Oxidative organic, aqueous or regioselective folding, what is the best strategy to unlock the potential of Conotoxins ?

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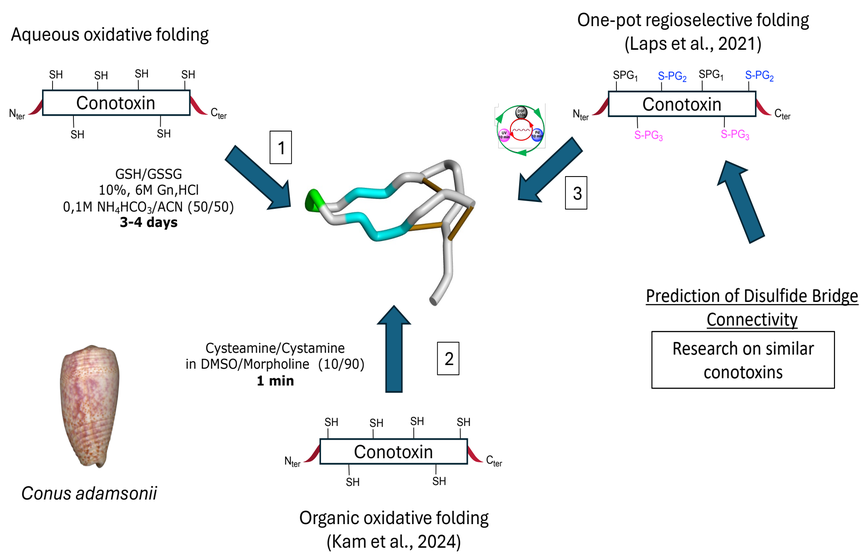
Introduction

Conotoxins, disulfide-rich peptides derived from cone snail venom, exhibit diverse biological activities and structural variations, leading to their classification into distinct subfamilies[1]. Each subfamily can target various pharmacological receptors. Due to their pharmacological significance, synthesizing these peptides is appealing but complicated by the presence of multiple disulfide bridges. Developing versatile chemical synthesis strategies for these compounds is essential to unlock their full potential[2]. In this work, we compare the results of three folding strategies applied to the unusual AdIIIB conotoxin, which features a single amino acid separating the fourth and fifth cysteine residues.

Conotoxin	Cone snail species	Sequence	Loop m/n/o	Disulfide linkage	Reference
AdIIIB	*Conus adamsonii*	KGCCSGVT–CPIYWKNVNICRCC–*	4/9/1	ND	This work
CnIIIF	*Conus consors*	–RCCGEGASCPRYFRNSQICSCC–*	5/9/1	ND	[3]
MIIIJ	*Conus magus*	ZKCCSGGS–CPLYFRDRLICPCC–*	4/9/1	ND	[4]
SIIID	*Conus striatus*	––CCGEGSSCPKYFKNNFICGCC–*	5/9/1	C1–C4, C2–C5, C3–C6	[5]
MrIIIE	*Conus marmoreus*	–VCC–PFGGC–––––HELCYCCD*	4/3/1	C1–C5, C2–C4, C3–C6	[6]

Table 1 : M1-branch of M conotoxin superfamily , * amidated , ND: not determined , Z: Pyroglutamate

Methodology



To synthesize these cyclic peptides, three methods have been compared:

1.Aqueous oxidative folding leads to the most stable isomer. In our case, we achieved optimal results using a 0.1M $NH4HCO_3$ buffer with acetonitrile as a co-solvent and reduced/oxidized glutathione[7].

2.Organic oxidative folding was performed in 90% morpholine as a solvent, with cysteamine/cystamine as disulfide bond mediators[8].

3.Regioselective oxidative folding combines several components. Disulfiram (DSF) acts as a disulfide bond oxidizing agent, ultraviolet light deprotects the nitrobenzyl (Nbzl) group, and palladium deprotects the acetamidomethyl (Acm) group. These elements enable chemoselective and regioselective cysteine activation, allowing for one-pot formation of multiple disulfide bonds in various peptides and proteins[9,10].

While oxidative folding methods generally lead to the most stable isomer without anticipating the specific arrangement of the disulfide bonds, directed folding allows for control over the formation of disulfide bonds, with the arrangement defined based on a literature survey.

Results

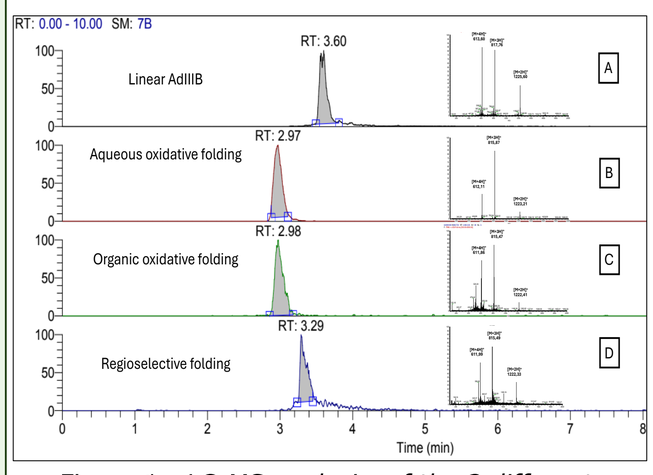


Figure 1: LC-MS analysis of the 3 different oxidative folding

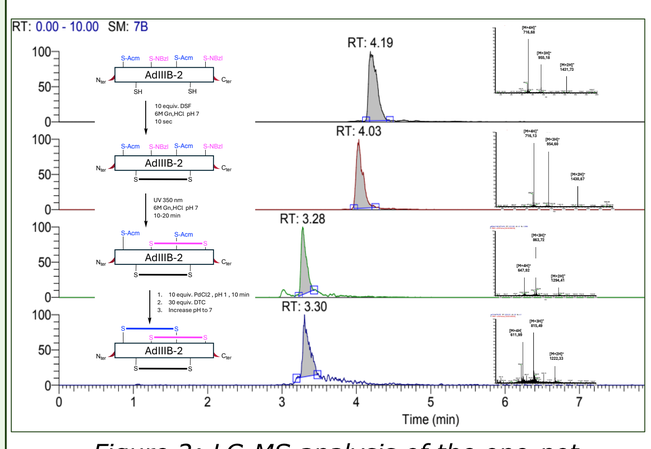


Figure 2: LC-MS analysis of the one-pot regioselective folding

1. Comparison of the three folding strategies.

Among the 15 different possible pairings starting from six cysteine residues, we obtained the following results:

•**Non-directed oxidative folding:** A single isomer, AdIIIB-1, containing three disulfide bonds, was obtained. This isomer elutes at 2.97 minutes, regardless of whether the folding occurred in aqueous or organic media (Figure 1: B, C).

•**Regioselective folding:** Starting from the linear precursor, we sequentially formed the disulfide bonds between C1–C4, C2–C5, and C3–C6, an arrangement based on the most active isomer of SIIID conotoxin, which has a sequence similar to AdIIIB. The elution time of this isomer, AdIIIB-2 (rt: 3.29 minutes, Figure 1: D), is different from that of the isomer obtained by non-directed oxidative folding.

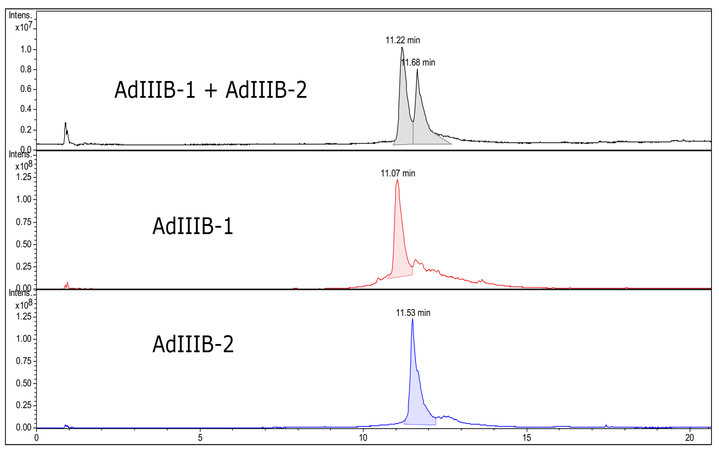


Figure 3: UHPLC analysis : Co-injection of the different isomer AdIIIB-1 & AdIIIB-2

Rat receptors	% Responce of current amplitude: 10 µM AdIIIB-1 Oocyte 1	Oocyte 2	Oocyte 3	10 µM AdIIIB-2 Oocyte 1	Oocyte 2	Oocyte 3
αβγδ(muscle typ.)	28%	14%	20%	78%	76%	71%
α2β4	97%	96%	86%	100%	100%	90%
α2β2	88%	86%	-	97%	98%	-
α3β4	96%	90%	84%	99%	96%	99%
α3β2	72%	68%	-	91%	89%	91%
α4β4	93%	92%	88%	100%	99%	97%
α7	92%	91%	-	100%	100%	-

Table 2: inhibitory potency on different nAChRs subtypes

2. Co-Injection of the Isomers

Co-injection of the isomers AdIIIB-1 and AdIIIB-2, obtained respectively through oxidative and regioselective folding, confirms their structural differences (Figure 3). This observation underscores the limitations of relying solely on literature predictions for disulfide bridge configurations.

3. Inhibitory Test

The inhibitory potency of AdIIIB-1 and AdIIIB-2 was assessed using two-electrode voltage clamp analysis on rat nAChR subtypes. Results indicated that AdIIIB-1, derived from oxidative folding, exhibited significantly higher biological activity compared to AdIIIB-2. Furthermore, AdIIIB-1 demonstrated a selective affinity for the α1β1γδ nAChR subtype over other subtypes.

Conclusion & perspective

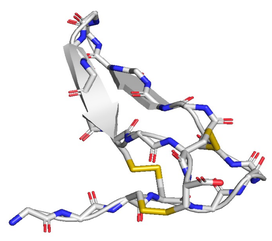
Comparing the three folding methods for conotoxin, the most prominent findings are:

•**Non-Directed Oxidation**: The isomer produced through non-directed oxidation exhibited the highest biological activity.

•**Organic Oxidative Folding**: This method was faster, but it requires improvements to simplify the purification of the cyclic peptide.

•**One-Pot Regioselective Oxidation**: This approach successfully yielded the desired isomer; however, the disulfide connectivity differed from that of the isomer obtained through non-directed methods.

•**Predictive Tools**: Utilizing tools like AlphaFold to predict disulfide bond formation could be beneficial in guiding the selection of folding strategies when applying the regioselective method.



AlphaFold structure prediction of AdIIIB

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