Novel substrates for efficient enzymatic transglycosylation by *Bacillus circulans*

Shiro Komba and Yukishige Ito

Abstract: To develop transglycosylation for efficient preparation of *N*-acetyllactosamine (Galβ(1→4)GlcNAc, LacNAc), β-galactosidase mediated transglycosylation using novel substrates *Bacillus Circulans* was explored. To make transglycosylation entropically favorable over hydrolysis, donor (lactose or galactose) and acceptor (*N*-acetylglucosamine, GlcNAc) components were connected to a single molecule. For that purpose, 1,2- and 1,3-benzenedimethanol and 2-hydroxy-5-nitro- and 5-hydroxy-2-nitro-benzyl alcohol were screened as linkers and enzymatic transglycosylation was examined under several conditions. In the case of 2-hydroxy-5-nitro-benzyl connected substrate **40**, an indication of the occurrence of intramolecular transglycosylation was observed, and the desired product (**58**) was obtained in 26% isolated yield. The same reaction in the presence of CMP sialic acid and α-(2→6)-sialyltransferase gave sialyl LacNAc **87** in one pot in 39% isolated yield. Additionally, the effect of the *C*-2 substituent of the acceptor component was briefly examined using substrates containing NHAlloc (**72**), NHTroc (**73**), and N_3 (**74**) groups. Although the occurrence of intramolecular transglycosylation was not clear in these cases, disaccharides **81**–**83** were obtained in reasonable yields.

Key words: galactosidase, intramolecular transglycosylation, *N*-acetyllactosamine, sialyltransferase.

Résumé : Faisant appel à de nouveaux substrats *Bacillus circulan*, on a étudié la réaction de transglycolisation catalysée par une β-galactosidase dans le but de développer une méthode de transglycosylation permettant de préparer la *N*-acétyllactosamine (Galβ(1→4)GlcNAc, LacNAc) d'une façon efficace. Afin de rendre la réaction de transglycosylation entropiquement favorable et de la rendre plus favorable que l'hydrolyse, on a relié les blocs donneur (lactose ou galactose) et accepteur (*N*-acétylglucosamine, GlcNAc) dans une seule molécule. À cette fin, on a utilisé les benzène-1,2- et 1,3-diméthanols ainsi que les alcools 2-hydroxy-5-nitro et 5-hydroxy-2-nitrobenzyliques comme connecteurs et on a étudié les réactions de transglycosylation sous plusieurs conditions. Dans le cas du substrat **40**, faisant appel à l'alcool 2-hydroxy-5-nitrobenzylique comme connecteur, on a observé des indications à l'effet de l'existence d'une réaction de transglycosylation intramoléculaire et on a obtenu le produit désiré, **58**, avec un rendement de 26% de produit isolé. La même réaction effectuée en présence de CMP de l'acide sialique et d'α-(2→6)-sialyltransférase dans une réaction monotope conduit à la formation de la LacNAc sialyée, **87**, avec un rendement de 39% en produit isolé. De plus, on a fait une brève étude de l'effet du substituant en *C*-2 du composant accepteur en utilisant des substrats portant des groupes NHAlloc (**72**), NHTroc (**73**) et N_3 (**74**). Même si les possibilités de réactions de transglycosylation intramoléculaire ne sont pas claires dans ces cas, on a obtenu les disaccharides **81**–**83** avec des rendements raisonnables.

Mots clés : galactosidase, transglycosylation intramoléculaire, *N*-acétyllactosamine, sialyltransférase.

[Traduit par la Rédaction]

Introduction

N-Acetyllactosamine (LacNAc) is a widespread component of glycoproteins (1), glycolipids (2), and glycosaminoglycans (3). It is particularly important as a core structure of ligands for cell adhesion molecules, for instance selectin (4) and CD22 (5). It was first isolated from porcine gastric mucin as a growth factor for *Lactobacillus bifidus var. pennysylvanicus* by Tomarelli et al. (6). A number of chemical approaches toward LacNAc have been reported (7–11). Most typically, it can be prepared from lactose (Lac) via lactal using azidonitration, developed by Wong and Lemieux (12) and Lemieux and Ratcliff (13), as the key transformation.

On the other hand, Wong et al. (14) developed a highly efficient LacNAc producing system, by using β-(1→4)-galactosyltransferase (EC 2.4.1.90), in combination with multiple enzymes for UDP–galactose recycling. This landmark achievement opened the way for glycosyltransferase catalyzed preparative scale oligosaccharide synthesis (15). Limitations in terms of the cost, availability, and stability of the enzymes and sugar nucleotides are still not negligible, however.

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Dedicated to the memory of late Professor Ray Lemieux in recognition of his greatest contribution to carbohydrate chemistry.

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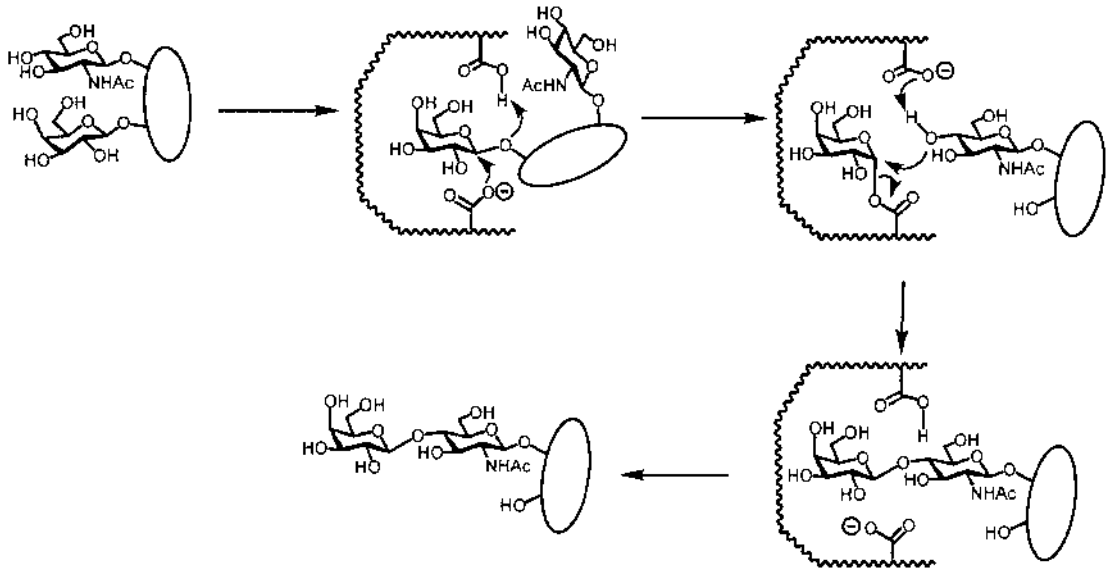
Scheme 1.



The glycosidase-based approach is attractive due to the following reasons: (*i*) an inexpensive (e.g., lactose) or easily obtainable (e.g., aryl β-D-galactoside) donor can be used; (*ii*) compared with glycosyltransferases, this group of enzymes are much more stable, easier to handle, and generally cheaper. Zilliken et al. (16) reported the first enzymatic synthesis of LacNAc from lactose and *N*-acetylglucosamine (GlcNAc) in the presence of extracts from *Lactobacillus bifidus*, although the yield was low from the standpoint of practical use. More recently, quite extensive studies on the use of β-D-galactosidase (EC 3.2.1.23) from *Bacillus circulans* have been reported by several groups (17–22).

Although these pioneering works clearly revealed the potential of galactosidase as a valuable tool for the synthesis of biologically relevant oligosaccharides, attainable yields have not been satisfactory. In most cases, a large excess of either glycosyl donor (e.g., lactose) or acceptor (*N*-acetylglucosamine) was used in order that the transglycosylation can compete with hydrolysis. It would be extremely difficult to suppress the hydrolysis to an extent that transglycosylation using an equimolar amount of glycosyl donor–acceptor can be achieved in a synthetically useful efficiency. One potential solution would be to perform the glycosyl transfer in an intramolecular manner by connecting the donor and acceptor components together into a single molecule (Scheme 1). Transglycosylation may be favorable over hydrolysis on the condition that the donor and acceptor portions of the substrate fit simultaneously into the binding pocket of the enzyme. In this paper, the first success in finding the substrate that, in part, fulfills these demands, is reported (23).

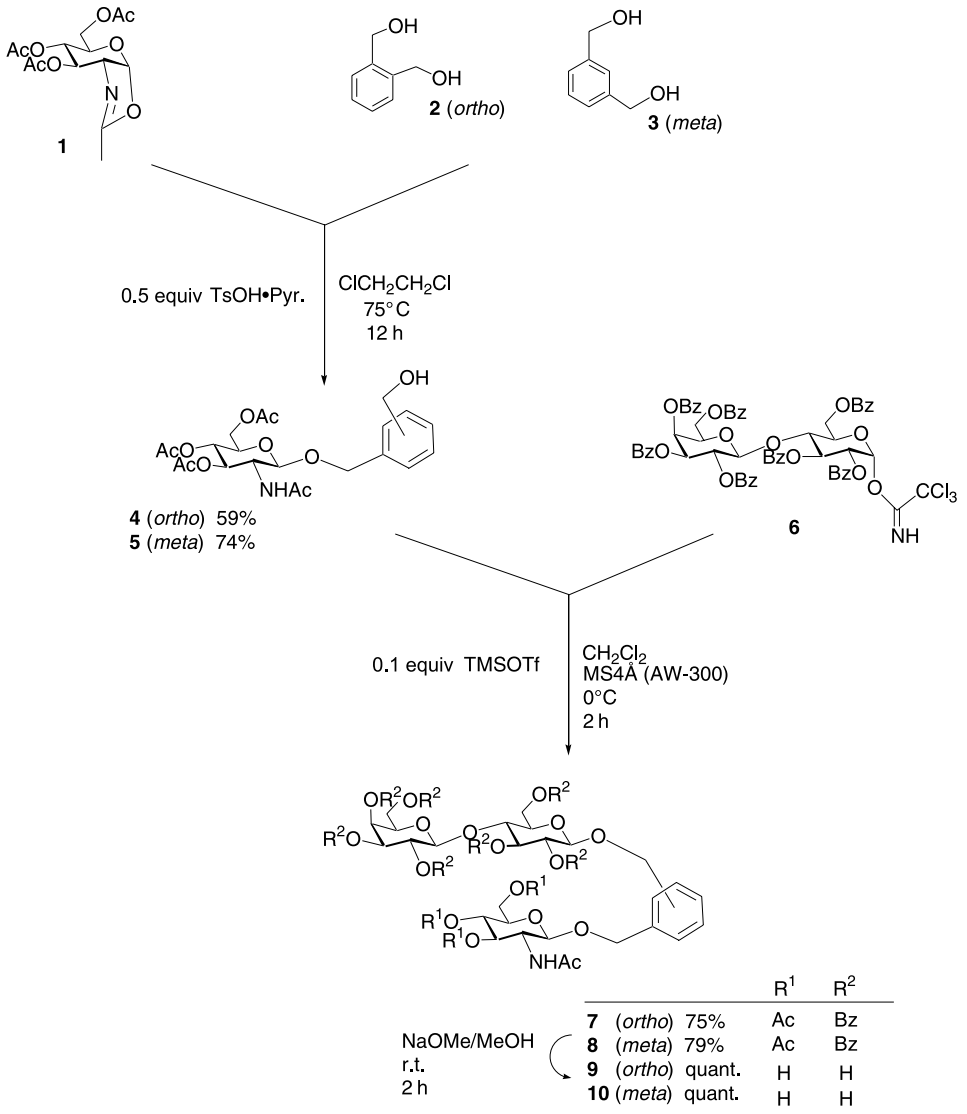
Results and discussion

As a tether to connect donor and acceptor components, our initial attention was focused on (**2**) 1,2-(*ortho*)-, and (**3**) 1,3-(*meta*)-benzenedimethanol, and substrates **9** and **10** were designed. These substrates were synthesized in a straightforward manner as depicted in Scheme 2. Although rather forcing conditions (75°C, 12 h) were required, the reactions of **2** and **3** with oxazoline **1** (24) selectively gave **4** and **5** in 59 and 74% yield, respectively, without noticeable contamination with diglycosylated products. Subsequent glycosylation with lactosyl trichloroacetimidate **6** (25) was effected in the presence of TMSOTf to afford **7** and **8** in 75 and 79% yield, respectively. Finally, *O*-acyl protecting groups were removed by sodium methoxide, and the designed substrates **9** and **10** were obtained in quantitative yield. These compounds were then examined as substrates for enzymatic transglycosylation using β-galactosidase from *B. circulans*, and the courses of the reactions were monitored by analytical HPLC (Fig. 1). Based on MALDI-TOF-MS and NMR analysis, peaks assignable as the desired products **15** and **16** (MALDI-MS *m*/*z*: ([M + Na]$^+$, 688) were identified, and their structures were rigorously confirmed by NMR analysis of corresponding acetates **17** and **18**. The yields of transglycosylated products **15** and **16** were rather low (~5%), however. While hydrolysis toward **11** and **12** was the overwhelmingly major pathway, it was noteworthy that the formation of bis-disaccharides **13** and **14** was dominant over **15** and **16** throughout the reaction.[2] Since the formation of **13** and **14** is possible only by intermolecular pathway, it is most probable that the intramolecular pathway made only a minor contribution, if any, to the formation of **15** and **16** (Scheme 3). Their formation would most likely be a result of sequential intermolecular transglycosylation and deglycosylation (i.e., (**9**, **10**)→(**13**, **14**)→(**15**, **16**)). This conclusion was supported by the extremely similar profiles of the *ortho* (**9**) and *meta* (**10**) isomers.

The above negative results obtained by **9** and **10** implied that the topological relationship of Gal and GlcNAc residues

[2] In addition, products that, based on MALDI-TOF mass, are assignable as multiply galactosylated, were detected. Their structures were not rigorously confirmed, however (see Figs. 1–5).

Scheme 2.



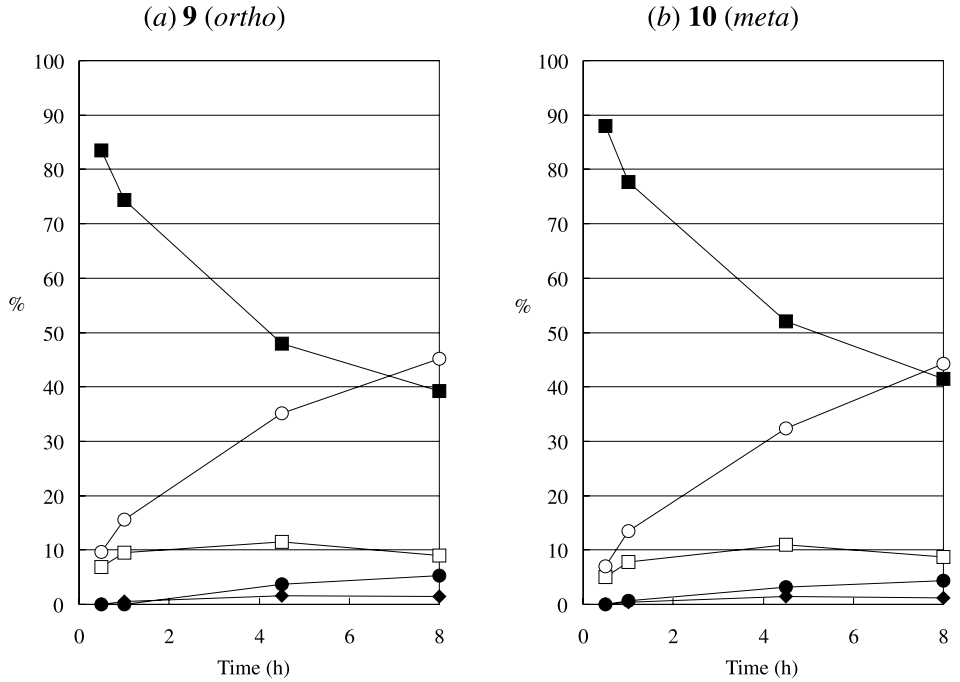
of these compounds does not fit the binding pocket of β-galactosidase. We therefore decided to attenuate the distance between these residues by changing the linker. To that end, 2-hydroxy-5-nitrobenzyl (*ortho*) (**20**) and 5-hydroxy-2-nitrobenzyl (*meta*) alcohol (**21**) were chosen, which each have one less methylene unit compared with **2** and **3**. Because they are unsymmetrical diols, regioisomers can be produced simply by changing the order of glycosylation (i.e., **36**–**37** and **38**–**39**). Additional diversity of the substrate can be generated by incorporating the galactose residue, instead of lactose, directly on the phenolic oxygen (i.e., **40** and **41**). In the latter case, the substrates can be viewed as derivatives of *p*-nitrophenyl galactoside, a well explored reactive donor for enzymatic transglycosylation.

Preparation of these substrates was achieved in a concise and divergent manner as depicted in Scheme 4. It commenced with a $BF_3{\cdot}OEt_2$-mediated chemoselective glycosylation of the primary hydroxy groups of **20** and **21**, with either lactosyl (**19**) (26) or glucosaminyl (**24**) (27) acetate to give **22** and **23** or **25** and **26**, respectively, in 67–85% yield. For the glycosylation of the unreactive phenolic hydroxy group, trichloroacetimidates **27** (28), **30** (25), and **33** (25) gave highly satisfactory results. To our surprise, addition of molecular sieves (MS), a common practise in glycosylation to ensure anhydrous conditions, deteriorated the efficiency of these particular reactions. Namely, when **22** and **23** were reacted with **27** in the presence of MS AW-300, the yields of **28** and **29** were less than 40%, while the same products were obtained in excellent yields in their absence. Reactions of **25** and **26** with **30** and **31** were likewise performed to give **31**, **32**, **34**, and **35** in 85–97% yield. Somewhat unexpectedly, in the case of the preparation of **34**, a significant amount of the corresponding α-isomer was also synthesized (α:β = 1:3.3). The obtained products were then subjected to deprotection, uneventfully, to give **36**–**41**.

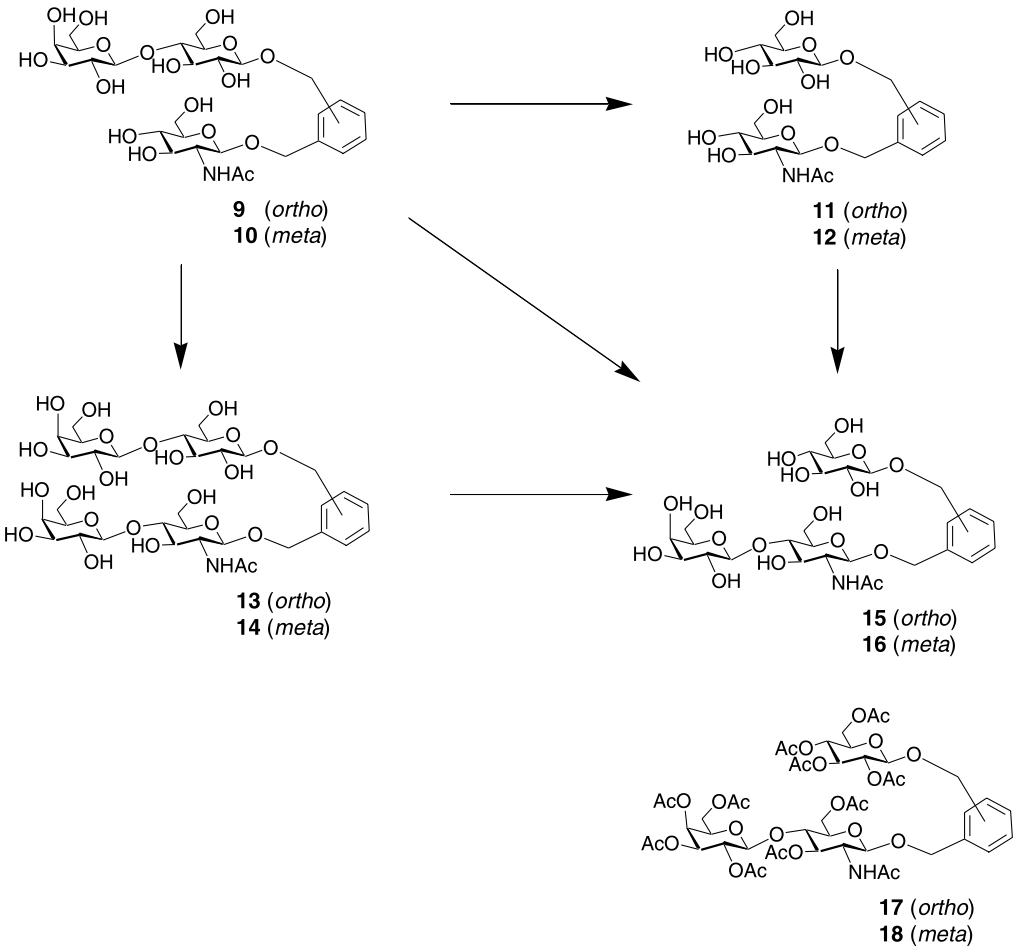
These compounds were examined as substrates for transglycosylation in a similar manner as that described for **15** and **16**. Namely, each substrate (8 mM) was incubated with *B. circulans* β-galactosidase in 50 mM acetate buffer (pH 5.0), and the reactions were followed by analytical HPLC (Fig. 2).

In the case of substrates carrying lactose as a donor component (**36**–**39**), severe peak overlaps retarded the interpretation

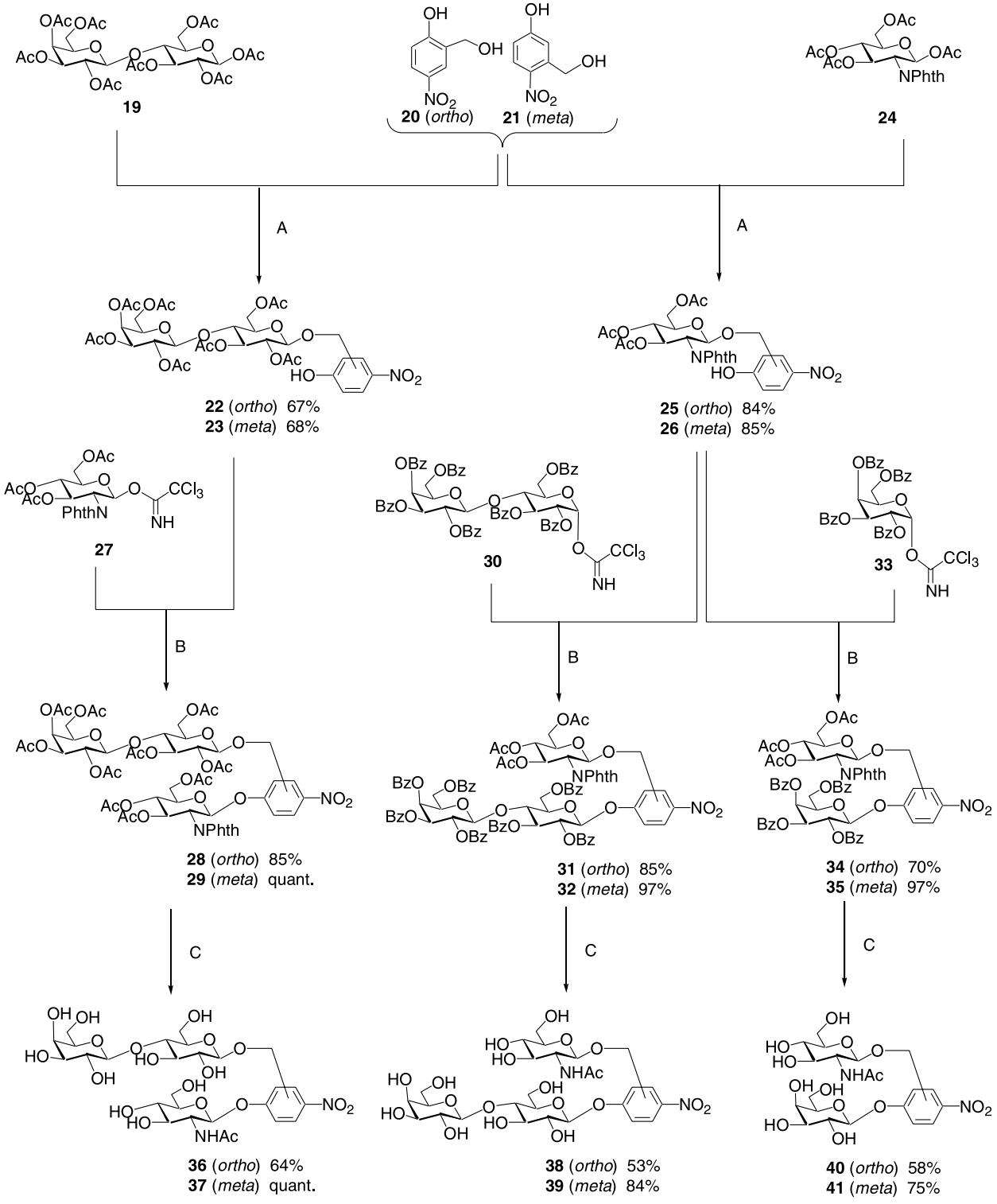
Fig. 1. Time course of β-galactosidase catalyzed reactions: (*a*) compound **9** (8 mM) was incubated with 0.5 U of β-galactosidase in 2.5 mL of acetate buffer (50 mM, pH 5.0) at 36°C; (*b*) compound **10** (8 mM) was incubated with 1.2 U of β-galactosidase under identical conditions as (*a*). Aliquots of the mixture were analyzed by HPLC. Peaks were assigned as follows based on MALDI-TOF-MS analysis: ■ starting material (**9** and **10**); ○ GlcNAcGlc (**11** and **12**); ● LacNAcGlc (**15** and **16**); □ LacNAcLac (**13** and **14**); ◆ GalLacNAcLac.



Scheme 3.

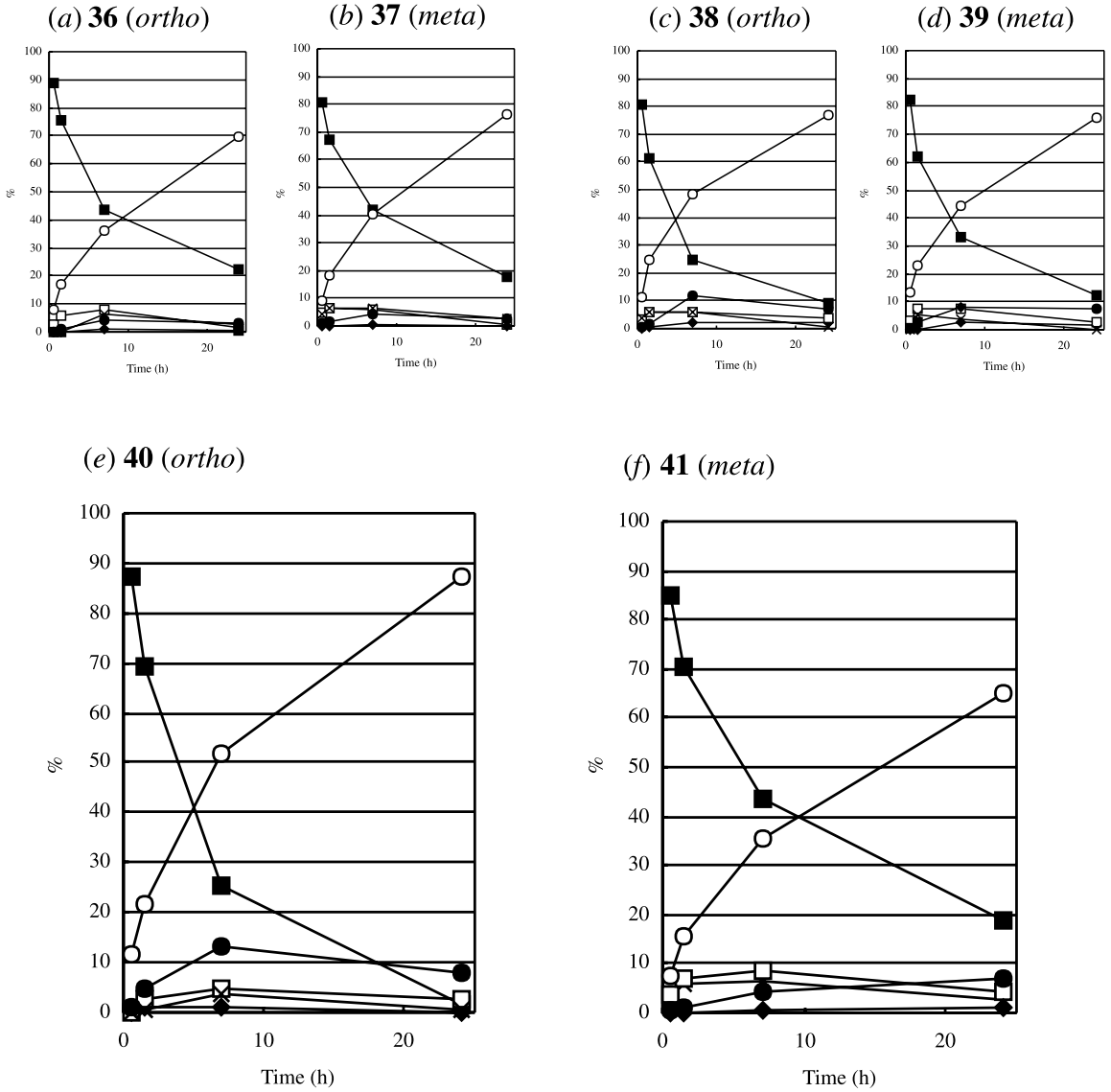


Scheme 4. Reagents and conditions: (*a*) 2 equiv $BF_3{\cdot}OEt_2$ in CH_2Cl_2 at rt for 1 h; (*b*) 0.5 equiv $BF_3{\cdot}OEt_2$ in CH_2Cl_2 at rt for 1 h; (*c*) (*i*) 75 equiv NH_2NH_2 in EtOH at 90°C for 3 h, (*ii*) Ac_2O in MeOH at rt for 8 h.



of the HPLC profiles. To enhance the peak assignments, aliquots of the mixtures were treated with β-*N*-acetylglucosaminidase from Jack beans (Sigma Co., Ltd.). After this treatment, galactosylated products, including the desired products (**54**–**57**) and bis-disaccharides (**48**–**51**), were readily distinguished from substrates and hydrolyzed products (**42**–**45**) by their resistance. In any event, no indication of intramolecular glycosyl transfer was obtained from these substrates; that is, at the early stage of incubation, the formation of **48**, **49**, **50**, and **51** was dominant over **54**, **55**,

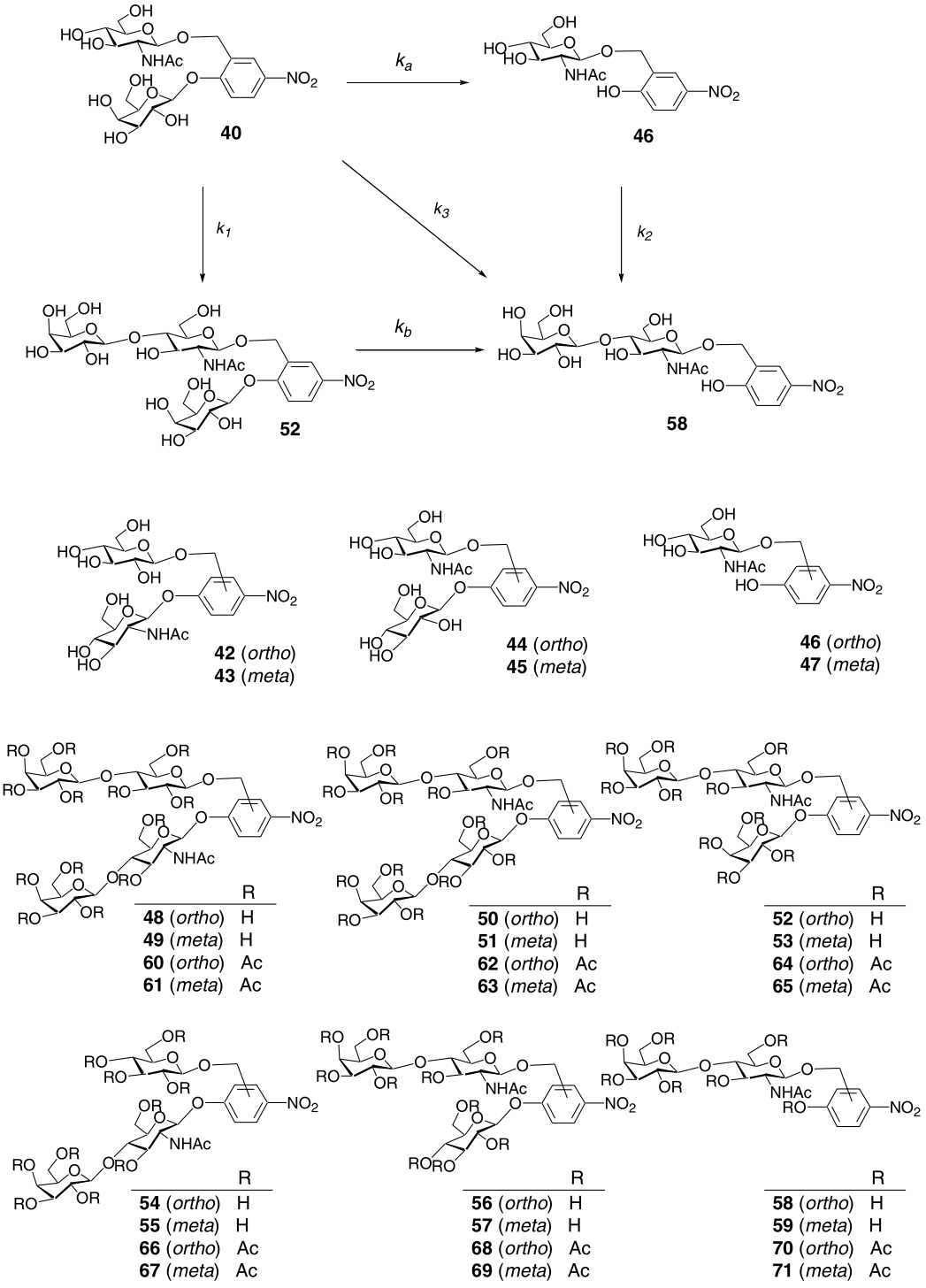
Fig. 2. Time course of β-galactosidase catalyzed reactions using substrates derived from the nitro-containing linker (Scheme 4). (*a*) Compound **36** (8 mM) was incubated with 0.34 U of β-galactosidase in 1.8 mL of acetate buffer (50 mM, pH 5.0) at 36°C; (*b*) compound **37** (8 mM) was incubated with 0.34 U of β-galactosidase, under identical conditions as (*a*) aliquots of the mixture were analyzed by HPLC. Peaks were assigned as follows based on MALDI-TOF-MS analysis: ■ starting material (**36** and **37**); ○ GlcNAcGlc (**42** and **43**); ● LacNAcGlc (**54** and **55**); □ LacNAcLac (**48** and **49**); × GlcNAcGalLac (regioisomer of **48** and **49**, structure not confirmed); ◆ $GlcNAcGal_2Lac$ (structure not confirmed); (*c*) compound **38** (8 mM) was incubated with 0.34 U of β-galactosidase in 1.8 mL of acetate buffer (50 mM, pH 5.0) at 36°C; (*d*) compound **39** (8 mM) was incubated with 0.10 U of β-galactosidase, under identical conditions as (*c*). Aliquots of the mixture were analyzed by HPLC. Peaks were assigned as follows based on MALDI-TOF-MS analysis: ■ starting material (**38** and **39**); ○ GlcNAcGlc (**44** and **45**); ● LacNAcGlc (**56** and **57**); □ LacNAcLac (**50** and **51**); × GlcNAcGalLac (regioisomer of **50** and **51**, structure not confirmed); ◆ $GlcNAcGal_2Lac$ (structure not confirmed).; (*e*) compound **40** (8 mM) was incubated with 0.13 U of β-galactosidase in 0.70 mL of acetate buffer (50 m M, pH 5.0) at 36°C; (*f*) compound **41** (8 mM) was incubated with 0.44 U of β-galactosidase under idential conditions as in (*e*). Aliquots of the mixture were analyzed by HPLC. Peaks were assigned as follows based on MALDI-TOF-MS analysis: ■ starting material (**40** and **41**); ○ GlcNAc (**46** and **47**); ● LacNAc (**58** and **59**); □ LacNAcGal (**52** and **53**); × $Gal_2GlcNAc$ (regioisomer of **52** and **53**, structure not confirmed); ◆ $Gal_3GlcNAc$ (structure not confirmed).



56, and **57**.[2] Nevertheless, it was observed that a significant amount of the transglycosylated product (**56**) was formed from *ortho* substrate **38**.

On the other hand, the profile of substrate **40** was markedly different. In this particular case, the formation of the LacNAc derivative (closed circle) was dominant over that of **52** even at the early stage of incubation ([**40**] > [**46**]).[2] Since the rates of intermolecular glycosyl transfer are likely to be similar between **40** and **46** ($k_1 \approx k_2$), it was inferred that the intramolecular pathway is functional ($k_3 > k_1, k_2$) (Scheme 5). By contrast, when starting from the *meta* oriented substrate **41**, it was found that the transglycosylation was far less efficient.

Scheme 5.



The formation of intermolecularly glycosylated product **53** preceded that of **59**, which means that the direct pathway was insignificant.

Using the preferred substrate **40**, the effects of substrate concentration (i.e., Table 1, entries 1–4), organic solvents (entries 6–12), and buffer pH (entry 5) were briefly examined. Contrary to several precedents on enzymatic transglycosylation (17, 20, 21), the beneficial effect of organic solvent was not observed (entries 6–12). A preparative scale reaction was performed at 20 mM concentration in acetate buffer (entry 3). Thus, using 50 mg (94 μmol) of compound **40**, incubation with β-galactosidase (430 mU) for 3 h afforded 26% yield (13.1 mg) of **58**, the structure of which was rigorously confirmed by NMR analysis of the corresponding acetate **70**. Subsequently, the effect of the glucosamine *C*-2 substituent was examined. Considering the potential synthetic utility of the expected products, three types of substrates were prepared, namely *N*-allyloxycarbonyl (Alloc) protected **72**,

Fig. 3. Time course of β-galactosidase catalyzed reactions using *C*-2 modified substrates (Scheme 7); (*a*) compound **72** (20 mM) was incubated with 86 mU of β-galactosidase in 86.5 μL of acetate buffer (50 mM, pH 5.0) at 36°C; (*b*) compound **73** (20 mM) was incubated with 86 mU of β-galactosidase under identical conditions as (*a*); (*c*) compound **74** (10 mM) was incubated with 172 mU of β-galactosidase under identical conditions as (*a*). Aliquots of the mixture were analyzed by HPLC. Peaks were assigned as follows based on MALDI-TOF-MS analysis: ■ starting material (**72** and **73** and **74**); ○ GlcNR (**75** and **76** and **77**); ● LacNR (**81** and **82** and **83**); □ LacNRGal (**78** and **79** and **80**); × Gal_2GlcNR (regioisomer of **78** and **79** and **80**, structure not confirmed); ◆ Gal_3GlcNR (structure not confirmed).

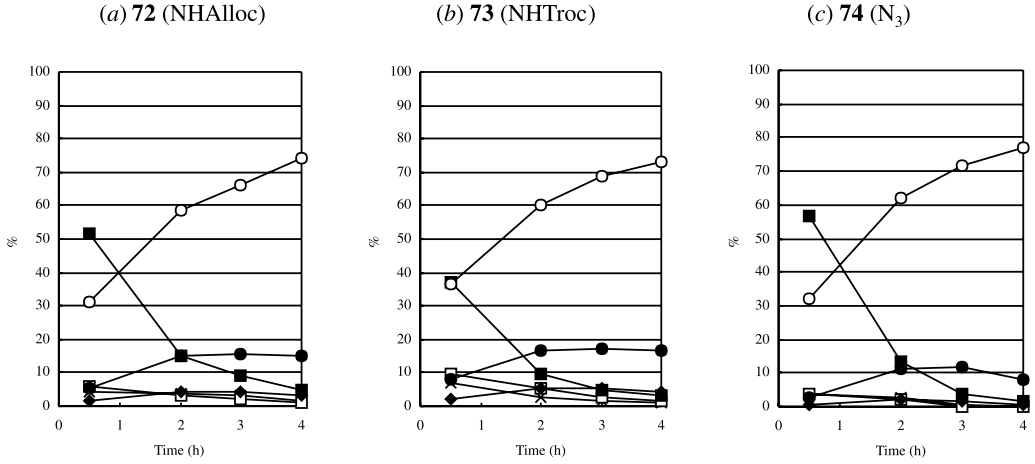


Table 1. Effects of solvent and substrate concentration.

Entry	**40** (mM)	Enzyme (mU)	Solvent (mL)[a]	Time (h)	Yield (%)
1	0.8	43	A (2.3)	48	4[b]
2	8	86	A (0.23)	8	15[b]
3	20	430	A (4.7)	3	26[c]
4	20	430	A (4.7)	4	18[b]
5	20	86	B (0.09)	4.5	15[c]
6	8	130	C (0.23)	24	7[b]
7	8	130	C (0.23)	72	14[b]
8	8	130	C (0.23)	96	14[b]
9	8	130	D (0.23)	24	2[b]
10	8	43	E (0.23)	8	10[b]
11	8	43	E (0.23)	24	13[b]
12	8	43	E (0.23)	72	1[b]

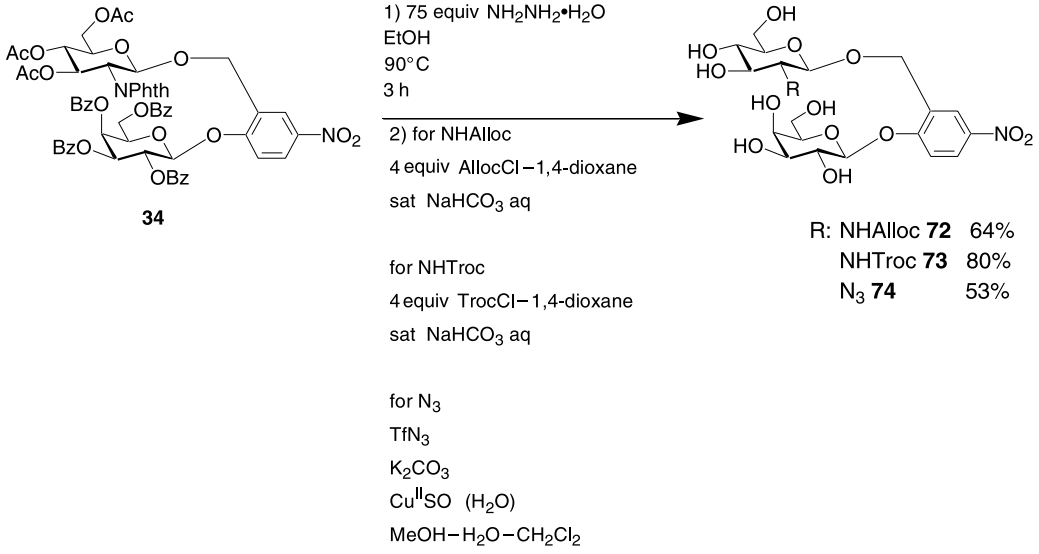
[a](A) 50 mM acetate buffer, pH 5.0; (B) 200 mM HEPES, pH 7.5; (C) 50% acetonitrile in A; (D) 60% triethyl phosphate in A; (E) 20% 2-ethoxyethyl ether in A.
[b]Based on HPLC relative peak integrations, quantified at 245 nm.
[c]Isolated yield.

N-trichloroethoxycarbonyl (Troc) protected **73**, and *C*-2 azide **74**. To synthesize these substrates, compound **34** was used as the common intermediate. Thus, **34** was treated with hydrazine hydrate in EtOH to remove *N*-phthalimido and *O*-acyl groups. The resultant amine was treated with either allyl chloroformate – $NaHCO_3$, trichloroethyl chloroformate – $NaHCO_3$, or TfN_3–Cu_2SO_4–K_2CO_3 (29), to afford **72**, **73**, and **74** in 64, 80, and 53% yield, respectively (Scheme 6).

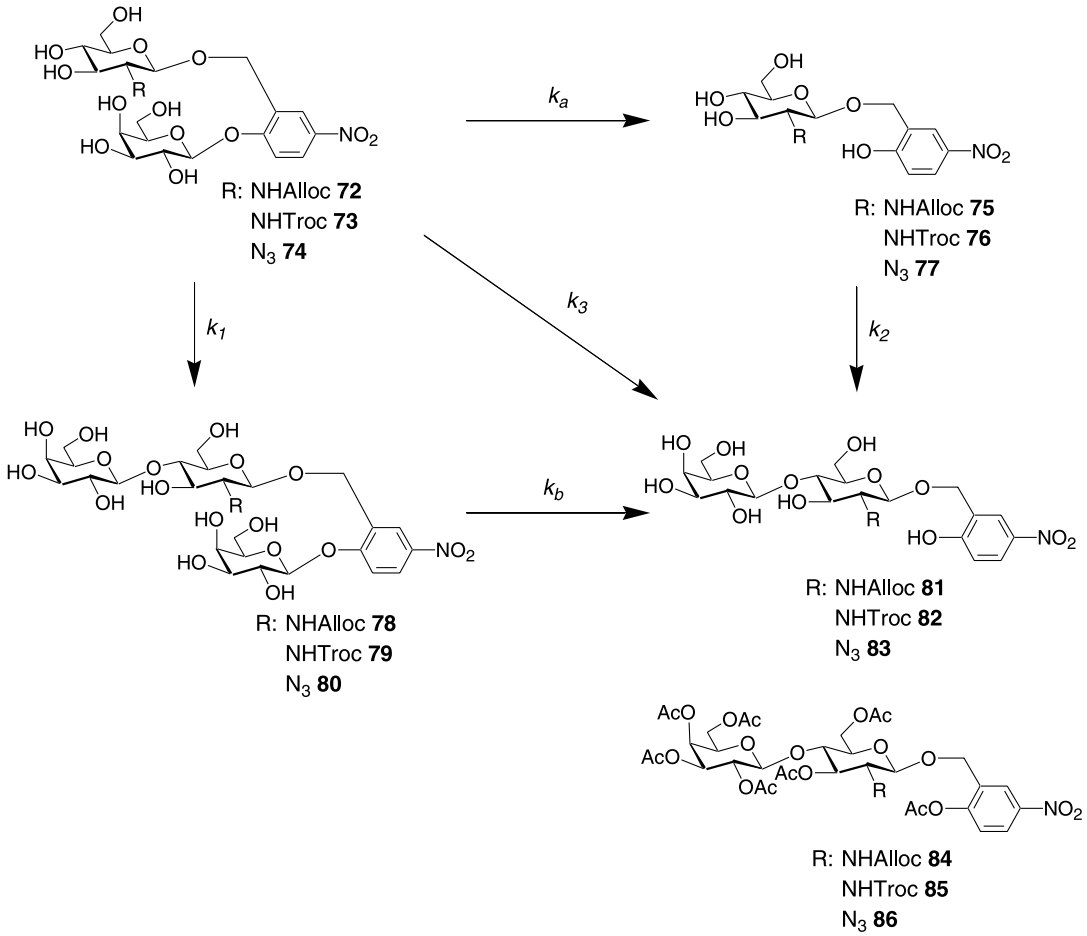
Enzymatic transglycosylations were examined under conditions optimized for **40** (20 mM substrates in 50 mM acetate buffer, pH 5.0, Fig. 3 (Scheme 7). In these cases, the dominancy of intramolecular transglycosylation was not as clear as **40**, because the formation of the expected products (**81**–**83**) was slightly behind the initial formation of intermolecularly transferred products (**78**–**80**);[2] nonetheless, carbamate-protected lactosamine derivatives **81** and **82** were formed with an efficiency comparable with **58**. These products were quite resistant to galactosidase mediated hydrolysis. Preparative scale reactions using **72** (17.3 μmol), **73** (15 μmol), and **74** (9.6 μmol) were performed, in the same manner as described for **40**, to give **81**, **82**, and **83** in 21, 21, and 11%, respectively (Scheme 8), the structures of which were rigorously confirmed by NMR analysis of the corresponding acetates (**84**, **85**, and **86**). Since *N*-Alloc- (30) and *N*-Troc-protected derivatives (31) and *C*-2 azide (13, 32) are useful as glycosyl donors for the β- and α-selective glycosylation of 2-amino sugars, respectively, this procedure may be useful for the chemoenzymatic synthesis of complex oligosaccharides.

We then turned our attention to the synthesis of sialyl LacNAc by using galactosidase mediated transglycosylation, in combination with sialyltransferase. Since it is obvious that the LacNAc product **58** is susceptible to further digestion by β-galactosidase (to provide **46**), its yield under standard conditions does not reflect the relative rates of intramolecular transglycosylation and hydrolysis (i.e., k_3 vs. k_a). In fact, the yield of **58** (26%) dropped to 18% after 4 h incubation (Table 1, entries 3 and 4). To minimize the extent of post-transglycosylation hydrolysis, and to gain a more accurate estimation of the efficiency of transglycosylation, α-(2→6)-sialyltransferase (rat, recombinant, *Spodoptera frugiperda*) (Calbiochem Co., Ltd.) (33, 34) and CMP sialic acid were added to the system to trap **58** as a galactosidase resistant product **87** (Scheme 9, Figs. 4 and 5). As expected, smooth formation of sialyl-α-(2→6)-LacNAc **87** (□) was observed, and its amount did not decrease after prolonged incubation. In a preparative scale run, substrate **40** was used (25 mg, 47 μmol), incubated in 200 mM HEPES buffer (2.4 mL, pH 7.5) in the presence of β-galactosidase (100 mU), α-(2→6)-sialyltransferase (60 mU), and CMP-sialic acid (30 mg,

Scheme 6.



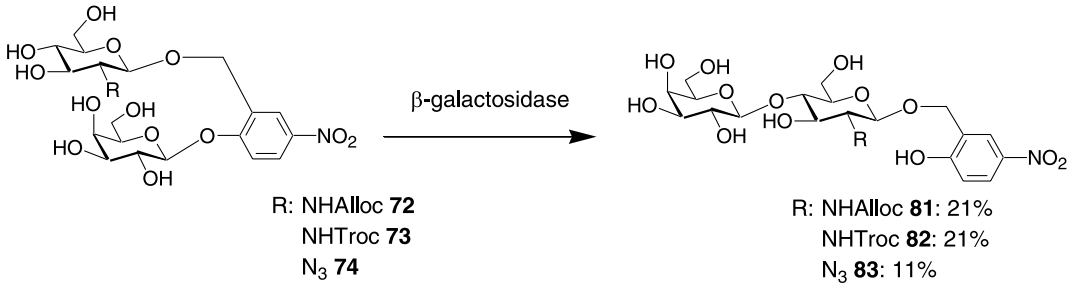
Scheme 7.



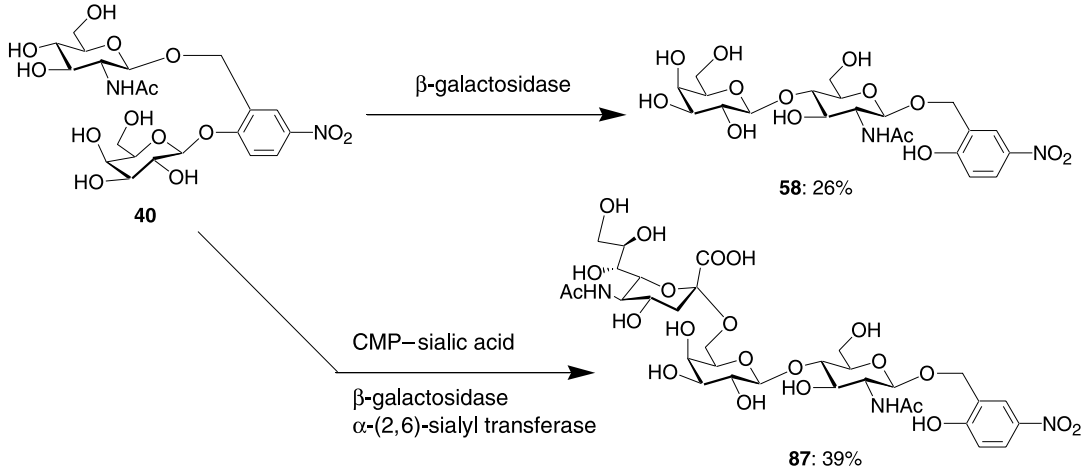
46 μmol). After 17 h, successive purification by Sephadex LH-20 and preparative HPLC afforded sialyl-α-(2→6)-LacNAc **87** in 39% yield (15.4 mg).

In conclusion, a novel method for the preparation of LacNAc and sialyl LacNAc was developed using β-galactosidase mediated intramolecular transglycosylation,

Scheme 8.



Scheme 9.



using substrates that have glycosyl donor and aglycon portions connected by a tether.

Experimental[3]

General methods

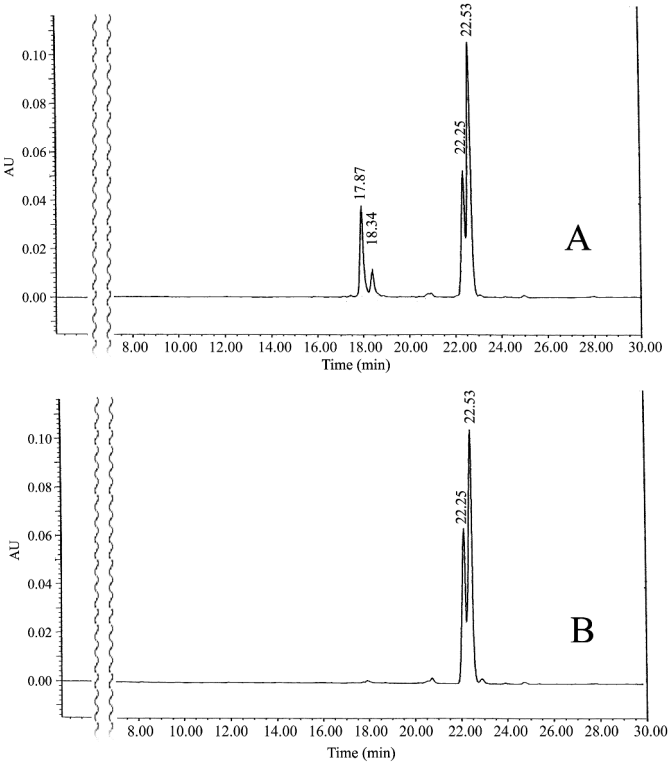
Column chromatography was performed on Silica Gel 60N (spherical, neutral) (Kanto chemical Co., Inc.). Preparative reverse-phase HPLC was performed on a Waters HPLC system (Millennium[32] operation system), using a Mightysil RP-18 GP 250–20 (5 μm) (Kanto chemical Co., Inc.), with a flow rate of 10 mL min^{-1}, and detected at 256 nm with a photodiode array detector (Waters 996). The solvent systems were: (*a*) TFA–water (1:1000) and (*b*) TFA–acetonitrile–water (1:900:100). Analytical reverse phase HPLC separations were performed on a Waters HPLC system, using a Mightysil RP-18 GP 250–3.0 (5 μm) (Kanto chemical Co., Inc.), with a flow rate of 0.6 mL min^{-1}, and detected at 256 nm with the same detector as above, using the same solvent system as above. MALDI-TOF-MS was performed in the positive ion mode on a Kompact MALDI IV tDE (Shimadzu Co., Ltd.), using a matrix of 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid, and an angiotensin I (MW = 1296.5) as a standard. FAB-MS was performed in the positive ion mode on a JMS-HX110 (JEOL), using a matrix mixture of glycerol and 3-nitrobenzyl alcohol. Optical rotations were measured with a DIP-370 digital polarimeter (Japan spectroscopic Co., Ltd.) at 22°C. ^{1}H NMR spectra were recorded at 400 MHz (JEOL JNM-AL400), or at 500 MHz (JEOL JNM-ECP500), in $CDCl_3$ (internal Me_4Si, δ: 0.00), DMSO-d_6, CD_3OD, or D_2O. ^{13}C NMR spectra were recorded at 100 or 125 MHz, respectively, on the same instruments in $CDCl_3$, DMSO-d_6, CD_3OD, or D_2O (internal MeOH, δ: 49.0). β-Galactosidase from *Bacillus circulans* was purchased from Daiwa Kasei K.K. (Japan). α-(2→6)-Sialyltransferase from rat, recombinant, *Spodoptera frugiperda* was purchased from Calbiochem Co., Ltd. β-*N*-Acetylglucosaminidase from Jack beans was purchased from Sigma Co., Ltd. All solvents and reagents were reagent grade and were used without further purification.

Enzymatic transglycosylation: typical procedure 1

A solution of substrate **9** (13.7 mg, 20.5 μmol, 8 mM) in 50 mM sodium acetate buffer (2.5 mL, pH 5.0) was incubated with β-D-galactosidase (0.5 U) at 36°C. After 30 min, 1 h, 4.5 h, and 8 h, aliquots of the reaction mixture were taken (ca. 200 μL) and terminated by heating at 95°C for 10 min. Each of them was separated by HPLC, first using 90% solvent A and 10% solvent B for 10 min, then the linear gradient 10–100% solvent B for 170 min. The eluted peaks were detected 254 nm UV absorption and the

[3] Experimental details for preparation of substrates were provided as Supplementary Material. Supplementary data may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Ottawa, ON K1A 0S2, Canada (http://www.nrc.ca/cisti/irm/unpub_e.shtml for information on ordering electronically).

Fig. 4. HPLC profiles of β-galactosidase – α-sialyltransferase reaction of **40**: (*a*) 1.5 h, (*b*) 6.5 h. Retention times of starting material (**40**), $Gal_2GlcNAc$ (**52**), NeuAcGalGlcNAc (**87**), and GlcNAc (**46**) are 17.87, 18.34, 22.25, and 22.53 min, respectively.



composition was calculated by integral curves. The fractions containing products were analyzed by MALDI-TOF-MS and each product was acetylated for extensive ^1H NMR analysis.

Enzymatic transglycosylation: typical procedure 2

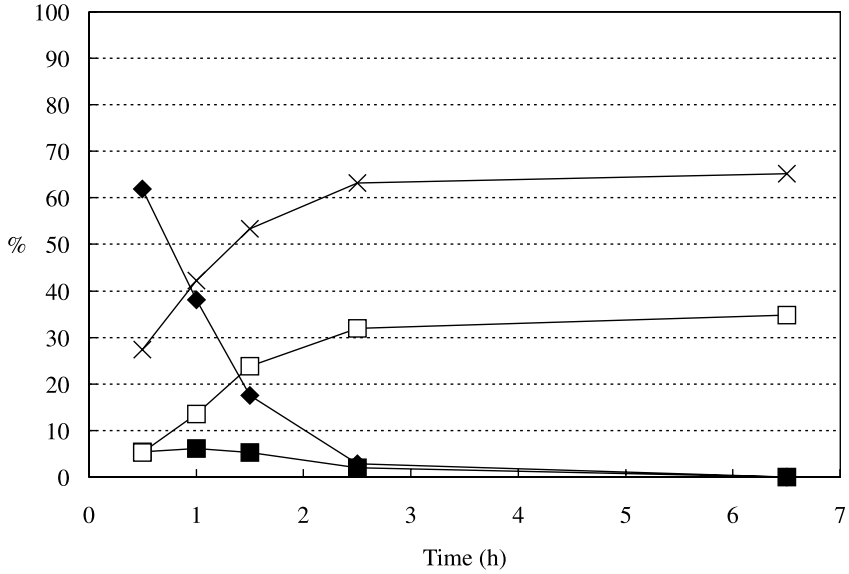
A solution of substrate **36** (10 mg, 14.3 μmol, 8 mM) in 50 mM sodium acetate buffer (1.8 mL, pH 5.0) was added to β-D-galactosidase (0.34 U) and incubated at 36°C. After 30 min, 1.5 h, 7 h, and 24 h, aliquots of the reaction mixture were taken and terminated by heating at 95°C for 10 min, and part of them were analyzed by analytical HPLC, first using 100% solvent A for 5 min, then the linear gradient 0–30% solvent B for 30 min.

Residual aliquots (180 μL) were treated with β-*N*-acetylglucosaminidase (62.5 mU) at 36°C for 2 days, then the reaction mixtures were terminated by heating at 95°C for 10 min. Each of them was analyzed by HPLC, 90% solvent A and 10% solvent B as isocratic mode. The eluted peaks were detected at 254 nm using UV absorption and the composition was calculated by integral curves.

Combined transglycosylation–sialylation: preparation of compound 87

To a solution of **40** (25 mg, 46.7 μmol, 20 mM) and CMP-Neu5Ac (30.8 mg, 46.7 μmol) in 200 mM HEPES buffer (2.3 mL, pH 7.5) containing 20 mM $MgCl_2$, 5.3 mM $MnCl_2$, and 20 mM KCl, was added α-(2→6)-sialyltransferase (60 mU), followed by β-D-galactosidase (2.2 U). The mixture was incubated at 36°C for 18 h, and terminated by heating at 95°C for 10 min. After concentration, the mixture was roughly purified by Sephadex LH-20 gel filtration column chromatography (H_2O). Fractions containing **87** were concentrated and the mixture was purified by preparative HPLC, first using 100% solvent A for 5 min, then the linear gradient 0–30% solvent B for 30 min to give sialylated compound **87** (15.4 mg, 39%), together with hydrolyzed compound **46** (10.8 mg, 61%). Compound **87**: ^1H NMR (500 MHz, D_2O and MeOH) δ: 8.06 (d, 1H, $J_{Ph4,Ph5}$ = 3.2 Hz, Ph6), 8.01 (dd, 1H, $J_{Ph3,Ph4}$ = 9.2 Hz, $J_{Ph4,Ph6}$ = 3.2, Ph4), 6.49 (d, 1H, $J_{Ph3,Ph4}$ = 9.2 Hz, Ph3), 4.75 (d, 1H, J_{gem} = 12.8 Hz, PhCH),

Fig. 5. Time course of β-galactosidase – α-sialyltransferase reaction of **40**. Compound **40** (20 mM) was incubated with 86 mU of β-galactosidase and α-(2→6)-sialyltransferase (2.4 mU) in 95 μL of HEPES (200 mM, pH 7.5), 20 mM $MgCl_2$, 5.3 mM $MnCl_2$, 20 mM KCl, and CMP-sialic acid (20 mM), at 36°C. Peaks were assigned as follows based on MALDI-TOF-MS analysis: ◆ starting material (**40**); × GlcNAc (**46**); □ NeuAcGalGlcNAc (**87**); ■ $Gal_2GlcNAc$ (**52**).



4.63 (d, 1H, J_{gem} = 13.3 Hz, PhCH′), 4.57 (d, 1H, $J_{1,2}$ = 8.2 Hz, H-1Gal), 4.40 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1GlcN), 2.64 (dd, 1H, $J_{3eq,4}$ = 4.1 Hz, J_{gem} = 11.9 Hz, H-3eq sialic acid), 2.00 and 1.95 (2s, 6H, 2NAc), 1.66 (dd, 1H, $J_{3ax,4}$ = J_{gem} = 11.9 Hz, H-3ax sialic acid). ^{13}C NMR (150 MHz, D_2O, NaOD, and MeOH) δ: 176.4 (C_{Ph2}), 175.1 (C_{NAc}=O GlcN), 174.8 (C_{NAc}=O sialic acid), 173.7 (C-1 sialic acid), 133.1 (C_{Ph5}), 127.6 (C_{Ph4}-H), 127.2 (C_{Ph6}-H), 126.5 (C_{Ph1}), 119.6 (C_{Ph3}-H), 103.8 (C-1 Gal), 100.5 (C-1 GlcN), 100.4 (C-2 sialic acid), 80.2 (C-4 GlcN), 74.8 (C-5 GlcN), 74.0 (C-5 Gal), 73.1 (C-3 Gal), 72.7 (C-3 GlcN), 72.7 (C-6 sialic acid), 71.9 (C-8 sialic acid), 70.8 (C-2 Gal), 68.9 (C-4 Gal), 68.6 (C-7 sialic acid), 68.4 (C-4 sialic acid), 67.0 (C_{Bn}-H_2), 63.6 (C-6 Gal), 62.8 (C-9 sialic acid), 60.7 (C-6 GlcN), 55.4 (C-2 GlcN), 52.2 (C-5 sialic acid), 40.5 (C-3 sialic acid), 22.3 and 22.2 ($2C_{NAc}$-H_3). MALDI-MS *m/z*: 848 ($[M + Na]^+$).

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