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Celogentin mimetics as inhibitors of tubulin polymerization

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Abstract

Bicyclic analogues of celogentin C have been synthesized in which the sidechain–sidechain crosslinks are replaced by thioether bonds. Several of the simplified bicyclic peptides displayed potent inhibition of tubulin polymerization.

Introduction

Several anti-mitotic agents, including paclitaxel,[1] ixabepilone,[2] vinblastine and vincristine[3] are used in the clinical treatment of cancer.[4-5] However, the development of resistance to current clinical cancer agents[6] is a major problem in anti-cancer treatment that encourages the development of new generation anti-mitotic agents.

The celogentin family of cyclic peptides were isolated from the seeds of the cockscomb plant, *Celosia argentea*, and exhibit potent inhibition of tubulin polymerization.[7] The most potent member of the family, celogentin C, exhibits greater inhibitory activity than vinblastine, yet further biological studies have been hampered by the small amounts of material available through natural production extraction[7] or total synthesis.[8-11]

The celogentins possess a central, highly functionalized tryptophan residue (Trp5) that is in involved in two unusual side-chain to side-chain cross-links; between the β-position of Leu2 and the indole C-6 of Trp5 and between the indole C-2 of Trp5 and the imidazole of the histidine side chain. Cross-linked aromatic amino acids occur commonly in bicyclic peptide natural products, wherein covalent links between side chain aromatic group and other amino

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acids generate a central aromatic core. Examples include, in addition to the core tryptophan of the celogentins, the central phenylglycine residue in vancomycin and the tryptophan residue in phalloidin (Figure 1).[12-13]

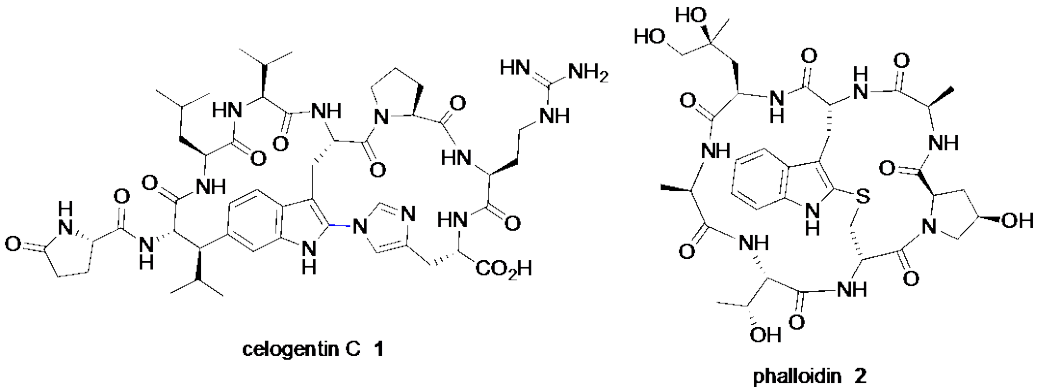


Figure 1: cross-linked cyclic peptides.

Though the development of analogues of biologically active cyclic peptide natural products has been achieved through semi-synthesis (e.g., through replacement of acyl groups),[14-15] modifications to the core scaffold of complex cyclic peptide natural products often require total synthesis,[16] which is lengthy and not cost effective. Modifications to introduce stable, non-reducible cross-links have been developed in recent years to improve pharmacodynamic properties of peptides.[17-19] Examples of such cross-links include olefins (through cross-metathesis), triazoles (through CuAAC reactions) and sulfides (through alkylation of cysteines with bis- or tris-electrophiles).[20-22] Though aromatic linkers have been used to constrain linear peptides through sidechain–sidechain cross-links, to the best of our knowledge very few examples (e.g. a vancomycin triazole mimic)[23] exist of cross-linking agents employed as replacements for the core scaffold of naturally occurring cyclic peptides.

We postulate that analogues of bicyclic peptide natural products such as celogentin C could be generated by replacement of the central aromatic core with aromatic moieties more routinely employed as stapling groups. Such bicyclic peptides should be much more synthetically accessible than the natural products themselves, and more amenable to the generation of libraries of analogues to assess structure-activity relationships and fine-tune biological properties. Recently, we reported the synthesis of bicyclic celogentin analogues in

which the core tryptophan indole was replaced by a mesitylene moiety, and demonstrated these peptides possess moderate anti-bacterial activity.[24] Herein, we report the tubulin polymerization inhibitory activities of these compounds, together with those of a range of novel cross-linked bicyclic peptides as celogentin mimics.

Materials and Methods

Solid phase peptide synthesis

The linear peptides were synthesized using standard Fmoc SPPS coupling methods, employing Wang or chlorotrityl resin for peptide C-terminal acids and Rink amide resin for C-terminal amides. The coupling steps were performed using a CEM Liberty Blue microwave peptide synthesizer. All peptides were synthesized on 0.1 mmol scale using a 4–5-fold molar excess of Fmoc-protected amino acid (0.4 or 0.5 mmol for a 0.1 mmol scale) that were activated using a 4–5-fold excess of HCTU in the presence of DIEA (8–10 equivalents). Fmoc deprotection was performed with 20% v/v piperidine in DMF.

Cleavage of the peptide from the solid support

After completion of solid phase synthesis, the peptides were cleaved from the solid support by treatment with a cleavage cocktail of trifluoroacetic acid (TFA):triisopropylsilane (TIPS):3,6-dioxa-1,8-octanedithiol (DODT):water (94:2.5:2.5:1, 15 mL/0.1 mmol of peptide) for 2 h. The cleavage cocktail was evaporated under nitrogen, and the remaining residue was precipitated with ice-cold diethyl ether and centrifuged at 3000 rpm for 3–5 min. Pellets were washed 3 × by resuspending in ice-cold diethyl ether followed by centrifugation.

Cleavage of side chain protected peptide from a solid support

Peptide-containing resin (0.1 mmol) was treated with 10% HFIP in CH_2Cl_2 (5 mL) for 30 min. The solvent was drained, and this process was repeated a further 2 ×. The filtrates were combined and the solvent was evaporated under vacuum.

Hydroxypyrroloindoline (Hpi)[25]

A 20 mM aqueous solution of Fmoc-Trp-OH (1.00 g, 2.35 mmol) was added in 22 % (v/v) acetone/ Milli-Q water and $NaHCO_3$ (3.94 g, 46.9 mmol) at 0 °C. Then an aqueous solution of Oxone® (40 mM, 120 mL) was added dropwise over 45 min at 0 °C. The mixture was diluted with saturated aqueous KH_2PO_4 and 2 percent (v/v) aqueous until pH 3 was reached. The aqueous layer was extracted with EtOAc (3 × 100 ml), the organic layers were combined, washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure. The crude product was purified by column chromatography in MeOH/DCM, to yield purified product (0.32 g, 31%). ESI MS *m/z* 443.160, $[M+H]^+$. ^1H NMR ($CDCl_3$) δ: 7.84 (dd, 2H, J 7.4 Hz,), 7.79-7.75 (m, 1.0H), 7.66 (dd, 2H, J = 7.5, 15.6 Hz), 7.61-7.52 (m, 2Ha), 7.48-7.24 (m, 6.0), 7.20-7.08 (m, 2.4H), 6.92 (dd, 0.4H, J = 7.5, 17.4 Hz), 6.82 (t, 0.4H, J = 7.5 Hz), 6.76 (t, 0.9H, J = 7.5 Hz), 6.66 (d, 0.43H, J = 7.9 Hz), 6.40 (d, 1Ha, J = 7.9 Hz), 5.50 (s, 0.38H), 4.86 (s, 1H), 4.85 (dd, 1H, J = 4.4, 10.9 Hz), 4.75 (dd, 1H, J = 3.7, 10.8 Hz), 4.50 (dd, 0.5H, J = 10.6, 6.1 Hz), 4.42 (dd, 0.5H, J = 10.6, 6.5 Hz), 4.39 (dd, 0.6H, J = 3.3; 7.8 Hz), 4.35 (dd, 2H, J = 3.5; 9.0 Hz), 4.25-4.19 (m, 1H), 2.70–2.58(m, 2H, CH,CH), 2.56 (dd, 2H, J 13.4, 9.6 Hz,CH_2), 2.38 (dd, 1H, J 7.4, 9.6 Hz, CH_2). ^{13}C NMR ($CDCl_3$) δ: 173.1(C), 172.2 (C), 155.5 (C), 155.4 (C), 148.5 (C), 147.9 (C), 144.4 (C), 144.3 (C), 142.0 (C), 141.7 (C), 141.5 (C), 130.6 (CH), 130.4 (CH), 130.2 (C), 129.8 (C), 128.3 (CH), 128.2 (CH), 128.1 (CH), 127.9 (CH), 127.8 (CH), 127.5 (CH), 125.4 (CH), 125.3 (CH), 125.1 (CH), 124.9 (CH), 123.5 (CH), 123.2 (CH), 120.5 (CH), 120.3 (CH), 119.9 (CH), 119.7 (CH), 110.9 (CH), 110.4 (CH), 88.1 (C), 87.3 (C), 85.5 (CH), 85.1 (CH), 68.2 (CH_2), 67.1 (CH_2), 60.1 (CH), 59.8 (CH), 47.6 (CH) 48.4 (CH), 43.8 (CH_2), 42.8 (CH_2).

Head-to-tail cyclization

The crude peptide **10** was dissolved in DMF (2 mL), then diphenylphosphoryl azide (5 equiv.) and DIPEA (5 equiv.) were added. The mixture was stirred for 12 h at room temp. The mixture was diluted with DCM (25 mL) and washed with 0.5M HCl (3 × 25 mL). The organic layer was separated, dried with Na_2SO_4 and evaporated in vacuo. The crude peptide **11** was used without further purification.

Trp–Cys cross-linking

The crude protected peptide **11** (0.067 g, 0.05 mmol) was dissolved in TFA (25 mL) and the pale yellow solution was stirred for 5 h. The solvent was removed in vacuo, then the peptide was precipitated with cold ether, and the crude peptide was purified by HPLC. Purified yield of compound **5** was 10% (from starting resin).

HATU assisted cyclization:

Peptide-containing resin (0.1 mmol) was treated with 1% TFA in CH_2Cl_2 (5 mL) for 15 min. The solvent was drained, and this process was repeated 2×. The filtrates were combined, and the solvent was evaporated under vacuum. The crude peptide was redissolved in DMF (10 mL) then HATU (0.15 mmol) and DIPEA (0.25 mmol) were added. The reaction mixture was stirred overnight at room temperature, then the solvent was evaporated under vacuum. The residue was treated with trifluoroacetic acid (TFA):triisopropylsilane (TIPS):water (95:2.5:2.5, 10 mL/0.1 mmol of peptide) for 1.5 h to effect deprotection. The solvent was evaporated under a stream of nitrogen, then the residue was treated with ice-cold diethyl ether to precipitate the peptide. The mixture was centrifuged at 3000 rpm for 3–5 min, then the precipitate was washed 3 × by suspending in ice-cold diethyl ether (15 mL) followed by centrifugation.

Purification

Purification of all peptides were performed using an Agilent RP-HPLC with a C18 Phenomenex 250×10 mm, 2μ column. The purity was assessed using an analytical C18 Phenomenex 150×4.6mm, 5μ column in a gradient mode with eluent (buffer) A; 0.1% aq. TFA and buffer B; 0.1% TFA in acetonitrile. RP-HPLC was performed using gradient elution with buffer B 0–40% over 40 min, monitoring at a wavelength of 214 nm.

Anti-mitotic assays

In vitro tubulin polymerization assays were performed using a tubulin polymerization assay kit (Cytoskeleton) per the manufacturer's instructions. Briefly, lyophilized porcine tubulin

was solubilized to a final concentration of 2 mg/mL in reaction buffer containing 80 mM PIPES (pH 6.9), 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP, and 20% glycerol and kept at 4 °C. Compounds (100× DMSO stock solutions) were added to prewarmed half-area 96-well plates at 37 °C. Cold tubulin solution was added to the wells. The plate contents were mixed by shaking, and the absorbance at 340 nm was measured every minute for 30 min. Serial two-fold dilutions were performed to generate compound concentrations of 200–12.5 μM for IC_{50} determination. Compounds that did not display any activity were assigned IC_{50} values greater than the highest experimental value tested. All assays were performed in duplicate.

Results and discussion

In our previous study,[24] analogues of celogentin C were designed based on the replacement of the crosslinked central tryptophan moiety with a mesitylene group. Thus, the three native cross-linked amino acid residues—leucine, tryptophan and histidine—were replaced with cysteine residues. Crosslinking of the Cys residues was achieved by treatment with tris(bromomethyl)benzene (TBMB) to give celogentin analogues **3** and **4** (Figure 2).

A new type of bicyclic celogentin mimic was conceived by analogy with the phallotoxins and amatoxins,[26-28] which contain a Trp–Cys crosslink. Thus, a bioinspired design in which the Trp–His crosslink is replaced with a Trp–Cys link, and the Leu–Trp link was replaced with a head-to-tail link was envisaged (compound **5**) (Figure 2).

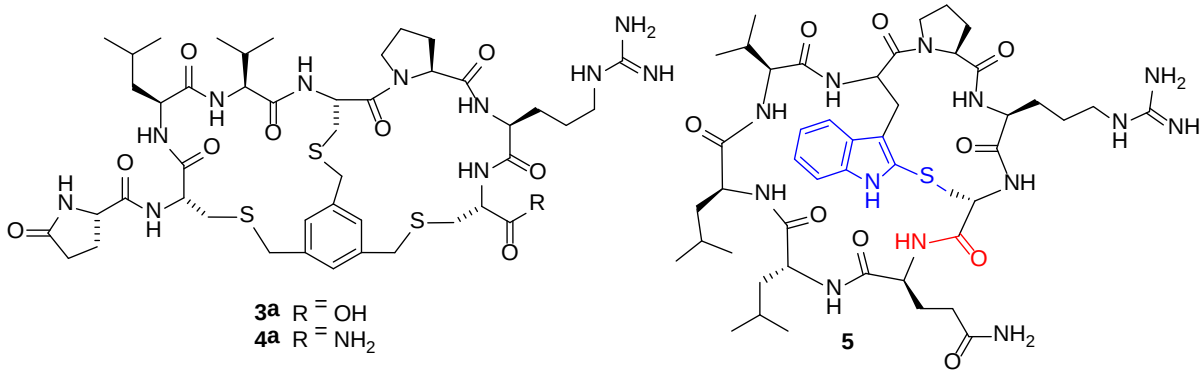
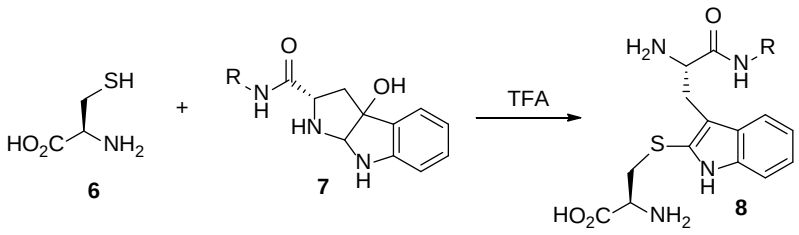


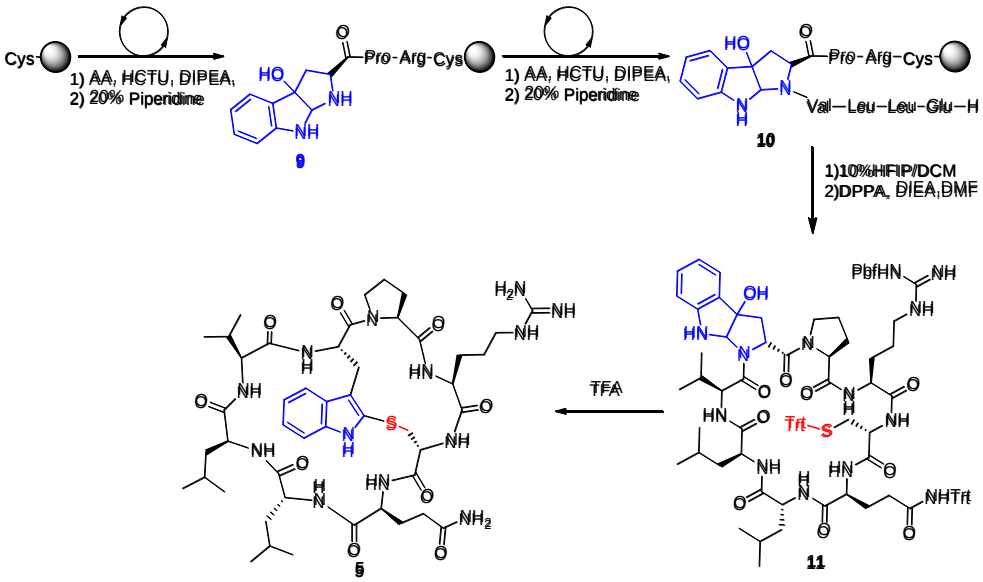
Figure 2: Celogentin C mimetics.

Several total syntheses of members of the phallotoxin/amatoxin familes have been reported,[29-30] with the two main methods employed for the construction of the tryptathionine Trp–Cys linkage being electrophilic aromatic substitution of the tryptophan residue with a cysteine-derived sulfenyl chloride,[26] or a Savige–Fontana reaction of cysteine and a Trp-derived hydroxypyrrolo[2,3-b]indole (Hpi) **7** under acidic conditions (Scheme 1). [30-31]



Scheme 1: Synthesis of tryptathionines

Accordingly, we sought to incorporate a Savige–Fontana reaction into the preparation of celogentin mimetic **5**. First, a Pro–Arg–Cys tripeptide sequence was assembled using standard SPPS protocols (Scheme 2). Fmoc-Hpi was prepared from Fmoc-Trp using the method of Perrin et al.,[25,30,32] then the Hpi residue was coupled to the tripeptide using COMU and Oxyma to give **9**. Extension of the tetrapeptide **9** to the octapeptide sequence **10** was completed using standard SPPS protocols.



Scheme 2: Synthesis of bicyclic peptide **9**

Cleavage of the peptide from the resin was achieved by treatment with hexafluoroisopropanol (HFIP), which furnished the peptide with side chain protecting groups intact. Head-to-tail macrocyclization was then performed using diphenyl phosphoryl azide (DPPA) in DMF/DIPEA to generate cyclic peptide **11**. Treatment of cyclic peptide **11** with TFA resulted in the removal of all side chain protecting group and subsequent Savige–Fontana reaction between the Cys and Hpi residues to generate the Trp–Cys cross-link in bicyclic peptide **5**, which was isolated in 10% overall yield from starting resin (Scheme 2).

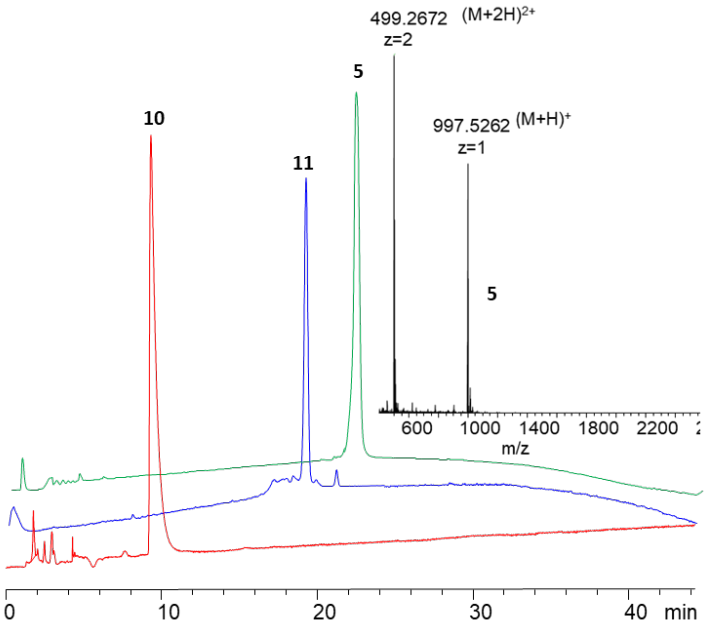
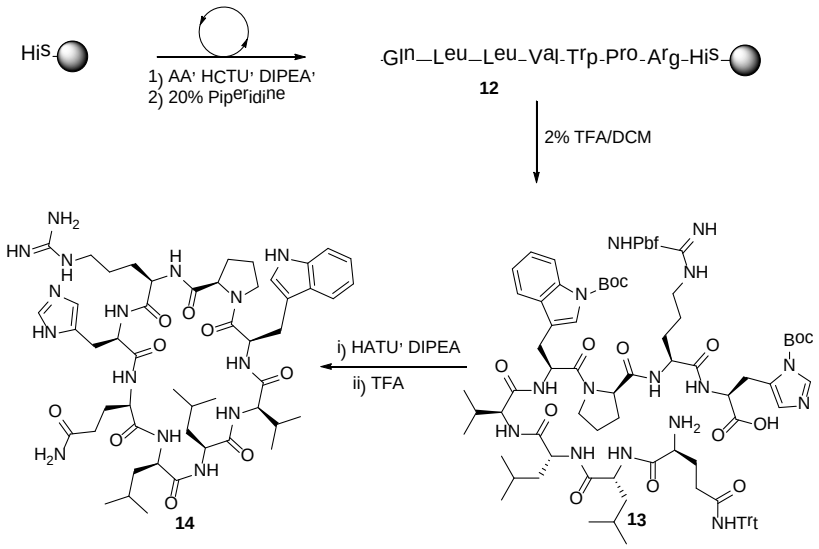


Figure 3: HPLC trace of crude linear peptide **10**, crude monocyclic peptide **11**, and purified bicyclic peptide **5**.

Bicyclic peptide **5** was characterized by LCMS (Figure 3), with a peak at *m/z* 997.5 indicating a 567-Dalton mass loss from monocyclic peptide **11** confirming the loss of the Trt and Pbf groups and water, indicating deprotection of the protecting groups and dehydration through a Savige–Fontana reaction to generate the bicyclic peptide **5**.



Scheme 3: Synthesis of head-to-tail cyclic peptide.

Lastly, a head-to-tail monocyclic analogue, as well as linear analogues containing either C-terminal amide or acid, were synthesized as controls. The linear peptide **12** was synthesized on chlorotrityl resin then the peptide was cleaved with dilute TFA to give protected linear peptide **13** (Scheme 3). Cyclization using a standard HATU coupling protocol generated the cyclic peptide **14**. Notably, cyclization was accompanied by epimerization (40%) and formation of the cyclic dimer (20%); common issues observed during peptide cyclization.[33-34] Recently, we have developed a novel Ag(I)-promoted head-to-tail cyclization method that generates cyclic peptides free of epimerization and cyclodimer.[35] Accordingly, peptide **14** was also synthesized in high yield using the Ag(I) promoted protocol. Linear peptide C-terminal acid **15** and C-terminal amide **16** were synthesized on chlorotrityl and Rink amide resins, respectively.

Tubulin polymerization inhibition

The celogentin analogues **3a**–**3f**, **4a**–**4g**, **5**, **14**–**16** were screened for potential anti-mitotic activity using a tubulin polymerization inhibition assay.[36-37] First, the effect of the peptides on tubulin polymerization was measured by a turbidity assay over 30 minutes (Figure 4A). The inhibition of tubulin polymerization was calculated for all compounds. Tubulin polymerization is enhanced in the presence of paclitaxel, which eliminates the nucleation phase and enhances the growth phase (Figure 4A, red). In contrast, anti-cancer drug vinblastine completely inhibits polymerization (Figure 4A, green). The thioether-linked bicyclic peptide **5** displayed moderate

inhibition of tubulin polymerization, as did the linear peptides **15** and **16**. Intriguingly, whilst most mesitylene-linked bicyclic peptides displayed negligible inhibition, peptides **3a** and **3b** exhibited potent inhibition of tubulin polymerization, with **3b** exhibiting near complete inhibition, similar to vinblastine (Figure 4B).

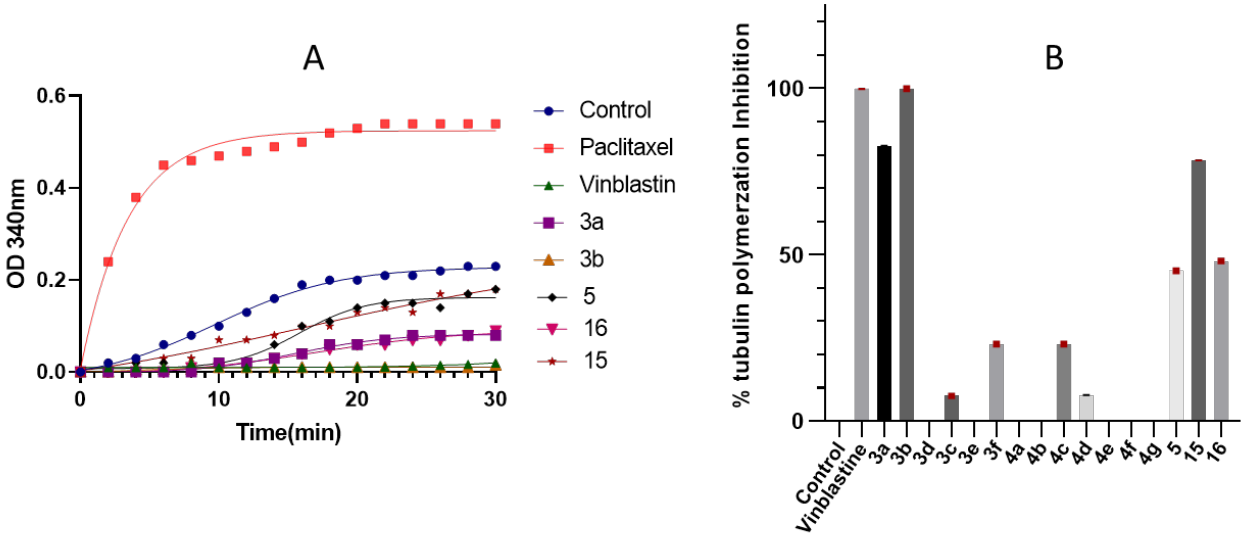


Figure 4. Inhibition of tubulin polymerization. A) polymerization of tubulin over 30 min. (measured by optical density). B) % inhibition of tubulin polymerization at 30 min relative to control

In order to determine the relative effectiveness of the various compounds, inhibition of tubulin polymerization was determined over a range of concentrations to determine IC_{50} values (Table 1). Compounds with little activity were assigned an IC_{50} of greater than the highest concentration tested. An alanine scan of the mesitylene-linked analogue **3a** shows that very few substitutions of the natural residues are tolerated, with Leu3, Val4 and Pro6 essential for activity. Intriguingly, when Arg7 was replaced with Ala (i.e. **3b**), inhibitory activity increased. Further C-terminal amides (i.e. **4a**–**g**) exhibited poor activity against tubulin polymerization. We speculate that negatively charged variants of these peptides exert greater inhibition of tubulin polymerization.

Table 1. Inhibition of tubulin polymerizationa) example of concentration curve b) table

Sequence	Compound	IC_{50} (μM) ± SD P value < 0.001
ZC*LVC*PRC*–OH	**3a**	45 ± 0.1
ZC*LVC*PAC*–OH	**3b**	2.2 ± 0.2
ZC*LVC*ARC*–OH	**3c**	>200
ZC*LAC*PRC*–OH	**3d**	>200
ZC*AVC*PRC*–OH	**3e**	>200
Ac–AC*LVC*PRC*–OH	**3f**	>200
ZC*LVC*PRC*–NH_2	**4a**	>200
ZC*LVC*PAC*–NH_2	**4b**	>200
ZC*LVC*ARC*–NH_2	**4c**	>200
ZC*LAC*PRC*–NH_2	**4d**	>200
ZC*AVC*PRC*–NH_2	**4e**	>200
AC*LVC*PRC*–NH_2	**4f**	>200
ZC*LVC*GRC*–NH_2	**4g**	>200
c(QLLVW$^{\#}$PRC$^{\#}$)	**5**	96 ± 0.5
c(QLLVWPRH)	**14**	99 ± 0.3
ZLLVWPRH–OH	**15**	6.2 ± 0.3
ZLLVWPRH–NH_2	**16**	50 ± 0.2

[a] Z = pyroglutamyl. [b] C* = Cys connected through 1,3,5- trismethylenylbenzene cross-link. [#] = thioether cross-link

Conclusion

In conclusion, we have synthesized a family of analogues of the peptide natural product, celogentin C, in which the cross-linked Leu–Trp–His core is replaced with a variety of non-natural cross-links: a 1,3,5-trisubstituted benzene moiety linked through three Cys residues, a Trp–Cys cross-link, a head-to-tail cyclization, or in which the sidechain–sidechain crosslinks have been omitted. Celogentin mimetic **3b** was shown to be a potent inhibitor of tubulin polymerization, exhibited greater activity against tubulin polymerization than the clinically used drug vinblastine.

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