Solid-Phase Glycosylation of Peptide Templates and On-Bead MAS-NMR Analysis: Perspectives for Glycopeptide Libraries

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Abstract: The efficient solid-phase glycosylation of amino acid side chains (serine (Ser), threonine (Thr), and tyrosine (Tyr)) in peptides was demonstrated with a variety of glycosyl trichloroacetimidate donors in high yields and purities. A novel photolabile linker, with no chiral centre, was introduced to facilitate analysis by both matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry and nanoprobe magic angle spinning (MAS) NMR spectroscopy. Product analysis by nanoprobe MAS NMR spectroscopy, LC-MS and MALDI-TOF mass spectrometry of the glycosylation reactions indicated that the reactivity order of the hydroxy side-chain functions of amino acids in peptides on the solid-phase was Tyr > Ser > Thr. The nearly quantitative glycosylation yields and the efficient on-bead product analysis by nanoprobe MAS NMR spectroscopy have made a truly solid-phase approach for the synthesis and analysis of glycopeptide libraries possible.

Keywords: glycopeptides · NMR spectroscopy · photolabile linker · solid-phase synthesis

Introduction

Nature employs glycoconjugates in various biological recognition processes such as intercellular communication, receptor–ligand interactions, cell adhesion, cell differentiation, growth and tumour metastasis.[1] The time consuming and cumbersome chemical synthesis of complex glycan structures has so far supplied the field of glycobiology with a limited number of compounds to study these biological phenomena. It has recently been demonstrated that simple, monosaccharide-containing glycopeptides can mimic large, naturally occurring glycan structures.[2] The synthesis of glycopeptides is simpler than that of complex oligosaccharides, and the synthesis of large and diverse libraries by the split and mix method can be achieved.[3] Application of pre-formed glycosylated amino acid building blocks during solid-phase peptide synthesis is currently the most efficient method to synthesise glycopeptides.[4] This method requires the synthesis of a new glycosyl amino acid building block for each carbohydrate incorporated.[5] It would be more efficient to couple carbohydrates directly and stereoselectively to free hydroxy groups of amino acid side chains of resin-bound peptides to generate a multitude of glycopeptides with variation in both the carbohydrate and peptide part. Glycosylation of preformed peptide templates in solution has only been partially successful in a few instances,[6] probably owing to the low solubility of the peptides in organic solvents which are required for the glycosylation reaction.[4b] A solid-phase approach would not suffer from this drawback and, when successful, would give access to large amounts of diverse ligands that could be used as possible mimics of oligosaccharides.

It has been demonstrated that hydroxy functions of solid-phase bound oligonucleotides[7] and oligosaccharides[8] can be efficiently glycosylated with different glycosyl trichloroacetimidates. Direct solid-phase glycosylations of hydroxy groups on amino acid side chains of peptides on a polystyrene support was possible, albeit in low reaction yields.[9] With the development of an inert polyethylene glycol (PEG) cross-linked resin, polyoxyethylene-polyoxypropylene (POEPOP),[10] glycosylation yields of 48–71% of Ser residues in tetra- and pentapeptides with galactose, fucose and glucosamine trichloroacetimidate donors have been reported.[11] The products obtained had to be cleaved from the resin before the structure could be analysed by conventional NMR techniques and MALDI-TOF mass spectrometry.

The analysis of glycopeptides still attached to the resin has obvious advantages. Firstly, cleavage conditions require strong acidic or alkaline conditions which often lead to undesirable side products. Secondly, direct information can be obtained on the solid-phase conformation of the glycopeptide.

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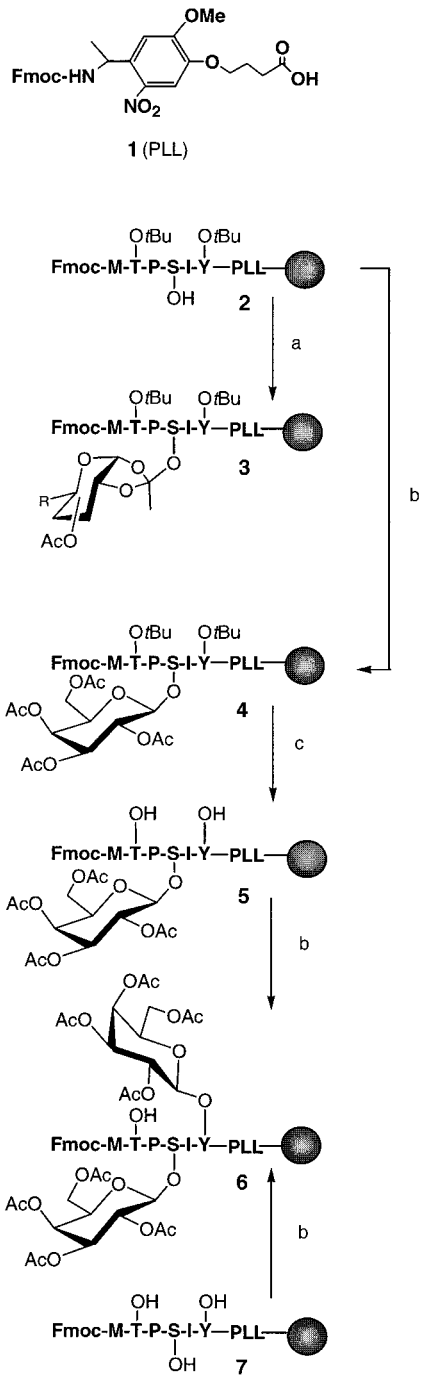
The acquisition of high-resolution NMR spectra of heterogeneous samples, like swollen beads, has only recently become possible with the development of high-resolution magic angle spinning probes.[12] The objective of spinning at the magic angle is to remove line broadening induced by the magnetic susceptibility differences between the sample components and at the sample cavity interfaces. The spectral quality obtained when studying resin-bound compounds is dependent on the resin used.[13] It has been shown that $POEPOP_{1500}$ gave well-resolved nanoprobe MAS NMR spectra, and was still stable to most chemical and mechanical manipulations.[14] With the nanoprobe MAS NMR technology, analysis of minute sample amounts can be carried out. It has been successfully used to monitor solid-phase reactions of resin-bound peptides and other compounds.[15]

Herein we demonstrate a new method for efficient, solid-phase glycosylations of the amino acid side chains of Ser, Thr and Tyr in peptide templates with a range of glycosyl trichloroacetimidate donors (galactose, glucose, mannose, fucose and glucosamine). The structures of the resin-bound glycopeptides were efficiently identified by nanoprobe MAS NMR spectroscopy. The ease of characterisation was improved by the introduction of a novel photolabile linker. Nanoprobe MAS NMR, LC-MS and MALDI-TOF MS techniques were used to obtain information on the reactivity of the side chains of amino acid acceptors on the solid phase (Tyr > Ser > Thr). The knowledge gained from this study can be used in the synthesis of large and diverse glycopeptide libraries. In addition, the ability to characterise resin-bound lead compounds by nanoprobe MAS NMR will increase the speed of the screening process and may give valuable information on the conformation of the biologically active lead compound on the bead.

Results and Discussion

Solid-phase glycosylation of resin-bound peptides: In a library format in which screening and analysis is performed on the solid phase, quantitative reaction yields are desirable for two reasons. Firstly, reaction mixtures may lead to confusing results in the solid-phase screening procedure. Secondly, the formation of single products simplifies the on-bead structural analysis by nanoprobe MAS NMR. To establish the best conditions for the solid-phase glycosylation of amino acid side chains in peptides, a model hexapeptide **2** was synthesised on $POEPOP_{1500}$ resin.[10] A photolabile linker[16] **1** was used in the model hexapeptide for two reasons. Firstly, it was stable towards both acidic and alkaline conditions that were used in the synthetic strategy. Secondly, it allowed the mild release of the glycopeptides without cleavage of labile glycosidic linkages (e.g. the fucose linkage). The solid-phase glycosylation of the model peptide was attempted using conditions described by Schleyer[11] (8 equiv trichloroacetimidate donor, 0.1 equiv trimethylsilyl trifluoromethanesulfonate (TMSOTf); Scheme 1). Initially, galactose donor **11** and mannose donor **13** (see Scheme 2) were used for the glycosylation of the model peptide. In both cases only the ortho-ester **3** of the corresponding product was formed. It was hoped that an increase of the reaction time of the glycosylation reaction would allow the ortho-ester to rearrange to the desired glycosidic linkage product. However, this approach was not successful, only starting peptide **2** could be detected by MALDI-TOF mass spectrometry. An increase in the amount of TMSOTf (0.1 equiv compared with donor **11** or **13**) resulted in the removal of the *tert*-butyl (*t*Bu) protecting groups of the Thr and Tyr residues, and their subsequent partial glycosylation. Although TMSOTf could yield quantitative glycosylations, it



Scheme 1. Synthesis of glycopeptides by solid-phase glycosylation of peptides **2** and **7**. a) 8 equiv donor **11** or **13** with 0.1 equiv TMSOTf; b) double glycosylation using 5 equiv donor **11**, 0.3 equiv BF_3OEt_2 compared with the donor at 0 °C; c) 50 % TFA in dichloromethane.

was evident that the product mixtures obtained were not useful for application in a library format that relied on the efficient on-bead characterisation of the lead compounds. Differences in the protecting groups in the donor molecule **11** (acetyl vs. benzoyl as applied by Schleyer et al.[11]) could be a partial explanation for the observed formation of the ortho-ester product. Since the mannose donor applied in both this study and in the paper by Schleyer et al.[11] was the same, differences in protecting groups was not the only factor determining the outcome of the reaction. Different acceptor molecules were used in these and the previous studies and therefore mismatching reactivities between the reaction partners may play an important role and explain the seemingly conflicting results between the present work and that of the previous[11] communication. The present results reveal that a higher concentration of TMSOTf will lead to the formation of the desired glycosidic linkage, however, the applied higher concentration was incompatible with the use of the *t*Bu protecting group in the peptide template. As a consequence, to allow efficient and selective glycosylation of peptide templates in a reproducible way, a Lewis acid was needed that would give satisfactory activation of the trichloroacetimidate donors without affecting the acid-labile protecting groups that are used in peptide synthesis. Since boron trifluoride diethyl etherate (BF_3OEt_2) is a milder Lewis acid than TMSOTf and is known to be an efficient activator of trichloroacetimidates, it was decided to use this catalyst for the glycosylation reaction of peptides. Fortunately, this change in Lewis acid resulted in the formation of the desired product **4** when galactosyl donor **11** was used, without affecting the *t*Bu protecting groups. The best results were obtained with the treatment of peptide **2** with donor **11** (5 equiv) at reduced temperature (0 °C) for 90 min with 0.3 equiv BF_3OEt_2 compared with the donor. Application of double-coupling reactions resulted in the formation of the desired compound **4** in a high yield, as shown by MALDI-TOF mass spectrometry LC-MS and nanoprobe MAS NMR spectroscopy (LC-MS and nanoprobe MAS NMR data are available in the Supporting Information).

In a report by Jensen et al.,[17] a *t*Bu protected Tyr was shown to be a better acceptor than an unprotected Tyr residue in silver trifluoromethanesulfonate (AgOTf) catalysed glycosylation reactions with Koenig–Knorr type donors. It was, therefore, postulated that a *t*Bu group could not be used for the protection of side-chain hydroxy groups of amino acids that were not involved in the glycosylation reactions. However, the present approach was completely compatible with the use of *t*Bu protecting groups in glycopeptide synthesis, because no side products were observed during the synthesis of **4**.

Removal of the *t*Bu protecting groups of product **4**, with 50% trifluoroacetic acid (TFA) in dichloromethane, gave a model peptide **5** that contained two possible glycosylation sites. The use of the same glycosylation conditions as described above resulted in the selective introduction of one more galactose residue. Surprisingly, analysis of the reaction product **6** by nanoprobe MAS NMR showed exclusive glycosylation of the Tyr moiety (see section below: analysis of resin-bound peptides and glycopeptides by nanoprobe MAS NMR spectroscopy; LC-MS and nanoprobe MAS NMR data are available in the Supporting Information). This was confirmed by amino acid sequencing of compound **6**. This analytical method showed the expected quantity of Thr, while the quantities measured for Ser and Tyr were negligible, and thus indicated that a modification of these two amino acids had occurred (amino acid sequencing data is available in the Supporting Information). Literature data on successful solution-phase glycosylations of peptide templates is scarce, owing to solubility problems of the peptides in the organic solvents that are necessary for the glycosylation reactions. However, results of solution-phase glycosylations of simple aryl moieties[18] and Tyr,[17, 19] under Lewis acid catalysed conditions, have demonstrated the problems involved with this reaction. The observed preference for the solid-phase glycosylation to occur on the Tyr instead of on the Thr in compound **5** was, therefore, surprising. To further investigate the unexpected reactivity order, the *t*Bu protecting groups of **2** were removed, to afford a structure containing three possible glycosylation sites **7**. Galactosylation of **7**, using BF_3OEt_2 at 0 °C, resulted in the formation of one product **6**. MALDI-TOF mass spectrometry of the released compound revealed that the mass corresponded to the introduction of two galactose residues. nanoprobe MAS NMR spectroscopy of the resin-bound product showed unambiguously that within the detection limit, the Ser and Tyr residues in **6** were glycosylated exclusively. Introduction of a third galactose residue in compound **6**, under the conditions described above, was not possible.

Whereas the selectivity of reaction between Ser, Thr and Tyr may in some cases be sufficient to sequentially introduce different glycosyl donors to different sites in a single OH-unprotected peptide, sequential quantitative glycosylation for use in library construction requires the protection of the hydroxy groups to be glycosylated in the second round.

To investigate if the observed reactivity order on the solid phase was general or sequence dependent, three different model peptides **8**, **9** and **10** were synthesised and glycosylated with a set of five different trichloroacetimidate donors (**11**, **12**, **13**, **14** and **15**; Scheme 2).[20]

The reactions were performed in a specially designed 20-well peptide synthesiser[21] that allowed reactions to be done under inert conditions. Each coupling was performed with two equivalents of trichloroacetimidate donor and 0.6 equivalents of BF_3OEt_2 in dichloromethane at 0 °C. The reaction products were analysed by MALDI-TOF mass spectrometry. As the glycosylation reactions are typically performed at the low micromolar level, the most useful analytical tool to routinely monitor reaction progress is MALDI-TOF mass spectrometry. To validate the quantification of the reactant/product ratio by MALDI-TOF peak heights, representative glycosylations were selected for large-scale synthesis and analysis by LC-MS. In all cases, the reaction progress as determined by integration of the HPLC UV chromatograms were in good agreement with the estimates obtained by MALDI-TOF analysis. Only a 5–10% deviation was observed between the analytical methods (data not shown). The data shown in Table 1 represent the progress of the glycosylation reactions in percentages, as established by MALDI-TOF mass spectrometry.

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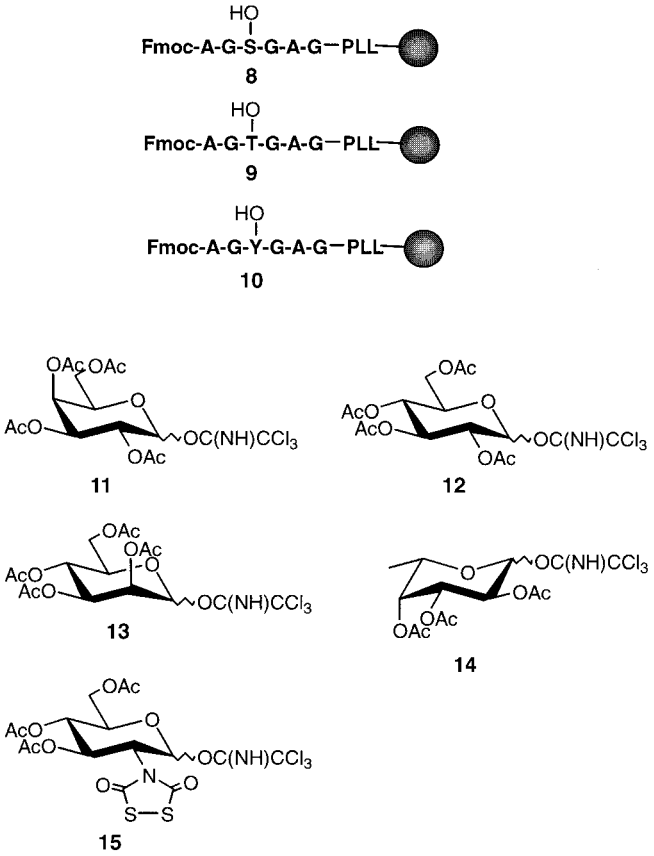

Scheme 2. Model peptides and trichloroacetimidate donors used for the establishment of the solid-phase reactivity order of the hydroxy functions of Ser, Thr and Tyr.

similar. The glucosamine donor **15** showed the lowest reactivity.

It should be pointed out that when double glycosylations (2×5 equiv donor) were applied all reactions with the fucose, mannose, galactose and glucose donors were quantitative, as judged from the MALDI-TOF mass spectra (an illustrative example is shown in Figure 1). In contrast, repeated glycosylations with glucosamine donor **15** gave only 40% conversion with acceptor peptide **10**, while no reaction products were observed when acceptors **8** or **9** were used. Since it was shown by Schleyer et al.[11] that hydroxy functions of serine could be efficiently glycosylated with donor **15**, it may be emphasised that the use of a milder catalyst (BF_3OEt_2 vs. TMSOTf) to avoid cleavage of acid-labile protecting groups also results in reduced activation by the Lewis acid.

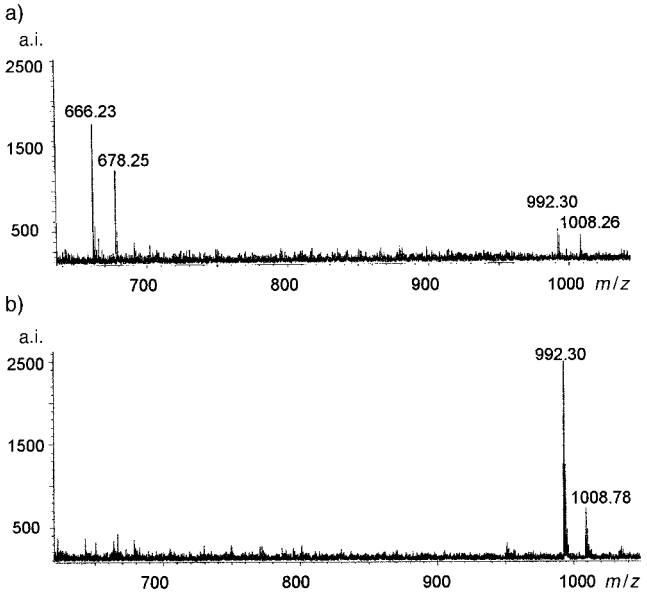

Figure 1. MALDI-TOF mass spectra showing glycosylation of acceptor **8** with donor **11**. a) Single glycosylation using two equivalents of donor **11**. b) Double glycosylation using each time five equivalents of donor **11**.

Comparison of the glycosylation yields of each donor with the different acceptor peptides indicated the reactivity order of the amino acid hydroxy functions on the solid phase as Tyr > Ser > Thr. An explanation for the observed higher reactivity on solid phase of Tyr, relative to Ser and Thr, lies in the inability of its hydroxy group to form a deactivating hydrogen bond with the peptide backbone.

Comparison of the glycosylation yields of each acceptor peptide (**8**, **9** or **10**) with the different glycosyl donors, under identical conditions for a fixed period of time, gave the reactivity order of the donors used in this study. It can be observed that the glycosylation yields with the fucose donor (**14**) were the highest, while the yields obtained with the galactose (**11**), glucose (**12**), and mannose (**13**) donors were

Table 1. Results of the glycosylation of three model peptides (**8**, **9** and **10**) with different trichloroacetimidate donors (**11**, **12**, **13**, **14** and **15**) to establish reactivity order of hydroxy side-chain functions of Ser, Thr and Tyr on solid-phase.

Donors	Acceptors **8** (MW_{calcd}/MW_{found})	**9** (MW_{calcd}/MW_{found})	**10** (MW_{calcd}/MW_{found})
11[a]	19% (992.66/992.3)[a]	15% (1006.69/1006.2)[a]	89% (1068.76/1068.3)[a]
12[a]	47% (992.66/991.8)[a]	23% (1006.69/1005.9)[a]	93% (1068.76/1067.9)[a]
13[a]	33% (992.66/992.1)[a]	9% (1006.69/1006.2)[a]	91% (1068.76/1068.3)[a]
14[a]	76% (934.66/934.4)[a]	31% (948.69/948.4)[a]	100% (1010.76/1010.4)[a]
15[a]	–	–	14% (1143.76/1143.1)

[a] Each glycosylation was performed with two equivalents of trichloroacetimidate donor and 0.6 equivalents of BF_3OEt_2.

Analysis of resin-bound peptides and glycopeptides by nanoprobe MAS-NMR spectroscopy: Both 1D and 2D homonuclear nanoprobe MAS spectra were acquired for the peptide **2a**, (peptide **2** synthesised without *t*Bu protection on Tyr) and glycopeptides **5** and **6**. The peptide **2a** was synthesised specifically for the NMR analysis (Figures 2 and 3), in particular, to compare the positions of tyrosine resonances in Figure 3a, b and c. Compound **2a** was not used for any further synthetic studies.

As can be seen in Figure 2, the 1D spectra of the three samples are well resolved although the compounds are resin bound. The introduction of acetyl-protected galactose can

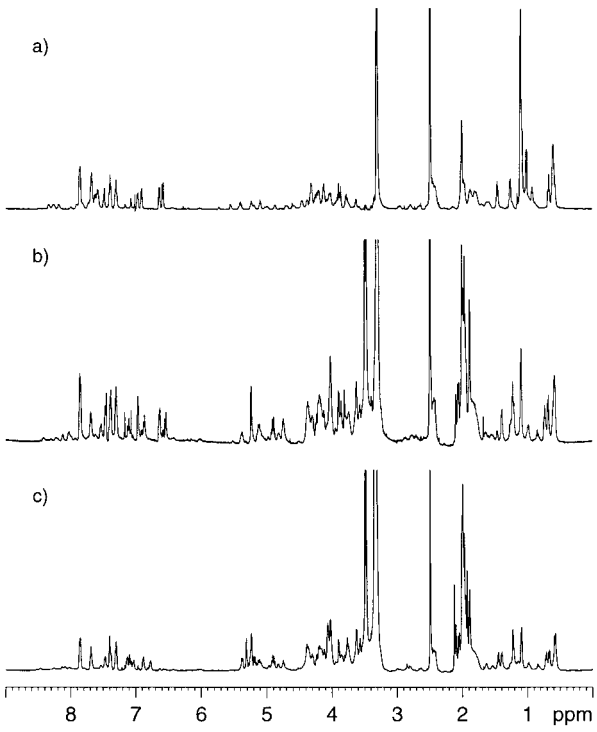

Figure 2. Spectra showing the a) peptide **2a**, b) glycopeptide **5**, c) glycopeptide **6**. The acquisition parameters are given in the Experimental Section.

easily be seen in the 1D spectra where new resonances appear in the region 5.4 – 4.0 ppm upon glycosylation of the peptide. Homonuclear 2D spectroscopy was needed to obtain a full assignment of the resonances. DQF-COSY and NOESY spectra were recorded and assigned for the peptide part according to the procedure described by Wüthrich.[22] The DQF-COSY spectra allowed for the identification of most of the resonances within the individual spin systems. The NOESY spectra were used to obtain the sequential assignment pathway through the peptide backbone from $H^{\alpha}(n)$–NH $(n+1)$ with a break in the sequence at the site of the Pro residue.

In all of the spectra, two sequential pathways can be traced through most of the peptide chain, with the largest difference in chemical shift for the two sets of resonances found at the residues closest to the linker. This was a result of the chiral centre present in photolabile linker, **1** (see section below: improving the characteristics of the photolabile linker for nanoprobe MAS-NMR analysis). From Figure 3 it can be noted that the spectra do give an indication about the completion of each glycosylation reaction. Although MALDI-TOF mass spectrometry of each reaction step indicated a quantitative yield, nanoprobe MAS NMR of the resin-bound compounds revealed the presence of a minor amount of starting material. This is evident from Figure 3b where, after the first round of glycosylation, four sets of resonances can be observed. The two dominant resonances resulted from the glycosylated product, and the two minor resonances originated from the TFA treated starting peptide **2a**. The 2D spectra of **6** acquired after glycosylation of **7** showed an almost quantitative reaction ($\approx$95%). The presence of this small amount of starting material did not interfere with the complete assignment of all resonances. In all glycosylation reactions investigated by MAS-NMR complete anomeric selectivity was obtained as determined by the total absence of anomeric protons with α-glycosidic shifts from the spectra. However, although this could be a general advantage of solid-phase glycosylations on PEG-based resins,[23] it has not been investigated in detail for the more reactive fucosyl donor, which could be less selective.

Assignment of the protected galactose resonances could be obtained from the 2D spectra for the majority of the resonances. The position of the acetyl-protected galactose residue in compound **5** was found from spectral data that showed the presence of a NOE from Ser H^{α} and H^{β} to the H-1 of the galactose residue. The spectra of the resin-bound glycopeptide **6** indicated that the second galactose residue was located at the tyrosine residue. As can be seen in Figure 3c, glycosylation of the Tyr resulted in a downfield shift of about 0.2 ppm for the Tyr aromatic resonances. This downfield shift was in agreement with results previously reported.[17] Additionally, a NOE from the Tyr aromatic protons to the H-1 of the acetylated galactose residue can be found (Figure 4). Owing to the chiral centre present in the photolabile linker two NOEs were observed.

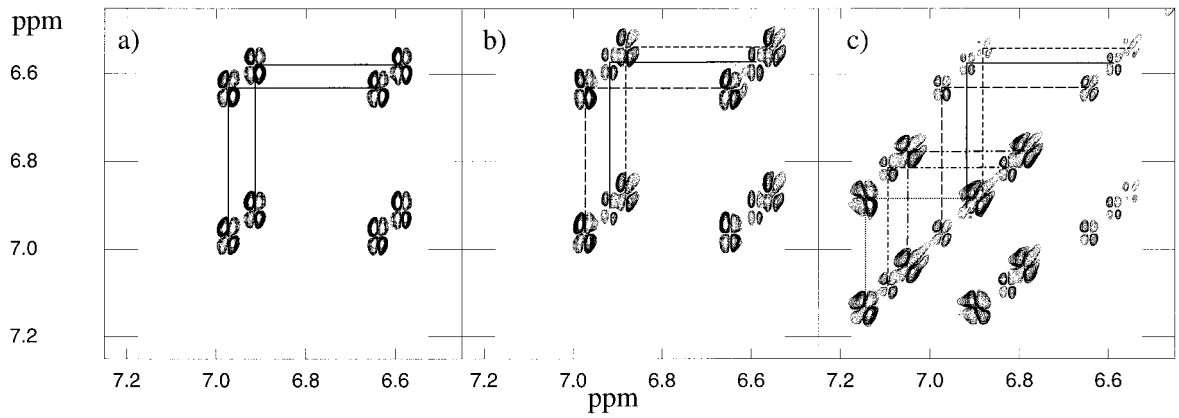

Figure 3. The Tyr aromatic area of 2D DQF-COSY spectra of a) peptide **2**, b) glycopeptide **5**, c) glycopeptide **6**. The acquisition parameters are given in the Experimental Section.

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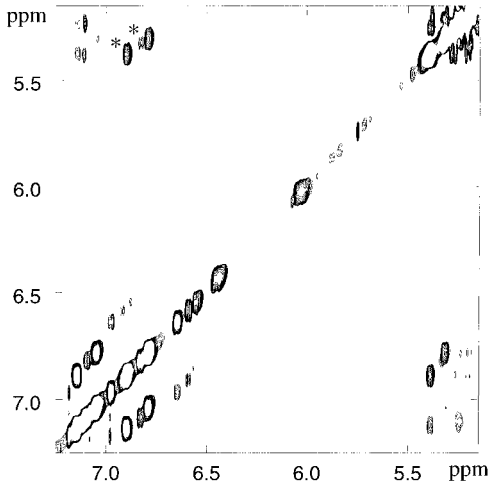

Figure 4. Part of the NOESY spectra of glycopeptide **6**, showing the area from the Tyr aromatics to the galactose H1 resonances. The * indicates the NOE connectivities from the Tyr aromatics to the H1 of the galactose attached to it.

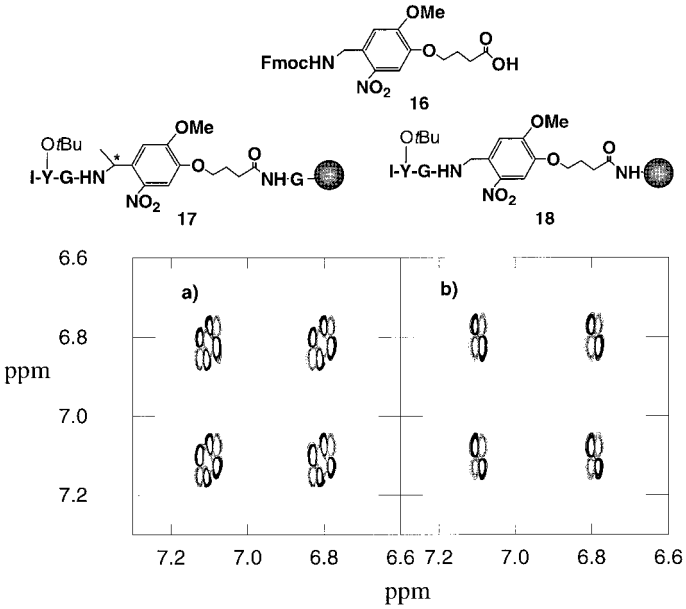

Figure 5. Use of novel photolabile linker **16** in peptide synthesis. The splitting of the Tyr aromatics showing two sets of resonances when the chiral photolabile linker **1** is used (a) and one set of resonances when the photolabile linker **16** is used (b).

Improving the characteristics of the photolabile linker for nanoprobe MAS-NMR analysis: In the nanoprobe MAS NMR spectra of the resin-bound model peptides **2a**, **5** and **6**, all signals appear as double sets of resonances. In principle, there could be two explanations for this observation. The slow exchange of two conformations on the NMR time scale or the presence of the chiral centre in the photolabile linker **1**. A photolabile linker **16** that did not contain a chiral centre was synthesised to test the second hypothesis. The synthetic route to **16** was the same as described for **1**,[16] except 4-hydroxy-3-methoxybenzaldehyde was used as the starting compound.[24] A simple peptide fragment was synthesised via photolabile linker **1** or **16** on super-permeable organic combinatorial-chemistry ($SPOCC_{1500}$) resin,[25] to obtain **17** or **18**, respectively (Figure 5).

Nanoprobe MAS NMR spectroscopy of the peptide fragments showed (Figure 5) that the double set of signals could be distinguished throughout the whole sequence of **17**, while in **18** only one set of signals could be observed. Therefore, it can be concluded that the chiral centre in **1** was responsible for the double set of signals in **2a**, **5** and **6**. Although the photolytic cleavage of compounds that are resin bound through linker **16** occurs in a slightly lower yield than with linker **1**, it still resulted in the release of plenty of material from single beads for the analysis by MALDI-TOF MS. As a consequence, photolabile linker **16** should be used in a library format to facilitate unambiguous structural assignment by nanoprobe MAS NMR spectroscopy.

In conclusion, it was demonstrated that the inert POEPOP and SPOCC resins perform excellently in solid-phase glycosylation reactions. Nearly quantitative glycosylation reactions of Ser, Thr and Tyr amino acid side chains of peptides were possible. It was established that the reactivity order of the hydroxy functions of amino acid side chains on the solid phase is Tyr > Ser > Thr. With the exception of glucosylamine donor **15**, nearly quantitative glycosylation of accessible hydroxy functions of amino acid side chains was achieved by using the standard conditions (double couplings with 5 equiv donor, 0.3 equiv BF_3OEt_2 to donor, 3/2 h at 0 °C) with all donors tested. These standard conditions were demonstrated to be compatible with the use of the *t*Bu protecting group, often used in (glyco) peptide synthesis. The described standard glycosylation conditions, together with the establishment of nanoprobe MAS NMR on minute amounts of resin-bound sample, have made synthesis and analysis of glycopeptide libraries feasible in a truly solid-phase approach.

Experimental Section

Materials and general methods: All solvents were of p. a. quality, and were distilled from appropriate drying agents when necessary. Solid-phase peptide coupling reactions were monitored by using the Kaiser test.[26] $POEPOP_{1500}$ and SPOCC1500 resin were synthesised as described by Grøtli et al.[14] and Rademann et al.,[25] respectively. Suitably protected N^{α}-Fmoc/Pfp amino acids and mesitylenesulfon-1-yl-3-nitro-1,2,4-triazole (MSNT) were purchased from NovaBiochem (Switzerland), 3-hydroxy-2,3-dihydro-4-oxobenzotriazine (Dhbt-OH) and 1-methylimidazole were purchased from Fluka (Switzerland).

Nanoprobe MAS-NMR spectroscopy: general methods: All spectra were recorded at 25 °C on a Varian Unity Inova 500 MHz spectrometer equipped with a 4 mm ^{1}H-observe Nano NMR-probe with a spin rate of approximately 2 kHz for all samples. 1D spectra were acquired as one-pulse experiments with presaturation of the main PEG signal (sweep width 6000–8500 Hz, acquisition time 1.4–2.0 s, presaturation delay 1.5 s, 64 scans). All 1D spectra were processed using a 0.3 Hz line broadening and zero filled to 64 K. 2D homonuclear ^{1}H–^{1}H NOESY[27] and DQF-COSY[28] spectra were acquired. All spectra were acquired on 5–10 beads, swollen in [D_6]DMSO and the total acquisition time for the 2D spectra was less than 18 h. The presaturation period for all the samples were divided between PEG and residual HDO. NOESY acquisition parameters: mixing times ranged from 150–600 ms, presaturation delay 1.3–1.5 s, 64 scans, 512 increments and a sweep width of 6000 Hz. The data matrix of 512 × 4096 was zero filled to 1024 × 4096 prior to Fourier transformation by application of a shifted sine-window function. 2D phase-sensitive double-quantum filtered (DQF-COSY) spectra were acquired with a presaturation delay 1.2–1.5 s, sweep width of 8500 Hz, 32 scans and 914–1024 incre-

ments. The data matrix of 1024 × 4096 was zero filled to 2048 × 4096 prior to Fourier transformation by application of a shifted sine-window function. All spectra were processed in XWINNMR Bruker version 2.1.

Mass spectrometry

General methods: MALDI-TOF mass spectra were recorded in the positive-ion reflectron mode on a Bruker Reflex III MALDI-TOF mass spectrometer. Spectra were obtained (1 – 100 pulses) using the lowest power required to facilitate desorption and ionisation. Ions were accelerated toward the discrete dynode multiplier detector with an acceleration voltage of 20 kV. The matrix α-cyano-4-hydroxycinnamic acid (CHC) was used to analyse both peptides and glycopeptides. The matrix was prepared by dissolving CHC (10 mg) in 30% aqueous acetonitrile. Bradykinin (1060.2 m.u.), substance P (1347.4 m.u.) or mellitin (28846.5 m.u.) were used as standards for external calibration of the masses.

Sample preparation: A bead, placed on the target, was irradiated for 30 min with a UV lamp (225 W) prior to measurement of the MALDI-TOF mass spectra. The released (glyco) peptide was extracted from the bead with 1 μL 30% aqueous acetonitrile, 0.5 μL of the matrix was added and the sample was dried at 40 °C.

Solid-phase synthesis of model peptides: The $POEPOP_{1500}$ or $SPOCC_{1500}$ resin (loading 0.23 mmol g^{-1}) was washed with CH_2Cl_2 (6 ×) then dried under vacuum (lyophiliser) for at least 24 h before use. After the introduction of the photolabile linker **1** or **16** (3 equiv photolabile linker to each hydroxy function, 3 equiv MSNT and 2.3 equiv 1-methylimidazole in dichloromethane), all manipulations of peptides and glycopeptides were carried out in subdued light (protected from UV radiation). All peptide syntheses were carried out in disposable syringes (2 mL) fitted with a Teflon filter on a 61 μmol scale using the Fmoc/Pfp ester methodology. Completion of acylation was monitored by using the Kaiser test. After the complete synthesis of each model peptide, a few beads were irradiated and the released products were analysed by MALDI-TOF mass spectrometry. Compound **2**: mass calcd: 1066.31 $[M+Na]^+$, 1082.43 $[M+K]^+$; mass found: 1066.0 $[M+Na]^+$, 1082.7 $[M+K]^+$; compound **7**: mass calcd: 958.13 $[M+Na]^+$, 974.24 $[M+K]^+$; mass found: 957.83 $[M+Na]^+$, 973.91 $[M+K]^+$; compound **8**: mass calcd: 662.66 $[M+Na]^+$, 678.77 $[M+K]^+$; mass found: 662.2 $[M+Na]^+$, 678.2 $[M+K]^+$; compound **9**: mass calcd: 676.69 $[M+Na]^+$, 692.79 $[M+K]^+$; mass found: 676.3 $[M+Na]^+$, 692.3 $[M+K]^+$; compound **10**: mass calcd: 738.76 $[M+Na]^+$, 754.87 $[M+K]^+$; mass found: 738.2 $[M+Na]^+$, 754.1 $[M+K]^+$; compound **17**: mass calcd: 651.77 $[M+Na]^+$, 667.87 $[M+K]^+$; mass found: 651.46 $[M+Na]^+$, 667.42 $[M+K]^+$; compound **18**: mass calcd: 651.77 $[M+Na]^+$, 667.87 $[M+K]^+$; mass found: 651.29 $[M+Na]^+$, 667.32 $[M+K]^+$.

Deprotection protocols: The Fmoc protecting groups were removed by the action of a 20% piperidine/DMF solution (2 + 18 min). The resin was washed with DMF (10 ×) before the next amino acid coupling was started. The *t*Bu esters were removed by the addition of a 50% TFA/dichloromethane solution to the resin (2+30 min). The resin was washed with dichloromethane (10 ×) and lyophilised overnight before it was used in the next glycosylation reaction.

Glycosylation protocol to evaluate the catalyst conditions: The glycosylations were performed with freshly distilled, dry dichloromethane under an argon atmosphere in a syringe fitted with a dry argon flow and an injection port. The reactions were carried out on $POEPOP_{1500}$ resin using model peptide MT(*t*Bu)PSIY(O*t*Bu)-Pll. The resin and the amount of tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate donor (4 equiv compared with the amount of hydroxy functions) were dried overnight under high vacuum in the syringe. The required amount of dichloromethane that allowed complete swelling of the resin was injected, and then the syringe kept at 20 °C or cooled to 0 °C. After 30 min, BF_3OEt_2 (0.3 equiv compared with the trichloroacetimidate donor) or TMSOTf (0.1 or 0.01 equivalents) was injected. Reactions were performed with and without ultrasound sonication but this had no influence on the reaction. After 90 min, suction was applied and the resin was washed with dichloromethane (10 ×). The resulting products were released by photolysis and analysed by high resolution MALDI-TOF mass spectrometry. Spectra were recorded over a range of laser attenuations to give reproducible spectra indicative of the course of reaction. When a considerable amount of starting peptide was present the reaction was repeated up to three times after which the reaction was always complete. The BF_3OEt_2-catalysed reaction was generally quantitative, clean and selective at only the unprotected hydroxy group using a single reaction, however, the TMSOTf-catalysed reactions were difficult to control. They generally gave a range of products resulting from either ortho-ester formation at low catalyst concentrations, as indicated by cleavage of the glycosyl unit by weak TFA treatment, or by cleavage of *t*Bu protecting groups and subsequent double and triple glycosylation. By-products were always present in TMSOTf-catalysed reactions and constituted, according to MALDI-TOF-MS and HPLC analysis, varying amounts from 10 – 60% depending on the conditions. Therefore the BF_3OEt_2-catalysed reaction was selected for the subsequent work.

General glycosylation protocol: All glycosylations were performed in dry dichloromethane under an argon atmosphere. The reactions were carried out in a special 20-well peptide synthesiser[21] that allowed the addition of solvents and catalyst under an inert atmosphere (Ar). The resin and the required amount of trichloroacetimidate donor (5 equiv compared with the amount of hydroxy functions) were dried overnight under high vacuum in the peptide synthesiser. The amount of dichloromethane that allowed complete swelling of the resin was injected, and then the synthesiser was cooled to 0 °C. After 30 min, BF_3OEt_2 (0.3 equiv compared with trichloroacetimidate donor) was injected. After 90 min, suction was applied and the resin was washed with dichloromethane (10 ×). To achieve quantitative reactions the described procedure was repeated. All compounds were analysed by MALDI-TOF mass spectrometry and nanoprobe MAS NMR spectroscopy. Compound **4**: mass calcd: 1396.32 $[M+Na]^+$, 1412.43 $[M+K]^+$; mass found: 1396.74 $[M+Na]^+$, 1412.72 $[M+K]^+$; compound **5**: mass calcd: 1284.27 $[M+Na]^+$, 1300.31 $[M+K]^+$; mass found: 1284.71 $[M+Na]^+$, 1300.69 $[M+K]^+$; compound **6**: mass calcd: 1615.11 $[M+Na]^+$, 1631.21 $[M+K]^+$; mass found: 1614.91 $[M+Na]^+$, 1630.88 $[M+K]^+$. The nanoprobe MAS NMR data are shown in Figure 2, 3, and 4 and the Supporting Information.

Glycosylation protocol to establish solid-phase reactivity order of Ser, Thr and Tyr, and the glycosyl donors: All glycosylations were performed with freshly distilled, dry dichloromethane under an argon atmosphere. The reactions were carried out in a special 20-well peptide synthesiser[21] that allowed the addition of solvents and catalyst under an inert atmosphere (Ar). The resin and the amount of trichloroacetimidate donor (2 equiv compared with the amount of hydroxy functions) were dried overnight under high vacuum in the peptide synthesiser. The required amount of dichloromethane that allowed complete swelling of the resin was injected, and then the synthesiser was cooled to 0 °C. After 30 min, BF_3OEt_2 (0.3 equiv compared with trichloroacetimidate donor) was injected. After 90 min, suction was applied and the resin was washed with dichloromethane (10 ×). The resulting reaction mixtures were analysed by MALDI-TOF mass spectrometry. The results are shown in Table 1 (% conversion is calculated as [peak height product/{peak height remaining starting material + peak height product}] × 100%).

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