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Intraannular Savige–Fontana Reaction: One-Step Conversion of One Class of Monocyclic Peptides into Another Class of Bicyclic Peptides

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Abstract: Cyclisation and cross-linking strategies are important for the synthesis of cyclic and bicyclic peptides. These macrolactams are of great interest due to their increased biological activity compared to linear analogues. Herein, we describe the synthesis of a cyclic peptide containing an Hpi toxicophore, reminiscent of phakellistatins and omphalotins. The first intraannular cross-linking of such a peptide is then presented: using neat TFA to catalyse a Savige–Fontana tryptathionylation, the Hpi-containing peptide is converted to a bicyclic amatoxin analogue. As such, this methodology represents an efficient cyclisation method for cross-linking peptides and exposes a heretofore unrealised relationship between two different classes of peptide natural products. This finding increases the degree of potential chemical space for library generation.

Keywords: cross-linking · indole · natural products · oxidation · peptides

Introduction

Omphalotins[1] and certain phakellistatins[2] are monocyclic peptides with antifungal and antimitotic activities, which were isolated from various marine organisms. These products, along with many others,[3–9] present a characteristic 3a-hydroxypyrrolo[2,3-*b*]indoline (Hpi) motif, the elaboration of which has been the subject of several synthetic investigations, including post-synthetic oxidation of the indole within the macrolactam. For example, phakellistatin 3 (Figure 1), a cyclic heptapeptide with anticancer activity isolated from *Phakellia carteri* marine sponge[10] was synthesised from phakellistatin 13 via a photo-oxidation of tryptophan. However, yields were low and separation of the diastereomeric products was problematic.[2]

Amatoxins and phallotoxins comprise two classes of chemically related bicyclic peptides (octa- and heptapeptides respectively), which are biosynthesised by the mushroom *Amanita phalloides*.[11,12] Both toxins show remarkable biological potency and bind to protein folds on their respective targets with subnanomolar dissociation constants; α-amanitin binds to RNA pol II,[13] whilst phalloidin binds to F-actin (Figure 1).[14] A characteristic structural feature of the amatoxin and phallotoxin peptide families is the tryptathionine linkage that effectively cross-links the main chain peptide cycle, and which has been synthetically introduced by at least three different approaches as part of several ongoing investigations.

Tryptathionine was first reported to have been introduced by formation of cysteine sulfenyl chloride and subsequent thiolation at the indole 2-position.[15] While this approach was successfully employed in solution phase syntheses, there has been but a single report of a solid-phase methodology towards phalloidin using this chemistry to form the tryptathionine.[16] Moreover, this approach is laborious as it involves extensive orthogonal protection strategies in conjunction with non-linear (bidirectional) peptide synthesis. Most recently, Shuresko and Lokey, in their synthesis of phalloidin, reported an elegant approach to tryptathionylation, coincidental with I_2-mediated detritylation of cysteine.[17] An alternative and well established route to a tryptathionine is via the Savige–Fontana reaction[18–20] in which, under acidic conditions, the cysteine thiol reacts with the Hpi moiety at the N-terminus of the linear peptide to afford the tryptathionine cross-link (Scheme 1). Concomitant with tryptathionine formation is the liberation of the N-terminus for continued peptide coupling, or in the cases of amanitin and phalloidin, macrolactamisation.

A priori there appears to be no biotic relationship between the Hpi-containing phakellistatins and omphalotins,

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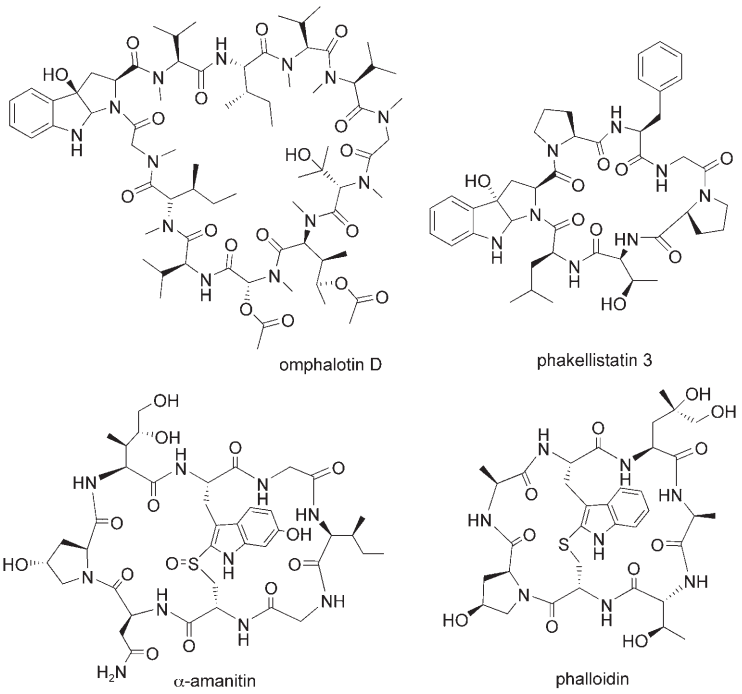
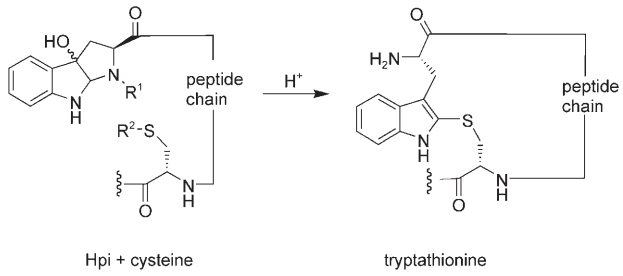


Figure 1. Omphalotin D, phakellistatin 3, α-amanitin and phalloidin.



Scheme 1. Savige–Fontana reaction in the construction of tryptathionine linkages, where R^1 = Boc or Tr, R^2 = Tr.

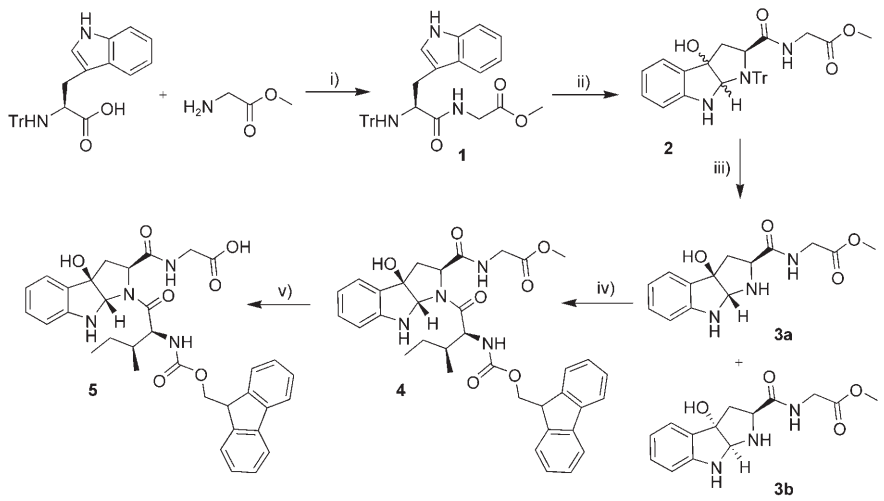
and the tryptathionine-containing amatoxins and phallotoxins. Nevertheless, since an Hpi can be converted to a tryptathionine bridge via the Savige–Fontana reaction, a potential synthetic relationship exists between both classes of natural products. Such a relationship might be exploited in the creation of "superfamilies" of synthetically related libraries of cyclic Hpi-containing peptides, which could be converted at will to libraries of bicyclic peptides containing tryptathionine bridges. The realisation of such a strategy is predicated on: a) the ability to cleanly introduce an Hpi within the macrocyclic peptide and b) that this Hpi would react intraannularly under acidic conditions with a suitably placed cysteine via the Savige–Fontana reaction, to afford a tryptathionine bridge.

Despite the potential link between macrolactams containing an Hpi and those containing a tryptathionine, to date the Savige–Fontana reaction has only ever been described in cases where the Hpi moiety is found as the N-terminal residue in a solution-phase peptide synthesis.[21–24] Immediately following Hpi incorporation, acid treatment cleaves the protecting groups and initiates tryptophanylation of cysteine. This method grows the peptide from C to N terminus in a conventional manner using simple protecting group chemistry, which is suited to solid-phase techniques. To the best of our knowledge, the Savige–Fontana reaction has never been described for cases where Hpi is contained internally within the peptide. In fact, Zanotti et al. reported on an attempted Savige–Fontana reaction with an N-acetylated Hpi; no tryptathionine formation was observed, and hence, it was concluded that the N-acylated Hpi was resistant to acid catalysed ring opening and tryptathionylation.[25,26] In contrast to that finding, here we describe the first synthesis of an intraannular tryptathionine linkage derived from an N-acylated Hpi moiety incorporated internally within a peptide chain (see below). In our study, an Hpi-containing tripeptide is prepared and purified as a single diastereomer prior to incorporation into longer peptides for subsequent cyclisation.[27] A solid-phase approach was used to generate an octapeptide with the Hpi moiety present in the middle of the peptide. Mild cleavage conditions and N–C backbone coupling yielded an octapeptide not dissimilar to a derivative of phakellistatin 3. Acid treatment affords high yielding conversion to the desired tryptathionine cross-linked bicyclic peptide.

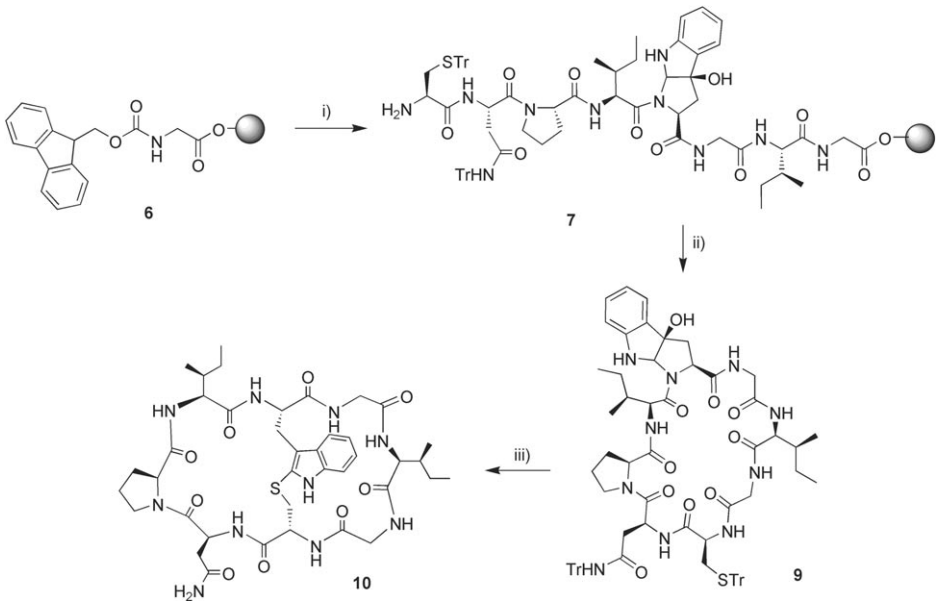
Results and Discussion

Pure H-Hpi-Gly-OMe dipeptide was prepared from Tr-Trp-Gly-OMe via dimethyldioxirane (DMDO) oxidation and subsequent detritylation, as previously reported.[27] Coupling of the *syn–cis* diastereomer to Fmoc-Ile-OH was carried out using (benzotriazol-1-yloxy)-tripyrrolidino-phosphonium hexafluorophosphate (PyBOP) to generate a fully protected tripeptide **4** (Scheme 2). For coupling to a growing peptide chain, selective deprotection of the methyl ester was achieved by using Me_3SnOH.[28] This left the base-labile Fmoc moiety untouched. Following purification, [*syn–cis*]-Fmoc-Ile-Hpi-Gly-OH **5** was ready for solid-phase synthesis.

The solid-phase chemistry was carried out using a 2-chlorotrityl resin due to its high acid lability (Scheme 3).[29] Fmoc-protected amino acids were used for the synthesis of the desired octapeptide (relevant side chains protected with trityl). Glycine was chosen as the C-terminal residue (at po-



Scheme 2. Synthesis of [*syn–cis*]-Fmoc-Ile-Hpi-Gly-OH (**5**). i) DCC, HOBt, NEt_3, CH_2Cl_2, RT, 18 h; ii) DMDO/acetone, CH_2Cl_2, −70 °C, 1 h (70 %); iii) HFIP, CH_2Cl_2, RT, 15 min (2 diastereomers 70 %); iv) Fmoc-Ile-OH, PyBOP, DIPEA, DMF, RT, 5 h (40 %); v) Me_3SnOH, CH_2ClCH_2Cl, 80 °C, 12 h (92 %).

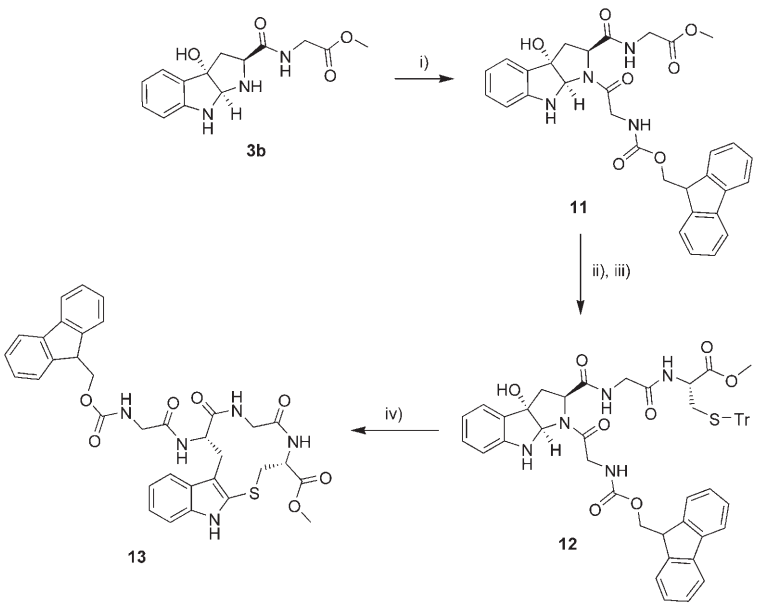


Scheme 3. Synthesis of Pro^2-Ile^3-*S*-deoxo-amaninamide (**10**). i) deprotection (a) and coupling (b) conditions were used in all cases except for addition of the tripeptide **5** where coupling conditions (c) were used: a) Piperidine, DMF, RT, 10 min; b) Fmoc-AA-OH (4 equiv), HBTU (4 equiv), DIPEA, DMF, RT, 20 min; c) Fmoc-Ile-Hpi-Gly-OH, HBTU, DIPEA, DMF, RT, 1 h; ii) HFIP/CH_2Cl_2 1:4, RT, 10 min; iii) PyBOP, DIPEA, DMF, RT, 18 h (10–16 % from **6**); iv) TFA, RT, 5 h (59 %).

sition 6 of amatoxins) to obviate any epimerisation, possible with other amino acids during cleavage or macrolactamisation. The resin was functionalised with Fmoc-Gly-OH in *N,N*-diisopropyldiethylamine (DIPEA)/CH_2Cl_2 for 2 h. Deprotection was effected with piperidine/DMF (20 %) for 10 minutes and all couplings were performed with Fmoc-AA-OH (4 equiv), 2-(1*H*-benzatriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (4 equiv) in DMF/DIPEA (0.5 %) for 20 min. The only exception to this was coupling of the Hpi tripeptide **5** which was performed with just 1.2 equiv of tripeptide and HBTU, for a longer period of 1 h. Hence, the resin-tethered octapeptide **7** was produced in this way.

Selective acid-mediated cleavage from the resin with hexafluoroisopropanol (HFIP) released the linear octapeptide **8** with trityl protection on the cysteine and asparagine residues.[30] Macrocyclisation was performed using PyBOP in DMF/DIPEA for 18 h with a yield of 40–60 % for macrocyclisation and an overall yield of 10–16 % based upon the resin loading, which is reasonable for an octapeptide solid-phase synthesis followed by a macrocyclic N to C coupling of this type. Hence a cyclic peptide **9** containing an Hpi moiety was synthesised. TFA treatment of this compound dispatched the remaining trityl groups and induced the Savige–Fontana reaction to afford a tryptathionine linkage in place of the cysteine and Hpi residues. This reaction proceeded in good yield and generated essentially one major product as observed by HPLC (see Supporting Information). Characterisation of this compound confirmed that this was indeed the desired bicyclic amatoxin derivative **10** (Pro^2-Ile^3-*S*-deoxo-amaninamide).[31–33] The yield is of particular note, despite the relatively harsh conditions, HPLC traces show almost 90 % purity in the crude mixture.

As the *anti–cis* form of **9** did not cleanly undergo tryptathionylation, the reactivity of both Hpi diastereomers was investigated in the context of Fmoc-Gly-Hpi-Gly-Cys(Tr)-OMe to verify that thioetherification would ensue with related substrates, irrespective of Hpi stereochemistry (*syn–cis* or *anti–cis*). Hence, [*anti–cis*]-Fmoc-Gly-Hpi-Gly-OMe was prepared as previously reported.[27] Following selective deprotection of the C-terminus and coupling of H-Cys(Tr)-OMe with HBTU, [*anti-cis*]-Fmoc-Gly-Hpi-Gly-Cys(Tr)-OMe (**12**) was afforded in good yield (Scheme 4). This linear tetrapeptide was then treated with neat TFA at room temperature for 4 h to yield the desired



Scheme 4. Synthesis of [*anti–cis*]-Fmoc-Gly-Hpi-Gly-Cys(Tr)-OMe (**13**). i) Fmoc-Gly-OH, PyBOP, DIPEA, DMF, RT, 8 h; ii) Me_3SnOH, CH_2ClCH_2Cl, 80 °C, 12 h; iii) H-Cys(Tr)-OMe, HBTU, DIPEA, DMF, RT, 3 h (2 steps 76%); iv) TFA, RT, 4 h (42%).

cyclic tryptathionine **13**. UV absorbance of this product showed an overlap of the Fmoc group with the characteristic absorption of the tryptathionine. A small amount of this was treated with piperidine to remove the protecting group and a clean tryptathionine UV absorbance spectrum was observed ($\lambda_{max} = 290$ nm, see Supporting Information). This finding suggests that the steric bulk of the Ile, and/or the conformational nature of the [*anti–cis*]-Hpi within a macrocyclic ring, prevented clean tryptathionylation in the case of the macrolactam. Although both N-glycylated Hpi *anti–cis* and *syn–cis* diastereomers, cleanly underwent tryptathionylation, the extent to which various steric/conformational effects might limit the generality of this reaction was not further investigated.

Conclusion

The salient points of this work are as follows: This work expands the reactivity of the Savige–Fontana reaction to intraannular systems, which did not appear possible according to earlier reports. As such, a circularly permuted approach to synthesizing tryptathionine bridged peptides is now feasible, in accordance with a synthetic strategy that will generally allow for macrolactamisation at almost any chosen amino-acid pair. Consequently, one avoids coupling at sterically constrained amino acids (e.g. Ile) that are prone to epimerisation. Indeed, the standard linear approach to synthesizing amanitin starts with an octapeptide that undergoes a Savige–Fontana cyclisation to install the tryptathionine and unmasks the terminal NH_2 of the tryptathionine, followed by macrocyclisation between Trp and Ile. In our hands, this approach has consistently provided two chromatographically distinct products with greatly differing CD spectra, which are due to an epimerisation of Ile to D-*allo*-Ile and not atropisomers,[33] as previously discussed for amanitin.[31,32] Of note, similar conformational concerns have been raised with regards to phalloidin.[16] Herein, we created an amatoxin by deliberately choosing a linear peptide synthetic route with Gly at the C terminus for macrolactamisation, thus obviating any possibility of epimerisation. Once cyclised, acidification afforded the tryptathionine bridge within a single bicyclic amanitin derivative exhibiting identical MS/HPLC characteristics to Pro^2-Ile^3-*S*-deoxo-amaninamide.[33]

Of note, Shuresko and Lokey published a formidable approach to the intraannular oxidative thioetherification of tryptophan using I_2 in DMF. Their findings complement the results presented here. In contrast to their elegant work, the results herein allow access to several classes of natural products (omphalotins, phakellistatins, amatoxins and phallotoxins) from minor modifications to the same library of compounds (phallotoxins have a deletion at Asx relative to amatoxins). This would appear to be significant in order to access a large area of chemical space with a directed combinatorial library approach. With this methodology one can imagine constructing a library of phakellistatins and related peptides that can be easily transformed into synthetic amatoxins, phallotoxins and related peptides, all from essentially the same linear resin-bound library of hepta- and octapeptides. The generally high yielding conversions we observe could be of particular importance in producing libraries of small quantities of these bicyclic peptides where high yields are necessary for sufficient activity to be observed in a subsequent screen. The construction of such libraries is currently being examined and will be reported in due course.

Experimental Section

General experimental details: All reagents were obtained from commercial sources and used without further purification unless otherwise stated. All amino acids were L-amino-acids unless otherwise stated. Thin layer chromatography was performed on Merck 60 F254 silica plates and viewed under UV radiation (254 nm), or staining with ninhydrin with gentle heating. Silicycle silica gel (230–400 mesh) was used for flash column chromatography. Further purification of peptides was performed using an Agilent 1100 HPLC. HPLC was carried out using a reverse-phase C18 column (8.5×15×1.5 mm) with gradients combining buffer A and B. Buffer A = (H_2O + 0.1% TFA); Buffer B = (MeCN + 0.05%

TFA). For all HPLC peptide purifications a photodiode array detector was used between 200 and 400 nm. ^{1}H NMR and ^{13}C NMR were performed at 300/400/600 MHz and 75/100/150 MHz, respectively. Chemical shifts for all spectra were reported in parts per million and referenced to the residual protic solvent peak. Mass spectrometry data was acquired on a Waters-Micromass ZQ system, using positive or negative ionisation mode in MeOH or MeCN.

[*syn–cis*]-Fmoc-Ile-Hpi-Gly-OMe (4): PyBOP (0.24 g, 0.5 mmol), [*syn–cis*]-H-Hpi-Gly-OMe (0.12 g, 0.4 mmol), and DIPEA (80 μL, 0.5 mmol) was added to a solution of Fmoc-Ile-OH (0.17 g, 0.5 mmol) in dry DMF (20 mL). The mixture was stirred for 18 h at room temperature. On completion of the reaction the mixture was evaporated to dryness and purified by flash chromatography on silica gel (hexane/EtOAc 1:1) to yield pure product as a white foam (0.10 g, 40 %). R_f=0.5 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ=7.72 (d, J=7.1 Hz, 2H, ArH^{Fmoc}), 7.61–7.51 (m, 2H, ArH^{Fmoc}), 7.40–7.19 (m, 6H, NHCO, ArH^4, ArH^{Fmoc}), 7.05 (t, J=7.6 Hz, 1H, ArH^5), 6.71 (t, J=7.6 Hz, 1H, ArH^6), 6.51 (d, J=7.6 Hz, 1H, ArH^7), 6.01–5.89 (m, 2H, NHCO, CH^{8a}), 4.59 (d, J=8.8 Hz, 1H, CH^2), 4.47 (dd, J=10.5, 7.3 Hz, 1H, CH^{Fmoc}), 4.35–4.05 (m, 4H, CH^{Fmoc}, CH^{Fmoc}, $CH^{\alpha Gly}$, $CH^{\alpha Ile}$), 3.93 (dd, J=18.2, 4.7 Hz, 1H, $CH^{\alpha Gly}$), 3.73 (s, 3H, CH^{OMe}), 2.35 (d, J=14.0 Hz, 1H, CH^3), 2.27 (dd, J=14.0, 8.8 Hz, 1H, CH^3), 1.93–1.70 (m, 1H, $CH^{\beta Ile}$), 1.64–1.51 (m, 1H, CH^{γ}), 1.15 (m, 1H, $CH^{\gamma Ile}$), 0.96–0.81 ppm (m, 6H, $CH^{\gamma Ile}$, $CH^{\delta Ile}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ=173.2 (C^{CONH}), 172.9 (C^{CONH}), 169.4 (C^{CO}), 156.6 (C^{OCONH}), 146.4 (C^{7a}), 143.6 (C^{Fmoc}), 141.2 (C^{Fmoc}), 129.6 (C^{3b}), 129.6 (CH^6), 127.7 (CH^{Fmoc}), 126.9 (CH^{Fmoc}), 124.9 (CH^{Fmoc}), 122.3 (CH^4), 119.9 (CH^{Fmoc}), 119.2 (CH^5), 109.9 (CH^7), 88.9 (C^{3a}), 87.2 (C^{8a}), 67.3 (CH_2^{Fmoc}), 61.6 (CH^2), 56.6 (CH^{Fmoc}), 52.4 (CH_3^{OMe}), 46.9 ($CH^{\alpha Ile}$), 41.4 ($CH^{\alpha Gly}$), 40.2 (CH_2^3), 38.0 ($CH^{\beta Ile}$), 24.7 (CH_2^{γ}), 14.7 ($CH_3^{\gamma'}$), 10.5 ppm (CH_3^{δ}); ES$^+$/MS: m/z: 649.3 [M+Na]$^+$; HRMS (ES$^+$): m/z: calcd for $C_{35}H_{39}N_4O_7$: 627.2819 [M+H)$^+$]$^+$, found 627.2796.

[*syn–cis*]-Fmoc-Ile-Hpi-Gly-OH (5): [*syn–cis*]-Fmoc-Ile-Hpi-Gly-OMe (0.10 g, 0.2 mmol) and trimethyltin hydroxide (0.14 g, 0.8 mmol) were weighed into a dry flask and 1,2-dichloroethane (10 mL) was added. The mixture was stirred overnight at 76 °C. TLC (CH_2Cl_2/MeOH 9:1) showed completion of the reaction and the mixture was concentrated in vacuo. Purification was with silica gel column chromatography (CH_2Cl_2/MeOH 9:1) and fractions were combined to yield pure product (0.90 g, 92 %). R_f=0.1 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ=7.75 (d, J=7.5 Hz, 2H, ArH^{Fmoc}), 7.61 (dd, J=7.1, 4.4 Hz, 2H, ArH^{Fmoc}), 7.38 (dd, J=7.1, 6.9 Hz, 2H, ArH^{Fmoc}), 7.34–7.21 (m, 4H, NHCO, ArH^4, ArH^{Fmoc}), 7.08 (t, J=7.6 Hz, 1H, ArH^5), 6.82 (d, J=9.9 Hz, 1H, NHCO), 6.76 (t, J=7.4 Hz, 1H, ArH^6), 6.58 (d, J=7.9 Hz, 1H, ArH^7), 5.88 (s, 1H, CH^{8a}), 4.65 (d, J=8.5 Hz, 1H, CH^2), 4.45 (dd, J=10.1, 7.6 Hz, 1H, CH^{Fmoc}), 4.38–4.25 (m, 3H, CH^{Fmoc}, $CH^{\alpha Gly}$, $CH^{\alpha Ile}$), 4.20 (t, J=7.4 Hz, 1H, CH^{Fmoc}), 3.92 (dd, J=18.1, 3.1 Hz, 1H, $CH^{\alpha Gly}$), 2.45 (d, J=14.2 Hz, 1H, CH^3), 2.37 (dd, J=14.2, 8.8 Hz, 1H, CH^3), 1.83–1.70 (m, 1H, $CH^{\beta Ile}$), 1.69–1.53 (m, 1H, CH^{γ}), 1.37–1.13 (m, 2H, OH, $CH^{\gamma Ile}$), 0.96–0.79 ppm (m, 6H, $CH^{\gamma Ile}$, $CH^{\delta Ile}$); ^{13}C NMR (100 MHz, $CDCl_3$): δ=174.6 (C^{CONH}), 172.6 (C^{CONH}), 171.4 (C^{CO}), 156.9 (C^{OCONH}), 146.3 (C^{7a}), 143.7 (C^{Fmoc}), 141.3 (C^{Fmoc}), 129.8 (C^{3b}), 129.6 (CH^6), 127.8 (CH^{Fmoc}), 127.1 (CH^{Fmoc}), 125.2 (CH^{Fmoc}), 122.5 (CH^4), 120.1 (CH^{Fmoc}), 119.5 (CH^5), 110.1 (CH^7), 89.0 (C^{3a}), 87.5 (C^{8a}), 67.6 (CH_2^{Fmoc}), 62.1 (CH^2), 56.8 (CH^{Fmoc}), 47.1 ($CH^{\alpha Ile}$), 41.8 ($CH^{\alpha Gly}$), 40.5 (CH_2^3), 37.9 ($CH^{\beta Ile}$), 24.9 (CH_2^{γ}), 14.7 ($CH_3^{\gamma'}$), 10.5 ppm (CH_3^{δ}); ES$^+$/MS: m/z: 635.2 [M+Na]$^+$; HRMS (ES$^+$): m/z: calcd for $C_{34}H_{36}N_4O_7Na$: 635.2482 [M+Na]$^+$, found 635.2485.

Compounds 6–7: 2-Chlorotrityl resin was functionalised with Fmoc-Gly-OH in the presence of DIPEA. After thorough washing the Fmoc-Gly-resin was dried in vacuo (resin loading was calculated to be 1.39 mmol g^{-1}). Fmoc deprotection was effected with piperidine (20 %) in DMF for 10 min. Coupling was effected with Fmoc-AA-OH (4 equiv), HBTU (4 equiv), DMF/DIPEA (0.5 %) agitating for 20 min (all standard L-amino acids). The tripeptide **5** (1.1 equiv) was coupled with HBTU (1.1 equiv), DMF/DIPEA (0.5 %), shaking for 1 h.

Cyclic peptide 9: The linear precursor was cleaved from the resin using HFIP/CH_2Cl_2 1:4 (10 mL) with stirring for 10 min. Solvent was removed by evaporation and the residue was dissolved in MeOH and filtered to remove resin. The filtrate was dried to yield a crude pale yellow residue, 10 mg. This residue was dissolved in dry DMF and PyBOP and DIPEA were added. The mixture was stirred for 18 h at room temperature. Solvent was removed in vacuo and the mixture was purified by silica gel column chromatography (CH_2Cl_2/MeOH 0→10 %). Pure fractions were combined to yield a white solid (3.0 mg, 16 % based upon resin loading). R_f=0.5 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ=9.52 (s, 1H, NH^{indole}), 8.78 (d, J=6.6 Hz, 1H, $CONH^{Cys}$), 8.72 (dd, J=4.6, 3.3 Hz, 1H, $CONH^{Gly}$), 8.65 (d, J=6.6 Hz, 1H, $CONH^{Ile}$), 8.51 (dd, J=8.0, 3.5 Hz, 1H, $CONH^{Gly}$), 8.01 (s, 1H, NH^{Asn}), 7.36–7.10 (m, 33H, $ArH^{Tr,indole}$, $CONH^{Asn,Ile}$), 7.04 (t, J=7.7 Hz, 1H, ArH^{indole}), 6.64 (t, J=7.7 Hz, 1H, ArH^{indole}), 6.46 (d, J=7.7 Hz, 1H, ArH^{indole}), 5.97 (s, 1H, OH), 5.22 (s, 1H, CH^{8a}), 4.43–4.30 (m, 2H, $CH^{\alpha Ile,\alpha Asn}$), 4.12 (dd, J=17.0, 8.0 Hz, 1H, $CH^{\alpha Gly}$), 4.03 (dd, J=8.6, 4.6 Hz, 1H, $CH^{\alpha Pro}$), 3.97 (dd, J=10.1, 8.8 Hz, 1H, $CH^{\alpha Ile}$), 3.81 (dd, J=11.3, 5.5 Hz, 1H, $CH^{\alpha Hpi}$), 3.60 (dd, J=15.1, 3.3 Hz, 1H, $CH^{\alpha Gly}$), 3.43–3.32 (m, 2H, $CH^{\alpha Cys,\alpha Gly}$), 3.19 (dd, J=17.0, 3.5 Hz, 1H, $CH^{\alpha Gly}$), 2.90 (t, J=11.4 Hz, 1H, $CH^{\beta Cys}$), 2.75 (t, J=12.3 Hz, 1H, $CH^{\beta Asn}$), 2.65–2.47 (m, 3H, $CH^{\delta Pro,\beta Cys,\beta Asn}$), 2.43 (dd, J=12.1, 5.5 Hz, 1H, $CH^{\beta Hpi}$), 2.28 (dd, J=12.1, 11.3 Hz, 1H, $CH^{\beta Hpi}$), 2.00–1.88 (m, 2H, $CH^{\beta Ile,\beta Pro}$), 1.78–1.72 (m, 1H, $CH^{\delta Pro}$), 1.66–1.58 (m, 1H, $CH^{\beta Ile}$), 1.54–1.46 (m, 1H, $CH^{\beta Pro}$), 1.41–1.34 (m, 1H, $CH^{\gamma Ile}$), 1.24–1.05 (m, 2H, $CH^{\gamma Ile,\gamma Pro}$), 1.05 (d, J=7.0 Hz, 3H, $CH_3^{\gamma' Ile}$), 1.02–0.72 (m, 2H, $CH^{\gamma Pro,\gamma Ile}$), 0.84–0.69 (m, 6H, $CH_3^{\gamma Ile,\delta Ile}$), 0.64–0.55 (m, 1H, $CH^{\gamma Ile}$), 0.35 ppm (t, J=7.4 Hz, 3H, $CH_3^{\delta Ile}$); ^{13}C NMR (150 MHz, [D$_6$]DMSO): δ=172.1, 171.5, 170.1, 169.8, 169.6, 168.9, 168.6, 168.3, 166.8, 151.7, 144.5, 144.4, 130.0, 129.1, 129.0, 128.7, 128.0, 127.3, 126.7, 126.3, 123.8, 117.8, 108.7, 85.9, 81.8, 69.3, 65.9, 60.4, 54.9, 54.0, 46.7, 46.0, 43.4, 42.2, 41.4, 40.8, 40.1, 35.5, 34.2, 30.8, 29.0, 24.9, 24.3, 24.1, 15.4, 13.8, 9.4, 8.0 ppm; ES$^+$/MS: m/z: 1364.9 [M+Na]$^+$; HRMS (ES$^+$): m/z: calcd for $C_{77}H_{84}N_{10}O_{10}SNa$: 1363.5990 [$M$+Na]$^+$, found 1363.5992.

Amatoxin 10: TFA (5 mL) was added to **9** (3.0 mg, 0.002 mmol) and stirred for 5 h at room temperature. The solvent was evaporated in vacuo and the residue was redissolved in water before evaporating again. This was repeated three times. The residue was dissolved in MeOH/H_2O, filtered with a 0.5 μm syringe filter then purified by HPLC. Pure product was combined to yield a white solid residue (1.1 mg, 59 %). This sample was co-eluted with a reference sample of Pro^2-Ile^3-*S*-deoxo-amanin-amide,[33] which gave an identical retention time. R_f=0.5 ($CHCl_3$/MeOH/water 90:13:1); HRMS (ES$^+$): m/z: calcd for $C_{39}H_{54}N_{10}O_9SNa$: 861.3696 [M+Na]$^+$, found 861.3694.

[*anti–cis*]-Fmoc-Gly-Hpi-Gly-Cys(Tr)-OMe (12): [*anti–cis*]-Fmoc-Gly-Hpi-Gly-OMe (60 mg, 0.10 mmol) was heated with Me_3SnOH (5 equiv) in dichloroethane (10 mL) as previously described.[27] On completion the reaction was evaporated to dryness and purified on a short silica plug. The white residue was dissolved in DMF and H-Cys(Tr)-OMe (42 mg, 0.11 mmol), HBTU (42 mg, 0.11 mmol) and DIPEA (39 μL, 0.22 mmol) were added. The reaction was stirred for 3 h and TLC (CH_2Cl_2/MeOH 9:1) showed consumption of starting material. The reaction was evaporated to dryness then redissolved in CH_2Cl_2 and washed with citric acid (5 %), sat. $NaHCO_3$, sat. NaCl and dried over anhydrous Na_2SO_4. The residue was purified by silica chromatography (CH_2Cl_2/MeOH 0→10 %) to give the title compound as a white solid (73 mg, 76 %). R_f=0.6 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=8.50 (d, 1H, J=7.6 Hz, NHCO), 8.00 (d, 1H, J=7.9 Hz, NHCO), 7.88 (d, 2H, J=7.3 Hz, ArH^{Fmoc}), 7.69 (d, 2H, J=7.6 Hz, ArH^{Fmoc}), 7.50–7.17 (m, 21H, ArH^{Tr}, ArH^{Fmoc}, NHCO, ArH^{indole}), 7.09 (t, 1H, J=7.3 Hz, ArH^{indole}), 6.69 (t, 1H, J=7.3 Hz, ArH^{indole}), 6.57 (d, 1H, J=7.9 Hz, ArH^{indole}), 4.91 (s, 1H, CH^{Hpi8a}), 4.32–4.15 (m, 4H, CH_2^{Fmoc}, $CH^{\alpha Gly}$, $CH^{\alpha Cys}$), 4.05–3.86 (m, 3H, $CH^{Fmoc,}$ $CH^{\alpha Gly}$, $CH^{\alpha Hpi}$), 3.69–3.20 (m, 6H, CH_3^{OMe}, $CH_2^{\alpha Gly}$, NH^8), 2.59–2.29 ppm (m, 4H, $CH^{\beta Cys}$, $CH^{\beta Hpi}$); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ=171.9, 171.7, 170.2, 169.7, 157.9, 149.2, 145.5, 145.3, 142.1, 132.2, 131.0, 130.5, 129.5, 129.2, 128.5, 128.4, 128.1, 126.7, 125.2, 121.5, 119.6, 110.6, 84.7, 79.2, 67.9, 67.2, 53.6, 52.9, 48.1, 47.5, 46.5, 44.7, 41.2, 34.3 ppm; ES$^+$/MS: m/z: 938.3 [M+Na]$^+$.

***N*-(9*H*-Fluoren-9-ylmethoxy)carbonylglycyl(2-mercapto-tryptophanyl-glycyl-cysteine cyclic sulfide) methyl ester (13)**: [*anti–cis*]-Fmoc-Gly-Hpi-Gly-Cys(Tr)-OMe (40 mg, 0.044 mmol) was stirred with TFA (4 mL) for 4 h at room temperature. Methanol was added and the solvent removed in vacuo, which was repeated three times. The residue was purified on

silica gel column chromatography (CH_2Cl_2/MeOH 0→15%) and purified further by HPLC to yield pure product (12 mg, 42%). R_f=0.4 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.25 (d, J=5.1 Hz, 1H), 8.12–8.05 (br s, 1H), 8.00 (d, J=7.4 Hz, 1H), 7.80 (d, J=7.4 Hz, 2H), 7.75 (d, J=7.9 Hz, 1H), 7.73–7.66 (m, 2H), 7.43–7.27 (m, 5H), 7.15 (t, J=7.9 Hz, 1H), 7.08 (t, J=7.4 Hz, 1H), 4.82–4.78 (m, 2H), 4.41 (d, J=6.5 Hz, 2H), 4.28 (t, J=6.5 Hz, 1H), 4.19 (dd, J=15.3, 5.7 Hz, 1H), 3.97–3.83 (m, 2H), 3.79 (s, 3H), 3.68–3.59 (m, 1H), 3.51–3.37 (m, 2H), 3.16–3.08 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=174.3, 171.4, 171.2, 170.9, 158.8, 144.8, 142.2, 139.1, 128.4, 128.1, 127.8, 125.3, 123.3, 120.5, 120.3, 119.6, 117.3, 113.1, 111.5, 67.8, 53.9, 53.2, 52.9, 49.5, 44.5, 37.3, 30.3 ppm; ES$^+$/MS: m/z: 678.1 $[M+Na]^+$.

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