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How to blast osteoblasts? Novel dicarba analogues of amylin-(1–8) to treat osteoporosis

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ABSTRACT

When administered in vivo, amylin (1–8) stimulates osteoblast proliferation increasing bone volume and bone strength. The native cyclic octapeptide amylin (1–8) is unstable, however, it provides an attractive framework for the creation of more stable, orally active synthetic analogues using various peptidomimetic techniques. On-resin ring closing metathesis (RCM) on the olefinic side chains of allylglycine residues and lysine moieties functionalized with an allyloxycarbonyl (Alloc) group, was used to prepare novel carba-bridged surrogates of the disulfide bridge between Cys/2 and Cys/7 in amylin-(1–8). Commercially available N^{α}-Fmoc N^{ε}-Alloc protected lysine was used as a convenient substrate for Grubbs' ring closing metathesis. Analogues of amylin-(1–8) prepared by cyclization of allylglycine residues that also contained proline residues at either position 4 or 6, or both, were also prepared to investigate the effect of proline as a 'kink-inducing' residue on the efficiency of the RCM reaction. Of the nine novel alkene-bridged analogues prepared, five showed promising biological activity in a proliferation study in primary foetal rat osteoblasts at physiological concentrations. Two of these analogues were chosen for further in vivo evaluation.

1. Introduction

Osteoporosis is a bone disease where bone loss occurs due to increased action of the bone resorbing cells (osteoclasts) over the action of bone forming cells (osteoblasts). Most of the current therapies to treat osteoporosis are antiresorptive and inhibit further bone loss by reducing osteoclast activity.[1] Preventive therapies that stimulate bone formation are still limited. The only therapy currently used that increases bone formation, is recombinant parathyroid hormone (PTH). However, PTH therapy is limited due to high cost, the requirement for daily self-injection and risk of development of osteosarcoma.[1,2] A new class of anabolic drug for the treatment of osteoporosis is therefore in great demand.

As we reported previously[3] a peptide hormone amylin-(1–37) which is co-secreted with insulin from the beta-pancreatic cells,[4] is active in fuel metabolism and also in bone.[5] It stimulates osteoblast proliferation and inhibits osteoclast action as shown by in vitro and in vivo studies.[5,6] We have identified an N-terminal octapeptide derived from amylin-(1–37), namely amylin-(1–8), as a promising but unstable candidate for therapeutic development due to its short sequence, lack of effect on fuel metabolism, and its retained anabolic effects on osteoblasts.[7] Both amylin-(1–37) and amylin-(1–8) contain a disulfide bond (Cys/2 and Cys/7) that may be an essential structural feature for osteoblast proliferation (Fig. 1). Since disulfide bridges are labile under certain chemical and enzymatic conditions, we investigated whether a disulfide bond replacement using peptidomimetic techniques would improve stability and/or the potency profile of amylin-(1–8). In our previous studies various chemical techniques for bridging Cys/2 and Cys/7 of amylin-(1–8), such as S-alkylation using maleimide, an aromatic ring or an alkyl chain, thiol-Michael addition and 1,3-dipolar cycloaddition ('click' reaction) were evaluated.[3] We identified one active analogue containing a *para*-xylyl linker as a substitute for the native disulfide bridge of amylin-(1–8). Based on this preliminary study, we envisaged that disulfide bond replacement by stable carba-bridges using the well established Grubbs' ring closing metathesis (RCM),[8,9] would afford analogues with improved stability and/or activity compared to native amylin-(1–8).

RCM has already proven to be a valuable tool in the peptidomimetics field and has been successfully used to prepare disulfide and thioether bond mimetics, to stabilize the spatial conformation of peptides, as a hydrogen bond surrogate (HBS), as a proline conformation mimetic or to generate cyclic peptides.[10–12] Moreover, it

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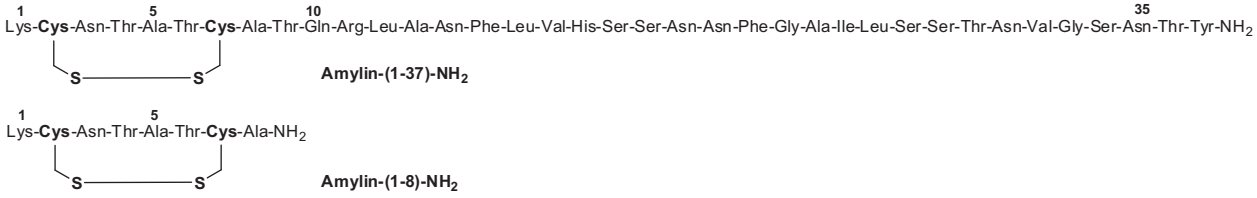


Figure 1. Primary structure of amylin-(1–37) and N-terminal octapeptide fragment amylin-(1–8).

has also been established that disulfide bond replacement by a hydrocarbon bridge can improve peptide potency and protease resistance.[13–16]

We therefore herein report the synthesis and biological evaluation of nine cyclic analogues of amylin-(1–8) prepared using RCM (Fig. 2). On-resin ruthenium-catalysed RCM was used to replace the native disulfide bond of amylin-(1–8) (Cys/2 and Cys/7) thus generating carba-based macrocycles (**1**–**5**). Allylglycine (Agl) or lysine (Alloc) residues were incorporated into the linear peptide precursors as replacements of Cys/2 and Cys/7 and used as bridging points to afford 20-membered macrocycles (**1–4**) and 34-membered macrocycles (**5a**, **5b**), respectively. In order to probe the conformational effect of the linear allylglycine-containing amylin-(1–8) derivative on the efficiency of peptide cyclization using RCM, a kink-inducing proline residue was also substituted in place of either Thr/4 or Thr/6, or both (analogues **2**–**4**) (Fig. 2).

2. Results and discussion

2.1. Synthesis of amylin-(1–8) peptide bridged analogues 1a and 1b by RCM of Agl residues at position 2 and 7

In order to prepare cyclic RCM analogues of amylin-(1–8) **1a** and **1b** (Fig. 2) initial synthesis of the linear precursor **7** containing

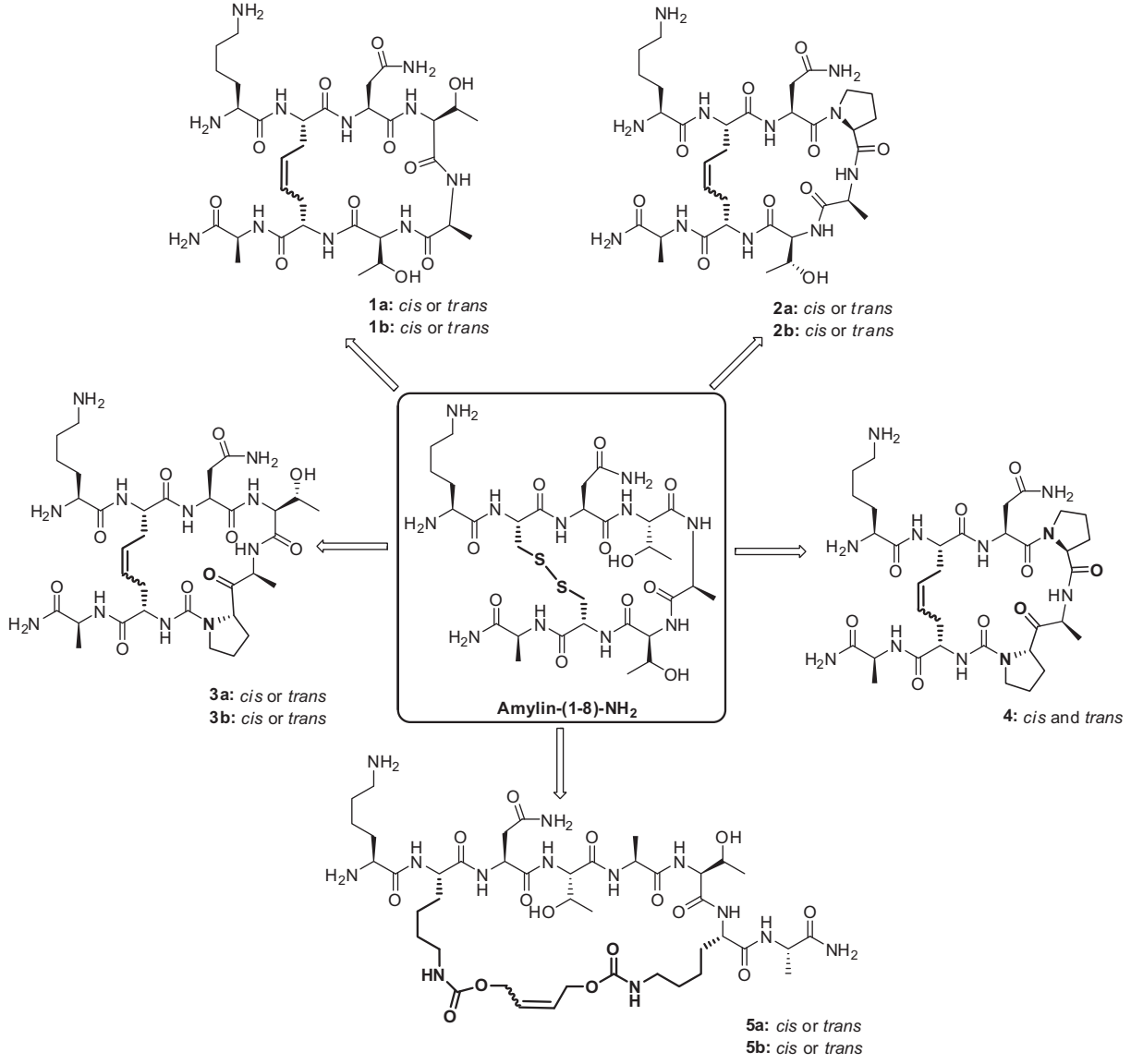


Figure 2. Chemical structures of the RCM analogues of amylin-(1–8) **1–5** synthesized.

allylglycine residues at Cys/2 and Cys/7 was required as summarized in Scheme 1. As the ruthenium metal may complex to polar sites present on the peptide chain, side chain protection and masking of the carboxylate and N^{α}-terminal group of the peptide was required for successful RCM.[17,18] It was therefore decided to perform ruthenium-catalysed cyclization on-resin.

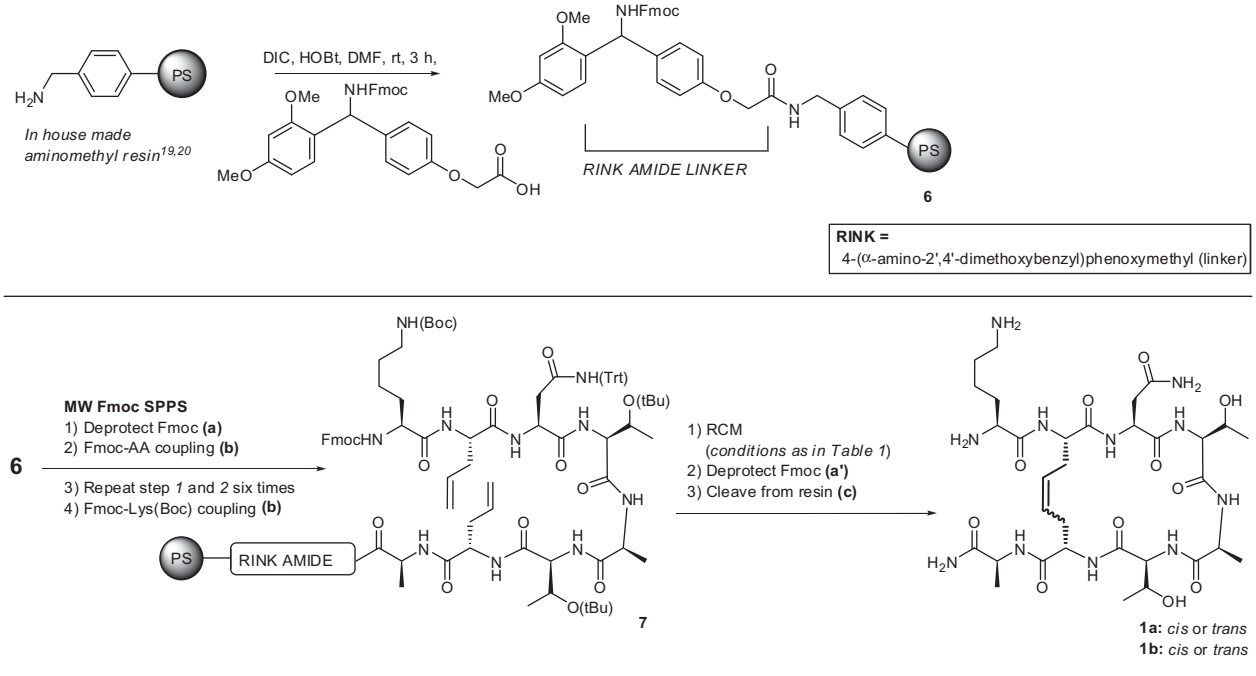
The resin-bound linear peptide **7** was initially synthesised by microwave enhanced (MW) Fmoc solid phase peptide synthesis (SPPS) using aminomethyl polystyrene resin bearing a Rink amide linker (**6**) (Scheme 1).[19,20] Repeated cycles of Fmoc-AA coupling (HBTU) and Fmoc protecting group removal (20% piperidine in DMF) afforded Fmoc-Lys(Boc)-Agl-Asn(Trt)-Thr(*t*Bu)-Ala-Thr(*t*Bu)-Agl-Ala-RINK-PS peptidyl resin (**7**) (see Fig. S1).

Factors such as the length, primary sequence and secondary structure of the metathesis precursor defines the spatial location of the alkene bridging points which subsequently impacts on the efficiency of macrocycle formation.[18,21–24] In the present work use of microwave heating[25,26] using either Grubbs' II or Hoveyda–Grubbs' II catalysts enabled successful RCM after optimization of reaction conditions (Table 1). After macrocyclization, the peptidyl resin was treated with DMSO to remove ruthenium-containing by-products,[27–29] the Fmoc protecting group removed and the peptide cleaved from the resin. The crude material was analysed using RP-HPLC and ESI-MS. Product formation is reported as ratio (%) of cyclic RCM product to corresponding linear and fully deprotected RCM precursor.

Initially the desired macrocyclic product was afforded as a mixture of *cis* and *trans* isomers **1a** and **1b** (56% yield) by treatment of **7** with 10 mol % of Grubbs' II catalyst in CH_2Cl_2/DMF (4:1) under MW heating for 2 h (entry 1, Table 1). RP-HPLC purification enabled separation of the individual novel *cis*/*trans* RCM analogues of amylin-(1–8) **1a** ($R_{tcis\ or\ trans}$ 7.00 min) as a major isomer and **1b** ($R_{tcis\ or\ trans}$ 7.54 min) as a minor isomer. NMR studies could not unequivocally assign the *cis*/*trans* stereochemistry to the individual products **1a** and **1b** and this was the case for all RCM products prepared in the present study. Increasing the catalyst loading to 30 mol %, together with prolonged reaction time (6 h) (entry 3, Table 1) resulted in improved conversion to alkenes **1a** and **1b** (ca. 68%) however, a substantial improvement to 87% (Fig. S2) was observed by increasing the catalyst concentration from 0.62 mM to 1.70 mM with a reaction time of 2 h (entry 4, Table 1). Use of dichlorobenzene[25] as the solvent for microwave enhanced RCM was ineffective in this case (entries 5 and 6, Table 1). or 2 h (entry 6, Table 1).

Use of 10 mol % and 30 mol % of Hoveyda–Grubbs' II in CH_2Cl_2/DMF (4:1) (entries 7 and 8, Table 1) did not offer any significant advantage. It was therefore established that use of 30 mol % Grubbs' II catalyst in CH_2Cl_2/DMF (4:1) under MW enhanced heating at 100 °C for 2 h was most effective to prepare carba-bridged analogues **1a** and **1b** of amylin-(1–8) (entry 4, Table 1). Chapman and Arora[25] demonstrated that the Grubbs' II catalyst performs best at 120 °C while Hoveyda–Grubbs' II is best at 200 °C. For this reason it was decided to use 30 mol % Grubbs' II at the lower temperature for the synthesis of additional analogues due to concerns about peptide degradation at a higher temperature.

Full conversion to the resin-bound precursor **7** to the macrocyclic carba-bridged *cis* and *trans* alkenes **1a** and **1b** could not be effected. Use of chaotropic salts, such as lithium chloride, to disrupt potential aggregates forming during RCM[17] which may prevent macrocyclization, was examined but no beneficial effect was observed. Incomplete conversion of metathesis precursor **7** to macrocycles **1a** and **1b** may be due to the fact that the Fmoc group masking the N^{α} amino group of Lys/1 was deprotected by *N*,*N*-dimethylamine resulting from decomposition of DMF at the elevated temperature.[30] In order to avoid undesired removal of the Fmoc protecting group during RCM it was henceforth decided to use a *tert*-butyloxycarbonyl protecting group (Boc) to mask the ter-



Reagents and conditions:
(a) 20% piperidine, DMF, 62 W, 75 °C, 1 x 0.5 min + 1 x 3 min; **(a')** 20% piperidine, DMF, rt, 1 x 5 min + 1 x 15 min; **(b)** HBTU, iPr$_2$EtN, DMF, 25 W, 73 °C, 5 min; **(c)** TFA, iPr$_3$SiH, H_2O, rt, 2 h.

Scheme 1. Synthesis of RCM analogues of amylin-(1–8) **1a** and **1b**.

Table 1
Conditions tested for the synthesis of RCM analogues **1a** and **1b** starting from fully protected RCM precursor 7

Entry	Catalyst loading	Catalyst concentration (mM)	Solvent	Conditions	ca. % by RP-HPLC at 210 nm	
					Cyclic **1a** and **1b**	Linear **7**
1	Grubbs' II 10 mol %	0.40	CH_2Cl_2/DMF 4: 1	MW, 100 °C, 2 h	56% (**1a**: 45% + **1b**: 11%)	31%
2	Grubbs' II 20 mol %	0.38	CH_2Cl_2/DMF 4:1	MW, 100 °C, 2 h	55% (**1a**: 47% + **1b**: 8%)	40%
3	Grubbs' II 30 mol %	0.62	CH_2Cl_2/DMF 4:1	MW, 100 °C, 6 h	68% (**1a**: 57% + **1b**: 11%)	25%
4	**Grubbs' II 30 mol %**	**1.70**	**CH_2Cl_2/DMF 4: 1**	**MW, 100 °C, 2 h**	**87% (1a: 67% + 1b: 20%)**	**10%**
5	Grubbs' II 20 mol %	0.35	Dichlorobenzene	MW, 120 °C, 3 min	No reaction	
6	Grubbs' II 20 mol %	0.36	Dichlorobenzene	MW, 100 °C, 2 h	No reaction	
7	Hoveyda–Grubbs' II 10 mol %	0.40	CH_2Cl_2/DMF 4:1	MW, 100 °C, 2 h	56% (**1a**: 42% + **1b**: 14%)	36%
8	Hoveyda–Grubbs' II 30 mol %	1.21	CH_2Cl_2/DMF 4:1	MW, 100 °C, 2 h	81% (**1a**: 61% + **1b**: 20%	8%

minal N^{α} amino group for subsequent generation of further analogues **2**–**5**.[31,32]

2.2. Effect of Pro residues on RCM of Agl residues in amylin-(1–8) to prepare peptide analogues 2–4

Introduction of turn-inducing residues,[33] such as pseudoprolines (ψPro),[34] to perform N to C peptide macrocyclization significantly improves the efficacy of the ring forming step when it does not proceed readily on the native linear peptide.[21,31] We were interested to see whether introduction of pseudo-proline residues[34] into the amylin-(1–8) peptide sequence would further improve the ruthenium-catalysed macrocycle formation step. Amylin-(1–8) conveniently contains Thr/4 and Thr/6 residues that could be substituted by the commercially available Thr($\psi^{Me,Me}$pro) moiety affording a native threonine residue upon TFA mediated peptide cleavage from the resin. Initially, it was decided to use a proline residue rather than Thr($\psi^{Me,Me}$pro) due to the high cost of this latter building block.

RCM precursors (**12–14**) were prepared containing a Boc protected N^{α}-terminal amino group, allylglycine residues incorporated in place of Cys/2 and Cys/7 in amylin-(1–8) and proline residues replacing either Thr/4 or Thr/6, or both, resulting in resin-bound peptides, **12**, **13** and **14**, respectively (Scheme 2). Fmoc-protected resin-bound peptides **9–11** were prepared using the same conditions as described above for the preparation of **7** (Figs. S5, S9 and S13, respectively). Interestingly, even though no explicit final Fmoc deprotection step had been performed, partial Fmoc deprotection was observed during the microwave enhanced SPPS as evidenced by the presence of deprotected product **15** and **17** (Figs. S5 and S13). However, Fmoc group removal was not observed for analogue **10**. This was not problematic as subsequent removal of the Fmoc protecting group of the N^{α} amino group of the Lys/1 residue was planned in order to exchange it for a Boc protecting group.

Manual deprotection of the remaining Fmoc group on the resin-bound peptides **9**, **10**, and **11** was undertaken followed by on-resin Boc reprotection affording the fully protected resin-bound macrocyclic peptide precursors **12**, **13** and **14** (Scheme 2).[35] Subsequent RCM cyclization of **12**, **13** and **14** using the previously optimised conditions was next carried out followed by treatment of the peptidyl-resins with DMSO and subsequent peptide cleavage from the resin (Table 2).

Excellent conversion to cyclised products **2a** and **2b** (Fig. S6), and **3a** and **3b** (Fig. S10) as a mixture of *cis* and *trans* isomers was observed. Interestingly, RP-HPLC analysis of the crude RCM product **4** with Pro residues incorporated in place of both Thr/4 and Thr/6, revealed unusual peak broadening which was not observed for RCM analogues where the proline residue was located only at Thr/4 (**2a**, **2b**) or at Thr/6 (**3a**, **3b**) (Fig. S14). Peak broadening for RCM products might be a consequence of spatial conformation restriction generated by the newly formed alkene bridge as previously reported by Robinson and co-workers.[22]

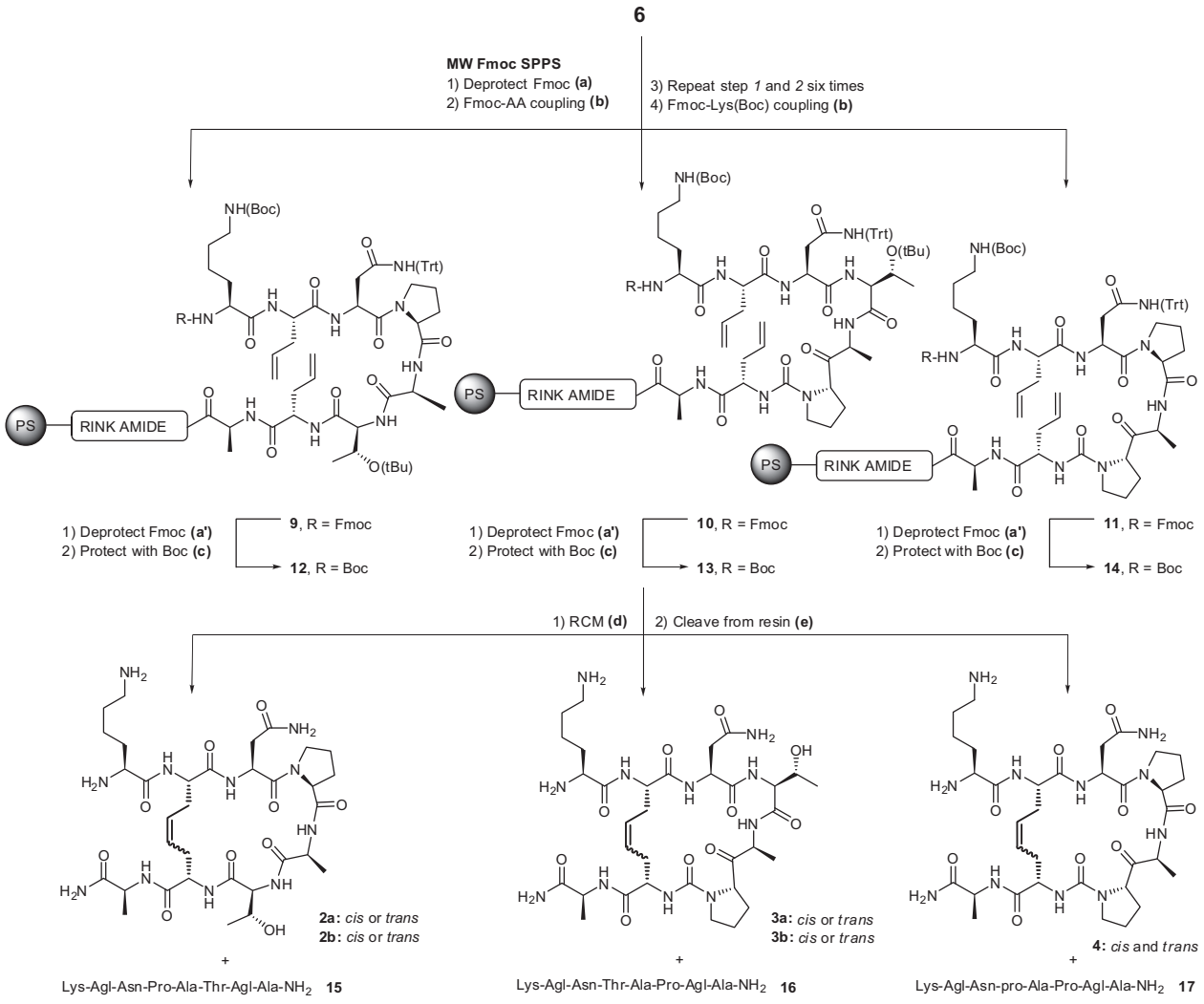
Semi-preparative RP-HPLC purification of the macrocyclic products afforded separable *cis*/*trans* isomers **2a** ($R_{tcis\ or\ trans}$ 7.57 min, major isomer, Fig. S7) and **2b** ($R_{tcis\ or\ trans}$ 8.60 min, minor isomer, Fig. S8), **3a** ($R_{tcis\ or\ trans}$ 7.40 min, minor isomer, Fig. S11) and **3b** (7.60 min, major isomer, Fig. S12), whereas analogue **4** was only isolated as an inseparable *cis*/*trans* isomer mixture (R_t 7.62 min (broad), Fig. S15).

The results described above indicate that replacement of the native threonine residues of amylin-(1–8) at position 4, or 6, or both by turn-inducing proline residues did not significantly improve the efficiency of macrocyclization via RCM as initially anticipated. Subsequent incorporation of Thr($\psi^{Me,Me}$pro) building blocks in place of Thr/4, Thr/6, or both, to give the native threonine residues was therefore not undertaken. However, sufficient material was obtained to evaluate the activity of RCM analogues (**2**–**4**) in osteoblast proliferation assays.

2.3. Synthesis of amylin-(1–8) peptide bridged analogues 5a and 5b by RCM of Lys(Alloc) residues at position 2 and 7

Having established that RCM of allylglycine residues embedded within amylin-(1–8) proceeded readily, we were interested to see whether the ruthenium-catalysed macrocyclization could be performed using an allyloxycarbonyl (Alloc) group on the N^{ε}-amino group of lysine residues that were substituted for Cys/2 and Cys/7. Using the commercially available Fmoc-Lys(Alloc)-OH building block in the RCM reaction would generate another peptidomimetic of amylin-(1–8) containing a 34-membered macrocyclic ring. The Alloc group has been employed previously to extend the carbon chain length using a cross metathesis reaction that proceeded in a moderate 40% yield.[36] To our knowledge, there are no reports to date on the use of Lys(Alloc) for RCM.

Synthesis of N^{α}-Boc protected peptidyl-resin **19** containing N^{ε}-Alloc protected lysine residues in place of Cys/2 and Cys/7 in the amylin-(1–8) sequence was next undertaken from **6** using the same conditions used for the synthesis of **12**, **13** and **14** (Scheme 3). The desired peptide sequence Fmoc-Lys-Lys(Alloc)-Asn-Thr-Ala-Thr-Lys(Alloc)-Ala-NH_2 that contained two Lys(Alloc) residues



Reagents and conditions:
(a) 20% piperidine, DMF, 62 W, 75 °C, 1 x 0.5 min + 1 x 3 min; **(a')** 20% piperidine, DMF, rt, 1 x 5 min + 1 x 15 min; **(b)** HBTU, *i*Pr_2EtN, DMF, 25 W, 73 °C, 5 min; **(c)** Boc_2O, DMF, rt, 30 min; **d)** Grubbs' II catalyst, CH_2Cl_2 : DMF (4 : 1), MW, 120 W, 100 °C, 2 h; **(e)** TFA, *i*Pr_3SiH, H_2O, rt, 2 h.

Scheme 2. Synthesis of RCM analogues of amylin-(1–8) **2**–**4**.

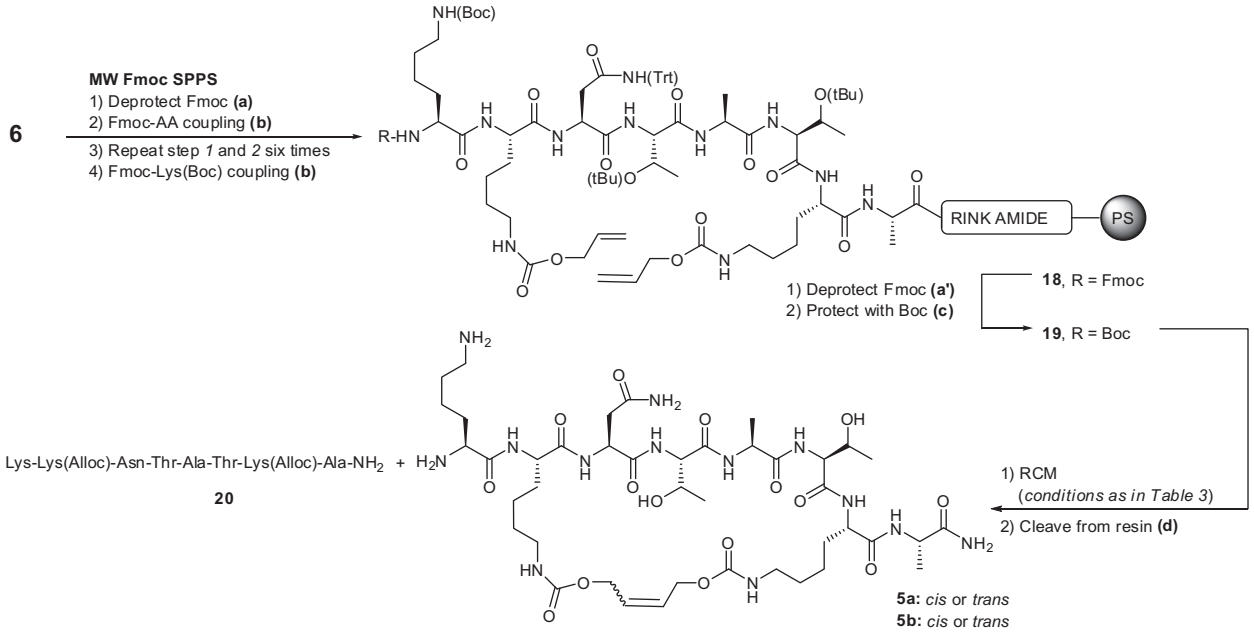
Table 2
RP-HPLC data of RCM amylin-(1–8) analogues **2**–**4** synthesised recorded at 210 nm

Entry	RCM on-resin precursor	ca. % by RP-HPLC at 210 nm		R_t of fully deprotected, linear, crude precursor (min)	R_t of crude RCM product (min)
		Cyclic	Linear		
1	**12**	**2a** and **2b**: 84%	**15**: 5%	**15**: 9.05 min	**2a**: $R_{tcis\ or\ trans}$ 7.65 (ca. 47%) **2b**: $R_{tcis\ or\ trans}$ 8.74 (ca. 37%)
2	**13**	**3a** and **3b**: 63%	**16**: 5%	**16**: 8.58 min	**3a**: $R_{tcis\ or\ trans}$ 7.52 (ca. 26%) **3b**: $R_{tcis\ or\ trans}$ 7.65 (ca. 37%)
3	**14**	**4**: appx. 86%	**17**: appx. 5%	**17**: 8.93 min	**4**: inseparable *cis/trans* isomer mixture R_t 7.60 (broad) (ca. 86%)

was formed in 94% (Fig. S16). Deprotection of the residual N-terminal Fmoc group in 18 followed by on-resin Boc protection afforded the fully protected peptidyl-resin **19**.[35]

Macrocyclization of **19** to *cis/trans* olefins **5a** and **5b** was then undertaken using the previously optimised conditions (entry 1, Table 3). The desired RCM product was formed as separable *cis* and *trans* isomers **5a** and **5b** albeit in ca. 29% (HPLC yield, $R_{tcis\ or\ trans}$ 9.90 min, $R_{tcis\ or\ trans}$ 10.47 min). Unreacted linear precursor **20** was isolated as the major product (ca. 46%, R_t 11.66 min) together with a by-product lacking an Alloc group from one of the Lys(Alloc) moieties.

The low yield of product obtained might be due to formation of a stable six-membered chelate between the ruthenium and the Alloc group that prevents metathesis from occurring.[37] Addition of a Lewis acid to the RCM reaction mixture may prevent formation of this complex.[38] Three separate additions of Grubbs' II catalyst were



Scheme 3. Synthesis of RCM analogues of amylin-(1–8) **5a** and **5b**.

Table 3
Conditions tested for the synthesis of RCM analogues **5a** and **5b** when starting from fully protected RCM precursor **19**

Entry	Catalyst loading	Catalyst Concentration (mM)	Solvent	Conditions	ca. % by RP-HPLC at 210 nm	
					Cyclic **5a** and **5b**	Linear **20**
1	Grubbs' II 30 mol %	1.26	CH_2Cl_2/DMF 4:1	MW, 100 °C, 2 h	29% (**5a**: 16% + **5b**: 13%)	46%
2	Grubbs' II 3 × 10 mol %	1.26	CH_2Cl_2/DMF 4:1 plus cat CDHB	MW, 100 °C, 2 h	39% (**5a**: 26% + **5b**: 13%)	26%
3	Hoveyda–Grubbs' II **2 × 15 mol %**	**1.44**	CH_2Cl_2/DMF 4:1 plus cat CDHB*	MW, 100 °C, 2 h	73% (**5a**: 58% + **5b**: 15%)	9%

* CDHB, dicyclohexylborane.

therefore carried out in the presence of chlorodicyclohexylborane[38] in CH_2Cl_2/DMF (4:1) under microwave conditions (100 °C) for 2 h (entry 2, Table 3). Pleasingly, the desired separable *cis* and *trans* isomers **5a** and **5b** containing a 34-membered macrocycle formed in an improved product ratio of ca. 39% together with unreacted metathesis precursor **20** (26%). Undesired Alloc group deprotection also took place under these conditions (data not shown).

Formation of products **5a** and **5b** was finally achieved in 73% conversion using two separate additions Hoveyda–Grubbs' II catalyst, in the presence of a catalytic quantity of chlorodicyclohexylborane[38] under microwave conditions (100 °C for 2 h) (entry 3, Table 3). A small amount of peptide precursor **20** (ca. 9%) together with a minor by-product lacking two Alloc groups (ca. 7%) was also observed (Fig. S17). Reverse-phase HPLC afforded two isomers **5a** ($R_{tcis\ or\ trans}$ 9.76 min, major isomer, Fig. S18), and **5b** ($R_{tcis\ or\ trans}$ 10.39 min, minor isomer, Fig. S19) that were subjected to biological assessment.

2.4. Osteoblast activity of amylin-(1–8) RCM analogues 1–5

Biological evaluation of the native amylin-(1–8) sequence and the more stable *cis*/*trans* RCM analogues **1–5** on proliferative activity in primary cell cultures of osteoblasts was undertaken and compared to control (Fig. 3). The osteoblast, bone-forming cell, culture system employed herein is an established in vitro bone cell assay which is an excellent tool for testing new drug compounds. Osteoblast precursor cells were isolated from neonatal rat calvarial bones using established digestion techniques. It was evident that for all the RCM analogues tested that one of the corresponding *cis*/*trans* isomers isolated exhibited better activity than the other. The reason for this was not investigated. It is postulated that the spatial conformation of one of the isomers (*cis* or *trans*) is more favourable thus giving rise to a more pronounced biological effect. Satisfyingly, five of the nine analogues prepared (**1a**, **3a, 3b**, **5a**, and **5b**) showed promising results on osteoblast activity exhibiting potency at a similar level to the native octapeptide. Interestingly, analogues **3a** and **3b** containing a proline residue incorporated in place of Thr/6 in amylin-(1–8) and using allylglycine moiety as the bridging point showed good activity. In contrast, analogues where a Pro residue was inserted at position 4 (compound **2a** and **2b**) and at position 4 and 6 (analogue **4**) did not exhibit activity within the level of significance. However, substitution of the disulfide bond of amylin-(1–8) with a carba-bridge constructed either via allylglycine residues (*cis* or

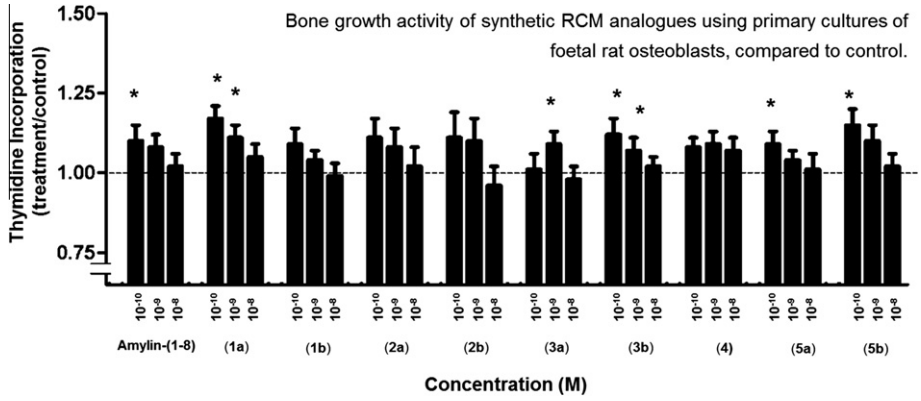


Figure 3. Osteoblast activity of the amylin-(1–8) and RCM analogues **1–5** prepared. Data are expressed as a ratio of treatment to control, mean ± SEM; *, significantly different from control ($p < 0.05$).

trans compound **1a**) or Lys(Alloc) (*cis* or *trans* compound **5b**) proved to be the most effective strategy affording analogues with the best activity. These two peptides were therefore chosen as good candidates for further in vivo study which will be undertaken in future.

3. Conclusions

Nine macrocyclic analogues **1–5** of native amylin-(1–8) were successfully prepared for evaluation as potential agents to treat osteoporosis. On-resin Grubbs' ring closing metathesis[8,9] was used to replace the native disulfide bond of amylin-(1–8) with a more stable carba-bridged macrocyclic ring. Allylglycine residues incorporated in place of Cys/2 and Cys/7 afforded a 20-membered macrocyclic ring analogues **1a** and **1b**. Using proline as a kink-inducing residue incorporated in place of Thr/4 or Thr/6, or both, enabled generation of a further five amylin-(1–8) analogues **2–4** however, the efficacy of the cyclization step was not improved. We have also demonstrated for the first time that Lys(Alloc) can be used for carba-bridging via RCM affording a further two peptidomimetic analogues of amylin-(1–8) that contain an impressive 34-membered ring (analogues **5a** and **5b**). This convenient 'off the shelf' building block may be used to prepare other peptide mimics requiring modification of peptide rigidity and stability. Addition of a Lewis acid[38] to reaction mixture was required in order to disrupt ruthenium chelates[37] formed with the Lys(Alloc) residues.

Five analogues (*cis* or *trans* **1a**, *cis* or *trans* **3a**, *cis* or *trans* **3b**, *cis* or *trans* **5a**, and *cis* or *trans* **5b**) exhibited activity in biological assays with significance estimated in post hoc Dunnett's tests. Two of the most promising compounds (*cis* or *trans* **1a** and *cis* or *trans* **5b**) have been selected for further in vivo study that will be reported upon in due course.

4. General methods

4.1. Chemistry

4.1.1. Materials

All reagents were purchased as reagent grade and used without further purification. Solvents were dried according to standard methods.[30] *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), was purchased from Advanced Chemtech (Louisville, KY), dimethylformamide (DMF) (AR grade), acetonitrile (HPLC grade) and hydrochloric acid (HCl) were purchased from Scharlau (Global Science). Trifluoroacetic acid (TFA) was purchased from Halocarbon (New Jersey), dichloromethane (CH_2Cl_2) was purchased from ECP Limited and dimethyl sulphoxide (DMSO) was purchased from Romil. 1-Hydroxybenzotriazole (HOBt), di-*tert*-butyl dicarbonate (Boc_2O), 4-[(*R*,*S*) -α-[1-(9*H*-fluoren-9-yl]methoxycarbonylamino]-2,4-dimethoxy]phenoxyacetic acid (Fmoc Rink linker), *N*-[(9*H*-fluoren-9-yl)methoxycarbonyl]-L-2-amino-4-pentenoic acid (Fmoc-Agl-OH) and Fmoc-amino acids were purchased from GL Biochem (Shanghai, China) with the following side chain protection: Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Thr(O*t*Bu)-OH. Fmoc-Lys(Alloc)-OH was purchased from NovaBiochem. *N*-Methylpyrrolidine (NMP), diisopropylethylamine (iPr_2EtN), piperidine, triisopropylsilane (iPr_3SiH), *N*,*N*′-dicyclohexylcarbodiimide (DIC), chlorodicyclohexylborane, 1,2-dichlorobenzene, Grubbs' II catalyst and Hoveyda–Grubbs' II catalyst were purchased from Sigma–Aldrich (Sydney, Australia).

4.1.2. Peptide synthesis, purification and analysis

Fmoc SPPS was performed on a Liberty Microwave Peptide Synthesiser (CEM Corporation, Mathews, NC) using the Fmoc/*t*Bu strategy as previously described.[39] The resulting peptides were cleaved from the resin with simultaneous side chain protecting group removal by treatment with TFA/iPr_3SiH/H_2O (v/v/v; 95/2.5/2.5), for 2 h at room temperature. Crude peptides were precipitated and triturated with cold diethyl ether, isolated (centrifugation), dissolved in 20% acetonitrile (aq) containing 0.1% TFA and lyophilized. Native amylin-(1–8)-NH_2 (disulfide bond) was synthesised according to previously described procedure.[3]

Analytical reverse phase high-performance liquid chromatography (RP-HPLC) was performed using a Dionex P680 (flow rate of 1 mL/min), using Waters XTerra® column (MS C_{18}, 150 mm × 4.6 mm; 5 μm) using gradient systems as indicated in the Supplementary data.

The solvent system used was A (0.1% TFA in H_2O) and B (0.1% TFA in acetonitrile) with detection at 210 nm, 254 nm, and 280 nm. The ratio of products was determined by integration of spectra recorded at 210 nm or 254 nm. A Bruker micrOTOF-Q II mass spectrometer was used for ESI-MS analysis (positive mode). Peptide purification was performed using a Waters 600E using a preparative Phenomenex Gemini C_{18}, 250 mm × 10 mm; 5 μm column. Gradient systems were adjusted according to the elution profiles and peak profiles obtained from the analytical RP-HPLC chromatograms. Fractions were collected, analysed by either RP-HPLC or ESI-MS, pooled and lyophilised 3 times from 10 mM aq HCl.

4.2. Bone growth activity assays

Osteoblasts were isolated from 20-day foetal rat calvariae, as previously described.[40] Briefly, calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue, were collected. The calvariae were sequentially digested using collage-

nase (Sigma) and the cells from third and fourth digests were collected, pooled and washed. Cells were grown in T75 flasks in 10% FBS/Dulbecco's modified eagle medium (DMEM) (Invitrogen) and 5 μg/mL L-ascorbic acid 2-phosphate (Sigma) for 2 days and then changed to 10% FBS/MEM (Invitrogen)/5 μg/mL L-ascorbic acid 2-phosphate and grown to 90% confluency. Cells were then seeded into 24 well plates in 5% FBS/MEM 5 μg/mL L-ascorbic acid 2-phosphate for 24 h. Cells were growth-arrested in 0.1% bovine serum albumin (BSA) (ICP, Auckland, New Zealand)/ 5 μg/mL L-ascorbic acid 2-phosphate for 24 h. Cells were pulsed with [^{3}H]thymidine 6 h before the end of the experimental incubation. The experiments were then terminated and thymidine incorporation assessed, as a measurement of cell growth. A Trilux counter (Wallac 1450 Microbeta counter) was used for the data collection. Each of the analogues was screened at three different concentrations. There were six wells in each group and each experiment was repeated 3 or 4 times. All treatments were compared to a vehicle control; data were analysed using analysis of variance with post-hoc Dunnett's tests for significant main effect. A 5% significance level (two-tailed) was used throughout.

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Supplementary data

Supplementary data (Experimental data for the synthesis of peptides **1**–**5**, RP-HPLC and ESI-MS traces is available free of charge in the online version) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.053.

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