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Carbohydrate Research 330 (2001) 439–443

CARBOHYDRATE RESEARCH

Large-scale production of the carbohydrate portion of the sialyl–Tn epitope, α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc, through bacterial coupling

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Received 9 November 2000; accepted 20 December 2000

Abstract

α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc, the carbohydrate portion of sialyl–Tn epitope of the tumor-associated carbohydrate antigen, was prepared by a whole-cell reaction through the combination of recombinant *Escherichia coli* strains and *Corynebacterium ammoniagenes*. Two recombinant *E. coli* strains overexpressed the CMP-Neu*p*5Ac biosynthetic genes and the α-(2→6)-sialyltransferase gene of *Photobacterium damsela*. *C. ammoniagenes* contributed to the production of UTP from orotic acid. α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was accumulated at 87 mM (45 g/L) after a 25-h reaction starting from orotic acid, *N*-acetylneuraminic acid, and 2-acetamide-2-deoxy-D-galactose.

Keywords: Enzymatic synthesis; Glycosyltransferase; Sugar nucleotide; Oligosaccharide

1. Introduction

Sialyl–Tn (STn) is an *O*-serine- or *O*-threonine-linked disaccharide expressed on mucins of most human adenocarcinomas.[1,2] STn is widely expressed on cancer cells of breast, prostate and ovarian origin, because its expression on normal tissues is restricted. Therefore, it is thought to be a target carbohydrate antigen for cancer immunotherapy,[3–6] and clinical trials with vaccine-induced antibody responses against STn antigen have been carried out.[7–14] A monoclonal antibody against STn could react with the envelope glycoprotein, gp120, of the human immunodeficiency virus (HIV), and the antibody was shown to be able to block infection by the virus.[15,16]

Although the importance of STn has been defined, there is no methodology for the large-scale preparation of the carbohydrate portion of STn epitope, α-Neu*p*5Ac-(2→6)-D-Gal*p*-NAc. Several chemical syntheses of the carbohydrate portion of the STn epitope have been well established;[17–21] however, the multiple protection and deprotection steps are indispensable because of the difficulty in the control of both stereo- and regiochemistry of bond formation. An alternative approach to synthesize the carbohydrate portion of the STn epitope would be an enzymatic method using sialyltransferases,[22,23] which is especially attractive since it is highly stereo- and regio-

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0008-6215/01/$ - see front matter
PII: S0008-6215(01)00007-6

selective. For instance, the multienzyme system for the synthesis of sialyl-*N*-acetyllactosamine (α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-D-Glc*p*-NAc) with in situ regeneration of CMP-Neu*p*5Ac would be applied.[24] Although the in situ regeneration of CMP-Neu*p*5Ac was established, the system still required several purified enzyme preparations and CMP and phosphoenolpyruvate. The poor availability of glycosyltransferases and expensive raw materials might limit the application to large-scale manufacture.

Recently, we reported a novel production system of sugar nucleotides and oligosaccharides through the coupling of recombinant *Escherichia coli* and *Corynebacterium ammoniagenes*.[25–27] In the case of CMP-Neu*p*5Ac production, two recombinant *E. coli* strains that overexpressed the genes of CMP-Neu*p*5Ac synthetase and CTP synthetase and *C. ammoniagenes* that contributed to the formation of UTP from orotic acid were used. When *E. coli* cells that highly expressed the gene of α-(2→3)-sialyltransferase from *Neisseria gonorrhoeae* were put into the CMP-Neu*p*5Ac production system, 3′-sialyllactose was produced from orotic acid, Neu*p*5Ac, and lactose.[27]

We report herein an efficient production system of the carbohydrate structure of STn epitope, α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc, through the coupling of recombinant *E. coli* and *C. ammoniagenes*. For the production of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc, the α-(2→6)-sialyltransferase from *Photobacterium damsela* was used.[28–30] The α-(2→6)-sialyltransferase from *P. damsela* showed a broad acceptor specificity compared with those of mammalian α-*N*-acetylgalactosaminide α-(2→6)-sialyltransferases and could transfer Neu*p*5Ac to *N*-acetylgalactosamine (Gal*p*NAc).[31,32]

2. Results and discussion

*Construction of the α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc production system.*—The basic system for the production of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was made up of a CMP-Neu*p*5Ac production system and a recombinant *E. coli* that overexpresses α-(2→6)-sialyltransferase gene (Fig. 1). The CMP-

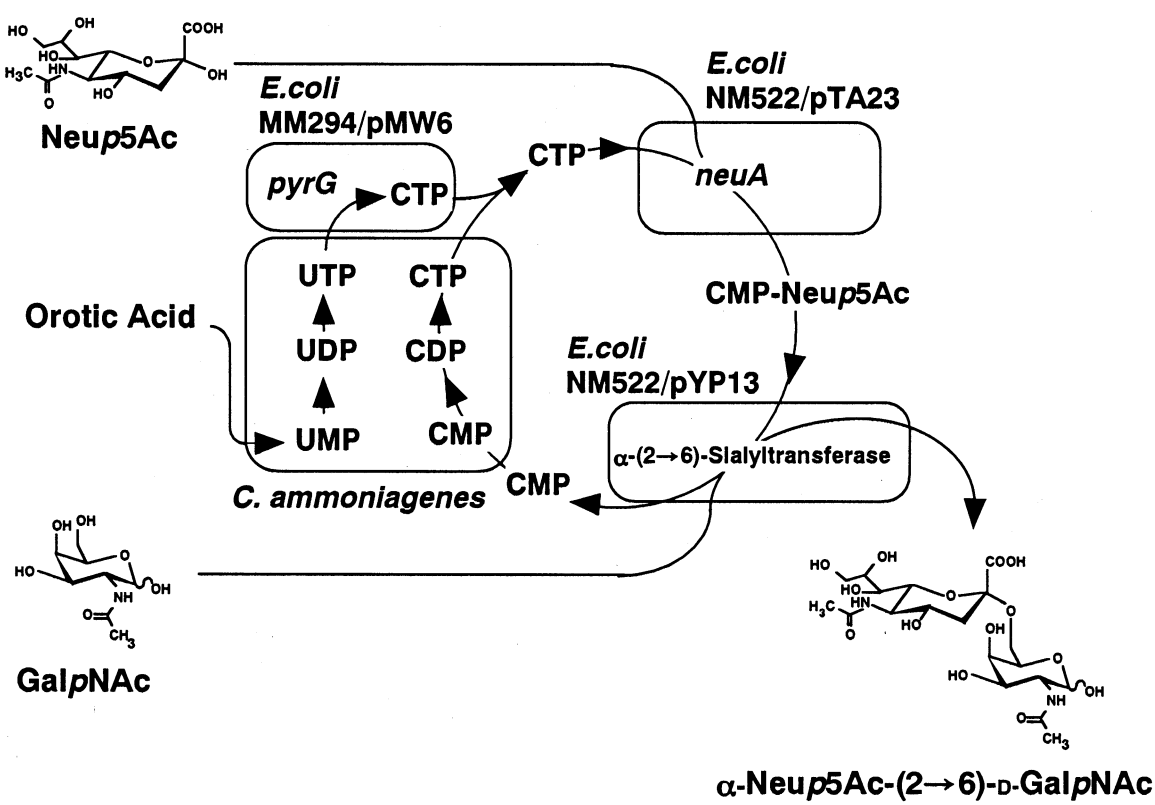

Fig. 1. Scheme of the production of carbohydrate portion of sialyl–Tn epitope, α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc.

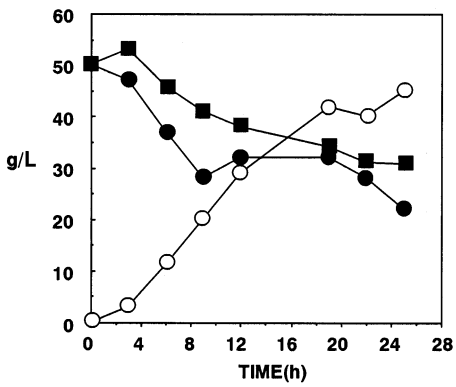

Fig. 2. Time course for α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc production on a 30 mL scale. The amount of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc (○), Gal*p*NAc (■) and Neu*p*5Ac (●) in the reaction mixture are indicated.

Neu*p*5Ac production system consisted of a recombinant *E. coli*, which overexpresses the CMP-Neu*p*5Ac synthetase (*neuA*) and CTP synthetase (*pyrG*), and *C. ammoniagenes*, which contributed to the production of UTP from orotic acid.[27] The α-(2→6)-sialyltransferase gene of *P. damsela* was cloned by PCR and inserted into pPAC31 under a P_L promoter to form pYP13. The gene was overexpressed through the inactivation of a temperature-sensitive cI857 repressor at 40 °C.

*Production of α-Neu*p*5Ac-(2→6)-*D*-Gal*p*-NAc.*—The production of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was examined as follows: The reaction was carried out on a 30-mL scale in a 200-mL beaker. The recombinant *E. coli* cells harboring pYP13 that overexpressed α-(2→6)-sialyltransferase gene of *P. damsela* were added to the CMP-Neu*p*5Ac production system. Neu*p*5Ac, Gal*p*NAc, and orotic acid were also added to the reaction mixture, as well as polyoxyethylene octadecylamine and xylene to permeabilize the cells. Fructose was added as an energy source. After 25 h, 45 g/L (87 mM) of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was produced from 162 mM Neu*p*5Ac, 370 mM fructose, and 226 mM Gal*p*NAc (Fig. 2). The yield of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was 54% from Neu*p*5Ac and 39% from Gal*p*NAc. Considering the remaining sugars in the reaction mixture, 68 mM Neu*p*5Ac and 135 mM Gal*p*NAc, the conversion yield of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was 93% from Neu*p*5Ac and 96% from Gal*p*NAc. Almost no peaks other than fructose, Gal*p*NAc, Neu*p*5Ac, and α-Neu*p*5Ac-(2→6)-D-Gal*p*-NAc were observed after 25 h, as analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) using a Dionex DX-500 system equipped with a Carbopac PA10 column. Because of the broad acceptor specificity of the α-(2→6)-sialyltransferase from *P. damsela*,[28–30] other sialyloligosaccharides such as α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-D-Glc*p* and α - Neu*p*5Ac - (2→6) - β - D - Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* were also produced by the system mentioned above (data not shown). Although the real structure of STn epitope is α-Neu*p*5Ac-(2→6)-α-D-Gal*p*NAc-(1→0)-Ser/Thr, the mass production of its carbohydrate portion, α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc, is of considerable importance for immunological studies and for the preparation of synthetic antitumor vaccines.

*Isolation of α-Neu*p*5Ac-(2→6)-D-Gal*p*-NAc.*—After the several purification steps, including activated charcoal chromatography and gel-filtration with Bio-Gel P2, α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc (338 mg; 98% purity) was isolated from the reaction mixture. With regard to large-scale purification, it should be possible to apply simulated moving bed systems that are used for the separation of carbohydrates.[33] The structure of the purified compound was identified as α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc by means of NMR spectra.

3. Experimental

Materials.—BioGel P-2 was purchased from BioRad Laboratories (Hercules, CA). Orotic acid used for the reaction was the product of Kyowa Hakko Kogyo Co., Ltd. All other chemicals used were commercially available and of analytical grade.

Plasmid construction.—DNA manipulations were performed according to the procedures described by Sambrook et al.[35] The gene encoding α-(2→6)-sialyltransferase was cloned from *P. damsela* ATCC33539. P_L promoter was amplified from pPAC31 by PCR using the primer, 5′-CAAGAATTCTCTCT-CACCTACCAA-3′, to introduce an *Eco*RI site, and 5′-AATCTCGAGATCGATACC-

CTTTTTTACG-3′, to introduce an *Xho*I site. The truncated α-(2→6)-sialyltransferase gene of *P. damsela* (ATCC 33539) was amplified by PCR using the primer, 5′-TAACTCGAGAT-GAAGAAAATACTGACAGTTC-3′, to introduce an *Xho*I site, and 5′-TAAGG-ATCCTTAAGCCCAGAACAGAACATC-3′, to introduce a *Bam*HI site which introduced a stop codon at 498 D.[30] The 0.3-kb PCR product digested with *Eco*RI and *Xho*I and the 1.5-kb PCR product digested with *Xho*I and *Bam*HI were inserted between the *Eco*RI and *Bam*HI sites of pPAC31 to give pYP13. The DNA sequence was confirmed by the dideoxy sequencing using the 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Bacterial strains and culture conditions.—*E. coli* strain NM522 was purchased from Stratagene (La Jolla, CA). The expression plasmids were used to transform *E. coli* NM522. *E. coli* strains were cultivated in a 5-L jar fermenter according to the method described before.[25] *C. ammoniagenes* DN510 cells were cultivated in a 5-L jar fermenter according to the method described before.[36] Cells were collected by centrifugation and stored at −20 °C until used.

*α-Neu*p*5Ac-(2→6)-*D*-Gal*p*NAc production.*—The production of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was carried out in a 200-mL beaker containing 30-mL of the reaction mixture, i.e., 50 g/L (wet weight) of *C. ammoniagenes* DN510 cells, 25 g/L (wet weight) of MM294/pMW6 cells overexpressing CTP synthetase gene,[27] 50 g/L (wet weight) of NM522/pTA23 cells overexpressing CMP-Neu*p*5Ac synthetase gene,[27] 50 g/L (wet weight) of NM522/pYP13 cells overexpressing α-(2→6)-sialyltransferase gene, 50 g/L of fructose, 50 g/L of Gal*p*NAc, 50 g/L of Neu*p*5Ac, 25 g/L of KH_2PO_4, 5 g/L of $MgSO_4 \cdot 7H_2O$, 5 g/L of orotic acid (potassium salt), 4 g/L of polyoxyethylene octadecylamine, and 10 ml/L of xylene. The reaction was carried out at 32 °C with agitation (900 rpm), and the pH was kept at 7.2 by the addition of 4 N NaOH.

Analyses.—α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc and other saccharides were analyzed by means of HPAEC–PAD using a Dionex DX-500 system equipped with a Carbopac PA10 column (Dionex, Sunnyvale, CA).[34] Inorganic phosphate was determined with Determiner IP-S Kit (Kyowa Medex, Tokyo).

*Isolation of α-Neu*p*5Ac-(2→6)-*D*-Gal*p*NAc.*—The reaction mixture was centrifuged at 7000 rpm for 30 min at 4 °C to remove the cells. The supernatant containing 1.3 g of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was applied to a column of activated charcoal (30 × 200 mm).[37] After the column was washed with 15 bed volumes of water, α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was eluted with five bed volumes of 30% EtOH. Fractions containing α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc were collected and freeze-dried, and 360 mg of α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc was obtained. Carbohydrates other than α-Neu*p*5Ac-(2→6)-D-Gal*p*-NAc were further removed by means of gel-filtration with a column of Bio-Gel P-2 (25 × 900 mm).[25] Fractions containing α-Neu*p*5Ac-(2→6)-D-Gal*p*NAc were collected and freeze-dried. NMR spectra were recorded in D_2O with a JEOL JNM-A400 instrument using residual HDO as the internal reference. The HRMSFAB mass spectra were obtained using a JEOL HX-110 double-focusing mass spectrometer operating in the negative-ion mode.

*Spectral data of α-Neu*p*5Ac-(2→6)-*D*-Gal*p*NAc.*—^{1}H NMR (400 MHz, 10 mg/0.6 mL D_2O): α anomer: 5.24 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 4.15 (dd, 1 H, $J_{1,2}$ 3.7, $J_{2,3}$ 11.2 Hz, H-2), 3.95 (m, 1 H, H-3), 4.04 (d, 1 H, $J_{3,4}$ 3.2 Hz, H-4), 4.22 (dd, 1 H, $J_{5,6a}$ 4.5, $J_{5,6b}$ 11.2 Hz, H-5), 3.66 (m, 1 H, H-6a), 3.93 (m, 1 H, H-6b), 2.08 (s, 3 H, 2-CH_3CO), 1.73*[1] (dd, 1 H, $J_{3'ax,3'eq}$ 12.2, $J_{3'ax,4'}$ 12.2 Hz, H-3′ax), 2.76 (dd, 1 H, $J_{3'ax,3'eq}$ 12.2, $J_{3'eq,4'}$ 4.6 Hz, H-3′eq), 3.71 (m, 1 H, H-4′), 3.86 (dd, 1 H, $J_{4',5'}$ 10.2, $J_{5',6'}$ 10.2 Hz, H-5′), 3.75 (dd, 1 H, $J_{5',6'}$ 10.2, $J_{6',7'}$ 1.7 Hz, H-6′), 3.61 (dd, 1 H, $J_{6',7'}$ 1.7, $J_{7',8'}$ 8.8 Hz, H-7′), 3.93 (m, 1 H, H-8′), 3.68 (m, 1 H, H-9a′), 3.92 (m, 1 H, H-9b′), 2.07 (s, 3 H, 5′-CH_3CO); β anomer:4.66 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1), 3.89 (m, 1 H, H-2), 3.73 (m, 1 H, H-3), 3.97 (m, 1 H, H-4), 3.80 (m, 1 H, H-5), 3.67 (m, 1 H, H-6a), 3.95 (m, 1 H, H-6b), 2.09 (s, 3 H, 2-CH_3CO) 1.72* (dd, 1 H, $J_{3'ax,3'eq}$ 12.2, $J_{3'ax,4}$ 12.2 Hz H-3′ax), 2.76 (dd, 1 H, $J_{3'ax,3'eq}$ 12.2, $J_{3'eq,4'}$ 4.6 Hz, H-3′eq), 3.71 (m, 1 H, H-4′), 3.86 (dd, 1 H, $J_{4',5'}$ 10.2, $J_{5',6'}$ 10.2

Hz, H-5′), 3.75 (dd, 1 H, $J_{5',6'}$ 10.2, $J_{6',7'}$ 1.7 Hz, H-6′), 3.61 (dd, 1 H, $J_{6',7'}$ 1.7, $J_{7',8'}$ 8.8 Hz, H-7′), 3.93 (m, 1 H, H-8′), 3.68 (m, 1 H, H-9a′), 3.92 (m, 1 H, H-9b′), 2.07 (s, 3 H, 5′-CH_3CO); ^{13}C NMR (100 MHz, 10 mg/0.6 mL D_2O): α anomer: 91.78 (C-1), 50.95 (C-2), 68.00 (C-3), 69.33 (C-4), 69.85 (C-5), 64.64 (C-6), 22.97 (2-CH_3CO), 175.40 (2-CH_3CO), 174.21* (C-1′), 101.21 (C-2′), 40.93* (C-3′), 68.96 (C-4′), 52.61(C-5′), 73.37 (C-6′), 69.01 (C-7′), 72.46 (C-8′), 63.40 (C-9′), 22.78 (5′-CH_3CO), 175.80 (5′-CH_3CO); β anomer: 96.13 (C-1), 54.34 (C-2), 71.72 (C-3), 68.65 (C-4), 74.32 (C-5), 64.45 (C-6), 22.72 (2-CH_3CO), 175.70 (2-CH_3CO), 174.17*[2] (C-1′), 101.21 (C-2′), 40.89*[3] (C-3′), 68.96 (C-4′), 52.61(C-5′), 73.37 (C-6′), 69.01 (C-7′), 72.46 (C-8′), 63.40 (C-9′), 22.78 (5′-CH_3CO), 175.80 (5′-CH_3CO)*: Assignments may be interchangeable. FABMS: *m*/*z* Anal. Calcd for $C_{19}H_{32}N_2O_{14}$ $[M-H]^-$ 511.1775; Found: *m*/*z* 511.1779.

Acknowledgements

We thank Ruriko Nishimura-Fujii, Yumino Kimura, and Hayumi Tsunoda for technical assistance.

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