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Aspartic Acid Side-Chain Benzyl Ester as a Multifunctionalization Precursor for Synthesis of Branched and Cyclic Arginylglycylaspartic Acid Peptides

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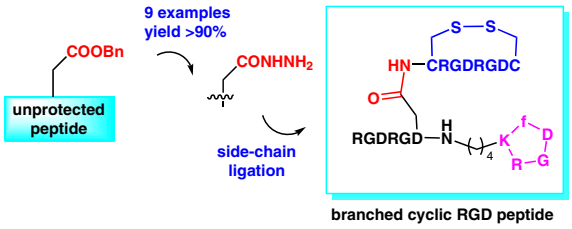

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Abstract Here, we report a peptide aspartic acid side-chain benzyl ester as a useful precursor that can be efficiently converted into various functional groups, including acid, amide, carbonyl hydrazide, carbonyl azide, or thio ester groups, without other protection for the peptide. With this strategy, we synthesized a series of novel branched and cyclic arginylglycylaspartic acid peptides through successive peptide C-terminal ligation and side-chain ligation based on a side-chain carbonyl azide or thio ester.

Key words peptide side-chain ligation, side-chain benzyl ester, peptide hydrazide, arginylglycylaspartic acid peptides, branched cyclic peptides

Branched and cyclic peptides have recently attracted increased research interest because of their biological functions in protein domain simulation,[1–4] vaccine design,[1,5,6] receptor-binding investigations,[7] and enhanced stability and optimized half-lives for therapeutic peptide drugs[8,9]. The arginylglycylaspartic acid (RGD) tripeptide unit is a common motif that is involved in interaction with integrins,[10] and previous research has shown that a series of cyclic RGD peptides[11] are selective inhibitors of particular integrins such as $\alpha_V\beta_3$, which is overexpressed in many cancers and mediates tumor metastasis.[12] The development of novel RGD peptides as tumor-targeting reagents or anticancer therapeutics has potential applications in cancer treatment.[13,14]

In our previous work,[15] we employed peptide side-chain hydrazide ligation for the synthesis of branched and cyclic peptides. This strategy permitted the assembly of a hydrazide functional group on the asparagine (Asn) side chain, and successive azidation with $NaNO_2$, thio ester formation with (4-sulfanylphenyl)acetic acid (MPAA), and peptide side-chain ligation with an N-terminal cysteine (Cys) peptide then gave the target branched peptides, following Liu and co-workers' approach of linear peptide hydrazide ligation.[16,17] Compared with other methods for branched-peptide synthesis, such as selective deprotection of side-chain carboxylic acid or amine groups[18,19] or incorporation of nonnatural amino acids for side-chain conjugation,[20,21] this method permits efficient ligation of unprotected peptides with a common amide linkage.

In our previous work, we used an Asn building block with a benzyloxycarbamate (Cbz)-protected side-chain hydrazine moiety [Fmoc-Asn(NHNHCbz)-OH] to introduce the hydrazide onto a peptide side chain. However, the use of the strong acid trifluoromethanesulfonic acid in the deprotection of the Cbz moiety led to a moderate yield and, consequently, this protecting strategy is not suitable for substrates with an acid-labile structure, such as the glycosidic bond in glycopeptides. Here, we present a better protection strategy that uses an Asp side-chain benzyl ester, which can be efficiently converted into various functional groups, including acid, amide, hydrazide, carbonyl azide, or thio ester groups, under mild conditions. With this strategy, we prepared a series of novel RGD branched and cyclic peptides through side-chain ligation using a carbonyl azide or thio ester. A dual-ligation approach involving successive C-terminal and side-chain ligations was employed to synthesize complicated RGD branched peptides.

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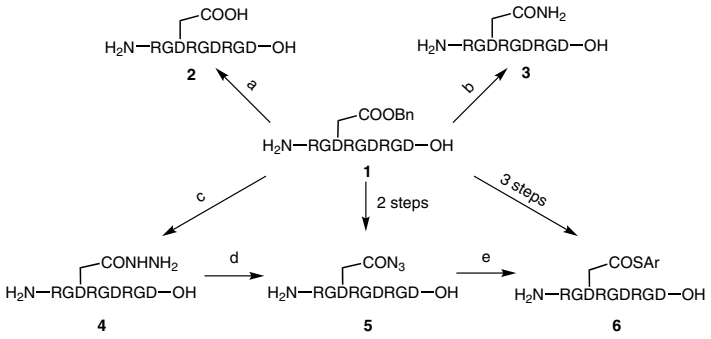

Scheme 1 Peptide side-chain functional-group conversions from a benzyl ester. *Reaction conditions*: (a) 10 mM aq K_2CO_3, r.t., 20 min, 90%; (b) 1% NH_3 in DMF, r.t., 30 min, 85%; (c) 5% N_2H_4 in DMF, r.t., 15 min, 95%; (d) $NaNO_2$, pH 2, –10 °C, 15 min, 90%; (e) MPAA, pH 5.5, –10 °C, 15 min, 95%.

We chose Fmoc-Asp(OBn)-OH as a building block for peptide synthesis. The benzyl ester exhibited good stability in Fmoc-based solid-phase peptide synthesis (SPPS) and later-stage ligation reactions. The RGD peptide **1** carrying a side-chain benzyl ester was prepared for side-chain functional-group conversion. As shown in Scheme 1 and Figure 1, the benzyl ester, as a useful precursor, could be efficiently converted into various functional groups. On hydrolysis with potassium carbonate or aminolysis with ammonia, the native Asp or Asn peptides **2** and **3**, respectively, were obtained in 85–90% yields within 20–30 min (Figures 1b and 1c). When treated with 5% NH_2NH_2 in DMF at r.t. for 15 min, benzyl ester **1** underwent complete hydrazinolysis to give the side-chain hydrazide peptide **4** in an excellent yield (Figure 1d). More examples of side-chain conversions of benzyl ester groups into hydrazide groups are available in the Supplementary Information. By following the approach of Liu and co-workers[16] and our previous method,[15] the side-chain hydrazide group was converted into the carbonyl azide **5** (Figure 1e) by treatment with $NaNO_2$ in acidic solution. Azide **5** was subsequently converted into the thio ester **6** (Figure 1f) by treatment with MPAA. These results demonstrated that a side-chain benzyl ester, acting as a multifunctionalization precursor, can be applied in temporary protection of Asp and Asn moieties or in introducing side-chain ligation handles such as hydrazide (one-step conversion), azide (two-step conversion), or thio ester (three-step conversion) in high yields under mild and expeditious conditions. The RGD peptides with a side-chain carbonyl azide group **5** or thio ester group **6** were able to perform ligations through direct amidation (using peptide **5**) with a peptide N-terminal amine or side-chain native chemical ligation (NCL) with an N-terminal Cys-peptide to give the corresponding branched RGD peptides.

The efficient conversion of the side-chain benzyl ester into a thio ester suggested that this might be a useful strategy for synthesizing branched peptides. Therefore, we employed the thio ester **6** to perform side-chain NCL with the N-terminal Cys-peptide CRGDRGDC, which contains two RGD units and a potential disulfide motif for cyclization. In our previous work,[15] a byproduct from the intramolecular cyclization of the side-chain thio ester with the neighboring amide nitrogen was observed. Here, we optimized the ligation condition in a pH 6.0 solution at a lower temperature of 4 °C to minimize the competitive intramolecular cyclization. Figure 2b shows the ligation of **6** and the Cys-peptide under these optimal conditions. Ligation product **7a** was

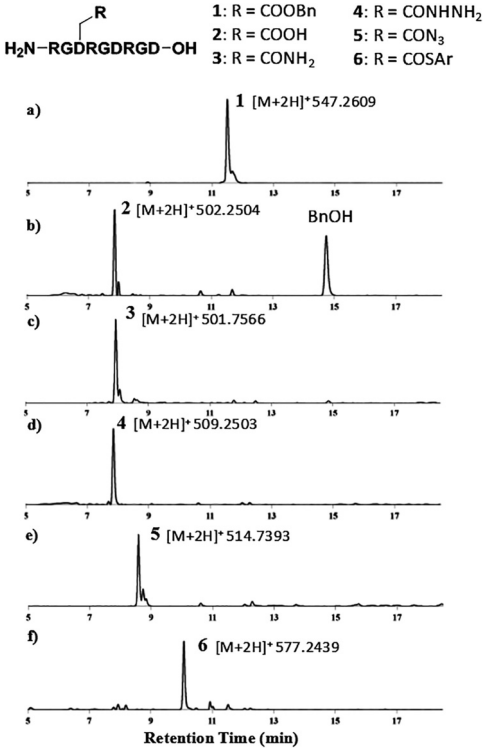

Figure 1 HPLC profiles of RGD peptide side-chain functional-group conversion of benzyl ester **1** into acid **2**, amide **3**, hydrazide **4**, azide **5**, and thio ester **6**. (a) HPLC profile of **1**; (b) **1** was treated with 10 mM K_2CO_3 at r.t. for 20 min; (c) **1** was treated with 1% ammonia in DMF at r.t. for 30 min; (d) **1** was treated with 5% $N_2H_4{\cdot}H_2O$ in DMF at r.t. for 15 min; (e) **4** was treated with $NaNO_2$ at –10 °C for 15 min; (f) **5** was treated with MPAA at –10 °C for 15 min.

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obtained in an improved yield (70%), with much less cyclization byproduct compared with the previous reported results.[15] With the purified branched peptide **7a** in hand, we conducted the disulfide-bond-formation reaction at a concentration of 0.25 mM in a sodium phosphate buffer (pH 7.5, 0.2 M) containing 20% DMSO and bubbled with air. After four hours, the branched cyclic peptide **7** was obtained in 95% yield. A general procedure for side-chain hydrazide conversion and successive ligation is described in the Notes and References section.[22]

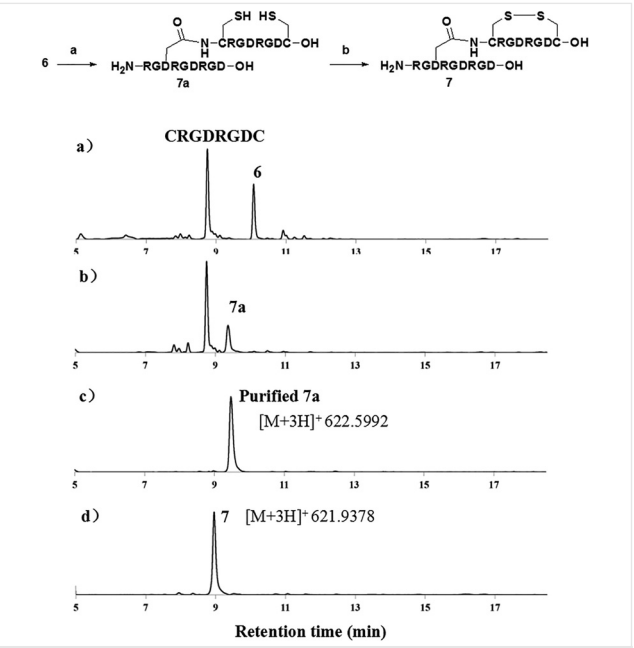
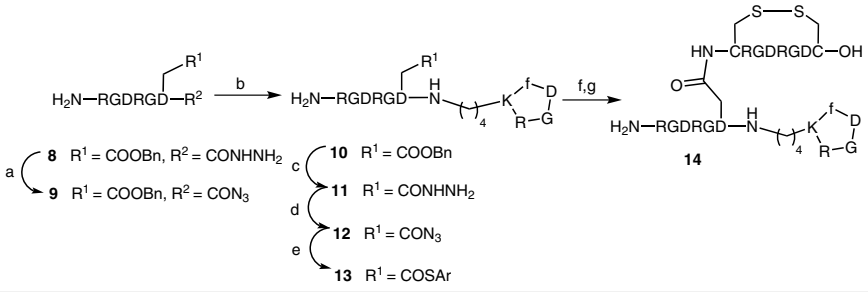


Figure 2 Synthesis of branched cyclic RGD peptide **7**. *Reaction conditions:* (a) Cys-peptide CRGDRGDC, pH 6.0, 4 °C, 8 h, 70%; (b) 20% DMSO, air, 4 h, 95%. HPLC profiles of the reactions: (a) ligation of thio ester **6** and peptide CRGDRGDC at 0 min; (b) ligation of thio ester **6** and peptide CRGDRGDC at 8 h; (c) purified branched peptide **7a**; (d) branched cyclic peptide **7**.

The successful synthesis of branched cyclic RGD peptide **7** from the side-chain benzyl ester **1** encouraged us to prepare more complicated dual-ring branched cyclic RGD peptides by a two-step ligation approach involving successive C-terminal and side-chain ligations. As shown in Scheme 2, an RGD hexapeptide **8** containing a C-terminal carbonyl hydrazide and an Asp side-chain benzyl ester was prepared as the starting material. It is noteworthy that in a previous study,[16] the peptide C-terminal Asp-hydrazide could not be obtained without side-chain protection because of cyclization during the peptide cleavage from the resin. However, with two-step cleavage or side-chain protection, this cyclization was blocked and C-terminal Asp-hydrazide ligation was possible.[23] The side-chain protection also blocked thio ester exchange between the side-chain thio ester and the C-terminal thio ester, consequently avoiding the formation of the corresponding byproduct by side-chain ligation.[23]

First, we performed a C-terminal ligation with **8**. The hydrazide was converted into the carbonyl azide **9**, which underwent direct amidation with the lysine side-chain amino group of the cyclic RGD peptide cyclo(KRGDf) (f = D-Phe)[11] in a pH 8.5 solution. The reaction was complete within 30 minutes, and gave the cyclic RGD peptide **10** in excellent yield (Figure 3b). We then attempted to perform a second ligation on the side chain of **10**. The side-chain benzyl ester of **10** was converted into the corresponding carbonyl hydrazide **11** by treatment with 5% hydrazine monohydrate in DMF (Figure 3c). Successive treatment with $NaNO_2$ and MPAA then gave the side-chain thio ester **13**. Side-chain ligation of **13** with the Cys-peptide CRGDRGDC provided the branched peptide **14a** in 60% yield (Figures 3d and 3e). The side-reaction between the side-chain thio ester and the nitrogen of the neighboring amino acid[15] gave a cyclic byproduct with a more significant rate of formation than that of thio ester **6**. This result is probably due to the lower steric hindrance of the amide in peptide **13** Asp-(side-chain)Lys compared with that of the amide of the Asp-Arg moiety in **6**, when these amide nitrogen atoms were involved in the cyclization side-reaction. Finally, disulfide-bond formation under oxidative conditions gave the dual-ring branched cyclic RGD peptide **14** in high yield (Figure 3f).



Scheme 2 Synthesis of dual-ring branched RGD peptide **14** by successive C-terminal and side-chain ligations. *Reaction conditions*: (a) $NaNO_2$, pH 2, –10 °C, 15 min, 95%; (b) cyclo(KRGDf) (5 equiv), pH 8.5, –10 °C, 30 min, 95%; (c) 5% NH_2NH_2 in DMF, r.t., 30 min, 95%; (d) $NaNO_2$, pH 2, –10 °C, 15 min, 95%; (e) MPAA, pH 5.5, –10 °C, 15 min, 95%; (f) CRGDRGDC (5 equiv), pH 6.0, 4 °C, 12 h, 60%; (g) 20% DMSO, air, r.t., 4 h, 95%.

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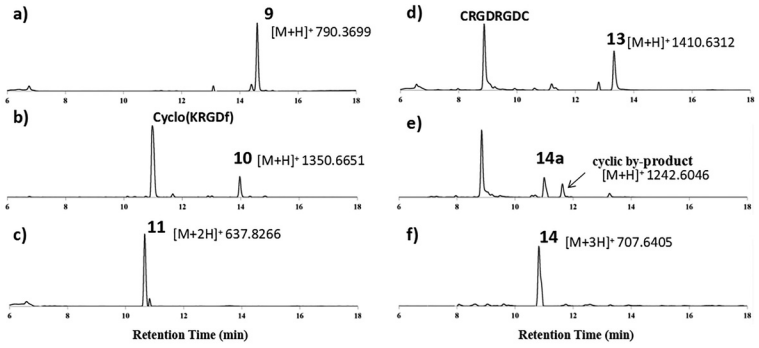
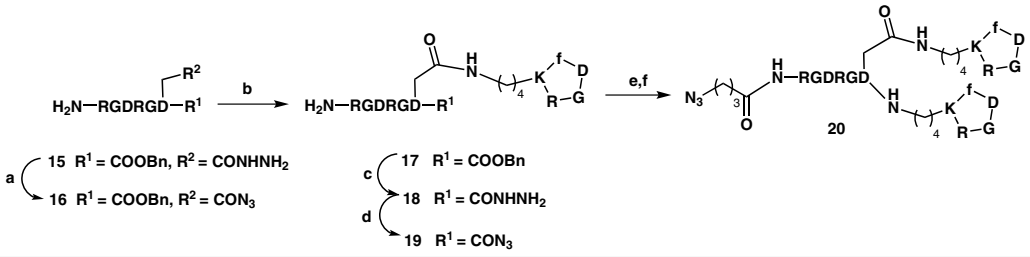


Figure 3 HPLC profiles of the procedures for the synthesis of the dual-ring branched cyclic RGD peptide **14** by successive C-terminal and side-chain ligations. (a) *In situ* hexapeptide carbonyl azide **9**; (b) C-terminal ligation of **9** and cyclo(KRGDf) for 30 min; (c) *in situ* carbonyl hydrazide peptide **11**; (d) side-chain ligation of thio ester **13** and CRGDRGDC for 0 min; (e) side-chain ligation of thio ester **13** and CRGDRGDC for 12 h; (f) *in situ* dual-ring branched cyclic RGD peptide **14** after disulfide-bond formation.

Next, we preformed successive side-chain and C-terminal ligations in the reverse order to prepare the homo-dual-ring branched cyclic RGD peptide **20**. As shown in Scheme 3, hexapeptide **15** containing a C-terminal benzyl ester and an Asp side-chain carbonyl hydrazide was employed as the starting material. Another building block, Fmoc-Asn(OH)-OBn, was used in SPPS for the synthesis of **15**, and the side-chain acid of the first amino acid Asp was coupled to the NH_2NH-CTC resin. To avoid the intramolecular cyclization side-reaction, we conducted ligation on the side chain prior to C-terminal ligation. All the processes are shown in Figure 4. The first step was to convert the side-chain hydrazide hexapeptide **15** into the carbonyl azide **16** (Figure 4a); this was followed by direct amidation with cyclo(KRGDf) to give the cyclic RGD peptide **17** (Figure 4b). Then, hydrazinolysis of the C-terminal benzyl ester on **17** was performed to give



Scheme 3 Synthesis of dual-ring branched RGD peptide **20** by successive side-chain and C-terminal ligations. *Reaction conditions*: (a) $NaNO_2$, pH 2, –10 °C, 15 min, 95%; (b) cyclo(KRGDf) (5 equiv), pH 8.5, –10 °C, 30 min, 85%; (c) 5% NH_2NH_2 in DMF, r.t., 30 min, 95%; (d) $NaNO_2$, pH 2, –10 °C, 15 min, 95%; (e) cyclo(KRGDf) (5 equiv), pH 8.5, –10 °C, 30 min, 80%; (f) $N_3(CH_2)_3COOSu$ (5 equiv), pH 7.5, r.t., 15 min, 95%.

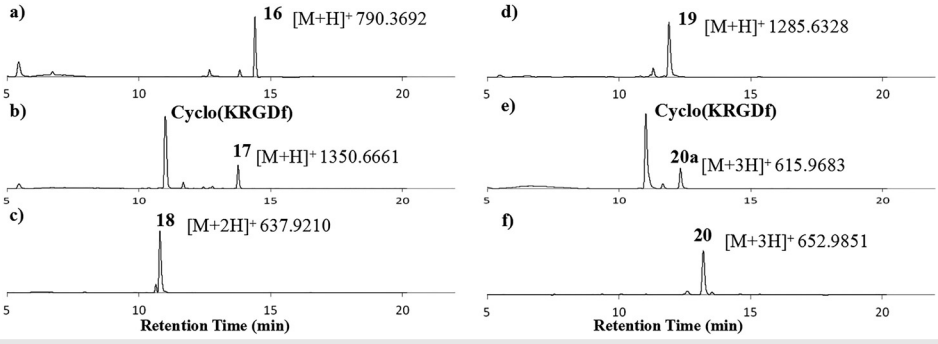


Figure 4 HPLC profiles of procedures in the synthesis of the dual-ring branched cyclic peptide **20**. (a) *In situ* RGD peptide **16** with a side-chain carbonyl azide; (b) side-chain ligation of **16** and cyclo(KRGDf); (c) *in situ* RGD peptide **18** bearing a C-terminal hydrazide synthesized by hydrazinolysis of **17**; (d) *in situ* RGD peptide **19** bearing a C-terminal carbonyl azide; (e) C-terminal ligation of **19** and cyclo(KRGDf); (f) azide labeling of dual-ring branched peptide **20** with $N_3(CH_2)_3COOSu$.

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the corresponding carbonyl hydrazide **18** in excellent yield (Figure 4c). After treatment with $NaNO_2$, the C-terminal carbonyl azide **19** was obtained (Figure 4d), which was treated with cyclo(KRGDf) for a second round of ligation (Figure 4e), affording the homo-dual-ring branched cyclic RGD peptide **20a**. Finally, to facilitate future bioactivity testing, an azide tag was added to the N-terminal amino group of **20a** by using $N_3(CH_2)_3CO_2Su$ (Su = succinimidyl), to give the azido-labeled branched cyclic RGD peptide **20**.

In conclusion, we present here a peptide side-chain benzyl ester that acts as a multifunctionalization precursor that can be efficiently converted into side-chain acid, amide, hydrazide, carbonyl azide, or thio ester groups in excellent yields under mild conditions. The resulting side-chain carbonyl azide and thio ester permit side-chain peptide ligation for branched peptide synthesis. With this strategy and successive C-terminal and side-chain ligations, a series of novel branched cyclic RGD peptides were prepared. Our method provides a simplified approach for the synthesis of branched cyclic peptides, with the advantage of selective activation of side-chain acids without other protection on the peptide chain. Furthermore, the convenient functional-group conversion on side-chain Asp in excellent yields and mild conditions makes this approach very robust in application. This method will facilitate the development of branched cyclic RGD peptides for therapeutic purposes.

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0036-1588870.

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(22) **Side-Chain Peptide Hydrazide Synthesis and Successive Side-Chain Ligation; General Procedure**
The side-chain benzyl ester peptide (2 mM) was treated with 5% N_2H_4 in DMF at r.t. for 15–30 mins until conversion was complete (HPLC). The product was purified by preparative HPLC, and the resulting side-chain peptide hydrazide was then treated with $NaNO_2$ (10 equiv) in a pH 2 buffer of 6.0 M guanidine hydrochloride and 0.2 M aq NaH_2PO_4 at –10 °C for 15 min to give the corresponding carbonyl azide. MPAA (50 equiv) was then added, the pH of the residue was adjusted to pH 5.5 with 1.0 M aq NaOH, and the mixture was kept at –10 °C for 15 min to give the side-chain thio ester. A Cys-peptide (2 equiv) or an amino-RGD peptide (5 equiv) was added then for side-chain ligation at r.t. for 4–8 h. The ligation product of the branched peptide was purified by preparative HPLC. For details and HPLC and MS data, see the Supplementary Information.
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