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High throughput screening of *O*-glycosylation conditions

Akihiro Ishiwata[a,b] and Yukishige Ito[a,b,*]

[a]*RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*
[b]*CREST, JST, Kawaguchi, Saitama 332-1102, Japan*

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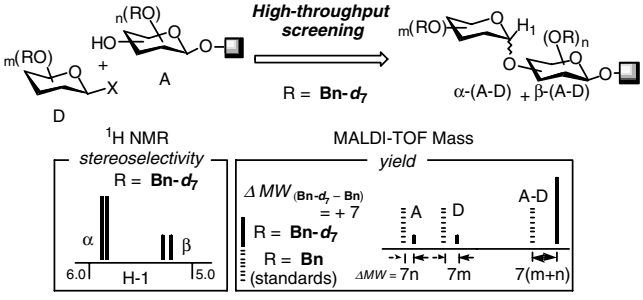
Abstract—We report a novel methodology for rapid and quantitative screening of *O*-glycosylation reactions of application to the analysis of parallel reaction systems. Our system exploits perdeuterated benzyl (Bn-d_7) ether, and stereoselectivity and yield are evaluated by ^{1}H NMR and MALDI-TOF MS, respectively. This paper summarizes over 240 screenings of 1 → 3 linkage formation between glucose residues targeting the α-isomer in high yield.

Optimization of reaction conditions is essential in multistep organic synthesis. In oligosaccharide synthesis, too, the efficiency of glycosylation is of primary importance.[1] Ideally, glycosylation should proceed in high yield and with complete stereoselectivity. Although there are a number of factors (i.e., solvent, temperature, molar ratio, leaving group, promoter, additives, concentration, etc.) that may affect the results, their effects are hardly predictable. To be meaningful, screening of reaction conditions should be conducted with each result evaluated quantitatively in terms of both yield and selectivity. In a conventional procedure, >0.05 mmol of substrates are typically required for each trial and results are evaluated after work-up, chromatographic isolation and spectroscopic analysis of products. The optimization processes, as a whole, tends to be time and material consuming. We report herein a novel methodology for rapid and quantitative screening of *O*-glycosylation reactions, which can be applied to the analyses of parallel reaction systems. Our system exploits perdeuterated benzyl (Bn-d_7) ether, and stereoselectivity and yield are evaluated by ^{1}H NMR and MALDI-TOF MS, respectively (Scheme 1).[2,3]



Scheme 1. Quantitative screening of *O*-glycosylation reactions by ^{1}H NMR and MALDI-TOF MS.

Bn ether[4] is one of the most widely used protective groups in carbohydrate chemistry.[5] It is stable under a variety of acidic, basic, oxidative, reductive, and organometallic conditions, and can be removed by catalytic hydrogenolysis under mild conditions. However, direct ^{1}H NMR analysis of oligosaccharides with multiple *O*-Bn groups is problematic. Benzylic methylene signals appear at 4–5 ppm as AB-quartets, obscuring the signals derived from anomeric protons. By employing Bn-d_7,[6] the benzylic methylene signals disappear and the isomeric ratio of glycosylated products can be determined easily by the relative integration of the anomeric signals. On the other hand, addition of Bn-d_7 increases the molecular weight (M.W.) by +7 Da as compared to Bn. For the yield, inspection of the MS spectrum of a reaction mixture supplemented with a defined amount of non-labeled substrate (donor or acceptor) or of product should provide a quantitative estimate[7] of the product yield and substrate recovery. Thus by combining MALDI-TOF MS and high-field NMR, reactions performed on micromolar scales can be analyzed rapidly.

Keywords: Stereoselective glycosylation; Rapid and quantitative screening; Deuterium-labeled compound; MALDI-TOF MS; ^{1}H NMR.
* Corresponding author. Tel.: +81 48 462 1111; fax: +81 48 462 4680; e-mail: yukito@riken.jp

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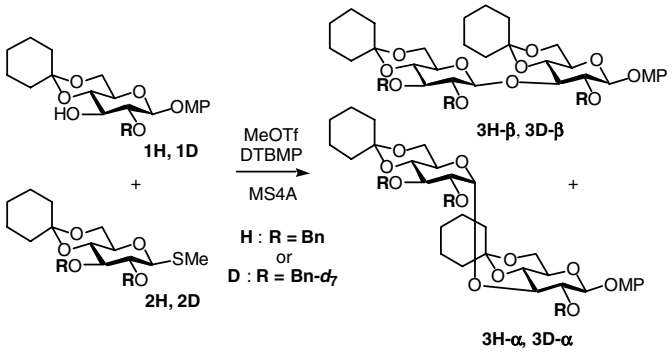

Scheme 2. Glycosylation of **1** with **2**.

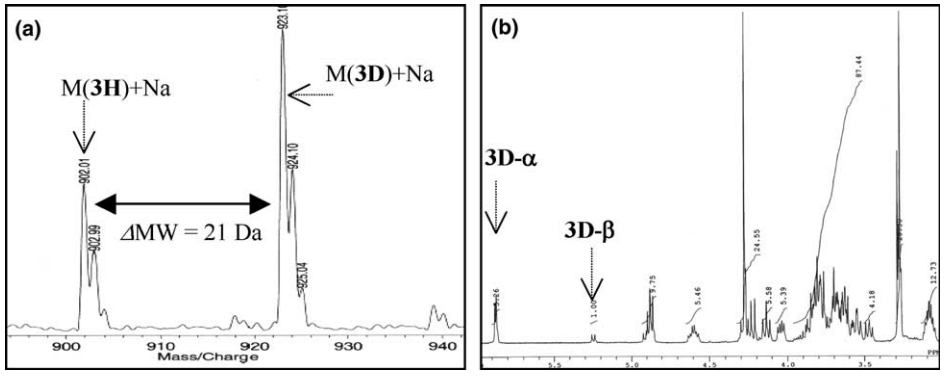

Figure 1. (a) MALDI-TOF MS spectrum of product **3D** (**3H**-α was used for the standard); (b)^{1}H NMR spectra of the crude mixture in C_6D_6. The mixture includes the products (**3D**-α and **3D**-β) concomitant with unreacted substrates (**1D** and **2D**).

As an initial demonstration of the strategy, the reaction depicted in Scheme 2 was examined with the aim of optimizing the glycosylation to form α1 → 3 linkage between glucose (Glc) residues.[8] This structure corresponds to the sub-terminal region of high-mannose type tetradecasaccharide $Glc_3Man_9GlcNAc_2$, a glycan that is transferred from dolichol diphosphophosphate (Dol-PP) to nascent polypeptide as the precursor of all types of asparagine (Asn)-linked glycoproteins.[9,10] As monosaccharide substrates, the thioglycoside **2** and the acceptor **1** were prepared in their Bn-d_7 and Bn protected forms from β-D-glucose pentaacetate.[11] To begin with, non-labeled substrates **1H** and **2H** (1.2 equiv) were reacted in the presence of 1.2 equiv of methyl trifluoromethanesulfonate (MeOTf),[12] 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP), and molecular sieves (MS) 4 Å in $(CH_2Cl)_2$ in order to obtain the mixture of the products and substrates. Under these conditions, an 80% yield of disaccharide **3H**[13] was obtained as a 5.0:1 mixture of α- and β-isomers with 33% and 20% recovery of donor (**2H**) and acceptor (**1H**), respectively. With a standard sample of the disaccharide **3H** in hand, the same glycosylation was conducted with deuterium-labeled compounds **1D** and **2D** in various solvents. In $(CH_2Cl)_2$, product **3D**[13] was obtained in identical yield and selectivity as **3H**. It was confirmed that the ionizing properties are nearly identical between deuterated/non-deuterated (**3H** vs **3D**) and α- and β-isomers (**3H**-α vs **3D**-β).[14] For easy quantitative estimation of the yield by MS analysis, it is essential to have the ion peaks of labeled and non-labeled standard samples widely separated with almost same ionizing properties (Fig. 1a). The ^{1}H NMR spectrum of **3D**-α is simpler than that of **3H**-α, especially in the anomeric region (4.5–6.0 ppm) as mentioned above (Fig. 1b).

As a next step, systematic screening was conducted in a parallel setting; reactions were performed with ~2 mg (~5 μmol) of each substrate. MALDI-TOF MS spectra of the crude mixtures were measured using stock solutions of **1H**, **2H**, and **3H** (1.0 mM each in CH_3CN) and yields were calculated from peak heights relative to the standards.[15] Anomeric ratios were estimated from relative integrations of H-1 signals (in C_6D_6) of α- (δ 5.89 ppm, J 3.6 Hz) and β- (δ 5.26 ppm, J 7.2 Hz) isomers.[15]

In total, 86 solvents were tested at ambient temperature (~23 °C) and 49 at 50 °C. The reactions were carried out in the presence of 4.2 equiv of MeOTf, DTBMP, and MS4A. Some of these results are summarized in Table 1, which displays the following features: (1) In contrast to general perception, the degrees of stereoselectivity are quite variable among halogenated hydrocarbons (entries 1–5). Among them, chloroform proved to be most effective, orienting the reaction to α-selectivity (α:β = 10.9:1, entry 3). (2) Compared to benzene, toluene, and *p*-xylene, substantially higher selectivity was observed for aromatics with electron-withdrawing substituents (entries 9–13). (3) Among ethereal solvents (en-

Table 1. Selected results of the effect of the solvent on the glycosylation of **1D** and **2D** at room temperature and 50 °C[a]

Solvent entry	Yield/% (α:β) at rt	Yield/% (α:β) at 50 °C
1. CH_2Cl_2	61 (5.89:1)	—
2. $(CH_2Cl)_2$	96 (3.68:1)	97 (4.18:1)
3. $CHCl_3$	60 (10.9:1)	100 (4.95:1)
4. CCl_4	25 (2.02:1)	85 (1.87:1)
5. $C_2Br_2F_4$	73 (1.86:1)	58 (2.54:1)
6. Benzene	65 (1.86:1)	100 (2.12:1)
7. Toluene	66 (1.46:1)	91 (1.84:1)
8. Xylene	68 (1.31:1)	97 (4.18:1)
9. PhF	69 (3.65:1)	100 (1.64:1)
10. PhCl	83 (3.13:1)	99 (4.85:1)
11. PhOMe	55 (3.11:1)	96 (3.13:1)
12. $PhCO_2Et$	68 (4.33:1)	98 (3.47:1)
13. *o*-$C_6H_4Cl_2$	94 (3.13:1)	100 (2.34:1)
14. Et_2O	76 (4.31:1)	—
15. *c*-C_5H_9OMe	78 (6.91:1)	42 (17.6:1)
16. *t*-BuOMe	57 (5.50:1)	1.4 (—)
17. THP	65 (3.33:1)	8.9 (4.18:1)
18. Dioxane	87 (4.11:1)	94 (1.60:1)
19. DME	43 (3.78:1)	90 (3.23:1)
20. EtOAc	77 (2.72:1)	96 (3.38:1)
21. Acetone	0 (—)	—
22. DMP	0 (—)	—
23. DEC	53 (2.80:1)	98 (2.31:1)
24. MeCN	7 (1:2.15)	33 (1:2.11)
25. EtCN	28 (1:1.06)	32 (1:1.48)
26. *n*-BuCN	99 (1.06:1)	60 (1.30:1)
27. CCl_3CN	93 (2.82:1)	100 (2.63:1)
28. DTP	30 (1:3.42)	27 (1:1.96)
29. DMF	0 (—)	—
30. DMSO	0 (—)	—

[a] Abbreviations: THP: tetrahydropyrane, DME: dimethoxyethane, DMP: 2,2-dimethoxypropane, DEC: diethyl carbonate, DTP: 2,6-di-*tert*-butylpyridine, DMF: dimethylformamide, DMSO: dimethylsulfoxide.

tries 14–18), cyclopentyl methyl ether gave the highest selectivity. (4) A number of liquids that are rarely used for glycosylation (e.g., EtOAc, CCl_3CN, *o*-$C_6H_4Cl_2$, *c*-C_5H_9OMe, DEC, ...) gave high yields of products, while dipolar (DMSO, DMF, ...), ketonic, and acetalic liquids proved to be unsuitable for *O*-glycosylation. (5) Although the yield was low, the substantial β-selectivity observed for 2,6-di-*tert*-butylpyridine (entry 28) may be indicative of the reverse anomeric effect.[16]

Mixed solvents systems (105 in total) were also screened; $CHCl_3$, toluene, *c*-C_5H_9OMe, *n*-BuCN, and EtOAc were selected as the principal solvents, each of which was mixed with 21 solvents in 1:1 ratio. Only 45 results are shown in Table 2. Overall, among the 240 reaction conditions screened, it was concluded that the optimum conditions were for entries 1–3 (3–1) in Table 2 ($CHCl_3$–*c*-C_5H_9OMe, 1:1, rt) in terms of both yield (quantitative) and selectivity (α:β = 11.4:1).

Our system enables the qualitative and facile screening of glycosylation reactions performed in parallel. Further refinements are possible to enhance the throughput. Firstly, MALDI-TOF MS may be used for the initial screening. The relatively time-consuming NMR analyses could be limited to high-yielding entries. Faster processing should be possible by automation and scaling-down with state-of-the-art instruments such as nano-probe NMR[17] and microreactors.[18]

Acknowledgments

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Table 2. Selected results of the effect of the solvent mixture (1:1) on the glycosylation of **1D** and **2D** at room temperature

Principal gradient	(1) $CHCl_3$			(2) Toluene			(3) *c*-C_5H_9OMe			(4) *n*-BuCN			(5) EtOAc		
Solvent (1:1)	Entry	Y./%	α:β	Entry	Y./%	α:β	Entry	Y./%	α:β	Entry	Y./%	α:β	Entry	Y./%	α:β
1. $CHCl_3$	1–1	60	10.9:1	2–1	78	3.64:1	3–1	100	11.4:1	4–1	51	1.69:1	5–1	42	3.65:1
2. Toluene	1–2	78	3.64:1	2–2	66	1.46:1	3–2	35	5.33:1	4–2	66	1.70:1	5–2	54	3.69:1
3. *c*-C_5H_9OMe	1–3	100	11.4:1	2–3	24	4.11:1	3–3	78	6.91:1	4–3	47	2.15:1	5–3	15	4.26:1
4. *n*-BuCN	1–4	51	1.69:1	2–4	76	2.51:1	3–4	47	2.15:1	4–4	99	1.06:1	5–4	95	1.19:1
5. EtOAc	1–5	42	3.65:1	2–5	55	3.48:1	3–5	15	4.26:1	4–5	95	1.19:1	5–5	77	2.72:1
6. CH_2Cl_2	1–6	81	11.3:1	2–6	49	4.79:1	3–6	98	8.26:1	4–6	97	1.77:1	5–6	49	3.67:1
7. Decaline	1–7	77	3.67:1	2–7	38	1.81:1	3–7	80	3.16:1	4–7	72	1.66:1	5–7	92	3.08:1
8. Dioxane	1–8	55	7.28:1	2–8	76	8.72:1	3–8	69	4.95:1	4–8	54	2.03:1	5–8	50	3.80:1
9. DME	1–9	100	3.23:1	2–9	51	4.09:1	3–9	84	4.73:1	4–9	42	1.95:1	5–9	92	3.56:1
10. DEC	1–10	85	3.36:1	2–10	80	1.16:1	3–10	89	3.67:1	4–10	75	2.18:1	5–10	99	4.25:1

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11. **1H** was synthesized from pentaacetyl-β-D-glucose as follows: (1) *p*-(MeO)C_6H_4OH, $BF_3{\cdot}OEt_2$, (2) NaOMe, (3) 1,1-dimethoxycyclo-hexanone, CSA, 90% in three steps, (4) TIPSCl, imidazole, (5) BnBr, NaH, and (6) TBAF, 59% in three steps. **2H** was synthesized from pentaacetyl-β-D-glucose as follows: (1) TMSSMe, TMSOTf, 66% (α:β = 1:5), (2) β-isomer, NaOMe, (3) 1,1-dimethoxycyclo-hexanone, CSA, 77% in two steps, (3) BnBr, NaH, 99%. **1D** and **2D** were synthesized according to the procedure for **1H** and **2H**, respectively, except benzyl-d_7 bromide was used instead of BnBr. Bn-d_7 was synthesized from toluene-d_8 following the procedure in Ref. 6.
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13. Spectroscopic data of alpha isomer of **3H**-α: ^{1}H NMR (C_6D_6, 400 MHz) δ 0.90–2.10 (10H, m, cyclohexyl × 2), 3.08 (1H, td, *J* = 10.0, 5.2 Hz, Glc1C5H), 3.27 (1H, s, MeO), 3.56 (1H, t, *J* = 10.8 Hz, Glc1C6H), 3.68–3.74 (2H, m, Glc1C6H, Glc2C2H), 3.76–3.89 (4H, m, Glc1C2H, Glc1C4H, Glc2C4H, Glc2C6H), 4.04 (1H, dd, *J* = 10.4, 5.2 Hz, Glc2C6H), 4.14 (1H, t, *J* = 9.2 Hz, Glc1C3H), 4.29 (1H, t, *J* = 8.8 Hz, Glc2C3H), 4.56–4.65 (1H, m, Glc2C5H), 4.86 (2H, s, Bn), 4.88 (1H, d, *J* = 8.0 Hz, Glc1C1H), 5.01 (1H, d, *J* = 11.6 Hz, Bn), 5.06 (1H, d, *J* = 10.8 Hz, Bn), 5.10 (1H, d, *J* = 10.8 Hz, Bn), 5.19 (1H, d, *J* = 11.6 Hz, Bn), 5.89 (1H, d, *J* = 3.6 Hz, Glc2C1H), 6.69 (2H, d, *J* = 9.2 Hz, MPM), 6.98 (2H, d, *J* = 9.2 Hz, MPM), 7.10–7.75 (15H, m, Ar); **3H**-β: ^{1}H NMR (C_6D_6, 400 MHz) δ 0.90–1.95 (10H, m, cyclohexyl × 2), 3.12–3.22 (1H, m, Glc1C5H), 3.20–3.28 (1H, m, Glc2C5H), 3.28 (1H, s, MeO), 3.62–3.77 (4H, m, Glc1C6H, Glc2C2H, Glc2C3H, Glc2C6H), 3.78–3.85 (3H, m, Glc1C42H, Glc1C6H, Glc2C4H), 3.89 (1H, t, *J* = 8.0 Hz, Glc1C2H), 3.95 (1H, dd, *J* = 9.2, 5.2 Hz, Glc2C6H), 4.17 (1H, t, *J* = 8.8 Hz, Glc1C3H), 4.88 (1H, d, *J* = 11.6 Hz, Bn), 4.91 (1H, d, *J* = 7.6 Hz, Glc1C1H), 4.93 (1H, d, *J* = 10.0 Hz, Bn), 4.96 (1H, d, *J* = 12.0 Hz, Bn), 5.07 (1H, d, *J* = 12.0 Hz, Bn), 5.10 (1H, d, *J* = 10.0 Hz, Bn), 5.11 (1H, d, *J* = 11.6 Hz, Bn), 5.26 (1H, d, *J* = 7.2 Hz, Glc2C1H), 6.69 (2H, d, *J* = 9.2 Hz, MPM), 7.00 (2H, d, *J* = 9.2 Hz, MPM), 7.10–7.50 (15H, m, Ar); **3D**-α: ^{1}H NMR (C_6D_6, 400 MHz) δ 0.90–2.10 (10H, m, cyclo-hexyl × 2), 3.08 (1H, td, *J* = 10.0, 5.2 Hz, Glc1C5H), 3.27 (1H, s, MeO), 3.55 (1H, t, *J* = 10.4 Hz, Glc1C6H), 3.68–3.74 (2H, m, Glc1C6H, Glc2C2H), 3.76–3.89 (4H, m, Glc1C2H, Glc1C4H, Glc2C4H, Glc2C6H), 4.05 (1H, dd, *J* = 10.8, 5.6 Hz, Glc2C6H), 4.14 (1H, t, *J* = 9.2 Hz, Glc1C3H), 4.29 (1H, t, *J* = 9.2 Hz, Glc2C3H), 4.57–4.65 (1H, m, Glc2C5H), 4.87 (1H, d, *J* = 8.0 Hz, Glc1C1H), 5.89 (1H, d, *J* = 3.6 Hz, Glc2C1H), 6.69 (2H, d, *J* = 9.2 Hz, MPM), 6.98 (2H, d, *J* = 9.2 Hz, MPM); **3D**-β: ^{1}H NMR (C_6D_6, 400 MHz) δ 0.90–1.95 (10H, m, cyclohexyl × 2), 3.10–3.20 (1H, m, Glc1C5H), 3.19–3.27 (1H, m, Glc2C5H), 3.27 (1H, s, MeO), 3.68–3.74 (4H, m, Glc1C6H, Glc2C2H, Glc2C3H, Glc2C6H), 3.78–3.85 (3H, m, Glc1C42H, Glc1C6H, Glc2C4H), 3.89 (1H, t, *J* = 8.0 Hz, Glc1C2H), 3.94 (1H, dd, *J* = 9.2, 5.2 Hz, Glc2C6H), 4.17 (1H, t, *J* = 8.8 Hz, Glc1C3H), 4.91 (1H, d, *J* = 7.6 Hz, Glc1C1H), 5.26 (1H, d, *J* = 7.2 Hz, Glc2C1H), 6.69 (2H, d, *J* = 9.2 Hz, MPM), 7.00 (2H, d, *J* = 9.2 Hz, MPM).
14. We checked the ionization ratio of labeled to non-labeled compound by comparing the apex values (mV) of the MALDI-TOF MS peaks; for (**3H**-α)/(**3D**-α), *y* = 1.04×; for (**3H**-α)/(**3D**-β), *y* = 0.967×.
15. General procedure for the small scale screening: each 100 μL of solution of acceptor **1D** (0.0966 g, 0.208 mmol), donor **2D** (0.1212 g, 0.250 mmol) and DTBMP (0.0768 g, 0.374 mmol) in 6.0 mL of CH_2Cl_2 were pipetted into multiplicate tubes, and the mixtures were evaporated by flashing with N_2 gas. In each tube, 3.47 μmol of acceptor, 4.17 μmol of donor and 6.23 μmol of DTBMP were prepared for the reaction. After MS4A (25 mg) and each solvent (200 μL) were added to the mixture, methyl trifluoromethanesulfonate (2.0 μL, 18 μmol) was added to each tube. The mixtures were magnetically stirred at room temperature for 24 h, and the reactions quenched by triethylamine. The mixtures were filtered through Celite, washed with aqueous satd $NaHCO_3$ solution, dried over Na_2SO_4, and evaporated by flashing with N_2 to give crude mixtures.
    Determination of the stereoselectivity by ^{1}H NMR: the anomeric ratios of crude mixtures were estimated from the relative intensities of H-1 signals (in C_6D_6) of α- (δ 5.89 ppm, *J* 3.6 Hz) and β- (δ 5.26 ppm, *J* 7.2 Hz) isomers. Quantitative MALDI-TOF MASS analysis: the crude mixtures were diluted with 700 μL of CH_3CN. A 4.0 μL measure of 1.0 mM standard solution of each of the three non-labeled compounds was pre-mixed with 2.0 μL of the crude solutions for MS analysis. The resulting solutions were measured by MALDI-TOF MASS using the RASTER function. The molar ratio of labeled to non-labeled compound was obtained from the ratio of each value (mV) at the apex of the ion peak of $[M+Na]^+$.
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