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Automated glycopeptide assembly by combined solid-phase peptide and oligosaccharide synthesis†

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Current strategies for the synthesis of glycopeptides require multiple manual synthetic steps. Here, we describe a synthesis concept that merges solid phase peptide and oligosaccharide syntheses and can be executed automatically using a single instrument.

Most human proteins are glycosylated. The glycan structure as well as the exact location and linkage to the protein influence the glycoprotein function. Establishing structure–activity relationships of glycoproteins is difficult since these molecules are typically obtained as heterogeneous mixtures of glycoforms. Contrary, synthetic glycopeptides are homogeneous and can serve to elucidate the biological significance of specific glycoprotein fragment.[1] In addition, synthetic glycopeptides have been explored as diagnostic tools and vaccine candidates.[1d,2]

Glycopeptide synthesis has been extensively developed in recent years.[3] *O*-Glycopeptides are usually synthesized *via* two main routes based on early work by Kaifu and Osawa.[4] One strategy incorporates glycosylated amino acid building blocks in solid phase peptide synthesis (SPPS) (Fig. 1A).[1b,c,5] Alternatively, a shortened glycan is grafted onto the peptide backbone and after completion of peptide synthesis, the glycan is further elongated by chemical or chemoenzymatic methods (Fig. 1B).[6] In both strategies, glycosylated amino acids are required for glycopeptide assembly by SPPS. The synthesis of glycosylated amino acids is time consuming, as it typically requires multistep solution phase procedures. Most building blocks are not commercially available or extremely expensive to purchase in large quantities. Lack of accessibility limits the use of these building blocks in solid phase glycopeptide synthesis wherein large amounts of building blocks are used for each coupling step.

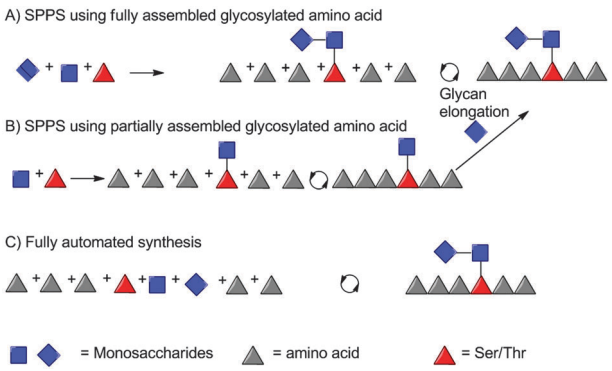

Fig. 1 Strategies for glycopeptide synthesis. Routes A and B utilize glycosylated amino acids as building blocks for the assembly of glycopeptides by SPPS. Route C requires only monomeric building blocks for automated assembly of glycopeptides by SPPS and SPOS.

Moreover, glycosylated amino acids may epimerize during SPPS and the purity of the synthesized glycopeptide needs to be ascertained.[7] Consequently, a method that minimizes the synthetic effort involved in glycopeptide synthesis is sought. An alternative synthetic route based on oligosaccharide assembly on a solid supported peptide platform was outlined but was not applied in automated practice.[8] This route is attractive as it may circumvent the use of glycosylated amino acids.

Recent advances in automated solid phase oligosaccharide synthesis (SPOS)[9] set the stage to combining both solid phase peptide and oligosaccharide assembly platforms to prepare glycopeptides. Here, we describe an automated, stepwise solid-phase approach to glycopeptide synthesis that relies exclusively on monosaccharide and amino acid building blocks (Fig. 1C).

The choice of a linker to tether the growing glycopeptide to the solid support is the crucial strategic decision for syntheses that require a multitude of protective groups. Photo-cleavable linkers are compatible with a variety of functional groups and were used for previous automated solid phase glycopeptide syntheses.[10] By using a photochemical flow reactor, inherent problems with cleavage efficiencies have been overcome.[11]

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† Electronic supplementary information (ESI) available: Detailed description of building block synthesis and characterization, automated synthesis protocols and modules, HPLC characterization of intermediates and NMR, HPLC and MS analysis of the final compound. See DOI: 10.1039/c3cc48761j

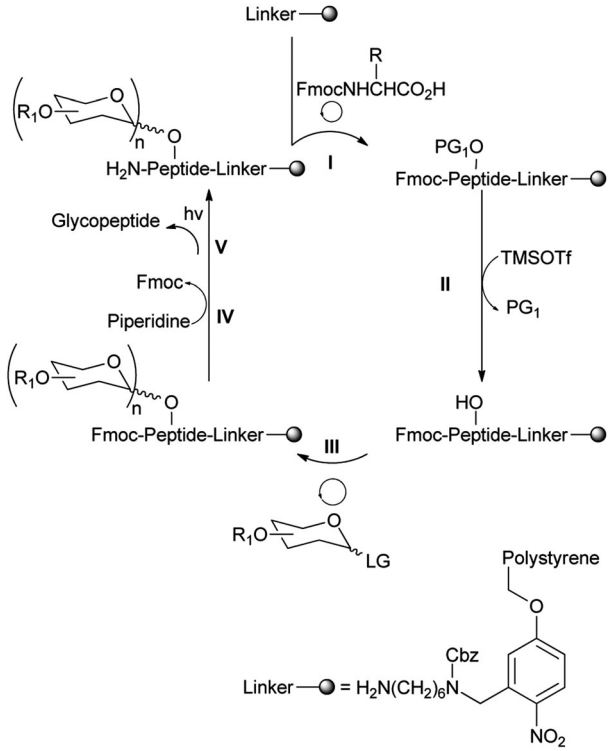

Fig. 2 Automated glycopeptide synthesis workflow. (I) Solid phase peptide synthesis. (II) Selective hydroxyl deprotection. (III) Solid phase oligosaccharide synthesis. (IV) Amine deprotection. (V) Photo-cleavage. PG_1 = *t*Bu or Trt, R = amino acid side chain (protected), LG = SEt, OC(*N*Ph)CF_3, R_1 = Bn, Ac, Lev, Bz, benzylidene.

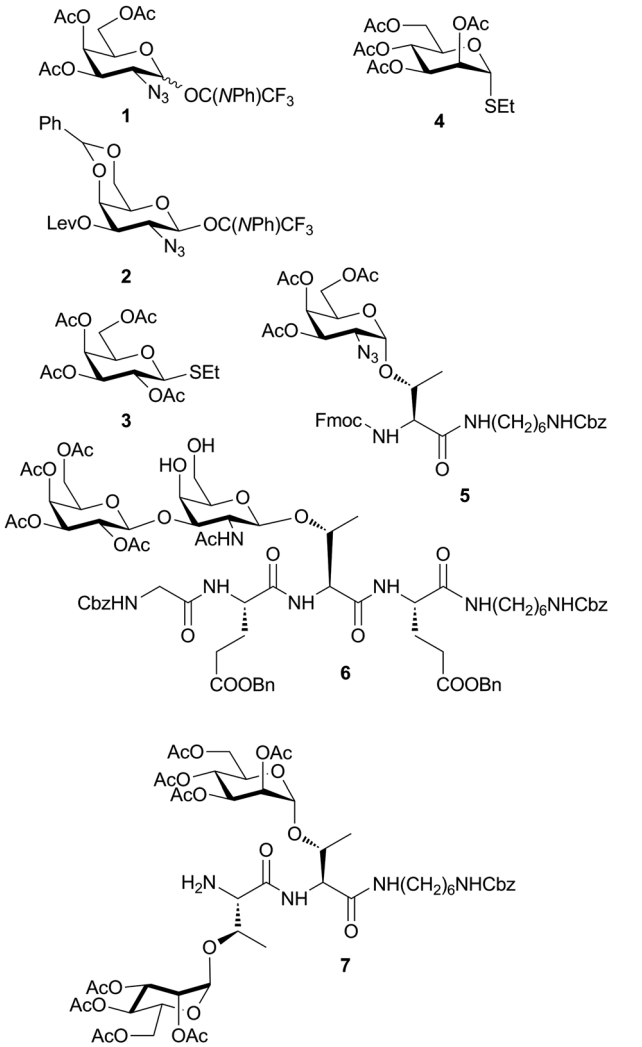

Fig. 3 Glycosylating agents **1–4** were used for the automated synthesis of glycopeptides **5–7**.

Since the automated assembly of oligosaccharides is instrumentally more demanding than the more mature peptide assembly, a recently developed oligosaccharide synthesizer that also accommodates SPPS was used for the synthesis (see ESI†).[9c]

The general workflow (Fig. 2) relies on four distinct phases for iterative step by step automated glycopeptide synthesis.

Attachment of a Fmoc-protected amino acid on the amino-functionalized solid support-bound linker was followed by standard peptide elongation using HBTU as an activator and piperidine as a deprotection reagent (Fig. 2, I). Amino acid side chain functional groups were permanently masked as benzyl ethers while either *tert*-butyl (*t*-Bu) or trityl (Trt) ethers were used as temporary protecting groups. The amino terminus of the peptide remained protected as an Fmoc carbamate during glycosylation.

Removal of the temporary *t*-Bu or Trt protecting groups from threonine or serine was achieved under mild acidic conditions using catalytic amounts of trimethylsilyl trifluoromethanesulfonate (TMSOTf) (Fig. 2, II). TMSOTf is also employed to activate glycosyl trichloroacetimidates and *N*-phenyl trifluoroacetimidates in the oligosaccharide synthesizer.[9c]

With a solid support bound peptide carrying a free side chain hydroxyl group in hand, an automated glycosylation sequence was initiated to place the desired glycan portion on the peptide (Fig. 2, III). Elongation of the peptide was simply possible by Fmoc removal from the amino terminus and standard SPPS (Fig. 2, IV). Repeating steps I–IV allows for the automated synthesis of peptides carrying multiple glycosylation sites. In the final step of the synthesis, the glycopeptide was removed from the solid support by irradiation.[11]

Three glycopeptides were prepared to illustrate the new automated synthesis approach (Fig. 3). Monosaccharide building blocks **1–4** that carry different protecting groups as well as anomeric leaving groups were utilized to demonstrate the variability of the approach. Commercially available amino acids were used without any modifications. The target glycopeptides were selected to challenge our approach in different ways. Glycopeptide **5** requires formation of a 1,2-*cis*-glycosidic linkage on a peptide backbone. Glycopeptide **6** requires elongation of the glycan side chain while peptide 7 requires incorporation of multiple side chain glycans (Fig. 3).

To install the *cis*-glycosidic linkage between the threonine/serine hydroxyl group and the GalNAc moiety on glycopeptide **5**, α-selective glycosylating agent **1** was used.[12] After coupling Fmoc-Thr-(*t*-Bu)-OH to the solid support, the *t*-Bu group was removed using TMSOTf. The free hydroxyl group was glycosylated

with **1** in the presence of TMSOTf. Cleavage from the resin and HPLC analysis revealed that the glycosylation reaction had proceeded with good selectivity (4 : 1, major α). The α-anomeric configuration of **5** was confirmed by comparison of the NMR spectra of **5** with that of Fmoc-Thr(Ac_3GalN_3)-O*t*-Bu.[12b]

After establishing that automated peptide glycosylation is possible, glycopeptide **6** bearing a (Galβ1-3GalNAcβ-*O*) disaccharide was prepared. Commercially available amino acids were used along with glycosylating agents **2** (prepared in three steps from a known intermediate)[13] and **3**.[14] The tetrapeptide backbone of **6** was assembled using SPPS. Selective removal of the *t*-Bu group was followed by glycosylation with glycosyl imidate **2** and TMSOTf as an activator at −10 °C. The glycosylation reaction proceeded with high conversion and yielded one major product. Removal of the levulinoyl protecting group was followed by formation of a second glycosidic linkage using galactosyl thioglycoside **3** activated by *N*-iodosuccinimide (NIS) and triflic acid (TfOH). Progress of the automated synthesis of **6** was monitored by cleaving small amounts of material from the solid support at different stages of the synthesis followed by HPLC-MS (see ESI†).

Deprotection of the galactosamine by reduction of the azide using AcSH,[15] and benzylidene removal using TFA were carried out manually after completion of the automated synthesis. Finally, glycopeptide **6** was cleaved from the resin under UV irradiation. The desired product **6** was obtained in 5.7% isolated yield after HPLC purification. ^{1}H-NMR coupling constants and coupled CH-HSQC analysis clearly indicate that two β-linkages were formed.

Since many glycopeptides harbor more than one glycosylation site, bis-glycosylated peptide **7** was targeted to challenge the method. Fmoc-Thr(Trt)-OH and mannose thioglycoside **4**[16] were employed twice. Placement of threonine on the solid support was followed by Trt removal. Mannose thioglycoside **4** was attached using the NIS–TfOH activator mixture. Fmoc removal and repetition of the assembly cycle gave glycopeptide **7** after light-induced cleavage from the solid support in 14% yield after HPLC purification. NMR analysis proved that both threonines were glycosylated in an α-selective manner.

An automated approach for the synthesis of *O*-glycopeptides based on the combination of solid phase peptide and oligosaccharide synthesis protocols can be executed on a single instrument. Employing Merrifield polystyrene resin equipped with a photocleavable linker allowed for the use of different leaving groups and activation systems. The examples presented in this study demonstrate the potential of a monomer based strategy for synthesizing glycopeptides. This conceptual advancement, combined with improved synthetic procedures, is expected to provide more expedient access to homogeneous glycopeptides in the future.

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