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Total synthesis of Crotogossamide using an on-resin concomitant cyclization/cleavage reaction

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Abstract:

Crotogossamide, a cyclic peptide isolated from the latex of *Croton gossypifolius*, has been synthesized by a rapid and efficient Boc solid-phase peptide synthesis. The strategy takes advantage of the oxime resin nucleophile susceptibility and comprises the synthesis of a linear precursor followed by on-resin head-to-tail concomitant cyclization/cleavage. In addition, we report the first antimicrobial and antibiofilm investigations on Crotogossamide.

Keywords: Crotogossamide, cyclic peptides, macrocycles, oxime resin, peptide synthesis

1. Introduction

Crotogossamide **1** is a cyclic nonapeptide isolated from the latex of *Croton gossypifolius* (Quintyne-Walcott et al., 2007). To our knowledge, only one report has so far described the total synthesis of Crotogossamide **1** using free-radical desulfurization as the key step (Wan and Danishefsky, 2007). Though several natural products have been isolated from the genus *Jatropha* of the *Euphorbiaceae* family, Crotogossamide is the first cyclic peptide isolated from a Croton species. Since the first report of a natural cyclic peptide isolation, Gramicidin S, in 1944 (Gause and Brazhnikova, 1944), macrocycles constitute a privileged scaffold for the scientific community (Marsault and Peterson, 2011; Nielsen et al., 2017; Peña et al., 2015; Russo et al., 2016; Tapeinou et al., 2015; Tsomaia, 2015; White and Yudin, 2011; Yudin, 2015). Over the years, a rich diversity of homodetic and heterodetic natural cyclic peptides have been isolated from different living organisms (Wessjohann et al., 2005). The first natural cyclic peptide drug, cyclosporine, (Borel et al., 1976) was launched on the market in 1983. The unique properties of cyclosporine prove that peptide cyclization could improve pharmaceutical properties by reducing polarity and conformational flexibility and increasing proteolytic stability (Marsault and Peterson, 2011).

Though peptide macrocycles are attractive for their unique properties, macrocyclization is always challenging (White and Yudin, 2011). Oligomerization and racemization often occur during macrocyclization processes, which require highly diluted conditions to counterbalance entropic loss by cyclization. Among the available synthetic strategies to cyclize peptides, our research group focused on the use of oxime resin (DeGrado and Kaiser, 1980; DeGrado and Kaiser, 1982). Previously, we reported parameters affecting the cyclization-oligomerization reactions favouring cyclic monomers of large peptide macrocycles (Blanchette et al., 2007). Cyclic hexa- to octa-peptides were prepared efficiently with amino acid sequence L-Pro-Gly-L-Pro-Gly to facilitate peptide folding (Figure 1) (Blanchette et al., 2007). To investigate the unknown pharmacological potential of Crotogossamide **1**, we report here the total synthesis, exploiting the key step developed in our lab (Bérubé et al., 2017; Bérubé et al., 2015; Bérubé and Voyer, 2016; Blanchette et al., 2007).

2. Results and discussion

Ring size is one of the most important factors in peptide cyclization processes. It is well documented (White and Yudin, 2011; Yudin, 2015) that cyclization of large peptides (more than seven amino acids) is easier than cyclization of shorter ones, due to their higher flexibility. In fact, natural peptide macrocycles with 14- to 18-member rings are the most common scaffold (Yudin, 2015). However, the primary sequence of Crotogossamide **1** contains nine amino acids, of which seven are hydrophobic. Consequently, the on-resin linear precursor could form β-sheets, decreasing the probability of cyclization occurring.

However, based on precedents in the literature about peptide macrocyclization, we anticipated that the linear precursor would promote the cyclization of Crotogossamide **1** in a head-to-tail fashion with minimal oligomerization/racemization side reactions. Indeed, examples of sequence-dependent cyclization/cleavages that favour the intramolecular process have been described on oxime resin (Blanchette et al., 2007; Nishino et al., 1992a; Nishino et al., 1992b; Smith et al., 1998).

Since the amino acid sequence influences the oligomerization/cyclization process, we examined the primary sequence of potential linear precursors of Crotogossamide **1**. As shown in Figure 1, the cyclic nonapeptide (**1**) contains six L-amino acids and three glycines with a primary sequence of *cyclo*(Gly-L-Ser-L-Ala-Gly-L-Leu-L-Asn-Gly-L-Ile-L-Phe). Absence of a β-turn-inducing proline in the sequence makes it more challenging to avoid oligomerization side reactions. Nevertheless, Crotogossamide **1** contains two amino acids between each glycine, which has more flexibility than branched amino acids, and less steric hindrance at the carbonyl group.

Hence, we chose to incorporate a glycine at the first position. Placing this achiral glycine at the linking position also avoids racemization during the formation of *O*-acetyl urea in the presence of diisopropylcarbodiimide (DIC) during the coupling of the first amino acid. On the other hand, Crotogossamide **1** contains one asparagine in its primary sequence. By the *N*-t-Boc strategy, asparagine can be incorporated without side-chain protection. Though

side reactions have been reported to occur with a variety of coupling agents, the addition of HOBt minimizes nitrile formation by carboxyamide dehydration. More importantly, weak acids catalyze intramolecular cyclization of glutamine into pyroglutamate, which can be avoided using a high TFA concentration for *N*-t-Boc removal (Dimarchi et al., 1982). Using 75% TFA/DCM deprotection conditions allows the deprotection to proceed without side reactions.

With the above considerations in mind, we synthesized Crotogossamide **1** on oxime resin using classical protocols with 0.3 mmol/g loading (Figure 2). The first amino acid was coupled for three hours using DIC as coupling reagent. The *N*-t-Boc protecting group was removed using a mixture of 1:1 trifluoroacetic acid (TFA)/dichloromethane, while the second amino acid was activated with hydroxybenzotriazole (6-Cl-HOBt) and 1-[*Bis*(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HCTU). After appropriate coupling/deprotection steps, the linear peptide was simultaneously cyclized and cleaved from the resin in the presence of diisopropylethylamine (DIEA; 2.5 equiv) and acetic acid (AcOH; 5 equiv) in dichloromethane at a 10^{-2} M precursor concentration, leading to a good 41% macrocyclization yield. The remaining of the linear precursor left on the resin could not be macrocyclized, probably due to its oligomerization on the resin.

HPLC-TOF-MS mass spectrometry was used to distinguish between the desired protected Crotogossamide and its cyclic dimer. Results revealed only two peaks, one at 7.47 min with 99% of the total surface area, and the second at 11.38 min, both with identical masses (Figure 3). However, the peak at 7.47 min possesses an isotopic m/z ratio equal to one, while the second peak at 11.38 has an isotopic m/z ratio equal to 0.5. The isotopic ratio distribution analysis allows us to assign the desired protected Crotogossamide **1** at 7.47 min. The peak at 11.38 min has been assigned to the Crotogossamide cyclic dimer (see ESI for details). Then, very low side-product (1%) arising from oligomerization were identified in the cyclization process. In addition, the cyclization of the linear precursor provided the desired protected Crotogossamide in high purity up to 99% after a simple trituration with

cold diethylether. Having confirm that the cyclization product is the right one, the final step was the serine benzyl deprotection. Even if this deprotection seems to be an easy task, the hydrogenolysis was a hard one. Indeed, performing hydrogenolysis in EtOH, MeOH and in THF provided Crotogossamide **1** in poor yields. However, Crotogossamide **1** was obtained by hydrogenolysis using 50 psi of H_2 in AcOH in 83% crude yield and 41 % pure isolated yield after preparative HPLC purification. Spectroscopic data of synthetic Crotogossamide **1** matched entirely those reported for the isolated natural product (see ESI for details).

As mentionned, peptide macrocycles exhibit a large diversity of biological properties and macrocyclic head-to-tail peptides are well-known powerful antimicrobial agents. In order to investigate the unknown pharmaceutical potential of Crotogossamide **1**, we evaluated antimicrobial properties towards *Escherischa coli* ATCC 15939, *Streptococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 10145 and *Candida albicans* ATCC 28366. In addition, its anti-biofilm activity was evaluated on both prokaryotic (*Streptococcus mutans* ATCC 25175) and eukaryotic (*Candida albicans* ATCC 28366) models. Unfortunately, no significant antimicrobial or antibiofilm activity was observed for concentrations of Crotogossamide **1** up to 96 ug/mL.

3. Conclusion

We achieved the total synthesis of Crotogossamide **1** on solid support by concomitant cyclization/cleavage with almost no oligomerization side reactions, and an overall yield of 34%. The cyclization of the linear precursor proceeded in an excellent yield and provided almost exclusively the desired protected Crotogossamide monomer, as confirmed by the isotopic ratio distribution in mass spectrometry. Access to sufficient amount of Crotogossamide **1** allowed us to perform the first antimicrobial investigation on that natural macrocyclic peptide, though no activity was observed. Work is currently underway to prepare a library of Crotogossamide analogs, as well as other natural macrocyclic peptides by head-to-tail, on-resin concomitant cyclization/cleavage using the oxime resin.

4. Experimental

4.1. General information

Oxime resin (0.30 mmol/g), coupling reagents, and *N*-Boc-protected amino acids were purchased from Matrix Innovation, Bachem, Advanced Chemtech and GL Shangai. Unless otherwise indicated, other starting materials were purchased from commercial sources (Sigma-Aldrich and VWR) and used without further purification. ^{1}H and ^{13}C-NMR spectra were recorded on an Agilent DD2 500 MHz spectrometer. The coupling constants are reported in hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), br (broad singlet), ddt (doublet of doublet of triplets), and m (multiplet). Mass spectra were obtained on an Agilent 6210 LC Time of Flight Mass Spectrometer in direct injection mode. HPLC purities were recorded on an Agilent 1260 Infinity instrument equipped with an auto sampler, a quaternary high-pressure mixing pump and a photodiode array detector. Chromatography analysis was performed with a Grace Vydac C18 column (5 µm, 250 x 4.6 mm i.d.). The gradient was performed at a flow rate of 4.1 mL/min with a mobile phase composed of H_2O containing 0.1% TFA (A) and CH_3CH (B). All solvents were degassed and a gradient of (100% H_2O) to (46.4% H_2O /54.4% CH_3CN) in 35 min followed with 100% H_2O between 35 to 42 min was used for Crotogossamide **1**. An isocratic gradient at 50% of B for 15 min was used to ensure a good separation of Crotogossamide **1**. The column temperature was kept at 22.5°C. The UV absorbance was recorded at 220 and 280 nm. Retention time is in minutes followed by the percentage integration of the total chromatogram. Semi-preparative HPLC was carried out using a HP 1260 system with a photodiode array detector using an Supercosil SPLC C18 column (5µm, 250 x 10.0 mm i.d). Optical rotations were measured at ambient temperature on a Jasco DIP-360 digital polarimeter using a sodium lamp. Optical density values for antimicrobial susceptibility and inhibition of biofilm formation assays were obtained using a BIO-RAD xMark Microplate spectrophotometer.

4.2 Preparation of Crotogossamide 1

4.2.1. Coupling of the first N-Boc glycine on oxime resin

2.5 g of oxime resin (0.30 mmol/g) was added to a peptide synthesis vessel. The resin was treated three times with CH_2Cl_2. Glycine (3.0 equiv, 0.75 mmol) and HOBt (3.0 equiv, 0.75 mmol) were dissolved in DMF (25 mL) in a 50 mL flask and the mixture was stirred for 10 minutes at 0 °C. DIC (3.0 equiv, 2.25 mmol), DIEA (6.0 equiv, 4.5 mmol) and DMAP (0.1 equiv, 0.075 mmol) were added and the mixture was introduced into the peptide synthesis vessel and stirred mechanically for 3h. The mixture was filtered under vacuum and the resin was washed [DMF (3 x 20 mL), MeOH (3 x 20 mL), DMF (3 x 20 mL), MeOH (3 x 20 mL)] and dried under reduced pressure.

4.2.3. Acetylation of unreacted sites on oxime resin

The resin was treated three times with DMF (3 x 25 mL). A solution of 50% v/v DMF/acetic anhydride (25 mL) and DIEA (1.25 mL) were added to the peptide synthesis vessel and shaken for 1 hour. Then, the mixture was filtered under vacuum and the resin was washed [DMF (3 x 25 mL), MeOH (3 x 25 mL), DMF (3 x 25 mL), MeOH (3 x 25 mL)] and dried under reduced pressure.

4.1.4. Removal of the N-Boc protecting group

The resin was treated three times with CH_2Cl_2 (25 mL). A 50% v/v solution (25 mL) of trifluoroacetic acid (TFA) in CH_2Cl_2 was added to the peptide synthesis vessel and shaken for 30 minutes. Then, the mixture was filtered under vacuum and the resin was washed with DMF (3 x 25 mL), MeOH (3 x 25 mL), DMF (3 x 25 mL), MeOH (3 x 25 mL) and with a solution of 10% v/v DIEA in CH_2Cl_2 (25 mL).

4.2.5. Coupling of the subsequent N-Boc protected α-amino acids

The amino acid (3.0 equiv, 2.25 mmol) was dissolved in DMF (25 mL) in a 50 mL flask. The solution was cooled to 0 °C, then HBTU (3.0 equiv, 2.25 mmol) and HOBt (3.0 equiv, 2.25 mmol) were added. The mixture was poured into the peptide synthesis vessel, in which the resin had been previously treated with CH_2Cl_2 (3 X 25 mL). DIEA (6.0 equiv, 4.5 mmol) was also added to the vessel and the mixture was shaken for 3 h. After filtration under vacuum, the resin was washed [DMF (3 x 25 mL), MeOH (3 x 25 mL), DMF (3 x

25 mL) and MeOH (3 x 25 mL)] and dried under reduce pressure. The Kaiser ninhydrin test was performed to monitor the efficiency of the coupling, and the coupling procedure was repeated if needed.

4.2.6. Cyclization/cleavage from the resin

First, the *N*-Boc group was removed using the procedure described in (4.1.4.), but without the 10% v/v DIEA/CH_2Cl_2 washing step. After drying, CH_2Cl_2 and DIEA (2.5 equiv, 1.875 mmol) were added to the peptide synthesis vessel and the mixture was shaken for 2 min. Acetic acid (5.0 equiv, 3.75 mmol) was then added and the contents were shaken for 24 h. The filtrate was collected and the resin was rinsed several times with CH_2Cl_2 and MeOH. All the filtrates were combined and evaporated. Trituration in a minimum of cold ether was performed and led to the desired compound (protected Crotogossamide) by simple filtration in a 31% macrocyclization yield.

4.2.7. Hydrogenolysis

The protected Crotogossamide **1** was poured into a high-pressure hydrogenation vessel and was suspended in AcOH. Pd/C 10% was then added. The vessel was introduced in the reactor and after 4 purges, the H_2 pressure was set to 50 psi and the reaction mixture was stirred for 24 h. The reaction mixture was filtered on a Celite® pad. The filtrate was concentrated *in vacuo* and the crude product was purified by preparative HPLC to afford pure Crototgossamide **1**. White powder. $[\alpha]^{22}$ -50.0° (c 0.1 in H_2O). **^{1}H-NMR** (500 MHz, DMSO-d_6): δ = δ 8.52 (br, 1H), 8.36 (br, 1H), 8.32 (br, 1H), 8.24 (d, *J* = 7.1 Hz, 1H), 8.16 (t, *J* = 6.2 Hz, 1H), 8.06 (t, *J* = 6.0 Hz, 1H), 7.92 (d, *J* = 7.5 Hz, 1H), 7.50 (d, *J* = 7.7 Hz, 1H), 7.38 (m, 2 H), 7.26 (t, *J* = 7.4 Hz, 2H), 7.19 (m, 3H), 6.86 (s, 1H), 5.01 (br, 1 H), 4.37 (m, 1 H), 4.29 (m, 3H), 4.02 (q, *J* = 5.7 Hz, 1H), 3.95 (m, 3H), 3.73 (dd, *J* = 6.1 and 17.5 Hz, 1H), 3.67 (m, 2 H), 3.64 (m, 1 H), 3.40 (m, 2 H), 3.16 (dd, *J* = 5.4 and 14.1 Hz, 1H), 2.96 (dd, *J* = 10.0 and 14.3 Hz, 1H), 2.72 (dd, *J* = 5.2 and 15.7 Hz, 1H), 2.54 (signal partially obscured), 1.76 (m, 1H), 1.59 (m, 1H), 1.50 (m, 1H), 1.28 (d, *J* = 7.0 Hz, 3H), 1.03 (m, 1H), 0.86 (d, *J* = 5.7 Hz, 3H), 0.83 (d, *J* = 5.7 Hz, 3H), 0.74 (t, *J* = 7.4 Hz, 3H), 0.57 (d, *J* = 6.8 Hz, 3H). **HRMS** (ESI-TOF, *m/z*): calcd for $C_{37}H_{57}N_{10}O_{11}$ $(M+H)^+$ =

817.4203, found 817.4217. **HPLC** (Retention time, purity): 10.80 min, 95%. UV (MeOH)$_{\lambda max}$: 220 nm.

4.3 Antimicrobial activity testing

The antimicrobial susceptibility of bacteria was assessed using the microdilution method as described by the Clinical and Laboratory Standards Institute. Briefly, two-fold dilutions of the test product in Mueller-Hinton Broth (MHB) were performed in sterile 96-wells plates to obtain final concentrations of Crotogossamide **1** ranging from 0 to 96ug/mL. Fresh cultures of microorganisms diluted in Mueller-Hinton Broth were added to the plates to obtain a final concentration of 5 x 10^5 CFU/mL. Microbial concentration was confirmed for each strain by plate count. Experiments were produced in triplicates and included positive control wells containing no test product (100% growth) as well as blanks containing no microorganism for every test product concentration (0% growth). Ampicillin was used as reference antibiotic. Plates were incubated aerobically for 24h at 37°C before results lecture. The same method was applied to determine antimicrobial activity against Candida *albicans* using Sabouraud medium at pH 7.0 instead of MHB.

The minimal inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that completely inhibits growth of the organism in the microdilution wells, as detected by the unaided eye and confirmed visually. The half maximal inhibitory concentration (IC_{50}) represents the concentration of a drug that is required for 50% growth inhibition in vitro. The optical density at 660 nm was measured to monitor bacterial growth in both inoculum preparation and results calculation.

The growth inhibition for each concentration (x) of Crotogossamide is calculated after incubation by reporting the mean absorbance ($A_{C(x)}$) as a percentage of the absorbance of wells containing no antibiotic ($A_{100\%\ growth}$), as follows:

$$Growth\ percentage\ (\%) = \left(\frac{[A_{C(x)}] - [A_{blank\ C(x)}]}{[A_{100\%\ growth}][A_{blank\ C(0)}]}\right) * 100\%$$

$$Growth\ inhibition\ (\%) = 100\% - Growth\ percentage\ (\%)$$

4.4 Inhibition of biofilm formation

Two-fold dilutions of Crotogossamide solution in 96-well plates were performed as for antimicrobial susceptibility testing, and included the same control wells, in culture media suitable for biofilm formation, as previously described (Ben Lagha et al., 2015). Candida *albicans* was grown in Sabouraud medium at pH 7.0, and Streptococcus *mutans* was grown in Castenholz tryptone-yeast extract media (TYE) supplemented with glucose (0.5%). Fresh cultures of microoganisms were added to obtain a final optical density of 0.1 at 660 nm in each well. The plates were incubated aerobically at 37°C for 24 hours to allow biofilm formation. Absorbance was measured at 660 nm to monitor bacterial growth, and then the culture media was removed from the wells, which were rinsed with water and dyed with 100 uL of an aqueous solution of crystal violet (0.001%) for 15 minutes. The wells were rinsed again after dye removal and allowed to dry. Solubilisation in 75% ethanol allowed measurement of biofilm formation by measuring absorbance at 550 nm. The biofilm formation percentage was calculated with this data using the same formula as for growth percentage. A compound is said to inhibit biofilm formation when significant reduction of biofilm formation is observed at concentrations that do not affect bacterial growth.

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Supplementary data

Supplementary data for compound **1** associated with this article can be found in the online version, including ^{1}H-NMR, COSY, HPLC, mass spectrometry analyses as well as anti-biofilm and antimicrobial results.

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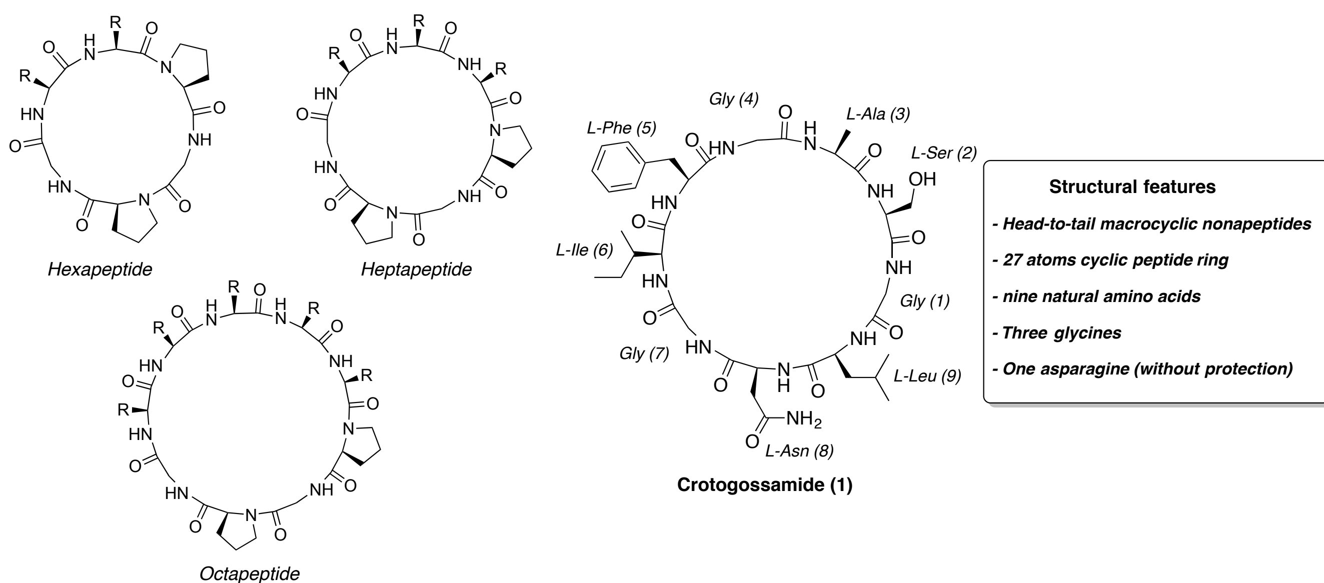

Fig. 1: Peptide macrocycles prepared on oxime resin and Crotogossamide **1** with its structural features

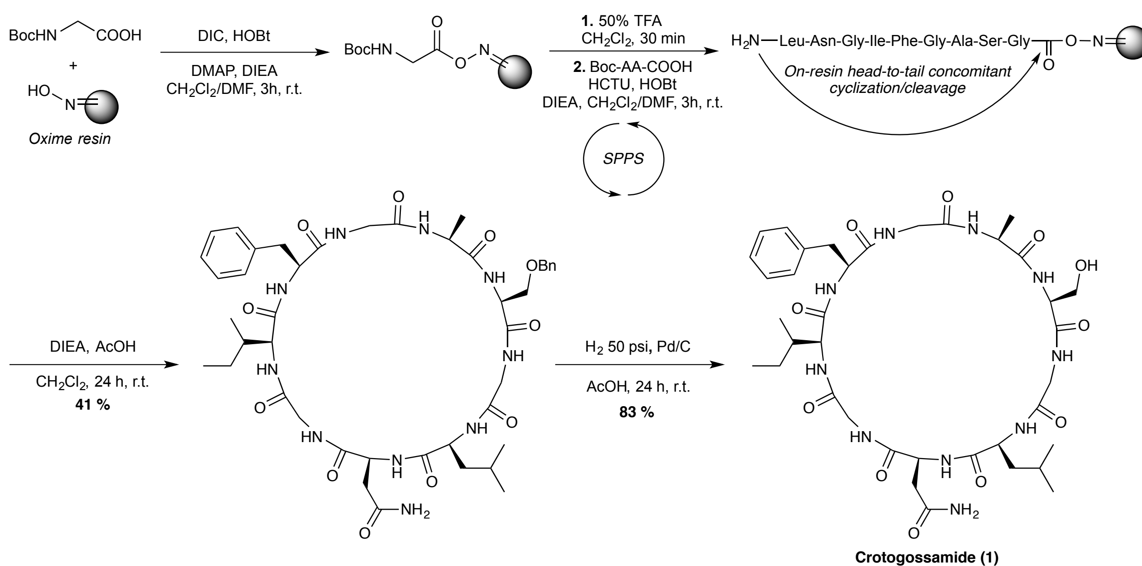

Figure 2: Synthetic route towards Crotogossamide **1**

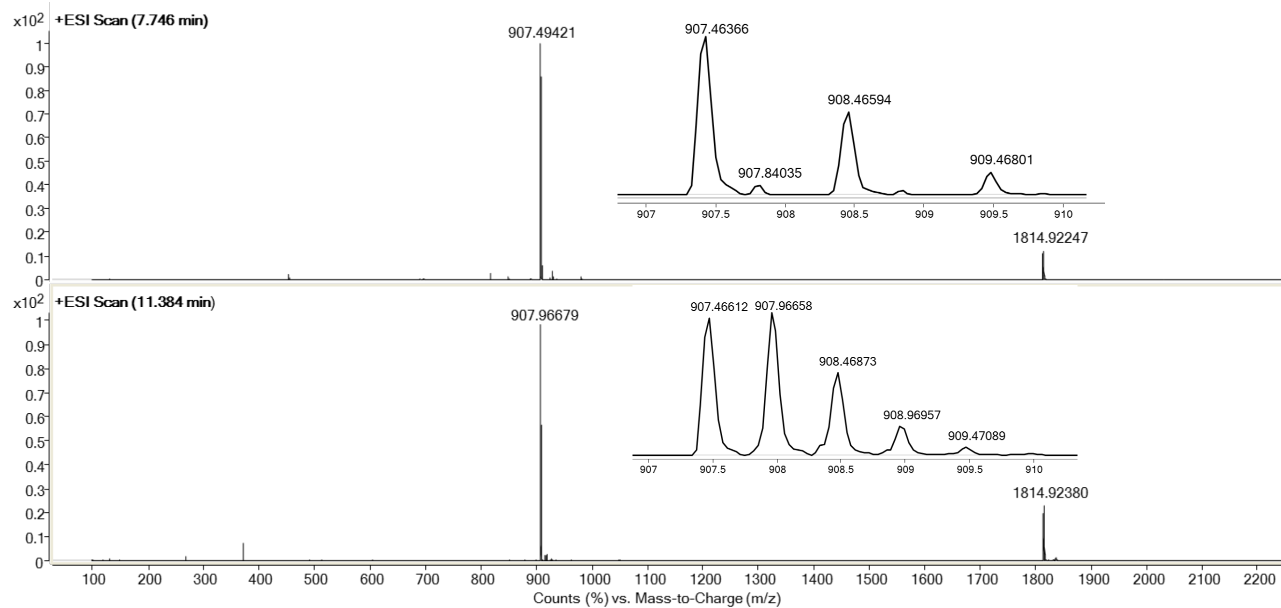

Figure 3: ESI-Mass spectrum of cyclized peptides obtained (above: monomer; below: dimer) with calculated monomer and dimer isotopic distributions

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