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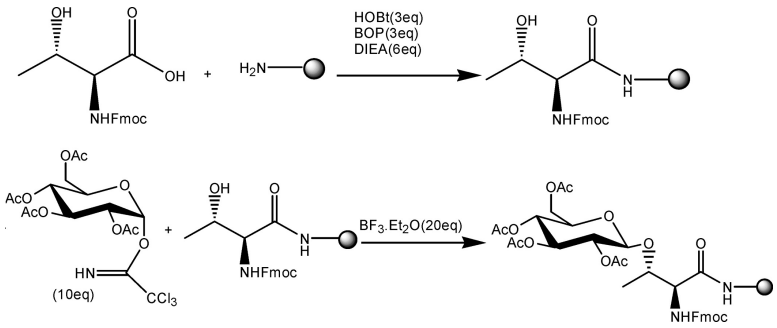

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Solid-Phase Synthesis of O-Glycosylated *N*α-Fmoc Amino Acids and Analysis by High-Resolution Magic Angle Spinning NMR

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Direct O-glycosylation of amino acids bound to TentaGel resin with a number of glycosyl trichloroacetimidate donors results in high yields. The glycosylation reaction can be easily monitored by analyzing the bead-bound amino acids with high-resolution magic angle spinning (HR-MAS) NMR. These studies pave a new way for the construction of "one-bead one-compound" *O*-glycopeptide libraries with standard amino acid building blocks and appropriate glycosyl trichloroacetimidate donors.

Introduction

Glycoproteins and glycopeptides are known to play important roles in immune response, intercellular recognition, cancer cell metastasis, and interflammation.[1−2] Glycosylation can sometimes promote the absorption of peptides from the lumen of the small intestine,[3] decrease their excretion through the kidney,[4] and provide protection of the peptides against enzymatic degradation.[3] There is a need for the development of methods to synthesize glycopeptide libraries efficiently. Several groups have reported the use of a solution-phase[5] or solid-phase method[6] to prepare carbohydrate or glycopeptide libraries. Despite these advances, many synthetic problems remain to be solved.[7−9]

The "one-bead one-compound" (OBOC) combinatorial library method is a highly efficient method for the rapid synthesis and screening of millions of compounds in a short time.[10] One major advantage of this method is that the compounds are spatially separable; therefore, all compounds can be tested concurrently but independently. TentaGel resin (polystyrene with grafted polyoxyethylene) is the most popular resin used in the OBOC library approach because of its favorable swelling characteristics both in organic and aqueous media, its uniform size, and its nonstick property.[10−11]

Rui et al. reported the use of preformed glycosyl amino acid building blocks to construct carbohydrate libraries.[6] The synthesis of glycosyl amino acid building blocks in a large quantity, however, has proven to be cumbersome[12,13] and low-yield. Direct solid-phase glycosylation of hydroxyl groups of Ser, Thr, or Tyr residues of peptides linked to POEPOP resin have been reported with an indirect quantitative method (MALDI-TOF) that requires a photo cleavable linker.[14−15] Moreover, glycosylation of free hydroxyl groups of Thr or Ser in peptides linked to PEGA resin, TentaGel, Polyhipe, and Macrosorb with a Rink linker as well as Polystyrene with Wang linker was promblematic.[16]

Our interest is to develop methods to construct OBOC combinatorial glycopeptide libraries on TentaGel resin, using peracetylated glycosyl trichloroacetimidates and commercially available Fmoc-amino acids. We also want to develop convenient methods to monitor the progress of the conjugation reactions on solid support during library construction. Recently, the high-resolution magic angle spinning (HR/MAS) NMR technique has been reported as a sensitive and nondestructive analytical method for obtaining detailed structure information of a compound covalently bound to resin support.[17] This technique can average out the inherent magnetic susceptibility difference over the heterogeneous sample to substantially reduce line-broadening. It has also been used to monitor the progress of the solid-phase reaction[18−21] and to determine the chemical structure of a compound bound on a single bead.[22−23] In this paper, we report on the use of peracetylated glycosyl trichloroacetimidate donors (galactose, glucose, mannose, fucose, glucosamine, lactose, maltose) to directly glycosylate amino acids (Ser, Thr, Tyr, and Hyp) on TentaGel resin. We also report the use of HR/MAS NMR spectroscopy to quantitatively monitor the incorporation of carbohydrates into these bead-bound amino acids.

Results and Discussion

Direct O-Glycosylation of Fmoc-Thr(OH)-CONH-TG with Carbohydrate 1,2-*trans*-Peracetates. Our initial experiments involved direct O-glycosylation of the free hydroxyl group of amino acids on TentaGel resin with increasing molar ratio of peracetated 1,2-*trans*-carbohydrates, because the starting materials are readily available and this method does not require additional steps. We selected Fmoc-Thr(OH)-COOH as the model compound and used a 10-fold excess of $BF_3{\cdot}Et_2O$ as catalyst and acetonitrile as solvent. O-acetylated Thr was only observed with β-D-glucose pentaacetate, owing to an ortho ester and acetyl

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Table 1. O-Glycosylation of Fmoc-Thr(OH)-COOH Using of $BF_3 \cdot Et_2O$ as Catalyst

entry	Fmoc amino acid	carbohydrate 1,2-*trans*-peracetate (equiv)	solvent	reaction time (h)	yield (%)
1	Fmoc-Thr-OH	Glu (10)	CH_2Cl_2	3	59
2	Fmoc-Thr-OH	Gal (10)	CH_2Cl_2	3	57
3	Fmoc-Thr-OH	Man (10)	CH_2Cl_2	3	25
4	Fmoc-Thr-OH	GluN (10)	CH_2Cl_2	3	0
5	Fmoc-Thr-OH	Lac (10)	CH_2Cl_2	3	31
6	Fmoc-Thr-OH	Mal (10)	CH_2Cl_2	3	17

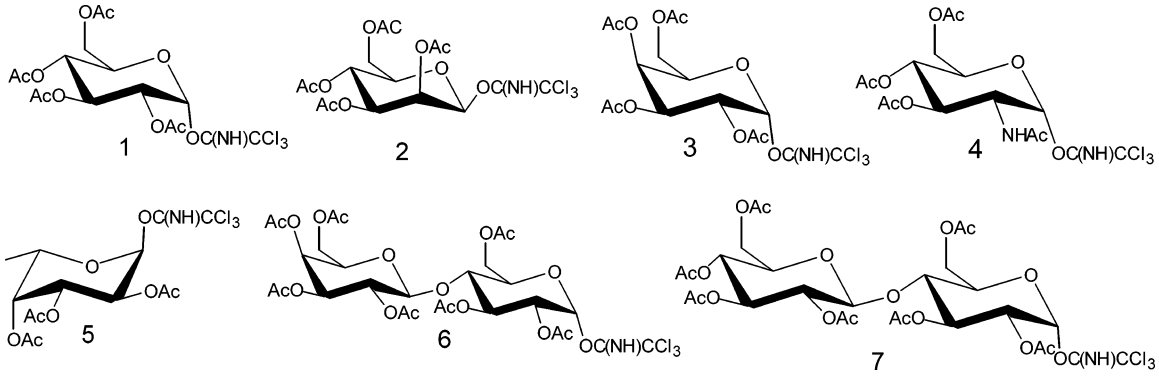

Figure 1. Trichloroacetimidate donors used for solid-phase synthesis of O-glycoylated Fmoc-AA(OH)-TG.

Scheme 1. Synthetic Route of O-Glycosylation of Fmoc-Thr(OH)-CONH-TG with Peracetylated Trichloroacetimidates of Glucose

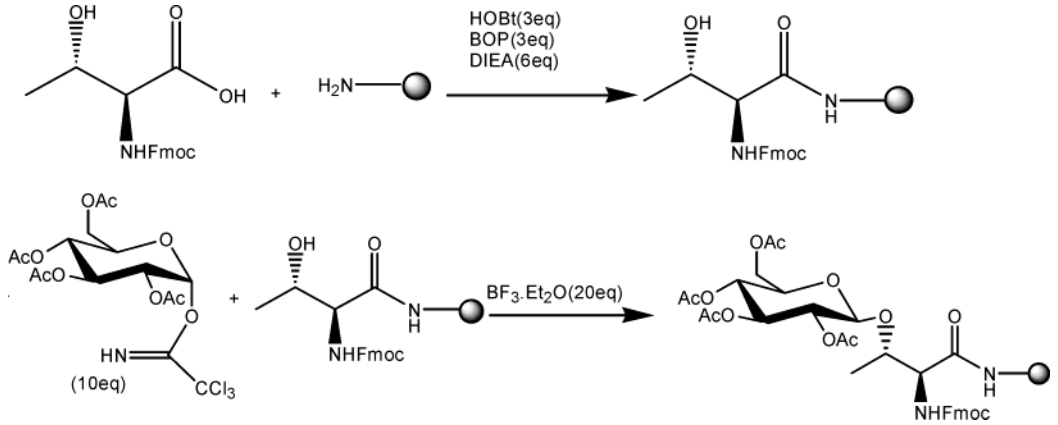

Table 2. O-Glycosylation of Fmoc-Thr(OH)-CONH-TG with Peracetylated Trichloroacetimidate of Fucose

entry	trichloroacetimidate donor (equiv)	$BF_3 \cdot Et_2O$ (equiv)	solvent	reaction time (h)	yield (%)
1	Fuc (10)	5	CH_2Cl_2	3	32.7
2	Fuc (10)	10	CH_2Cl_2	3	25.9
3	Fuc (10)	20	CH_2Cl_2	3	79.4
4	Fuc (10)	30	CH_2Cl_2	3	56.9
5	Fuc (10)	50	CH_2Cl_2	3	25.3

transfer.[12] Instead of acetonitrile, dichloromethane could give O-glycosylated Thr; however, the yields were low (Table 1), that is, not useful for OBOC combinatorial library construction.

O-Glycosylation of the Fmoc-AA(OH)-CONH-TG with Trichloroacetimidate Donors. We then explored the possibility of using trichloroacetimidate donors as the starting material, in which the α-hydroxyl group of the sugars is preactivated. Figure 1 shows the chemical structures of the seven peracetylated trichloroacetimidate donors that were presynthesized with a solution method.[24]

The synthetic route for O-glycosylation of Fmoc-Thr(OH)-TG with peracetylated trichloroacetimidates of glucose is outlined in Scheme 1. Fmoc-protected hydroxyamino acid was first coupled to TentaGel resin (0.27 mmol/g) using standard Fmoc chemistry.[25] Peracetylated trichloroacetimidate donors were then reacted with the Fmoc-AA(OH)-CONH-TG in DCM by using $BF_3 \cdot Et_2O$ as the catalyst. O-Glycosylation with peracetylated trichloroacetimidate of fucose in solution has been reported to give high yield.[15] We therefore selected O-glycosylation of Fmoc-Thr(OH)-CONH-TG with peracetylated trichloroacetimidate of fucose to optimize the reaction conditions. Table 2 indicates that the optimal level of $BF_3 \cdot Et_2O$ needed was ~20-fold excess. Further increase in $BF_3 \cdot Et_2O$ led to a decrease in the final yield of the desired product. Ten-fold excess of peracetylated trichloroacetimidate donors in DCM was used, and the

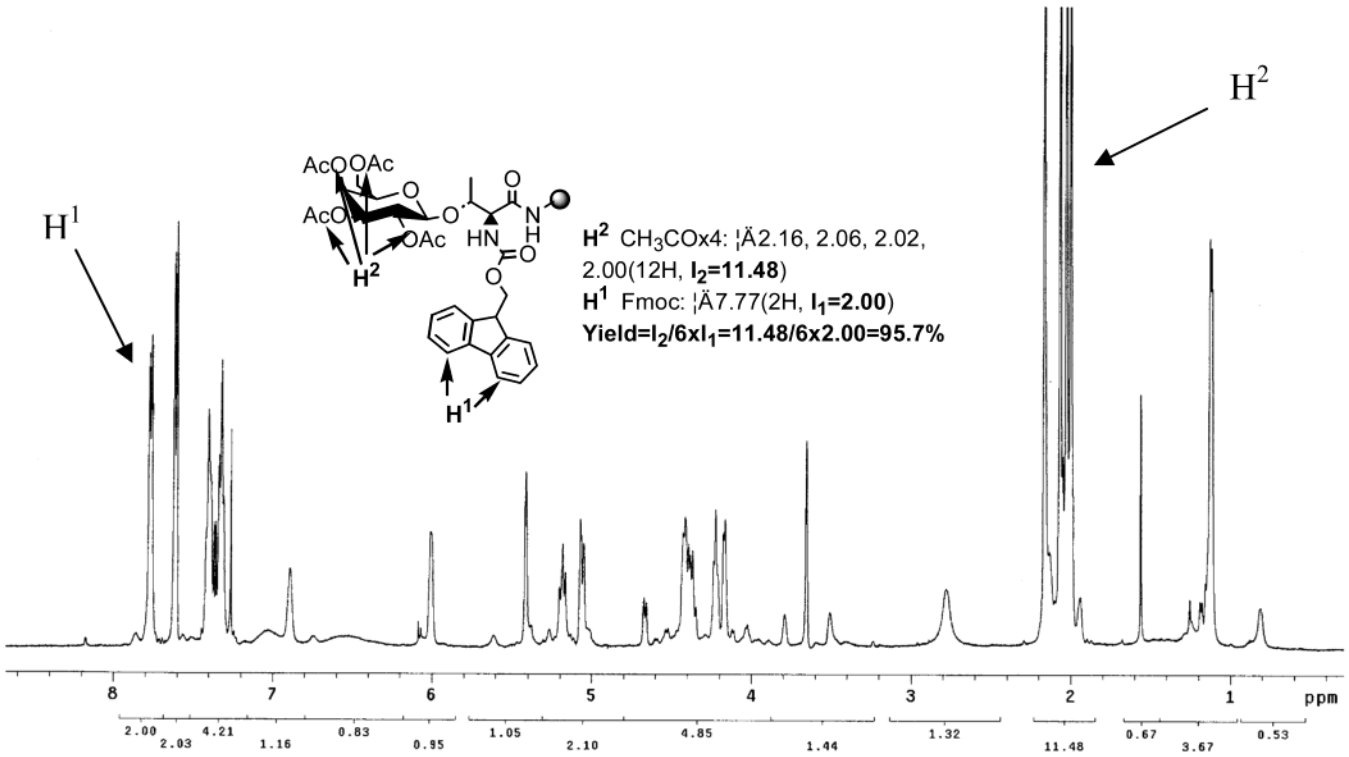

Figure 2. HR/MAS ^{1}H-NMR of tetra-O-acetyl-β-D-galactopyranosylated Fmoc-Thr-CONH-TG and its H^1 and H^2 protons assignments.

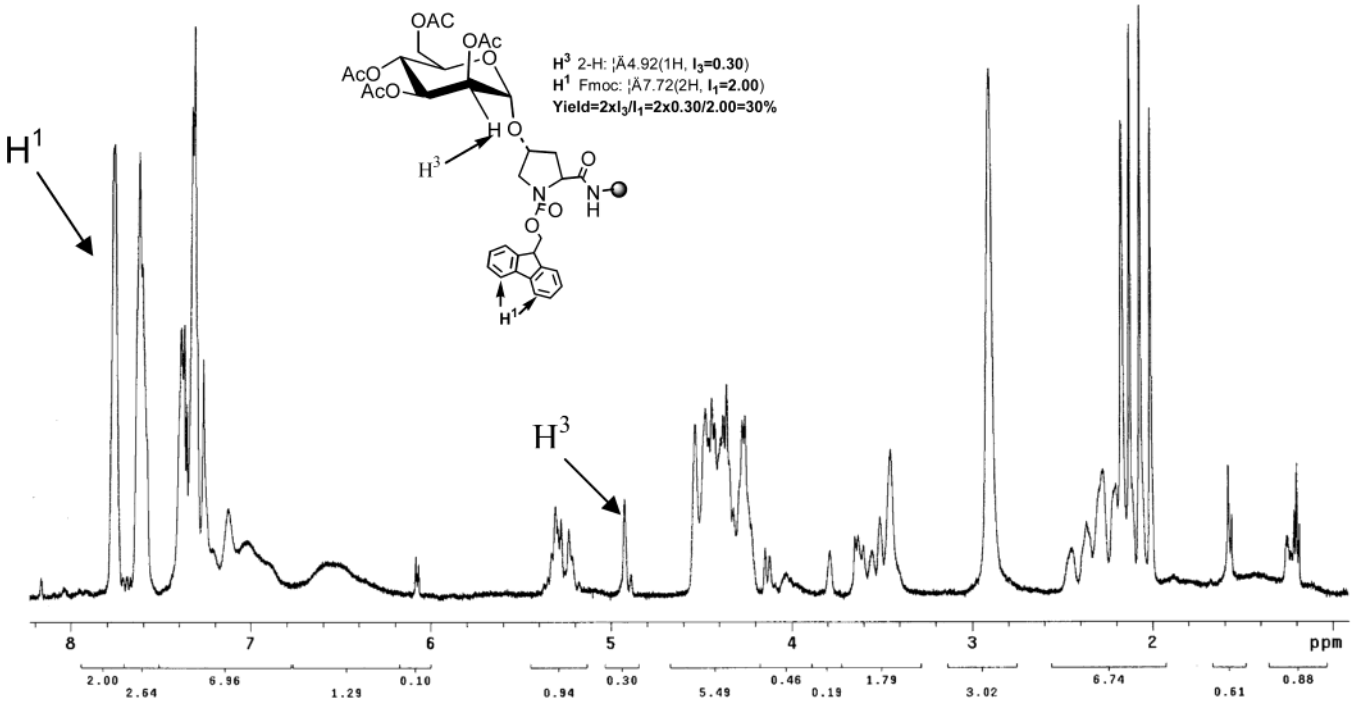

Figure 3. HR MAS ^{1}H NMR of tetra-O-acetyl-β-D-mannopyranosylated Fmoc-Hyp-CONH-TG and its $H^1$and H^3 protons assignments.

reaction proceeded at 0 °C for 3 h. We have found that double glycosylation did not increase the yield significantly.

Quantitative Monitoring of O-Glycosylation of Amino Acid on TentaGel Resin By High-Resolution Magic Angle Spinning (HR/MAS) NMR. Generally, the resin structure is the dominant factor that influences the ^{1}H line widths, and the solvent is playing the secondary role in the high-resolution MAS NMR. Paul[26] reported that presaturation of the methylene singlet at δ 3.55 of TentaGel resin is particularly effective at reducing all resonance arising from the resin, including the broad aromatic resonance. $CDCl_3$ was used as the solvent for the NMR studies because it can be removed easily during sample preparation and TentaGel swells well in this solvent. In a typical NMR experiment, 5−10 mg dried TentaGel resin was allowed to swell in 40 μL of $CDCl_3$. The methylene singlet at δ 3.55 was presaturated. The narrowest line widths and the high-resolution spectrum are obtained. Since proton (H^1) singlets of the Fmoc group stay in both starting material and product, we picked them as an internal standard indicated in Figure 2. The

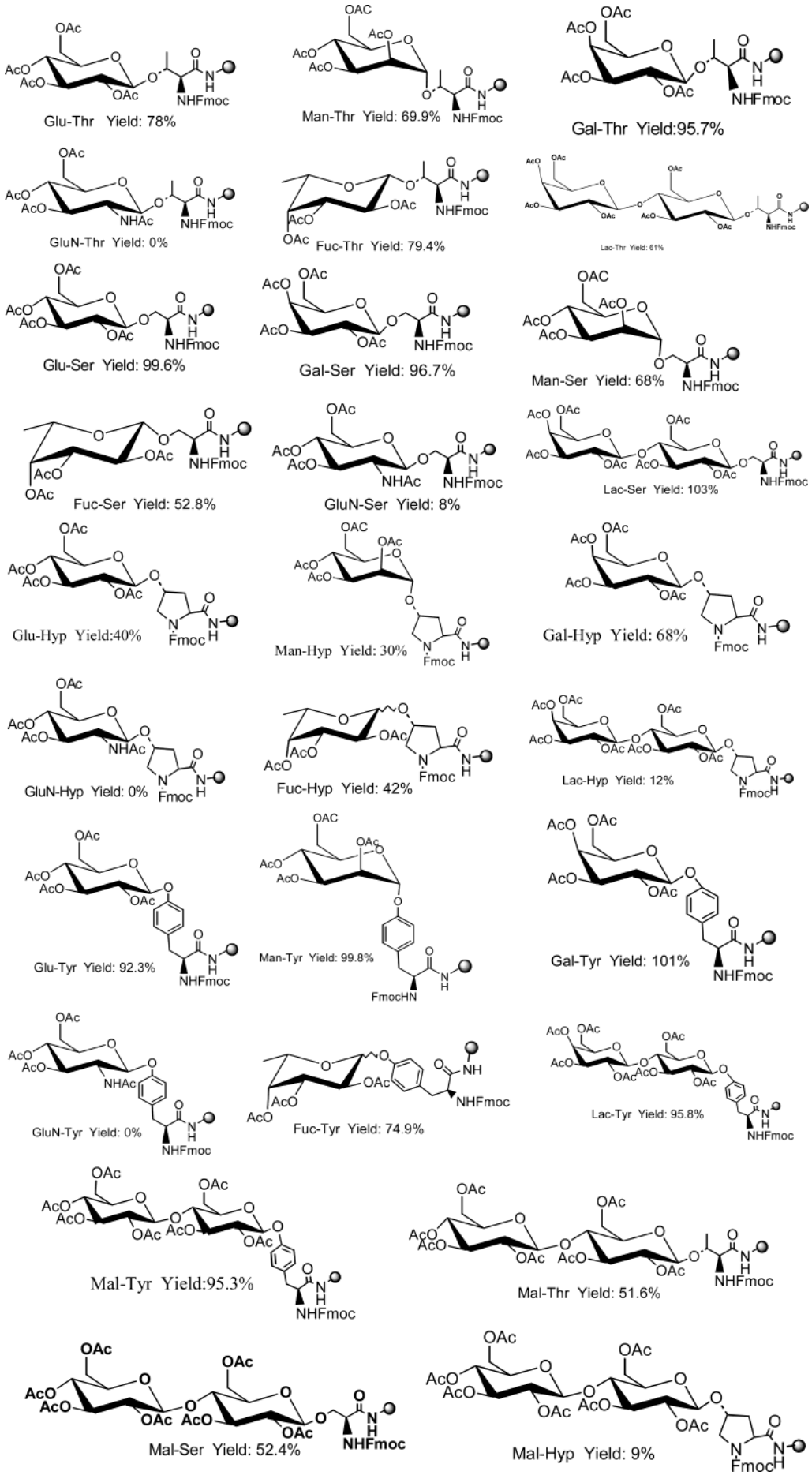

Figure 4. The O-glycosylated Fmoc-AA(OH)-CONH-TG and its yields.

increasing singlets of protons (H^2) from the acetyl group of the product located at $\sim\delta$ 2.0 without interfering of other proton signals. Proton (H^2) was then treated as a reporter of the reaction progress in this experiment, in comparison with protons (H^1). The yield of Glu, Man, Gal, and GluN in corporation with Thr, Ser, Tyr and Fuc in corporation with Thr, Ser, Tyr and Lac, Mal in corporation with Thr, Ser, Tyr are calculated by the formula

$$I_2/6 \times I_1, \quad 2 \times I_2/9 \times I_1, \quad 2 \times I_2/21 \times I_1$$

where I_1 and I_2 represent the peak volumes of two H^1 protons of the Fmoc group and 12, 9, and 21 H^2 protons from the acetyl group of product, respectively. Typical assignments of H^1 protons and 12 H^2 of Fmoc-Thr(Gal)-TG are indicated in Figure 2. Since singlets of methylene of the Hyp ring and acetyl group of sugar are overlapped, the 2-H (H^3) of sugar located at $\sim\delta$ 5.0 is alternatively selected as another reporter for comparing with the proton (H^1, Figure 3). The yield is then calculated by the formula $2 \times I_3/I_1$ in that case, where I_3 represents the peak volumes of the 2-H of sugar. The yields of all of the amino acid and sugar combinations are given in Figure 4.

Conclusions

Fmoc-protected amino acids bound to TentaGel resin are successfully O-glycosylated with a number of peracetylated glycosyl trichloroacetimidate in high yields. Our studies have shown that the preparation of Gal-Thr, Glu-Ser, Gal-Ser, Lac-Ser, Glu-Tyr, Man-Tyr, Lac-Tyr, and Mal-Tyr on TentaGel resin produced over 90% yield. It is conceivable that OBOC combinatorial glycopeptide libraries can be prepared using this approach. The yield for Glu-Thr, Man-Thr, Fuc-Thr, Lac-Thr, Man-Ser, Fuc-Ser, Gal-Hyp, Fuc-Tyr, and Mal-Ser ranged between 50 and 80% and will require further optimization prior to employment in OBOC library synthesis. Except for Hyp-Gal with a 68% yield, all the other Hyp-sugars had a low yield. Peracetylated trichloroacetimidate of glucosamine is not reactive under the current conditions. Some disaccharides (e.g., lactose, maltose) gave good results, but need to be further optimized. We have found that high-resolution MAS ^{1}H NMR is a valuable tool for quantitative monitoring of solid-phase glycosylation of amino acids bound to TentaGel resin.

Experimental Section

TentaGel resin (loading 0.27 mmol/g, 1% DVB cross-linked: 90 μm) was purchased from Rapp Polymere (Tübingen, Germany). Amino acids were purchased from Chem-Impex International, Inc. (Wood Dale, IL). 1,2-Trans peracetated carbohydrates were purchased from Sigma (St. Louis, MO). Dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF) were distilled from appropriate drying agents. All ^{1}H NMR experiments were performed on a Varian-Unity 500 MHz NMR spectrometer equipped with a 4 mm ^{1}H-observe Nano NMR-probe with a spin rate of $\sim$2 K Hz for all samples. Five to ten milligrams of beads was transferred into a nano NMR tube, and 40 μL of $CDCl_3$ was then added. The spectra were acquired at room temperature and with a presaturation at δ 3.55.

General Procedure for the Synthesis of Trichloroacetimidate Donors. Hydrozine acetate was added to a solution of 1,2-trans preacetated carbohydrates (5 g) in DMF (20 mL). After stirring for 24 h at room temp, ethyl acetate (100 mL) was added. The mixture was thoroughly washed with saturated sodium hydrogen carbonate and saturated NaCl and dried over $MgSO_4$. Concentrated residue was then dissolved in CH_2Cl_2 (40 mL), followed by the addition of trichloroacetonitrile (8 mL, 6 equiv) and DBU (0.25 mL, 0.13equiv). After stirring for an additional 24 h at room temperature, the reaction mixture was concentrated, and the final peracetylated glycosyl trichloroacetimidate was purified by silica gel column chromatography (petroleum ether/ethyl acetate (2:1), containing 0.1% NEt_3) (**1**−**7**).

General Coupling Procedure for the Preparation of Resin-Bound Fmoc Amino Acids. TentaGel resin (1.0 g) was swollen in DMF for 30 min at room temperature in a reaction column fitted with a frit filter. Amino acids (3.0 equiv), HOBt (3.0 equiv), BOP (3.0 equiv), and DIEPA (6.0 equiv) were mixed with 12 mL of DMF for 15 min and then added to TentaGel resin. The final mixture was agitated in a shaker for at least 3 h at room temperature until a Kaiser test became negative. The resin was then thoroughly washed with DMF (×3), DCM (×3), MeOH (×3), and DCM (×3) and dried in a vacuum prior to the next O-glycosylation reaction.

General O-Glycosylation Procedure. The glycosylation was performed with freshly distilled DCM in a flask under an argon atmosphere. Fmoc-AA(OH)-CONH-TG(75 mg) and trichloroacetimidate donors (10 equiv) were first added to the flask and dried in a vacuum overnight. A 2.0-mL portion of DCM was then added for additional 30 min at room temperature to completely swell the resin, and 60 μL of BF_3·Et_2O (20 equiv) was subsequently added. After the reactants were stirred for 3 h at 0 °C, the resin was washed thoroughly with DMF (×3), 20% DIPEA in DMF (×2), DCM (×3), MeOH (×3), DCM(×3), and Et_2O (×3), and dried enough in a vacuum for the ^{1}H NMR experiment.

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