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Synthesis and evaluation of disulfide bond mimetics of amylin-(1–8) as agents to treat osteoporosis

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ABSTRACT

Osteoporotic fracture is a significant public health problem, resulting in fractures in >50% of women and in almost one third of men age 65 and older. Most of the existing therapies act by slowing bone loss, through inhibiting the action of bone resorbing cells. However, more substantial reductions of fracture numbers will only result from treatments that can rebuild bone. Our own animal studies demonstrated the anabolic potential of the small but unstable octapeptide fragment of amylin-(1–37), namely amylin-(1–8) containing one disulfide bridge (Cys/2 and Cys/7) [*Am. J. Physiol. Endocrinol. Metab.* **2000**, *279*, E730]. Herein, we describe the synthesis of amylin-(1–8) octapeptide and seven analogues thereof wherein the disulfide bridge is modified either via insertion of different linkers or bridges of a different nature in order to improve the stability and/or bone anabolic activity of the parent peptide. The peptide analogues were screened for proliferative activity in primary foetal rat bone-forming cells or osteoblasts at physiological concentrations. One such analogue showed promising biological activity.

1. Introduction

Osteoporosis is a skeletal disorder of aging leading to bone fragility and resulting in increases in bone fractures.[1] Bone is maintained throughout life by being continually replaced, mainly via the action of the bone forming cells (osteoblasts) and the bone resorbing cells (osteoclasts). In osteoporosis bone resorption exceeds bone formation resulting in bone loss. The majority of current treatments for osteoporosis are anti-resorptive, decreasing the osteoclast activity. However, a therapy capable of rebuilding bone by acting on the osteoblasts would have a major influence on treatment and prevention of osteoporosis. The only such anabolic therapy currently used is parathyroid hormone, and this is limited by the need for daily injections and an annual cost of $10,000 per patient. Moreover, therapy involving parathyroid hormone is restricted to 20 months due to safety concerns.[2] As a result, there is an unmet medical requirement for a new class of orally active bone anabolic drugs for the treatment of osteoporosis.

Amylin-(1–37) is a pancreatic hormone that is co-secreted with insulin from the beta-islet cells (Fig. 1).[3] Like insulin it belongs to the calcitonin family and is active in fuel metabolism. We have shown that amylin-(1–37) stimulates osteoblast proliferation in vitro, thus activating bone growth,[4] and similar to calcitonin inhibits osteoclasts activity, thus reducing bone resorption.[5] The bone activity of amylin-(1–37) makes it an attractive candidate for the osteoporosis treatment, however due to its large molecular weight and other non-osteogenic effects, it is not an ideal therapeutic agent. We have also demonstrated that the N-terminal octapeptide fragment of amylin-(1–37), namely amylin-(1–8), is inactive on carbohydrate metabolism and does not lead to amyloid formation like the full length peptide; however it still retains its anabolic effects on osteoblasts (Fig. 1). When administered in vivo, amylin-(1–8) stimulates osteoblast proliferation increasing bone volume and bone strength.[1] Analogous to the parent peptide, amylin-(1–8) requires both the intact disulfide bridge (Cys/2 and Cys/7) and C-terminal amidation to maintain osteogenic activity. This smaller but unstable cyclic peptide (stable for 6 months at −80 °C under argon) is a more attractive candidate for pharmaceutical development and might be used as a model for the creation of orally active analogues which could be potential candidates for the treatment of osteoporosis. The aim of the present study is to engineer more stable synthetic analogues of amylin-(1–8) that are osteogenic using various peptidomimetic techniques.

The group of Ellegaard[6] have recently questioned that amylin-(1–8) is active towards osteoblast proliferation. This observed lack

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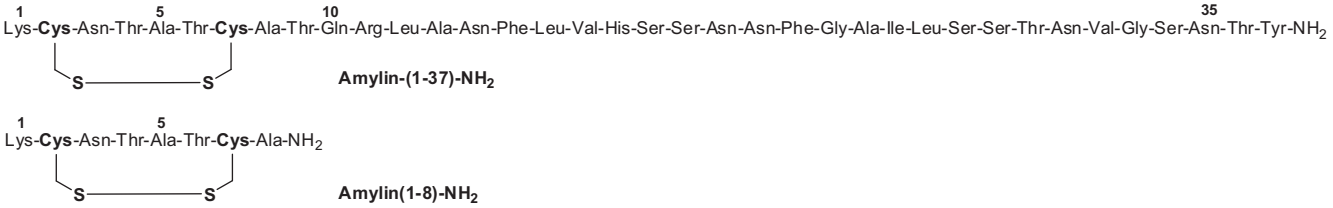


Figure 1. Primary structure of amylin-(1–37) and amylin-(1–8) fragment.

of activity may be due to incorrect handling or storage of the unstable amylin-(1–8) peptide as the authors were unable to show the proliferative effect of the full molecule of amylin as well as amylin-(1–8). Moreover, the receptor that amylin binds to in osteoblasts is not known. A putative receptor for amylin, calcitonin receptor/receptor activity-modifying proteins (CTR/RAMP), is not the receptor in the osteoblast as osteoblasts are devoid of the CTR receptor. Thus it remains unknown what the effective receptor for amylin-(1–8) is on osteoblasts. The authors use cAMP signaling to screen amylin activity and they report a strong response, yet many other researchers have shown that amylin is only a very moderate activator of cAMP. We therefore still consider that amylin-(1–8) is effective stimulator of osteoblasts.

Disulfide bonds are natural conformational protein constraints that stabilize tertiary structures of peptides and proteins. They are responsible for the biological activity of many natural compounds such as hormones, enzymes, toxins, and growth factors. They are prone however to both enzymatic and chemical cleavages under reducing, nucleophilic or basic conditions or by the action of disulfide reductase, thus limiting their use in therapeutic agents.

Therefore replacement of disulfide bridges by more stable synthetic bridges can increase metabolic stability or receptor-binding affinity of bioactive peptides. Different approaches including replacement of native disulfide bridges by diselenocysteine bridges,[7] lactam,[8] thioether[9] and carba bridges[10,11] have been explored. Meldal et al.[12] have recently introduced two triazole rings via a Cu(I) catalysed intramolecular cycloaddition 'click' reaction, as a mimetic of disulfide bonds of Tachyplesin I. In addition, various bridging reagents have been used for disulfide bond modifications. Baker et al.[13] reported the use of dibromomaleimide for disulfide bond bridging on somatostatin, while α,α′-dibromoxylenes were explored in the study of Timmerman et al.[14] for cyclization of peptides via two cysteine residues present in the linear peptide.

We envisaged that introduction of various disulfide bridge replacements (Cys/2 and Cys/7) of amylin-(1–8) would lead to improved stability and activity of the peptide. We herein report the synthesis of the bioactive amylin-(1–8) peptide and several analogues thereof wherein the disulfide bridge is modified either by insertion of different linkers or bridges of a different nature(Fig. 2).

These novel chemical entities may lead to the development of therapeutic compounds for use in the oral treatment of osteoporosis or for promoting fracture healing.

2. Results and discussion

2.1. Synthesis of amylin-(1–8) peptide 1 and amylin-(1–8) peptide analogues 2–8

Synthesis of the native amylin-(1–8) peptide **1**, containing a natural disulfide bridge between the cysteine residues at position 2 and 7, was successfully undertaken using manual Fmoc solid phase peptide synthesis (SPPS), starting from commercially available Pal-PEG-PS resin (Scheme 1). Twenty percentage of piperidine was used for Fmoc deprotection and HBTU/*i*Pr$_2$EtN as coupling reagents. Acid mediated cleavage of the peptide from the resin with side chain deprotection using TFA/*i*Pr$_3$SiH/DODT/H$_2$O afforded the key intermediate, reduced amylin-(1–8) **9**. This was followed by the direct oxidation of crude **9** to form the intramolecularCys2/Cys7 disulfide bond without prior purification. This was undertaken by air oxidation in 50 mM Tris–HCl buffer at pH 8.8 following the procedure of Abedini and Raleigh.[14] Aliquots from the reaction mixture were monitored every few hours by analytical reverse-phase HPLC (RP-HPLC), and after 21 h full conversion to the oxidised amylin-(1–8) peptide **1** was achieved (Fig. S3). Purification by RP-HPLC afforded the desired product **1** in 30% yield (Fig. S4).

Our initial approach for the generation of amylin-(1–8) analogues was to substitute the disulfide bond of Cys/2 and Cys/7 using thioalkyl bridges that vary in structure and length. Based on the work of Baker et al.[13] where mono- and dibromomaleimides were used for cysteine modification on the SH2 domain of the Grb2 adaptor protein, a maleimide bridge was inserted into the disulfide bond of purified amylin-(1–8) **9** using 2,3-dibromomaleimide. Thus, reaction of **9** with 2,3-dibromomaleimide was undertaken in H$_2$O/acetonitrile (2:1), using 250 mM potassium phosphate buffer (pH 7.0) (Scheme 1). Reaction was complete after 30 min as evidenced by RP-HPLC analysis (Fig. S5). Purification by RP-HPLC afforded peptide **2** in 15% yield, Fig. S6.

Another approach to generate bridged amylin-(1–8) analogues was based on the procedure of Timmerman et al.[14] (later exploited by Hartman et al.[15]) who cyclized linear peptides containing multiple free cysteines onto poly(bromomethyl)-functionalized scaffolds. With this strategy in mind peptide **9** was subjected to intramolecular cyclization using α,α′-dibromo-*p*-xylene or α,α′-dibromo-*o*-xylene as bridging reagents in H$_2$O/acetonitrile (1:1) in 20 mM ammonium bicarbonate buffer (Scheme 1). Rapid formation of the desired products **3** and **4** was observed by RP-HPLC and ESI-MS analysis after 40 min and 30 min, respectively (Figs. S7 and S9). RP-HPLC purification afforded the desired product **3** and **4** in 67% and 29% yield, respectively (Figs. S8 and S10).

Encouraged by these successful syntheses we next investigated the feasibility of intramolecular cyclization using alkyl chains of variable length to afford macrocyclic rings of different size. In order to minimise purification steps we preferred to use crude peptide **9** for the synthesis. Thus, alkylation of crude amylin-(1–8) **9** using 1,2-dibromoethane or 1,3-dibromopropane (for compound **5** and **6**, respectively) was performed in H$_2$O/acetonitrile (1:1), using 200 mM ammonium bicarbonate as a buffer. Significantly longer reaction times were required to obtain the desired products **5** and **6** when using these less reactive alkyl-bridging reagents. To avoid disulfide bond formation during these long reactions *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) as reducing agent was added (Scheme 1). Successful conversion to the desired alkylated product **5** (22-membered ring) and **6** (23-membered ring) was thus observed after 42 h and 96 h, respectively (Figs. S11 and S13). Purification by RP-HPLC afforded the required products **5** and **6** in 24% and 10% yield, respectively (Figs. S12 and S14). The long reaction times and reduced overall yields for

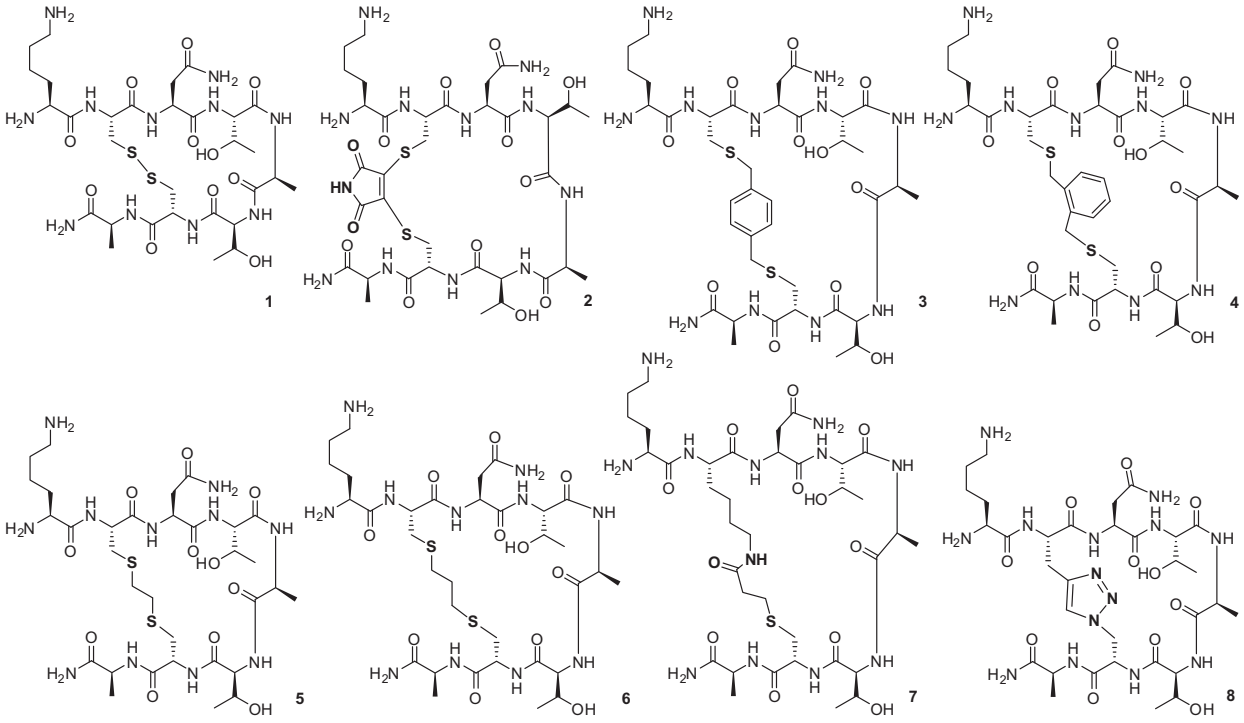


Figure 2. Chemical structures of amylin-(1–8) (**1**) and the analogues **2–8** synthesised.

bridged analogues **5** and **6** can be explained by the decreased reactivity of either 1,2-dibromoethane or 1,3-dibromopropane compared to α,α′-dibromo-*p*-xylene or α,α′-dibromo-*o*-xylene.

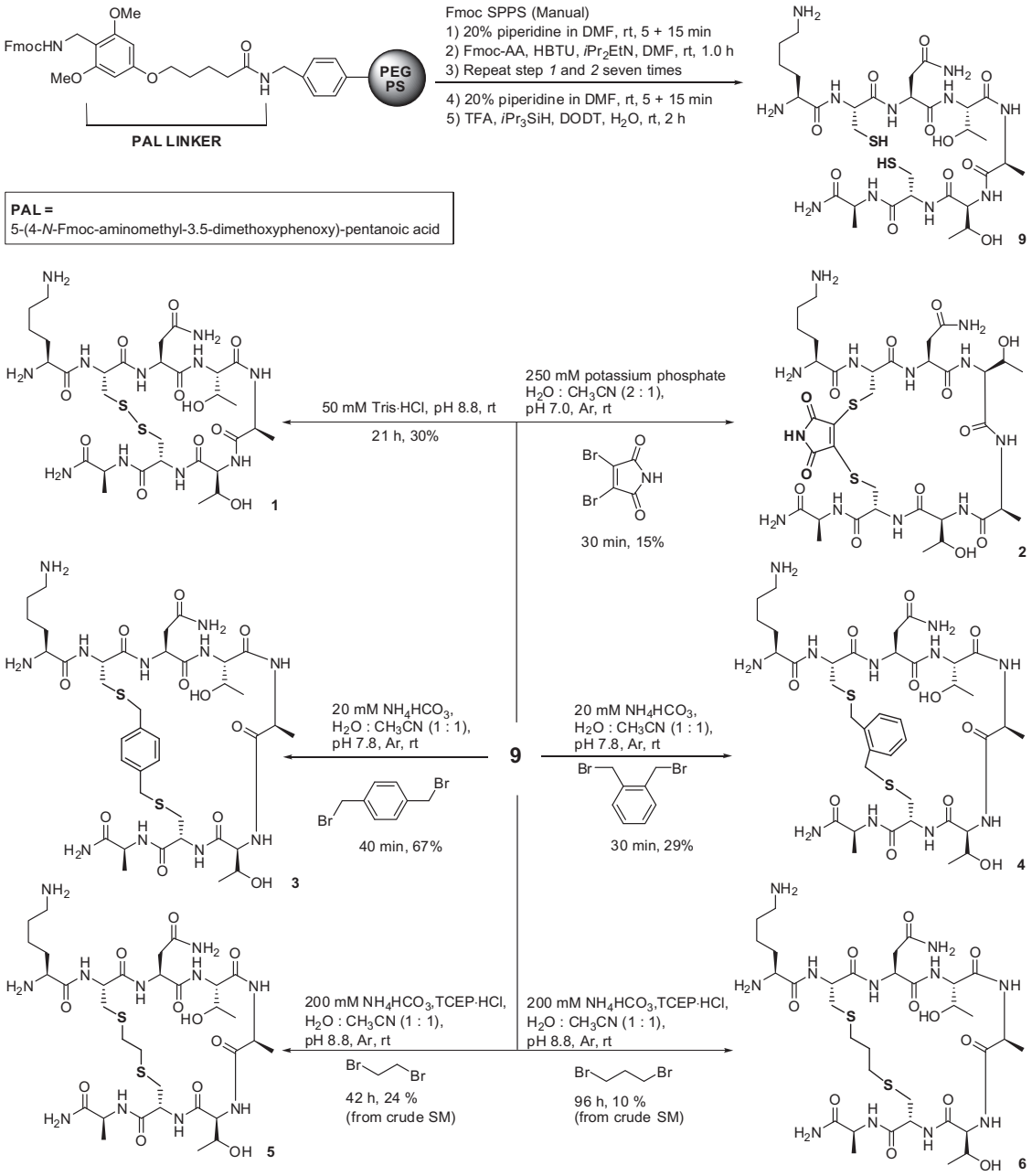
Due to these difficulties encountered, a different synthetic approach for generation of large macrocyclic rings was investigated. Thus, synthesis of peptide **7** containing a 26-membered ring was undertaken which required that a lysine residue was incorporated in place of Cys/2 in the amylin-(1–8) sequence. Functionalisation of the ε-NH_2 group of this residue with a suitable electrophile, for example, 3-bromopropanoic acid, may allow intramolecular S-alkylation on Cys/7.

For the synthesis of analogue **7**, microwave enhanced Fmoc SPPS was employed (Scheme 2). Commercially available Pal-PEG-PS resin was used in conjunction with HBTU and *i*Pr_2EtN as coupling reagents for elongation of the peptide. Subsequent steps of Fmoc removal using 20% piperidine in DMF and incorporation of fully protected Fmoc amino acids afforded peptidyl-resin **10** with the Dde protecting group (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) at the N^{ε} of the Lys/2 residue. Selective removal of the Dde moiety using hydroxylamine hydrochloride[16] in DMF/NMP/CH_2Cl_2 (0.5:5:0.5) for 3 h afforded peptidyl-resin **11**. Having the N^{α}-terminal Fmoc intact enabled further branching at the N^{ε} site of the Lys/2 residue. Manual incorporation of 3-bromopropanoic acid was thus undertaken using HBTU and *i*Pr_2EtN in DMF at room temperature, affording peptidyl-resin **12**.

Disappointingly, subsequent removal of the N-terminal Fmoc (5% piperazine in DMF) and cleavage from the resin (TFA/*i*Pr_3SiH/H_2O) resulted in the isolation of Michael donor/acceptor **13**, instead of expected peptide **14**, as confirmed by RP-HPLC and ESI-MS analysis (Fig. S15). Apparently, treatment of **12** with piperazine resulted in elimination of HBr to afford the α,β-unsaturated compound **13**. However, with Michael donor/acceptor **13** in hand, we decided to explore direct cyclization of this peptide using thiol-Michael addition.[17]Thus, **13** was stirred in 250 mM potassium phosphate buffer affording the cyclic product **7** quantitatively after 48 h as judged by the disappearance of the peak corresponding to the starting material **13**, (R_t 7.96 min), and the formation of an earlier eluting product (**7**) (R_t 7.35 min) on RP-HPLC (Fig. S16). Due to the long reaction time required for reaction to complete (48 h) it was decided to also use TCEP·HCl in the reaction mixture to ensure that the sulfhydryl group of the cysteine residue in the reduced state is available for Michael addition.

However, as starting material **13** and desired product **7** have the same mass therefore mass spectrometry could not be used to confirm the success of the reaction. If the reaction was uncompleted, the thiol group present on the cysteine residue of the peptide **13** would undergo S-alkylation in the presence of alkylating reagent affording a product with a different retention time than the starting material **13**. Thus, iodoacetamide was added to the reaction mixture and allowed to react for 30 min (Scheme 2). Pleasingly, S-alkylated peptide **15** was not observed and the retention time of the product **7** remained unchanged suggesting that the desired peptide **7** was obtained (Fig. S17). Subsequent RP-HPLC purification afforded the required product **7** in 9% yield (Fig. S18).

We next turned to explore the Cu(I)-catalysed Huisgen azide–alkyne 1,3-dipolar cycloaddition reaction or so called 'click' reaction[18,19] which has recently gained considered attention in peptidomimetic studies.[20–23] The triazole ring has already shown to be a potent inhibitor of cysteine[24] and HIV proteases[25] demonstrating that this amide bond surrogate may significantly improve the proteolytic stability of peptides. The 'click' reaction[18,19] has previously been explored for the synthesis of disulfide bond mimetics,[12,26] therefore we sought to replace the disulfide bond with a triazole linkage. The azide and alkyne functionalities were introduced onto the polypeptide using *N*-Fmoc-L-3-azido-alanine (Fmoc-N_3Ala)[27] and *N*-Fmoc-L-propargylglycine (Fmoc-Pra), respectively. L-Propargylglycine was synthesised from L-proline using a Ni(II) template following established procedures.[28–30] Subsequent Fmoc protection of the N^{α} amino group of L-propargylglycine using Fmoc-OSu[31] afforded the required *N*-Fmoc-L-propargylglycine building block.[32]



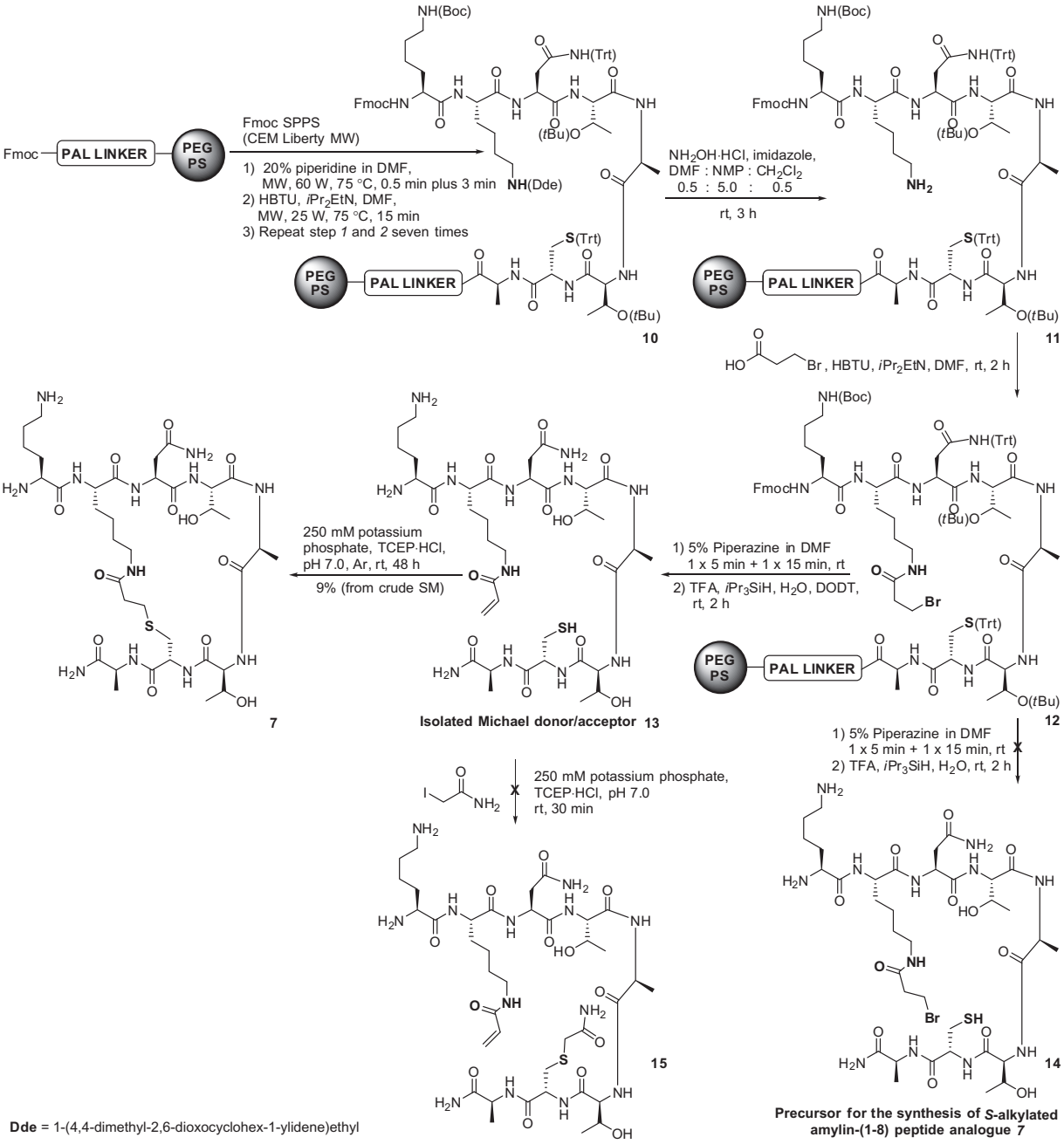
Scheme 1. Synthesis of native amylin-(1–8) peptide **1** and analogues **2**–**6**.

Fmoc-N_3Ala and Fmoc-Pra were incorporated in place of Cys/2 and Cys/7, in the native amylin-(1–8) octapeptide using microwave enhanced Fmoc SPPS starting from Pal-PEG-PS resin as depicted in Scheme 3 (Path A). This procedure afforded two 'click' peptide precursors ready for subsequent 'click' cyclisation either on resin (precursor **16**) or in solution (precursor **17**).[22]

After chain assembly and peptide cleavage using TFA/iPr$_3$SiH/H_2O the expected linear peptide **17** was obtained as the major product. Unfortunately, the presence of two by-products, a deletion by-product lacking the L-3-azido-alanine moiety and a by-product in which elimination of the azide group had taken place, were also detected (as confirmed by RP-HPLC at 210 nm and ESI-MS analysis, Fig. S19). It is proposed that due to the position of N_3Ala close to the C-terminus of the peptide chain (position 7) elimination of the azide group occurred upon multiple treatments of the peptidyl-resin with the 20% piperidine solution in DMF required for Fmoc group removal (Scheme 3). Moreover, microwave heating used for the peptide synthesis may have influenced the extent of azide group elimination.

Thus, manual Fmoc SPPS of peptidyl-resin **18** at room temperature in which the Fmoc-N_3Ala required was placed closer to N-terminus of the peptide chain (position 2) was next undertaken (Scheme 3, Path B). HBTU was used as the coupling reagent with iPr$_2$EtN as base and the Fmoc group was removed using 20% piperidine in DMF. Pleasingly, cleavage of a sample of peptidyl-resin **18** (TFA/iPr$_3$SiH/H_2O) afforded Lys-N_3Ala-Asn-Thr-Ala-Thr-Pra-Ala-NH_2 as the major product and the dehydroalanine by-product was not observed (Fig. S23).

To further confirm that the azide group was prone to elimination under the basic conditions used for Fmoc deprotections a sample of peptidyl-resin **18** was left overnight in 20% piperidine solution in DMF (Scheme 3, Path C). Subsequent TFA cleavage of

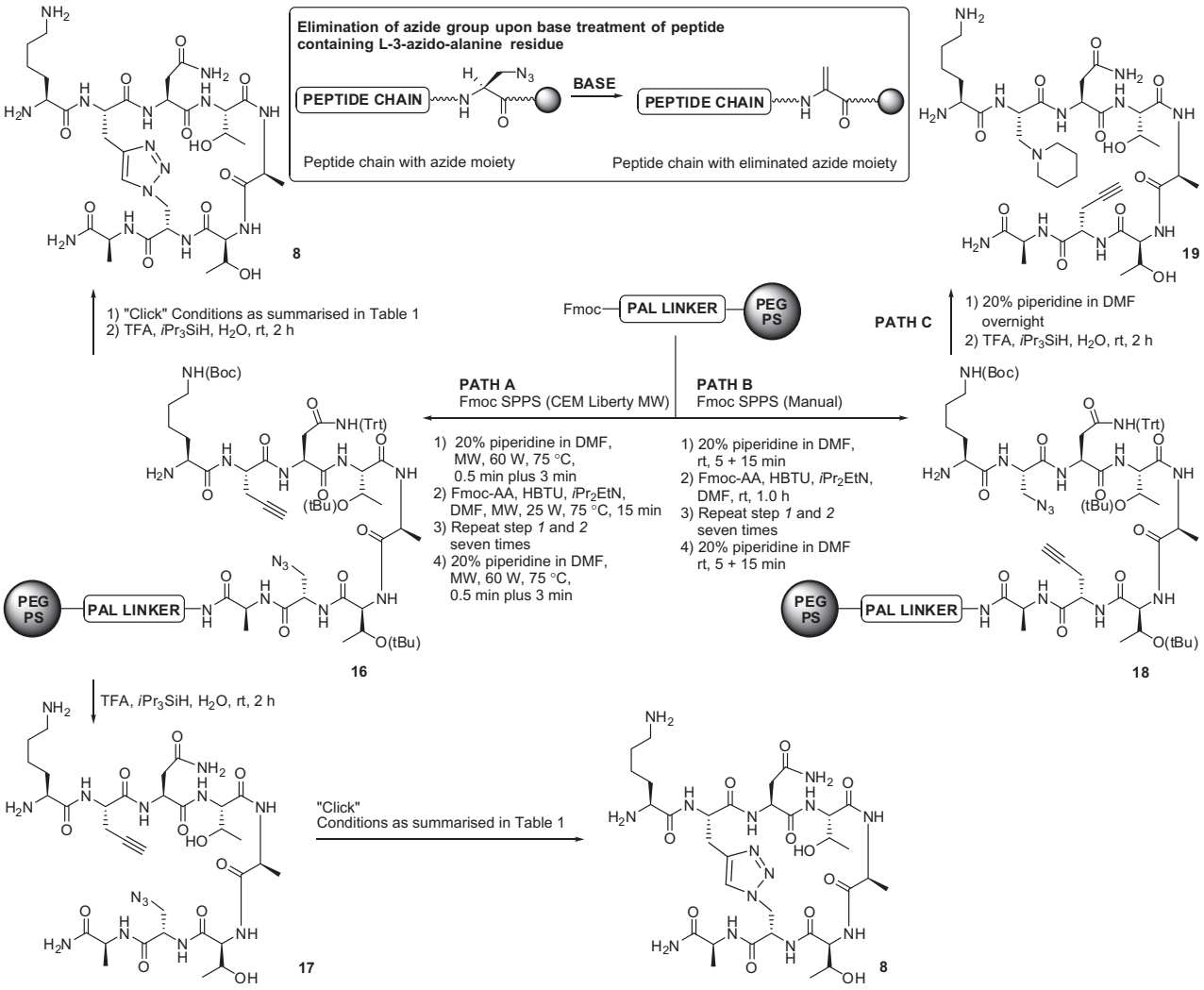


Scheme 2. Synthesis of analogue **7** by thiol-Michael addition.

the peptide from the resin followed by analytical RP-HPLC and ESI-MS analysis revealed the presence of peptide **19** lacking an azide group but containing a piperidide by-product (Fig. S24). It was thus confirmed that upon prolonged treatment with piperidine the elimination of azide group from N_3Ala moiety takes place affording a dehydroalanine moiety. Subsequent Michael addition leads to formation of a peptide–piperidide adduct. Similar reactions occur during the synthesis of C-terminal cysteine peptides when base-catalysed elimination of the sulfhydryl-protected side chain of the cysteine moiety takes place which is followed by nucleophilic addition to the olefin.[33] The elimination of the azide group may be prevented by using milder conditions than 20% piperidine in DMF for Fmoc group deprotection (e.g., 5% piperazine in DMF) when synthesising peptides containing N_3Ala residues.

Next, synthesis of a cyclic 1,2,3-triazole linked amylin-(1–8) peptide **8** using 'click' chemistry was undertaken. The success of 'click' chemistry for the synthesis of cyclic peptides strongly depends on the solvent system, type of the resin, proximity of the alkyne moieties to the resin (if reaction is performed on resin) and the length of the peptide.[22] For these reasons, a number of conditions were evaluated during this study varying the solvent and catalyst, and conducting the reaction either at room temperature or using microwave heating. Two approaches were evaluated and the results are summarised in Table 1 and Scheme 3; synthesis on resin (Table 1, entries 1–2) and synthesis in solution (Table 1, entries 3–8).

The initial experiments to form the 'click' cyclic product **8** were carried out on resin (Table 1, entry 1 and 2) using copper(I) iodide and sodium ascorbate as catalysts in acetonitrile/DMSO/H_2O (8:2:1).[34] The reaction was undertaken either at room temperature (entry 1) or at 40 °C (conventional heating, entry 2). Subsequent cleavage from the resin (TFA/iPr$_3$SiH/H_2O) and analysis using



Scheme 3. Synthesis of amylin-(1–8) peptide 'click' analogue **8**.

Table 1
Conditions tested for the synthesis of cyclic analogue **8** using 'click' chemistry

Entry	Starting material	Catalyst	Solvent	Conditions
1	**16**	0.5 equiv CuI 1.0 equiv Na ascorbate 2.0 equiv lutidine	$CH_3CN/DMSO/H_2O$ 8:2:1	RT, 16 h
2	**16**	0.5 equiv CuI 1.0 equiv Na ascorbate 2.0 equiv lutidine	$CH_3CN/DMSO/H_2O$ 8:2:1	40 °C, 21 h oxygen excluded
3	**17** (CRUDE)	20 equiv $CuSO_4$ 50 equiv Na ascorbate	$tBu/CH_2Cl_2/H_2O$ 1/1/1	RT, 23 h
4	**17** (CRUDE)	20 equiv $CuSO_4$ 50 equiv Na ascorbate	$tBu/CH_2Cl_2/H_2O$ 1/1/1	MW, 80 °C, 5 min
5	**17** (CRUDE)	20 equiv $CuSO_4$ 50 equiv Na ascorbate	$tBu/CH_2Cl_2/H_2O$ 1/1/1	MW, 80 °C, 10 min
6	**17** (CRUDE)	2 mol % $CuSO_4$ 5 mol % Na ascorbate	$tBu/CH_2Cl_2/H_2O$ 1/1/1	MW, 80 °C, 20 min in total, progress checked every 5 min
7	**17** (CRUDE)	2 mol % $CuSO_4$ 5 mol % Na ascorbate	tBu/H_2O 1:1	MW, 80 °C, 20 min in total, progress checked every 5 min
8	**17** PURIFIED	1 equiv $CuSO_4$ 2.5 equiv Na ascorbate	tBu/H_2O 1:1	MW, 80 °C, 20 min

LC–MS revealed that the desired product **8** was not formed and only minor amounts of products possibly due to intermolecular reactions were detected (data not shown). As the reaction progress could not be followed by mass spectrometry (mass of the 'click' precursor is the same as the cyclic product) and no major shifts in retention times were observed after reaction, it was difficult to confirm the presence of the product. In addition, in order to monitor the progress of the reaction using RP-HPLC, small-scale peptide cleavages had to be performed which was time consuming. For these reasons, further investigations into the 'click' reaction were undertaken in solution (Table 1, entries 3–8).

Various catalyst loadings (copper(II) sulphate and sodium ascorbate), solvent systems and microwave heating were tested (Table 1). Use of a large excess of catalyst (Table 1, entry 3–5) lead to almost quantitative conversion to an unidentified product with an earlier retention time (R_t 6.62 min) than the starting material (R_t 7.89 min) (data not shown). Disappointingly, the product was impossible to be identified using ESI-MS analysis as neither the crude reaction mixture nor the purified sample ionized. However, FT-IR analysis (entries 3–5) revealed the absence of an azide stretch at ν_{max} cm^{-1} 2100–2270 suggesting that the azide group on the peptide chain had in fact reacted.

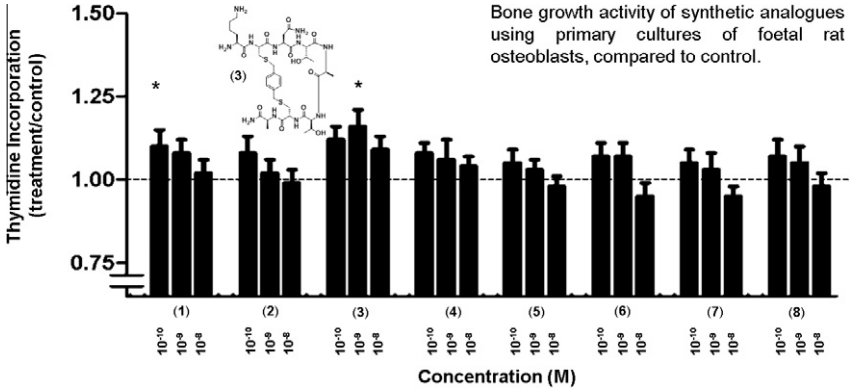


Figure 3. Assessment of the amylin-(1–8) **1** and synthetic analogues **2**–**8** prepared on the stimulating effect on osteoblasts. Data are mean ± SEM; *significantly different from untreated control (p <0.05).

Encouraged by these results and assuming that a lack of ionisation was due to a large excess of copper catalyst coordinating to the peptide it was decided to undertake experiments using less catalyst (Table 1, entries 6–7). In order to accelerate the reaction rate, the reaction was performed under microwave heating. Attempts to form the cyclic 'click' analogue starting from crude peptide **17** (Table 1, entries 6–7) again resulted in no retention time shift on RP-HPLC compared to **17**, and lack of ionization (ESI-MS, APCI-MS) was observed once again. FT-IR analysis of the products formed however, showed the presence of an azide stretch at ν_{max} cm^{-1} 2113 and 2111 (entry 6 and 7, respectively) establishing that the azide group on the peptide chain was still present and the desired 1,2,3-triazole ring had not formed (data not shown).

Finally, it was rewarding to observe that microwave enhanced reaction of a purified peptide **17** (as an inseparable mixture with dehydroalanine by-product) activated using copper sulfate(II) and sodium ascorbate in *tert*-butanol/H_2O (1:1) afforded the desired product **8** (Table 1, entry 8) as confirmed by the absence of an azide absorbance in the FT-IR spectrum (Fig. S21). Under these conditions intermolecular reaction did not occur. RP-HPLC purification afforded the desired product **8** in 18% yield that also contained some dehydroalanine by-product (Fig. S22).

2.2. Proliferative activity of amylin-(1–8) synthetic analogues 2–8

The seven synthetic analogues of amylin-(1–8) **2**–**8** were evaluated for bone growth activity and compared to a control (Fig. 3). Gratifyingly, analogue **3** containing a planar benzyl ring with the *para* xylyl linker incorporated showed a promising result that was comparable with osteoblast activity of native amylin-(1–8). However, additional biological evaluation to assess the nature and the extent of its activity is still required. A positive biological result was only observed for the analogue containing a relatively short and rigid bridge system, such as the *para* xylyl linker incorporated. No improvement in peptide activity was observed with the analogue containing *ortho* xylyl linker inserted (analogue **4**) or with the analogues bearing flexible alkyl chains (peptide **5** and **6**) or a large macrocyclic ring (analogue **7**). These observations suggest that further work should focus on generating cyclic analogues containing systems with similar ring size to the native disulfide bond. It was disappointing to note that the 1,2,3-triazole ring (analogue **8**) gave no promising results when tested for bone anabolic activity.

3. Conclusions

The successful preparation of the parent amylin-(1–8) peptide **1** and the seven analogues **2**–**8**, wherein the native disulfide bond was modified or replaced as a peptidomimetic and their biological evaluation on osteogenic activity, is described. Analogues **2**–**6** vary in the nature of the bridging linkers (maleimide and benzyl ring or alkyl chains) that were introduced by S-alkylation of the two native cysteine residues at position 2 and 7. Peptide **7** was synthesised via thiol-Michael addition of Cys/7 to α,β unsaturated amide formed upon substitution of Cys/2 with lysine residue followed by attempted coupling of 3-bromopropanoic acid to the N^{ε} of thereof Lys residue. This latter reaction afforded a cyclic peptide with a large, 26-membered ring. Introduction of non-natural amino acid residues (*N*-Fmoc-L-propargylglycine and *N*-Fmoc-L-3-azido-alanine) in place of Cys/2 and Cys/7, respectively, enabled the synthesis of triazole containing peptide **8**.

Importantly, analogue **3** containing a *para* xylyl linker joining Cys/2 and Cys/7, has shown promising bone forming activity. The other synthetic analogues (**2**, **4**–**8**) did not exhibit promising enough osteogenic activity when comparing to the control. All the synthetic analogues of amylin-(1–8) were bench stable in contrast to native octapeptide which is stable for 6 months only at −80 °C under an argon atmosphere. Further work aimed at generation of an extended family of analogues is currently underway in our laboratory.

4. General methods

4.1. Chemistry

4.1.1. Materials

All solvents and reagents were used as supplied. *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*″-tetramethyluronium hexafluorophosphate (HBTU), was purchased from Advanced Chemtech (Louisville, KY), dimethylformamide (DMF) (AR grade) and acetonitrile (HPLC grade) were purchased from Scharlau. Trifluoroacetic acid (TFA) was purchased from Halocarbon (New Jersey), the Fmoc-PAL-PEG-PS resin (5-(4-*N*-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)-pentanoic acid) was purchased from Applied Biosystems. Fmoc-amino acids were purchased from CEM or GL Biochem 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(Dde)–OH was purchased from Iris Biotech. *N*-Fmoc-L-3-azido-alanine (Fmoc-N_3Ala) was synthesised according to previously described procedures[27] and *N*-Fmoc-L-propargylglycine (Fmoc-Pra) was prepared using known procedures.[28–32]

1,2-Dibromoethane and 1,3-dibromopropane were purchased from Merck. *N*-methylpyrrolidine (NMP), diisopropylethylamine (*i*Pr_2NEt), piperidine, piperazine, lutidine, imidazole, 3,6-dioxa-1,8-octanedithiol (DODT), triisopropylsilane (*i*Pr_3SiH), hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$), 2-mercaptoethanesulfonic acid sodium salt (MESNA), sodium ascorbate (Na ascorbate),

α,α′-dibromo-*p*-xylene, α,α′-dibromo-*o*-xylene, 2,3-dibromomaleimide, 3-bromopropanoic acid, *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) and iodoacetamide were purchased from Sigma–Aldrich.

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[35] or manually starting from PAL-PEG polystyrene resin (0.21 mmol/g). For manual synthesis the following steps were undertaken: (a) Fmoc deprotection with 20% piperidine for 5 min, then 15 min, washing with DMF 5×; (b) coupling of the Fmoc amino acid (5 equiv) in the presence of HBTU in DMF (4.9 equiv) and iPr$_2$NEt (10 equiv) for 1 h and washing with DMF 5×. For coupling of Fmoc-Pra (1.5 equiv) and Fmoc-N$_3$Ala (1.5 equiv), 1.45 equiv of HBTU and 4.5 equiv of iPr$_2$NEt were used. The progress of the acylation step was monitored by the Kaiser test. A minimum amount of DMF was used for dissolution of the Fmoc amino acid. The resulting peptides were cleaved from the resin with simultaneous side chain protecting group removal by treatment with either TFA/iPr$_3$SiH/DODT/H$_2$O (v/v/v/v; 94/1/2.5/2.5), or with TFA/iPr$_3$SiH/H$_2$O (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.

Analytical RP-HPLC was performed using a Dionex P680 (flow rate of 1 mL/min), or Dionex Ultimate U3000 system (flow rate of 0.5 mL/min or 0.2 mL/min) using Waters XTerra® column (MS C$_{18}$, 150 mm × 4.6 mm; 5 μm) or, Phenomenex Aqua column (C$_{18}$, 250 mm × 4.6 mm; 5μ), or Phenomenex, Gemini column (C$_{18}$, 50 mm × 2.0 mm, 5μ), using gradient systems as indicated in the Supplementary data.

The solvent system used was A (0.1% TFA in H$_2$O) 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. Peptide masses were confirmed by an inline Thermo Finnegan MSQ mass spectrometer using ESI in the positive mode. When appropriate, a Bruker micrOTOF-Q II mass spectrometer was used for ESI-MS analysis (positive mode). Infrared spectra were obtained using a Perkin Elmer Spectrum One Fourier Transform infrared spectrometer with a universal ATR sampling accessory.

Peptide purification was performed using a Waters 600E or Dionex Ultimate U3000 system using a Waters XTerra® column (C$_{18}$, 300 mm × 19 mm; 10 μm), or 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 three times from 10 mM aq HCl.

4.2. Bone growth activity assays

Osteoblasts were isolated from 20-day foetal rat calvariae. Briefly, calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue, were collected. The calvariae were sequentially digested using collagenase (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. Each of the analogues was screened at three different concentrations. There were six wells in each group and each experiment was repeated three or four times. All treatments were compared to an untreated control, data was analyzed using analysis of variance with post-hoc Dunnet's tests for significant main effect. A 5% significance level (two-tailed) was used throughout.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.030.

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