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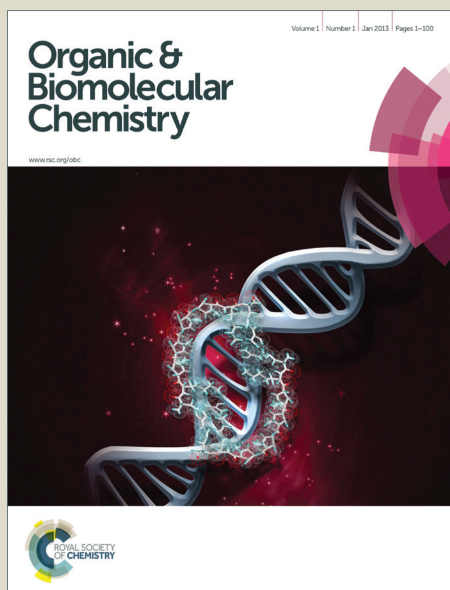

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Convergent chemoenzymatic synthesis of a library of glycosylated analogues of pramlintide:

structure-activity relationships of amylin receptor agonism.

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Abstract

Pramlintide (Symlin®), a synthetic analogue of the naturally occurring pancreatic hormone amylin, is currently used with insulin in adjunctive therapy for type 1 and type 2 diabetes mellitus. Herein we report a systematic study into the effect that *N*-glycosylation of pramlintide has on activation of amylin receptors. A highly efficient convergent synthetic route, involving a combination of solid phase peptide synthesis and enzymatic glycosylation, delivered a library of *N*-glycosylated variants of pramlintide bearing either GlcNAc, the core *N*-glycan pentasaccharide [$Man_3(GlcNAc)_2$] or a complex biantennary glycan [$(NeuAcGalGlcNAcMan)_2Man(GlcNAc)_2$] at each of its six asparagine residues. The majority of glycosylated versions of pramlintide were potent receptor agonists, suggesting that *N*-glycosylation may be used as a tool to optimise the pharmacokinetic properties of pramlintide and so deliver improved therapeutic agents for the treatment of diabetes and obesity.

Introduction

Diabetes mellitus (DM) is a chronic disease that results from defects in either insulin secretion, or action, or both, that culminates in disturbances of carbohydrate, fat and protein metabolism.[1] An estimated 350 million people were affected by DM worldwide in 2008, and this number is likely to rise to ~440 million by 2030.[2, 3] There are three major types of diabetes: type 1, type 2, and gestational diabetes mellitus. Type 1 diabetes results from autoimmune-mediated destruction of pancreatic beta-cells, leading to insufficient insulin production; treatment requires an exogenous insulin supply. The most common form of diabetes is type 2, which is characterised by defects in insulin secretion and/or action, and resistance to insulin activity, leading to high blood sugar levels. The onset of gestational diabetes mellitus, the third most predominant subtype of diabetes, occurs during pregnancy, and is characterised by sugar intolerance resulting in hyperglycaemia.[1] Therapies to treat diabetes mainly focus on lowering excessive blood sugar levels by subcutaneous injections

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of biosynthetic human insulin and insulin analogues.[4, 5] Insulin therapy itself has limitations, including the risk of recurrent severe hypoglycaemia and weight gain.[6] Islet amyloid polypeptide (IAPP), also referred to as amylin, is a 37-amino acid regulatory hormone that is co-secreted with insulin from the beta-cells of the pancreatic islets of Langerhans in response to food intake (Figure 1).[7,8] Amylin, together with insulin and glucagon, plays an important role in glucose homeostasis in healthy individuals.[6] Amylin controls insulin and glucagon action by modulating gastric emptying, preventing postprandial spikes in blood glucose levels;[9, 10] it is also associated with increased satiety leading to decreased food intake and weight loss.[10]

In principal amylin replacement therapy could therefore be a useful treatment for diabetes. However, significant issues with the therapeutic use of amylin arise from the tendency of human amylin to self-associate into toxic amyloid fibres, which are associated with beta-cell death.[10, 11] Interestingly, rat amylin is not amyloidogenic and therefore non-toxic,[11] a property which has been attributed to the presence of three proline residues (Pro-25, Pro-28 and Pro-29) in the peptide that impedes the formation of amyloid fibrils.[12] Pramlintide (or Symlin®) was therefore developed by exchanging the corresponding residues of human amylin (Ala-25, Ser-28 and Ser-29) for proline, to afford a more stable and soluble, non-aggregating and yet equipotent peptide in comparison with human amylin (Figure 1).[10, 13, 14]

Figure 1. Primary sequence of human and rat amylin and amylin analogue pramlintide.

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Pramlintide is currently used as an adjunctive therapy for type 1 and type 2 diabetes.[4, 13] Despite the therapeutic benefits of pramlintide, including diminished food intake, delayed gastric emptying, smoothing of blood glucose levels, and reduction in excessive glucagon release, the pharmacokinetic properties of the peptide are not ideal. For example it has a circulatory half-life of only 48 minutes, and must be injected three times daily. Additionally, its solubility is highly pH dependent (it has been reported to precipitate above pH 5.5), and therefore co-formulation with insulin is currently not possible.[10, 15] Chemical modification of pramlintide to afford a better therapeutic agent is therefore an attractive option. Hansen *et al.*[16] have investigated the biological activity of various pramlintide analogues containing polyethylene glycol or various hydrocarbon chains, incorporated *via* lysine or cysteine residues at various positions within the pramlintide sequence. Some of these analogues exhibited similar or improved amylin receptor affinity profiles, together with improved solubility as compared to the parent pramlintide.[16]

Glycosylation of peptides and proteins has proven to be an effective tool to improve their physiochemical and pharmacokinetic profiles, including protection from oxidation and cross-linking.[17, 18] Reduced unfolding and denaturation upon changing the pH or freezing, reduced aggregation, and increased solubility and proteolytic stability can also be achieved by glycosylation.[17-20] Several examples of therapeutic drugs which display increased stability due to glycosylation have been reported.[17] Improved proteolytic resistance has been reported following the glycosylation of glucagon-like peptide 1 (GLP-1),[19] insulin[21] and exendin-4,[22] all of which are therapeutic agents that may be used to treat diabetes. Interestingly, the naturally occurring *O*-glycosylated forms of amylin (*O*-glycans attached to Thr-4 and Thr-6) are not biologically active;[23] this lack of activity may result from steric effects caused by the presence of glycans close to the *N*-terminal receptor binding region.[24] We recently reported[25] the first synthesis of glycosylated variants of pramlintide in which different *N*-glycans, namely a monosaccharide (GlcNAc), a pentasaccharide (the core *N*-glycan pentasaccharide, $Man_3(GlcNAc)_2$) and an undecasaccharide

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(the complex biantenary glycan, $(NeuAcGalGlcNAcMan)_2Man(GlcNAc)_2$) were attached to Asn-3 or Asn-21. Agonist activity at an amylin receptor (AMY_1) was retained for all three variants in which Asn-21 was glycosylated. Whilst the peptide bearing GlcNAc at Asn-3 was also a potent agonist, the presence of larger glycans at Asn-3 decreased biological activity, indicating that the presence of bulky sugars close to the *N*-terminus of the peptide sterically hindered the receptor binding region of pramlintide, in line with the lack of activity observed for naturally *O*-glycosylated amylin.[24] An *in vivo* investigation of pramlintide analogues containing either GlcNAc or the complex biantennary glycan at Asn-21, by means of a glucose tolerance test in Sprague Dawley rats, demonstrated equal, if not more effective, smoothing of blood glucose levels when compared to pramlintide.[25] These promising results encouraged a more extensive study into the effects of glycan structure and the position of *N*-glycosylation on *in vitro* activity towards amylin receptors. Pramlintide contains six possible sites for *N*-glycosylation, Asn-3, Asn-14, Asn-21, Asn-22, Asn-31 and Asn-35. A comprehensive systematic study was initiated in which each of these six sites was glycosylated with three carbohydrate structures. Whilst incorporation of carbohydrates at any of these positions may reduce peptide aggregation, improve solubility or inhibit proteolytic cleavage, reduced agonist activity could also be observed. The objective of this investigation was to determine the site- and glycan-specific nature of the effects that glycosylation of pramlintide has on receptor agonism *in vitro*, prior to embarking on detailed *in vivo* biological studies.

Amylin acts *via* a G protein-coupled receptor – the calcitonin receptor (CT), which has enhanced affinity for amylin in the presence of receptor activity-modifying proteins (RAMPs).[26] There are three RAMPs (RAMP1,2,3) and several splice variants of the calcitonin receptor, creating many different amylin receptor subtypes. The best characterised of these are $AMY_{1(a)}$ and $AMY_{3(a)}$, which contain the $CT_{(a)}$ splice variant of the calcitonin receptor.[27, 28] Pramlintide is as active at these receptors as human or rat amylin.[29]

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The synthesis of *N*-linked glycopeptides and glycoproteins is a particularly challenging task. Amongst various reported approaches,[30] the convergent assembly of glycopeptides by enzymatic mediated addition of *N*-glycans, which are activated at the reducing terminus as oxazolines,[31-43] is a particularly powerful method for the construction of a glycopeptide library, the objective of the current study. Glycopeptides bearing different *N*-glycan structures at the same asparagine residue can all be prepared from the same peptide glycosyl acceptor by glycosylation with different oxazolines. Additionally the site of glycosylation can also be varied by incorporation of the GlcNAc asparagine building block at pre-designed sites of the peptide sequence allowing ready access to a library of materials in which both the site of glycosylation and the nature of the attached glycan are systematically varied. Critical to the current study was the ability to access a reliable and efficient synthetic method to access *C*-terminally amidated pramlintide with the requisite Cys-2/Cys-7 disulfide bond in place, structural features which are both crucial for biological activity of pramlintide.[44]

The synthetic platform reported herein enabled the preparation of a comprehensive series of pramlintide analogues containing a GlcNAc at each of the six asparagine residues. These peptides were then used as acceptor substrates for endo-β-N-acetylglucosaminidases (ENGase) mediated glycoside extension, in combination with either fully synthetic or naturally-derived *N*-glycan oxazoline donors.[32, 43, 45, 46] Accordingly, native pramlintide **1** and eighteen *N*-glycosylated analogues containing GlcNAc **2-7**, the core *N*-glycan pentasaccharide [$Man_3(GlcNAc)_2$] **8-13**, and a complex biantennary *N*-glycan undecasaccharide [$(NeuAcGalGlcNAcMan)_2Man(GlcNAc)_2$] **14-19** at position 3, 14, 21, 22, 31 or 35, were synthesised in a convergent fashion using a combination of synthetic chemistry and enzymatic glycosylation (Figure 2). The biological activity of pramlintide and its eighteen *N*-glycosylated analogues was screened against amylin receptors. An overview of the synthesis of pramlintide and its *N*-glycosylated variants, and the biological activity of these compounds is reported herein.

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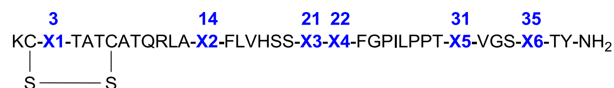

1 : X1-X6 = Asn = Pramlintide

2 : X1 = Asn(**GlcNAc**); X2-X6 = Asn
3 : X2 = Asn(**GlcNAc**); X1, X3-X6 = Asn
4 : X3 = Asn(**GlcNAc**); X1, X2, X4-X6 = Asn
5 : X4 = Asn(**GlcNAc**); X1-X3, X5, X6 = Asn
6 : X5 = Asn(**GlcNAc**); X1-X4, X6 = Asn
7 : X6 = Asn(**GlcNAc**); X1-X5 = Asn

8 : X1 = Asn(**Man_3**(**$GlcNAc)_2$**)); X2-X6 = Asn
9 : X2 = Asn(**Man_3**(**$GlcNAc)_2$**)); X1, X3-X6 = Asn
10 : X3 = Asn(**Man_3**(**$GlcNAc)_2$**)); X1, X2, X4-X6 = Asn
11 : X4 = Asn(**Man_3**(**$GlcNAc)_2$**)); X1-X3, X5, X6 = Asn
12 : X5 = Asn(**Man_3**(**$GlcNAc)_2$**)); X1-X4, X6 = Asn
13 : X6 = Asn(**Man_3**(**$GlcNAc)_2$**)); X1-X5 = Asn

14 : X1 = Asn[(**NeuAcGalGlcNAcMan**$)_2$**Man**(**GlcNAc**$)_2$]; X2-X6 = Asn
15 : X2 = Asn[(**NeuAcGalGlcNAcMan**$)_2$**Man**(**GlcNAc**$)_2$]; X1, X3-X6 = Asn
16 : X3 = Asn[(**NeuAcGalGlcNAcMan**$)_2$**Man**(**GlcNAc**$)_2$]; X1, X2, X4-X6 = Asn
17 : X4 = Asn[(**NeuAcGalGlcNAcMan**$)_2$**Man**(**GlcNAc**$)_2$]; X1-X3, X5, X6 = Asn
18 : X5 = Asn[(**NeuAcGalGlcNAcMan**$)_2$**Man**(**GlcNAc**$)_2$]; X1-X4, X6 = Asn
19 : X6 = Asn[(**NeuAcGalGlcNAcMan**$)_2$**Man**(**GlcNAc**$)_2$]; X1-X5 = Asn

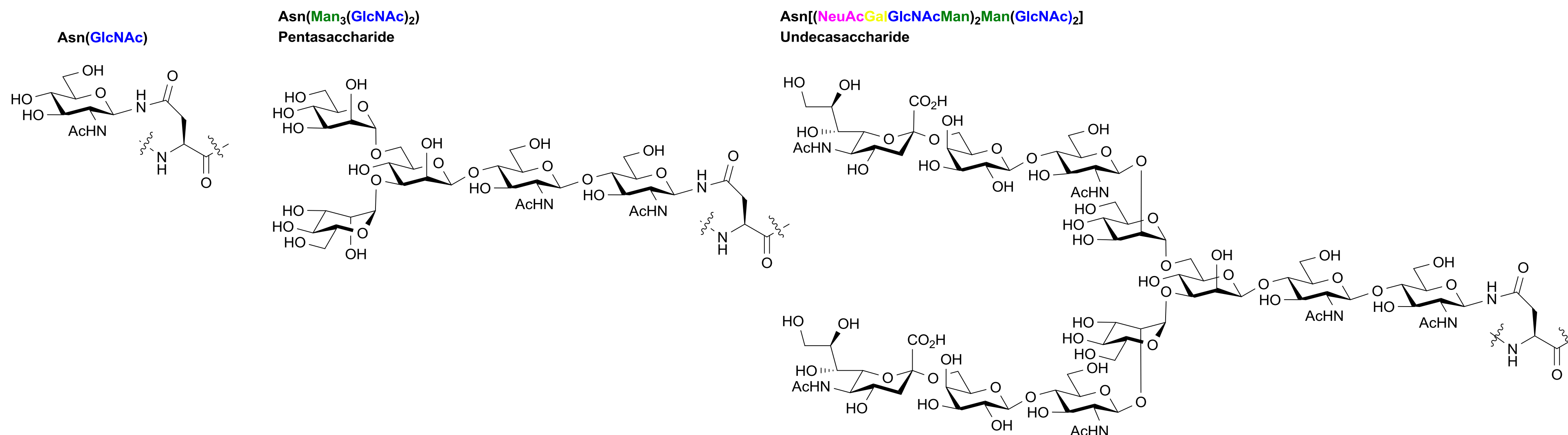

Figure 2. Pramlintide **1** and *N*-glycosylated pramlintide analogues **2**-**19** synthesised and tested.

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Results and Discussion

Synthesis of pramlintide 1

Although several synthetic approaches have been reported for the preparation of pramlintide,[47-50] none of these have used microwave conditions. Precise heating of the reaction mixture *via* microwave irradiation during peptide coupling or 9-fluorenylmethoxycarbonyl (Fmoc) removal generally results in more efficient peptide synthesis, and microwave-enhanced Fmoc Solid Phase Peptide Synthesis (Fmoc SPPS) has shown to be effective for the synthesis of glycopeptides, phosphopeptides, *N*-methylated peptides, β-peptides and so-called "difficult" sequences,[51] including human amylin.[52-54] Microwave-enhanced Fmoc SPPS of linear pramlintide **20** was therefore employed in the present work. Aminomethyl polystyrene resin,[55, 56] functionalised with a 4-[(R,S)-α-[1- (9H-floren-9-yl)]methoxycarbonylamino]-2,4-dimethoxy]phenoxyacetic acid (Rink amide linker), was used to prepare the linear peptide **20** in combination with a CEM Liberty 12 automated peptide synthesiser (Scheme 1).[54] *O*-(6-Chlorobenzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethyluronium hexafluorophosphate (HCTU) and *N*,*N*-diisopropylethylamine ($i\mathrm{Pr_2EtN}$) were employed as the coupling reagents, and 20% piperidine in *N,N*-dimethylformamide (DMF) was used to remove the N^{α}-Fmoc protecting groups. Finally, cleavage from the resin and global removal of side-chain protecting groups was carried out using trifluoroacetic acid/ triisopropylsilane/ water/ 3,6-dioxa-1,8-octane-dithiol (TFA/$i\mathrm{Pr_3SiH}$/H_2O/DODT) at room temperature, which afforded linear pramlintide **20** with a crude purity of 49% (Figure S1). Notably this crude purity was higher than the synthesis reported by Mohe *et al*.[50] (37%), which used a pseudoproline building block.[57, 58] Disulfide bond formation was performed directly on crude **20** using a 0.1 M tris(hydroxymethyl)aminomethane (Tris·HCl) buffer (pH 8.4) in water at various concentrations (1 mg/mL, 3 mg/mL and 5 mg/mL), which afforded the desired product **1** after 24 h (as evidenced by liquid chromatography-mass spectrometry (LC-MS), data not shown).

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However, the poor solubility of the starting material resulted in low isolated yields of peptide **1**. Disulfide bond formation was subsequently attempted using a mixture of 0.1 M Tris·HCl buffer (pH 8.4) in a mixture of dimethyl sulphoxide (DMSO) and water (1 : 3), at a concentration of 5 mg/mL. Whilst the use of DMSO as co-solvent did improve the solubility, a long reaction time (4 h) was required for the reaction to go to completion. Previously the use of 2,2'-dipyridyl disulfide (DPDS)[59] had effected rapid disulfide bond formation during the syntheses of amylin[54] and vesiculin,[60] carried out in our laboratory, and again this proved to be an effective reagent; cyclization of crude linear pramlintide **20** was complete in 20 min when treated with DPDS in DMSO at a high concentration (10 mg/mL), (as confirmed by LC-MS, Figure S 2). Subsequent purification using semi-preparative reverse phase high-performance liquid chromatography (RP-HPLC) afforded pramlintide **1** in 99% purity, with the desired Cys-2/Cys-7 disulfide bond in place.

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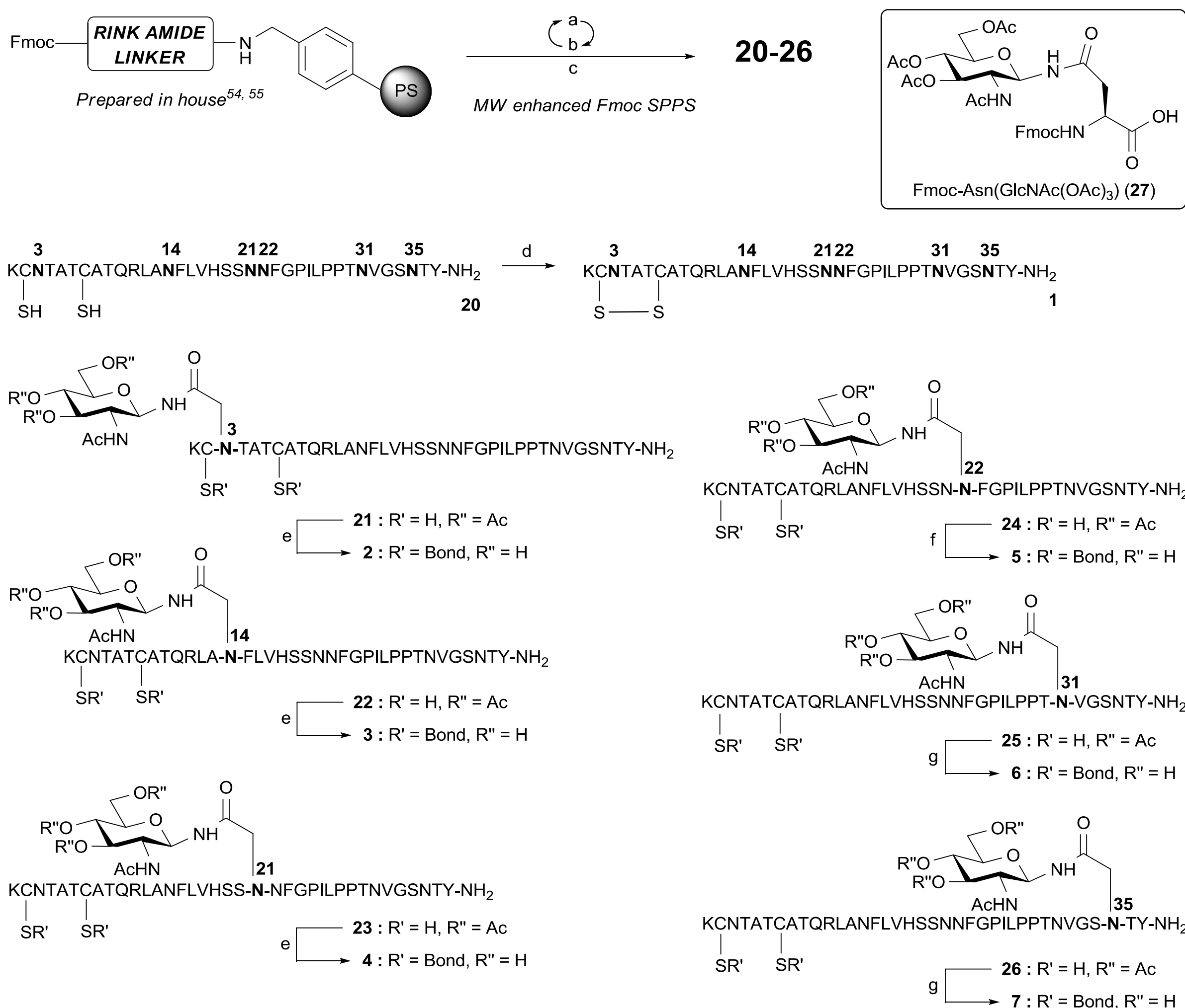

Reagents and Conditions: (a) 20% piperidine, DMF, 0.5 min then 3 min cycle, 62 W, 75 ºC; (b) Fmoc-amino acid, HCTU, *i*PrNEt, 5 min, 25 W, 75 ºC *or* Fmoc-Arg(Pbf), HCTU, *i*PrNEt, 25 min, rt followed by 3 min, 25 W, 75 ºC *or* **27,** HATU, collidine, 15 min, 25 W, 75 ºC; (c) TFA, *i*Pr_3SiH, DODT, H_2O, 2 h, rt; (d) DPDS, DMSO, rt; (e) DPDS, DMSO; then 13% NH_2NH_2·1.5 H_2O, rt; (f) 5% NH_2NH_2·1.5 H_2O, 10% DMSO, 85% 6 M Gu·HCl, 17 h, rt; (g) DPDS, DMSO; then 5% NH_2NH_2·1.5 H_2O, rt.

Scheme 1. Synthesis of pramlintide **1**, and pramlintide analogues **2**-**7** containing a GlcNAc residue at Asn-3, Asn-14, Asn-21, Asn-22, Asn-31 or Asn-35.

Synthesis of GlcNAc pramlintide analogues 2-7

With an established procedure for the synthesis of pramlintide **1** in hand, attention focused on the preparation of *N*-glycosylated analogues **2**-**7**, containing a GlcNAc attached to the asparagine residue at position 3, 14, 21, 22, 31 or 35. Microwave-enhanced Fmoc SPPS generated linear pramlintide analogues **21**-**26** with free thiols at Cys-2 and Cys-7, in which the

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GlcNAc residue was acetate protected (Scheme 1). The GlcNAc(OAc)$_3$ moiety was installed by incorporation of the glycosyl amino acid building block **27**[61] into the microwave-assisted SPPS synthesis, using *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) and collidine activation.

Glycopeptides **25** and **26**, in which the GlcNAc(OAc)$_3$ was incorporated close to the *C*-terminus of pramlintide (Asn-31 and Asn-35, respectively), were readily accessed in high purity. The corresponding glycopeptides containing partially de-protected sugar hydroxyls (42 Da and 84 Da less) were the only impurities detected by LC-MS (Figure S 19 and Figure S 23, respectively) and their presence was not problematic as acetate removal was performed at a later stage during the synthesis. The synthesis of *N*-glycosylated analogues **21**-**24**, in which the glycosyl amino acid was incorporated at positions 3, 14, 21 or 22, proved to be slightly more difficult. In these cases the crude glycopeptide products contained other impurities as well as partially de-protected GlcNAc(OAc)$_3$ units. The unexpected formation of a truncated and *N*-terminally Thr-4 acetylated minor by-product (Ac-TATCATQRLANFLVHSSNNFGPILPPTNVGSNTY-NH_2; ESI-MS, 730.3 ([M + 5H]$^{5+}$ requires 730.41) was observed during the synthesis of the Asn-3 glycopeptide **21** (Figure S 4). Similarly, an *N*-acetylated truncated peptide, terminating at Phe-15 (Ac-FLVHSSNNFGPILPPTNVGSNTY-NH_2) was detected during the synthesis of glycopeptide **22** (ESI-MS, 839.6 ([M + 3H]$^{3+}$ requires 839.60, Figure S 8). However the most significant formation of undesired truncated *N*-acetylated by-products, namely Ac-NFGPILPPTNVGSNTY-NH_2 and Ac-FGPILPPTNVGSNTY-NH_2, occurred during the synthesis of **23** and **24** (Figure S 12 and Figure S 16, respectively). Significantly, the truncated peptides were all formed before incorporation of the glycosylated amino acid **27**. Moreover, truncated by-products were not observed during the synthesis of the *N*-glycopeptides containing sugars towards the *C*-terminus (Asn-31 and Asn-35). These observations led us to conclude that *N*-acetylation occurred during incorporation of the sterically demanding Fmoc-

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Asn(GlcNAc(OAc)$_3$) building block (**27**), presumably resulting from acyl transfer of acetate protecting groups present on **27** to the *N*-terminus of the peptide. Alternatively *N*-Acetylation could also possibly result from partial acetate hydrolysis of **27** leading to the presence of acetic acid.

With the linear *N*-glycosylated pramlintide analogues **21-26** in hand, attention turned to removal of the acetate protecting groups and formation of the Cys-2/Cys-7 cyclic disulfide. Although subjection of **24** to a 'one-pot' de-protection / cyclisation procedure, using 5% hydrazine in 10% DMSO aqueous solution (6 M guanidinium hydrochloride),[62] gave pramlintide analogue **5** in sufficient quantities for subsequent studies (Scheme 1), long reaction times were required. A more efficient process was arrived at in which both reactions were carried out sequentially in the same vessel. Treatment of **26** with DPDS in DMSO gave the cyclic disulfide after 1.5 h, and the subsequent addition of 5% hydrazine hydrate then afforded the desired pramlintide analogue **7** after a further 4 h. This procedure was also used to prepare pramlintide analogue **6** (Scheme 1). The use of a higher concentration of hydrazine hydrate (13%) resulted in faster acetate removal (2 h) and was used to access glycopeptides **2-4**. Finally RP-HPLC purification enabled the isolation of pure samples of the desired *N*-GlcNAc pramlintide analogues **2**, **3**, and **5-7** in excellent purities ranging from 97% to 99%.*

Synthesis of N-pentasaccharide and N-undecasaccharide pramlintide analogues 8-19

The use of the ENGases, such as Endo A[63, 64] and Endo M[65-68] in combination with oxazolines as activated donors has proven to be extremely powerful method for the synthetic of a wide variety of glycoconjugates bearing *N*-glycan structures.[33-43,72] The inherent synthetic utility of these enzymes is further enhanced by the production of glycosynthase mutants[69-73] (enzymes

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* RP-HPLC purification of pramlintide analogue **4** (GlcNAc at Asn-21), afforded only a trace amount of pure material that was insufficient for subsequent enzymatic glycosylation and receptor binding studies. Therefore, a commercial sample of **4** (sourced from Mimotopes)[25] was used for this study.

that are capable of performing synthetic reactions but which are incapable of product hydrolysis), such as the commercially available N175Q mutant[71] of Endo M developed by Wang and Yamamoto.

Endo A transfers high mannose glycans to variety of acceptors bearing a GlcNAc acceptor and the kinetics of the wild-type enzyme are such that, when using truncated glycan structures, high yields of product can be achieved by using short reaction times. Endo M is additionally capable of transferring complex biantennary glycans to acceptors, and the yields that are achievable using this enzyme are significantly enhanced by the use of the N175Q mutant. Pramlintide analogues **8-19**, containing either a pentasaccharide or an undecasaccharide at Asn-3, 14, 21, 22, 31 or 35 were prepared by enzymatic syntheses using either tetrasaccharide oxazoline **28** (Scheme 2) or decasaccharide oxazoline **29** (Scheme 3) as the glycosyl donor, in conjunction with either Endo A or Endo M N175Q, respectively. Tetrasaccharide **28** was accessed by total synthesis as previously described,[43] whilst decasaccharide **29** was prepared from the corresponding reducing sugar, which in turn was itself isolated from hens' eggs, using known procedures.[74, 75] Endo A effected the enzymatic transfer of **28** to glycosyl acceptor peptides **2-7**, to afford pramlintide analogues **8**-**13**, in which the core *N*-glycan pentasaccharide was attached to the corresponding asparagine residue (Scheme 2).

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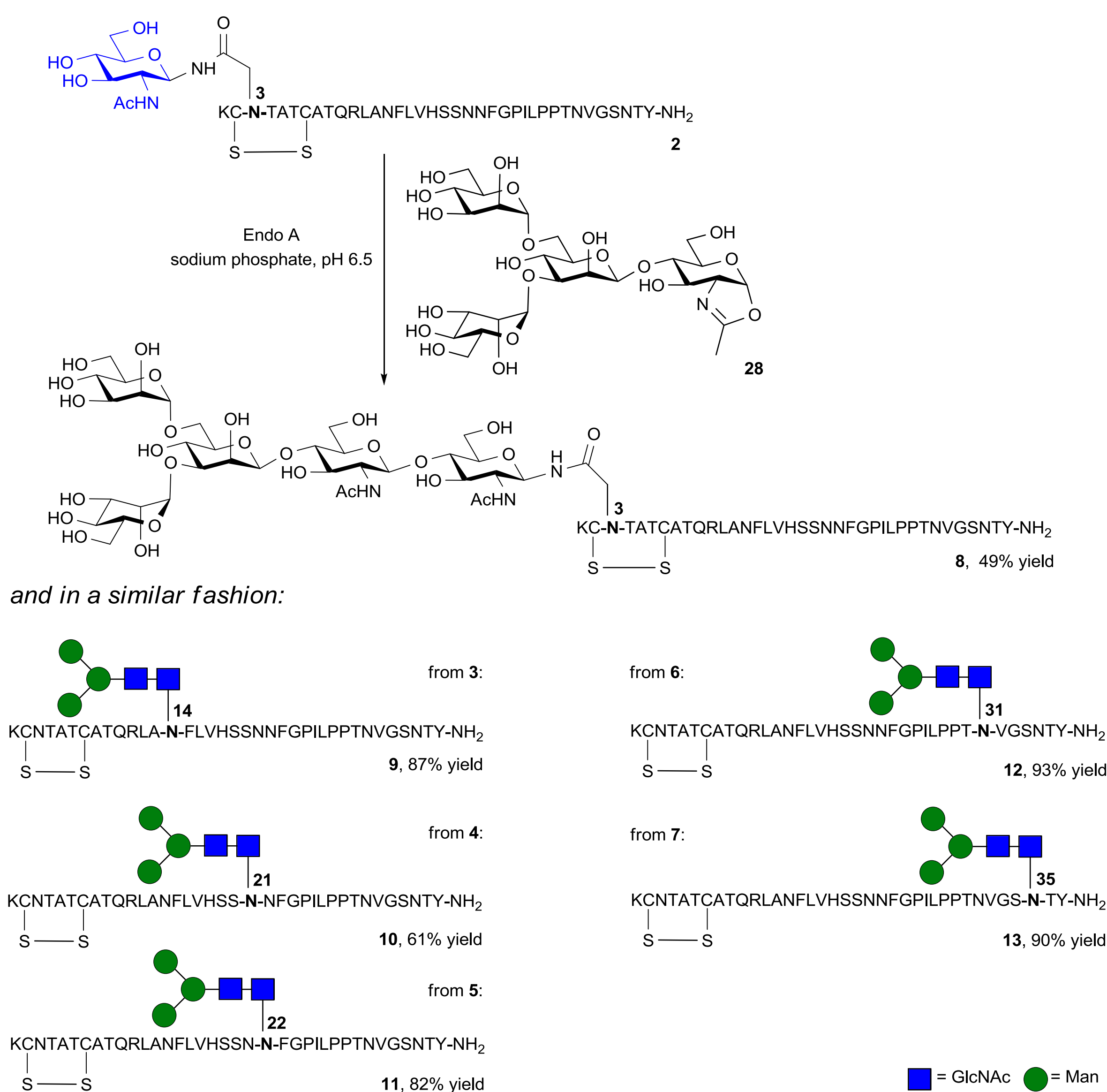

Scheme 2. Synthesis of pramlintide analogues **8**-**13** containing the core *N*-glycan pentasaccharide [$Man_3(GlcNAc)_2$] at position 3, 14, 21, 22, 31 or 35.

In a similar fashion, the use of **29** as the glycosyl donor, in conjunction with the N175Q mutant of Endo M effected conversion of GlcNAc peptides **2-7** into the more elaborate glycopeptides **14-19**, each bearing a complex bi-antennary glycan at the designated asparagine residue (Scheme 3). Subsequent purification by RP-HPLC and high-resolution mass spectrometry (HRMS) analysis confirmed the structures of the synthetic glycopeptides **8-19**.

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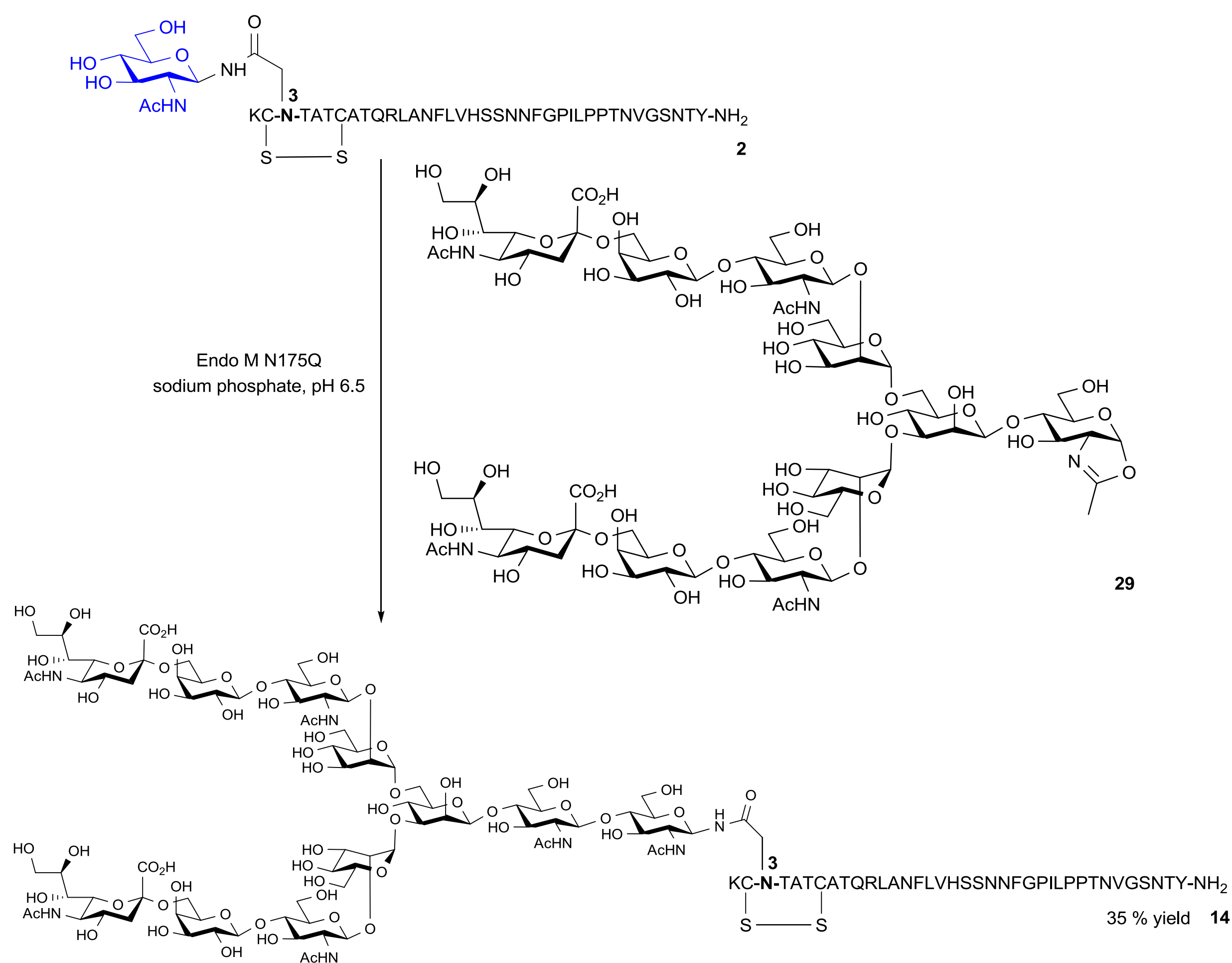

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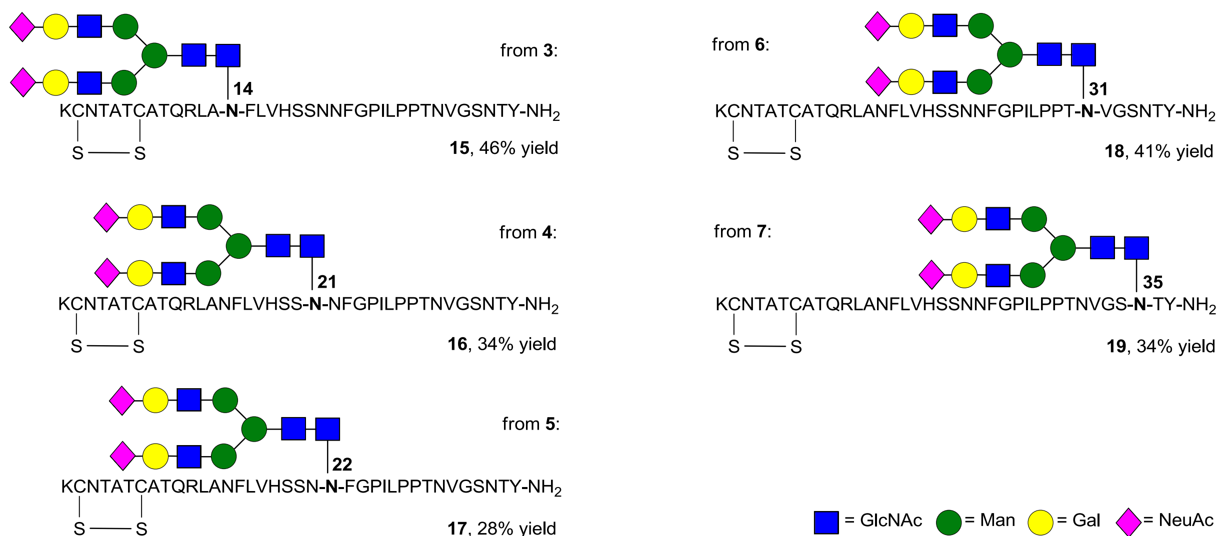

Scheme 3. Synthesis of pramlintide analogues **14-19** containing a complex biantennary glycan [(NeuAcGalGlcNAcMan)$_2$Man(GlcNAc)$_2$] at position 3, 14, 21, 22, 31 or 35.

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The apparent ease with which this small library of highly complex glycoconjugates was assembled is a clear demonstration of the power of this convergent chemoenzymatic approach to complex glycopeptides.

In order to investigate the effects of both the nature of the glycan structure and the site of *N*-glycosylation, the activity of the synthetic pramlintide **1** and glycopeptides **2-19** was screened at amylin receptors. Cos 7 cells were transiently transfected with the necessary receptor components, and cyclic AMP production was measured according to our published methods.[76, 77]

Pramlintide **1** and glycopeptides **2-7** were tested at three receptors: $CT_{(a)}$, $AMY_{1(a)}$ and $AMY_{3(a)}$ and the data are summarised in Table 1. In line with our earlier study, analogues **8-19** were only tested at $AMY_{1(a)}$, and were directly compared to **1** in each experiment (Table 2). The glycosylated pramlintide analogues **2-6** generally exhibited slightly lower potency when compared to the parent pramlintide **1** at all three receptors. However, statistical significance was only observed consistently for analogue **3**, which contained a GlcNAc moiety at Asn-14. Its potency was reduced by approximately 3-fold (Table 1). Attaching larger sugar moieties to Asn-3, Asn-14 and Asn-22 also caused reductions in potency, ranging between ~5 and ~40-fold, depending on the analogue. However, at Asn-21, Asn-31 and Asn-35, even the largest, complex biantennary glycan was well-tolerated (Table 2). Figure 3 shows the effect of each of the sugar moieties at Asn-3 and Asn-35. The decreased activity of some analogues is presumably due to unfavourable steric interactions, arising from the proximity of the bulky glycan and interference with the binding of the peptide to the receptor. In the cases where the glycan is attached towards the *N*-terminus (Asn-3, Asn-14), this may be due to impaired binding within the transmembrane bundle or extracellular loops of the receptor.[24, 78] In addition, the bulky sugar moieties incorporated into pramlintide may have influenced the conformation of pramlintide leading to decreased activity of some of the glycosylated analogues.

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A clear pattern was observed in which activity of the analogues at the receptors decreased inversely with the size of the sugar incorporated (i.e. undecasaccharide < pentasaccharide < GlcNAc). This pattern was more pronounced when the sugar was incorporated towards the *N*-terminus of pramlintide. We have therefore demonstrated that receptor activity (at $AMY_{1(a)}$) is maintained when *N*-glycosylation of pramlintide was performed towards the *C*-terminal site of the peptide (Asn-21, Asn-31 and Asn-35).

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	$AMY_{1(a)}$			$AMY_{3(a)}$			$CT_{(a)}$		
	Control pEC_{50}	**Analogue pEC_{50}**	**Fold change from control**	**Control pEC_{50}**	**Analogue pEC_{50}**	**Fold change from control**	**Control pEC_{50}**	**Analogue pEC_{50}**	**Fold change from control**
2	9.28 ± 0.07 (3)	8.89 ± 0.07 (3)	-2.5	9.01 ± 0.09 (3)	8.91 ± 0.30 (3)	-1.3	8.20 ± 0.17 (3)	7.70 ± 0.24 (3)*	-3.2
3	9.12 ± 0.19 (3)	8.69 ± 0.21 (3)**	-2.7	8.93 ± 0.18 (3)	8.38 ± 0.17 (3)**	-3.5	8.48 ± 0.25 (4)	7.88 ± 0.31 (4)*	-4.0
4	9.03 ± 0.09 (3)	8.70 ± 0.17 (3)	-2.1	8.91 ± 0.11 (3)	8.52 ± 0.22 (3)	-2.5	8.10 ± 0.37 (3)	7.78 ± 0.12 (3)	-2.1
5	9.12 ± 0.19 (3)	8.65 ± 0.16 (3)	-3.2	8.93 ± 0.18 (3)	8.60 ± 0.17 (3)	-2.1	8.69 ± 0.20 (3)	8.40 ± 0.03 (3)	-1.9
6	9.19 ± 0.15 (4)	8.78 ± 0.23 (4)*	-2.6	8.93 ± 0.18 (3)	8.47± 0.13 (3)	-2.9	8.62 ± 0.16 (4)	8.39 ± 0.24 (4)	-1.7
7	9.18 ± 0.16 (3)	9.18 ± 0.06 (3)	0	8.66 ± 0.29 (4)	8.96 ± 0.32 (4)	+2.0	8.18 ± 0.20 (4)	8.12 ± 0.14 (4)	-1.1

Table 1. Summary of potency (pEC_{50}) values of pramlintide analogues **2-7** at the $CT_{(a)}$, $AMY_{1(a)}$, and $AMY_{3(a)}$ receptors. Values are mean ± SEM. Numbers in parentheses represent the number of independent experiments analysed. Paired t-tests were performed to compare to **1** (control); $*P < 0.05$, $**P < 0.01$.

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	AMY$_{1(a)}$				AMY$_{1(a)}$		
	Control pEC$_{50}$	**Analogue pEC$_{50}$**	**Fold change from control**		**Control pEC$_{50}$**	**Analogue pEC$_{50}$**	**Fold change from control**
8	9.21 ± 0.27 (4)	8.10 ± 0.11 (4)**	-12.9	**14**	9.52 ± 0.16 (4)	7.89 ± 0.11 (4)**	-42.7
9	9.30 ± 0.29 (4)	7.99 ± 0.33 (4)*	-20.4	**15**	9.26 ± 0.17 (4)	8.55 ± 0.20 (4)*	-5.1
10	9.30 ± 0.29 (4)	9.22 ± 0.21 (4)	-1.2	**16**	9.06 ± 0.12 (4)	8.62 ± 0.20 (4)	-2.8
11	9.25 ± 0.26 (4)	8.58 ± 0.27 (4)***	-4.7	**17**	9.06 ± 0.12 (4)	8.17 ± 0.11 (4)*	-7.8
12	9.44 ± 0.20 (4)	9.25 ± 0.13 (4)	-1.5	**18**	9.06 ± 0.12 (4)	8.77 ± 0.12 (4)	-1.9
13	9.32 ± 0.19 (4)	9.21 ± 0.30 (4)	-1.3	**19**	8.89 ± 0.12 (4)	9.17 ± 0.18 (4)	+1.9

Table 2. Summary of potency (pEC$_{50}$) values of pramlintide analogues **8-19** at the AMY$_{1(a)}$ receptor. Values are mean ± SEM. Numbers in parentheses represent the number of independent experiments analysed. Paired t-tests were performed to compare to **1** (control); *P < 0.05, **P < 0.01.

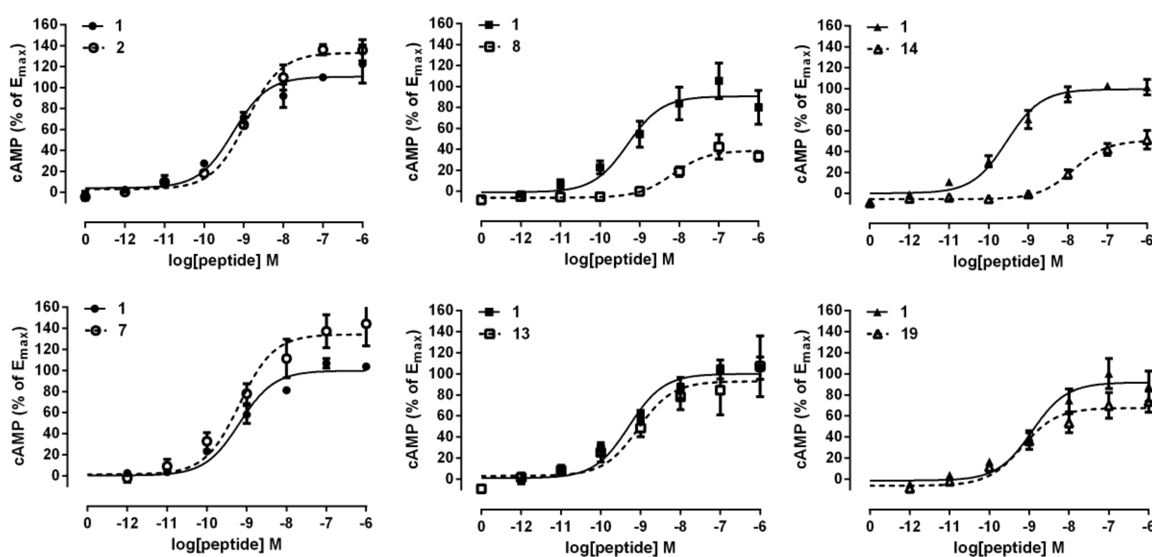

Figure 3. Activity of **1**, **2**, **7**, **8**, **13**, **14** and **19** at the AMY$_{1(a)}$ receptor. Data are combined from at least three independent experiments and show the mean ± S.E.M.

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Conclusions

This report details the first systematic study of the effect of asparagine glycosylation of pramlintide on its activity at amylin-responsive receptors. Although glycosylation with large glycans close to the *N*-terminus resulted in reduced response, agonist activity was maintained for most analogues where the glycans were incorporated towards the *C*-terminus. Since *in vitro* activity was maintained, these results support the hypothesis that *N*-glycosylation may be an effective tool for improvement of the pharmacokinetic properties of therapeutic peptides.

Additionally this work established a reliable and efficient synthetic platform that was used to access pramlintide and eighteen *N*-glycosylated analogues. Central to the convergent synthesis was the use of a microwave-enhanced Fmoc SPPS protocol that did not require the use of a pseudoproline building block,[57, 58] and employed only one RP-HPLC purification step. Subsequently the enzymatic extension of peptides bearing GlcNAc residues using different oxazoline donors demonstrated the utility of ENGases for the convergent assembly of glycopeptide libraries in which the structural features were varied systematically. The development of *N*-glycosylated analogues of pramlintide that possess improved pharmacokinetic properties should improve both treatment regimes and the lives of diabetes sufferers. Further investigations into the *in vivo* biological effects of these, and other glycosylated analogues of pramlintide, are currently in progress, and the results will be reported in due course.

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Supplementary data

Experimental data for the synthesis and receptor activity testing of compounds **1-19** is available free of charge in the online version, at doi:

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