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Preparation of *de Novo* Globular Proteins Based on Proline Dendrimers

Glòria Sanclimens,[†,‡] Laia Crespo,[‡] Ernest Giralt,[*,†,‡] Fernando Albericio,[*,†,‡] and Miriam Royo,[*,‡,§]

Barcelona Biomedical Research Institute, Barcelona Science Park, University of Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain, Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1, 08028 Barcelona, Spain, Combinatorial Chemistry Unit, Barcelona Science Park, University of Barcelona, Josep Samitier 1, 08028 Barcelona, Spain

mroyo@pcb.ub.es; albericio@pcb.ub.es; egiralt@pcb.ub.es

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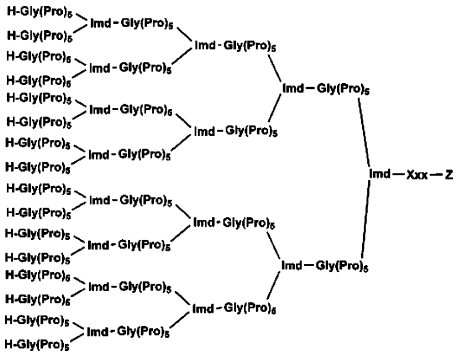

A synthetic method for the preparation of protein-like globular dendrimers derived from a combination of proline, glycine and imidazolidin ring as branching unit is described. The methodology allows the synthesis of novel peptide dendrimers up to fourth generation. Dendrimers were synthesized by a convergent solid-phase peptide synthesis approach. The conformational properties of branched polyproline peptides and proline dendrimers were studied by CD experiments. CD data suggest conformational plasticity of branched peptides for PPI and PPII, and a stable well-defined secondary structure of proline dendrimers for PPII.

Introduction

Dendrimer chemistry has become a rapidly expanding research area at the interface of traditional synthetic organic chemistry and polymer science.[2,3] There is an increasing interest in the synthesis of peptide-based dendrimers owing to their structural similarity to globular proteins.

Most natural proteins in solution assume spherical shapes and are hence generally classified as globular proteins. Several millions of years of evolution were required to achieve the highly compact and well defined folds encountered in the proteins of modern organisms. This evolution involved the iterative mutation of amino acids at positions which ultimately led to globular-shaped proteins, whereby hydrophilic residues are concentrated at the protein surface and hydrophobic side-chains are densely packed in the interior.[4] The fractal geometry of proline dendrimers or other peptide dendrimers results in spherical macromolecules with high surface density and tightly packed congregation of amino acids in their inner core. Thus, compactness, an evolutionary advantage in natural proteins, is artificially achieved in peptide

[†] Barcelona Biomedical Research Institute, Barcelona Science Park.
[‡] Department of Organic Chemistry, University of Barcelona.
[§] Combinatorial Chemistry Unit, Barcelona Science Park.

(1) [1]Abbreviations: AB, 3-(4-hydroxymethylphenoxy)propionic acid; AM, p{(*R*,*S*)-amino-2,4-dimethoxybenzyl}-phenoxyacetic acid; Boc, t-butoxycarbonyl; CD, circular dichroism; DBU, 1,8-diazabicyclo[5.4.0]-undec-7-ene; DCM, dichloromethane; DIEA, *N*,*N*′-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMAP, 4-dimethylaminopiridine; DMF, *N*,*N*-dimethylformamide; EtOH, ethanol; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; G2−4, second, third and fourth generation; AcOH, acetic acid; HOBt, 1-hydroxybenzotriazole; Imd, imidazolidine-2-carboxylic acid; MBHA resin, 4-methylbenzhydrylamine resin; MALDI-TOF, matrix-assisted laser desorption/ ionization-time-of-flight mass spectrometry; MeOH, methanol; NMP, 1-methyl-2-pyrrolidone; PyAOP, 7-aza-benzotriazol-1-yl-N-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate; RP-HPLC, reverse-phase high performance liquid chromatography; SEC, size exclusion chromatography; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid.

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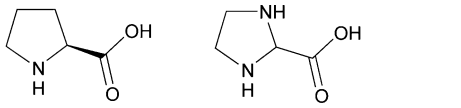
dendrimers via the layering of sequences based on natural amino acids outward from a symmetrical core.

The folding of one protein into a specific three-dimensional structure is caused by an intramolecular self-organization that is driven by a cooperative interaction of different molecular recognition processes such as hydrogen-bonding, van der Waals, electrostatic and solvophobic interactions.[4,5] These relations organize the protein structure in a manner that induces conformational cooperativity to occur over large distance causing small energetic differences relating to conformational states to be magnified, leading to highly stable folded structures. The tight paking of the protein interior in the folded conformational state is thought to provoke conformational cooperativity, and therefore protein stability.[6] For that reason, improvements in the packing protein efficiency increase its stability.[7] In dendrimers, the dendritic scaffold provides a highly branched connectivity that becomes the basis of a increasingly packing at higher generations.[8] Although steric packing on the surface increases at higher generation, the interior of dendritic structures tend to have larger amounts of void space as the generation increases.

If the dendrimer is composed by a peptide sequence that can adopt different conformations such as polyproline, one of them could be stabilized at higher generations due to a packing dendritic effect.

Polyproline oligomers exist in two distinct conformations. In organic solvents, they adopt a conformation known as polyproline I (PPI), a right-handed helix in which all peptide bonds are *cis*-oriented ($\omega = 0°$).[13] In aqueous solvents, they adopt the conformation known as polyproline II (PPII), a left-handed helix in which all peptide bonds are *trans*-oriented ($\omega = 180°$).[14] The transition from PPI to PPII considerably increases the long dimension of the helix from 1.9 to 3.1 Å. Proline-rich regions are found in several proteins due to the active role played by proline in protein folding, affinity, stability and transition. Proline-rich regions on the surface of proteins solubilize their hydrophobic region and also participate in signaling processes.[15] The solubility of polyproline sequences in both aqueous and organic solvents combined with their conformational plasticity make proline compounds promising candidates for biomedical applications.[16−19] The *de novo* proteins described in the present paper marry the aforementioned properties, derived from their proline-rich nature sequences, with those characteristics intrinsic to all dendritic structures, such as well-defined size, shape and topology and enhanced proteolytic resistance.[20] The result is a new type of globular *de novo* proteins in which the branches adopt a stable canonical polypeptide secondary structure, in this particular case, PPII.



L-proline (Pro) imidazolidine-2-carboxylic-acid (Imd)

FIGURE 1. Structure of proline vs branching unit.

Previous work[16,22] from our laboratory has illustrated the complexity of synthesizing polyproline branched peptides and short dendrimers (generation 1 and 2). In the present paper, we report a straightforward approach for the preparation up to four generations of a new type of proline dendrimer, overcoming those problems found early that prevented to pass of generation 2.

Results and Discussion

Dendrimer Synthesis and Surface Modification. Proline dendrimers have been achieved following two key points: (1) the use of a proline-like sterically nonhindered imidazolidin ring as branching unit (Figure 1) and (2) the incorporation of a flexible glycine residue at the *N*-terminal position of each building block.

The key building block **1** [(Fmoc-Gly-Pro_5)$_2$-Imd-OH], which was repeatedly coupled to render the target structures, was prepared using a stepwise solid-phase protocol starting from the incorporation of Fmoc-Imd-(Boc)-OH onto hydroxymethyl Merrifield resin, followed by successive Boc-Pro-OH elongation, and terminated by the incorporation of Fmoc-Gly-OH. After semipreparative RP-HPLC purification, **1** was obtained with a purity of 95−99%. The purity obtained can be considered excellent, because the impurities are deletions from the same amino acid, in our case proline, and show very similar retention times by HPLC, difficulting the purification. On purified **1** only around 2% of two Pro deletion peptides were detected by MS,[23] illustrating its high quality for use as a building block.

Dendrimers were prepared using state of the art Fmoc SPPS methodology as indicated in Scheme 1. While **3a** (G2), **4a** (G3), and **5a** (G4) were prepared on a Wang type

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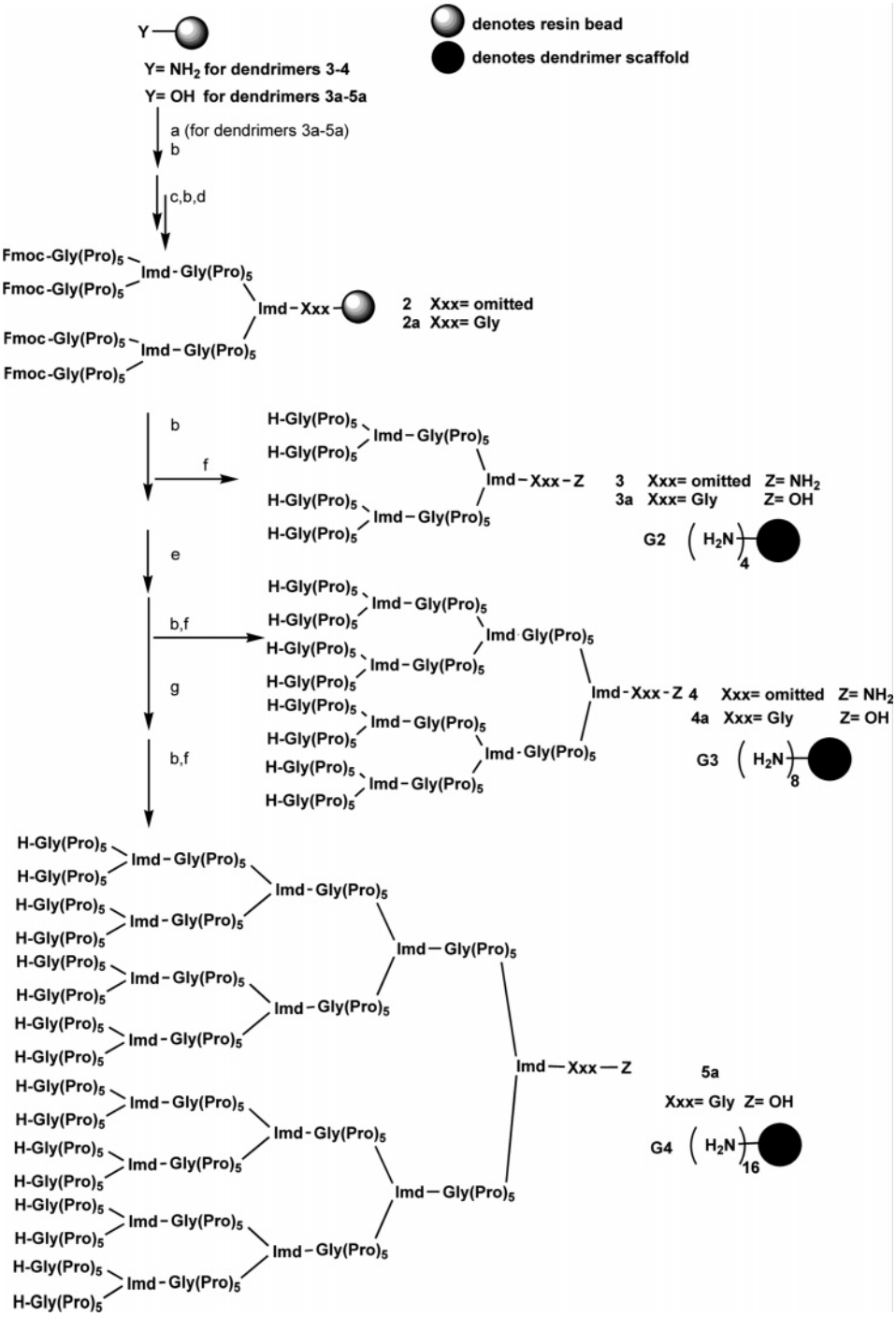
(23) Although MS is considered a qualitative analytical method, in this case it is useful to determine small amount of deletion peptides, which are formed from the same residue, and therefore should have a response very similar to MS. Other techniques such as HPLC are not useful due to structural similarity of impurities.

SCHEME 1. General Strategy for the Synthesis of Proline Dendrimers[a]



[a] Reaction conditions: (a) Fmoc-Gly-OH (10 equiv), DIPCDI (10 equiv), DMAP (0.1 equiv), DMF; (b) piperidine-DMF (1:4) or DBU/piperidine/toluene/DMF (5:5:20:70); (c) **1** (1.5 equiv), DIPCDI (1.5 equiv), HOBt (1.5 equiv), DMF; (d) **1** (3 equiv), DIPCDI (3 equiv), HOBt (3 equiv), DMF; e) **1** (6 equiv), DIPCDI (6 equiv), HOBt (6 equiv), DMF; f) TFA-H_2O (95:5); (g) **1** (12 equiv), DIPCDI (12 equiv), HOBt (12 equiv) or **1** (12 equiv), PyAOP (12 equiv), DIEA (12 equiv) (double additions), DMF.

resin, dendrimers **3** and **4** were made on a Rink type resin. In both cases, **1** was repeatedly coupled on the resin with DIPCDI/HOBt in anhydrous DMF for 3 h at 25 °C (Scheme 1). In the case of the last coupling of **1** to reach **5a**, NMP-DMF (1:1) was used instead DMF to facilitate the coupling, because NMP is used as a solvent in couplings of large fragments,[24] and 7-aza-benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)/DIEA was used as the coupling method.[25] Due to the low stability of OAt esters in solution for long period of times, a second portion of PyAOP is added to ensure carboxylic acid activation.[26]

The simultaneous use of NF31[27] and Kaiser[28] tests to assess the coupling efficiencies were crucial and recouplings were occasionally required. On high generations Fmoc-protected peptidyl resins, after the treatments with

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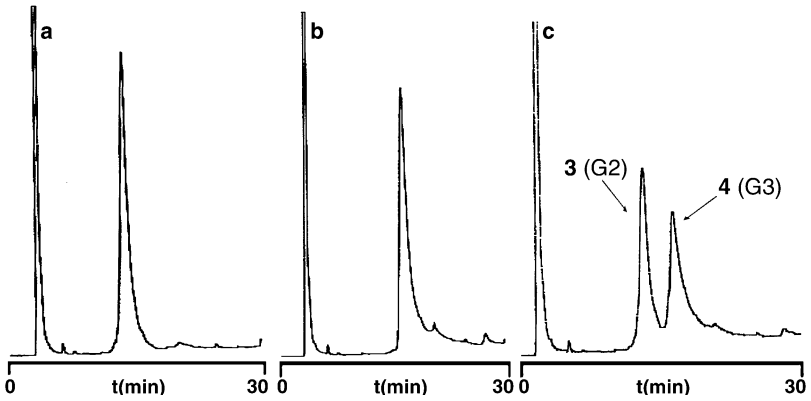

FIGURE 2. Analytical RP-HPLC of crude dendrimers **3** and **4** using a C_4 column and a gradient of 10−50% CH_3CN. **a**: dendrimer **3**. **b**: dendrimer **4**. **c**: Coelution of dendrimers **3** and **4**.

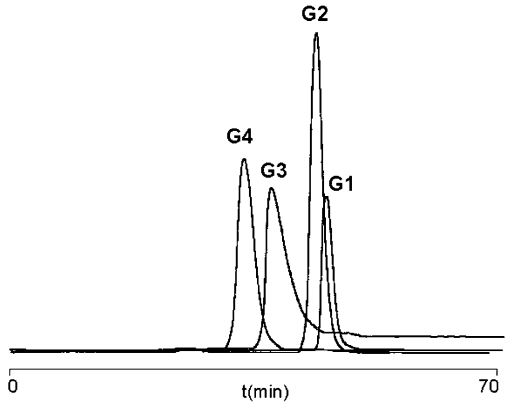

FIGURE 3. Comparison of crude dendrimers by analytical SEC from G1 to G4 corresponding to building block **1x** (G1) and crude peptide dendrimers **3a** (G2), **4a** (G3), and **5a** (G4) (Ultrahydrogel 250 and 500 columns, 5% aq. NaH_2PO_4 containing 3% CH_3CN,[30] isocratic).

piperidine-DMF (1:4) to remove the Fmoc groups, these test gave negative or slightly positive. In these cases, the standard protocol was replaced by treatments with DBU/piperidine/toluene/DMF (5:5:20:70).

The *N*-terminal amino group was kept free to increase solubility in H_2O. Crude dendrimers were cleaved from the resin by acidolytic treatment with TFA-H_2O (95:5).

Purity was verified by analytical RP-HPLC, RP-HPLC-MS, MALDI-TOF, and SEC (Figures 2 and 3).

All compounds gave results consistent with the desired products, showing in MS spectra the expected MW together with peaks belonging with signals corresponding to those dendrimers where just a residue of Pro ismissing,[29] confirming that all compounds are size monodisperse.

Once a synthetic methodology was established, modifications of the dendrimer surface were performed, because these changes can determine the bulk properties of a dendritic system and can lead to functional macromolecules useful in future applications such as drug delivery. The surface of **2** was successfully transformed into free guanidine (dendrimer **6**) and carboxylic acid (dendrimer **7**) groups as shown in Scheme 2. The reaction of dendrimer-resin **2** with 1,3-di-Boc-2-methylisothiourea (4.4 equiv) and DIEA (12 equiv) for 2 h at 25 °C provided dendrimer **6**. Several recouplings were carried out to ensure reaction completion. Dendrimer **7** was obtained from dendrimer **2** via reaction with succinic anhydride (60 equiv) and DIEA/HOBt (60 equiv) in anhydrous DMF for 2 h at 25 °C (Scheme 2). In the aforementioned reaction, coupling efficiencies were monitored using Kaiser and malachite green[31] tests to detect free carboxylic acids.

Circular Dichroism Measurements. The conformational properties of polyproline are well-known.[9] They can adopt two helical conformations, PPI and PPII, with very different physicochemical and spectroscopic properties. PPI helices are more compact than PPII helices and are favored in *n*-propanol and other aliphatic alcohols.[10] Water, acetic acid, and other acidic solvents favor the formation of PPII helix.

The presence of PPI and PPII forms and their interconversion in solution can be analyzed by CD spectroscopy.[11−12,32] The CD spectrum of poly-L-proline type I helix is characterized by a medium intensity negative band at 199 nm, a strong positive band at 215 nm and a weak peak at 232 nm. The CD spectrum of poly-L-proline type II helix exhibits a strong negative band at 206 nm and a weak positive band at 226 nm.

To establish the influence of the Gly residue and the branching unit (Imd) in the building blocks secondary structure and conformational plasticity, CD measurements were carried out in different solvents (water and *n*-propanol). First of all, CD spectra of building blocks **1x**[(H-Gly-Pro_5)$_2$-Imd-OH] and **1y** [(H-Pro_5)$_2$-Imd-OH][33]

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(26) For these kind couplings where excess of coupling reagent is present, phosphonium salts such as PyAOP are preferred to aminum/uronium salts, because the latter can give guanidinium formation. Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *26*, 9678−9683.

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(29) These signals came from the low percentage of Pro deletion in building block **1**, and its intensity was amplified due the dendritic architecture itself.

(30) Several buffers were studied as mobile phases.

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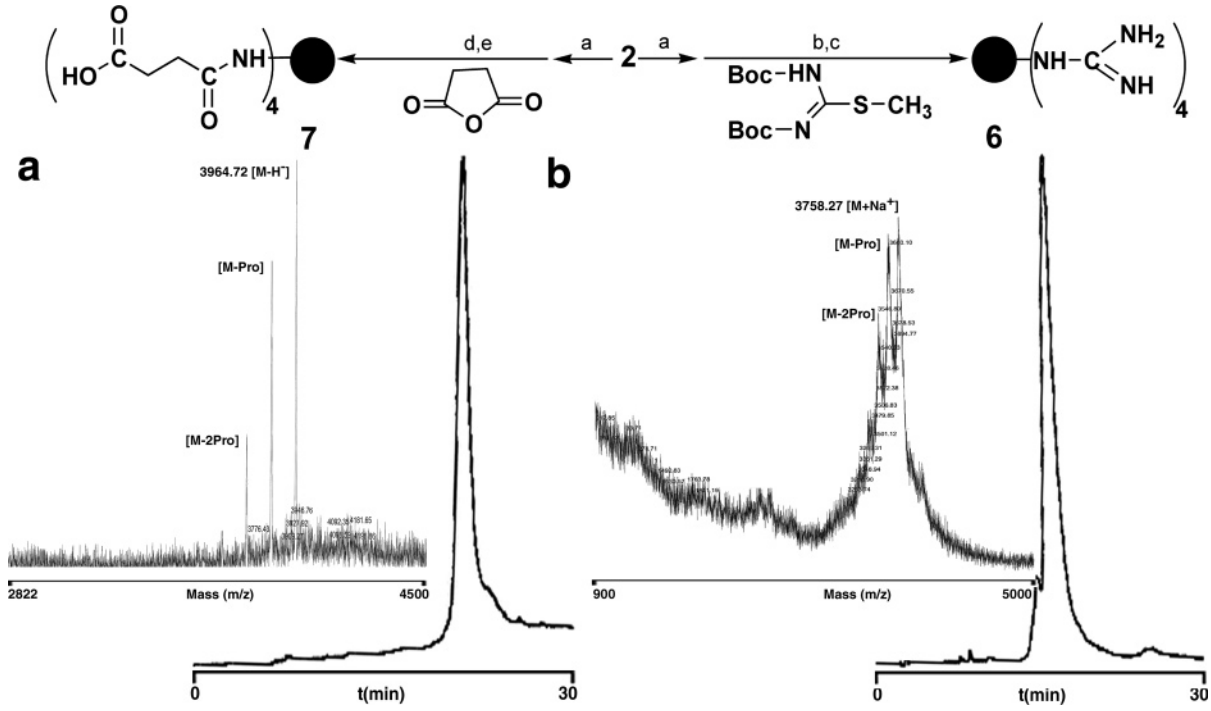
SCHEME 2. Synthesis of Dendrimers 6 and 7[a]



[a] Reaction conditions: (a) piperidine-DMF (1:4); (b) 1,3-di-Boc-2-methylisothiourea (4.4 equiv), DIEA (12 equiv), DMF; (c) TFA-H_2O (95:5); (d) succinic anhydride (60 equiv), HOBt (60 equiv), DIEA (60 equiv), DMF; (e) TFA-H_2O (95:5). **a**: Analytical RP-HPLC on a C_{18} column of crude dendrimer **7** using 15−40% CH3CN as a gradient and its corresponding MALDI-TOF. **b**: Analytical RP-HPLC on a C_{18} column of crude dendrimer **6** using 0−50% CH_3CN as a gradient and its corresponding MALDI-TOF.

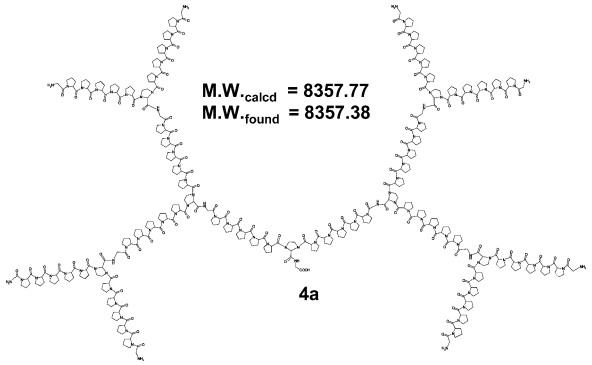


FIGURE 4. Representation of third generation dendrimer **4a**.

with the *N*-terminal group free were recorded in water at different peptide concentrations, from 60 to 550 μM. Both cases showed similar profiles characteristic of PPII helix exhibiting a strong negative band at 205 nm and a weak positive band at 227 and 226 nm for **1x** and **1y**, respectively, and were concentration no dependent. PPII conformation was also observed for both after 192 h at 100 μM, verifying the stability of the structure (Figure 6).

CD spectra of **1x** and **1y** in PPI-promoting conditions [*n*-propanol−water (99:1), at peptide concentration: 100 and 500 μM], were characteristic of the PPI helix with a medium intensity band at 198 nm, a strong positive band at 214 and 213 nm for **1x** and **1y**, respectively, and a weak negative band at 233 nm. PPI conformation was slightly enhanced for both compounds when incubated for longer periods of time (48 and 192 h, respectively) (Figure 6). As a result, Imd as a branching unit and Gly as residue at the *N*-terminal position of proline-rich branched peptides **1x** and **1y** do not disrupt helix formation. PPII and PPI structures are thus formed, revealing the conformational plasticity characteristic of proline-rich peptides.

G2 dendrimers **3**, **6**, and **7** were also studied in both solvents at 50 μM. CD spectra of **3**, **6**, and **7** in water were characteristic of the PPII helix with a strong negative band at 205 nm and a weak positive band at 229 nm. Thus, guanidine (**6**) or carboxylic acid (**7**) surface dendrimers did not disrupt the stability of the PPII helix. Samples were incubated at 25 °C for 360 h without a shift in the CD signature. In contrast to **1x** and **1y**, no PPI conformation was detected for these G2 dendrimers in *n*-propanol−water (99:1). Samples incubated in 99% *n*-propanol for 360 h showing strong PPII helix (Figure 7).

CD spectra of **3**, **6**, and **7** in other PPI promoting solvents such as *n*-propanol, 2,2,2-trifluoroethanol or EtOH at different temperatures, from 25 to 85 °C, were recorded for study their effects on the transition from PPII→PPI (data not shown). Nevertheless, PPI was not observed for G2 dendrimers, exhibiting no solvent dependence, hence confirming the predilection of these macromolecules for PPII conformation.

From these results, it's easy to hypothesized that the dendrimer scaffold amplify the energetic differences between PPII and PPI conformational states and just the increase of only one generation level (from generation 1 to 2) on the dendrimer structure is enough to block the

(33) This was taken as a reference to show that the presence of an additional residue of Gly does not disrupt the expected polyproline secondary structure.

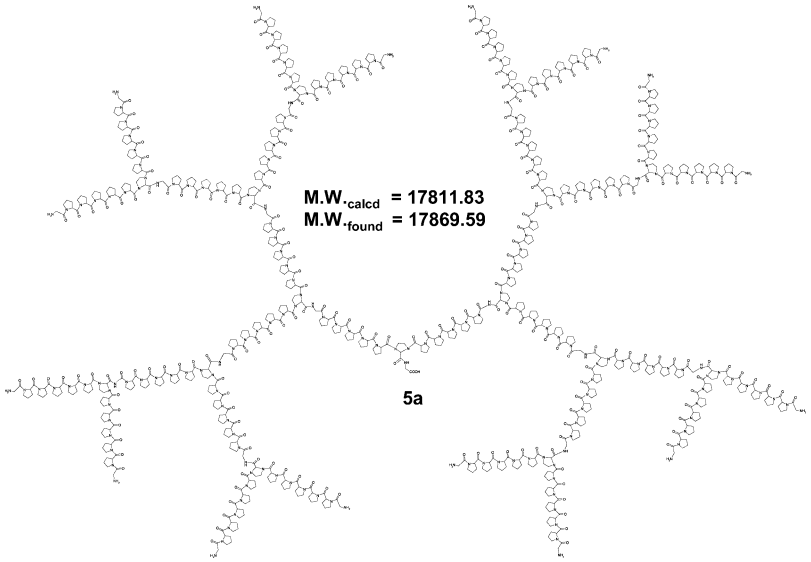


FIGURE 5. Representation of fourth generation dendrimer **5a**.

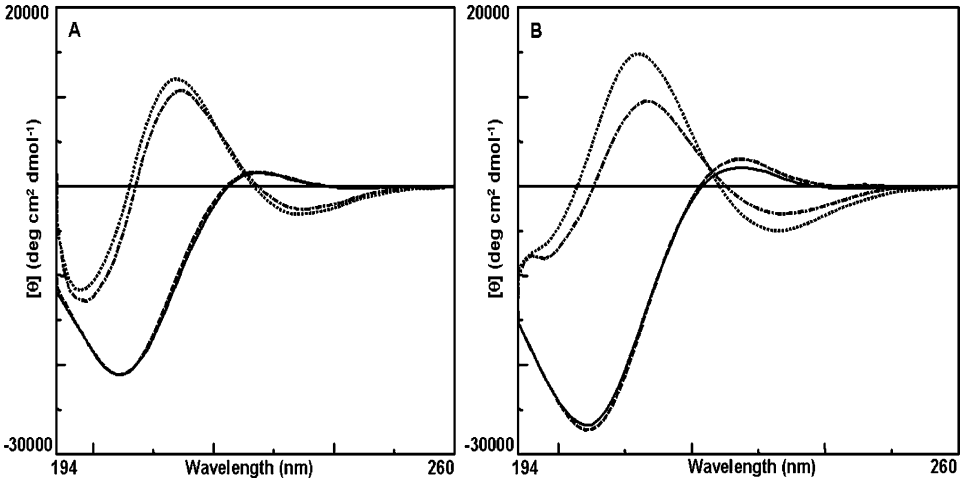


FIGURE 6. Representative CD spectra of building blocks **1x** and **1y** at 100 μM at 25 °C. **A**: **1x** recorded in water for 1 h (−), for 48 h (---); **1x** recorded in *n*-propanol/ water (99:1) for 1 h (−•−•), 48 h (•••). **B**: **1y** recorded in water for 1 h (−), for 192 h (--); **1y** recorded in *n*-propanol/water (99:1) for 1 h (−•−•), for 192 h (•••). The same CD spectra profiles were observed for **1x** and **1y** at 500 μM.

transition from PPII to PPI conformation. This, results in a macromolecule with a stable secondary structural order (PPII conformation) and it's is a clear example of the dendritic effect on the stabilization of the secondary structure of a peptide dendrimer.[8a]

Polyproline dendrimers could thus be considered as novel protein-like globular dendrimers due to their stable and well-defined secondary structure.[34]

Conclusions

Protein dendrimers up to generation 4 based on polyproline sequences were successfully obtained via iterative condensation of the building block **1**. The use of the sterically unhindered imidazolidin ring as a branching unit and the incorporation of a flexible glycine residue at the *N*-terminal position of the building block were crucial to the success of the synthesis. This strategy provides access to a new class of protein-like globular biopolymers in which compactness is created by dendrimeric composition and a well defined branched secondary structure is maintained by proline-rich sequences, outcoming an example of the so-called dendritic effect, with the stabilization of a specific helical structure due to the dendrimer. It is expected that variations in amino acid sequence, building block composition and surface modification of these proline dendrimers will ultimately yield a structurally diverse family of compounds with interesting bioapplications.

To the best of our knowledge, this is the first case where a peptide sequence is part of the dentritic structure and that shows a clear dendritic effect on its secondary structure. In other examples reported in the literature, peptides are decorating the surface of a non-peptide dendritic scaffold, taking advantage of its compactness to stabilize its secondary structure.[19,35−37]

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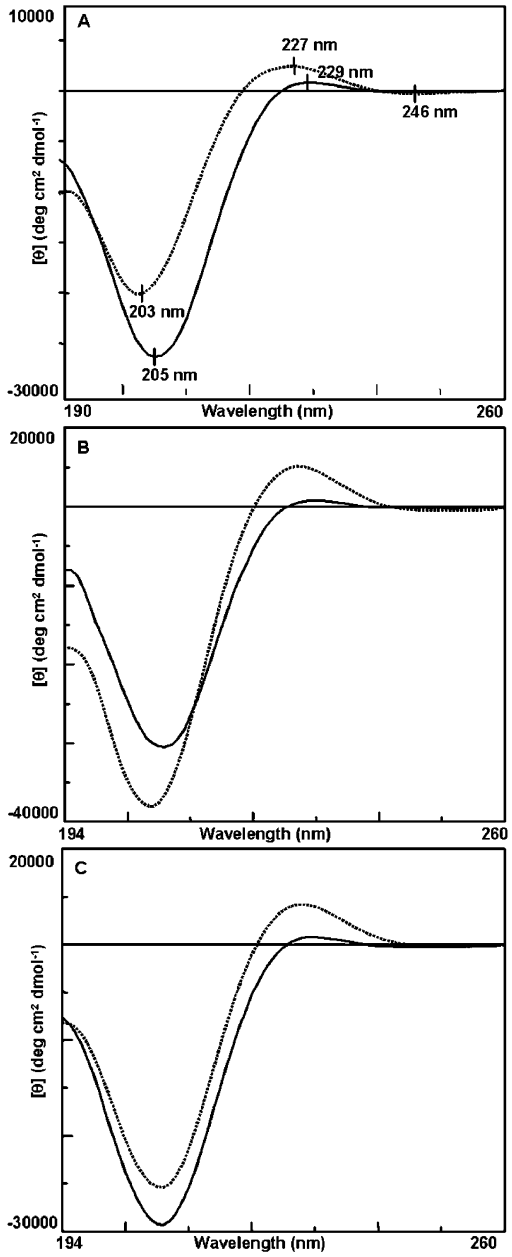

FIGURE 7. Representative CD spectra of dendrimers **3**, **6**, and **7** at 50 μM at 25 °C. A: **3** recorded for 7 h in water (−); for 360 h in *n*-propanol/water (99:1) (···). **B**: **6** for 7 h in water (−); in *n*-propanol/water (99:1) (···). **C**: **7** for 7 h in water (−); in *n*-propanol/water (99:1) (···).

Experimental Section

General Procedures. Optimization of the Resin Loading. The substitution of the resin was reduced using glycine or alanine. Thus, Boc-Ala-OH or Boc-Gly-OH (0.25 equiv) was incorporated with DCC (0.25 equiv) in CH_2Cl_2 (1 × 1 h). After coupling, the resin was washed with CH_2Cl_2 (4 × 1 min), MeOH (4 × 1 min) and dried, and peptide-resin aliquots (5 mg) were hydrolyzed in 12 N aqueous HCl-propionic acid (1:1) at 155 °C for 1−3 h. Resin loading was monitored by amino acid analysis. Once the desired loading was accomplished, the remaining reactive groups on the resin were capped with acetic anhydride (5 mmol) and DIEA (5 mmol) in DMF (1 × 30 min).

Incorporation of Building Block [(Fmoc-Gly-Pro$_5$)$_2$-Imd-OH] 1. (1) **1** (1.5 equiv) was coupled with DIPCDI (1.5 equiv) in the presence of HOBt (1.5 equiv) in anhydrous DMF (1 × 3 h); (2) CH_2Cl_2 (4 × 1 min); (3) DMF (4 × 1 min). A mixture of anhydrous NMP-DMF was used for the incorporation of large amounts of **1** to reach high generations. **1** was coupled with PyAOP in the presence of DIEA (double additions with 1.5 equiv each time) when recouplings were required to complete the reaction. Longer reaction times, such as overnight, were also employed.

Removal of Fmoc Groups. (1) DMF (4 × 1 min); (2) piperidine−DMF (1:4) (1 × 1 min + 1 × 20 min); (3) piperidine−DMF (5:5) (1 × 15 min); (4) DMF (4 × 1 min). Standard protocol was replaced by treatments with DBU−piperidine−toluene−DMF (5:5:20:70) (1 × 3 min + 1 × 30 min) when the chloranil test gave negative or slightly positive. This was especially useful for higher generations.

Monitoring of Coupling Efficiencies. Coupling efficiencies were monitored using the Quiñoa[38] test for hydroxyl groups, the chloranil[39] and NF31[27] tests for secondary amines, the Kaiser[28] test for primary amines, and the malachite green test[31] for carboxylic acids. The NF31 test was also used to follow difficult couplings of primary amines.

Acidolytic Cleavage with TFA. Prior to acidolytic cleavage, all peptide dendrimer resins were washed with DMF (4 × 1 min), CH_2Cl_2 (4 × 1 min), MeOH (4 × 1 min) and dried. Cleavage was achieved with TFA−H_2O (95:5) for 2 h at 25 °C. Upon resin cleavage all crude peptides were subsequently precipitated with anhydrous *tert*-butylmethyl ether, dissolved in AcOH and lyophilized.

Acidolytic Cleavage with HF. The peptide resin was washed with MeOH (4 × 1 min), dried, and treated with HF in the presence of 10% anisole for 1 h at 0 °C. The crude peptide was precipitated with anhydrous *tert*-butylmethyl ether, dissolved in AcOH and lyophilized.

Analysis by RP-HPLC. The purity of the crude dendrimers was verified by analytical RP-HPLC with different linear gradients of H_2O (containing 0.045% TFA) and CH_3CN (containing 0.036% TFA) at a flow rate 1 mL/min. Appropriates aqueous-acetonitrile gradients were found to be: A: 0−100% CH_3CN; B: 10−70% CH_3CN; C: 15−50% CH_3CN; D: 15−45% CH_3CN; E: 10−50% CH_3CN; G: 15−40% CH_3CN; H: 0−50% CH_3CN.

Purification. Only building blocks **1**, **1x**, and **1y** were purified. Crude dendrimers were not purified because they were considered sufficiently pure for CD experiments. Semipreparative RP-HPLC purifications were performed using linear gradients A−G of H_2O (containing 0.1% TFA) and CH_3CN (containing 0.1% TFA) at a flow rate of 25 mL/min and detection at 220 nm.

SEC. Analytical SEC was carried out using an isocratic gradient of 5% NaH_2PO_4 with 3% CH_3CN at a flow rate of 0.5 mL/min and detection at 220 nm. Ultrahydrogel 250 and 500 were used consecutively for all samples in order to achieve good separation.

Synthesis of (Y-Gly-Pro$_5$)$_2$-Imd-OH, Y = Fmoc or H (1). The building block (Fmoc-Gly-Pro$_5$)$_2$-Imd-OH, **1**, was prepared following the protocol described in previous works.[22] In the case of (H-Pro$_5$)$_2$-Imd-OH, **1y**, the last amino acid Fmoc-Gly-OH was not added. The Fmoc group was ultimately removed as required (for **1x** and **1y**). Peptide resins were then cleaved with HF and lyophilized as described in general procedures. Crude peptides were purified by semipreparative RP-HPLC using linear gradients of H_2O/CH_3CN. Fractions were analyzed by RP-HPLC and combined to afford 95−99% pure peptide.

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(**1**) For *N*-terminally Fmoc-protected compounds with Imd as branching unit: (Fmoc-Gly-Pro_5)$_2$-Imd-OH MALDI-TOF: calcd [M + H^+] 1646.89; found [M + H^+] 1646.48, [M + Na^+] 1668.47, [M + K^+] 1684.47.

(**1x**) For *N*-terminal Gly compounds: (H-Gly-Pro_5)$_2$-Imd-OH MALDI-TOF: calcd [M + H^+] 1202.40; found [M + H^+] 1202.46, [M + Na^+] 1224.47, [M + K^+] 1240.44.

(**1y**) For *N*-terminal compounds without Gly: (H-Pro_5)$_2$-Imd-OH MALDI-TOF: calcd [M + H^+] 1088.30; found [M + Na^+] 1111.60, [M + K^+] 1128.60.

Synthesis of ((Fmoc-Gly-Pro_5)$_2$-Imd)$_2$-(Gly-Pro_5)$_2$-Imd-AM-Gly-MBHA Resin (2). The loading of MBHA resin was reduced (from the initial loading 0.7 mmol/g to 0.50 mmol/g) using Gly as described in general procedures. Fmoc-AM-OH handle was then coupled (10 equiv) with DIPCDI (10 equiv) in the presence of HOBt (10 equiv) in DMF for 16 h at 25 °C. Afterward, **1** was repeatedly incorporated as described in general procedures, on AM-Gly-MBHA-resin (0.05 g, 0.5 mmol/g) in which the Fmoc group had previously been removed.

Synthesis of G2 Free-Guanidine Modified Dendrimer (6). Starting from **2** (0.015 g, 0.16 mmol/g), Fmoc groups were removed then DIEA (4 equiv) was added. After 1 min preactivation 1,3-di-Boc-2-methylisothiourea (4.4 equiv) was incorporated with DIEA (12 equiv) in anhydrous DMF (3 × 2 h + 1 × 16 h) at 25 °C. Peptide resin was subsequently cleaved with TFA and the purity of the crude G2 **6** dendrimer was determined by analytical RP-HPLC to be 70% pure.

MALDI-TOF: calcd [M + H^+] 3736.35; found [M + Na^+] 3758.27.

Synthesis of G2 Free Carboxylic Modified Dendrimer (7). Starting from **2** (0.015 g, 0.16 mmol/g), Fmoc groups were removed and succinic anhydride (60 equiv) was added with DIEA/HOBt (60 equiv) in anhydrous DMF (1 × 2 h) at 25 °C. Peptide resin was then cleaved with TFA and the purity of the crude G2 **7** dendrimer was determined by analytical RP-HPLC to be 70% pure.

MALDI-TOF: calcd [M − H^-] 3964.45; found: [M − H^-] 3964.72.

Typical *C*-terminal Carboxylic Acid Dendrimer Synthesis for G2-G4 (3a−5a). The loading of MBHA resin was reduced (from the initial loading of 0.7 mmol/g to 0.19 mmol/g) using Ala as described in general procedures. Then, AB handle was coupled (5 equiv) with DIPCDI/HOBt (5 equiv) in DMF for 1 h at 25 °C. Afterward, Fmoc-Gly-OH was coupled (10 equiv) with DIPCDI in the presence of DMAP (0.1 equiv) in DMF (2 × 1 h) at 25 °C. Consequently, **1** was repeatedly incorporated, as described in general procedures, on resin (0.045 g, 0.19 mmol/g) in which the Fmoc group had previously been removed. Once the desired generation was reached, Fmoc groups were removed and compounds were cleaved from the resin with TFA. The purity of the crude dendrimers was determined by analytical RP-HPLC, MALDI-TOF, MS and SEC.

(**3a**) Purity: >95% by analytical SEC and 86% by analytical RP-HPLC.

RP-HPLC-ESI-MS by gradient C: MW calcd 3625.2; found (M + 2H^+)/2 1813.7, (M + 3H^+)/3 1209.8, (M + 4H^+)/4 907.6, (M + 5H^+)/5 726.6; MW average 3626.5 ± 0.5.

(**4a**) Purity: ∼95% by analytical SEC and 70% by analytical RP-HPLC.

MALDI-TOF: calcd [M − H^-] 8357.77; found [M − H^-] 8357.38.

ESI-MS: MW calcd 8358.8; found (M + 7H^+)/7 1194.9, (M + 8H^+)/8 1046.1, (M + 9H^+)/9 929.8, (M + 10H^+)/10 837.0; MW average 8359.35 ± 1.90.

(**5a**) Purity: >90% by analytical SEC and 55% by analytical RP-HPLC.

ESI-MS: M. W. calcd 17811.83; found (M + 11H^+)/11 1625.4, (M + 12H^+)/12 1491.0, (M + 13H^+)/13 1375.5, MW average 17869.59 ± 7.44.

CD Spectropolarimetry. CD spectra were recorded at 25 °C with a Jasco J700 spectropolarimeter at a spectral bandwidth of 2 nm, with a time constant of 4 s (scan speed 10 nm/min) and a step resolution of 1 nm. Building blocks **1x** and **1y** were analyzed with varying concentrations as indicated, and dendrimers **3**, **5**, and **6** were analyzed at 50 μM. Samples were incubated at 30 °C at different times before recording the CD spectrum. The spectrum of the solvent was subtracted from the spectra of the samples. PPII samples were dissolved in Milli-Q reagent grade water (conductivity 18.2 MΩ cm^{-1}). PPI samples were prepared by first dissolving them in water (1 part), then diluting them with spectral-grade *n*-propanol (99 parts). Cylindrical quartz cells with a path length of 1 mm, 0.1 mm and 0.5 mm were used. Data are presented in residue molar ellipticities (θ, deg cm^2 $dmol^{-1}$).

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Supporting Information Available: Material and Equipment; Analytical Data for Building Block [(Fmoc-Gly-Pro_5)$_2$−Imd-OH: HPLC and MALDI-TOF; Synthesis of G2 (**3**) and G3 (**4**) dendrimers. This material is available free of charge via the Internet at http:// pubs.acs.org.

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