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Short Communication

Microwave-assisted RCM for the synthesis of carbocyclic peptides

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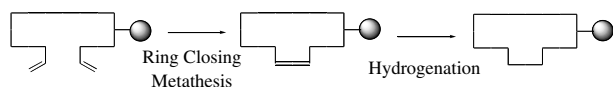School of Chemistry, Monash University, Clayton 3800, Victoria, Australia

Abstract: Microwave irradiation dramatically improves the efficiency of ring closing metathesis (RCM) reactions of resin-attached peptides and the technology is illustrated by the highly selective synthesis of dicarba analogues of α-conotoxin IMI. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: microwave; ring closing metathesis; dicarba peptides; conotoxins

Disulphide bridges are metabolically and chemically unstable structural features in many naturally occurring and synthetic peptides. Replacement of cystine by the dicarba isostere (S,S)-2,7-diaminosuberic acid ((S,S)-2,7-diamino octanedioic acid) [1–3] has been used to increase the metabolic stability in vasopressin [4], natriuretic β-ANP [5], oxytocin [6–8], calcitocin [9], bradykinin antagonists [10] and haematoregulatory peptides [11]. We have recently reported the synthesis of dicarba analogues of the cystine-containing octapeptides octreotide, vapreotide and lanreotide using a single pot, on-resin, tandem ruthenium-catalysed ring closing metathesis (RCM) followed by rhodium-catalysed hydrogenation (Scheme 1) [12,13]. Although high purity products were obtained, variable yields of products were isolated and the RCM reactions required high catalyst loadings (20 mol% first-generation Grubbs' catalyst) and long reaction times (48–96 h). In this communication, we report that microwave irradiation accelerates RCM reactions of resin-attached peptides resulting in quantitative cyclisation under lower catalyst loadings (5–10 mol%) and much shorter reaction times (1–2 h).

α-Conotoxin IMI **1** is a disulphide-rich peptide isolated from the venom of the vermivorous conus species *Conus imperialis* [14]. The microwave-accelerated catalysis is illustrated with the synthesis of two dicarba-cystino hybrid CTx-IMI analogues, bicyclic peptides **2** and **3** (Figure 1). Native α-conotoxins are amidated at

their *C*-termini. Rink amide resin was therefore chosen to facilitate linear peptide construction and generate the required *C*-terminal carboxamide upon resin cleavage. Standard SPPS using HATU-NMM activation and Fmoc-protected amino acids were used to construct the two linear peptides **4** and **5** (Scheme 2). Both of these sequences possess two strategically placed non-proteinaceous L-allylglycine (Hag) residues to facilitate construction of the dicarba bridge. Intermediates were carried through without purification or characterisation up to the dodecapeptides **4** and **5**. A sample of each linear peptide was obtained by cleavage from the resin and determined to be of >95% purity by reverse-phase analytical chromatography. Mass spectral analysis gave molecular ion peaks at m/z 783.5 $[1/2(M + 2H)^+]$ and 805.4 $[1/2(M + 2HCOO)^+]$, which are consistent with the structures of the isomeric side chain de-protected linear peptides **4** and **5**.

Microwave irradiation of a mixture of Rink amide bound-peptide **4** and second-generation Grubbs' catalyst (**6**, 10 mol%) in DCM containing 10% LiCl in DMF [7,8] resulted in *complete* ring closure in only 1 hour [15,16]. Complete conversion could also be achieved using a 5 mol% loading of catalyst and 2 h of microwave irradiation. Mass spectral analysis of the product mixture showed the required molecular ion with m/z 769.4 $[1/2(M + 2H)^+]$ for the TFA-cleaved, unsaturated [2,8] -dicarba peptide **7** and no starting linear peptide. Analogous reaction conditions also resulted in a *complete* cyclisation of **5** to the isomeric [3,12] -dicarba analogue **8** (Scheme 2). Fmoc-de-protection followed by aerial oxidation of resin-cleaved **7** and **8** in 10% DMSO/aqueous $(NH_4)_2CO_3$ (0.1 M. pH 8) then afforded the unsaturated cystino-dicarba CTx-IMI analogues **9** and **10**, respectively (Figure 2). Each peptide was obtained in 25% crude yield and purified by reverse-phase preparative chromatography (>99% purity).

Scheme 1 Catalytic sequence for dicarba bridge formation.

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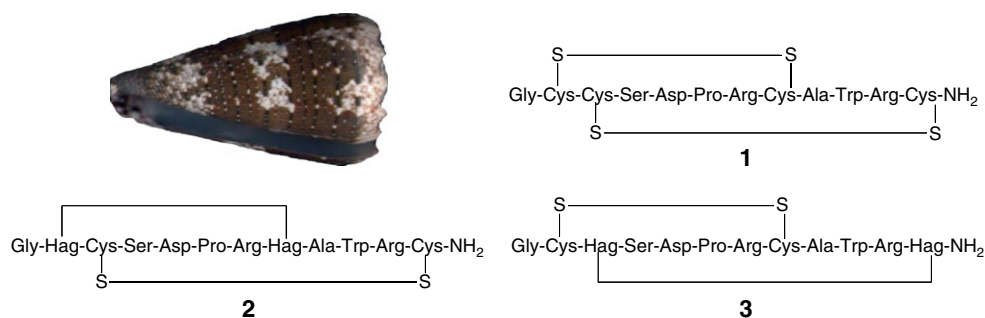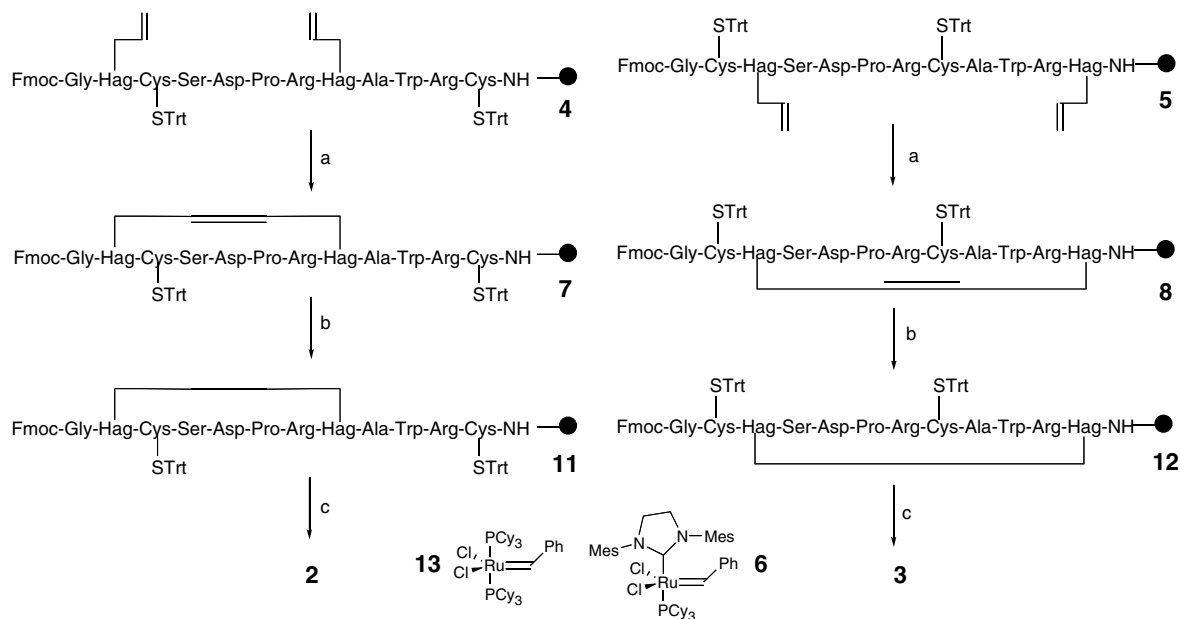WILEY InterScience®
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Figure 1 *Conus imperialis*, α-CTx IMI **1** and carbocyclic analogues **2** and **3**.

Scheme 2 *Reagents and conditions:* Rink amide resin (●, 0.52 mmol/g loading). (a) 5–10 mol% second-generation Grubbs' catalyst, DCM-10% 0.4 M LiCl/DMF, rt → 100 °C, 1–2 h, microwave irradiation; (b) Rh(I)(PPh$_3$)$_3$Cl, 80 psi H$_2$, rt, 10% MeOH in DCM, 14 h; (c) Fmoc-de-protection and resin cleavage.

Hydrogenation of carbocycles **7** and **8** in 10% MeOH-DCM using sulphur-tolerant Wilkinson's catalyst and low hydrogen pressure (80 psi) facilitated quantitative reduction at room temperature to give resin-bound peptides **11** and **12** (Scheme 2). Interestingly, the resin-cleaved crude product from each of these reactions existed in the oxidised form (**2, 3**).

Significantly, attempted RCM reactions without microwave irradiation were less successful. Exposure of peptide **4** to the first-generation Grubbs' catalyst **13** (50 mol%) in DCM at 50 °C for 72 h gave only trace amounts (<10%) of cyclised product **7**. While RCM progressed further (∼70%) using the more reactive second-generation Grubbs' catalyst **6**, conditions could not be found to affect full cyclisation to **7** even under high catalyst loading (50 mol%). Changes in solvent, catalyst loading and reaction time had no positive effect on conversion. The addition of chaotropic salts to the reaction medium to disrupt aggregation also had no effect on RCM yield. Similarly, RCM of a dicrotylglycine

analogue of the primary sequence of **4**, which avoids catalytic cycling through an unstable ruthenium-methylidene species [17] and could be expected to give a higher yield, also failed to achieve a complete conversion to the cyclic target **7**.

Construction of the isomeric [3,12]-dicarba CTx-IMI **8** was found to be even more problematic under conventional heating conditions. Exposure of the resin-bound peptide **5** to both first- and second-generation Grubbs' catalysts under a variety of experimental conditions failed to yield the unsaturated carbocycle **8**. Possible reasons for the poor reactivity of **5** included the diminished influence of the proline residue in assisting the formation of the larger carbocycle (32-membered ring), and the close proximity of the C-terminal allylglycine residue to the bulky Rink amide linker. The sequence was subsequently reconstructed on BHA-resin bearing a linear HMBA-Gly-Gly linker (Exposure of HMBA-linked peptides to ammonia vapour causes cleavage and the generation of C-terminal
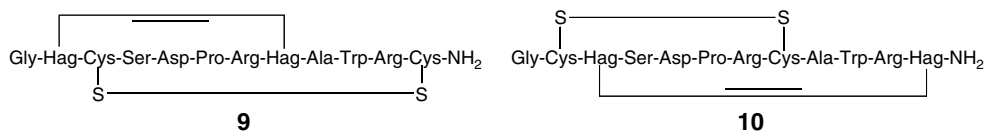
Gly-Hag-Cys-Ser-Asp-Pro-Arg-Hag-Ala-Trp-Arg-Cys-NH$_2$

9

Gly-Cys-Hag-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Hag-NH$_2$

10

Figure 2 Unsaturated [2,8]- and [3,12]-dicarba-α-CTx IMI analogues.

carboxamides). Cyclisation of the BHA resin-bound peptide was attempted in the presence of 20 mol% of second-generation Grubbs' catalyst **6** and chaotrophic salts, but mass spectral analysis of the product mixture again showed only the starting peptide **5**.

Hence, the microwave-assisted RCM methodology, described above, provides a highly efficient, on-resin route to carbocyclic peptides with quantitative conversions, fast reaction times (typically 1–2 h), high resin loadings and lower Grubbs' catalyst loading (5–10 mol%). Importantly, the use of a dielectric heating facilitates the RCM of sequences that are otherwise *completely inert* to cyclisation under conventional heating methods [18]. Cystine-dicarba hybrids of biologically active cyclic peptides, such as those described above, can serve as useful probes for the delineation of functional (redox active) and structural roles of native disulphide bonds. Significantly, the on-resin homogeneous catalytic protocol, described in this paper, can be used to generate these analogues. Conotoxins are currently attracting widespread interest and are being assessed as therapeutic agents for the treatment of cancer, stroke, epilepsy and also the control of pain [19]. The biological activity of the cystine-dicarba CTx-IMI analogues **2**, **3**, **9** and **10**, described herein, is currently being examined and will be reported in due course.

EXPERIMENTAL

Peptide Materials and Procedures

Peptides were synthesised in polypropylene Terumo syringes (10 ml) fitted with a polyethylene porous (20 µm) frit. Solid phase peptide synthesis (SPPS) was performed using a Visprep SPE DL 24-port model vacuum manifold supplied by Supelco. Coupling reactions and cleavage mixtures were shaken on a KS125 basic KA elliptical shaker, supplied by Labortechnik at 400 motions per minute. Cleaved peptides were centrifuged on a Hermle Z200A centrifuge, supplied by Medos at a speed of 4500 cycles per minute. **General SPPS procedure:** Peptides were prepared using the general Fmoc-SPPS methodology on Rink amide resin. After synthesis of the linear peptide, a small aliquot was subjected to TFA cleavage. In a fritted syringe, Rink amide resin was washed with DCM (7 ml, 3×1, 1×60 min) and DMF (7 ml, 3×1, 1×30 min) and de-protected with 20% piperidine in DMF (7 ml, 1×1, 2×10 min) and washed again with DMF (7 ml, 5×1 min). NMM (6 equiv.) was added to a solution of the protected amino acid and Fmoc-Xxx-OH (3 equiv.) and HATU (2 equiv.) in DMF (3 ml) and shaken gently for 1 min. The activated amino acid solution was added

to the resin-tethered amino acid and shaken gently for the reported period of time. At the end of the reaction period, the resin-peptide was washed with DMF (7 ml, 3×1 min), de-protected with 20% piperidine in DMF (7 ml, 1×1, 2×10 min) and washed again with DMF (7 ml, 5×1 min). The above procedure was repeated until the desired peptide sequence was constructed. The quantity of amino acids (3 equiv.), coupling agents HATU (2 equiv.) and NMM (6 equiv.) remained constant throughout the synthesis. After coupling the final amino acid, the resin-peptide was washed with DMF (7 ml, 3×1 min), DCM (7 ml, 3×1 min), MeOH (7 ml, 3×1 min) and dried on the SPPS manifold for 1 h. **General cleavage procedure:** A small aliquot of the resin-peptide ($\sim$1 mg) was added to the cleavage solution (5 ml, 90% TFA: 5% thioanisole: 2.5% EDT: 2.5% water and phenol, 1.6 g/5 ml of cleavage solution) and shaken gently for 1.5 h. The mixture was then filtered and the resin beads were rinsed with TFA (3×1 ml). The filtrate was concentrated with a constant stream of air to yield oil. The peptide was precipitated with ice-cold Et$_2$O (2 ml) and the mixture was centrifuged (3×10 min). The supernatant liquid was decanted and the resultant residue was collected and analysed by RP-HPLC and mass spectroscopy.

Catalytic Materials and Procedures

Tricyclohexylphosphine[1,3-bis(2,4,6-trimethylphenyl)-4,5 -dihydroimidazol-2-ylidene]-(benzylidene)ruthenium(II) dichloride (second-generation Grubbs' catalyst) and tris(triphenyl-phosphine) rhodium(I) chloride (Wilkinson's catalyst, Rh[(PPh$_3$)$_3$Cl]) were used as supplied by Aldrich and stored under argon in a dry box. Argon and hydrogen were supplied by BOC gases and were of high purity ($<$10 ppm oxygen). Additional purification was achieved by passage of the gases through water, oxygen and hydrocarbon traps. DCM, MeOH and lithium chloride in DMF (LiCl/DMF), used for metal-catalysed reactions, were de-gassed with high purity argon prior to use. **Microwave-assisted RCM procedure:** Microwave reactions were carried out on a Personal Chemistry (now Biotage) Smith Synthesiser. The instrument produces a continuous focussed beam of microwave irradiation at 2.45 GHz with a maximum power delivery of 300 W, which reaches and maintains a selected temperature (100 °C). Reactions were performed in high-pressure quartz microwave vessels fitted with self-sealing Teflon septa as a pressure relief device, which were crimped in place. The vessels contained magnetic stirrer beads and the pressure and temperature of each reaction was monitored continuously with an inbuilt pressure transducer (located in the lid) and infrared pyrometer, respectively. Reaction times were measured from the time the microwave began heating until the reaction period had elapsed (cooling periods were not inclusive). In a dry box, a high-pressure quartz microwave vessel was loaded with resin-peptide, catalyst and solvent (DCM and LiCl/DMF). The vessel was capped and irradiated at 100 °C for 1 h. At the end of the reaction period, the

resin-peptide was washed with DMF (3 ml, 3 × 1 min), DCM (3 ml, 3 × 1 min) and MeOH (3 ml, 3 × 1 min) and dried on the SPPS manifold for 1 h. A small aliquot of resin-peptide (∼1 mg) was subjected to the TFA-mediated cleavage procedure and the isolated peptide was analysed by RP-HPLC and mass spectroscopy. Metathesis experiments are described in the following format: resin-peptide (mg), solvent (ml), catalyst, reaction temperature (°C) and reaction time (h). **Wilkinson's hydrogenation procedure:** In a dry box, a Fischer-Porter tube was charged with substrate, Wilkinson's catalyst and dry deoxygenated solvent (10% MeOH/DCM). The apparatus was connected to a hydrogenation manifold and purged three times using a vacuum and an argon flushing cycle before being pressurised with hydrogen gas to the reported pressure. The reaction was then stirred at ambient temperature for the reported reaction time. The hydrogen gas was vented and the mixture was then filtered, washed with DCM (3 ml, 3 × 1 min), MeOH (3 ml, 3 × 1 min) and dried on the SPPS manifold for 1 h. A small aliquot of resin-peptide (∼1 mg) was subjected to the TFA-mediated cleavage procedure and the isolated peptide was analysed by RP-HPLC and mass spectroscopy. Analytical liquid chromatography-mass spectrometry (LC-MS) was conducted on a Gilson (Middleton, USA) instrument equipped with a Gilson 215/819 injector module, 306 gradient pumps and an Agilent 1100 diode array detector. Analysis was performed on a Phenomenex Luna 5-μm C8(2) analytical column (50 × 3 mm) using a gradient of 10–60% MeOH (0.1% formic acid) at a flow rate of 1.1 ml min^{-1} for 10 min. Peptides were detected with UV irradiation between 200 and 300 nm and a Micromass (Manchester, UK) ZMD mass spectrometer in positive electrospray ionisation (ESI$^+$) mode. Inlet flow to the source was restricted with a flow splitter (100 μl min^{-1}) and the entire instrument was controlled by MassLynx v3.5 software (Micromass). Peptide purification was performed by reverse-phase high performance liquid chromatography (RP-HPLC) on the Gilson/Micromass LC-MS instrument described above, equipped with a Gilson FC 405 fraction collector that was triggered by mass detection in the ESI Source. All runs were performed on a Phenomenex Luna 5-μm C8(2) preparative column (50 × 21.2 mm) using a solvent gradient of 10–60% MeOH (0.1% formic acid) at a flow rate of 10 ml min^{-1} for 15 min. The flow to the detectors was reduced with an LC Packing (San Francisco, USA) post-column splitter. Peptides were detected by ESI-MS with the doubly charged ion used as the collection trigger.

(2,8)-Dicarba-(3,12)-Cystino Conotoxin Transformations Linear(2,8)-Hag-(3,12)-Cys Conotoxin IMI

Fmoc-Gly-Hag-Cys-Ser-Asp-Pro-Arg-Hag-Ala-Trp-Arg-Cys-NH$_2$ 4. The linear peptide **4** was synthesised using the general SPPS procedure outlined above with Rink amide resin (740 mg, loading 0.52 mmol g^{-1}, 0.39 mmol), NMM (255 μl, 2.31 mmol, 6 equiv.), the protected C-terminal amino acid, Fmoc-L-Cys(Trt)-OH (676 mg, 1.15 mmol, 3 equiv.) and HATU (293 mg, 0.77 mmol, 2 equiv.) in DMF (3 ml). The mixture was gently shaken for 2.5 h. At the end of the reaction period, the resin-peptide was washed with DMF (7 ml, 3 × 1 min), de-protected with 20% piperidine in DMF (7 ml, 1 × 1, 2 × 10 min) and washed again with DMF (7 ml, 5 × 1 min). The above procedure was repeated

until the desired peptide sequence was constructed. A small aliquot of the resin-peptide (∼1 mg) was subjected to the TFA-mediated cleavage procedure and mass spectral analysis confirmed the formation of the linear conotoxin analogue **4**. Mass spectrum (ESI$^+$, MeCN/H$_2$O): m/z 783.5 [1/2(M + 2H)]$^+$, 1/2(C$_{71}$H$_{98}$N$_{20}$O$_{17}$S$_2$); m/z 1565.7 (M + H)$^+$, C$_{71}$H$_{97}$N$_{20}$O$_{17}$S$_2$. LC-MS (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid): t_R = 8.86 min.

(2,8)-Dicarba-(3,12)-Cys Conotoxin IMI

Fmoc-Gly-c(Hag-Cys-Ser-Asp-Pro-Arg-Hag)-Ala-Trp-Arg-Cys-NH$_2$ 7. The linear Fmoc-Gly-Hag-Cys(Trt)-Ser(O^{t}Bu)-Asp(O^{t}Bu)-Pro-Arg(Pbf)-Hag-Ala-Trp(Boc)-Arg(Pbf)-Cys(Trt)-peptidyl-resin was subjected to the general microwave-assisted RCM procedure under the following conditions: Resin-peptide (158 mg, 82.2 μmol), DCM (3 ml), LiCl/DMF (0.4 M, 0.3 ml), second-generation Grubbs' catalyst (7.0 mg, 8.2 μmol, 10 mol%), 100 °C, 1 h, 100% conversion into **7**. At the end of the reaction period, a small aliquot of peptidyl-resin (∼1 mg) was subjected to the TFA-mediated cleavage procedure. Mass spectral analysis of the isolated residue confirmed the formation of the cyclic peptide **7**. Mass spectrum (ESI$^+$, MeCN/H$_2$O): m/z 769.4 [1/2(M + 2H)]$^+$, 1/2(C$_{69}$H$_{94}$N$_{20}$O$_{17}$S$_2$); m/z 1537.7 (M + H)$^+$, C$_{69}$H$_{93}$N$_{20}$O$_{17}$S$_2$. LC-MS (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid): t_R = 8.59 min.

(2,8)-Dicarba-(3,12)-Cystino Conotoxin IMI

NH$_2$-Gly-c(Hag-Cys-Ser-Asp-Pro-Arg-Hag)-Ala-Trp-Arg-Cys-NH$_2$ 9. The resin-bound carbocyclic peptide (100 mg, 52.0 μmol) was swollen in DCM (3 × 1, 1 × 30 min) and DMF (3 × 1, 1 × 30 min) and de-protected with 20% piperidine in DMF (1 × 1, 2 × 20 min). The resin was then washed with DMF (5 × 1 min), DCM (3 × 1 min), MeOH (3 × 1 min) and dried on the SPPS manifold for 1 h. The Fmoc-de-protected peptidyl-resin (47.0 mg, 24.4 μmol) was subjected to the TFA-mediated cleavage procedure. The residue was then lyophilised for 18 h to give the fully de-protected carbocyclic peptide as a colourless solid (20.0 mg, 15.2 μmol). A sample of lyophilised peptide (10.1 mg, 7.7 μmol) was dissolved in an aqueous solution of (NH$_4$)$_2$CO$_3$ (0.1 M, 80 ml) containing 5% DMSO (4 ml). The reaction was stirred at room temperature and monitored by the Ellman's test [20]. After 3 days, the reaction mixture was lyophilised and mass spectral analysis of the isolated residue confirmed the formation of the cystine-oxidised peptide **9**. The peptide was purified by RP-HPLC (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid) and the unsaturated [2,8]-dicarba-[3,12]-cystino conotoxin hybrid **9** was isolated as a colourless solid (1.8 mg, 5%) in >99% purity. Mass spectrum (ESI$^+$, MeCN/H$_2$O): m/z 657.4 [1/2(M + 2H)]$^+$, 1/2(C$_{54}$H$_{82}$N$_{20}$O$_{15}$S$_2$); m/z 668.3 [1/2(M + H + Na)]$^+$, 1/2(C$_{54}$H$_{81}$N$_{20}$NaO$_{15}$S$_2$); m/z 1313.5 (M + H)$^+$, C$_{54}$H$_{81}$N$_{20}$O$_{15}$S$_2$. LC-MS (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid): t_R = 5.50 min.

(2,8)-Saturated Dicarba-(3,12)-Cystino Conotoxin IMI

NH$_2$-Gly-c(Hag-Cys-Ser-Asp-Pro-Arg-Hag)-Ala-Trp-Arg-Cys-NH$_2$ 2. The carbocyclic-Fmoc-Gly-Hag-Cys(Trt)-Ser(O^{t}Bu)-Asp(O^{t}Bu)-Pro-Arg(Pbf)-Hag-Ala-Trp(Boc)-Arg(Pbf)-Cys

(Trt)-peptidyl-resin was subjected to the general Wilkinson's hydrogenation procedure under the following conditions: Resin-peptide (200 mg, 0.14 mmol), DCM : MeOH (5 ml, 9 : 1), Wilkinson's catalyst (3 mg), 80 psi, 22 °C, 22 h. At the end of the reaction period, a small aliquot of peptidyl-resin was Fmoc-de-protected (20% piperidine in DMF, 1×1, 2×10 min) and washed with DMF (5×1 min), DCM (5×1 min), MeOH (5×1 min) and dried on the SPPS manifold for 1 h. The Fmoc-de-protected peptidyl-resin was then subjected to the TFA-mediated cleavage procedure. Mass spectral analysis of the isolated residue indicated the presence of the cystine-oxidised **2** form of the saturated product. Mass spectrum (ESI$^+$, MeCN/H$_2$O): m/z 658.3 $[1/2(M + 2H)]^+_{\text{oxidised}}$, $1/2(C_{54}H_{84}N_{20}O_{15}S_2)$. LC-MS (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid): t_R **2** = 6.01 min.

(2,8)-Dicarba-(3,12)-Cystino Conotoxin Transformations Linear (2,8)-Cys-(3,12)-Hag Conotoxin IMI

Fmoc-Gly-Cys-Hag-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Hag-NH$_2$ 5. The linear peptide **5** was synthesised using the general SPPS procedure outlined above with Rink amide resin (730 mg, loading 0.52 mmol g^{-1}, 0.38 mmol), NMM (250 µl, 2.27 mmol, 6 equiv.), the protected *C*-terminal amino acid, Fmoc-L-Hag-OH (384 mg, 1.14 mmol, 3 equiv.) and HATU (289 mg, 0.76 mmol, 2 equiv.) in DMF (3 ml). The mixture was shaken gently for 2.5 h. At the end of the reaction period, the resin-peptide was washed with DMF (7 ml, 3×1 min), de-protected with 20% piperidine in DMF (7 ml, 1×1, 2×10 min) and washed again with DMF (7 ml, 5×1 min). The above procedure was repeated until the desired peptide sequence was constructed. A small aliquot of the resin-peptide (~1 mg) was subjected to the TFA-mediated cleavage procedure and mass spectral analysis confirmed the formation of the linear conotoxin analogue **5**. Mass spectrum (ESI$^+$, MeCN/H$_2$O): m/z 783.5 $[1/2(M + 2H)]^+$, $1/2(C_{71}H_{98}N_{20}O_{17}S_2)$; m/z 1565.7 $(M + H)^+$, $C_{71}H_{97}N_{20}O_{17}S_2$. LC-MS (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid): t_R = 9.13 min.

(2,8)-Cys-(3,12)-Dicarba Conotoxin IMI

Fmoc-Gly-Cys-c(Hag-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Hag)-NH$_2$ 8. The linear Fmoc-Gly-Cys(Trt)-Hag-Ser(O^{t}Bu)-Asp(O^{t}Bu)-Pro-Arg(Pbf)-Cys(Trt)-Ala-Trp(Boc)-Arg(Pbf)-Hag-peptidyl-resin was subjected to the general microwave-assisted RCM procedure under the following conditions: Resin-peptide (840 mg, 0.44 mmol), DCM (5 ml), LiCl/DMF (0.4 M, 0.5 ml), second generation Grubbs' catalyst (74.3 mg, 87.5 µmol, 20 mol%), 100 °C, 1 h, 100% conversion into **8**. At the end of the reaction period, a small aliquot of peptidyl-resin (~1 mg) was subjected to the TFA-mediated cleavage procedure. Mass spectral analysis of the isolated residue confirmed the formation of the cyclic peptide **8**. Mass spectrum (ESI$^+$, MeCN/H$_2$O): m/z 769.4 $[1/2(M + 2H)]^+$, $1/2(C_{69}H_{94}N_{20}O_{17}S_2)$; m/z 1537.7 $(M + H)^+$, $C_{69}H_{93}N_{20}O_{17}S_2$. LC-MS (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid): t_R = 8.99 min.

(2,8)-Cystino-(3,12)-Dicarba Conotoxin IMI

Fmoc-Gly-Cys-c(Hag-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Hag)-NH$_2$ 10. The resin-bound carbocyclic peptide (100 mg,

52.0 µmol) was swollen in DCM (3×1, 1×30 min) and DMF (3×1 min, 1×30 min) and de-protected with 20% piperidine in DMF (1×1, 2×20 min). The resin was then washed with DMF (5×1 min), DCM (3×1 min), MeOH (3×1 min) and dried on the SPPS manifold for 1 h. The Fmoc-de-protected peptidyl-resin (61.7 mg, 32.1 µmol) was subjected to the TFA-mediated cleavage procedure. The residue was then lyophilised for 18 h to give the fully de-protected carbocyclic peptide as a colourless solid (15.1 mg, 11.5 µmol). A sample of lyophilised peptide (11.2 mg, 8.5 µmol) was dissolved in an aqueous solution of $(NH_4)_2CO_3$ (0.1 M, 80 ml) containing 5% DMSO (4 ml). The reaction was stirred at room temperature and monitored by the Ellman's test [20]. After 3 days, the reaction mixture was lyophilised and mass spectral analysis of the isolated residue confirmed the formation of the cystine-oxidised peptide **10**. The peptide was purified by RP-HPLC (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid) and the unsaturated [2,8] -cystino- [3,12] -dicarba conotoxin hybrid **10** was isolated as a colourless solid (2.3 mg, 5%) in >99% purity. Mass spectrum (ESI$^+$, MeCN/H$_2$O): m/z 657.3 $[1/2(M + 2H)]^+$, $1/2(C_{54}H_{82}N_{20}O_{15}S_2)$; m/z 668.3 $[1/2(M + H + Na)]^+$, $1/2(C_{54}H_{81}N_{20}NaO_{15}S_2)$; m/z 1313.6 $(M + H)^+$, $C_{54}H_{81}N_{20}O_{15}S_2$. LC-MS (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid): t_R = 4.46 min.

(2,8)-Cystino-(3,12)-Saturated Dicarba Conotoxin IMI

Fmoc-Gly-Cys-c(Hag-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Hag)-NH$_2$ 3. The carbocyclic-Fmoc-Gly-Cys(Trt)-Hag-Ser (O^{t}Bu)-Asp(O^{t}Bu)-Pro-Arg(Pbf)-Cys(Trt)-Ala-Trp(Boc)-Arg(Pbf)-Hag-peptidyl-resin was subjected to the general Wilkinson's hydrogenation procedure under the following conditions: Resin-peptide (200 mg, 0.14 mmol), DCM : MeOH (5 ml, 9 : 1), Wilkinson's catalyst (3 mg), 80 psi, 22 °C, 18 h. At the end of the reaction period, a small aliquot of peptidyl-resin was Fmoc-de-protected (20% piperidine in DMF, 1×1, 2×10 min) and washed with DMF (5×1 min), DCM (5×1 min), MeOH (5×1 min) and dried on the SPPS manifold for 1 h. The Fmoc-de-protected peptidyl-resin was then subjected to the TFA-mediated cleavage procedure. Mass spectral analysis of the isolated residue indicated the presence of the cystine-oxidised **3** form of the saturated product. Mass spectrum (ESI$^+$, MeCN/H$_2$O): m/z 658.4 $[1/2(M + 2H)]^+_{\text{oxidised}}$, $1/2(C_{54}H_{84}N_{20}O_{15}S_2)$; m/z 1315.7 $(M + H)^+_{\text{oxidised}}$, $C_{54}H_{83}N_{20}O_{15}S_2$. LC-MS (Luna C8 RP-column, 10–60% MeOH and 0.1% formic acid): t_R **3** = 7.02 min.

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