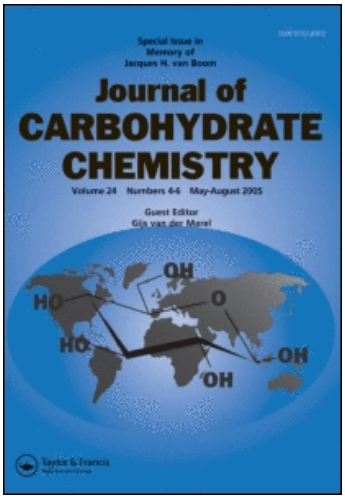

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Practical Synthesis of a Manβ(1-4)GlcNTroc Fragment via Microfluidic β-Mannosylation

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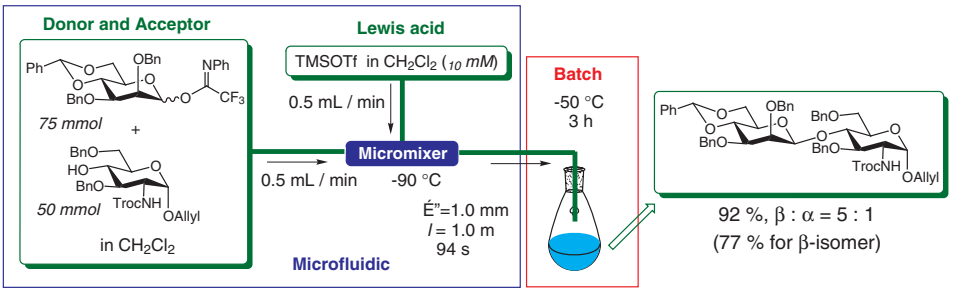
Graphical Abstract



Combined microfluidic/batch conditions were applied to β-mannosylation, a key glycosylation for the synthesis of the Manβ(1-4)GlcNAc motif in *N*-glycan structures. By applying the advantageous features of microfluidic conditions (i.e., efficient mixing and fast heat transfer), the Manβ(1-4)GlcNTroc fragment was practically and reproducibly synthesized on the gram scale.

Keywords β-Mannosylation; Manβ(1-4)GlcNAc fragment; Microreactor; *N*-glycan

Stereoselective formation of the β-mannoside linkage, a key glycosylation in the synthesis of the Manβ(1-4)GlcNAc unit of *N*-linked glycoproteins,[1,2] is a challenging topic in oligosaccharide synthesis. Various methods,[1,3–11]

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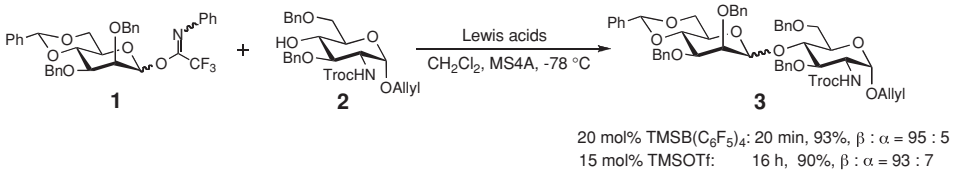
such as intramolecular aglycon delivery (IAD)[12–14] or glycosylation with 4,6-*O*-benzylideneacetal-protected α-mannosyltriflates,[15] have recently been reported and successfully applied to β-mannoside synthesis. We have been investigating the efficient synthesis of the Manβ(1-4)GlcNAc motif as part of our research on solid-phase synthesis of *N*-glycan[16]. We have achieved an excellent β-selectivity in the reaction of 4,6-*O*-benzylidene-mannopyranosyl-*N*-phenyltrifluoroacetimidate **1** (C_4–OB_n or C_4–OR_ZClB_{n4} see respective structures) with *N*-Troc-glucosamine acceptor **2** (93% yield, $\beta{:}\alpha = 95:5$) (Sch. 1)[17] using the bulky and dual Lewis acid/cation trap reagent TMSB$(C_6F_5)_4$.[18]

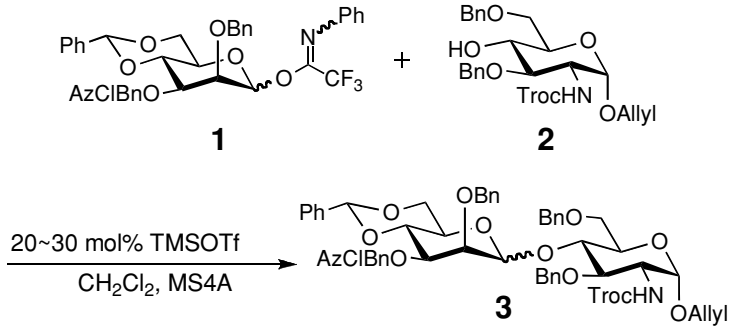


Scheme 1: Previous β-mannosylation using TMSB$(C_6F_5)_4$ and TMSOTf as activators. Glycosylation is performed using 1.5 equiv of donor **1** relative to acceptor **2**.

Nevertheless, it is difficult to apply our β-mannosylation protocol to a few gram-scale synthesis of Manβ(1-4)GlcNTroc fragment **3** because the scaled-up glycosylation requires a large quantity of the bulky TMSB$(C_6F_5)_4$ activator, which has limited commercial availability.[17,18] Therefore, from a practical viewpoint for preparing Manβ(1-4)GlcNTroc as a starting material, we refocused on applying more common TMSOTf as a glycosyl activator because our earlier experiments[17] indicated that TMSOTf shows a good yield and β-selectivity on a 20-mg scale (90% yield, $\beta{:}\alpha = 93{:}7$) (Sch. 1).

However, the efficiency of glycosylation is extremely sensitive to the reaction scale as well as the addition speed of the Lewis acid (Table 1). When TMSOTf was added dropwise to a solution of mannosyl donor **1**[19] and acceptor **2** at –78°C, the yield of β-mannoside **3** gradually decreased as the reaction scale increased (entries 1–3). On a 50-mg scale, 63% of β-disaccharide **3** was isolated, whereas only 27% of β-isomer **3** was obtained on a 500-mg scale (entries 1 and 3; yield for isolated β-mannoside is shown in the table). For an unknown reason, slowly adding a Lewis acid in the larger-scale reactions inhibited the glycosylation process at an earlier stage.[20] Moreover, even the subsequent addition of the TMSOTf catalyst did not activate the glycosylation between the remaining starting materials. On the other hand, when the acid was added to the initial solution of **1** and **2** in one portion, mannosylation proceeded smoothly (entry 4). However, the β-selectivity decreased to 4.9:1, presumably due to the exothermic nature of the reaction—that is, heat is generated while rapidly mixing, which leads to an overall decrease in the isolated yield of β-disaccharide **3** (61% on 900-mg scale).

Table 1: β-Selective mannosylation using TMSOTf as an activator[a]



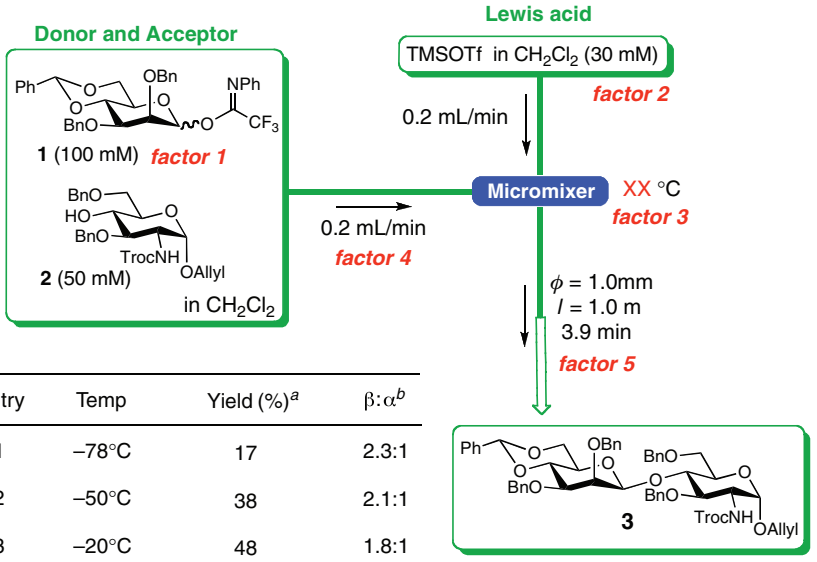
Entry	Addition of LA	Scale (mg)	Yield (%)[b]
1	dropwise	50	63
2	dropwise	300	48
3	dropwise	500	27
4	In one portion	900	61[a]

[a]Reaction is performed using 1.5 equiv. of donor **1** relative to acceptor **2**.
[b]Isolated yields for β-isomer.
[c]$\beta:\alpha = 4.9:1$ based on ^{1}H NMR analysis.

Therefore, we decided to examine the microfluidic conditions based on the observations shown in Table 1, which indicate that the current glycosylation is sensitive to the addition speed of the Lewis acid (i.e., slow or fast mixing), as well as the reaction scale. A continuous-flow microreactor, which has been reported to realize efficient mixing and a fast heat transfer, has been recognized as innovative technology in recent organic syntheses.[21,22] In addition, a flow system allows the residence time to be controlled. Hence, this method is well-suited for reactions with unstable intermediates. Once reaction conditions are optimized for a small-scale operation, the same conditions are directly applicable to large-scale synthesis under the flow process. By taking advantage of these aspects, we have recently applied a microfluidic system to cation-mediated reactions[23] and have realized improvements for α-sialylation,[23(b)] dehydration,[23(c)] and reductive opening of the benzylidene acetal groups in sugar.[23(d)] Because the inefficiency of these cation-mediated reactions under the batch process is due to inefficient mixing with the acid reagents on a large scale, we envisioned that microfluidic conditions might also circumvent the scaling-up problems associated with the β-mannosylation observed in Table 1. Herein, we report the practical synthesis of the Manβ(1–4)GlcNTroc fragment via microfluidic β-mannosylation catalyzed by TMSOTf as a common glycosyl activator. This method was used to prepare β-mannoside disaccharide **3** on a few gram scales, but could be used on a larger scale and readily be applicable to *N*-glycan synthesis.

As shown in Table 2, we initially constructed the microfluidic system based on our previous experiences with microfluidic α-sialylation.[23(b)] In addition to

Table 2: β-Mannosylation under microfluidic conditions



Entry	Temp	Yield (%)[a]	β:α[b]
1	–78°C	17	2.3:1
2	–50°C	38	2.1:1
3	–20°C	48	1.8:1

[a] Isolated yields as a mixture of β- and α-isomers.
[b] ^{1}H NMR and HPLC analyses determined the α/β-ratio.

the aspects mentioned above, an attractive feature of the microfluidic reaction is that the reaction can be readily optimized under the flow process;[22(d)] the optimal conditions are rapidly determined using a small quantity of materials, that is, concentrations of the substrates, mixing speed, temperature, and residence time (optimization factors 1–5 in Table 2). Thus, a dichloromethane solution of mannosyl donor **1** and glucosaminyl acceptor **2** with various concentrations (optimization factor 1) was mixed with a TMSOTf solution in dichloromethane to determine the optimal concentration (factor 2) at the appropriate temperature (factor 3) using a Comet X-01 micromixer[24] at various flow rates (factor 4). After the reaction mixture was allowed to flow at an appropriate time interval (factor 5) through a reactor tube ($\Phi = 1.0$ mm), the mixture was quenched by introducing a triethylamine solution in dichloromethane.

By using such a microfluidic apparatus, we investigated more than 30 conditions in a combinatorial fashion. Table 2 shows representative data when the flow rate was fixed at 0.20 mL/min, and the concentrations of donor **1**, acceptor **2**, and TMSOTf were adjusted to 100 mM, 50 mM, and 30 mM, respectively. When micromixing and the flow reaction occurred at –78°C, a mixture of α/β-disaccharide **3** was obtained in only 17% yield (β:α = 2.3:1, entry 1). Although increasing the temperature further improved the yield of **3**, the β-selectivity decreased (entries 2 and 3); α/β mixture of **3** was obtained in 38% at –50°C with β:α = 2.1:1, but in 48% at –20°C with β:α = 1.8:1.

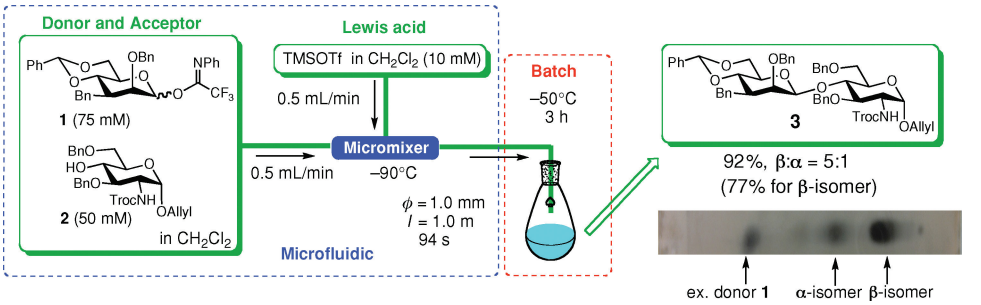


Figure 1: β-Mannosylation using an integrated microfluidic/batch system. Yield and β/α ratio are analyzed by ^{1}H NMR, HPLC (column: nacalai tesque 5C$_{18}$-AR300. 4.6 × 250 mm; MeCN in H$_2$O(55–100% gradient over 60 min); retention time of the β-isomer: 46.3 min. α-isomer: 48.6 min), as well as TLC stain contrast detected by *Image J 1.40* (eluent; toulene:AcOEt:10:1).

The preliminary optimization performed in Table 2 demonstrated that both the β-selectivity and yield may further be improved by (1) micromixing the substrates and acid solutions at a lower temperature and (2) a longer reaction time (residence time) at a higher temperature after micromixing. Although it is theoretically possible to maintain an indefinite residence time by increasing the reactor tube length, in certain conditions (i.e., when the reaction has to proceed for more than an hour), employing an extremely long tube is impractical. Therefore, as shown in Figure 1, we constructed an apparatus where the microfluidic system is integrated with a conventional batch apparatus. Namely, the reaction solution through the micromixing system was subsequently inserted into the batch system, and then was conventionally stirred in a flask for a few hours to complete the reaction.

Thus, glycosylation trials in the integrated microfluidic/batch apparatus led to the determination of the optimal conditions: micromixing at –90°C and a batch reaction at –50°C for 3 h. Further adjustments of the microfluidic parameters (i.e., concentration of substrates and flow speed), as depicted in Figure 1, provided α/β-mannoside **3** in 92% yield and with a moderate β-selectivity (β:α = 5.0:1).[25]

It should be noted that although the β-selectivity was somewhat lower than that observed in the small-scale batch reaction (Sch. 1), β-mannoside **3** could be obtained in a similar efficiency (77% for microfluidic reaction versus 84% for 20-mg scale batch reaction). Moreover, under the microfluidic mannosylation conditions, Manβ(1–4)GlcNTroc fragment **3**, which is easily separated from the α-isomer by column chromatography, was reproducibly obtained even in the scaled-up synthesis (Experimental section) by simply preparing stock solutions of substrates and reagents, and then continuously pumping them into the integrated microfluidic/batch system.

In summary, we have established a practical β-mannosylation under an integrated microfluidic/batch system. In addition to realizing efficient mixing and precise temperature control for β-mannosylation, an advantage of

utilizing microfluidic reactions is the rapid determination of the optimal conditions. Although perfect β-glycosyl bond formation was not realized by adjusting the microfluidic parameters, the reproducibility and scaled-up preparation of Manβ(1-4)GlcNTroc fragment **3** under the flow process is noteworthy from the viewpoint of preparing an important starting material for complex oligosaccharide synthesis.

EXPERIMENTAL SECTION

Allyl 4-*O*-[3-*O*-(4-Azido-3-chlorobenzyl)-4,6-*O*-benzylidene-2-*O*-benzyl-α-D-mannopyranosyl]-3,6-di-O-benzyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (3)

A solution of TMSOTf (92.7 μL, 0.513 mmol, 13.5 mM) in CH_2Cl_2 (38.0 mL) was injected, in advance, into the micromixer by a syringe-pump at a flow rate of 0.95 mL/min. Then a solution of donor **1** (C4-OAzClBn, 1.98 g, 2.85 mmol, 75.0 mM) and acceptor **2** (1.09 g, 1.90 mmol, 50.0 mM) dissolved in CH_2Cl_2 (38.0 mL) was injected into the micromixer by another syringe-pump at a flow rate of 0.5 mL/min. The reaction was mixed at –90°C. After the reaction mixture was allowed to flow at –90°C for an additional 94 s through a Teflon tube reactor (Φ = 1.0 mm, l = 1.0 m), the mixture was introduced into a flask, which was previously cooled to –50°C. The reaction mixture was stirred for 4 h at this temperature, and the mixture was quenched by triethylamine at –50°C. The resulting mixture was extracted with ethyl acetate, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The residue was purified by column chromatography on silica gel (13% ethyl acetate in toluene) to give β-mannoside **3** as a colorless oil (1.34 g, 67%): ESI-MS m/z calcd for $C_{53}H_{55}Cl_4N_4O_{12}$ $(M+H)^+$ 1079.2, found 1079.2; ^{1}H NMR (400 MHz, $CDCl_3$, data for β-anomer) δ 7.40–7.03 (m, 23H, aromatic), 5.95–5.86 (m, lH, $-C\underline{H}_2-CH=CH_2$), 5.46 (s, lH, PhC$\underline{H}$-), 5.30 (dd, J = 17.3, 1.5 Hz, 1H, $-CH_2-CH=C\underline{H}_2$), 5.24 (dd, J = 10.3, 1.0 Hz, 1H, $-CH_2-CH=C\underline{H}_2$), 5.04 and 4.67 (each d, J_{gem} = 11.5 Hz, 2H, $-C\underline{H}_2Ph$), 5.01 (d, $J_{N,2}$ = 10.0 Hz, 1H, N$\underline{H}$), 4.92 (d, J = 3.4 Hz, 1H, H-1), 4.82 (s, 2H, $-C\underline{H}_2Ph$), 4.73 and 4.37 (each d, J_{gem} = 12.0 Hz, 2H, $-C\underline{H}_2Ph$), 4.67 and 4.64 [each d, J_{gem} = 12.2 Hz, 2H, $-C\underline{H}_2$-($C_6H_3N_3Cl$)], 4.62 and 4.59 (each d, J_{gem} = 12.7 Hz, 2H, $-NH-COO-C\underline{H}_2-CCl_3$), 4.67 (s, 1H, H-1′), 4.09 (dd, J = 12.3, 5.1 Hz, 1H, $-C\underline{H}_2CH=CH_2$), 4.06–3.93 (m, 5H, $-C\underline{H}_2-CH=CH_2$, H-2, H-4, H-4′,H-6′a), 3.70–3.57 (m, 5H, H-3, H-4, H-6a, H-6b, H-2′), 3.45 (t, J = 10.3 Hz, 1H, H-6′b), 3.37 (dd, J =9.5, 2.9 Hz, 1H, H-3′), 3.07 (td, J = 9.5, 4.9 Hz, 1H, H-5′).

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19. Differences in reactivity were not observed between the Bn- and AzClBn-protecting group at the C3-hydroxyl of donor **1.**

20. As shown in Table 1, a rather unusual phenomenon in which the slow addition of TMSOTf inhibits glycosylation in the middle of the reaction is observed, and cannot be clearly explained based on presently available data. However, it is possible that the different active species from imidate **1** may be involved in our mannosylation.

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25. Although the selectivity slightly increased to β:α = 6.0:1 when a batch reaction was performed at -55°C, the reaction stopped in a few hours, and the yield of β/α-mannoside **3** decreased to 42% yield (36% for β-mannoside).