data was confirmed by the transfection experience with *siRNA* inhibiting the expression of the p53 gene evaluated after 48 hours of transfection by cytofluorometric analysis after marking cells with propidium iodide (Figure 6), a fluorescence molecule capable of crossing the membrane of the dead cells, and Annexine, a fluorescent protein structure capable of binding sphingolipids expressed on the membrane of the apoptotic cells, in order to discern dead cells, apoptotic cells, and living cells.

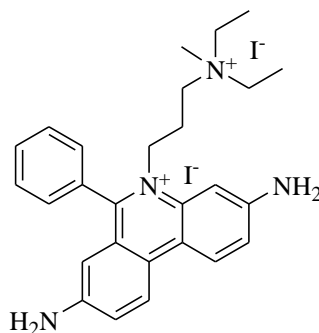


Figure 8. Structure of propidium iodide.

Figure S64 in Supporting Information (page 67) illustrates as dendrimer **27** was slightly cytotoxic, because 83% of cells are still alive, and able to enter the cells but the only 3% of apoptotic cells was a signal of poor transfection efficiency. Similar results were obtained with dendrimers **59** and **61**.

4. Conclusion

Versatile protocols for the step-wise synthesis of dendrimers containing peripheral amino acid residues have been setup. Fourth generation dendrimers were obtained by anchoring *N*-BOC-protected amino acids directly onto the polyester dendrimer **10** containing 48 hydroxyl groups at the periphery prepared from 2,2-*bis*(hydroxymethyl)propanoic acid (*bis*-HMPA).

The fifth and sixth generation dendrimers were obtained by functionalizing first and second generation dendrons **2** and **5** respectively, with *N*-BOC-protected amino acids and successively, after debenzoylation, anchoring the functionalized dendrons onto **10**.

In both the routes the peripheral protecting group was removed by treatment protected dendrimers with HCl generated in anhydrous conditions from acetyl chloride and ethanol affording dendrimers with polycationic nature at the periphery. The possibility of preparing *hetero*-dendrimers was also proved by the synthesis of fifth generation dendrimer containing lysine and histidine residues.

The structures of the dendrimers **9** and **10** and the results of the successive chemical modifications were confirmed by ^1H and ^{13}C NMR spectra and Elemental analysis. Further structural

and composition evidence for the dendrimers in the form of hydrochlorides **23-28** and **56-64** came from the determination of molecular weights by titration with HClO₄ solutions in AcOH in the presence of mercuric acetate which were in accordance with the calculated ones in respect of composition assessed by NMR analysis or estimated by the integrals ratio of proper peaks in ¹H NMR spectra.

Potentiometric titrations showed that dendrimers containing lysine or histidine residues had buffer capacity (β) close to or higher than branched PEI-b taken as reference and that all the prepared dendrimers were better than G4-PAPAM derivatives.

Biological assays on a selection of dendrimers representative of all the prepared samples revealed that only dendrimers containing GABA were more toxic than *Jet*PEI taken as reference, while the majority was well tolerated by all the cell lines used for the assays (mortality below 3%). Both *p*DNA and smaller *si*RNA binding assays gave very satisfactory results: 3 out of 4 samples showed of binding genetic materials even at very low N/P ratio values (2.5). The samples tested in transfection assay proved to be able to enter the cells but for some reasons such as endosome delayed escape, lysosomal or nuclease degradation, difficulty in passing the nuclear membrane, the amount of genetic material expressed was still unsatisfactory.

It is designed to be increased by preparing hybrid *hetero*-dendrimers containing for example *L*-arginine residues, known to improve the cellular uptake, tertiary amine groups, known for their contribution to the proton sponge effect which favors endosomal escape and lipophilic residues to improve interactions with cell membranes.

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Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/OC>

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