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Microwave-Assisted Solid-Phase Synthesis of side-chain to side-chain lactam-bridge cyclic peptides

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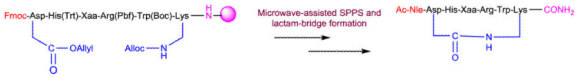
Abstract

Side-chain to side-chain lactam-bridged cyclic peptides have been utilized as therapeutic agents and biochemical tools. Previous synthetic methods of these peptides need special reaction conditions, form side products and take longer reaction times. Herein, an efficient microwaveassisted synthesis of side-chain to side-chain lactam-bridge cyclic peptides SHU9119 and MTII is reported. The synthesis time and efforts are significantly reduced in the present method, without side product formation. The analytical and pharmacological data of the synthesized cyclic peptides are in accordance with the commercially obtained compounds. This new method could be used to synthesize other side-chain to side-chain lactam-bridge peptides and amenable to automation and extensive SAR compound derivatization.

Graphical Abstract



Keywords

Cyclic peptides; Microwave assisted synthesis; Lactam-bridge cyclic peptides; SHU9119; MTII

Cyclic peptides offer great potential as therapeutic agents because they can exhibit membrane permeability,[1] resistance to proteolytic degradation,[2] and metabolic stability[3]. Cyclic peptides also serve as biochemical tools in studying protein-protein interactions and molecular probes.[4-7] A wide variety of methodologies have been reported for syntheses of cyclic peptides using side-chain to side-chain bridging techniques, which include disulfide formation,[8] lactamization,[9] all-hydrocarbon linkage.[10] Among these peptides, lactam-bridge peptides are finding an increasing number of applications in protein biology, which include

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Supplementary Material

Detailed procedure for the syntheses of peptides, analytical HPLC chromatograms, analytical data of peptides and AlphaScreen® cAMP assay are given in the supporting information.

protein folding, protein aggregation, peptide ligand–receptor recognition, and development of potent peptide therapeutics.[11]

In general, side-chain to side-chain lactam-bridged cyclic peptides can be synthesized by cyclizing the side-chain amino group of a lysine residue with the side-chain carboxyl group of a glutamic acid or aspartic acid residue of the peptide. Previous syntheses of these peptides used Nα-Boc strategy, which requires strong acid like trifluoroacetic acid (TFA) for repetitive removal of the Boc groups, while often relying on corrosive and toxic hydrofluoric acid (HF) for release of the assembled peptide from the support.[12-15] Later approaches have used Nα-Fmoc strategy to synthesize this class of cyclic lactam-bridge peptides, where the Fmoc group can be removed under basic conditions.[4,16-21] But, these methods require specialized reaction conditions such as inert atmosphere and closed reaction vessel without the presence of oxygen for selective deprotection of alloc, allyl protecting groups.[16] These methods are also prone to form side products,[19] which result in cumbersome purification steps and take longer time to achieve completion for peptide coupling.[4,16,19,20] Hence, an efficient synthesis of side-chain to side-chain lactam-bridge cyclic peptides in shorter reaction time, without need for specialized reaction conditions and no side products formation is highly desirable.

The utilization of microwave heating can be advantageous in organic synthesis to synthesize diverse compounds, which increases reaction yields and shortens reaction times.[22] The microwave-assisted solid-phase peptide synthesis have also made improvements to both the speed of the peptide coupling and Nα-Fmoc deprotection as well as increased the purity of the crude peptides.[23] Here an efficient synthesis of side-chain to side-chain lactam peptides SHU9119, Ac-Nle-c[Asp-His-DNal(2')-Arg-Trp-Lys]-NH_2 and MTII, Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2 using microwave irradiation in an open vessel is reported. This method increases overall efficiency and significantly reduces the reaction time of each step including side-chain to side-chain lactam-bridge formation. No considerable side product formation was observed in this method.

The cyclic SHU9119 and MTII peptides were selected as examples of side-chain to side-chain lactam-bridge peptides, because of their tremendous applicability as biochemical tools for *in vivo* and *in vitro* characterization of the melanocortin receptor system.[15,17,18,24-28] Melanocortin receptors (MC1R-MC5R) belong to the family of G-protein coupled receptors (GPCRs) and have been known to mediate numerous physiological processes including energy homeostasis,[29] steroidogenesis,[30] feeding behavior,[28] sexual function,[31] and skin pigmentation.[32] The β-lactam containing SHU9119 peptide is an antagonist and partial agonist at the mMC3R and a complete antagonist at the mMC4R.[13] The MTII peptide is a potent agonist at the MC1, MC3-5 receptors.[12,14]

The SHU9119 and MTII peptides, which are C-terminal amidated lactam-bridge cyclic peptides were synthesized efficiently by microwave-assisted Fmoc solid phase peptide synthesis using Rink Amide MBHA resin (0.35 meq/g loading) in a manual microwave synthesizer (Discover SPS™, CEM Corp., supporting information). Initially, these peptides were elongated up to the Asp residue to afford linear peptide resins **1a** and **1b** respectively (Scheme 1) under microwave irradiation. Aspartimide formation is predominant in the

deprotection of Fmoc group of the aspartic acid in the peptide, when an allyl ester is present as the side chain protection for aspartic acid.[33] To avoid aspartimide formation, the alloc and allyl groups were selectively deprotected and the lactam cyclization was performed prior to the removal of the Fmoc of the Asp residue. Traditionally, allyl and alloc groups were selectively removed with $Pd(PPh_3)_4/PhSiH_3$ at room temperature for 30 min (2 times).[16] This reaction was done in a sealed reaction vessel under argon atmosphere with a complete absence of oxygen, which poisons the catalyst.[16] Deprotection of the allyl and alloc functional groups under microwave-assisted conditions was presented at the American Peptide Symposium.[34] However, the presently described method utilized modified microwave conditions to selectively deprotect alloc and allyl functional groups. While on resin, peptides **1a** and **1b** were treated with 20 equiv. of $PhSiH_3$, and 0.25 equiv. of $Pd(PPh_3)_4$, in 1,2-dichloroethane under microwave conditions (30 W, 35 °C) for 2 min in an open reaction vessel and the above process was repeated. After the deprotection, a Kaiser test[35] indicated the presence of free amino group.

Traditionally, lactam-bridge formation was reported in the presence of HBTU/PyBOP/HATU, HOBt and DIEA for 2-24 h, at room temperature.[4,16,19,20] This coupling was repeated until a negative Kaiser test resulted.[16,19] In the present method, the peptides were successfully cyclized on the resin under microwave conditions in 10 min by treating with HBTU and DIEA in DMF to afford **2a** and **2b** (Scheme 1). Subsequent Nα-Fmoc deprotection, coupling of the final Fmoc-Nle-OH amino acid and final Nα-Fmoc removal were performed using same conditions described above.

The resulting free N-terminal peptide resins were acetylated under microwave conditions in 4 min to afford final peptide resins **3a** and **3b**. Cleavage of the final cyclic peptides from resin was done in 10 min under microwave irradiation using modified conditions of a previously published method.[36] Treatment of final cyclic peptide resins **3a** and **3b** with a mixture of trifluoroacetic acid, thioanisole, triisopropylsilane and water (9.1:0.3:0.3:0.3) under microwave conditions in 10 min gave crude peptides **SRT5-134** (SHU9119) and **SRT5-148** (MTII). A complete comparison of synthetic conditions of these cyclic peptides under traditional Fmoc/t-Bu approach at room temperature and microwave-assisted solid-phase peptide synthetic conditions used in the current methodology are summarized in Table 1.

HPLC profiles of these crude peptides **SRT5-134** (SHU9119) and **SRT5-148** (MTII) showed 91% and 85% crude peptide purity respectively (supporting information). In the traditional room temperature method, the reported crude peptides purity were in the range of 65-72%.[33] After purification of the crude peptides using semi-preparative HPLC and lyophilization, mass analysis found that major peak for each peptide corresponded to the expected product (supporting information). Resulted peptides were found to be ≥ 99% pure (RP-HPLC) and the yields of the peptides were comparable to that of room temeperature method.[16] Microwave-assisted peptide synthesis and resin loading could be the factors in obtaining lactam-bridge cyclic peptides with high crude peptide purity. Co-injection of synthesized SHU9119 (SRT5-134) and commercially obtained SHU9119 (Bachem) into analytical RP-HPLC resulted in elution of both compounds as one peak, matching the chromatograms of both synthesized SHU9119 (SRT5-134) and commercially obtained

SHU9119 (supporting information). The methodology presented here allows the formation of the cyclic lactam peptides on solid support in an efficient manner with shorter reaction times compared to that of room temperature methods,[4,16,19,20] without requiring any specialized reaction conditions and no considerable side product formation.

The synthesized SHU9119 and MTII peptides were tested for functional activity at the mouse MCRs using the cAMP-based AlphaScreen® assay[37] (PerkinElmer) according to the manufacturer's instructions. The results obtained are illustrated for the synthesized and commercially obtained SHU9119 and MTII peptides in Table 2. Since, the AlphaScreen® assay is a competition assay with loss of signal at higher concentrations, the concentration-activity curves were normalized for illustration purposes similar to Elster et al (Figure 1).[38] These results support the hypothesis that the synthetic route do not alter functional potency or efficacy as anticipated.

In conclusion, an efficient microwave-assisted synthetic strategy for the syntheses of side-chain to side-chain lactam-bridged cyclic peptides on a solid support without aspartimide formation is reported. This synthetic route makes the purification of these peptides efficient and the synthesis amenable to rapid SAR compound generation and purification. Utilization of microwave irradiation in each step of the peptide synthesis, which include i) deprotection of Fmoc group, ii) coupling of amino acid, iii) selective deprotection of alloc and allyl protecting groups, iv) formation of lactam cyclic-bridge, v) N-terminal acetylation, and vi) cleavage of peptide from resin, reduces the reaction times at each step. The current method also permits the syntheses of these peptides efficiently in an open reaction vessel without need for complete absence of oxygen. The methodology presented here could be automated and applied to the synthesis of other side-chain to side-chain lactam-bridge cyclic peptides, decreasing the synthetic times and improving the synthetic efficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MWA-SPPS	microwave-assisted solid phase peptide synthesis
MBHA	methylbenzhydryl
MCR	melanocortin receptors
NDP	[Nle4, DPhe7]-αMSH
MTII	melanotan-II
RP-HPLC	reverse phase high performance liquid chromatography
TFA	trifluoroacetic acid

TIS	triisopropylsilane
H_2O	water
HBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro-phosphate
DIEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DCM	dichloromethane
HATU	*O*-(7-azabenzotrizol-1-yl)-*N*,*N*,*N*’,*N*’-tetramethyluronium hexafluoro-phosphate
PyBOP	(benzotriazol-1-yloxy)tripyrrolidinophospho-nium hexafluoro-phosphate
HOBt	*N*-hydoxybenzotriazole
TES	triethylsilane
NMP	*N*-methyl-2-pyrrolidone.

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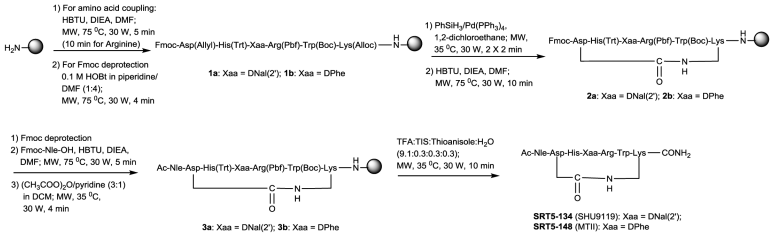
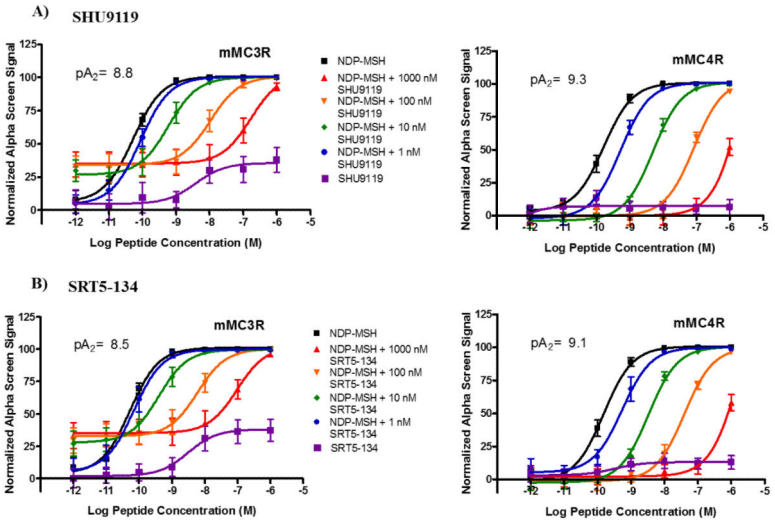


Figure 1.

Antagonist Pharmacology of SHU9119 synthesized (SRT5-134) and commercial using Schild analysis at the mouse MC3R and MC4R.



Scheme 1.

Microwave-assisted syntheses of **SRT5-134** (SHU9119) and **SRT5-148** (MTII)

Table 1

Comparison of the traditional Fmoc/tBu SPPS[4,16,19,20] and MWA-SPPS experimental conditions.

Step	Traditional SPPS (room temperature)	Microwave-assisted SPPS
Fmoc deprotection	20-25% Piperidine/DMF; 5 min and 30 min	0.1 M HOBt in piperidine /DMF (1:4) solution; 2 min at rt; MW, 75 °C, 30 W, 4 min
Coupling	Fmoc-amino acid (3 equiv.), HBTU, HOBt, DIEA, 2 h	Fmoc-amino acid (3 equiv.), HBTU, DIEA; MW, 75 °C, 30 W, 5 min (10 min for Arginine)
Alloc and Allyl group deprotection	$PhSiH_3$ (24 equiv.) or DMBA (10 equiv.), $Pd(PPh_3)_4$ (0.1-0.25 equiv.); Argon atm.; 2 X 30 min	$PhSiH_3$ (20 equiv.), $Pd(PPh_3)_4$ (0.25 equiv.); MW, 35 °C, 30 W, 2 X 2 min
Lactam-bridge Formation	HOBt (6 equiv.), HBTU/PyBOP/HATU (1-6 equiv.), DIEA (3-12 equiv.) in NMP/THF for 2-24 h (repeat the process until a negative Kaiser test resulted)	HBTU, (6 equiv.) DIEA (12 equiv.) in DMF; MW, 75 °C, 30 W, 10 min
N-Acetylation	Acetic anhydride: pyridine (3:1), 30 min	4 mL of acetic anhydride: pyridine (3:1) mixture in 4 mL DCM; MW, 35 °C; 30 W, 4 min
Cleavage from Resin	TFA:TES:H_2O (9:0.5:0.5), 3 h	TFA:TIS:Thioanisole:H_2O (9.1:0.3:0.3:0.3); MW, 35 °C, 30 W, 10 min

Table 2

Pharmacology of commercial and synthesized SHU9119 and MTII at the mouse melanocortin receptors.[a]

Peptide	mMC1R	mMC3R	mMC4R	mMC5R
		EC50 (nM)		
NDP-MSH	0.04±0.01	0.23±0.03	0.47±0.21	0.18±0.07
SHU9119 (Commercial)	0.98±0.17	Partial agonist PA_2 = 8.8, K_i = 1.6 nM	Antagonist PA_2 = 9.3, K_i = 0.5 nM	Partial agonist
SHU9119 (SRT5-134)	1.43±0.33	Partial agonist PA_2 = 8.5, K_i = 3.2 nM	Antagonist PA_2 = 9.1, K_i = 0.79 nM	Partial agonist
MTII (Commercial)	0.06±0.02	0.18±0.04	0.15±0.06	0.14±0.05
MTII (SRT5-148)	0.05±0.01	0.15±0.04	0.14±0.02	0.12±0.02

* The pA2 values were calculated by a Schild analysis. [39]

[a]The indicated errors represents the standard error of the mean determined from at least three independent experiments.