Supporting Information

Arthrobacter Endo-β-N-acetylglucosaminidase Shows Transglycosylation Activity on Complex Type N-Glycan Oxazolines. One-pot Conversion of Ribonuclease B to Sialylated Ribonuclease C

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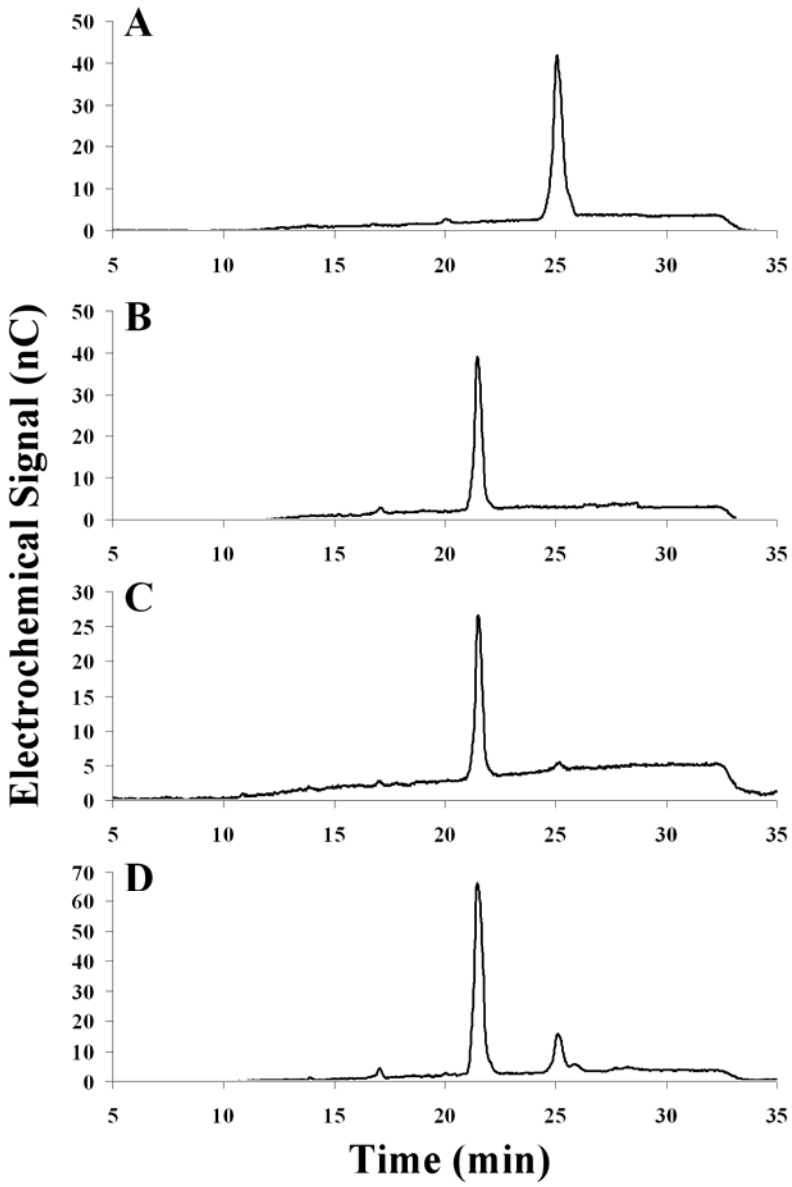
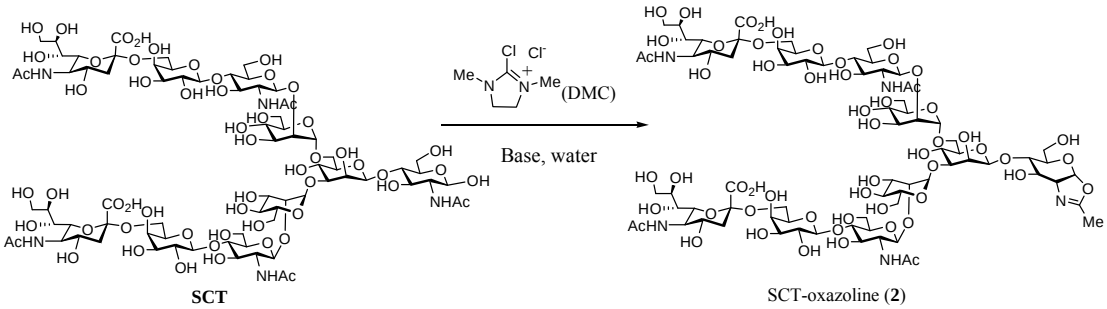


Figure S1. HPAEC monitoring of sialoglycan oxazoline formation using different reaction conditions. A mixture of the sialoglycan (SCT), DMC, and a base in water was stirred at 4 ^{o}C for 15 min. Then an aliquot was taken and subject to HPAEC analysis. (A) the standard sialoglycan SCT; (B) SCT: DMC : Et_3N (1 : 15 : 42); (C) SCT : DMC : NaOH (1 : 15 : 25); and (D) SCT : DMC : K_2CO_3 (1 : 20 : 40).

Table S1



Entry	DMC	base	Yield[a]
1	15 eq.	Et_3N, 42 eq.	100 %
2	12 eq.	Et_3N, 25 eq.	91 %
3	8 eq.	NaOH, 20 eq.	45 %
4	12 eq.	NaOH, 20 eq.	84 %
5	15 eq.	NaOH, 25 eq.	95 %
6	10 eq.	K_2CO_3, 20 eq.	45 %
7	20 eq.	K_2CO_3, 30 eq.	75 %
8	20 eq.	K_2CO_3, 40 eq.	81 %

[a] Reaction yields were calculated on the basis of measurement by HPAEC (DIONEX)

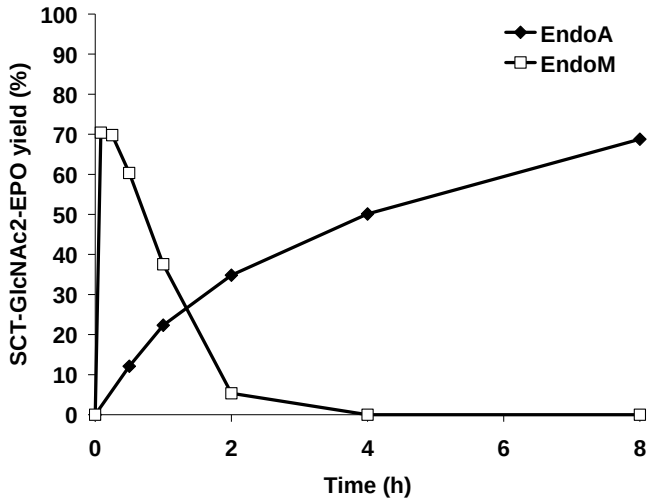


Figure S2. Time course of enzymatic transglycosylation of sialoglycan oxazoline (**2**) and GlcNAc-pentapeptide (**3**) catalyzed by Endo-A (◆) and EndoM (□).

Experimental Procedures

Materials and methods. Natural bovine ribonulcease B (RNase B) was purchased from Sigma-Aldrich and purified by the reported method (K. Witte, et al, *J. Am. Chem. Soc*., **1997**, *119*, 2114-2118). Endo-A was overproduced following the literature (Fujita, eta al, *Biochem. Biophys. Res. Commun.,* **2000**, *267*, 134-138). The hydrolytic activity of Endo-A was defined as following: 1 unit of hydrolytic activity of Endo-A is the amount of enzyme required to hydrolyze 1 μmol of $Man_9GlcNAc_2Asn$ (at 10 mM) in 1 min at 30 ^oC in a phosphate buffer (50 mM, pH 6.5). EndoM-N175A was overproduced according to the previously reported method (M. Umekawa et al, *J. Biol. Chem*., **2008**, *283*, 4469-4479). The transglycosylation activity of Endo-A or Endo-M-N175A was defined as follows: 1 mU of transglycosylation activity was defined as the amount of Endo-A or EndoM-N175A needed for transferring 1 nmol of $Man_3GlcNAc$-oxazoline to GlcNAc-PNP in 1 min at 23 ^oC in a phosphate buffer (50 mM, pH 7.0).

Analytical RP-HPLC was performed on a Waters 626 HPLC instrument with a Symmetry300^{TM} C18 column (5.0 μm, 4.6 × 250 mm) at 40°C. The column was eluted with a linear gradient of MeCN at a flow rate of 1 mL/min using one of the following two gradient methods: (A) 0-30% MeCN containing 0.1% trifluoroacetic acid (TFA) for 18 min; (B) 23-29% MeCN containing 0.1% TFA for 30 min. Preparative HPLC was performed on a Waters 600 HPLC instrument with a preparative C18 column (Symmetry300^{TM}, 7.0 μm, 19 × 300 mm). The column was eluted with a suitable gradient of water/acetonitrile containing 0.1% TFA.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed on a DIONEX DX600 chromatography system (DIONEX Corporation, Sunnyvale, CA) equipped with an electrochemical detector (ED50) and an anion exchange column (CarboPac PA100, 4 × 250 mm). The mobile phase (flow rate, 1.0 mL/min) was composed of deionized water (eluent A), 1 M NaOAc (eluent B), and 0.2 M NaOH (eluent C).The gradient used was as follows: 0 min, 50% eluent A, 0% eluent B, 50% eluent C; 5.0 min, 50% eluent A, 0% eluent B, 50% eluent C; and 25.0 min, 35% eluent A, 15% eluent B, and 50% eluent C.

NMR spectra were measured with JEOL ECX 400 MHz and/or Inova 500 MHz NMR spectrometers.

ESI-MS Spectra were measured on a Waters Micromass ZQ-4000 single quadruple mass spectrometer.

MALDI-TOF MS spectra were recorded on an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics). The instrument was calibrated by using ProteoMass Peptide MALDI-MS calibration kit (MSCAL2, Sigma/Aldirich). The matrix used for glycans is 2,5-dihydroxybenzoic acid (DHB) (10 mg/mL in 50% acetonitrile containing 0.1% trifluoroacetic acid). The measuring conditions in detail: 337 nm nitrogen laser with 100 μJ output; laser frequency 50.0 Hz; laser power 30-45%; linear mode; positive polarity; detection range 1000-10000; pulsed ion extraction: 70ns; high voltage: on; realtime smooth: high; shots: 500-2000.

Synthesis of the sialylated complex-type N-glcyan oxazoline (2). SCT (10 mg, 5 μmol) and 2-chloro-1,3-dimethylimidazolinium chloride (DMC, 12.6 mg, 75 μmol) were dissolved in 0.2 mL of water at 4 ^{o}C. Then Et_3N (30 μL, 215 μmol) was added and the mixture was shaken at 4 ^{o}C for 30 min. HPAEC indicated the completion of reaction. The residue was subject to gel filtration on a Sephadex G-15 column. The column was eluted by water containing 0.05% Et_3N. The fractions containing the sialoglycan-oxazoline were combined. The fractions were mixed with an aqueous solution of NaOH (0.1 M, 10 μL) and were then lyophilized to give the sialoglycan-oxazoline (**2**) as a white powder (9.8 mg, quantitative yield). The inclusion of a catalytic amount of NaOH ensures an alkaline situation for the oxazoline for its stability after freeze-dry.. ^{1}H NMR (D_2O, 500 MHz) of oxazoline **2**: δ 6.09 (d, 1 H, *J* = 7.5 Hz, H-1 of oxazoline), 5.11 (s, 1 H, H-1 of α-Man), 4.95 (s, 1 H, H-1 of α-Man), 4.74 (s, 1 H, H-1 of β-Man), 4.58 (m, 2 H, H-1 of two β-GlcNAc), 4.42 (d, 2 H, *J* = 9.0 Hz, H-1 of two β-Gal), 4.37 (m, 1 H), 4.16 (m, 4 H), 2.65 (m, 2 H, H-3_{ax} of sialic acid), 2.06 (s, 3 H, Ac), 2.05 (s, 6 H, Ac, CH_3 of oxazoline), 2.01 (s, 6 H, 2 Ac), 1.71 (t, 2 H, *J* = 12.0 Hz, H-3_{eq} of sialic acid); ^{13}C NMR (D_2O, 125 MHz, from HSQC) δ 103.5 (C-1 of Gal), 101.2 (C-1 of β-Man), 100.3 (C-1 of oxazoline), 99.7 (C-1 of α-Man), 99.5 (C-1 of GlcNAc), 96.4 (C-1 of α-Man), 40.2 (C-3 of sialic acid), 22.7-21.3 (CH_3 of Ac), 13.1 (CH_3 of oxazoline); HPAEC-PAD: t_R = 21.4 min; ESI-MS: calculated for $C_{76}H_{123}N_5O_{56}$, M = 2001.69 Da; found (m/z), 1001.44 $[M + 2H]^{2+}$.

Endo-A catalyzed transglycosylation with sialoglycan-oxazoline (2). Synthesis of sialylated glycopeptide (4). A solution of sialoglycan oxazoline **2** (1.5 mg, 0.75 μmol) and GlcNAc-pentapeptide (**3**) (200 μg, 0.25 μmol) in a phosphate buffer (50 mM, pH 7.0, 10 μL) was incubated with Endo-A (200 mU/μL) at 23 ^{o}C for 8 h. The transglycosylation product was then purified by

preparative HPLC to give glycopeptide **4** (472 μg, 68%). Analytical HPLC (Method A): t_R = 13.7 min. ESI-MS: calculated for $C_{108}H_{179}N_{13}O_{70}$, M = 2778.08 Da; found (m/z), 1390.68 $[M + 2H]^{2+}$, 927.67 $[M + 3H]^{3+}$.

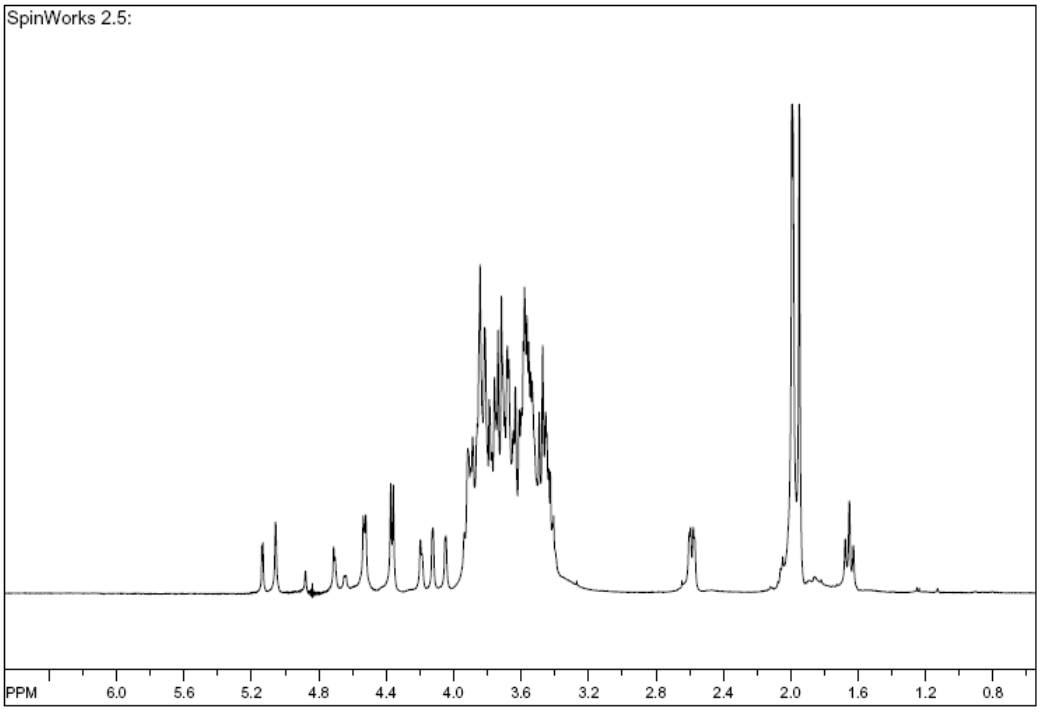
One-pot conversion of RNase B into sialylated RNase C by Endo-A. A solution of sialoglycan oxazoline **2** (1.4 mg, 0.72 μmol) and RNase B (840 μg, 0.06 μmol) in a phosphate buffer (50 mM, pH 7.0, 10 μL) was incubated with Endo-A (700 mU/μL) at 23 ^oC for 4 h. The transglycosylation product was then purified by preparative HPLC to give the sialylated RNase C (240 μg, 25%). Analytical HPLC (Method B): t_R = 20.8 min. ESI-MS: calculated, M = 15886 Da; found (m/z), 1766.86 $[M + 9H]^{9+}$, 1590.27 $[M + 10H]^{10+}$, 1445.39 $[M + 11H]^{11+}$, 1325.05 $[M + 12H]^{12+}$, and 1223.20 $[M + 13H]^{13+}$. The deconvolution data, M = 15888.35 ± 0.63 Da.

One-pot conversion of RNase B into sialylated RNase C by a combined use of Endo-A and EndoM-N175A. A solution of sialoglycan oxazoline **2** (1.4 mg, 0.72 μmol) and RNase B (840 μg, 0.06 μmol) in a phosphate buffer (50 mM, pH 7.0, 10 μL) was incubated with Endo-A (10 mU/μL) and EndoM-N175A (10 mU/μL) at 23 ^oC for 4 h. The transglycosylation product was then purified by preparative HPLC to give sialylated RNase C (678 μg, 70%). The product was confirmed to be the same as described above for using the Endo-A alone.

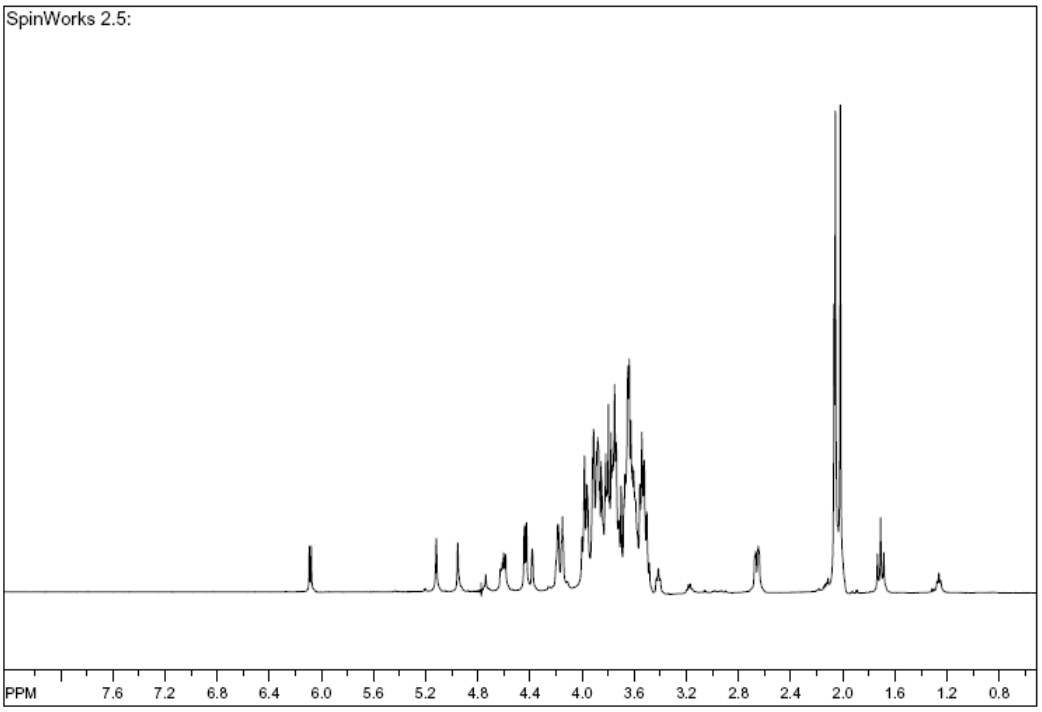
Characterization of glycopeptide (4)

The ESI-MS deconvolution data (M = 2779.36) of glycopeptide **4** was in good agreement with the calculated data (M = 2779.07); treatment of **4** with pronase, which hydrolyzes all the peptide bonds, gave the expected Asn-linked sialoglycan SCT-Asn (ESI-MS: calculated, M = 2336.83; found (m/z), 2360.32 $[M+Na^+]$), indicating that the glycan was attached to the Asn-linked GlcNAc moiety in the peptide during transglycosylation; and, finally, treatment of **4** with Endo-M, which is specific for cleaving the GlcNAcβ1→4GlcNAc linkage in complex type N-glycans, gave the free sialoglycan (SCT) and the GlcNAc-pentapeptide (**3**). These results confirm that the newly formed glycosidic bond in product **4** is the expected GlcNAcβ1→4GlcNAc linkage.

^{1}H NMR of the sialoglycan (SCT)



^{1}H NMR of the sialoglycan-oxazoline (**2**)



^{1}H-^{13}C HSQC of the sialoglycan-oxazoline (**2**)