Tetrahedron Letters 53 (2012) 1066-1070

Contents lists available at SciVerse ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Efficient solid-phase synthesis of cyclic RGD peptides under controlled microwave heating

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ARTICLE INFO

Article history: Received 21 November 2011 Accepted 16 December 2011 Available online 23 December 2011

Keywords: Microwave-assisted synthesis Solid-phase peptide synthesis Cyclic RGD peptides COMU

ABSTRACT

Cyclic RGD peptides are potent antagonists for the $\alpha_v\beta_3$ integrin receptor. In this Letter, microwaveassisted solid-phase synthesis of cyclic RGD peptides is described. In a coupling reaction between Fmoc-Arg(Pbf)-OH and high-loading H-Gly-Trt(2-Cl) resin, multiple coupling reactions were required for completion under the conventional HBTU activation. We found that the use of COMU, a new coupling reagent, under microwave heating to 50 °C accelerated the reaction even inside the resin. This method was applicable to the synthesis of linear pentapeptides, H-Asp(OtBu)-Xxx-Yyy-Arg(Pbf)-Gly-OH (Xxx = D-Phe(*p*-Br) or D-Tyr, Yyy = Lys(Boc) or MeVal). Cyclization of these peptides followed by deprotection gave the desired cyclic RGD peptides with high purity.

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Cyclic RGD peptides are potent antagonists for the $\alpha_{v}\beta_{3}$ integrin receptor.¹ To date, various cyclic RGD peptides conjugated to a radioactive tracer or fluorophore have been developed for the non-invasive imaging of $\alpha_{v}\beta_{3}$ receptor-overexpressed tumors.^{2,3} Our research interest has been aimed to develop new radiohalogen-labeled cyclic RGD peptides for positron emission tomography (PET) imaging of tumor in vivo. For this purpose, we were pursuing an efficient synthetic protocol for **1**, *cyclo*(Asp-p-Phe(*p*-Br)-Lys-Arg-Gly (Fig. 1), an useful precursor and non-radioactive standard of radiobromine-labeled PET tracer. According to the improved synthetic protocol for cyclic RGD peptides,⁴ side-chain protected linear peptides could be prepared using acid-labile 2-chlorotrityl resin (Trt(2-Cl)-resin). In our attempt for synthesizing a linear RGD peptide using commercially available high-loading H-Gly-



Figure 1. Structure of 1.

Trt(2-Cl)-resin (100-200 mesh, substitution: 0.74 mmol/g resin), we were suffering from the contamination of many impurities in the desired peptide. (RP-HPLC chromatogram of the crude of linear RGD peptide was shown in Figure S1 in the Supplementary data.) It is well known that conjugation of Arg-containing peptides is difficult due to the steric hindrance of Pbf in a side-chain as a protecting group, resulting in low peptide yield or even synthesis failure.⁵ Exactly, we found that triple coupling reactions between Fmoc-Arg(Pbf)-OH and the resin were required for completion of the reaction when using the conventional uronium-based reagent HBTU or the more potent coupling reagent HATU. Although microwave (MW) heating is an efficient tool for accelerating coupling reactions in solid-phase peptide synthesis (SPPS),⁶⁻¹⁵ activation of Fmoc-Arg(Pbf)-OH under MW irradiation causes γ -lactam formation.5a To avoid this side reaction, Fmoc-Arg(Pbf)-OH should be activated under mild conditions, that is, low power of MW



Abbreviations: Trt(2-Cl)-resin, 2-chlorotrityl resin; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PET, positoron-emission tomography; RP-HPLC, reversed-phase high performance liquid chromatography; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxy-benzotriazole; COMU, (1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholinocarbenium hexafluorophosphate; MW, microwave; SPPS, solid-phase peptide synthesis; MeVal, N^{α} -methyl-L-valine; Boc, *tert*-butoxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; tBu, *tert*-butyl; TIS, triisopropylsilane.

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Figure 2. Structure of COMU, HBTU and HATU.

irradiation at room temperature.^{5b,c} Very recently, a novel coupling reagent COMU (Fig. 2) was shown to be more effective than either HBTU or HATU for MW-assisted SPPS of hindered peptides.¹⁶ Furthermore, COMU had advantage that it was free from significant side reactions such as N-terminal capping,^{16b} leading to reduce the by-products and so on. Therefore, we considered to evaluate the performance of COMU in MW-assisted SPPS of Arg-containing peptides. In this paper, we describe the synthesis of cyclic RGD peptides using COMU under controlled MW heating.

Scheme 1 shows MW-assisted SPPS of **1**. According to our previous studies on MW-assisted SPPS of an acetylated Core2 glycosidedipeptide conjugation, coupling reactions proceeded smoothly under MW irradiation only at 50 °C for 10 min to afford a product

up to a 95% vield.^{7b} Therefore, the coupling reaction in this study was conducted under the same condition. H-Glv-Trt(2-Cl) resin (50 mg, substitution: 0.74 mmol/g) was swollen in DMF for over 2 h before use. Each Fmoc-amino acid (0.185 mmol, 5 equiv) was coupled by a coupling reagent (0.185 mmol, 5 equiv) and DIEA (0.37 mmol, 10 equiv) in DMF with MW heating (50 °C, 10 min). The Fmoc group was removed using 20% piperidine in DMF with MW heating (50 °C, 3 min). Each coupling and deprotection was conducted only once except Fmoc-Arg(Pbf)-OH and was monitored by chloranil test.¹⁷ Following completion of the sequence, the linear pentapeptide was cleaved from resin by the treatment with AcOH/ TFE/DCM = 1:1:4 (v/v/v) for 90 min at room temperature. The solution was filtered and concentrated in vacuo, followed by lyophilization to afford the crude product of the protected linear precursor **2**. H-Asp(OtBu)-D-Phe(p-Br)-Lys(Boc)-Arg(Pbf)-Gly-OH. For comparison, the synthesis using HBTU as the coupling reagent was conducted similarly. The yields and purity of 2 after chromatographic purification are shown in Table 1. Reversed-HPLC analysis of the crude product shown in Figure 3 indicated that some minor by-products were formed but not quantified. On contrary, MW irradiation made the amount of the by-products reduced as to compare to the reaction without MW irradiation. Especially, the synthesis using COMU afforded 2 in good yield and purity. When using HBTU under MW irradiation, a major by-product was observed $(t_{\rm R}$ = 4.7 min in RP-HPLC), which was estimated to be the deletion



Scheme 1. MW-SPPS of the linear and cyclic RGD peptides (1-3).

Table 1

Isolated yield and purity of 2 after chromatographic purification^a

	COMU	HBTU
MW(-) (rt, 30 min) ^a	73% (>95%) ^b	69% (>95%) ^b
MW(+) (50 °C, 10 min) ^a	84% (>95%) ^b	39% (>95%) ^b

^a H-Gly-(Cl-Trt)-resin (50 mg, substitution: 0.74 mmol/g resin) was used. Coupling reaction was carried out using Fmoc-AA (5 equiv), coupling reagent (5 equiv) and DIEA (10 equiv).

^b The value in the parentheses indicates the purity of **2** estimated by RP-HPLC peak area%, UV absorbance at 220 nm.

peptide as H-D-Phe(*p*-Br)-Lys(Boc)-Arg(Pbf)-Gly-OH by spectroscopic analyses (ESI-MS: m/z 939.0 [M+H]⁺, ¹H NMR spectra of **2** and the by-product were shown in Figure S2 in Supplementary data). Subsequently, isolated yield of **2** was significantly decreased (39%).

The linear pentapeptide **2** was subjected to head-to-tail cyclization in solution. The reaction using PyBOP under the conditions of high dilution in DCM¹⁸ ([**2**]: <1 mM) afforded the desired fullyprotected cyclic RGD peptide *cyclo*(Asp(OtBu)-D-Phe(*p*-Br)-Lys (Boc)-Arg(Pbf)-Gly) (**3**) in a good yield. Characterization of **3** by MALDI-TOF-MS and RP-HPLC (Fig. 4) showed the product to be highly pure, with no detectable dimerized side-product, *cyclo* (Asp(OtBu)-D-Phe(*p*-Br)-Lys(Boc)-Arg(Pbf)-Gly)₂. Final deprotection by TFA-triisopropylsilane (TIS)-H₂O afforded **1** in an 85% yield.¹⁹

High-yielding synthesis of the linear RGD peptide **2** was accomplished by the combination of MW irradiation and COMU. In order to evaluate the effectiveness of the activation of Fmoc-Arg(Pbf)-OH by COMU, microscopic images of the resin beads after the coupling between Fmoc-Arg(Pbf)-OH and H-Gly-Trt(2-Cl) resin was observed (Fig. 5). Aliquots of the beads were stained with 2% chloranil/acetaldehyde solution in DMF to detect amino groups. Single coupling of Fmoc-Arg(Pbf)-OH using HBTU or COMU (5 equiv) without MW irradiation (MW(–)) was insufficient (Fig. 5: upper panel)., and double coupling with HBTU with MW irradiation (MW(+)) provided some improvement but was



Figure 3. Comparison of reversed-phase HPLC profiles between the crude products obtained using COMU and HBTU. The peak marked by asterisk corresponds to the desired linear pentapeptide **2**. Left panel: using COMU; right panel: using HBTU. The following conditions were applied; column: YMC-pack Pro C18 (4.6 × 150 mm); eluent: 40–70% MeCN aq containing 0.1% TFA (30 min analysis); flow rate: 1 mL/min; detection, λ 220 nm.



Figure 4. Analysis of the crude cyclic peptide **3.** Left panel: comparison of reversed-phase HPLC profiles between **3** and its linear peptide, **2.** Following conditions were applied; Column: Inertsil ODS-3 (4.6×250 mm), eluent: 40-70% MeCN aq containing 0.1% TFA (30 min analysis), flow rate: 1 mL/min, detection: λ 220 nm. Right panel: MALDI-TOF-MS spectrum, calcd. for $C_{49}H_{72}BrN_9O_{12}S$ 1089.42, mass found m/z 1090.2 [M+H]⁺, 1112.2 [M+Na]⁺, 1128.2 [M+K]⁺.



Figure 5. Microscopic images of the resin beads after coupling of Fmoc-Arg(Pbf)-OH to H-Gly-Trt(2-Cl) resin. Upper panel: coupling without MW irradiation (MW(-)) using HBTU (left) and COMU (right); lower panel: coupling with MW irradiation (MW(+)) using HBTU (left) and COMU (right). The resin beads were stained by chloranil/ acetaldehyde in DMF for 2 min.

inadequate for complete acylation (Fig. 5: left and lower panels). In contrast, the use of COMU (5 equiv) under MW irradiation was more effective than HBTU even in a single coupling (Fig. 5: right and lower panels), and double coupling resulted essentially to completion (Fig. 5: right and lower panels). In the case of incomplete acylation, microscopic images of the beads showed staining of the inside of the resin but not the surface (Fig. 5), which suggested that the incomplete acylation between Fmoc-Arg(Pbf)-OH and the resin was mainly due to insufficient permeation of the activated species into the resin beads.

Although the effectiveness of MW irradiation has not been comprehensively understood at present, we conjecture the MW effect for this improvement of poor resin penetration of the activated species as follow. As our previous report of a MW-assisted glycosylation at low temperature.²⁰ Shimizu et al. found that MW irradiation was effective for glycosylation reactions at low temperature (-10 °C) to afford oligosaccharides though no MW irradiation at the same temperature had led only to side reactions, and which indicated that MW plays a more significant roles than heating. They considered that a glycosyl acceptor might exist as clusters such as micelle formation and therefore reactive hydroxyl groups on the acceptor would be hidden inside the cluster.²¹ As a result, an activated glycosyl donor could not react with the hydroxyl groups. In our case, Fmoc-Arg(Pbf) molecules might also exist as micelle-like clusters due to the bulky and hydrophobic side chain protecting group (Pbf) and Fmoc group. The clustered molecules would be too large to permeate into the resin beads under the normal condition but were broken by MW irradiation, enabling the activated Fmoc-Arg(Pbf) molecules to permeate and react with the amino groups in the beads. The efficacy of COMU would be due to increased resin permeability of the activated species derived from Fmoc-Arg(Pbf)-OH and COMU. Little is known about MW irradiation effects on the diffusion of organic molecules in cross-linked polystyrene, but MW irradiation may change the swelling characteristics of the beads, allowing the COMU-activated Arg(Pbf) molecules to permeate the beads effectively. Another several experiments will be required to confirm this hypothesis, that is, studying the coupling between Fmoc-Arg(Pbf)-OH and resins with excellent swelling properties under MW irradiation will provide useful information.

To address further usefulness and limitation of the MW/COMU method for synthesizing cyclic RGD peptides, we synthesized side-chain protected derivative of N-methylated cyclic peptide cilengetide (4. cvclo(Asp(OtBu)-p-Tvr(tBu)-MeVal-Arg(Pbf)-Glv)).^{1d} In general, acylation to N-terminal MeVal residue resulted in a low yield, which is mainly due to its steric hindrance.²² In this study, coupling reactions using HATU were also conducted to compare the reactivity of COMU with HATU. The synthetic protocol for 4 is shown in Figure 6. The reaction between Fmoc-Arg(Pbf)-OH and H-Gly-Trt(2-Cl) resin by COMU was completed after double coupling. On contrary, triple coupling reactions were required for completion when using HATU. MeVal residue was successfully introduced by a single coupling. The coupling of Fmoc-D-Tyr (tBu)-OH, however, required triple coupling, indicating that both COMU and HATU activations under MW irradiation did not significantly accelerate the acylation to MeVal residue. After the



Figure 6. (a) Structure of 4, (b) graphical synthesis outline for H-Asp(OtBu)-D-Tyr(OtBu)-MeVal-Arg(Pbf)-Gly-OH (5). Each coupling reaction was carried out under MW irradiation at 50 °C for 10 min.

elongation was completed, the peptide was cleaved by the same procedure described above (AcOH/TFE/DCM = 1:1:4 (v/v/v) for 90 min at room temperature) to afford H-Asp(OtBu)-D-Tyr(OtBu)-MeVal-Arg(Pbf)-Gly-OH (**5**). RP-HPLC analysis revealed that the syntheses by both protocols did not form any significant impurities. Finally, cyclization of **5** by PyBOP in DMF afforded **4** in a good yield (see Fig. S3 in the Supplementary data).

In summary, we accomplished the efficient solid-phase synthesis of cyclic RGD peptides by utilizing COMU under controlled MW heating. We demonstrate that the use of COMU is effective for the coupling of Fmoc-Arg(Pbf)-OH under MW irradiation at 50 °C for 10 min. In general, coupling reaction in SPPS is monitored by coloration of the beads using the amine-reactive dyes such as ninhydrin and chloranil. In our study, microscopic analysis of the stained beads enabled us to evaluate the MW effect for the coupling reaction. The purity of the crude pentapeptide was over 90%, and no significant side-reactions or amino acid residue deletions were observed. Our method should greatly contribute to the development of RGD-based pharmaceutics and molecular probes for detecting $\alpha_v \beta_3$ integrin-overexpressed tumors in vivo.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.12.069.

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 - Procedure for cyclization: To a stirred solution of PyBOP (6.2 mg, 0.022 mmol) and DIEA (6.1 μL, 0.036 mmol) in DCM (20 mL) at 0 °C was added 1 (20 mg, 0.018 mmol) in DCM (4 mL) dropwise. The reaction mixture was stirred for 1 h. The solvent was evaporated off and the residue was lyophilized to afford 2. (17 mg, 87% yield).
 - Procedure for the final deprotection: 2 (6.2 mg, 0.0057 mmol) was dissolved in TFA-TIS-H₂O (95:2.5:2.5 (v/v/v), 5 mL) and the solution was placed at room temperature for 2 h. The solution was concentrated in vacuo and the residue was lyophilized to afford the crude. Gel filtration chromatography (Sephadex LH-20, DMF) gave the desired cyclic RGD peptide 3 as a TFA salt (3.9 mg, 85% yield). ESI-MS: calcd. for C₂₇H₄₁BN₉O₇ 682.2, mass found *m/z* 681.9 [M+H]^{*}.
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