Peptide Dendrimers from Natural Amino Acids

Yoonkyung Kim, Fanwen Zeng, and Steven C. Zimmerman

Abstract: The high-yielding cyanoethylation – hydrogenation strategy was used to prepare simple AB₂ monomers from natural amino acids. Third-generation peptide dendrimers were assembled from these monomers on a polyethylene glycol (PEG) resin by standard Boc peptide coupling methods. Preliminary conformational studies were conducted on these chiral peptide dendrimers by size-exclusion chromatography, optical activity measurements, and investigation of the solid-state properties. These peptide dendrimers have potential applications as drug-delivery agents, asymmetric catalysts, peptido- and protein mimetics, and new biomaterials.

Keywords: amino acids · dendrimers · peptides · solid-phase synthesis

Introduction

Recent advances in dendrimer syntheses have made available dendrimers with a wide range of sizes, functionalities, and properties. Of the many monomer structures possible, those that contain natural or unnatural amino acids are particularly appealing because they are chiral and have the potential to produce dendrimers with enhanced biocompatibility and diversity. These properties may render such dendrimers suitable for use as drug-delivery agents, asymmetric catalysts, and peptido- and protein mimetics. Furthermore, defined three-dimensional structures might be attained through specific folding of the constituent amino acid units. The construction of a combinatorial library of these dendrimers by variation of the amino acid monomers and dendrimer generation number might create a pool of proteinlike synthetic macromolecules which could be screened for desired properties.

Since Denkewalter’s report of a polylsine dendrimer in 1983, only a few dendrimers have been reported that are made from natural amino acid based building blocks. We disclose herein an efficient and general method to prepare monomers containing natural amino acids and the corresponding third-generation peptide dendrimers. Preliminary studies of the chiroptical and solid-state properties of these dendrimers are reported.

Results and Discussion

Our goal in designing a monomeric unit for the natural amino acid based dendrimers was to minimally modify the natural amino acid structures in an effort to maximize the structural resemblance to natural proteins. Thus, the high-yielding cyanoethylation – hydrogenation procedure of Meijer and co-workers was adopted to make branched monomers from natural amino acids. To obviate the tedious purifications involved in many dendrimer syntheses, a liquid-phase peptide synthesis method using polyethylene glycol (PEG) as a support was employed. Chapman and co-workers recently reported the iterative divergent synthesis of an eighth-generation polylsine dendrimer using this approach.

The synthetic utility of our approach was demonstrated using l-valine and l-leucine, amino acids containing relatively bulky side chains, which provide a stringent test of the synthetic methodology. Double Michael addition of acrylonitrile to amino acid in a refluxing aqueous sodium hydroxide (NaOH) solution gave bis(cyanoethyl)amino acid as a crude product. Diamino acids were easily obtained on a 100 g scale through the above two-step process. Tert-butoxycarbonyl (Boc) protection of the amino groups, precipitation with dicyclohexylamine (DCHA), and removal of DCHA gave analytically pure activated monomers for the peptide dendrimer synthesis.

The peptide dendrimers were synthesized on a poly(ethylene glycol) monomethyl ether (MeO-PEG-OH) resin derivatized with glycine to form a protected amino acid monomer. Each generation of dendrimer growth comprises a deprotection, coupling, and capping reaction.

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Scheme 1. Synthesis of activated amino acid monomers 5: a) 1 N NaOH (1.00 equiv), 0 °C, then add acrylonitrile (3.00 equiv), 25 °C, 6 h; 100 °C, 24 h (2a, 100%; 2b, 89%); b) H2, Raney Co, MeOH, 1000 psi, 70 °C, 7 h; c) 2 N NaOH (2.10 equiv), (Boc)2O (2.20 equiv), tBuOH, 0 °C, 16 h; DCHA (1.02 equiv), 1:10 Et2O/hexane, 25 °C, 5 min; d) 2 N HSO4, (1.20 equiv), EtOAc, 0 °C, 10 min (4a, 41% for three steps; 4b, 46% for three steps); e) pentafluorophenol (1.09 equiv), DCC (1.03 equiv), EtOAc, 0—25 °C, 17 h (5a, 95%; 5b, 53%).

Scheme 2). The reactions were monitored by 1H NMR spectroscopy; the average molecular weights were obtained from MALDI-TOF mass spectrometry. The Boc groups were removed by treatment with 1:1 trifluoroacetic acid (TFA)/methylene chloride (CH2Cl2). The coupling reaction[13] was performed in a 4:1 mixture of N,N-dimethylformamide (DMF) and CH2Cl2 by preactivation of monomer 5 with 1-hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIEA) introduced in the in situ neutralization protocol.[14] Any uncoupled free amino groups were capped with acetic anhydride (Ac2O). Third-generation resin-bound peptide dendrimers 10a and 10b obtained through three such iterations were purified by size-exclusion chromatography (SEC).

Scheme 3. Cleavage of dendrimers from PEG resin: a) 1 N NaOH, MeOH, 25 °C, 6 h (11b, 51%; 12a, 84%; 12b, 75%).

dendrimers 10a and 10b obtained through three such iterations were purified by size-exclusion chromatography (SEC).

Cleavage from the resin with a dilute methanolic sodium hydroxide solution (Scheme 3) produced dendrimers 12a and 12b, which were characterized by 1H NMR spectroscopy, including COSY experiments, 13C-APT, MALDI-TOF mass spectrometry, and SEC. The third-generation dendrimers were pure as judged by 1H NMR (> 95%). Compound 10b was converted in a final iteration to the fourth-generation resin-bound dendrimer 13. Despite attempts to optimize the yield of this last cycle, 13 contained defects (1H NMR).

Some of the peptide dendrimers were further examined by SEC and chiroptical methods. Interestingly, in the SEC, 12a (lower molecular weight, MW) eluted earlier than 12b (higher MW) (Figure 1), a fact suggesting that 12b has a more compact structure, possibly as a result of folding. Alternatively, the valine-based dendrimer may exhibit a larger hydrody-
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**Figure 1.** SEC traces in THF for the third-generation dendrimers 12a and 12b. SEC was performed on a Waters Styragel HR3 column (MW range 500–30000; flow rate: 1 mL min⁻¹).

**Figure 2.** A) Cross-polarized optical micrograph at 25°C and B) DSC trace measured on a Perkin–Elmer DSC7 at a heating rate of 5°C min⁻¹ for the fourth-generation leucine-based dendrimer attached to PEG, 13.

The third- and fourth-generation PEG-bound dendrimers slowly crystallized at room temperature from a 1:1 mixture of dimethyl sulfoxide (DMSO) and chloroform (CHCl₃). A polarized optical micrograph of a representative crystalline sample of 13 is shown in Figure 2A. Supporting evidence for the crystalline behavior was obtained by differential scanning calorimetry (DSC, Figure 2B). Although the crystalline nature of these compounds might suggest a discrete folded structure for the peptide dendrimer, Fréchet reported crystallinity in a polybenzyl ether dendrimer attached to a PEG group; suggesting that the PEG group may be responsible for the crystallinity.

**Table 1.** Chiroptical data for peptide dendrimers in CH₂Cl₂.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>Generation</th>
<th>χ[^a]</th>
<th>[α]°[^b]</th>
<th>[Φ]°[^c]</th>
<th>[Φ]°/n[^d]</th>
</tr>
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<tr>
<td>4a[^e]</td>
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<td>1</td>
<td>1.8</td>
<td>−28.7</td>
<td>−124</td>
<td>−124</td>
</tr>
<tr>
<td>11a</td>
<td>1115.51</td>
<td>2</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>12a</td>
<td>2369.27</td>
<td>3</td>
<td>1.5</td>
<td>−44.1</td>
<td>−1044</td>
<td>−149</td>
</tr>
<tr>
<td>4b[^e]</td>
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<td>1</td>
<td>2.0</td>
<td>+10.5</td>
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<td>1157.59</td>
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<td>+14.9</td>
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<td>3</td>
<td>1.5</td>
<td>+10.5</td>
<td>+259</td>
<td>+37</td>
</tr>
</tbody>
</table>

[^a]: Concentration in g/100 cm⁻³.  
[^b]: Specific rotation in 10⁻¹° cm² g⁻¹.  
[^c]: Molar rotation in 10⁻⁸ cm² mol⁻¹.  
[^d]: Molar rotation per chiral center n.  
[^e]: Unlike the second- and third-generation dendrimers, first-generation dendrimers did not contain the additional glycine unit in the molecule.

The third- and fourth-generation PEG-bound dendrimers, but do not show evidence of structural order in the peptide dendrimer segment alone. Efforts to enhance the crystallinity of peptide dendrimers through the modification of dendrimer structures are currently under investigation.

**Conclusion**

In summary, we developed a general method to prepare simple branched amino acid monomers in a highly efficient fashion and synthesized the corresponding optically active peptide dendrimers using an established liquid-phase peptide synthesis strategy. The fact that the approach works well with valine and leucine bodes well for the construction of peptide dendrimer libraries using additional amino acids. The structural resemblance of our dendrimers to proteins and the ability to construct libraries may provide a novel approach to the discovery of new biomaterials.

**Experimental Section**

**Materials:** Glassware was flame-dried and cooled to room temperature in a nitrogen atmosphere before use. All reactions were carried out under a dry nitrogen atmosphere. CH₂Cl₂ was freshly distilled from calcium hydride.
DMF was dried over calcium sulfate, then distilled under reduced pressure, and stored over 4 Å molecular sieves. Ethyl acetate (EtOAc) was stored over 4 Å molecular sieves before use. DMSO was distilled from ninhydrin.

**Methods**

Hydrogenation was performed with a large reactor equipped with a thermostate at the high-pressure laboratory in the School of Chemical Sciences, University of Illinois. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity 400 or 500NB spectrometer. Chemical shifts are reported in parts per million (δ). When neat [D$_6$]DMSO or 1:1 [D$_6$]DMSO/CDCl$_3$ was used as a solvent, chemical shifts were measured relative to the center of the residual water peak (H δ = 4.80), and chemical shifts of 13C NMR spectra were uncorrected. Chemical shifts of 19F NMR spectra were uncorrected. COSY experiments were performed on a Waters Styragel HR3 column (MW range 500 –30000 Da). THF was used as eluent at a flow rate of 1.0 mL min$^{-1}$. TFA was purchased from Aldrich as HPLC grade and redistilled. Ac$_2$O was provided by Grace Davison Chemical and rinsed three times with methanol before use. Polyethylene glycol monomethyl ether (MeO-PEG$_3$) was used without further purification.

**General procedure for hydrogenation**

The reaction was performed following the general procedure described above with t-leucine (6.5 g, 0.45 mol). After removal of acrylonitrile, the mixture in water was extracted with ether (3 × 150 mL), and the combined organic extracts were washed with brine (200 mL), then dried over Na$_2$SO$_4$, and concentrated to ca. 150 mL. Solid precipitated from the viscous oil upon standing 3–4 days at room temperature. The product 2b (105.6 g, 0.45 mol) was filtered and collected as a pure solid. Yields: 89%; m.p. 64–66°C; 1H NMR (400 MHz, [D$_6$]DMSO): δ = 1.20 (s, 1H, COH), 2.76 (m, 4H, C(H$_2$)CH$_2$), 2.88 (m, 4H, C(H$_2$)CH$_2$), 2.96 (m, 2H, NCH$_2$), 3.87 (m, 3H, CH$_2$OH), 4.80 (J = 6.5 Hz, 3H, CH(CH$_3$)$_2$); 13C NMR (100 MHz, [D$_6$]DMSO): δ = 174.6, 119.8, 60.9, 47.0, 38.8, 24.0, 23.1, 21.8, 17.6; HRMS (FAB) calcd for C$_{16}$H$_{20}$N$_2$O$_4$: [M⁺]$: 238.1556$, found: 238.1556.

**General procedure for Boc protection**

An aqueous solution of NaOH (2N, 21 mL, 42 mmol) was added dropwise at 0°C to a solution of 3 (20 mmol) in tert-butyl alcohol (30 mL). The mixture was left to stir at room temperature for 20 min. The reaction mixture was extracted with EtOAc (5 × 100 mL). Combined organic layers were washed with brine (200 mL), dried over Na$_2$SO$_4$, and concentrated under reduced pressure, and dried in vacuo to give 1H-t-butyl alcohol. The mixture was dissolved in EtOAc (5 × 100 mL), and the combined organic layers were washed with brine (200 mL), dried over Na$_2$SO$_4$, and concentrated under reduced pressure, and dried in vacuo to give an off-white solid. The solid was dissolved in EtOAc (5 × 100 mL), and the combined organic layers were washed with brine (200 mL), dried over Na$_2$SO$_4$, and concentrated under reduced pressure, and dried in vacuo to give a white solid.
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General procedure for preparation of pentafluorophenol activated esters: A solution of DCC (2.13 g, 10.3 mmol) in EtOAc (10 mL) at 0°C under N2 was cannulated into a solution of 4 (10 mmol) and pentafluorophenol (2.01 g, 10.9 mmol) in EtOAc (40 mL). The mixture was stirred for 17 h while it warmed slowly to room temperature. The reaction flask was cooled to 0°C, then quickly filtered through a plug of Celite, and the filtrate was concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on a deactivated silica gel (prewashed with CH2Cl2) to give 5a as a colorless oil.

General procedure for flash column chromatography on a deactivated silica gel (prewashed with CH2Cl2): Yield: 5a (327.3 mg, 44.5 mmol) gave 1a (287.8 mg, 37.4 mmol) as a colorless oil. Yield: 96%.

General procedure for peptide dendrimer synthesis on PEG: Poly(ethylene glycol) monomethylether (MeO-PEG-OH) was derivatized with Boc-protected glycine to give 7 following the procedure of Zamboni.[17b] Amino groups were deprotected by stirring the PEG-bound dendrimer with 1 M TFA/ CH2Cl2 (5–10 mL per 1 g resin) at room temperature for 3 h. Peptide coupling was carried out in 4:1 DMF/CH2Cl2 by precipitating monomer 5 with HOBt and DIAE for 5 min, then cannulating this mixture under N2 into a solution of the peptide dendrimer in 4:1 DMF/CH2Cl2. Actual amounts of reagents used relative to the number of the coupling sites are the following: 3 equiv for the monomer; 3 equiv plus the equivalent amount of TFA salt present for DIAE (4 equiv for the first-generation, 9 equiv for the second generation, and 19 equiv for the third-generation); 10 mL per gram resin for 4:1 DMF/CH2Cl2. The coupling reaction was completed in 12–24 h. Longer reaction times increased the amount of the unidentified side products. The uncoupled free amino groups were capped by stirring the PEG-bound dendrimer with Ac2O (4 equiv) and DIAE (1 equiv) in CH2Cl2 at room temperature for 20 h. After each reaction, the mixture was poured into a 100-fold excess of cold anhydrous ether, filtered, and washed with ether to obtain the product. Additional washing with cold ethanol was necessary after each coupling step to remove any remaining HOBt. Before cleavage of PEG, third-generation dendrimer was recrystallized, purified by preparative HPLC on a reverse-phase column. The mass balances for each step were essentially quantitative.

General procedure for the cleavage of PEG: The dendrimers were cleaved from the resin through the modified procedure of Chapman and Mahan.[9b] Third-generation PEG-bonded dendrimer 10 (327.3 mg) was dissolved in MeOH (250 mL), then an aqueous solution of NaOH (16.5 mL, 36 mmol) was added at 0°C. A white precipitate was detected instantly, but the reaction mixture became a clear, homogenous solution upon stirring for 2–3 min. Additional MeOH (10 mL) was added and the mixture was stirred at room temperature for 6 h. The solution became cloudy by diluting with water (250 mL), then at 0°C was neutralized with citric acid powder to pH 6. The cloudy mixture was further diluted with water (300 mL) and kept at 4°C overnight. The white solid was filtered off and washed with plenty of water to ensure the removal of PEG. The collected solid was dissolved in CH2Cl2, dried over Na2SO4, then concentrated under reduced pressure, and dried in vacuo to give 12 as a white solid.
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[9] No racemization was detected at this step as determined by the chiral HPLC analysis of 3,5-dimethylanilide derivatives of 2 (both L and D isomers of valine and leucine) which were prepared by (benzotriazole-1-ylxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (Py-BOP) peptide coupling of 2 and 3,5-dimethylaniline with DIEA in CH2Cl2. A Whelk-O chiral HPLC column was used for the analysis and co-injection of L and D isomers of each amino acid gave baseline separation. W. H. Pirkle, C. J. Welch, J. Chromatogr. 1994, 685, 347 –353.

[10] Other amino acids have been successfully prepared using this method. The yields for the cyanethylation and hydrogenation are the following: cyanethylation of glycine (94 %), L-alanine (91 %), L-phenylalanine (74 %), L-serine (98 %); hydrogenation of glycine (100 %), L-alanine (100 %), L-phenylalanine (97 %), L-serine (81 %). The hydrogenation products were contaminated with a trace amount of cobalt.


[12] Although DMF was required as a major solvent for this step to disrupt any possible aggregation through hydrogen bonding, addition of a small amount of CH2Cl2 was necessary to get a homogeneous solution and thus drive the reaction to >95 % (1H NMR) for each generation.


[14] For the fourth-generation leucine-based dendrimer with PEG, the molecular weight ratio of the dendrimer portion and the PEG was approximately 1:1.
