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Solid-phase synthesis of tailed cyclic RGD peptides using glutamic acid: unexpected glutarimide formation

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Abstract: To provide multiple conjugating sites on cyclic peptides for their increasing biomedical applications, a tailed cyclic RGD peptide, c[RGDfE(GGGKK-NH_2)] was designed with c(RGDfE) linked through Glu to a tail consisting of a spacer of three Gly residues and a linker of two Lys residues. The spacer is used to increase the mobility and binding ability of the c(RGDfE) ligand, and the linker is used to proved multiple active sites for conjugating other molecules or biomaterials. We found that the sequence of Glu(Gly)-OAll leads to glutarimide formation, which disrupts the formation of cyclic RGD peptides. However, our results show that glutarimide formation is sequence dependent and can be inhibited by incorporating an amino acid like Lys(Boc) with steric hindrance from the protecting group. To prevent glutarimide formation, Ser(*t*Bu) was used to replace the glycine in the GGG spacer adjacent to the residue of Glu, and a tailed cyclic RGD peptide, c[RGDfE(SGGKK-NH_2)] was successfully obtained.

Keywords: glutarimide formation; cyclic RGD peptide; solid-phase peptide synthesis; glutamic acid; allyl protecting group

INTRODUCTION

Cyclic peptides are important synthetic targets because of their importance in biomedical uses as cell-specific adhesive ligands, protein mimics, antibiotics and other therapeutic agents [1–3]. The constraint imposed by cyclization results in increased stability in solution and resistance to proteolysis as well as higher specificity and enhanced biological activity in comparison with linear analogs [4,5]. Cyclic peptides can be synthesized by cyclizing linear peptides either in solution or on resin. The on-resin method has been more efficient to prepare cyclic peptides than the solution-phase approach. The principle of on-resin cyclization is based on anchoring the peptide to the resin through a side chain and applying orthogonal protecting groups for ring closure. It possesses advantages, such as timesaving, less side reactions and easy purification.

Cyclic RGD peptides, like cyclo(Arg-Gly-Asp-D-Phe-Val) [c(RGDfV)], efficiently compete with native adhesion proteins like vitronectin and fibronectin for specific binding to integrins [6]. To provide conjugating sites for their increasing applications in drug delivery, tumor imaging, surface modification and tissue engineering [7–10], a tailed cyclic peptide of c[RGDfE(tail)] was designed in our laboratory with c(RGDfE) linked to a tail consisting of a spacer and a linker, as shown in Figure 1. The spacer is used to increase the mobility of the c(RGDfE) ligand, which may lead to enhanced binding ability. The linker may consist of multiple active sites for conjugating with other molecules or biomaterials. For example, a tailed cyclic peptide with a linker consisting of multiple Lys residues, has the potential use for coupling with acryloyl-PEG-NHS to make bioactive PEG diacrylate (PEGDA) [11]. Compared with c(RGDfK) with limited spacer length and only one free amino group on Lys for conjugation, the design of tailed cyclic RGD peptides have the advantage to control the flexibility through the spacer and provide multiple conjugating sites through the linker.

Allylic groups have been used as common orthogonal protecting groups for on-resin cyclization with Fmoc strategy [12,13]. For example, All and Aloc groups, for protecting carboxylic and amino groups, respectively, enable the construction of a lactam bridge selectively between carboxylic and amino groups while the peptide is still attached to the resin. One major problem with using aspartic acid for orthogonal peptide synthesis is the formation of aspartimide [14–17]. Glutamic acid has a similar structure to aspartic acid. However, to the best of our knowledge, there is little research on the study of glutarimide formation [18,19].

Here, we report on unexpected glutarimide formation in the orthogonal solid-phase synthesis of tailed cyclic peptides using Fmoc-protected glutamic acid α-allyl ester (Fmoc-Glu-OAll). Initially, c[RGDfE(GGGKK-NH_2)] was designed with c(RGDfE) linked through Glu to a tail consisting of a spacer of three Gly residues

Abbreviations: As recommended in *J. Pep. Sci.* 2006; **12**: 1–12, with the following additions and variations: All, allyl; Aloc, allyloxycarbonyl; Apd, 3-amino piperidine 2,5-dione; c(RGDfE), cyclo(Arg-Gly-Asp-D-Phe-Glu); DEDTC, sodium diethyldiethio-carbamate trihydrate; DIPEA, diisopropylethylamine; EDT, 1,2-ethanedithiol; NHS, *N*-hydroxyl succinimide; NMP, 1-methyl-2-pyrrolidinone; $Pd(PPh_3)_4$, tetrakis-(triphenyl phosphine) palladium (0); RGD, Arg-Gly-Asp; *t*Bu, *t*-butyl ether; TIPS, triisopropylsilane.

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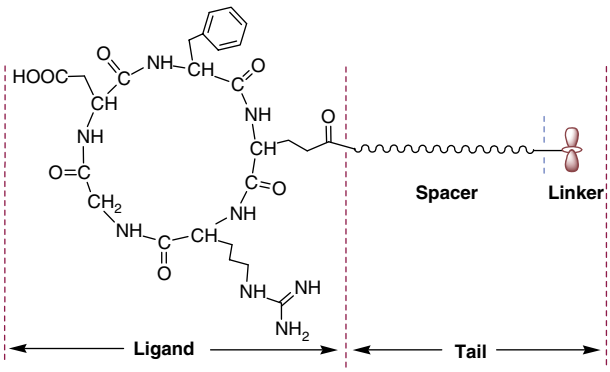


Figure 1 Tailed cyclic peptide of c[RGDfE(tail)] with c(RGDfE) linked to a tail consisting of a spacer and a linker.

(GGG) and a linker of two Lys residues (KK). We found that the sequence of Glu(Gly)-OAll leads to glutarimide formation, which disrupts the formation of cyclic RGD peptides. MALDI-TOF MS and ^{1}H NMR were used to confirm glutarimide formation. The prevention of glutarimide formation was studied by incorporating amino acids with steric hindrance next to Glu.

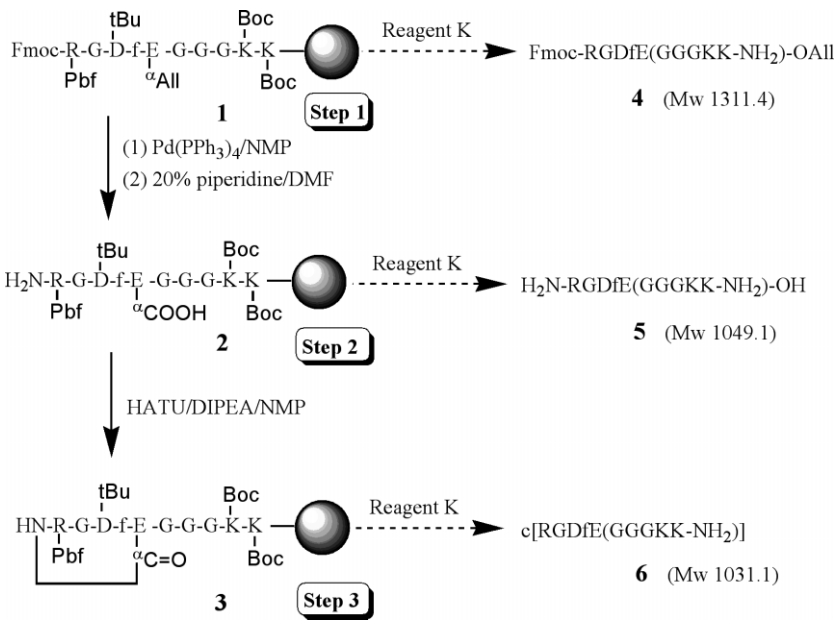
MATERIALS AND METHODS

Fmoc-protected amino acids were purchased from AnaSpec Inc. (San Jose, CA). PAL resin with a loading of 0.36 mmol/g was purchased from PE Biosystems (Foster City, CA). HATU was purchased from GenScript Corp. (Piscataway, NJ). The cleavage cocktail of Reagent K was prepared prior to use according to TFA/phenol/H_2O/EDT/TIPS (85/5/5/2.5/2.5). Mass spectrometry was performed on a Bruker Biflex III MALDI-TOF mass spectrometer by dissolving the sample and the matrix of 2,5-dihydroxybenzoic acid in 1 : 1 (v/v) ethanol and water, and mass spectra were typically accumulated from 200 laser shots. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer.

Peptides were synthesized by a three-step synthesis method in a 0.25 mmol scale with PAL resin to produce an amide *C*-terminus on a 433A automatic peptide synthesizer (Applied Biosystems). An example for synthesizing designed c[RGDfE(GGGKK-NH_2)] was shown in Scheme 1. Standard Fmoc strategy was used in Step 1. Removal of the All group in Step 2 was performed manually on Peptide Synthesizer by 3 equiv of $Pd(PPh_3)_4$ in a mixture solvent of acetic acid/NMM/DCM (1.6/0/8/30) for 2 h, followed by washing sequentially by 0.5% DEDTC in DIPEA/NMP, DCM and NMP. Then, 20% piperidine in DMF was transferred to the reaction vessel to remove Fmoc. The resulting linear peptides were cyclized in Step 3 using 4 equiv of HATU in DIPEA/NMP (1 : 9). Peptides were cleaved by Reagent K at room temperature for 2–3 h, followed by filtration, precipitation in cold ether, centrifugation and drying under vacuum. Crude peptides were further purified by reverse phase-HPLC on a Waters 2690 Alliance system equipped with a Waters 2487 UV detector and an Alltech Hyperprep PEP 100A C18 column (250 × 22 mm, 8 μm), using a 30–50% acetonitrile gradient in water with 0.01% TFA (v/v).

RESULTS AND DISCUSSION

In the initial experiment, a spacer of three Gly residues (GGG) was used to extend the linker out, and a linker of two Lys residues (KK) was used to provide two free amine groups as active sites for further conjugation with other biomaterials. The solid-phase synthesis of tailed cyclic peptides included three major steps, as shown in Scheme 1. First, a protected linear peptide on PAL resin **1** was synthesized. Then, All and Fmoc groups were removed sequentially by $Pd(PPh_3)$ and



Scheme 1 Three-step synthesis of designed c(RGDfE) linked with a tail of GGGKK.

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piperidine to produce a deprotected linear peptide on the resin **2**. Finally, cyclization of **2** was carried out using HATU for the expected formation of the cyclic peptide of c[RGDfE(GGGKK-NH_2)] on the resin **3**. Theoretically, three free peptides, including an Fmoc/All protected linear peptide [**4**, molecular weight (MW) 1311.4], a deprotected linear peptide (**5**, MW 1049.1) and a cyclic peptide (**6**, MW 1031.1) would be obtained from their cleavage from the peptide-bound resins **1–3**, respectively, by using Reagent K containing 85% TFA.

MALDI-TOF MS analysis was performed to verify the peptides obtained in each step in Scheme 1. Unexpectedly, as shown in Figure 2, the MW of the peptides obtained in Steps 1 and 2 were 1253.8 and 1031.3, respectively. They did not match with the desirable structures of peptides **4** and **5**. Further analysis shows that the MW of the obtained peptide in Step 1 is 58 less than the expected structure of **4**, which is equal to the MW of allyl alcohol. This indicates that the All group may be eliminated during Step 1. The peptides obtained in Step 3 show two major products with MW of 1083.8 and 1115.9, with ΔMW_{23} of 52.1 and 84.5, respectively. We did combinational calculations including adducts with the possible hydrolytic products from HATU, but still did not find a satisfied explanation due to the complexity of the hydrolysis of HATU [20,21].

To confirm the elimination of the All group, ^1H NMR analysis was carried out. Fmoc-Glu-OAll in $CDCl_3$ shows two characteristic proton peaks for the double bond of the All group at 5.25 (2H, $-CH{=}C\boldsymbol{H}_2$) and 5.95 ppm (1H, $-C\boldsymbol{H}{=}CH_2$), which disappeared in the NMR spectrum of the peptide in D_2O obtained in Step 1 in Scheme 1. No allyl proton signals were observed from the peptide obtained in Step 1 in Scheme 1, suggesting the elimination of the All group in this step. This is also consistent with ΔMW_{12} of 222 (Table 1), which equals the MW of one Fmoc leaving group. As we know, aspartimide is easy to form when using the sequence of Asp-Gly in peptide synthesis. Our results indicate that the sequence of Glu(Gly)-OAll (**7**), as shown in Scheme 2, follows the same trend to form a cyclic imide, glutarimide (**9**). In the transition state (**8**), the attack of the NH group of Gly on the O-All protected α-carboxyl group of Glu is catalyzed by bases like piperidine. The leaving of the All group and the closure of a six-member ring proceed simultaneously to form glutarimide with a structure of Apd. The MW of the resulting Apd-Gly (**9**) is 58 less than that of Glu(Gly)-OAll due to the elimination of one molecule of allyl alcohol (**10**), which is consistent with the MW difference observed in Step 1.

Based on the above analysis, the actual structures of the peptides formed in Steps 1 and 2 in Scheme 1 are **11** and **12**, as shown in Figure 3. They have an Apd unit instead of a Glu residue. HPLC analysis, as shown in Figure 4(a) demonstrates that Fmoc-RGDfE(GGGKK)-OAll resulted in a quantitative Apd formation (100%) in Step 1, and the yield results are listed in Table 2. Mass spectra (Figure 2) shows that **12** is the major products in Step 2, and there were no further addition of products with piperidine. Thus, glutarimide is stable in basic conditions like piperidine due to its six-member ring structure, but **12** may react with byproducts from HATU hydrolysis to form peptide adducts in Step 3 [19]. Glutarimide

Table 1 MW of peptides obtained in the three-step synthesis of tailed cyclic RGD peptides of c[RGDfE(tail)]

Tail	MW from MALDI-TOF MS			ΔMW_{12}[a]	ΔMW_{23}[b]
	Step 1	Step 2	Step 3		
GGGKK	1253.8	1031.7	N/A[c]	222.1	N/A[c]
KK	1139.8	878.0	860.1	261.8	17.9
SGGKK	1340.8	1078.8	1060.7	262.0	18.1

[a] MW difference between the peptides obtained in Steps 1 and 2.
[b] MW difference between the peptides obtained in Steps 2 and 3.
[c] Not applicable.

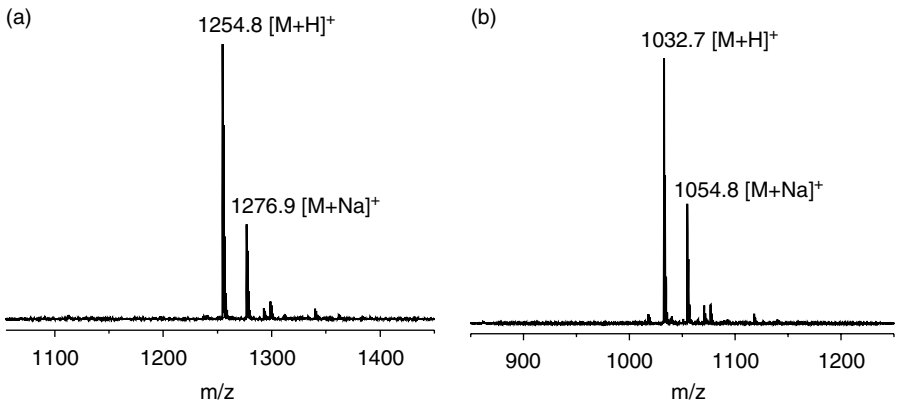

Figure 2 MALDI-TOF MS spectra of peptides obtained in Step 1 (a) and Step 2 (b) in the three-step synthesis of peptides with a GGG spacer.

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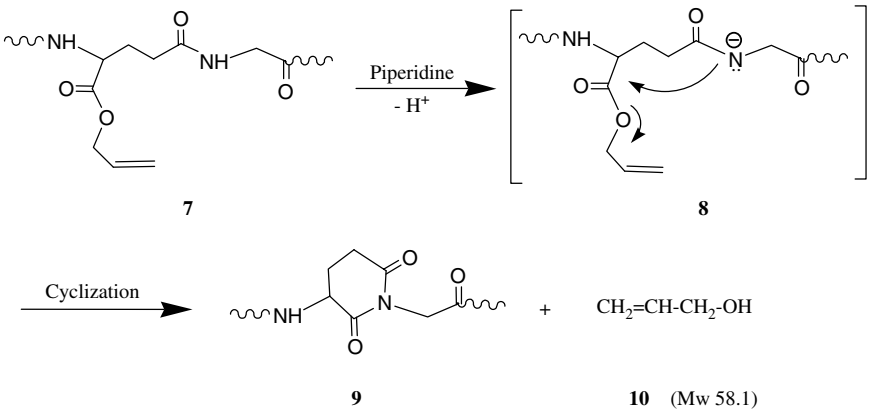

Scheme 2 Mechanism of glutarimide formation in Glu(Gly)-OAll.

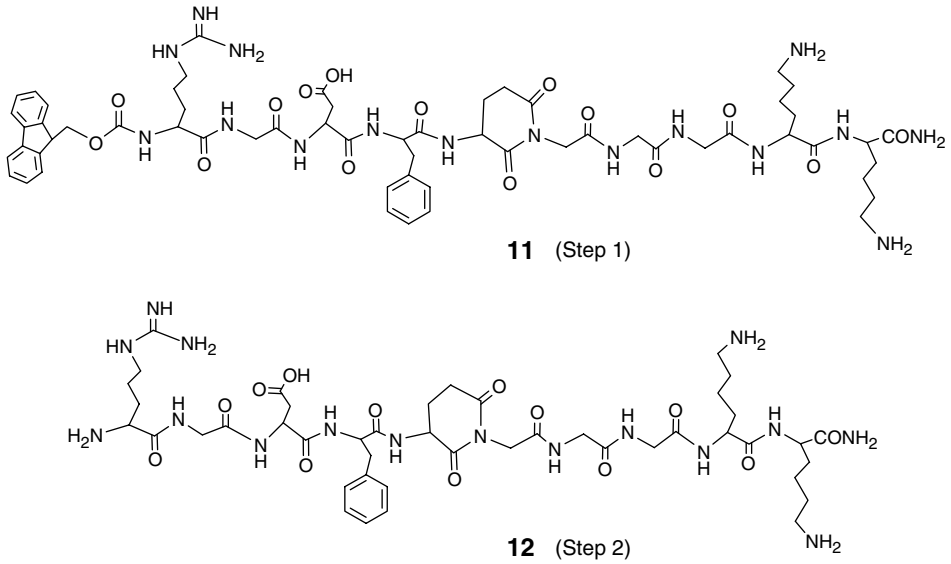

Figure 3 Peptide structures obtained in Step 1 (**11**) and Step 2 (**12**) in the three-step synthesis of designed c(RGDfE) with a GGG spacer.

Table 2 Peptide yields in the first step synthesis of tailed cyclic RGD peptides determined by HPLC

Peptide	Expected MW	Glu-containing peptide (%)	Apd-containing peptide (%)	Found MW
Fmoc-RGDfE(GGGKK)-OAll	1311.4	0.0	100[a]	1253.5
Fmoc-RGDfE(KK)-OAll	1140.0	99.1	0	1139.8
Fmoc-RGDfE(SGGKK)-OAll	1341.4	93.1	0	1341.3

[a] MW of Apd-containing peptide is 1253.4.

formation from the sequence of Glu(Gly)-OAll in Step 1 results in no carboxylic group available from Glu in **12**, and thereafter disrupts the formation of cyclic RGD peptides between Arg and Glu in Step 3. This analysis provides a reasonable explanation on what happened in the orthogonal solid-phase synthesis of designed cyclic RGDfE peptides with a GGGKK tail through Glu.

A control peptide, c[RGDfE(KK-NH_2)] without the GGG spacer, was synthesized in the similar three-step synthesis as shown in Scheme 1, in order to elucidate the sequence effect of glycine on glutarimide formation. Mass analysis shows that all three peptides had the MW as expected. The MW difference between **13** and **14** (ΔMW_{12}) is 262, equal to one Fmoc group

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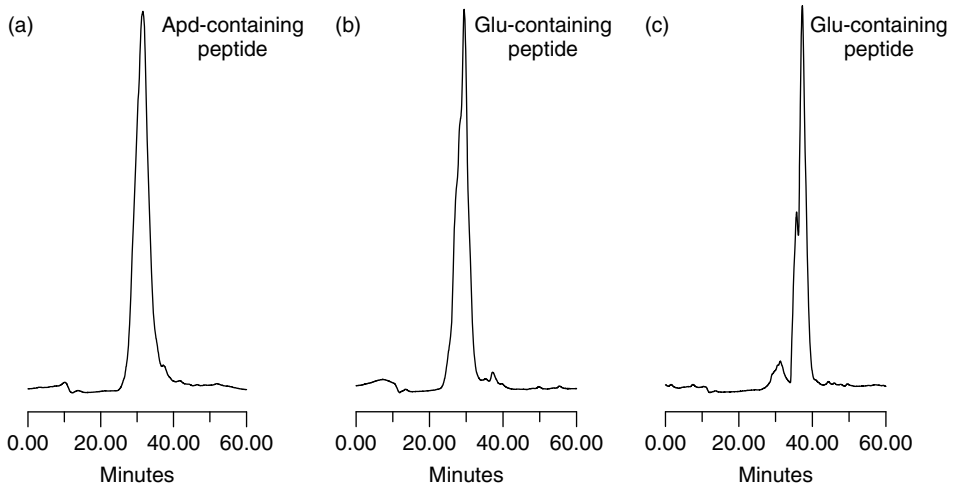

Figure 4 HPLC profiles of crude products obtained in Step 1 in the synthesis of cyclic RGDfE peptides with tails of GGGKK (a), KK (b) and SGGKK (c).

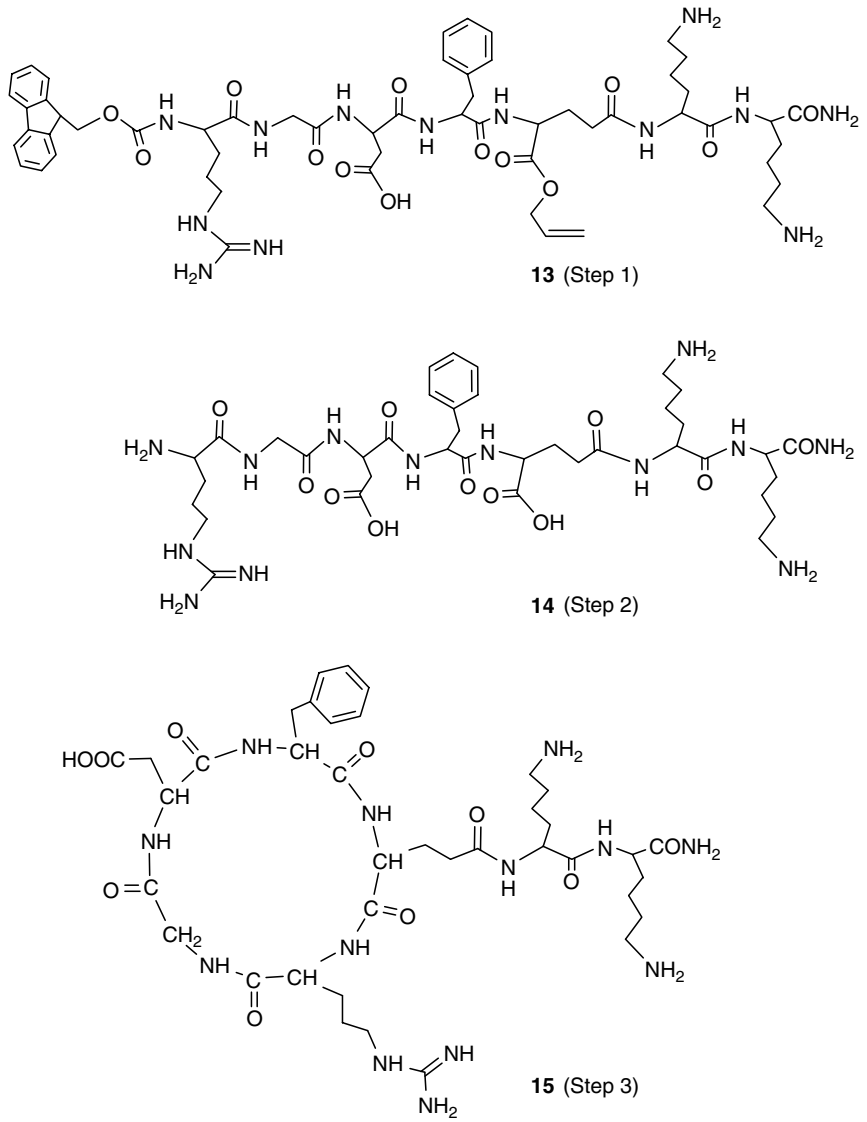

Figure 5 Peptide structures obtained in the three-step synthesis of designed c[RGDfE(KK-NH_2)] without the GGG spacer.

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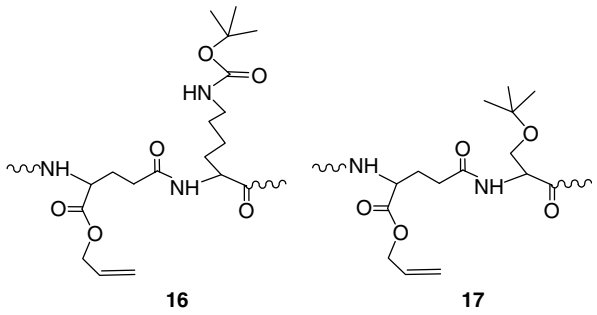
plus one All group while the MW difference between **13** and **15** (ΔMW_{23}) is 18, equal to one molecule of water (Table 1). These results show that both Fmoc and All groups remained in Step 1 and were removed in Step 2, and formed a ring structure in Step 3. Their structures are shown in Figure 5. HPLC analysis of the peptides obtained in Step 1 also shows no glutarimide formation as shown in Figure 4(b). It demonstrates that the hindrance from the Boc protecting group, as shown in Figure 6 (**16**), can prevent the NH of lysine to attack the O-All protected carboxyl of Glu, leading to no glutarimide formation in Step 1 (Table 2). Therefore, glutarimide formation is sequence dependent and can be inhibited by an amino acid with steric hindrance.

To prevent glutarimide formation in the sequence of Glu(Gly)-OAll while still preserving a spacer between the ligand and the linker, c[RGDfE(SGGKK-NH_2)] was designed by using Ser(*t*Bu) to replace the glycine in the GGG spacer adjacent to the residue of Glu. The sequence of Glu[Ser(*t*Bu)]-OAll (**17**) (Figure 6) is expected to provide a similar steric hindrance to prevent glutarimide formation as Glu[Lys(Boc)]-OAll (**16**). HPLC analysis of the peptides obtained in Step 1 also shows no glutarimide formation, as shown in Figure 4(c). The results from MALDI-TOF mass analysis (Tables 1 and 2) show that the Fmoc and All groups still remained in the Step 1 peptide (**18**), as shown in Figure 7. The peptides



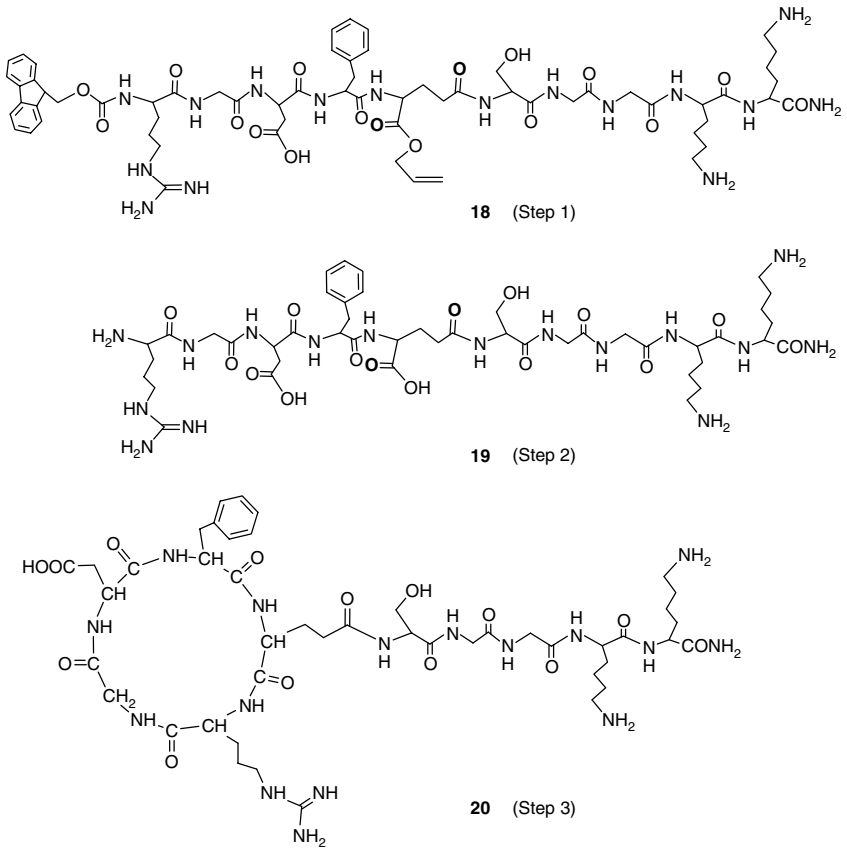
16 **17**

Figure 6 Sequence structures of Glu[Lys(Boc)]-OAll (**16**) and Glu[Ser(*t*Bu)]-OAll (**17**).

18–20 obtained in the three-step synthesis had the expected MW with ΔMW_{12} of 262 and ΔMW_{23} of 18. It indicates that the *t*Bu protective group of serine, like the Boc protective group of lysine, can provide steric hindrance to prevent glutarimide formation. As a result, cyclic RGD peptide **20** with a SGGKK tail was formed in Step 3 from the cyclization of 19 between Arg and Glu.

CONCLUSIONS

We present here the occurrence of glutarimide formation in orthogonal solid-phase synthesis of cyclic RGD



18 (Step 1)

19 (Step 2)

20 (Step 3)

Figure 7 Peptide structures obtained in the three-step synthesis of c[RGDfE(SGGKK-NH_2)].

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peptides linked to a GGGKK tail using Fmoc-Glu-OAll. The Glu(Gly)-OAll sequence leads to glutarimide formation, which disrupts the formation of cyclic RGD peptides. The results show that glutarimide formation is sequence dependent and can be prevented by incorporating a sterically hindered amino acid like Lys(Boc) adjacent to the residue of Glu. To prevent glutarimide formation while still preserving a spacer between the c(RGDfE) ligand and the linker of KK, Ser(*t*Bu) was used to replace the glycine in the GGG spacer adjacent to the residue of Glu, and a tailed cyclic RGD peptide, c[RGDfE(SGGKK-NH_2)] was successfully obtained.

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REFERENCES

1. Lambert JN, Mitchell JP, Roberts KD. The synthesis of cyclic peptides. *J. Chem. Soc., Perkin Trans. 1* 2001; **2001**(5): 471–484.
2. Davies JS. The cyclization of peptides and depsipeptides. *J. Pept. Sci.* 2003; **9**: 471–501.
3. Schaffner P, Dard MM. Structure and function of RGD peptides involved in bone biology. *Cell. Mol. Life Sci.* 2003; **60**: 119–132.
4. Rizo J, Gierasch LM. Constrainted peptides: models of bioactive peptides and protein substructures. *Annu. Rev. Biochem.* 1992; **61**: 387–418.
5. Hruby VJ, Al-Obeidi F, Kazmierski W. Emerging approaches in the molecular design of receptor-selective peptide ligands: conformational, topographical and dynamic consideration. *Biochem. J.* 1990; **268**: 249–262.
6. Thumshirn G, Hersel U, Goodman SL, Kessler H. Multimeric cyclic RGD peptides as potential tools for tumor targeting: solid-phase peptide synthesis and chemoselective oxime ligation. *Chem. Eur. J.* 2003; **9**: 2717–2725.
7. Nasongkla N, Shuai X, Ai H, Weinberg BD, Pink J, Boothman DA, Gao J. cRGD-functionalized polymer micelles for targeted doxorubicin delivery. *Angew. Chem. Int. Ed. Engl.* 2004; **43**: 6323–6327.
8. Liu S. Radiolabeled multimetric cyclic RGD peptides as integrin $\alpha_v\beta_3$ targeted radiotracers for tumor imaging. *Mol. Pharm.* 2006; **3**: 472–487.
9. Auernheimer J, Dahmen C, Hersel U, Bausch A, Kessler H. Photoswitched cell adhesion on surfaces with RGD peptides. *J. Am. Chem. Soc.* 2005; **127**: 16107–16110.
10. Jeschke B, Meyer J, Jonczyk A, Kessler H, Adamietz P, Meenen NM, Kantlehner M, Goepfert C, Nies B. RGD-peptides for tissue engineering of articular cartilage. *Biomaterials* 2002; **23**: 3455–3463.
11. Zhu J, Beamish JA, Tang C, Kottke-Marchant K, Marchant RE. Extracellular matrix-like cell-adhesive hydrogels from RGD-containing poly(ethylene glycol) diacrylate. *Macromolecules* 2006; **39**: 1305–1307.
12. Brase S, Kirchhoff JH, Kobberling J. Palladium-catalysed reactions in solid phase organic synthesis. *Tetrahedron* 2003; **59**: 885–939.
13. Kates SA, Daniels SB, Albericio F. Automated allyl cleavage for continuous-flow synthesis of cyclic and branched peptides. *Anal. Biochem.* 1993; **212**: 303–310.
14. Tam JP, Riemen MW, Merrifield RB. Mechanisms of aspartimide formation: the effects of protecting groups, acid, base, temperature and time. *Pept. Res.* 1988; **1**: 6–18.
15. Mergler M, Sax DB, Weiler P, Vorherr T. The aspartimide problem in Fmoc-based SPPS. Part I. *J. Pept. Sci.* 2003; **9**: 36–46.
16. Flora D, Mo H, Mayer JP, Khan MA, Yan LZ. Detection and control of aspartimide formation in the synthesis of cyclic peptides. *Bioorg. Med. Chem. Lett.* 2005; **15**: 1065–1068.
17. Stathopoulos P, Papas S, Kostidis S, Tsikaris V. α- and β- Aspartyl peptide ester formation via aspartimide ring opening. *J. Pept. Sci.* 2005; **11**: 658–664.
18. Battersby AR, Robinson JC. Studies on specific chemical fission of peptide links. Part I. The rearrangement of aspartyl and glutamyl peptides. *J. Chem. Soc.* 1955; **77**: 259–269.
19. Feinberg RS, Merrifield RB. Modification of peptides containing glutamic acid by hydrogen fluoride-anisole mixtures. γ-Acylation of anisole or the glutamyl nitrogen. *J. Am. Chem. Soc.* 1975; **97**: 3485–3757.
20. Albericio F, Bofill JM, El-Faham A, Kates SA. Use of onium salt-based coupling reagents in peptide synthesis. *J. Org. Chem.* 1998; **63**: 9678–9683.
21. Alsina J, Barany G, Albericio F, Kates SA. Pyrrolidide formation as a side reaction during activation of carboxylic acids by phosphonium salt coupling reagents. *Lett. Pept. Sci.* 1999; **6**: 243–245.