ARTICLE

On-resin synthesis of the somatostatin venom analog Consomatin Ro1

Ramoncito Luis B. de Boda*[1], Zildjian G. Acyatan[2], Gisela P. Concepcion[2], and Aaron Joseph L. Villaraza[1]

[1]Institute of Chemistry, College of Science, University of the Philippines, Diliman, Quezon City, 1101, Philippines
[2]Marine Science Institute, College of Science, University of the Philippines, Diliman, Quezon City, 1101, Philippines

ABSTRACT

Consomatin Ro1 is a disulfide-containing peptide derived from the venom of the cone snail *Conus rolani* with a sequence that is similar to the vertebrate peptide hormone somatostatin. It has been shown to preferentially activate human somatostatin receptor subtypes 1 and 4, and to exhibit antinociceptive and antihyperalgesic properties making it an interesting peptide to study and develop as a chemical probe or an analgesic drug. Here, we describe the synthesis of Consomatin Ro1 using an on-resin approach wherein the disulfide bond is formed while the peptide is still attached to the resin. This was achieved by selectively removing the methoxytrityl protecting group of Cys residues with a weak acidic mixture, and treating the resulting thiol-containing peptidyl resin with the mild oxidant *N*-chlorosuccinimide. The strategy yielded a considerably higher amount of the peptide when compared with the previously reported in-solution disulfide formation method.

*Corresponding author
Email Address: alvillaraza@up.edu.ph
Date received: March 31, 2023
Date revised: July 28, 2023
Date accepted: August 14, 2023

INTRODUCTION

Consomatin Ro1 (Figure 1A) is a 13-residue peptide derived from the venom of the cone snail *Conus rolani* with post-translational modifications that include: a γ-carboxylated Glu (abbreviated as Gla or γ), a Trp in the D-configuration (abbreviated as D-Trp or small letter w), a hydroxylated Pro (abbreviated as Hyp or O), and two Cys that form an intramolecular disulfide bond (Ramiro et al. 2022). Its sequence bears similarity with somatostatin (Figure 1B), a peptide hormone secreted throughout the nervous system, gastrointestinal tract, and pancreas that regulates hormone secretion, neurotransmission, smooth muscle contractility, nutrient absorption, and cell division (Kumar and Grant 2010). Bioactivity studies by Ramiro et al. (2022) found that the venom peptide preferentially activates human somatostatin receptor subtypes 1 and 4 (SST_1 and SST_4)—SST_4, in particular, is associated with pain modulation (Helyes et al. 2009)—and exhibits antinociceptive and antihyperalgesic activities in mouse pain models. These properties make Consomatin Ro1 a novel lead compound that can be developed as an SST_1 and SST_4 signaling pathway chemical probe and as an analgesic drug.

KEYWORDS

Consomatin Ro1, cone snail venom peptide, on-resin disulfide formation, solid phase peptide synthesis, organic chemistry

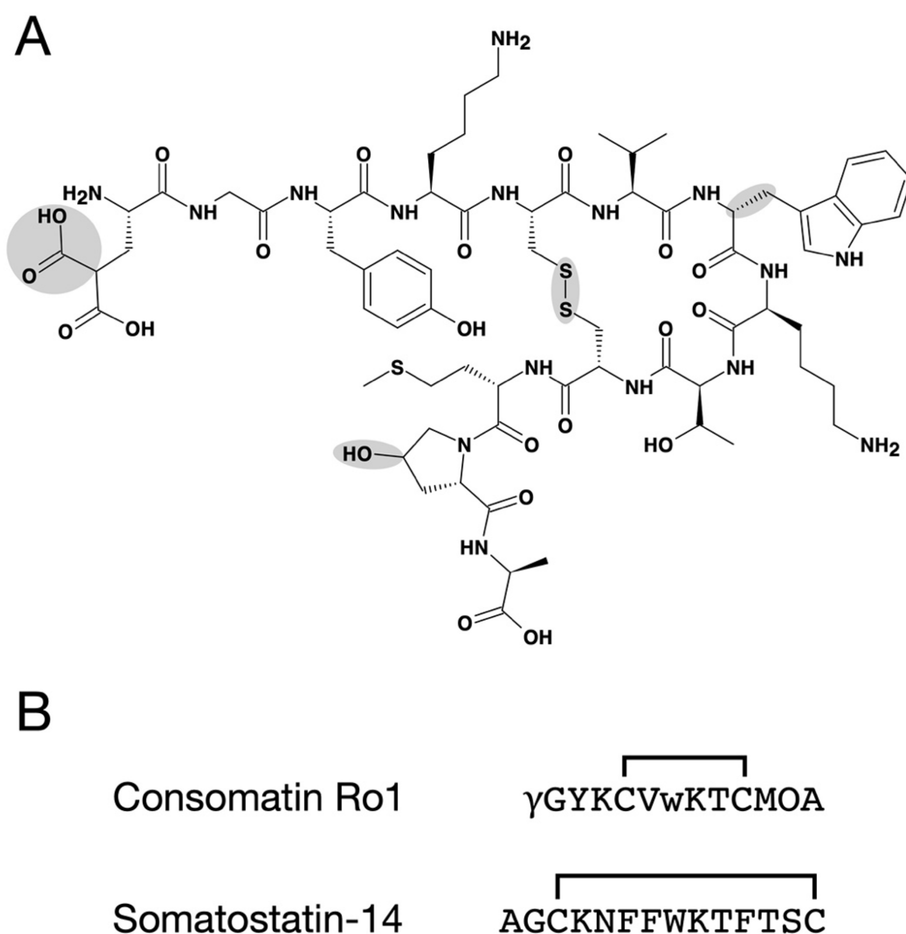


Figure 1: (A) Chemical structure of Consomatin Ro1 with post-translational modifications shaded gray. (B) Comparative sequence alignments of Consomatin Ro1 (top) and somatostatin-14 (bottom). Black brackets indicate disulfide connectivity. γ, γ-carboxyglutamic acid; w, D-tryptophan; O, hydroxyproline.

Synthesis of Consomatin Ro1 was previously achieved using an in-solution folding strategy that involved the following steps: (1) sequence assembly of the linear precursor on a solid support or resin, (2) peptide cleavage from the resin, (3) reversed-phase high-performance liquid chromatography (RP-HPLC) purification and lyophilization of the linear peptide, (4) solution phase disulfide bond formation via oxidation, and (5) RP-HPLC purification and lyophilization of the folded peptide (Ramiro et al. 2022). Drawbacks of this approach are peptide oxidation is performed under high dilution to avoid disulfide-mediated dimerization and oligomerization, and multiple purification steps are required which is costly and time-consuming.

Here, we describe an alternative method of synthesizing Consomatin Ro1 in which the disulfide bond is formed while the peptide is still anchored to the resin. This on-resin strategy takes advantage of the pseudodilution phenomenon that occurs in resin-supported reactions wherein bound dithiol-containing peptide molecules are sequestered from each other, creating a high dilution environment that favors intramolecular disulfide bond formation over intermolecular side reactions (Albericio et al. 1991; Annis et al. 1998). On-resin folding allows easy removal of excess oxidation reagents and side products by filtration and reduces RP-HPLC purification steps since crude disulfide-containing peptides are already obtained after cleavage. It has been used to synthesize oxytocin (Albericio et al. 1991; Postma and Albericio 2013; Kondasinghe et al. 2017; Kobayashi et al. 2021; Spears et al. 2022), tumor-homing peptide analogs (Galande et al. 2005), conotoxins (Galanis et al. 2009; Postma and Albericio 2013; Kondasinghe et al. 2019; Kobayashi et al. 2021), atosiban (Yang et al. 2020), melanin-concentrating hormone (Kobayashi et al. 2021), and microcionamide analogs (Inocentes et al. 2023) with acceptable yields.

For the on-resin folding of Consomatin Ro1 reported in this study, the disulfide bond was formed by removing the Cys protecting group of the resin-bound peptide using a mild acid solution and then treating the free thiol group with a mild oxidant. We confirmed the identity of the synthetic product by analyzing its mass with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and by comparing it with native Consomatin Ro1 via RP-HPLC and mouse behavioral assay.

MATERIALS AND METHODS

Materials

Wang resin and the following *N*-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids were purchased from AAPPTec: Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Val-OH. *N*-Ethyldiisopropylamine (DIPEA) and Fmoc-Cys(Mmt)-OH were purchased from Merck. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Fmoc-D-Trp(Boc)-OH, and Fmoc-Gla(OtBu)$_2$-OH were purchased from GL Biochem. Oxyma, *N*,*N'*-diisopropylcarbodiimide (DIC), 4-methylpiperidine, benzoic anhydride, pyridine, *N*-chlorosuccinimide (NCS), triisopropylsilane (TIPS), and 3,6-dioxa-1,8-octanedithiol (DODT) were purchased from Sigma-Aldrich. Fmoc-Hyp(tBu)-OH and benzotriazol-1-yloxytripyrrolidinophosphonium

hexafluorophosphate (PyBOP) were purchased from Chem-Impex. Dichloromethane (DCM), dimethylformamide (DMF), and trifluoroacetic acid (TFA) were purchased from J.T.Baker. Methyl tert-butyl ether (MTBE) was purchased from Scharlau. HPLC-grade water and acetonitrile (ACN) were purchased from DUKSAN.

Fmoc-Ala-OH loading

Wang resin (0.9 mmol/g) was swollen in DCM for 1 h and washed with DMF. Fmoc-Ala-OH (4 eq) was attached manually onto the resin at rt for 2 h in a DMF solution containing DIPEA (8 eq), HBTU (4 eq), and Oxyma (3.8 eq) that was preactivated for 5 min before coupling. The loaded resin was washed 5 times with DMF and another 5 times with DCM before drying overnight. Actual resin loading (0.5 mmol/g) was determined via Fmoc release measurement according to the method described in Note 3 by Amblard et al. (2006). Unreacted sites were capped by adding a solution of benzoic anhydride (5 eq) and pyridine (1 eq) in DMF to the resin and stirring for 30 min as described in the protocol of Amblard et al. (2006) for Fmoc-amino acid attachment to hydroxymethyl-based resins. After capping, the resin was washed with 5 × DMF and 5 × DCM, and dried overnight.

Peptide assembly

The subsequent eleven amino acid residues (GYKCVwKTCMO) of Consomatin Ro1 were assembled on 0.1 g Fmoc-Ala-loaded Wang resin with a scale of 0.05 mmol using a CEM Liberty Blue™ automated microwave peptide synthesizer according to the method of Collins et al. (2014) with some modifications. Fmoc protecting group was removed using 20% (v/v) 4-methylpiperidine in DMF at 90°C for 50 s. Fmoc-amino acids (5 eq) were coupled once using DIC (10 eq) and Oxyma (5 eq) in DMF at 90°C for 110 s except for Fmoc-Val-OH, Fmoc-D-Trp(Boc)-OH, and Fmoc-Met-OH which were coupled twice as recommended in the Liberty Blue™ user guide (CEM Corporation 2018). After assembly, the resin was washed with DMF and DCM as described previously.

Fmoc-Gla(OtBu)$_2$-OH (5 eq) was incorporated manually onto the peptide chain at rt for 1.5 h using DIPEA (10 eq) and PyBOP (5 eq) in DMF as coupling reagents with 5 min preactivation. The resin was then washed with DMF and DCM as described previously. The Fmoc protecting group was removed by treatment with 20% (v/v) 4-methylpiperidine in DMF at rt for 3 min under sonication. After deprotection, the resin was washed with DMF and DCM as described previously.

On-resin disulfide bond formation

On-resin disulfide bond formation of Consomatin Ro1 was achieved according to the method of Postma and Albericio (2013) with some modifications. The resin was treated with 2% (v/v) TFA in DCM at rt for 5 min 10 times to remove the methoxytrityl (Mmt) protecting group from the Cys residues. It was then washed with 5 × DCM and 5 × DMF. NCS (1.05 eq) in DMF was added to the resin and agitated at rt for 15 min to form the intramolecular disulfide bond. The resin was washed with DMF and DCM, as described previously, and dried overnight.

Peptide cleavage and side chain deprotection

Cleavage and side chain deprotection of Consomatin Ro1 was accomplished by treating the resin with a cleavage mixture of 92.5:2.5:2.5:2.5 (v/v/v/v) TFA/TIPS/DODT/water at rt for 1.5 h. The mixture was filtered and added dropwise to cold MTBE to precipitate the peptide. The suspension was then centrifuged and decanted. The crude peptide was washed once with cold MTBE, dissolved in 50% ACN in water, and lyophilized overnight.

Peptide purification and purity determination

Purification of crude Consomatin Ro1 was performed via RP-HPLC on a Shimadzu Prominence UFLC system using an Inertsil ODS-3 C18 preparative column (20 × 250 mm, 5 µm particle size) as the stationary phase. 0.1% TFA in water (solvent A) and 0.1% TFA in ACN (solvent B) were used as HPLC solvents. The crude peptide was purified using a linear gradient of 10–50% solvent B over 40 min at a flow rate of 8 mL/min. The peptide was further purified on the same RP-HPLC system using a Phenomenex Luna C18 semi-preparative column (10 × 250 mm, 5 µm particle size) with the same gradient as described above at a flow rate of 3 mL/min. Purity was assessed on the same RP-HPLC system using a Phenomenex Luna C18 analytical column (4.6 × 250 mm, 5 µm particle size) with a linear gradient of 35–45% solvent B over 10 min at a flow rate of 1 mL/min. Eluents were monitored by measuring absorbance at 220 nm.

MALDI-TOF MS analysis

MALDI-TOF MS analysis was conducted using a Shimadzu Biotech Axima Confidence MALDI-TOF mass spectrometer. 1 µL of 10 mg/mL ferulic acid (FA) in 30% (v/v) solvent B was spotted on a stainless steel MALDI plate before spotting 0.5 µL (~2 nmol) of the pure peptide. MALDI-TOF mass spectrum was acquired in reflectron positive operating mode with a laser power set at 50. External calibration was performed by spotting 0.5 µL of LaserBio Labs Peptide Calibration Mix 4 (500–3,500 Da) to a FA matrix-spotted well.

Peptide quantitation

Pure Consomatin Ro1 was quantified by ultraviolet absorbance at 280 nm using an extinction coefficient of 6990 $M^{-1}{\cdot}cm^{-1}$ as described by Ramiro et al. (2022).

RP-HPLC comparison of synthetic and native Consomatin Ro1

Synthetic and native Consomatin Ro1 were separately loaded on a Phenomenex Luna C18 analytical column (4.6 × 250 mm, 5 µm particle size) using the same gradient and flow rate that were applied for purity determination. A co-elution experiment was also done by mixing both synthetic and native peptides in a 0.25:1 ratio and injecting the mixture on the same C18 analytical column with the same conditions.

Intracranial mouse bioassay

Intracranial mouse bioassay was performed according to the method reported by Clark et al. (1981) with minor modifications. Briefly, synthetic and native Consomatin Ro1 were dissolved in 20 µL normal saline solution (NSS; 0.9% NaCl), and injected intracranially into 14-day old ICR mice using a 29-gauge insulin syringe. Control mice were injected with NSS only. General mouse behaviors such as roaming and grooming, and response to tactile stimuli such as prodding were documented for at least 3 h. All samples were tested at n = 2. Behavioral differences between the treated and control groups were recorded. The protocol for this assay was approved by the Institutional Animal Care and Use Committee of the University of the Philippines Diliman (AP-2020-12; approved 3 April 2020).

RESULTS AND DISCUSSION

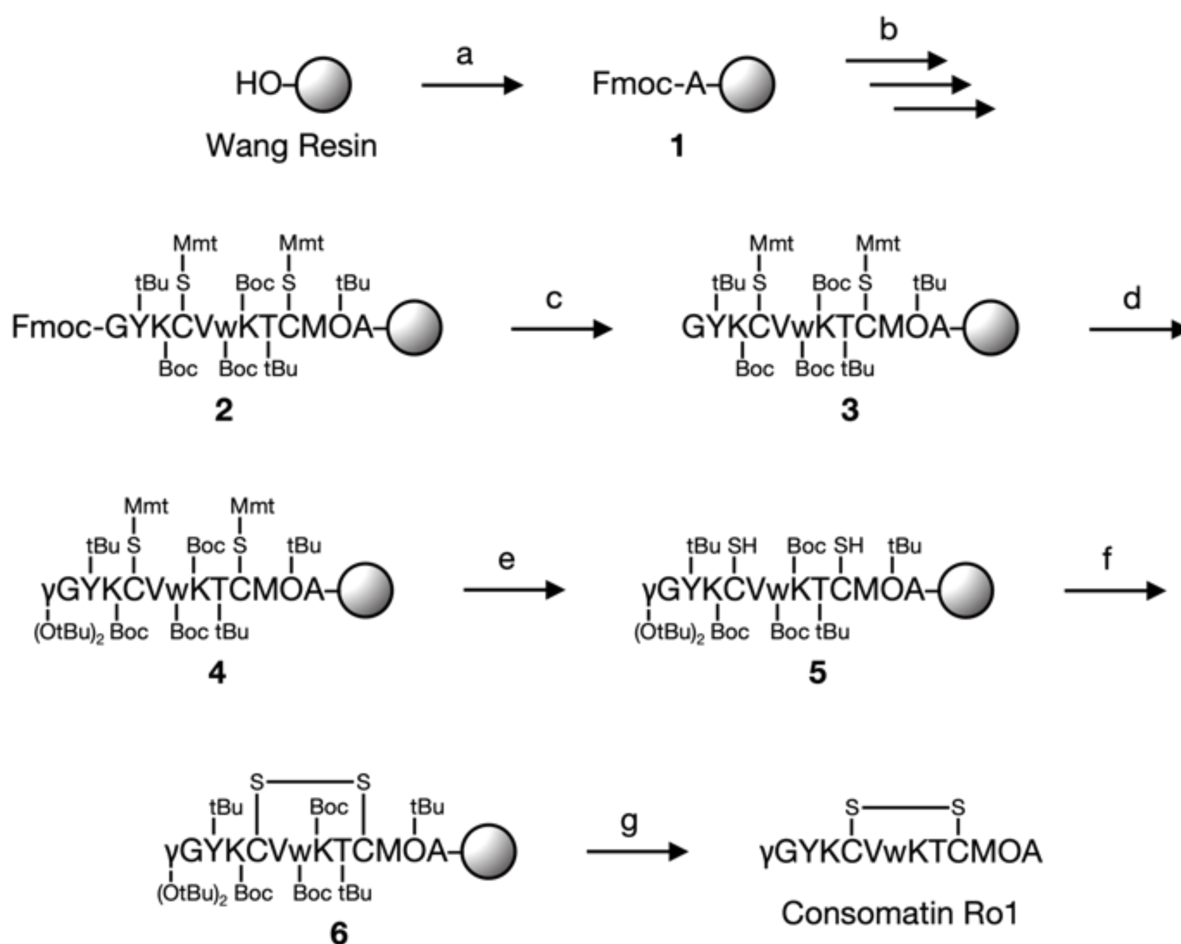
Scheme 1 outlines the synthetic route of Consomatin Ro1 using an on-resin strategy. Fmoc-Ala was attached manually to Wang resin by reacting the Fmoc-amino acid with the resin only once with HBTU, DIPEA, and Oxyma as coupling reagents. Fmoc-Ala-Wang resin **1** was used despite the actual loading being only 55% of the expected loading to prevent any intermolecular reactions that may occur with a high-loading resin. Peptidyl resin **2** was then assembled on **1** using an automated microwave

peptide synthesizer with DIC and Oxyma as coupling reagents. After removing the Fmoc protecting group of **2**, Fmoc-Gla(OtBu)$_2$-OH was coupled manually to **3** by means of the same activating reagents used by Ramiro et al. (2022) to ensure that only the needed amount of the expensive Fmoc-amino acid was prepared. The resulting peptidyl resin **4** was treated with a dilute acid solution of TFA in DCM to yield the free thiol-containing peptidyl resin **5**. This was achieved by attaching Cys residues with Mmt as the thiol-protecting group to the peptide chain. The high sensitivity of Mmt to acid allows its selective deprotection under mild acid conditions (Barlos et al. 1996) without cleaving the peptide from the less acid labile Wang resin. Intramolecular disulfide bond was formed by treatment of **5** with NCS, a mild oxidant, in DMF. The crude product was finally generated by treating the disulfide-containing peptidyl resin **6** with a TFA/TIPS/DODT/water cocktail that cleaves the peptide from the resin and removes the side chain protecting groups.

A chromatogram with a major peak at t_R 23.32 min which constitutes 18% of the crude product (Figure 2A) was obtained from preparative C18 RP-HPLC purification. MALDI-TOF MS analysis of the fraction corresponding to the major peak revealed a monoisotopic mass that is almost identical to Consomatin Ro1 (observed $[M+H]^+$: 1573.67 Da; calculated $[M+H]^+$: 1573.65 Da); furthermore, the linear precursor was not observed in the crude product. Purification of the fraction containing the major peak by semi-preparative C18 RP-HPLC (Figure 2B) yielded a peptide with 98% purity (Figure 2C) and a $[M+H]^+$ mass of 1573.64 Da (calculated $[M+H]^+$: 1573.65 Da).

The identity of the synthesized product was validated as Consomatin Ro1 by comparing its HPLC profile and bioactivity with the native peptide (Consomatin Ro1 isolated from the venom of *C. rolani*). As shown in Figure 3, the synthetic product and native Consomatin Ro1 exhibited similar retention times (synthetic Consomatin Ro1 t_R 15.26 min; native Consomatin Ro1 t_R 15.27 min) and displayed a single peak upon injection of a 0.25:1 mixture of the synthetic and native peptides (t_R 15.23 min). Moreover, different HPLC retention times of the folded and linear Consomatin Ro1 and the presence of two peaks in the chromatogram upon their co-injection (Supplementary Figure 1) further prove that a disulfide bond was formed in the on-resin folded peptide.

When injected intracranially into 14-day old mice, 2.5 nmol of the synthetic peptide elicited behavioral phenotypes (i.e., lethargy, loss of balance, and sedation for about 3 h) similar to what were observed in the native peptide at the same dose (Table 1). These observations were also reported in the first isolation of Consomatin Ro1 (Ramiro et al. 2022).



Scheme 1: On-resin synthesis of Consomatin Ro1. Reagents and conditions: (a) Fmoc-Ala-OH (4 eq), DIPEA (8 eq), HBTU (4 eq), Oxyma (3.8 eq) in DMF, rt, 2 h; (b) (i) 20% (v/v) 4-methylpiperidine in DMF, 90°C, 50 s; (ii) Fmoc-amino acid (5 eq), DIC (10 eq), Oxyma (5 eq) in DMF, 90°C, 110 s; (c) 20% (v/v) 4-methylpiperidine in DMF, 90°C, 50 s; (d) (i) Fmoc-Gla(OtBu)$_2$-OH (5 eq), DIPEA (10 eq), PyBOP (5 eq) in DMF, rt, 1.5 h; (ii) 20% (v/v) 4-methylpiperidine in DMF, rt, 3 min; (e) 2% (v/v) TFA in DCM, rt, 10 × 5 min; (f) NCS (1.05 eq) in DMF, rt, 15 min; (g) 92.5:2.5:2.5:2.5 (v/v/v/v) TFA/TIPS/DODT/water, rt, 1.5 h.

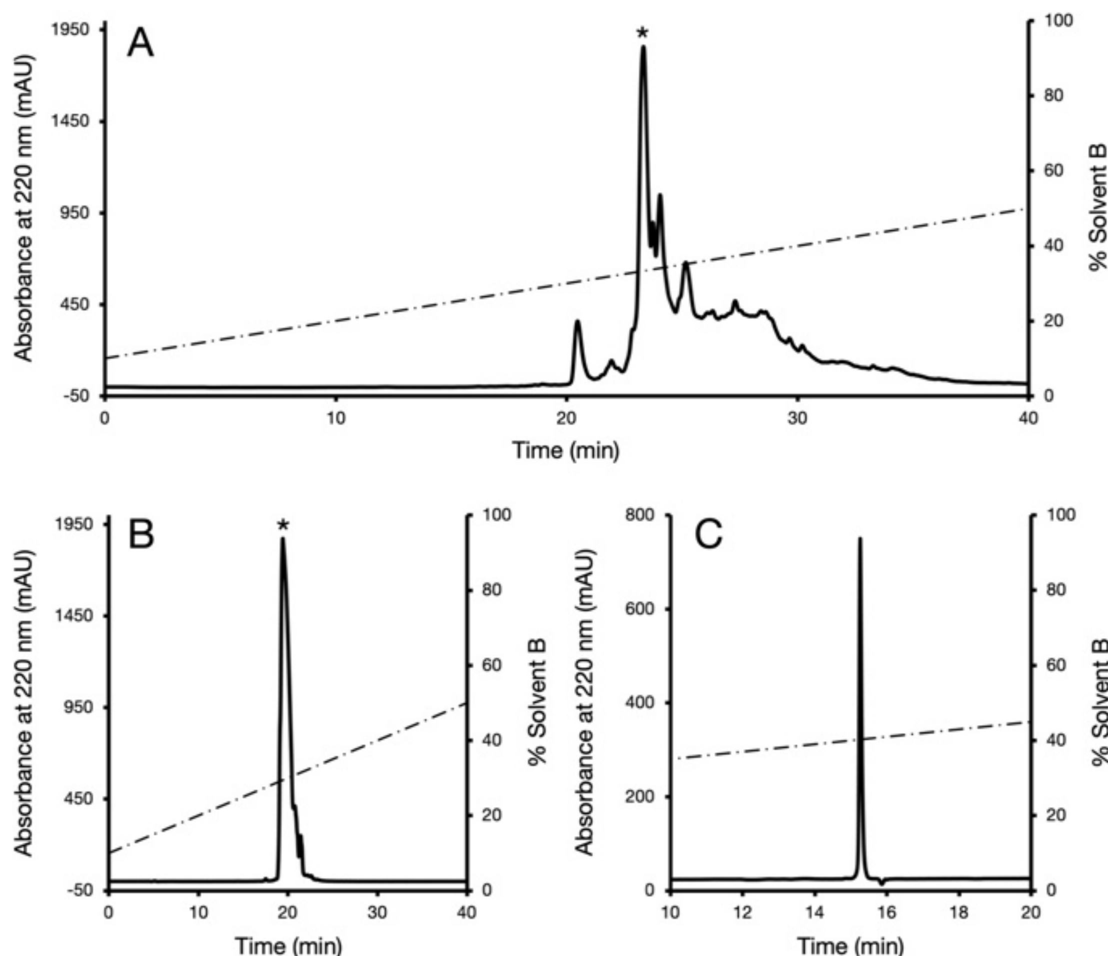


Figure 2: (A) HPLC chromatogram of crude Consomatin Ro1 using a C18 preparative column with a linear gradient of 10–50% solvent B over 40 min (dash-dotted line) at a flow rate of 8 mL/min. The peak corresponding to the collected fraction is indicated by an asterisk (*). (B) HPLC chromatogram of the collected fraction using a C18 semi-preparative column with a linear gradient of 10–50% solvent B over 40 min (dash-dotted line) at a flow rate of 3 mL/min. The peak corresponding to Consomatin Ro1 is indicated by an asterisk (*). (C) HPLC chromatogram of pure Consomatin Ro1 using a C18 analytical column with a linear gradient of 35–45% solvent B over 10 min (dash-dotted line) at a flow rate of 1 mL/min.

Overall, our results show a successful synthesis of the somatostatin venom analog, Consomatin Ro1 using an on-resin strategy. Moreover, this method produced 0.00426 mmol or 6.7 mg of the peptide which corresponds to a yield of 8% with respect to the 0.05 mmol synthesis scale used. This is a considerable improvement when compared with the yield (5%) calculated from the amounts derived from the previously reported in-solution disulfide formation method (Ramiro et al. 2022).

CONCLUSION

In this study, we have presented an on-resin disulfide formation strategy for the synthesis of Consomatin Ro1 that involves selective deprotection of Cys(Mmt) residues in a mild acid condition and the use of NCS to form the disulfide bond. HPLC, MS, and mouse bioassay results further confirmed that the strategy successfully synthesized the peptide. Its higher yield compared with the in-solution approach makes it an attractive method to use for the synthesis of Consomatin Ro1 analogs and other somatostatin-like peptides.

ACKNOWLEDGMENTS

This work was funded by the Department of Science and Technology through Project 1. Anti-Pain and Anti-Neurodegeneration Drug Candidates: Discovery and Development under the Discovery and Development of Health Products - Marine Component (Phase II) program. The authors would like to thank the Philippine Council for Health Research and Development for monitoring the research project.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

RLBdB designed the synthesis of the peptide, synthesized and purified the peptide, performed RP-HPLC and MALDI-TOF MS analyses, and wrote the manuscript.

ZGA provided the native peptide, performed the intracranial mouse bioassay, and edited the manuscript.

GPC supervised the intracranial mouse bioassay, and edited the manuscript.

AJLV supervised the experiments on peptide synthesis and characterization, and edited the manuscript.

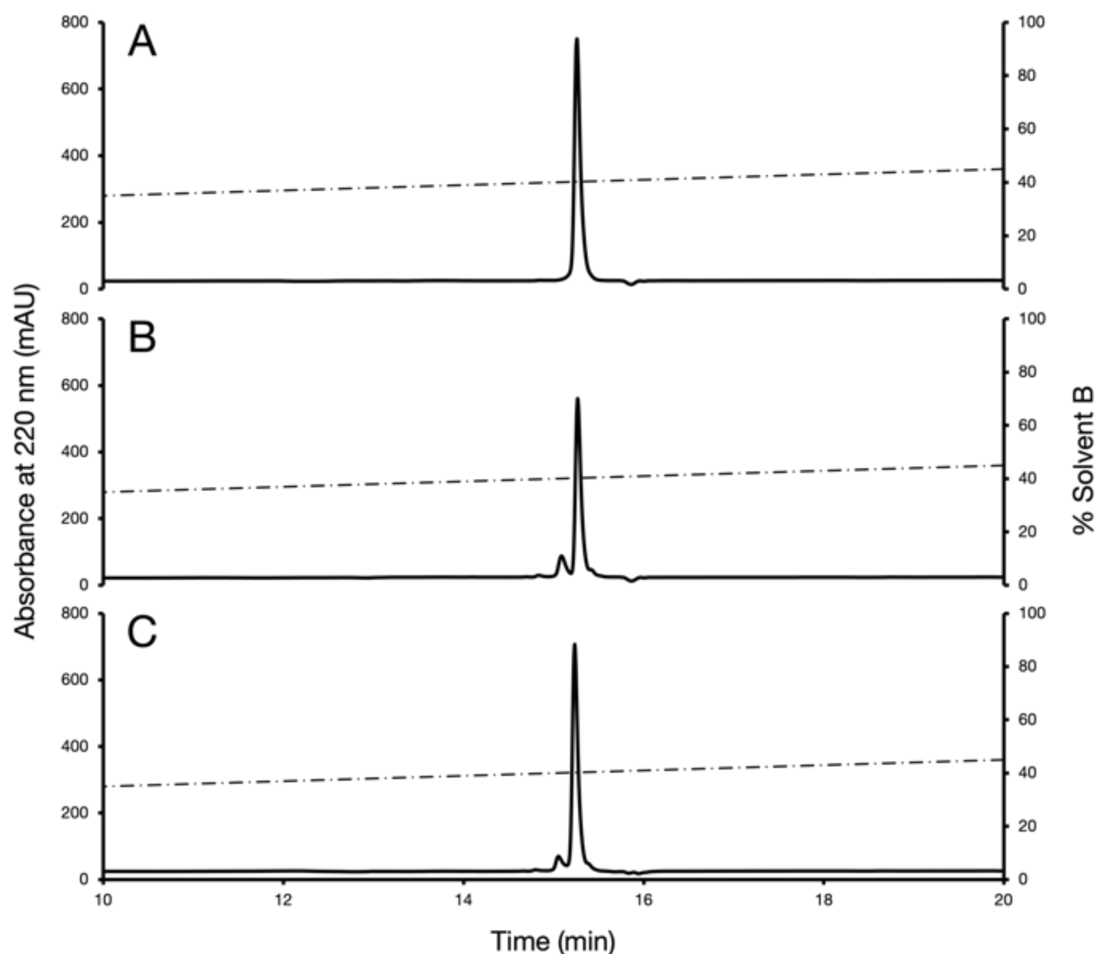


Figure 3: HPLC chromatogram of (A) synthetic (t_R 15.26 min) and (B) native (t_R 15.27 min) Consomatin Ro1, and (C) 0.25:1 mixture of the synthetic and native peptides (t_R 15.23 min) using a C18 analytical column with a linear gradient of 35–45% solvent B over 10 min (dash-dotted line) at a flow rate of 1 mL/min.

Table 1: Effects of intracranially injected Consomatin Ro1 on mice (n = 2).

Sample (dose)	Mice age (days)	Mice weights (g)	Observations post injection
Synthetic Consomatin Ro1 (2.5 nmol)	14	6.41, 6.47	Hypoactivity, leaning on one side; low tactile response at 38 min lasting ~2.5–3 h.
Native Consomatin Ro1 (2.5 nmol)	14	6.29, 6.35	Hypoactivity, leaning on one side; low tactile response at 37 min lasting ~2.5–3 h.
Control (NSS only)	14	5.00, 5.14	Normal grooming, walking, rearing; responsive to prodding.

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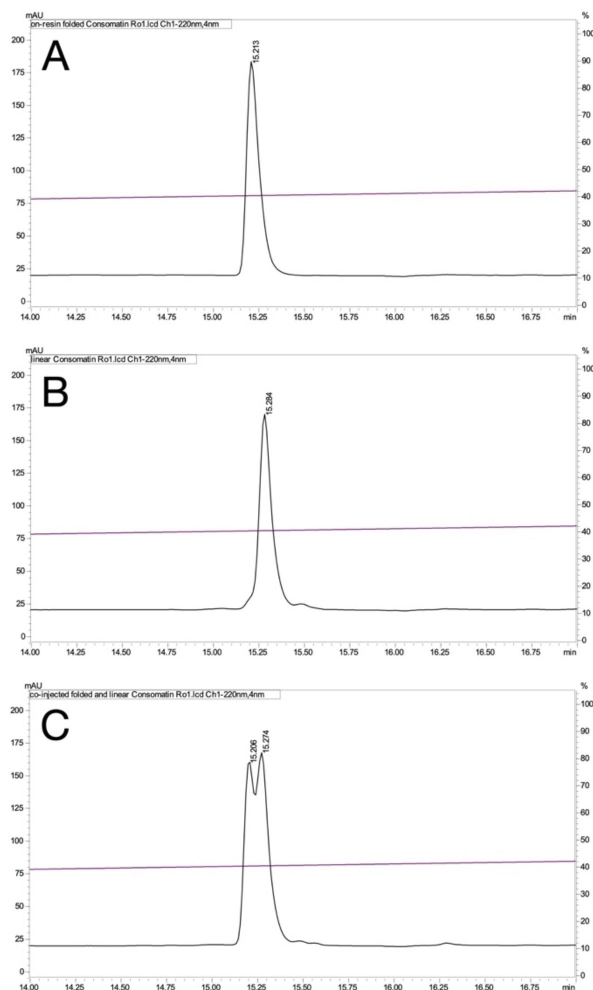
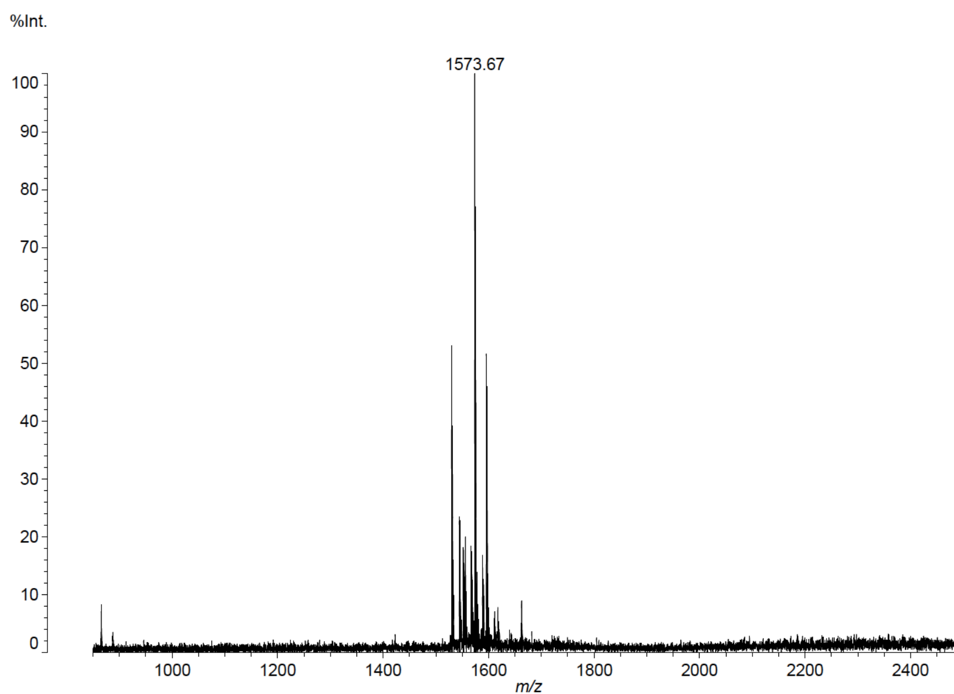
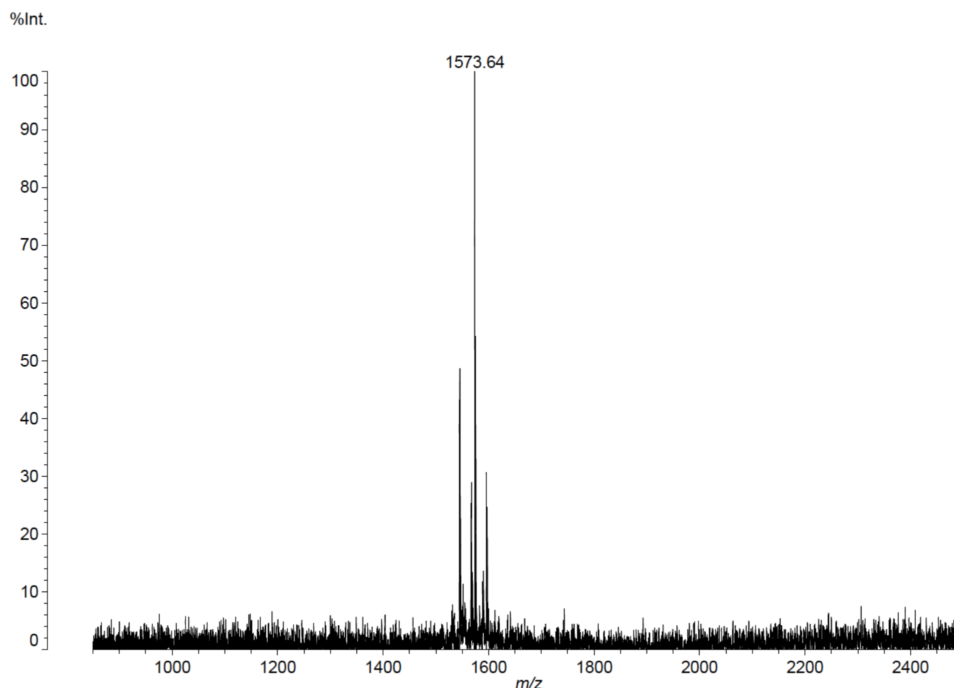
SUPPLEMENTARY DATA



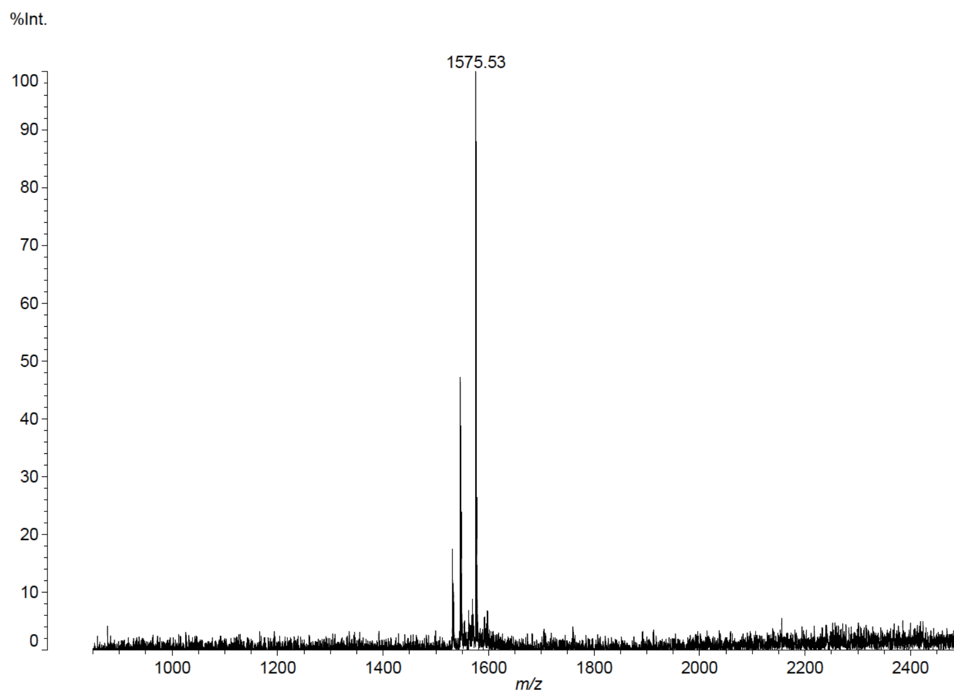
Supplementary Figure 1: HPLC chromatogram of (A) folded and (B) linear Consomatin Ro1, and (C) mixture of the folded and linear peptides using a C18 analytical column with a linear gradient of 35–45% solvent B over 10 min (purple line) at a flow rate of 1 mL/min.



Supplementary Figure 2: MALDI-TOF mass spectrum of peak collected at t_R 23.32 min from the preparative RP-HPLC purification of the crude product (observed $[M+H]^+$: 1573.67 Da; calculated $[M+H]^+$: 1573.65 Da).



Supplementary Figure 3: MALDI-TOF mass spectrum of pure Consomatin Ro1 (observed $[M+H]^+$: 1573.64 Da; calculated $[M+H]^+$: 1573.65 Da).



Supplementary Figure 4: MALDI-TOF mass spectrum of linear Consomatin Ro1 (observed $[M+H]^+$: 1575.53 Da; calculated $[M+H]^+$: 1575.66 Da).