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Unprotected Peptides as Building Blocks for the Synthesis of Peptide Dendrimers with Oxime, Hydrazone, and Thiazolidine Linkages

Jun Shao and James P. Tam*

Contribution from the Department of Microbiology and Immunology, Vanderbilt University, Nashville, Tennessee 37232-2363

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Abstract: A general approach for forming peptide dendrimers with oxime, hydrazone, and thiazolidine linkages was developed using unprotected peptides as building blocks and selective ligation between an aldehyde and a weak base. To illustrate the generality of this approach, a branched lysine core matrix with an aldehyde was used to ligate four copies of unprotected peptides containing a weak nucleophilic base such as aminooxy, hydrazide, or cysteine 1,2-aminothiol groups at their N-termini to form synthetic branched proteins. Various parameters affecting the ligations were studied, and optimal conditions gave 12−27-fold rate increases and shortened the reaction time from 24−60 to 2−8 h. Among the three reactions studied, ligation by thiazolidine appeared to be superior to ligation by oxime or hydrazone in reaction rate and product stability. The purified dendrimeric products gave single peaks on reverse phase HPLC and size exclusion HPLC. Their macromolecular structures were also characterized by mass spectrometry and amino acid analysis. Circular dichroism spectra of the dendrimers showed that they have an increased ordered helical structure. Ligation reactions using a mutually reactive weak base and aldehyde pair should provide a useful approach for the synthesis of peptide dendrimers and artificial proteins.

Introduction

Since the introduction of solid phase peptide synthesis by Merrifield,[1] cumulative advances have made it possible to synthesize large peptides and small proteins through stepwise synthesis. However, the synthesis of novel artificial proteins, such as peptide dendrimers with branched structure and high molecular weights over 20 000, is generally difficult to accomplish by a stepwise approach in high purity. Peptide dendrimers with branched structure have been found to be useful in developing vaccines,[2,3] diagnostic products,[4] and artificial enzymes.[5] They have recently been employed to mimic the pore structure of ionic channels[6] and in the development of antibiotics.[7] The synthesis of these branched proteins can be

* To whom correspondence should be addressed: James P. Tam, Department of Microbiology and Immunology, Vanderbilt University, A5119 MCN, Nashville, TN 37232-2363. Telephone: (615) 343-1465. Fax: (615) 343-1467.

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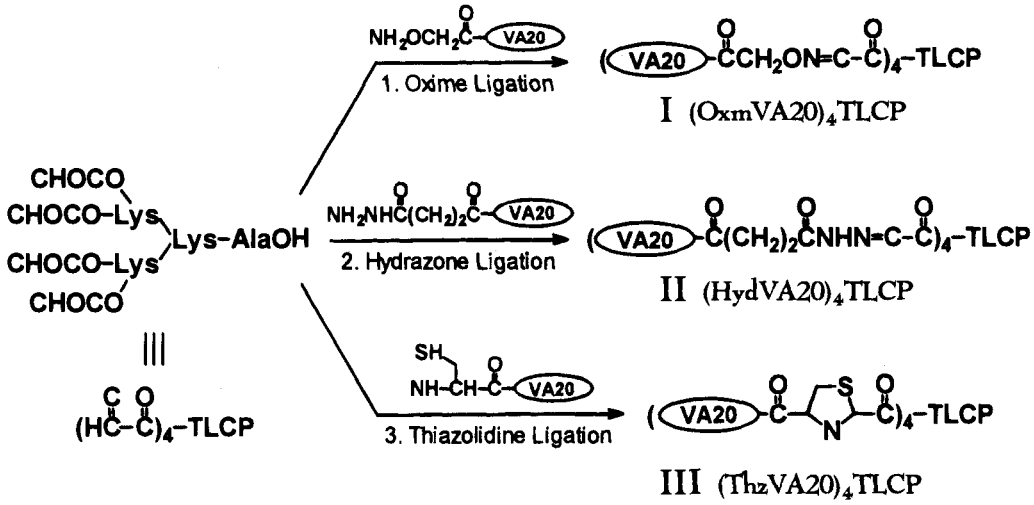

Figure 1. Synthesis of peptide dendrimers with formation of (1) oxime, (2) hydrazone, and (3) thiazolidine linkage. Peptide sequence: VA20 = VMEYKARRKRAAIHVMLALA. Reaction products: (I) (OxmVA20)$_4$TLCP, (II) (HydVA20)$_4$TLCP, (III) (ThzVA20)$_4$TLCP.

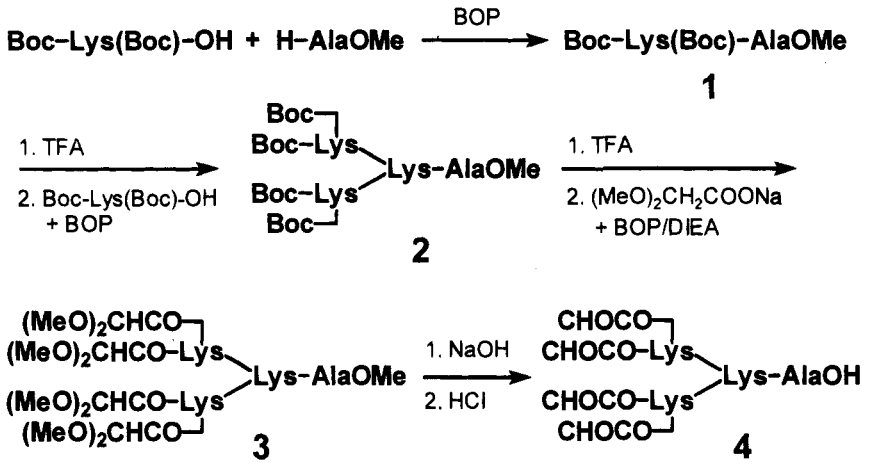

Figure 2. Scheme for preparation of tetravalent glyoxylyl-lysinyl core peptide.

accomplished by using condensation of protected peptide segments. But the limitations of this approach are the poor solubility of protected segments and sluggish coupling rates.[8] These limitations could be overcome by using unprotected peptide segments as building blocks to form peptide dendrimers in aqueous media. We and others have successfully used this approach for the syntheses of such artificial proteins[3,9] with branched structure that can be classified as peptide dendrimers.[10] In this approach, unambiguous products were produced by using a two-stage procedure in which purified peptide segments and a lysinyl core peptide were ligated together through mutually reactive functional groups to attain the desired macromolecule.[3]

A key requirement in the ligation of unprotected peptides is chemoselectivity so that only the intended sites can react with each other in the presence of many side-chain functionalities. This requirement can be met by a general approach using carbonyl chemistry in which a weak base is ligated with an aldehyde under acidic conditions. Under acidic conditions, basic side-chain nucleophiles are protonated to be excluded from the reaction. Weak bases including thiols, hydrazides, and aminooxy groups are suitable for our purpose. They are good nucleophiles and highly reactive toward aldehyde in the acidic pH range of 4−5.5.[11−13] Previous works using thiol as a weak base in the ligation of unprotected peptides focused on thioether formation at pH 7−8.5[3] or thioester formation at pH 4[9a] with the haloacetyl group. Thioether formation, at neutral or basic conditions, has the limitation of giving an oxidative disulfide side product. Thioester suffers from instability in neutral or basic conditions. We therefore seek other alternative and mild reactions for the synthesis of peptide dendrimers. Thiazolidine formation appears to be a suitable choice for this purpose. The cysteine 1,2-aminothiol moiety forms a stable thiazolidine ring with an aldehyde at pH 4−5 within 10 min.[12,13] Under the same condition, an amino group gives a reversible Schiff base. Other weak bases including aminooxy and hydrazide also form stable oxime or hydrazone with aldehyde at pH 4−5.5. As a group, these weak but nucleophilic bases can be exploited for ligation under acidic conditions to utilize the protonation of side chains as protection. Oxime has been used in protein conjugation

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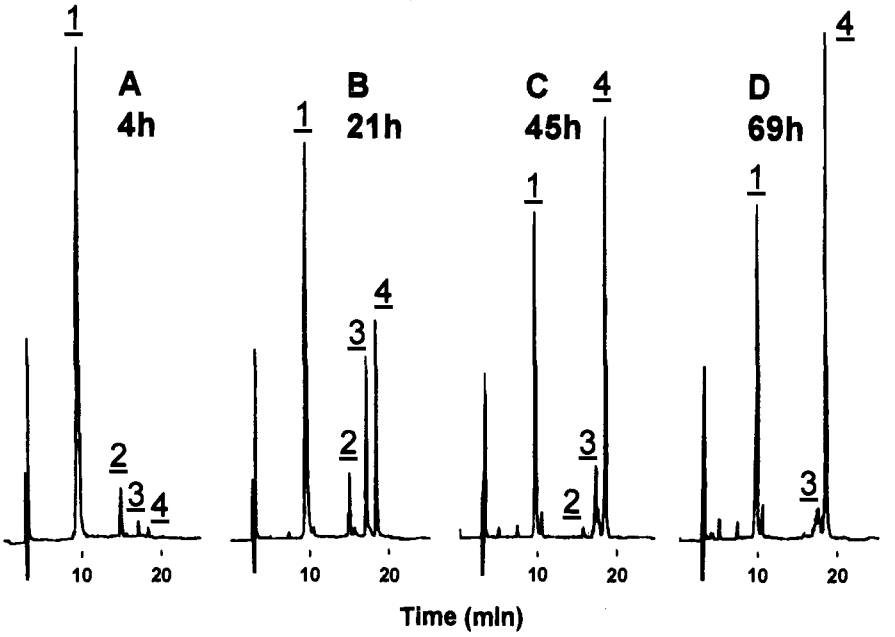

Figure 3. RP-HPLC analysis of dendrimer formation through oxime linkage. Unprotected peptide was marked as 1. Core peptides linked with two, three, or four copies of peptide were labeled as 2, 3, or 4, respectively. HPLC conditions are described in the Experimental Section.

reactions[14] and in the synthesis of peptide dendrimers.[9b] Hydrazone has also been employed in conjugation reactions through the reactions of aldehyde and C-terminal hydrazide.[15,16] In our synthetic scheme, the aldehyde group was placed on the core peptide, and the nucleophilic aminooxy, hydrazide, and cysteinly moieties were introduced onto the N-terminus of the peptide (Figure 1).[17] These nucleophiles are generally highly accessible. For example, (aminooxy)acetic acid and succinic acid monohydrazide are introduced onto peptides similar to protected amino acids. Furthermore, the resulting linkages are small in size and possess good water solubility. In this paper, we report our results in synthesizing peptide dendrimers on a branched lysinyl core using weak base–aldehyde ligation chemistry (Figure 1). In our model studies, a branched tetravalent core peptide was used for the ligation with an unprotected peptide of 20 residues to form tetrameric peptide dendrimers. Peptide dendrimers with higher branches have also been prepared in this laboratory.[18]

Results and Discussion

Synthesis of Functionalized Peptides and the Tetravalent Lysinyl Core Peptide. Synthesis of the functionalized tetravalent lysinyl core peptide containing an aldehyde (Gx_4TLCP, **4**) was accomplished by the solution method (Figure 2).[19] The acetal derivative was obtained from saponification of the commercially available ester and directly coupled onto the tetravalent lysinyl core peptide. Cleavage of dimethyl acetal required an acid stronger than trifluoroacetic acid (TFA)[13,20] because of the stabilization effect of the adjacent amide bond. However, the use of concentrated hydrochloric acid gave satisfactory results.

For the synthesis of functionalized peptides containing a weak base suitable for ligation with an aldehyde group of the tetravalent lysinyl core peptide, (aminooxy)acetyl, monohydrazide succinyl, and Cys were attached to the N-terminus of model peptide VA20, which is derived from the surface protein of feline immunodeficiency virus and consists of 20 amino acid residues. The assembled peptide dendrimer is being used as a prototypic vaccine. Because this peptide contains basic, acidic, and aromatic side-chain varieties, it is also suitable for our study as a model compound. VA20 was synthesized by the stepwise solid phase method employing Fmoc chemistry on *p*-(benzyloxy)benzyl alcohol resin (Wang-resin). After the peptide sequence assembly was completed, the functional residues for ligation—(aminooxy)acetyl, monohydrazide succinyl, or cysteinyl—were introduced successively onto the N-terminus. Cleavage of the peptides was performed with TFA[21] and the crude peptide products were purified by reverse phase HPLC (RP-HPLC). The purified peptides gave the expected results of amino acid analysis and mass spectrometric analysis.

Ligation of Peptides with the Tetravalent Lysinyl Core Peptide. Typically, the ligation reaction was performed in an aqueous medium, and a "standard condition" using 2.5 equiv of unprotected peptide ligand was used as a starting point for optimization and comparison with other conditions. First, the optimal pH's of 4.7, 5.2, and 4.5 for oxime, hydrazone, and thiazolidine ligation, respectively, were used according to previously reported similar reactions.[11–13]

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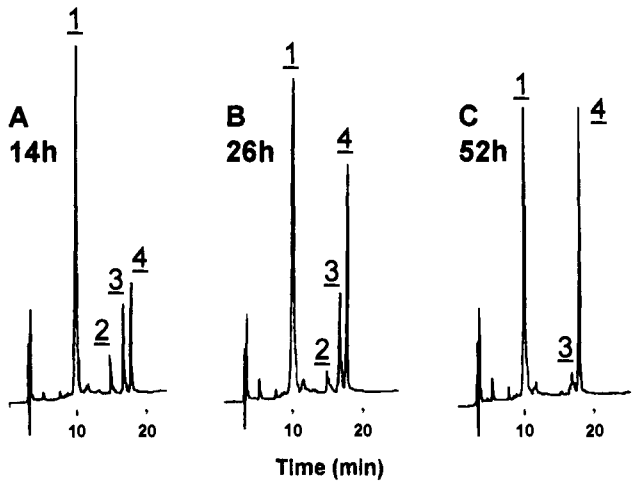

Figure 4. RP-HPLC analysis of dendrimer formation through hydrazone linkage. Unprotected peptide derivative was marked as 1. Core peptides linked with two, three, or four copies of peptide were labeled as 2, 3, or 4, respectively. HPLC conditions are described in the Experimental Section.

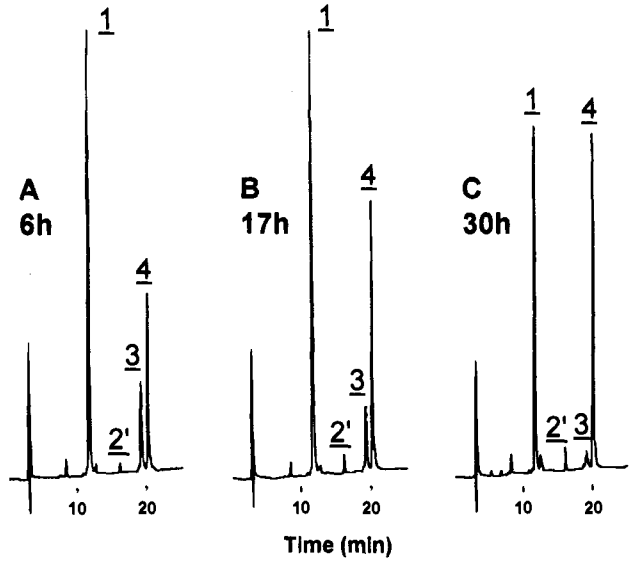

Figure 5. RP-HPLC analysis of dendrimer formation through thiazolidine linkage. Unprotected peptide derivative was marked as 1. Core peptides linked with three or four copies of peptide were labeled as 3 or 4, respectively. The peak marked with 2′ is the dimeric product through disulfide formation. HPLC conditions are described in the Experimental Section.

The reaction process was followed by HPLC analysis (Figures 3−5). In Figures 3−5, all peptide segment peaks were marked as 1. In oxime and hydrazone ligation, reaction intermediates with one copy of the peptide attached on the core peptide were not observed and only the core peptide with two (2), three (3), and four (4) copies of peptide was observed during the course of ligation. Similar findings were also reported by Dawson and Kent in the synthesis of the TASP molecule in thioester ligation.[9a] In thiazolidine ligation (Figure 5), only products containing three (peak 3) and four copies of the peptide (peak 4) were observed shortly after mixing the two components. The minor peak marked with 2′ is a dimer of the peptide linked with a disulfide bond. In this reaction, 0.01 M EDTA was added to the reaction mixture with argon purging through the reaction solution to suppress disulfide formation. Under this reaction condition, less than 2% of the total starting cysteinyl-peptide was oxidized to disulfide dimer (Figure 5, peak 2′). This result demonstrates a significant advantage of ligation under acidic conditions over other thiol conjugations under neutral or basic conditions which would give disulfide formation as one of the major side reactions. Under acidic conditions, all three reactions produced clean ligation products in good yield, which fulfilled our original objective of overcoming the side reactions that occur in basic media. The final ligation products, as well as the ligation intermediates, were characterized by matrix-assisted laser desorption ionization MS (MALDI-MS, Table 1). Amino acid analysis results of the final ligation products also gave the expected compositions. However, the completion of thiazolidine (pH 4.5), oxime (pH 4.7), and hydrazone (pH 5.2) formation in aqueous solution required 1−3 days.

Table 1. MALDI-MS Analysis of VA20−TLCP Dendrimers

peptide dendrimers	mass found $(M + H)^+$	mass calcd (av)
$(OxmVA20)_4TLCP$	10 233 ± 10	10 230
$(HdzVA20)_4TLCP$	10 393 ± 10	10 395
$(ThzVA20)_4TLCP$	10 347 ± 10	10 351

Optimization of the Ligation Reactions. Since these ligation reactions required 1−3 days for completion, it is desirable to increase the reaction rate and thus shorten the reaction time. Various measures, including changing the pH of the reaction medium, using a higher excess of peptide ligand, performing the reactions at elevated temperature, and using organic cosolvents, were studied. The results are summarized in Tables 2−4.

Effects of pH and Stoichiometry. Because all three ligations involved the participation of weak bases and their reaction rates were pH dependent, the effects of pH on reaction rates were investigated (Tables 2−4). The pH values tested ranged from 4.2 to 5.7 for oxime ligation, from 4.7 to 5.7 for hydrazone ligation, and from 4.0 to 5.0 for thiazolidine ligation. In general, the rate of all three reactions increased as the pH increased. In the oxime ligation, although the reaction rate increased with increasing pH, the product yield decreased due to side reactions. Thus, the optimal pH appeared to be around 5.0. In the hydrazone conjugation, changing the pH of the reaction medium did not result in a significant change in the reaction rate and yield. Although the reaction rate increased with increasing pH in the thiazolidine ring formation, the thiol group was also more readily oxidized at higher pH. pH 4.5 was optimal for thiazolidine formation because oxidation of the thiol group at higher pH consumed the peptide to give the disulfide product and consequently diminished the reaction rate. In contrast to the pH effect, the effect of stoichiometry of the unprotected peptide on the core peptide was insignificant. When a 5-fold peptide derivative excess was used for ligation, reaction rates increased less than 2-fold.

Effect of Organic Cosolvents. The mechanism common to all three ligations is the elimination of water molecules after nucleophilic addition. Reduction of water content in the reaction media would favor the reactions to completion. Several water miscible organic solvents were therefore tested. For the ligation involving oxime and hydrazone formation, acetonitrile, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were used as organic cosolvents. Although the cosolvent acetonitrile did not improve the oxime formation rate, addition of DMF and DMSO significantly accelerated the ligation reaction. In 50% DMF at pH 5.7, the reaction rate was 3 times that under the standard condition, while in DMSO, the rate increased 6.5-fold (Table 2). The side reactions that occurred at pH 5.7 were not observed in media containing DMF or DMSO. For hydrazone formation in 50% DMSO, a significant 20-fold improvement in the reaction rate was observed. More than 90%

Table 2. Rate of $(OxmVA20)_4TLCP$ Formation

	pH (22 °C)				50% organic cosolvents (pH 5.7, 22 °C)			pH (37 °C)		
								H_2O	50% DMSO	
reaction condition	4.7[c]	4.2	5.2	5.7	CH_3CN	DMF	DMSO	4.7	4.7	5.7
time (h)[a]	52	64	38	32	35	18	8	23	16	4.5
relative rate[b]	1.0	0.8	1.4	1.6	1.5	2.9	6.5	2.3	3.3	12

[a] Time for reaching 90% of completion of ligation reaction based on HPLC analysis. [b] Relative rate is the relative reaction rate based on standard condition. [c] Defined as standard condition.

Table 3. Rate of $(HdzVA20)_4TLCP$ Formation

	pH (22 °C)			50% organic solvents (pH 5.7, 22 °C)			pH (37 °C)		
							H_2O	50% DMSO	
reaction condition	5.2[c]	4.7	5.7	CH_3CN	DMF	DMSO	5.2	5.2	5.7
time (h)[a]	40	44	34	76	16	2	26	8	1.5
relative rate[b]	1.0	0.9	1.2	0.5	2.5	20	1.5	5	27

[a] Time for reaching 90% of completion of ligation reaction based on HPLC analysis. [b] Relative rate is the relative reaction rate based on standard condition. [c] Defined as standard condition.

Table 4. Rate of $(ThzVA20)_4TLCP$ Formation

	pH (22 °C)			50% organic solvents (pH 4.5, 22 °C)			37 °C (pH 4.5)	
reaction condition	4.5[c]	4.0	5.0	CH_3CN	TFE	DMF	H_2O	50% DMF
time (h)[a]	24	30	16	18	30	5	8	2
relative rate[b]	1.0	0.8	1.5	1.3	0.8	4.8	3	12

[a] Time for reaching 90% of completion of ligation reaction based on HPLC analysis. [b] Relative rate is the relative reaction rate based on standard condition. [c] Defined as standard condition.

of the final ligation product was formed within 2 h, and the ligation was completed in less than 4 h (Table 3). In thiazolidine formation, DMSO was not used because it oxidized the thiol group to disulfide. Organic solvents such as trifluoroethanol (TFE), acetonitrile, and DMF were tested. Only DMF showed a notable reaction rate improvement, and the reaction was completed in 6 h, compared with 30 h under the standard conditions (Table 4). In summary, polar aprotic solvents such as DMSO (in oxime and hydrazone ligation) or DMF (in thiazolidine ligation) led to the most significant rate improvements. Our results show that the addition of water miscible polar aprotic organic cosolvents is an effective way to increase the rate of ligations. It should be mentioned that the use of organic cosolvents has two additional advantages in preventing aggregation of various peptide dendrimers and in ligation of water insoluble organic molecules with peptides or proteins.

Temperature Effect. Because physiological temperature is often used in biochemical studies, ligations were also performed at 37 °C to accelerate the reaction rate. For oxime and thiazolidine formations, a 2−3-fold rate acceleration was achieved while a less significant temperature effect was observed for the hydrazone reaction (Tables 2−4). The combined effects of elevated temperature and organic cosolvent were also studied. DMSO was used in oxime and hydrazone formation, and DMF was tested for thiazolidine formation. The additive effect on oxime formation rate at pH 4.7 was over 3-fold. At pH 5.7, the reaction rate increased 12-fold and 90% of the ligation product was formed in less than 5 h (Table 2), while at 37 °C and pH 5.7, the rate of hydrazone ligation was 27 times that under the standard condition at 22 °C (Table 3). The formation of thiazolidine in 50% DMF at 37 °C was 90% complete in 2 h, showing a 12-fold rate improvement (Table 4). In short, all three ligations were completed within 6 h under such conditions.

Stability of Oxime, Hydrazone, and Thiazolidine Linkages. The stability of three branched peptide dendrimers was tested for 24 h at four discrete pH values—3, 5, 7, and 9—in aqueous media. The oxime bond was stable in acidic and neutral media, but 21% of the ligation product decomposed at pH 9 to the peptide ligand and the lysinyl core peptide with three copies of the peptide. The hydrazone linkage was stable at pH 5 and 7, while 32 and 26% of the product was decomposed at pH 3 and 9, respectively. The thiazolidine linkage showed the highest stability, being stable between pH 3 and 9. These results are consistent with the previous knowledge that thiazolidine is a stable aldehyde derivative.[12,13]

Secondary Structure of the Peptide Dendrimers. The effect of tetravalent lysinyl scaffolding on the secondary structure of the dendrimers was also studied by means of circular dichroism (CD) spectra. The CD measurement was performed in a mixture of 50% water/TFE. The spectra of the peptide and peptide dendrimers are shown in Figure 6. The ligated molecules [$(OxmVA20)_4TLCP$, curve 2; and $(ThzVA20)_4TLCP$, curve 3] are more helical in structure compared with peptide VA20 (curve 1). Under identical conditions, the tetravalent lysinyl core peptide Ac_4TLCP does not show any ordered structure (curve 2). The maximum around 191 nm and two minima at 207 and 222 nm with a zero crossover at 201 nm suggest the helical structure of the peptide dendrimers. This result was similar to those of peptide dendrimers on a template.[9a,22,23] It is of interest that in our model, the peptides are attached to the core peptide through the N-terminus, in contrast to those dendrimers formed through C-terminal linkages.[9a,22,23] Our results are also consistent with the fact that the repeating sequence suggests the possibility of a regular periodic structure.[24]

Conclusion

Ligation using weak base−aldehyde chemistry and unprotected peptide building blocks to form oxime, hydrazone, or thiazolidine linkages provides a useful general approach for

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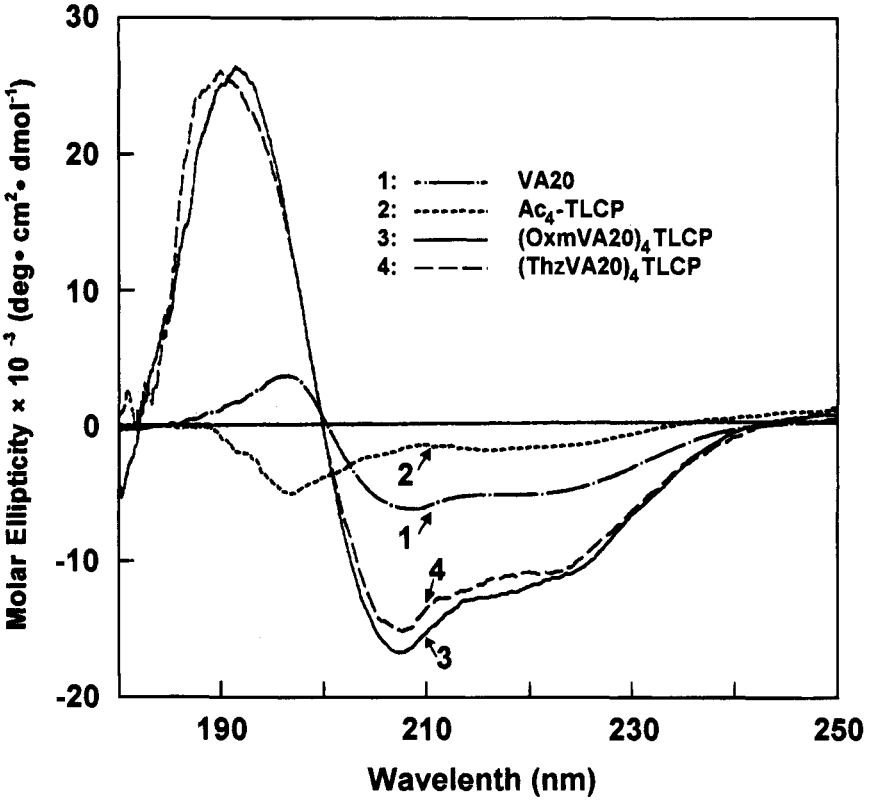

Figure 6. Circular dichroism (CD) spectra of peptide dendrimers, peptide VA20, and core peptide Ac_4TLCP in 50% TFE/water. Curve 1, VA20; curve 2, Ac_4TLCP; curve 3, $(OxmVA20)_4$TLCP; curve 4, $(ThzVA20)_4$TLCP.

synthesizing peptide dendrimers of high molecular weights. The ligation rates can be increased by manipulating reaction conditions such as changing the pH and temperature and by adding organic cosolvents. The optimal reaction pH range is 4.7−5.2 for oxime and hydrazone ligations and 4.5 for thiazolidine ligation. At 37 °C, the reaction rates increased 2−3-fold. Addition of organic cosolvents further accelerated reaction rates. DMSO is the most useful cosolvent for oxime and hydrazone formation, while DMF is best for thiazolidine formation. When reactions were performed at 37 °C in media containing the appropriate organic solvents, rate increases greater than 12-fold were observed for all three reactions. Ligations were completed within 6 h, compared to 1−3 days under unoptimized conditions. Addition of organic cosolvents also has the effect of preventing peptide aggregation and solubilizing organic molecules. The thiazolidine ligation showed the fastest reaction rate among the three reactions tested, and its ligation product was stable in a wide pH range of 3−9. The rapid reaction rate and stable product make thiazolidine ligation the most attractive ligation reaction for the synthesis of large peptide dendrimers.[18] Another advantage of this ligation strategy is that peptide dendrimers can be readily produced in high purity. The weak base−aldehyde ligation chemistry is also applicable to the synthesis of cyclic peptides, the semisynthesis of proteins, and the bioconjugation of reporter groups to proteins. Combined with other ligation methods,[3,9,14−16] our results will provide an array of useful tools in generating a diversity of peptide dendrimers for both biochemical and biophysical studies.

Experimental Section

Materials and Methods. Solid phase peptide syntheses were performed on a CSBIO-536 automated synthesizer using CSBIO software (Foster City, CA). Peptides were purified on a Waters 600 multisolvent delivery system equipped with Vydac C18 reverse phase columns (size 25 × 2.2 cm i.d. and 25 × 1 cm i.d.). Analytical HPLC was performed on Shimadzu instruments including a SCL-10A system controller, two LC-10AS pumps, a SIL-10A auto injector, a SPC-10A UV-VIS detector, and a CR501 integrator. The analyses were performed on a Vydac C18 reverse phase column (25 × 0.46 cm i.d.) at a flow rate of 1.0 mL/min, monitoring at 225 nm. Eluents used were (A) 0.046% TFA in water and (B) 0.039% TFA in 60% acetonitrile. The gradients used in the analyses are as follows: (1) oxime ligation (Figure 3), 0−1 min, 38% B, 1−21 min, linear gradient from 38 to 60% B; (2) hydrazone ligation (Figure 4), 0−1 min, 38% B, 1−21 min, linear gradient from 38 to 64% B; and (3) thiazolidine ligation (Figure 5), 0−1 min, 35% B, 1−21 min, linear gradient from 35 to 60% B. For analytical size exclusion HPLC (SE-HPLC), a Bio-Sil TSK-250 column (Bio-Rad) was used. The chromatographic conditions were as follows: mobile phase, 0.1 M phosphate buffer (pH 7.0); and flow rate, 1 mL/min. The OPA/2-mercaptoethanol method was used for amino acid analysis.[25] The molecular weight of the peptides was determined on a Kratos MALDI-MS III instrument. CD spectra were recorded on a JASCO Spectropolarimeter J720.

Solid Phase Peptide Synthesis. Peptide VA20 with the sequence of VMEYKARRKRAAIHVMLALA was synthesized on an automated synthesizer using the Fmoc/tBu strategy on *p*-(benzoyloxy)benzyl alcohol resin (Wang-resin). Coupling was accomplished by the DCC/1-hydroxybenzotriazole (HOBt) method with 2.5 equiv of amino acids, and the Fmoc group was deprotected by 20% piperidine in DMF. The protected weak bases for ligation were introduced onto the peptide through coupling 3 equiv of Boc-$NHOCH_2COOH$, Boc-$NHNHCOCH_2$-CH_2COOH, or Fmoc-Cys(Trt)-OH with (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent.[26] Final cleavage of peptides from the resin was performed with 90% TFA/6% thioanisole/3% ethanedithiol/1% anisole (50 mL/g resin) for 3 h.[21] The resin was removed by filtration, and the filtrates were concentrated in vacuo. After the peptide products were precipitated with dry ether, they were filtered and further washed with dry ether. The precipitates were taken up in 100 mL of 10% acetic acid. Insoluble residues were

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removed by centrifugation. After lyophilization, these crude products were purified by preparative RP-HPLC. The purified peptides were characterized by MALDI-MS and amino acid analysis. MALDI-MS: NH_2OCH_2CO-VA20, 2402 ± 2.4 (calcd for M + H^+, 2402); NH_2NH-$(CH_2)_2CO$-VA20, 2444 ± 2.4 (calcd for M + H^+, 2443); Cys-VA20, 2431 ± 2.4 (calcd for M + H^+, 2432). Amino acid analysis gave expected results for all peptides.

Preparation of Glyoxylyl Tetravalent Lysinyl Core Peptide. Boc-Lys(Boc)-Ala-OCH_3 (1). H-Ala-OCH_3·HCl (1.40 g, 10 mmol) was suspended in a solution of Boc-Lys(Boc)-OH (3.46 g, 10 mmol) in DMF (12 mL) and DCM (6 mL) and cooled in an ice bath. To this mixture were added BOP (4.42 g, 10 mmol) and DIEA (2.84 g, 22 mmol). The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 20 h. DCM and DMF were then removed in vacuo, and the residue was taken up in ethyl acetate (100 mL). The ethyl acetate phase was washed with saturated NaCl solution (2 × 15 mL), 2% $KHSO_4$ (2 × 15 mL), 5% $NaHCO_3$ (3 × 15 mL), and water (3 × 15 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated to dryness. Recrystallization from ethyl acetate/hexanes gave dipeptide product (**1**). Yield: 4.37 g (91.6%). MALDI-MS: 480.7 ± 0.4 (calcd for M + H^+, 480.6). Anal. Calcd for $C_{24}H_{37}N_3O_7$ (479.57): C, 60.11; H, 7.78; N, 8.76. Found: C, 60.31; H, 7.92; N, 8.48.

Boc-Lys(Boc)-Lys[Boc-Lys(Boc)]-Ala-OCH_3 (2). Dipeptide **1** (0.96 g, 2 mmol) was dissolved in 50% TFA/DCM (20 mL). After the mixture was stirred at room temperature for 20 min, TFA and DCM were removed in vacuo. The residue was washed with dry ether (4 × 10 mL) and then dissolved in DMF (20 mL). After the addition of DIEA (1.55 g, 12 mmol) and Boc-Lys(Boc)-OH (1.44 g, 4.2 mmol), the mixture was cooled at 0 °C. BOP (1.86 g, 4.2 mmol) was added to this solution in 0.5 min. The mixture was stirred at 0 °C for 30 min and then at room temperature for 24 h. The peptide was worked up as described in the previous preparation of dipeptide **1**. Recrystallization from ethyl acetate/hexanes gave the title compound. Yield: 1.65 g (92.6%). MALDI-MS: 910.4 ± 0.9 (calcd for M + Na^+, 911.1). Anal. Calcd for $C_{42}H_{77}N_7O_{13}$ (888.11): C, 56.80; H, 8.74; N, 11.04. Found: C, 57.01; H, 8.88; N, 10.68.

$(CH_3O)_2CHCO$-Lys(($(CH_3O)_2CHCO$)-Lys[($(CH_3O)_2CHCO$-Lys-($(CH_3O)_2CHCO$)]-Ala-OH (3). (A) To a stirred solution of methyl dimethoxyacetate (671 mg, 5 mmol) in methanol (5 mL) was added 0.5 N NaOH (10.5 mL, 5.25 mmol). The reaction was completed after 2 h according to TLC monitoring. Methanol was removed in vacuo, and the remaining aqueous solution was diluted to 25 mL with water. The aqueous solution was washed with ethyl acetate (3 × 8 mL), concentrated to 10 mL, and then lyophilized to dryness.

(B) Branched tetrapeptide **2** (0.89 g, 1 mmol) was dissolved in 50% TFA/DCM (20 mL), and the solution was stirred at room temperature for 20 min. After the removal of TFA and DCM, the residue was washed with dry ether (3 × 10 mL) and dissolved in DMF (10 mL). To this solution were added the powder obtained in A (5 mmol), BOP (1.86 g, 4.2 mmol), and DIEA (0.65 g, 5 mmol). After the reaction mixture was stirred for 20 h at room temperature, DMF was removed in vacuo. To the residue was added ethyl acetate (10 mL), and the solution was allowed to stand at 4 °C overnight. A small amount of product was precipitated and collected by filtration. The mother liquor was concentrated to dryness and then dissolved in water (3 mL). After the addition of acetic acid (4 drops), the emerging precipitate (HOBt) was filtered off. The filtrate was concentrated to dryness. After the residue was washed with dry ether (4 × 10 mL), a white powder was obtained and combined with the precipitate obtained from ethyl acetate (see above). The combined product was purified on a silica gel (40 g, 130−270 mesh, 60 Å, Aldrich) column using $CHCl_3$/EtOAC/MeOH (60:25:15) as eluent. The title compound was obtained after combination of pure fractions. Yield: 680 mg (75.9%). MALDI-MS: 919.2 ± 0.9 (calcd for M + Na^+, 918.99). Anal. Calcd for $C_{38}H_{69}N_7O_{17}$ (896.00): C, 50.94; H, 7.76; N, 10.94. Found: C, 50.82; H, 8.10; N, 10.77.

CHOCO-Lys(CHOCO)-Lys[CHOCO-Lys(CHOCO)]-Ala-OH (4). To the solution of peptide **3** (134.4 mg, 0.15 mmol) in water (10 mL) and methane (2 mL) was added NaOH (1.8 mL of 0.1 N, 0.18 mmol). After the mixture was stirred at room temperature for 2 h, the hydrolysis reaction was completed according to TLC analysis. Methanol was removed in vacuo, and the pH of the remaining aqueous solution was brought to 7.5 by adding 0.1 N HCl (0.25 mL). After lyophilization, the white powder was dissolved in water (1.5 mL). Five hundred microliters (50 μmol) of this solution was taken out and added to concentrated HCl (5 mL). After stirring at room temperature for 3 min, the solution was concentrated to dryness in vacuo on a water bath at 35 °C. The residue was purified by RP-HPLC. After lyophilization, the glyoxylyl core peptide was obtained. Yield: 22.5 mg (64.5%). MALDI-MS: 699.0 ± 0.9 (calcd, 698.7).

Reaction Conditions of Peptide Dendrimer Formation through Oxime, Hydrazone, and Thiazolidine Ligations. All peptides were dissolved in water to give a 5 mM stock solution. The glyoxylyl core peptide was dissolved in water as a 5 mM stock solution. For different ligation experiments in aqueous media, peptide stock solution (50 μL) was mixed with water (50 μL), 0.2 M Na/HOAc buffer (100 μL), and glyoxylyl core solution (5 μL). The final concentration was 1.25 mM for peptide and 0.125 mM for glyoxylyl core. For ligation in 50% organic cosolvent, peptide stock solution (50 μL) was mixed with 0.4 M Na/HOAc buffer (50 μL), individual organic solvent (100 μL), and glyoxylyl core solution (5 μL). For ligation using 5 equiv of peptide, peptide stock solution (100 μL) was mixed with 0.2 M Na/HOAc buffer (100 μL) and glyoxylyl core solution (5 μL). The final concentration was 2.5 mM for peptide and 0.125 mM for glyoxylyl core peptide. All the reactions were followed by RP-HPLC analysis; 5 μL of reaction solution was taken at various time intervals and analyzed through RP-HPLC. The calculated rates of product formation are summarized in Tables 2−4.

Synthesis of Peptide Dendrimers through Oxime, Hydrazone, and Thiazolidine Ligations. To peptide stock solution (400 μL, 2 μmol) were added 0.4 M Na/HOAc buffer (400 μL) and glyoxylyl core solution (40 μL, 0.2 μmol). DMSO (800 μL, in the cases of oxime and hydrazone ligation) or DMF (800 μL, in the case of thiazolidine ligation) was added to the solution. The pH of the solutions was adjusted to the following values: oxime ligation, 5.7; hydrazone ligation, 5.7; and thiazolidine ligation, 4.5. The reactions solutions were incubated at room temperature for 10, 5, and 8 h for the oxime, hydrazone, and thiazolidine reactions, respectively. The ligation products were purified by RP-HPLC and then lyophilized. In the case of hydrazone products, the solution pH was adjusted to 5.5 before lyophilization. Yield: oxime ligation, 1.1 mg (53.8%); hydrazone ligation, 7.1 mg (containing sodium trifluoroacetate); thiazolidine ligation, 1.2 mg (58.0%). All products gave a single peak in both analytical RP-HPLC and SE-HPLC. The products were characterized by MALDI-MS (Table 1). The amino acid analyses of the ligation products also gave the expected compositions.

Circular Dichroism. Peptide VA20 (2 × 10^{-4} M), tetravalent lysinyl core peptide Ac_4TLCP (5 × 10^{-5} M), and peptide dendrimers $(OxmVA20)_4$TLCP (5 × 10^{-5} M) and $(ThzVA20)_4$TLCP (5 × 10^{-5} M) were dissolved in 50% TFE/H_2O. CD measurements were performed in a 0.4 mL cuvette of 0.2 cm path length.

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Supplementary Material Available: RP-HPLC and SE-HPLC of final dendrimeric products and LD-MS analysis of the dendrimeric products (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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