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Raising the Bar On-Bead: Efficient On-Resin Synthesis of α -Conotoxin LvIA

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Abstract

α 4/7-Conotoxin LvIA is an isoform-selective inhibitor of the α 3 β 2 nicotinic acetylcholine receptor. An efficient strategy for the synthesis of this toxin is critical to advancing its utility as a probe for receptor function and as a potential pharmaceutical lead target. On-resin methods for peptide synthesis offer potential synthetic advantages; however, strategies for on-resin formation of multiple disulfides have historically been low-yielding. Here, we harness the reactivity of the Allocam protecting group and employ 3-amino acid spacer strategy to synthesize α 4/7-conotoxin LvIA via three different on-resin strategies, each of which results in an isolated yield higher than prior fully on-resin approaches.

Graphical Abstract

α 4/7-Conotoxin LvIA (α -LvIA, **1**) is a selective inhibitor of the α 3 β 2 isoform of the rat nicotinic acetylcholine receptor (nAChR, Figure 1A).¹ This neuronal receptor is implicated in a variety of neurological disorders as well as some carcinomas.^{2,3} Since the initial report of its rare selectivity,^{3a} synthetic α -LvIA has enabled important biochemical studies,⁴ including the development of a selective inhibitor of human α 3 β 2 and α 6 β 2 nAChRs.⁵ These studies highlight the critical importance of efficient and straightforward methods for the synthesis of conotoxins and their analogs. Known methods for controlled folding of α -conotoxins require multiple solution-phase deprotection and oxidation steps, often mandating that the peptide be repeatedly extracted,⁶ precipitated and/or purified.⁷ Because

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Supporting Information. Peptide synthesis procedures and supporting analytical RP-HPLC-MS data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interests.

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of the convenience of solid-phase methods, a strategy wherein the entire total synthesis is performed on the solid support would be ideal. Such an approach would enable ready removal of all necessary reagents, avoid potential issues with toxicity of intermediate peptides, avoid loss of yield due to aggregation of partially folded intermediates, and minimize purification steps. Currently, the limiting step in the synthesis of these targets is controlling the connectivity of the two disulfide linkages.⁸ As a first step toward expanding the toolbox of on-resin disulfide-forming methods,⁹ we recently reported a new strategy for on-resin disulfide formation by Pd-mediated¹⁰ removal of the allyloxycarbonyl aminomethyl (Allocam, Figure 1B) protecting group with in situ oxidation.¹¹ In this manuscript, we harness the utility of the Allocam group to develop three new on-resin methods for the synthesis of the two-disulfide polypeptide α 4/7-conotoxin LvIA.

There are relatively few methods for formation of multiple disulfides on resin,^{12a} likely because of early reports of interstrand disulfide formation during solidsupported reactions.¹³ However, the pseudo-dilution effect is known to favor macrocyclization reactions,¹⁴ and recent reports of on-resin oxidation using low-loading resins have not reported this issue.^{12a} Previous strategies for native α -conotoxin syntheses have resulted in a maximum yield of <5%.^{15,16} Other approaches led to crude folded conotoxins showing good purity,¹² but with no yields reported, it is difficult to predict the amenability of these strategies to production of useful quantities of peptide. An interesting alternative is the synthesis of diseleno analogs of conotoxins; however, this strategy is limited by unpredictable conformational biases during on-resin oxidative folding and the cost of selenocysteine analogs.¹⁷ We envisioned that application of our Allocam chemistry to the synthesis of α -LvIA would be strategically advantageous because the Pd(0)-mediated cleavage mechanism presumed to be operative in Allocam removal would be orthogonal to groups cleaved by other pathways.

To determine the stability of common protecting groups to our Allocam removal conditions, we synthesized model peptides **2-TGT** bearing four common protecting groups and subjected them to our previously optimized Allocam removal/oxidation conditions (Scheme 1).^{11,18} Treatment of the mono- methoxytrityl (Mmt)-protected peptide **2-TGT** led to a mixture of partial cleavage products, disulfide **3**, and starting peptide **2-OH**. The acetamidomethyl (Acm) group was also unstable, leading mainly to oxidized peptide **3**. Presumably, the instability of these groups is due to the presence of Pd(II) in the reaction mixture, which can function as a soft Lewis acid.¹⁰ Not surprisingly, the trityl (Trt) and *t*-Bu groups were unmodified by these conditions, and only peptide **2-OH** was observed. Either of these groups could potentially be viable for protection of the second pair of Cys residues in the synthesis of α -LvIA.

We next investigated the stability of Allocam to conditions for the removal of four other protecting groups and oxidation to the disulfide. Although Allocam is sensitive to high percentages of the TFA needed for peptide cleavage, it is stable to the low percentages (<30% TFA) required for the removal of Trt and Mmt.¹¹ Thus, if Allocam is stable to oxidation of free thiols to disulfides, Trt and Mmt would be viable groups for the first disulfide. We tested several conditions using a model peptide bearing Allocam-protected cysteines (Table 1).¹⁸ Exposure of model peptide **4-TGT** to *N*-chlorosuccinimide (NCS),^{12,19} resulted in a 1:1 ratio of oxidized (**3**) and unreacted peptides (**4-OH**, entry 1).

Increasing the reaction time resulted in complete conversion to the oxidized product. Iodine can be employed for oxidative cleavage of peptides that contain Trt-, Mmt-, or AcM-protected Cys.²⁰ Exposure of model peptide **4-Wang** to iodine conditions for oxidative cleavage led only to oxidized product **3** (entry 3). The sensitivity of Allocam to NCS and I₂ piqued our interest because the analogous AcM-protected peptide requires longer I₂ exposures to reach complete conversion, and the sensitivity of peptides to prolonged oxidant exposure is well established.²¹ We did not attempt oxidation of free thiols or AcM-protected peptide **4** using TI(III) salts²² because of the exceptional toxicity of TI(I),²³ which would be formed during the reaction. We next considered the compatibility of Allocam with the S*t*Bu/Mmt protection strategy,^{15,21,24} which requires initial reduction of the Cys(S*t*Bu) side chain using β -mercaptoethanol followed by activation of the free thiols using 2,2'-dithiobis(5-nitropyridine) (DTNP). To induce cyclization, a low percentage solution of TFA and triisopropylsilane (TIPS) is used to deprotect Cys(Mmt) and liberate a free thiol for the disulfide bond formation with pre-activated thiol group. Peptide **4-TGT** was exposed to the conditions for conversion of S*t*Bu and Mmt to a disulfide, and we did not observe any Allocam removal or oxidation (entry 3).

Our test studies indicated that Allocam could be partnered with Trt, with Allocam removed first, or with the S*t*Bu/Mmt three-step disulfide formation, with Allocam removed last via Pd(0), *N*-chlorosuccinimide, or I₂-mediated methods. Previous studies of α -conotoxin folding suggested that the small loop is easier to form on resin.¹⁵ Thus, we initiated our studies with Allocam on Cys(1) and Cys(3) and Trt protection on Cys(2) and Cys(4). We assembled α -LvIA precursor **5** via Fmoc-SPPS on a Rink-amide (RA)-linked resin (Scheme 2).¹⁸ Application of our previously optimized conditions¹¹ (1.5 equiv Pd(OAc)₂,²⁵ 5% AcOH, 3% NMM in DMSO) provided the desired mono-disulfide intermediate in 6 h without any observable loss of either S-Trt group. Next, oxidative removal of the S-Trt groups using 5.0 equiv I₂,²⁵ removal of the side-chain protecting groups and cleavage from the resin, and then HPLC purification afforded native α -LvIA (**1**) in 1% isolated yield relative to the final loading of the peptide.

We considered that the low yield in this synthesis might arise from insufficient space for the peptide to reach the necessary conformation for oxidation in each disulfide formation. Thus, we modified our resin selection, opting for the better swelling properties of the poly(ethylene glycol)-based aminomethyl-ChemMatrix (CM) resin.²⁶ Additionally, we modified the linker strategy to incorporate a tripeptide spacer before installation of the Rink amide linker (Scheme 2). Interestingly, the synthesis of the small loop proceeded faster on this substrate, requiring only 3 h to complete.¹⁸ The iodine oxidation proceeded uneventfully under the same conditions as the synthesis on Rink amide-PS.²⁷ We were pleased to observe an overall isolated yield of 8% via this approach. The correct folding of the isolated product was confirmed by co-injection with a sample prepared according to the approach described in the structural elucidation manuscript.^{3a,28}

We next turned our attention to the synthesis of α -LvIA via the second strategy supported by our studies in Scheme 1 and Table 1, protection of the first loop by a combination of S*t*Bu and Mmt with Allocam on the second loop. The three-step synthesis of the first disulfide via this approach required significant optimization, ultimately leading to the conditions outlined

in Scheme 3.¹⁸ First, disulfide exchange with β -mercaptoethanol required microwave irradiation over 3 h to proceed to completion (peptide **6** $\rightarrow$ **7**). Next, a second disulfide exchange was performed with the oxidant DTNP at ambient temperature over 6 h to afford activated peptide **8**. On small scale (50 mg resin), this step could be performed for 1 h at 60 °C in the microwave, but those conditions proved to be unreliable on larger scale (>50 mg resin). Removal of the monomethoxytrityl (Mmt) protecting group from peptide **8** proved challenging because of premature cleavage of the peptide from the Rink amide linker when either higher acid concentrations or longer reaction times were employed. Complete removal of Mmt was observed with minimal loss of peptide **8** in the presence of 3% trifluoroacetic acid and 5% triisopropylsilane in CH_2Cl_2 for two 15-min exposures. With mono-disulfide precursor **9** in hand, we investigated the Allocam removal by treatment with $\text{Pd}(\text{OAc})_2$. A variety of reaction times and Pd-loadings were attempted,¹⁸ with the best result giving a 1:1 ratio of the target peptide **1** and the partial cleavage product bearing one Allocam (**mono-Allocam-1**). We hypothesized that the C-terminal Cys(Allocam) was the problematic residue because of the steric crowding caused by the resin.

Repetition of the Fmoc-SPPS on Rink amide-GGG-CM was followed by optimization of the reaction conditions for each step (Scheme 3).¹⁸ All reaction times were shortened using this resin/spacer strategy. Peptide **6** was treated with 30% β -mercapto-ethanol in DMF at 60 °C under microwave conditions for 1.5 h to afford peptide **7**. Activation with DTNP for 1 h provides peptide **8**, and then treatment with 5% trifluoroacetic acid, 5% triisopropylsilane in CH_2Cl_2 for five 1-min exposures affords disulfide linked peptide **9**. Upon treatment of **9** with 3.0 equiv $\text{Pd}(\text{OAc})_2$ for 6 h at ambient temperature followed by resin cleavage with global side chain deprotection, and then purification, native α 4/7-conotoxin LvIA was isolated in 5% yield relative to the final loading of peptide **6** (HPLC trace shown in Figure 2). This result establishes that Pd-mediated formation of the larger loop, which forms more slowly, is a viable approach. Additionally, the GGG spacer is a critical design element that provides conformational mobility to facilitate folding and shorten reaction times, thereby minimizing any non-specific peptide decomposition that might be induced by prolonged exposure to oxidants.

Based on our experiments in Table 1, we knew that the Allocam group was also labile to both NCS and iodine. Because of the convenience and simplicity of these reagents, we were excited by their potential use for oxidative removal of Allocam in a complex setting. Based on the improvement in reactivity observed when the GGG spacer was used in Scheme 2 and Scheme 3, this spacer was retained in the syntheses using other oxidants for the final loop. Upon treatment of peptide **9** (Scheme 4) with 5.0 equiv NCS for 5 hours, we observed the desired peptide in the analytical trace,¹⁸ but no product could be isolated by semi-preparative HPLC when this reaction was repeated on a larger scale. Gratifyingly, in the presence of 8 equiv I_2 for 6 h, the desired Allocam cleavage and disulfide formation were observed, and the final conotoxin was isolated in 12% yield from the final loading of peptide **6** (HPLC trace shown in Figure 3).¹⁸ Importantly, this result represents the highest yielding on-resin synthesis of an α -conotoxin reported to date.^{12,15}

In summary, we have established the utility of the Allocam group in facilitating the on-resin synthesis of the subtype-selective nAChR antagonist α 4/7-conotoxin LvIA. The Allocam

group is stable to the removal of groups cleaved by low concentrations of TFA or by disulfide exchange reactions, including Mmt, Trt, and *S*tBu. Conversely, protective groups not readily removed by Lewis acid conditions, such as Trt and *t*Bu, are stable to Pd-mediated Allocam removal. Allocam is expected to be advantageous in strategies where AcM would typically be employed because it can be removed faster than AcM using iodine, thus reducing the potential for undesired oxidation of sensitive amino acids. We also designed and implemented a spacer strategy that improves the yield of on-resin folding and reduces reaction times. By harnessing the multimodal reactivity of the Allocam group, we have established three new protecting group strategies for the synthesis of α -LvIA. Each of these approaches represents a significant improvement on existing strategies for on-resin α -conotoxin synthesis. Importantly, the strategy employing I₂-mediated conditions for Allocam removal constitutes the first on-resin synthesis that results in a synthetically useful isolated yield of a 2-disulfide conotoxin. Application of the technologies described herein to other 2-disulfide and higher-order conotoxins is ongoing in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

RP-HPLC	Reverse-Phase High Performance Liquid Chromatography
MS	Mass Spectroscopy
TGT	PEG-linked trityl resin
PEG	poly(ethylene glycol)
PS	polystyrene resin
CM	ChemMatrix resin
Allocam	Allyloxycarbonylaminomethyl
AcM	Acetamidomethyl
Trt	trityl
Mmt	Monomethoxy trityl
NCS	<i>N</i> -chlorosuccinimide
DMSO	dimethylsulfoxide

TFA	trifluoroacetic acid
TIPS	triisopropylsilane
DTNP	2,2'-dithiobis(5-nitropyridine)
DMF	<i>N,N</i> -dimethylformamide

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- (28). In our hands, the previously published synthesis^{3a} of this target provided the final pure compound in 22% yield from the purified linear peptide following resin cleavage. See the Supplemental Information for details.

Figure 1.

A) Ribbon structure³ and schematic representation of $\alpha 4/7$ -conotoxin LvIA. B) Structure of Allocam group.

Figure 2. HPLC trace and MS data for on-resin folded (via Scheme 3 with GGG spacer) and in-solution folded α -LvIA²⁸ with co-injection.¹⁸

Figure 3. HPLC trace and MS data for on-resin folded (via Scheme 4 with GGG spacer) and in-solution folded α -LvIA²⁸ with co-injection.¹⁸

Scheme 2.

Allocam/Trt strategy for on-resin synthesis of native α4/7-conotoxin LvIA.²⁵

Scheme 3.
Implementation of *S*-Bu/Mmt protecting strategy in combination with Allocam.²⁵

Scheme 4.

Alternative oxidative cleavage conditions provide high yield of final product.²⁵

Table 1.

Stability of Allocam to on-resin removal and/or oxidation conditions for various cysteine functional groups.

Entry	Resin	Cys Group	Conditions	Solvent	Time (min)	3:4-OH ^d
1. ^b	TGT	H	2 equiv NCS	DMF	15	54:46
2. ^c	Wang	Trt, Mmt, Acn	8 equiv I ₂	CH ₂ Cl ₂	60	100:0
3. ^b	TGT	StBu/Mmt	20% HS(CH ₂) ₂ OH then excess DTNP then, 1 % TFA, 5% TIPS	DMF CH ₂ Cl ₂	180	0:100

^a Unless otherwise noted, reaction was performed using 10 mg of resin and 500 μ L total volume, $rt = 24 \pm 1$ °C, LCMS analysis is performed directly on crude peptide following removal of solvents from resin cleavage.

^b NovaSyn PEG-PS copolymer resin with TGT linker, preloaded with Fmoc-Thr(O t Bu), resin cleaved with 1:1:3 TFE:AcOH:CH₂Cl₂.

^c Novabiochem copoly (styrene-1% DVB) resin with Wang linker, 100–200 mesh, pre-loaded with Fmoc-Tyr(O t Bu), resin cleaved with 30:5:65 TFA:TIPS:CH₂Cl₂.

^d Ratio determined by integration of relevant peaks in HPLC-MS.