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A New Multi-Enzyme System for a One-Pot Synthesis of Sialyl Oligosaccharides: Combined Use of β-Galactosidase and α(2,6)-Sialyltransferase Coupled with Regeneration *in situ* of CMP-Sialic Acid†

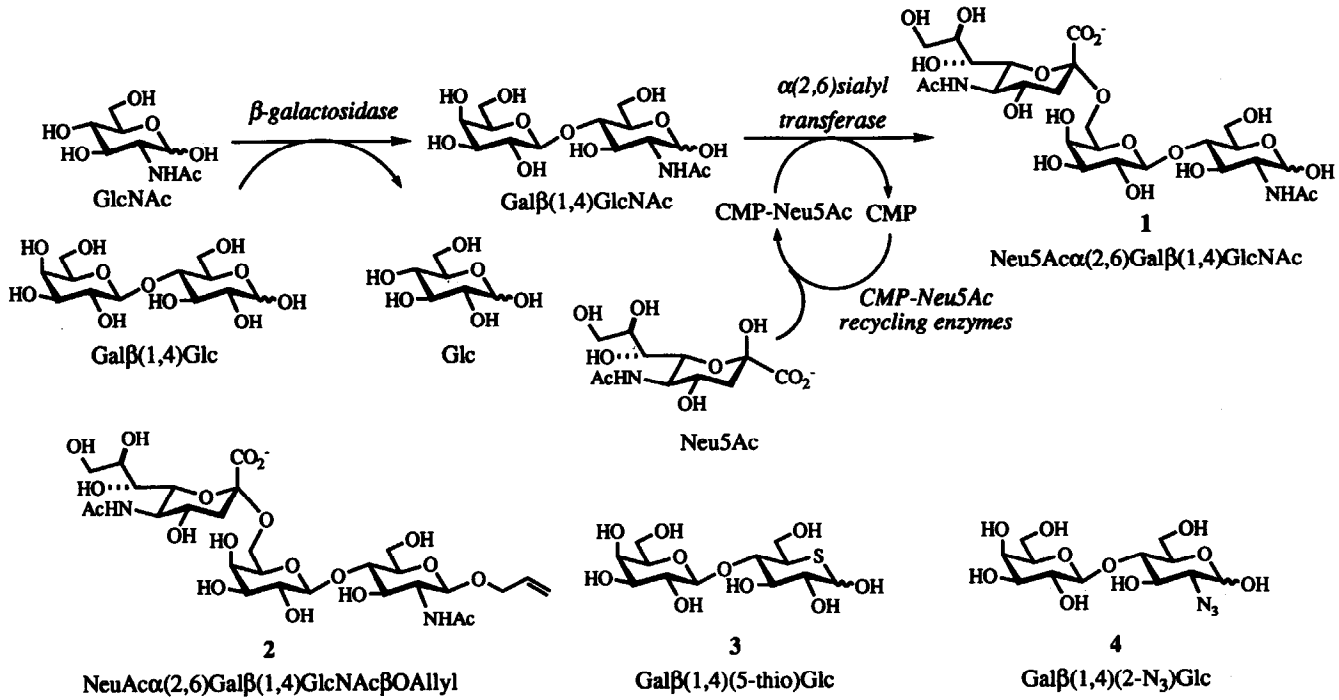
Guido F. Herrmann,[1,2] Yoshitaka Ichikawa,[1] Christian Wandrey,[2] Federico C.A. Gaeta,[3] James C. Paulson,[1,3] and Chi-Huey Wong[1]*

[1]Department of Chemistry, The Scripps Research Institute,
10666 North Torrey Pines Road, La Jolla, CA 92037
[2]Research Center Jülich, Institute of Biotechnology 2, P.O. Box 1913,
D-5170 Jülich, Germany
[3]Cytel Corporation, 3525 John Hopkins Court, San Diego, CA 92121

Abstract: An irreversible one-pot enzymatic synthesis of sialyl oligosaccharides has been achieved with a β-galactosidase-catalyzed galactosylation of an acceptor followed by a sialyltransferase-catalyzed sialylation with regeneration *in situ* of CMP-sialic acid.

Sialyl-oligosaccharides are of growing importance due to their function as ligands of selectins[1] and as important recognition elements on the surface of a variety of cells.[2] Enzymatic approaches to the synthesis of these compounds have proven to be useful,[3] especially that based on glycosyltransferases coupled with regeneration *in situ* of sugar nucleotides.[4-7] By using galactosyltransferase and sialyltransferase, sialyl *N*-acetyllactosamine, for example, was obtained in moderate yields.[6] Glycosidases have also been utilized in glycosylation. The yields, however, are relatively low and the regioselectivity is often difficult to control,[8] though certain glycosidases catalyze regioselective transglycosylation.[8e,f] Another problem with the use of glycosidases is that the product is subject to the enzymatic cleavage, making the process difficult to control. We envisioned that a sequential formation of two glycosidic bonds with a glycosidase followed by a glycosyltransferase in one pot would give an oligosaccharide, which is no longer a substrate for the glycosidase. This communication illustrates the synthesis of α(2,6)sialyl-LacNAc and analogs based on the β-galactosidase from *Bacillus circulans*[8e] and pig liver α(2,6)-sialyl-transferase coupled with regeneration *in situ* of CMP-sialic acid (CMP-Neu5Ac) (Scheme 1). The synthesis of LacNAc catalyzed by the β-galactosidase (0.02 cents/unit) starts with inexpensive lactose[11] and GlcNAc, and the process does not require the expensive β(1,4)-galactosyltransferase ($13/unit). Although the β-galactosidase reaction is reversible, the formed LacNAc is irreversibly sialylated with the sialyltransferase, because the sialylated saccharide is no longer a substrate for the β-galactosidase.

In a representative synthesis of Neu5Acα(2,6)-LacNAc (**1**), to a 1.07 mL HEPES buffer (0.2 M, 20 mM $MgCl_2$, 5.3 mM $MnCl_2$, 20 mM KCl, pH 7.5) containing 12.3 mg Neu5Ac (20 mM), 180 mg Lac (250 mM), 265 mg GlcNAc (600 mM), 30.2 mg PEP (trisodium salt, 40 mM), CMP (2 mM) and ATP (0.2 mM) were added myokinase (MK, EC 2.7.4.3, 6 U), pyruvate kinase (PK, EC 2.7.1.40, 80 U), inorganic



Scheme 1. Coupling of β-galactosidase and α(2,6)sialyltransferase reaction for the synthesis of sialylated oligosaccharides **1** and **2**. Compounds **3** and **4** were prepared similarly with the β-galactosidase. *Abbreviations*: Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Neu5Ac, N-acetylneuraminic acid; CMP, cytidine 5'-monophosphate; CMP-Neu5Ac, cytidine 5'-monophospho-N-acetylneuraminic acid.

pyrophosphatase (PPase, EC 3.6.1.1, 4 U), CMP-Neu5Ac synthetase (EC 2.7.7.43, 0.32 U), α(2,6)sialyltransferase (EC 2.4.99, 0.052 U) and 1 mg of crude β-galactosidase (EC 3.2.1.23) from *Bacillus circulans*. The total volume was adjusted to 2 mL. The reaction was conducted for 91 h under argon at room temperature and monitored by TLC on silica gel 60 (R_f: Lac, 0.16; LacNAc, 0.27; Gal, 0.29; Glc, 0.37; α(2,6)sialyl-LacNAc, 0.45; NeuAc, 0.54; GlcNAc, 0.54 in 7:2:1 (v/v/v) iPrOH/H_2O/NH_4OH). The reaction mixture was centrifuged and the supernatant was directly applied to a BioGel P2 Column (200-400 mesh, 43x2 cm) with water as the eluent. The trisaccharide containing fractions were pooled and lyophilized to give 6.8 mg of Neu5Acα(2,6)Galβ(1,4)GlcNAc (26% yield).[9] The ^{1}H NMR spectrum was identical to that reported.[5] No other sialylated product could be detected. Neither Lac, one of the starting materials, nor Galβ(1,6)GlcNAc, a by-product of the β-galactosidase reaction[8e] is a substrate for α(2,6)sialytransferase[10] due to the high K_m (390 mM) and low V_{max} (3%), respectively. Of several galactosides tested, lactose was found to be the best substrate for the galactosidase.[11] In a similar manner, the sialylated saccharide **2** was also prepared.[12] Compounds **3** and **4** were prepared[12] separately in ~20% yield with the galactosidase; however, **3** was not a substrate for the sialyltransferase and **4** was a very weak substrate.

In summary, we have shown a new efficient procedure for the synthesis of a sialyl-trisaccharide from GlcNAc, NeuAc and Lac. The synthesis employed a β-galactosidase and a sialyltransferase, and each enzyme worked sequentially and selectively, in one-pot, to form a sialyl *N*-acetyllactosamine. Although the glycosidase catalyzes the reversible reaction (i.e., glycosidic bond-hydrolyzing and -forming reactions), a combined usage of these two enzymes in one pot allows the synthesis of sialyl trisaccharides in an irreversible manner. In addition, regeneration of CMP-Neu5Ac from CMP catalyzed by the two kinases and CMP-Neu5Ac synthetase in the sialylation reaction reduces the cost of the process and the problem of product inhibition caused by CMP. It demonstrates another strategy for oligosaccharide synthesis based on a multi-enzyme system with regeneration of CMP-Neu5Ac.

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9. ^{1}H NMR NeuAcα(2,6)Galβ(1,4)GlcNAc (**1**): δ 1.677 (1H, t, J=12.5 Hz, H-3$_{ax}$ of NeuAc), 1.985 (3H, s, NHAc of GlcNAc), 2.022 (3H, s, NHAc of Neu5Ac), 2.628 (1H, dd, J=5 Hz, 12.5 Hz, H-3$_{eq}$ of NeuAc), 4.409 (1H, d, J=8 Hz, H-1 of Gal), 4.780 (0.5 H, d, J=8 Hz, H-1β of GlcNAc), 5.154 (0.5 H, d, J=2.5 Hz, H-1α of GlcNAc).
10. For detailed kinetic study of α(2,6)sialyltransferase, see Paulson, J.C.; Rearick, J.I.; Hill, R.L. *J. Biol. Chem.* **1977**, *252*, 2363; Galβ(1,4)GlcNAc: V_{max} = 1.00, K_m = 12 mM; Galβ(1,6)GlcNAc: V_{max} = 0.03, K_m = 140 mM; Galβ(1,4)Glc: V_{max} = 1.02, K_m = 390 mM.
11. The β-galactosidase accepts the following substrates with the indicated relative rate: Lac (100), *o*-nitrophenyl β-galactoside (10.6), methyl β-galactoside (1).
12. ^{1}H NMR NeuAcα(2,6)Galβ(1,4)GlcNAcβOAllyl (**2**): δ 1.672 (1H, t, J=12.5 Hz, H-3ax of Neu5Ac), 1.984 (3H, s, NHAc of GlcNAc), 2.015 (3H, s, NHAc of Neu5Ac), 2.623 (1H, m, J=4.5, 12.25 Hz, H-3eq of Neu5Ac), 4.130 (1H, m, allyl), 4.294 (1H, m, allyl), 4.403 (1H, d, J=8 Hz, H-1 of Gal), 4.569 (0.5 H, d, J=8.5 Hz, H-1 β of GlcNAc), 5.221 (1H, m, allyl), 5.268 (1H, m, allyl), 5.872 (1H, m, allyl), R_f 0.50 in 7:2:1 (v/v/v) iPrOH/H_2O/NH_4OH.
^{1}H NMR spectrum of **3** was identical with that reported previously: Gautheron-Le Narvor, C.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1991**, 1130.
^{1}H NMR Galβ(1,4)(2-N_3)Glc (**4**): 3.27 (dd, J=8.32, 9.82 Hz, H-2β), 4.42 (d, J=7.75 Hz, H-1'β), 4.69 (d, J=8.72 Hz, H-1β), 5.31 (d, J=3.50 Hz, H-1α). The ^{1}H NMR spectrum of **4** was in good agreement with that of allyl glycoside derivative of **4**.[7]

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