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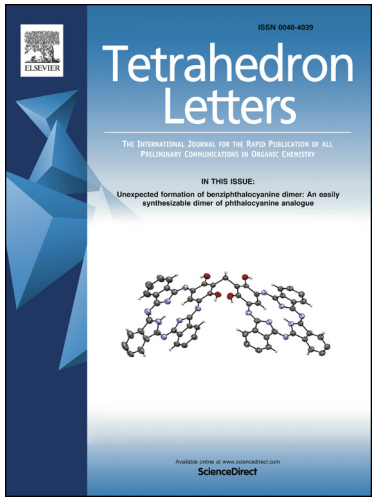

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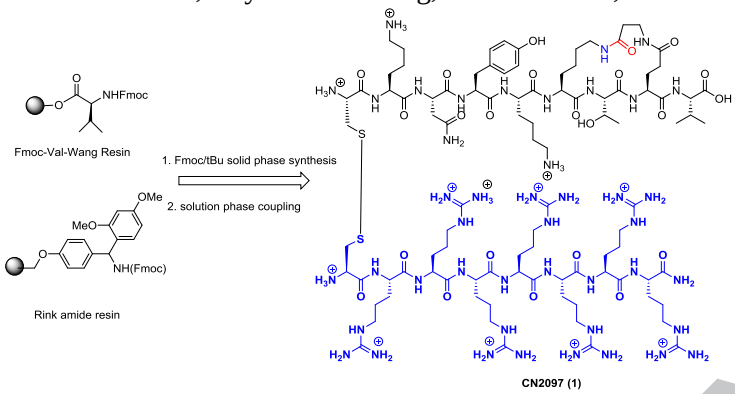

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Efficient synthesis of CN2097 using in situ activation of sulfhydryl group

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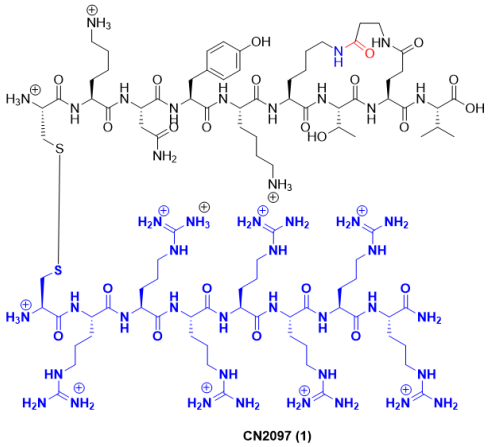
ABSTRACT

CN2097 (R_7Cs-sCYK[KTE(β-Ala)]V) is a rationally designed peptidomimetic that shows effectiveness in preclinical models for the treatment of neurological disorders, such as Angelman syndrome, traumatic brain injury (TBI) and stroke. Because of its therapeutic activity for the treatment of human CNS disorders, there was an urgent need to develop an efficient strategy for large-scale synthesis of CN2097. The synthesis of CN2097 was accomplished using Fmoc/tBu solid phase chemistry in multiple steps. Two different peptide fragments (activated polyarginine peptide Npys-sCR_7 and CYK[KTE(β-Ala)]V) were synthesized, followed by solution phase coupling in water. Activation of the polyarginine (CR_7) was achieved in situ during cleavage of protected peptide (C(Trt)R(Pbf)$_7$) from the Rink amide resin using 5 equiv. of 2,2-dithopyridine in TFA:TIS:H_2O (95:2.5:2.5, v/v/v) for 4 h. The disulfide coupling was efficient which provided a 60% yield.

1. Introduction

Postsynaptic density protein-95 (PSD-95), Discs-large, and zonula occludens-1 (PDZ) domains are 90 amino acid residue modular protein interaction domains found in scaffolding and adaptor proteins.[1] PSD-95, which consists of three PDZ domains (PDZ1-3), is an abundant scaffold protein at excitatory synapses. The PDZ domains of PSD-95 bind to membrane receptors and ion channels via a C-terminal binding sequence, tSXV.[2, 3] The PDZ domains of PSD-95 have been targets for the development of compounds for therapeutic application in ischemic brain injury,[4-8] psycho-stimulant addiction,[9] and Angelman syndrome.[10] For example, the engineered peptide IETAV, containing the backbone of the five *C*-terminal amino acids of GluN2B, was shown to be a more potent inhibitor to the PDZ1 and PDZ2 domains of PSD95, than the native peptide.[11] Similarly, a nine amino acid peptide from the CRIPT protein, which binds to the PDZ3 domain of PSD-95[12], was modified to develop a more efficient inhibitor.[13] In addition to PSD-95, other PDZ domain-associated proteins have also been found to be important therapeutic drug targets, including syntenin in metastatic breast cancer,[14] parkin in Parkinson's diseases,[15] and E6 protein in oncogenic human papillomavirus.[16, 17]

More recently a rationally designed peptidomimetic (designated CN2097) consisting of a polyarginine disulfide-linked cyclic PDZ binding motif through a lactam ring and β-alanine linker; (R_7Cs-sCYK[KTE(β-Ala)]V) was generated. The cyclic PDZ binding motif was designed based on last 5 residues of *C*-terminus of CRIPT scaffolding protein as found in the x-ray analysis of PDZ3 complex with CRIPT.[12] These residues were modified to improve bonding interaction using beta-alanine. Polyarginine was added to improve cell-permeability. In preclinical animal studies, CN2097 was found to be effective in the treatment of Angelman syndrome,[10] TBI[18], and neuroprotective in an excitotoxicity animal model that mimics a stroke.[19] One of the major drawbacks in proceeding to preclinical trial studies is that the reported methodology for the synthesis of CN2097, based on solid phase and microwave chemistry, provides a low yield (~5%)[20] during conjugation of polyarginine and cyclic PDZ peptide to form a disulfide bond. The synthesis consists of an activated cysteine on solid-phase that requires a large amount of cyclic-peptide for disulfide coupling on the resin. This, and the greater potential for denaturation of the peptide and/or inducing a change in the secondary structure under



CN2097 (1)

Figure 1. Chemical structure of CN2097 (**1**).

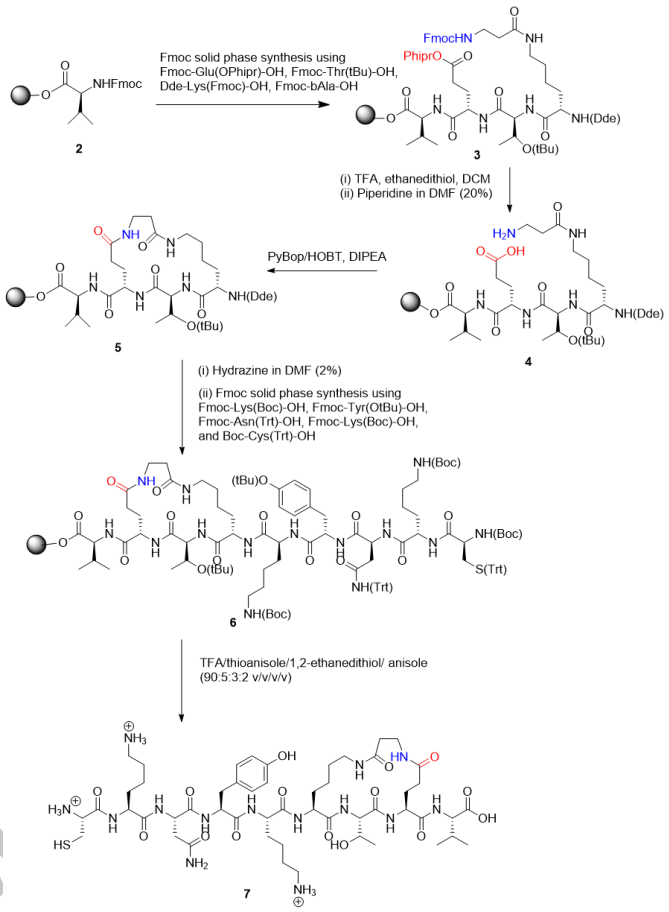
microwave heating during synthesis make scaling up the process impractical.[20] Therefore, an efficient methodology for the synthesis of CN2097 is urgently required for generating a large amount of compound for the clinical studies. Herein, we report an efficient and novel synthesis of CN2097 in a higher scale.

Results and discussion

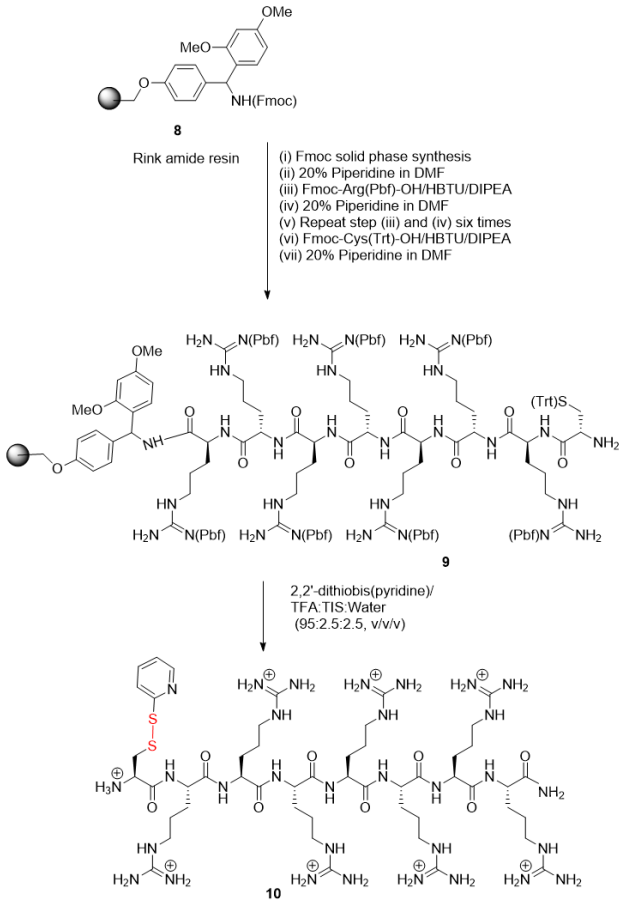
CN2097 consists of a PDZ macrocyclic peptide and polyarginine peptide that are linked by a disulfide linkage resulting from each peptides *N*-terminal cysteine residue. The individual peptides were synthesized independently and then coupled in the solution phase to produce a faster and more efficient coupling and for large-scale synthesis. Standard Fmoc-based methodology was used to synthesize each peptide.

The synthesis of a macrocycle targeting the PDZ domain of PSD-95 was previously reported[13] without a *N*-terminal cysteine residue. The peptide, $K_1N_2Y_3K_4K_5T_6E_7V_8$, was based on the C-terminal residues of CRIPT and was synthesized using Fmoc/tBu solid-phase chemistry. A cysteine residue was added at the N-terminus for disulfide coupling. The peptide sequence was assembled on the Fmoc-Val-Wang resin (**2**) using Fmoc-Glu(Ophipr)-OH, Fmoc-Thr(tBu), Dde-Lys(Fmoc)-OH, and Fmoc-β-Ala-OH with coupling and deprotection cycles with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/ *N*,*N*-diisopropylethylamine (DIPEA) and piperidine in *N*,*N*-dimethylformamide (DMF) (20% v/v), respectively. After the synthesis of the linear protected peptide on the solid phase (Dde-K(β-Ala-NHFmoc)-T(tBu)-E(OPhipr)-V-Wang resin, **3**) was confirmed using ESI mass after cleavage of small amount of resin, the Phipr group from glutamic acid was selectively deprotected using 2.5% (v/v) TFA in (5% v/v ethanedithiol (EDT) in dichloromethane (DCM)) for 10 minutes 3 times, which showed incomplete removal of Phipr group. Therefore, the TFA concentration was increased to 5% in the EDT (5%) in DCM. Subsequently, the Fmoc group from β-alanine was removed using 20% piperidine in DMF followed by washing. The resin appeared to aggregate as observed during washing, which may be due to electrostatic charges between COOH and NH_2 group as depicted in Scheme 1 for compound **4**. On resin cyclization was carried out using PyBOP/HOBt (3 equiv.), DIPEA (6 equiv.) under nitrogen and using DMSO:NMP (1:4 v/v) for 4 h and found to be complete as monitored by electrospray ionization mass spectrometer which showed a peak of 693.4215 corresponding to $[M + H]^+$ for cyclic compound **5**. The resin was washed and used for elongation of peptide chain by deprotection of Dde group. The peptidyl-resin was treated with hydrazine in DMF (2% v/v) for 15 min followed by washing and coupling subsequent Fmoc-protected amino acids, Fmoc-Lys(Boc)-OH, Fmoc-Tyr(OtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Lys(Boc)-OH and Boc-Cys(Trt)-OH, respectively, to generate **6**. The deprotection of the side chains, final cleavage from the solid support using cleavage cocktail R (trifluoroacetic acid (TFA)/thioanisole/1,2-ethanedithiol/ anisole 90:5:3:2 v/v/v/v, 2 h), purification with HPLC, and lyophilization afforded macrocyclic peptide **7** containing a cysteine residue. The chemical structure of peptide **7** was confirmed using a high-resolution matrix-assisted laser desorption/ionization (MALDI) spectrometer.

In a separate synthesis, the polyarginine-cysteine peptide **10** was synthesized using Fmoc/tBu solid-phase chemistry and Rink amide resin **8**. Fmoc-Arg(Pbf)-OH was coupled after deprotection of Fmoc group from the resin. Six more arginine residues were coupled under similar conditions followed by

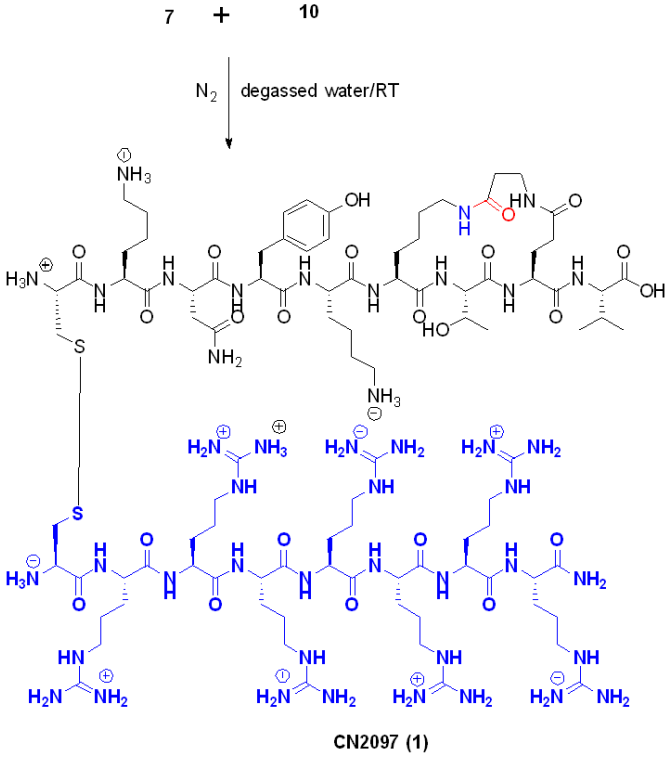


Scheme 1. Synthesis of macrocyclic PDZ peptide **7**.



Scheme 2. Synthesis of activated polyarginine peptide **10**.

addition of a cysteine residue using Fmoc-Cys(Trt)-OH. Fmoc group at the *N*-terminal was deprotected to afford peptidyl resin **9**. Cysteine was added at the *N*-terminus for disulfide coupling to the macrocyclic peptide. The cleavage of a small amount of the resin confirmed the formation of linear CR_7 peptide in the MALDI spectrometer. The disulfide coupling was facilitated by adding the activated cysteine in the polyarginine chain rather than macrocyclic peptide **7** since the linear peptide was easily synthesized as compared to macrocyclic peptide. The use of Boc-Cys(Npys)-OH in CR_7 peptide to get activated sulfhydryl provided a very small amount of conjugated peptide CN2097 (around 5% yield) in our attempt.[20] Thus, we activated sulfhydryl group in the peptide using 2,2′-dithiodipyridine (DTP). The activation of sulfhydryl group failed under normal solution phase conditions using DTP and polyarginine peptide with the acidic condition. Therefore, we followed in situ activation of sulfhydryl group during cleavage of the peptide from the resin and removal of the protecting group. The final deprotection and cleavage of the polyarginine peptide from the resin using the 5 equivalent of 2,2′-dithiodipyridine in cleavage cocktail (water/triisopropylsilane/TFA (2.5:2.5:95, v/v/v) for 4 h afforded the activated cysteine generated in situ within the polyarginine peptide **10** that was purified by HPLC and lyophilized. It is worthwhile to note that 4 h cleavage time removed most of the Pbf protecting groups from the side chain of arginine and provided activated pyridyl-s-sCR_7 peptide. However, cleavage time longer than 4 h resulted in the loss of s-pyridyl group, leaving only the CR_7 peptide.



Scheme 3. Synthesis of CN2097(1).

The final coupling of the two synthesized peptides (PDZ macrocyclic peptide **7** and polyarginine peptide **10**) to form disulfide bond was carried out in solution phase using an equimolar ratio of macrocyclic peptide **7** and the polyarginine peptide **10** under a nitrogen atmosphere with degassed water for 18 h.[21] (Scheme 3) MALDI-TOF analysis of the reaction after 2 h showed the formation of the product. After final HPLC purification and lyophilization, CN2097 (**1**) containing the disulfide bond was obtained (Figure 1). The conjugation reaction proceeds with high yield (>60% yield after conjugation of two peptides and HPLC purification). We synthesized 250 mg of CN2097 (**1**) using in situ sulfhydryl activation of polyarginine peptide (**10**) and obtained compound **1** in overall 25% yield after multiple HPLC purification. The chemical structure of CN2097 was confirmed using a high-resolution time-of-flight electrospray mass spectrometer.

Conclusions

We have developed an efficient method for the large-scale synthesis of CN2097 required for clinical studies. After solid-phase synthesis of the macrocyclic and polyarginine peptides, a solution phase condition with an equimolar ratio of macrocyclic and polyarginine peptide was used to synthesize CN2097 in the final step. The in situ activation of cysteine in the polyarginine peptide provides the activated cysteine for disulfide conjugation. Our methodology provides higher yield (>60%) and high purity 98% of the synthesized CN2097.

Acknowledgments

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21. Procedure for the synthesis of CN2097 (**1**) by solution-phase coupling reactions of **7** and **10**: The final disulfide coupling was performed by using polyarginine peptide (10, 20.0 mg, 15.12 mmol) dissolved in 2 mL of degassed water and addition of peptide **7** (17.6 mg, 15.12) at room temperature. After addition of cyclic peptide **7**, the color of the reaction was turned to light yellow. After stirring for 18 h, the reaction was diluted with ethyl acetate (2 mL). The aqueous phase was separated and extracted with ethyl acetate (3 × 5mL). The aqueous phase was lyophilized and the residue was purified by reverse phase HPLC (C18 column using 1-20 % acetonitrile gradient over 30 min) to afford CN2097 (**1**) in >60% overall yield (Figure 1). The chemical structure of CN2097 was determined using a high-resolution MALDI-TOF (m/z) $C_{96}H_{174}N_{44}O_{23}S_2$: calcd. 2375.3240 found 1215.0348 $[M + H - cyclic]^+$, 1166.9063 $[M + 2H - CR_7]^+$, 2378.5848 $[M + 3H]^+$.

Supplementary Material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/.

Highlight

- An efficient synthesis of CN2097 was developed to overcome low yield and purity issue.
- CN2097 is under preclinical studies and require a large amount of compound.
- Insitu sulfhydryl activation and solution phase coupling of both peptides provided good yield and purity.