Synthesis and in vitro bone cell activity of analogues of the cyclohexapeptide Dianthin G

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Supporting information
General methods

Materials

All reagents were acquired as reagent grade and used without further purification. Solvents for RP-HPLC were purchased as HPLC grade and used without further purification. 6-Chloro-1-hydroxybenzotriazole (6-Cl-HOBt) was purchased from Aapptec (Louisville, Kentucky). O-(Benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium hexafluorophosphate (HBTU), 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 2-chlorotrityl chloride resin, 4-(hydroxymethyl)phenoxyacetic acid (HMP linker), di-tert-butyl dicarbonate (Boc₂O), Fmoc-allylglycine-OH (Fmoc-Agl), Fmoc-Pro-OH, Fmoc-Leu-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, and Fmoc-Gly-OH were purchased from GL Biochem (Shanghai, China). N,N-Dimethylformamide (DMF) (AR grade), and acetonitrile (CH₃CN) [high-performance liquid chromatography (HPLC) grade] were purchased from Scharlau (Barcelona, Spain). N,N’-Diisopropylethylamine (iPr₂EtN), N,N’-diisopropylcarbodiimide (DIC), 2-mercaptetoethanol, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 2,4,6-trimethylpyridine (sym-collidine), Hoveyda-Grubbs’ II catalyst, 2,2,2-trifluoroethanol (TFE), dimethylsulfate (DMS), 2-nitrobenzenesulfonyl chloride (o-NBS-Cl), formic acid, 1-methyl-2-pyrrolidinone (NMP), and piperidine were purchased from Sigma–Aldrich (Sydney, Australia). Dichloromethane (CH₂Cl₂) was purchased from ECP Limited (Auckland, New Zealand). Triisopropylsilane (iPr₃SiH) was purchased from Alfa Aesar (Lankashire, U.K). Dimethyl sulfoxide-d₆ (DMSO-d₆) was purchased from Cambridge Isotope Laboratories (Massachusetts, USA). Trifluoroacetic acid (TFA) was purchased from Halocarbon (New Jersey, USA). Dimethyl sulfoxide (DMSO) was purchased from Romil Ltd (Cambridge, UK). Fmoc-Gly-O-CH₂-Phi-OCH₂-CH₂-COOH (Fmoc-Gly-HMPP) was purchased from PolyPeptide Laboratories Group (Strasbourg, France). Aminomethyl polystyrene resin was purchased from Rapp Polymere (Tuebingen, Germany).

HPLC, MS and NMR

Analytical RP-HPLC spectra were performed on a Dionex (California, USA) Ultimate 3000 System equipped with a two-channel UV detector using an analytical column
(XTerra® MS C₁₈ column, 4.6 mm x 150 mm, 5 µm) and a linear gradient of 5% to 75%B over 35 mins (ca. 2%B per minute) at a flow rate of 1 mL min⁻¹. The solvent system used was A (0.1% trifluoroacetic acid in H₂O) and B (0.1% trifluoroacetic acid in CH₃CN). Peptide masses were confirmed by a Bruker microTOF-Q II mass spectrometer (Bremen, Germany) or a Hewlett Packard (HP) 1100 series mass spectrometer (California, USA) using direct flow injection at 0.3 mL min⁻¹ into an ESI source in the positive mode.

Peptides were purified using a Waters (Massachusetts, USA) S600E system using a semi-preparative column (Waters XTerra® C₁₈, 300 mm x 19 mm, 10 µm) at a flow rate of 10 mL min⁻¹ and eluted using a one-step slow gradient protocol with detection at 210 nm. Fractions were collected, analysed by analytical RP-HPLC or ESI-MS, pooled and lyophilised three times from 10 mM aq HCl.

Nuclear magnetic resonance spectra were recorded on a Bruker AVANCE 600 spectrometer (Bremen, Germany) using DMSO-d₆ as a solvent. Assignments were made with the aid of HSQC, TOCSY, and NOESY experiments.

**Peptide synthesis**

**Loading of the C-terminal amino acid to the resin**

For peptides 1-5:

A solution of Fmoc-Gly-O-CH₂-phi-OCH₂-CH₂-COOH (190.2 mg, 0.4 mmol) and DIC (62 µL, 0.4 mmol) in CH₂Cl₂/DMF (ν/ν; 2:1, 3 mL) was added to pre-swollen (CH₂Cl₂, 3 mL, 20 min) aminomethyl polystyrene resin (220.0 mg, 0.2 mmol) and the mixture gently agitated for 5 h, at room temperature, filtered and washed with DMF (4 x 3 mL). A negative Kaiser test³ confirmed the coupling.

For peptide 6:

A solution of Fmoc-Gly-OH (119.0 mg, 0.4 mmol) and iPr₂EtN (70 µL, 0.4 mmol) in CH₂Cl₂/DMF (ν/ν; 2:1, 3 mL) was added to pre-swollen (CH₂Cl₂, 3 mL, 20 min) 2-CITtrtCl resin (150.0 mg, 0.2 mmol) and the mixture was shaken for 1 h at room temperature, filtered, and washed with DMF (4 x 3 mL).

For peptides 7, 8, 9, and 10:

A solution of HMP linker (109.3 mg, 0.6 mmol) and DIC (93 µL, 0.6 mmol) in CH₂Cl₂/DMF (ν/ν; 2:1, 3 mL) was added to pre-swollen (CH₂Cl₂, 3 mL, 20 min)
aminomethyl polystyrene resin (220.0 mg, 0.2 mmol) and the mixture was shaken for 5 h at room temperature, filtered, and washed with DMF (4 x 3 mL). A negative Kaiser test\(^3\) confirmed the coupling.

For peptides 7, 8, and 9, a mixture of Fmoc-allylglycine (202.4 mg, 0.6 mmol), DIC (93 µL, 0.6 mmol) and DMAP (2.44 mg, 0.02 mmol) in DMF (3 mL) was then added to the resin and the reaction mixture was shaken for 1 h at room temperature. This procedure was repeated once with fresh reagents. For peptide 10, a mixture of the Fmoc-Gly-OH (178.4 mg, 0.6 mmol), DIC (93 µL, 0.6 mmol) and DMAP (2.44 mg, 0.02 mmol) in DMF (3 mL) was then added to the resin and the reaction mixture was shaken for 1 h at room temperature (repeated once).

**Elongation of the peptide sequence**

Extension of the C-terminal amino acid on the resin was performed at room temperature using manual Fmoc-SPPS. Deprotection of the Fmoc group was accomplished using 20% v/v piperidine/DMF (3 mL) for 5 min twice with consecutive DMF washes after each addition. A solution of Fmoc-amino acid (0.8 mmol), HATU (281.4 mg, 0.74 mmol), iPr\(_2\)EtN (278.7 µL, 1.6 mmol) in DMF (3 mL) was then added to peptidyl-resin and the mixture was shaken at room temperature for 45 min, then filtered and washed with DMF (4 x 3 mL).

Following on-resin elongation of the linear precursor of RCM peptides (peptidyl-resin 18, 19, and 22), Boc\(_2\)O (436.5 mg, 2.0 mmol) in DMF (3 mL) was added to the peptidyl-resin and the mixture was shaken at room temperature for 2 h, filtered, and washed with DMF (4 X 3 mL), CH\(_2\)Cl\(_2\) (4 x 3 mL), and dried under vacuum.

**Peptide cleavage and isolation**

The resulting peptides were released from the resin with concomitant removal of the threonine side chain protecting group by treatment with TFA/iPr\(_3\)SiH/H\(_2\)O (v/v/v; 95:2.5:2.5, 5 mL) at room temperature for 3 h. The resin was removed by filtration, washed with TFA (2 x 3 mL) and the combined filtrates were concentrated, resuspended with H\(_2\)O/CH\(_3\)CN (v/v; 1:1) and lyophilised.
Peptide cyclisation

Cyclisation was carried out in solution using the pseudo-high dilution conditions described by Brimble et al.⁴ Peptide 2 is used here as an example of the synthetic procedure followed for macrolactamisation. To a stirring solution of iPr₂EtN (139.4 μL, 0.8 mmol) in CH₂Cl₂ (150 mL) was added a mixture of the linear peptide (105.0 mg, 0.16 mmol, 0.8 mM), HBTU (182.0 mg, 0.48 mmol), and 6-Cl-HOBt (81.40 mg, 0.48 mmol) in CH₂Cl₂/DMF (v/v; 9:1, 50 mL) dropwise at a rate of 2.0 mL h⁻¹. After complete addition of the reagents, the reaction mixture was concentrated under reduced pressure, diluted with H₂O (15 mL) and lyophilised. The crude peptide was purified by RP-HPLC to yield peptide 2 as a white fluffy solid (49.0 mg, 38% overall yield, 98% purity).
A. Dianthin G (1)

Dianthin G (1) was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol), which following the cleavage conditions in the general procedure afforded the crude linear precursor as a white solid (110 mg, 85% yield). The crude linear precursor (110 mg, 0.17 mmol) was cyclised using solution-phase macro lactamisation as outlined in the general methods section. RP-HPLC purification afforded peptide 1 as a white fluffy solid (65.4 mg, 52% overall yield, 99% purity); Rf 20.40 min; m/z (HR-MS) 629.3663 ([M+H]+ requires for C₃₂H₄₉N₆O₇: 629.3584).

![Dianthin G (1)](image)

**Figure S1.** Analytical RP-HPLC and HR-MS data of dianthin G (1).
B. Peptide 2

Peptide 2 was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol), which following the cleavage conditions in the general procedure afforded the crude linear precursor (105 mg, 79% yield). The crude linear precursor (105 mg, 0.16 mmol) was cyclised using solution-phase macrolactamisation as outlined in the general methods section. RP-HPLC purification afforded peptide 2 as a white fluffy solid (49.0 mg, 38% overall yield, 98% purity); \( R_t \) 21.56 min; \( m/z \) (HR-MS) 643.3815 ([M+H]+ requires for \( C_{33}H_{51}N_6O_7 \): 643.3741).

![Peptide 2 structure](image1)

**Figure S2.** Analytical RP-HPLC and HR-MS data of peptide 2.
C. Peptide 3

Peptide 3 was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol), which following the cleavage conditions in the general procedure afforded the crude linear precursor (67.4 mg, 51% yield). The crude linear precursor (67.4 mg, 0.1 mmol) was cyclised using solution-phase macrolactamisation as outlined in the general methods section. RP-HPLC purification afforded peptide 3 as a white fluffy solid (28.3 mg, 22% overall yield, 97% purity); $R_t$ 23.17 min; $m/z$ (HR-MS) 643.3820 ([M+H]$^+$ requires for C$_{33}$H$_{51}$N$_6$O$_7$: 643.3741).

![Peptide 3 structure](image)

**Figure S3.** Analytical RP-HPLC and HR-MS data of peptide 3.
Peptide 4 was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol), which following the cleavage conditions in the general procedure afforded the crude linear precursor (95.2 mg, 72% yield). The crude linear precursor (95.2 mg, 0.14 mmol) was cyclised using solution-phase macrolactamisation as outlined in the general methods section. RP-HPLC purification afforded peptide 4 as a white fluffy solid (65.6 mg, 51% overall yield, 99% purity); R<sub>t</sub> 20.78 min; m/z (HR-MS) 643.3796 ([M+H]<sup>+</sup> requires for C<sub>33</sub>H<sub>51</sub>N<sub>6</sub>O<sub>7</sub>: 643.3741).

**Figure S4.** Analytical RP-HPLC and HR-MS data of peptide 4.
E. Peptide 5

Peptide 5 was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol), which following the cleavage conditions in the general procedure afforded the crude linear precursor (84.6 mg, 64% yield). The crude linear precursor (84.6 mg, 0.13 mmol) was cyclised using solution-phase macrolactamisation as outlined in the general methods section. RP-HPLC purification afforded peptide 5 as a white fluffy solid (55.3 mg, 43% overall yield, 98% purity); $R_t$ 20.80 min; $m/z$ (HR-MS) 643.3815 ([M+H]$^+$ requires for $C_{33}H_{51}N_6O_7$: 643.3741).

![Peptide structure](image)

**Figure S5.** Analytical RP-HPLC and HR-MS data of peptide 5.
F. Peptide 6

Peptide 6 was synthesised using manual Fmoc-SPPS on a 2-chlorotrityl chloride resin (0.2 mmol), which following the cleavage conditions in the general procedure afforded the crude linear precursor (80.6 mg, 61% yield). The crude linear precursor (80.6 mg, 0.12 mmol) was cyclised using solution-phase macrolactamisation as outlined in the general methods section. RP-HPLC purification afforded peptide 6 as a white fluffy solid (50.1 mg, 39% overall yield, 98% purity); $R_t$ 22.09 min; $m/z$ (HR-MS) 643.3803 ([M+H]$^+$ requires for C$_{33}$H$_{51}$N$_6$O$_7$: 643.3741).

![Peptide 6 structure]

**Figure S6.** Analytical RP-HPLC and HR-MS data of peptide 6.
G. Peptide 7

The peptidyl-resin 18 was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol) and the peptidyl-resin (67.2 mg, 32.5 x 10^{-3} mmol) was subjected to microwave-assisted ring closing metathesis as outlined in the general methods section. Following cleavage from the resin, RP-HPLC purification afforded peptide 7 as a white fluffy solid (4.84 mg, 16% yield from resin-bound peptide 18, 99% purity); $R_t$ 18.75 min; $m/z$ (HR-MS) 756.4262 ([M+H]^+ requires for C_{38}H_{58}N_{7}O_{9}: 756.4218).

![Peptide 7 structure](image)

**Figure S7.** Analytical RP-HPLC and HR-MS data of peptide 7.
H. Peptide 8

The peptidyl-resin 19 was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol) and the peptidyl-resin (66.5 mg, $3.25 \times 10^{-3}$ mmol) was subjected to microwave-assisted ring closing metathesis as outlined in the general methods section. Following cleavage from the resin, RP-HPLC purification afforded peptide 8 as a white fluffy solid (6.55 mg, 25% yield from resin-bound peptide 19, 98% purity); $R_t$ 17.6 min; $m/z$ (HR-MS) 655.3810 ([M+H]$^+$ requires for $C_{34}H_{51}N_{6}O_{7}$: 655.4218).

![Peptide Structure]

**Figure S8.** Analytical RP-HPLC and HR-MS data of peptide 8.
I. Peptide 9

Peptide 8 (6.0 mg, 9.2 μmol) was cyclised in solution, as outlined in the general methods, to yield the crude peptide 9. RP-HPLC purification afforded peptide 9 as a white fluffy solid (4.07 mg, 68% yield from peptide 8 and 16% overall yield, 99% purity); Rₜ 21.80 min; m/z (HR-MS) 637.3706 ([M+H]⁺ requires for C₃₄H₄₉N₄O₆: 637.3605).

Figure S9. Analytical RP-HPLC and HR-MS data of peptide 9.
J. Peptide 10

The peptidyl-resin 22 was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol) and the peptidyl-resin (56.2 mg, 32.5 x 10⁻³ mmol) was subjected to microwave-assisted ring closing metathesis as outlined in the general methods section. Following cleavage from the resin, RP-HPLC purification afforded peptide 10 as a white fluffy solid (4.52 mg, 20% yield from resin-bound peptide 22, 99% purity); \( R_t \) 13.00 min; \( m/z \) (HR-MS) 565.3346 ([M+H]⁺ requires for \( C_{27}H_{45}N_{6}O_{7} \): 565.3271).

Figure S10. Analytical RP-HPLC and HR-MS data of peptide 10.
K. Peptide 23

Peptide 23 was synthesised using manual Fmoc-SPPS on an aminomethyl polystyrene resin (0.2 mmol), which following the cleavage conditions in the general procedure afforded the crude linear precursor as a white solid (77.0 mg, 65% yield). The crude linear precursor (77.0 mg, 0.13 mmol) was cyclised using solution-phase macrolactamisation as outlined in the general methods section. RP-HPLC purification afforded peptide 23 as a white fluffy solid (45.98 mg, 40% overall yield, 96% purity); Rt 21.20 min; m/z (HR-MS) 575.3547 ([M+H]+ requires for C_{29}H_{47}N_{6}O_{6}: 575.3479).

**Figure S11.** Analytical RP-HPLC and HR-MS data of peptide 23.
**Figure S12.** Partial $^1$H NMR spectrum of 7 in DMSO-d$_6$ at 298K.

**Figure S13.** Partial 600 MHz TOCSY spectrum of 7 in DMSO-d$_6$ at 298K.
Figure S14. Amide and alkene regions of the $^1$H NMR spectra of (A) dicarba analogue 8 at 298 K with a 2:1 ratio of major:minor signals for the olefinic hydrogens, (B) bicyclic analogue 9 at 298 K showing the presence of only one diastereomer.
**Figure S15.** Partial 600 MHz TOCSY spectrum of 8 in DMSO-d6 at 298K.
Figure S16. Selected 600 MHz NOESY spectra of the dicarba analogue 8 showing the olefinic-H connectivities with the cis-isomer isomer exhibiting strong nOe.
**Figure S17.** Partial $^1$H NMR spectrum of 10 in DMSO-d6 at 298K.

**Figure S18.** Partial 600 MHz TOCSY spectrum of 10 in DMSO-d6 at 298K.
Figure S19. Effects of dianthin G (1) (10⁻⁸ M) and the dicarba analogue 8 (10⁻⁸ M) in primary human osteoblasts on cell differentiation, assessed by percentage of mineralisation. Data are expressed as a ratio of treatment to control, mean ± SEM from a representative experiment. Dexamethasone [Dex] (10⁻⁸ M) is included as a positive control. ****significantly different from control (P < 0.0001).

Circular dichroism spectroscopy
All CD spectra were recorded using a Pi Star-180 (Applied Photophysics, Surrey, UK) spectrometer at 20 °C with a cell of 0.1 cm path length in the range from 190 nm to 300 nm at 0.5 nm intervals with a 5 s response time. Each CD spectrum measurement represents the average of four scans obtained with a 2 nm optical bandwidth. Baseline spectrum was collected with the solvent alone (30% TFE in water) and then subtracted from the raw peptide spectra. The measurements were performed at peptide concentrations of 86 μM in 30% TFE in water, in 1 mm quartz cuvettes (Hellma Analytics, Mullheim, Germany). Data are expressed as mean residue ellipticities [θ] in (deg cm² dmol⁻¹), calculated as follows:

\[ \theta = \frac{S}{(10 \times c \times L \times n)} \]

Where S is the raw CD signal in millidegrees, c is the peptide concentration (M), L is the cuvette path length (cm), and n is the number of peptide bonds.

References