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The use of porcine liver (2→3)-α-sialyltransferase in the large-scale synthesis of α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-D-Glc*p*NAc, the epitope of the tumor-associated carbohydrate antigen CA 50*

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ABSTRACT

α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-D-Glc*p*NAc (**2**) and, α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAcOMBn were prepared on a large scale by the action of β-D-Gal*p*-(1→3)-D-Gal*p*NAc (2→3)-α-sialyltransferase (partially purified from porcine liver) on β-D-Gal*p*-(1→3)-D-Glc*p*NAc and β-D-Gal*p*-(1→3)-β-D-Glc*p*NAcOMBn, respectively. The trisaccharide **2** is the epitope of the tumor-associated carbohydrate antigen CA 50, highly expressed in human pancreatic adenocarcinoma.

INTRODUCTION

Sialyloligosaccharides, which occur as terminal sequences in glycoproteins and glycolipids of the cell surface, are involved in cellular recognition phenomena. Such sequences are recognized as receptors for viruses, toxins, lectins, and mycoplasma, and also as antigenic determinants which have been identified as tumor markers[1]. Monoclonal antibodies have been developed against these tumor-associated carbohydrate antigens isolated from biological material and are now being exploited in the diagnosis and therapy of cancer. Moreover, antibodies directed against synthetic antigens would be helpful for screening a panel of antibodies, thus defining more precisely the epitope and eventually increasing the sensitivity of cancer diagnosis.

In a research program related to synthetic antigens, we were interested in the chemical synthesis of α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-D-Glc*p*NAc (**2**), the epitope of the tumor-associated carbohydrate antigen CA 50, which is highly expressed in human pancreatic adenocarcinoma[2], and has been detected in human malignant glioma[3] and serum of patients with prostate cancer[4]. A difficult problem in this synthesis is the introduction of a sialyl group at O-3 of the D-galactose residue with the required α stereoselectivity[5]. One of us recently reported the preparation of sialyloligosaccharides by the use of an immobilized (2→6)-α-sialyltransferase[6]. This result prompted us to

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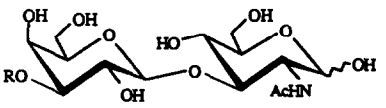
further investigate the synthetic potentiality of sialyltransferases and we now report the use of partially purified porcine liver, β-D-Gal*p*-(1→3)-D-Gal*p*NAc:CMP-Neu*p*5Ac-(2→3′)-α-sialyltransferase (EC 2.4.99.4) in sialyloligosaccharide synthesis. Whereas some glycosides of trisaccharide **2** have been prepared on a few-mg scale[7-9], the present enzyme-catalyzed sialylation of β-D-Gal*p*(1→3)-D-Glc*p*NAc (**1**), isomer of the real substrate, and of its glycoside, β-D-Gal*p*-(1→3)-D-Glc*p*NAcOMBn (**3**) led to the first synthesis of the free trisaccharide **2** and its 4-methoxybenzyl glycoside **4**.

RESULTS AND DISCUSSION

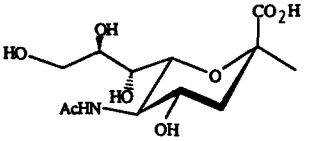
The enzymic transfer of *N*-acetylneuraminic acid from CMP-Neu*p*5Ac to the oligosaccharide acceptor is catalyzed by sialyltransferases. Sialyltransferases are stereo- and regio-specific, and generally exhibit a high selectivity towards the oligosaccharide acceptor. Starting from the observation that β-D-Gal*p*-(1→3)-Gal*p*NAc (**5**), the "T" antigen, turned out to be a good acceptor of *N*-acetylneuraminic acid with a crude extract of porcine liver, we partially purified the β-D-Gal*p*-(1→3)-D-Gal*p*NAc:CMP-Neu*p*5Ac-(2→3′)-α-sialyltransferase from this source. The enzyme was extracted with 1.4% Triton X-100, and the crude preparation was passed through a cationic exchanger, a Zeta-Prep Sp capsule. The enzyme, which was not retained at pH 6.0, was collected in the filtrate and washings, and applied to a column of CDP-hexanolamine–agarose, eluted with a stepwise gradient of NaCl. Most of the enzymic activity was recovered in the 0.4 and 0.6M NaCl eluates. These were dialyzed and again purified by a second affinity chromatography on CDP-hexanolamine–agarose, eluted with a linear gradient of CTP. The (2→3)-α-sialyltransferase was obtained in 30% overall yield and, thus, was purified 350-fold. The purification of this enzyme from porcine submaxillary gland[10] and human placenta[11] has been published, but the purification from porcine liver only appeared in abstract form[12].

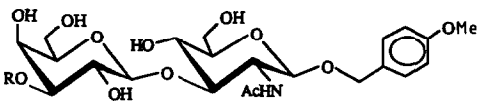
To confirm that the enzyme preparation catalyzes the transfer of sialic acid from CMP-Neu*p*5Ac to OH-3′ of **5**, 30 μmol of substrate **5** were incubated. The product of sialylation was isolated in 35% yield after ion-exchange and Bio-Gel P-2 chromatography, and characterized as compound **6**; ^{1}H-n.m.r. data were in good accordance with those reported[7,13]. However, some discrepancy from the reported data was observed for the optical rotation, owing to a different ratio of α and β anomers, as shown by the ^{1}H-n.m.r. spectrum. The K_m value for the "T" antigen **5** was found to be 0.38mM, which is close to the value of 0.21mM reported for the porcine submaxillary gland enzyme[10]; pH 7.5 was determined as the optimum pH. Various oligosaccharides were tested as acceptors of this (2→3)-α-sialyltransferase. Although, at a concentration of 5mM, these compounds were very poor substrates, they were good acceptors at a higher concentration (100mM). The initial rates observed with each oligosaccharide expressed as a percent of the initial rate with compound **5** are presented in Table I. Compound **7**, the "T" antigen having a sialyl group linked to the 2-acetamido-2-deoxy-D-galactose residue, was a good acceptor. Of special interest was disaccharide **1**, which was the best substrate of the three tested compounds, **1**, **9**, and **10**. The initial rate for **1** and for its



1 R = H

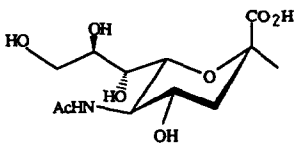
2 R =

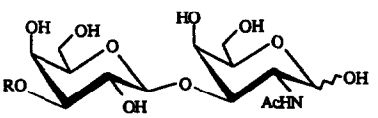




3 R = H

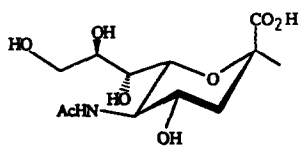
4 R =





5 R = H

6 R =



β-D-Gal*p*-(1→3)-[α-Neu*p*5Ac-(2→6)]-D-Gal*p*NAc

7

β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-D-Glc*p*NAc

8

β-D-Gal*p*-(1→4)-D-Glc*p*NAc

9

β-D-Gal*p*-(1→4)-D-Glc*p*

10

TABLE I

Acceptor specifity of partially purified porcine liver (2→3)-α-sialyltransferase

Compound	*Relative initial rate*[a] *(%)*
1[b]	18
3	11
5[b]	100
7	38
8	0
9	10
10	6

[a] Determined at 100mM acceptor concentration and 280μM CMP-Neu*p*5Ac. [b] K_m for this compound was 0.3mM; V_{max} was 0.36 μmol/min.

glycoside **3** were, respectively, 18 and 11% of the one measured for the preferred substrate. On the other hand, disaccharide **8** having an α-L-fucosyl group linked to the 2-acetamido-2-deoxy-D-glucose residue turned out to be totally inactive towards the enzyme, not a surprising result in view of the biosynthetic pathway for the sialyl-Le[a] determinant[14]; a similar behavior of commercial porcine submaxillary gland (2→3′)-α-sialyltransferase has been reported[8].

It seem that sialyltransferases have a wider substrate specificity than accepted until now. Thus, commercial β-D-Gal*p*-(1→4)-D-Glc*p*NAc-(2→6)-α-sialyltransferase has recently been shown, by van Pelt *et al.*[15], to transfer a sialyl group to D-mannose instead of D-galactose. In our case, however, we cannot completely exclude the possibility of a contamination of the enzyme preparation by another sialyltransferase having the β-D-Gal*p*-(1→3)-Glc*p*NAc specificity.

The cross-reactivity of the porcine liver β-D-Gal*p*-(1→3)-D-Gal*p*NAc:CMP-Neu*p*5Ac-(2→3′)-α-sialyltransferase allowed the sialylation of compounds **1** and **3** on a preparative scale. The enzymic incubation was performed on one mmol of **1** with 0.7 unit of sialyltransferase and 1.2 equiv. of CMP-Neu*p*5Ac, enzymically prepared[6] in the presence of $MnCl_2$ to eliminate the inhibition by CTP present in the enzymic preparation. After purification by chromatography on a silica gel column, incubation with sialyl aldolase and pyruvate decarboxylase immobilized on Eupergit C and chromatography on a Sephadex G-15 column, pure trisaccharide **2** was obtained in a 20% overall yield according to ^{1}H- and ^{13}C-n.m.r. data, which were compared with the values reported[7,8] for the β-glycosides of **2**. The use of immobilized enzymes was advantageous, both for stabilizing the enzyme during incubation and facilitating reaction-mixture purification. The incubation with the disaccharide, 4-methoxybenzyl glycoside **3**, was done in the same way on 0.2 mmol of substrate. After purification, the pure trisaccharide glycoside **4** was obtained in 50% yield based on CMP-Neu*p*5Ac.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Jasco digital micropolarimeter. ^{1}H-N.m.r. spectra were recorded, at 250 MHz, with a Bruker AM-250 spectrometer, the chemical shifts being given relative to the signal of tetramethylsilane as external standard (0.2% solution in $CDCl_3$) for solutions in D_2O. ^{13}C-N.m.r. spectra were recorded at 50 MHz with a Bruker AM-200 spectrometer; 1,4-dioxane was used as the external standard (δ 66.64 from the signal of tetramethylsilane). Zeta-prep SP capsules were obtained from Cuno. CDP-hexanolamine-agarose was prepared as described earlier[10]. CTP and CMP-Neu*p*5Ac were synthesized from CMP according to a published procedure[6]. CMP-[9-^{3}H]Neu*p*5Ac (specific activity, 1.14 TBq/mmol) was from New England Nuclear. β-D-Gal*p*-(1→3)-D-Gal*p*NAc (**5**) was synthesized according to the procedure of Lubineau and Bienaymé[16]. β-D-Gal*p*-(1→3)-D-Glc*p*NAc (**1**), β-D-Gal*p*-(1→3)-β-D-Glc*p*NAcOMBn (**3**) and β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc (**8**) were prepared as described earlier[17,18]. β-D-Gal*p*-(1→3)-[α-Neu*p*5Ac-(2→6)]-D-Gal*p*NAc (**7**) was synthesized according to Lubineau *et al.*[19]. Sialyl aldolase was obtained from Toyobo and pyruvate decarboxylase from Sigma.

Partial purification of the β-D-Galp-(1→3)-D-GalpNAc:CMP-Neup5Ac-(2→3')-α-sialyltransferase from porcine liver. — Glassware was siliconized[20]. Fractions from the column were collected in plastic tubes. All operations were performed at 4°. Proteins were estimated with the bicinchoninic acid reagent[21]. The activity of the sialyltransferase was determined by a radiochemical assay: assay mixtures (25 μL) contained β-D-Gal*p*-(1→3)-D-Gal*p*NAc (**5**; 0.125 μmol), bovine serum albumin (25 μg), enzyme extract, and CMP-[^{3}H]Neu*p*5Ac (40 000 d.p.m., 7 nmol) in 50mM sodium cacodylate buffer (pH 7.5) containing 0.4% of Triton X-100. After incubation at 37° (5–30 min), the mixture was passed through a Pasteur pipette column of Dowex 1-X8 (PO_4^{2-}) anion-exchange resin (200–400 mesh). The radiolabeled reaction product was eluted with 5mM sodium phosphate buffer (pH 7.0, 2mL) and quantitatively determined as d.p.m. in 10 mL of liquid scintillation cocktail with an Intertechnique scintillation counter. One unit of enzyme was defined as the amount of enzyme that catalyzes the transfer of 1 mmol of *N*-acetylneuraminic acid/min under the aforementioned incubation conditions. When column fractions containing CTP were assayed, 20mM $MnCl_2$ was added to the standard assay in order to prevent inhibition of activity by CTP.

Fresh porcine liver (300 g) was homogenized with 25mM Na cacodylate buffer (pH 6.0) containing 20mM $MnCl_2$ (450 mL) in a Waring blendor for 4 periods of 10 s each with intervals of 10 min; the homogenate was centrifuged at 7 500 r.p.m. for 60 min, the supernatant was discarded, the pellet was resuspended in the same buffer (400 mL), and the treatment was repeated. After centrifugation, the pellet was homogenized as described above with 25mM Na cacodylate buffer (pH 6.0) containing 10mM $MnCl_2$, 75mM NaCl (buffer *A*). The concentration of the homogenate in Triton X-100 was brought to 1.4% by the addition of 20% (w/v) Triton X-100 (31.5 mL). After being stirred for 45 min, the suspension was centrifuged as described above for 60 min to give the Triton extract I (500 mL, 2.4 U). The pellet was treated again in the same way to give

Triton extract II (450 mL, 1.8 U), and then Triton extract III (350 mL, 0.5 U). Triton extract I was filtered through a Zeta-Prep SP capsule. After being washed with buffer *A*, the washing and the filtrate were pooled and adsorbed at the rate of 100 mg of protein/mL of gel on a column (4 × 4 cm) of CDP-hexanolamine–agarose (7 μmol·mL^{-1} of gel) previously equilibrated in 10mM Na cacodylate buffer (pH 6.0) containing 0.1M NaCl, 1% Triton X-100, and 25% glycerol (buffer *B*). The sialyltransferase was eluted with a stepwise gradient of NaCl (0.2, 0.4 and 0.6M) in buffer *B*. All eluates containing enzyme activity were pooled (500 mL, 1.7 U), dialyzed against buffer *B* containing 50mM NaCl (2 × 3 l), and applied to a column (2 × 14 cm) of CDP-hexanolamine–agarose (7 μmol·mL^{-1} of gel), equilibrated in buffer *B* containing 50mM NaCl. After washing with this buffer, the sialyltransferase was eluted with a linear CTP gradient (0–2mM) formed from 75 mL of 10mM Na cacodylate buffer (pH 6.0) containing 50mM NaCl and 0.5% Triton X-100 as starting buffer, and 75 mL of this buffer with 2mM CTP added as limit buffer. Fractions containing (2→3)-α-sialyltransferase were pooled (70 mL, 0.66 U, specific activity ~ 0.04 U·mg^{-1} of protein). Triton extracts II and III were treated in the same way, affording together 0.64 U of enzyme. Bovine serum albumin was added to the whole enzymic preparation (1.3 U, 220 mL) to a concentration of 0.15 mg · mL^{-1} to stabilize the sialyltransferase. The solution was lyophilized and the residue stored at 4° until used.

Sialyltransferase kinetics and acceptor specificity. — Kinetic studies were carried out in analogous manner to the enzymic assay but with concentrations of **5** varying from 0.037 to 0.200mM. The activity of the β-D-Gal*p*-(1→3)-D-Gal*p*NAc-(2→3′)-α-sialyltransferase towards various oligosaccharides was determined by use of the standard assay procedure, but at a concentration in acceptor 20-fold higher (100mM) than the one described in the enzymic assay for the normal substrate **5**.

O-*(5-Acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonic-acid)-(2→3)-O-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose* (**6**). — Disaccharide **5** (12 mg, 0.031 mmol), CMP-Neu*p*5Ac bis(triethylammonium) salt (20 mg, 0.021 mmol), and bovine serum albumin (7 mg) were dissolved in 50mM Na cacodylate buffer (pH 7.5, 7 mL) containing 10mM $MnCl_2$, 50mM NaCl, 0.5mM CTP, 1% Triton X-100, and sialyltransferase (0.04 U). The mixture was incubated at 37° and monitored by t.l.c. on silica gel (5:5:1:3 ethyl acetate–pyridine–acetic acid–water). After 6 h, more CMP-Neu*p*5Ac (14 mg, 0.015 mmol) was added and the incubation was allowed to continue for 15 h. The mixture was then diluted with water and applied to a column (1 × 12 cm) of Dowex 1-X2 (PO_4^{2-}) anion-exchange resin (200–400 mesh). After washing with water, the product was eluted with 5mM Na phosphate buffer, pH 7.0. Fractions containing the product were freeze-dried, redissolved in water (0.5 mL), and applied to a column (1 × 34 cm) of Bio-Gel P-2 (200–400 mesh) to afford **6** (8 mg, 35%), $[\alpha]_D^{20}$ + 34° (*c* 0.6, water; at equilibrium, α:β ratio 3:2); lit.[13] $[\alpha]_D^{20}$ + 19° (*c* 0.27, water, α:β ratio 1:2); ^{1}H-n.m.r. (D_2O) : δ 1.75 (t, 1 H, $J_{3''a,4''} = J_{3''a,3''e}$ 12.5 Hz, H-3″*a*), 1.97 (s, 6 H, 2 NAc), 2.72 (dd, 1 H, $J_{3''e,4''}$ 4.5 Hz, H-3″*e*), 4.47 (d, 0.4 H, $J_{1',2'}$ 8 Hz, H-1′β), 4.54 (d, 0.6 H, $J_{1',2'}$ 8 Hz, H-1′α), 4.65 (d, 0.4 H, $J_{1,2}$ 8 Hz, H-1β), and 5.20 (d, 0.6 H, $J_{1,2}$ 3.5 Hz, H-1α).

O-*(5-Acetamido-3,5-dideoxy-α-*D-glycero-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-*O-*β*-D-*galactopyranosyl-(1→3)-2-acetamido-2-deoxy-*D-*glucopyranose* (**2**). — Disaccharide **1** (383 mg, 1 mmol) and CMP-Neu*p*5Ac bis(triethylammonium) salt (200 mg, 0.210 mmol) were dissolved in 60mM Na cacodylate buffer (pH 7.5, 50 mL) containing 200mM NaCl, 2mM CTP, 2% Triton X-100, and sialyltransferase (0.7 U); $MnCl_2$ (150 mg, 0.75 mmol) was added to the mixture which was incubated at 37°. The reaction was monitored by t.l.c. on silica gel (5:5:1:3 ethyl acetate–pyridine–acetic acid–water). CMP-Neu*p*5Ac was added at the rate of 200 mg/day during the incubation time (7 days). The mixture was then adsorbed on silica gel (20 g) and chromatographed on a silica gel column (4 × 15 cm, flash chromatography) which was first washed with 6:6:1 2-propanol–ethyl acetate–water until all Triton X-100 had been eluted. The elution was started with 4:1 propanol–water to afford, in fractions 18–30, unreacted starting material **1**, in fractions 31–55 a mixture of **1** and 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, and in fractions 60–90 a mixture (600 mg) of the sialylated disaccharide **2**, *N*-acetylneuraminic acid, and salts.

This mixture was incubated with sialyl aldolase (17 U)and pyruvate decarboxylase (10 U), both immobilized on Eupergit C, in 50mM Na cacodylate buffer (pH 6.5, 10 mL) at 37° until complete disappearance of *N*-acetylneuraminic acid (t.l.c. in 4:1 propanol–water). Eupergit C was filtered off and washed with water, and the solution was concentrated under reduced pressure and divided into four portions, which were successively applied to a column (4 × 90 cm) of Sephadex G-15, equilibrated and eluted with water, to afford pure **2** (140 mg, 20%), $[\alpha]_D^{20}$ +4° (*c* 0.5, water); ^{1}H-n.m.r. (D_2O): δ 1.77 (t, 1 H, $J_{3''a,4''e}$ 12.5 Hz, H-3″*a*), 2.03 (s, 6 H,2 NAc), 2.72 (dd, 1 H, $J_{3''e,4''}$ 4.5 Hz, H-3″*e*), 4.45 (d, 0.4 H, $J_{1',2'}$ 8 Hz, H-1′*β*), 4.49 (d, 0.6 H, $J_{1',2'}$ 8 Hz, H-1′α), 4.70 (d, 0.4 H, $J_{1,2}$ 8 Hz, H-1*β*), and 5.14 (d, 0.6 H, $J_{1,2}$ 3.5 Hz, H-1α); ^{13}C-n.m.r. (D_2O): δ 22.83 ($NHCOCH_3$), 40.54 (C-3″), 52.42 (C-5″), 53.62 (C-2α), 56.25 (C-2*β*), 61.34 (C-6α), 61.48 (C-6*β*), 61.77 (C-6′), 63.23 (C-9″), 68.02 (C-4′), 68.80 (C-7″), 69.16 (C-4″), 69.53 (C-2α,*β*), 69.93 (C-2′), 72.03 (C-5α), 72.61 (C-8″), 73.56 (C-6″), 75.82 (C-5′), 76.23 (C-5*β*), 81.05 (C-3α), 83.49 (C-3*β*), 91.80 (C-1α), 95.47 (C-1*β*), 100.41 (C-2″), 104.17 (C-1′), 174.65 (C-1″), 175.29 (CO), and 175.73 (CO).

4-Methoxybenzyl O-*(5-acetamido-3,5-dideoxy-α-*D-glycero-D-galacto-*2-nonulopyranosylonic acid)-(2→3)-*O-*β*-D-*galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-*D-*glucopyranoside* (**4**) — Disaccharide glycoside **3** (96 mg, 0.2 mmol) and CMP-Neu*p*5Ac bis(triethylammonium) salt (20 mg, 0.021 mmol) were dissolved in 50 mM Na cacodylate buffer (pH 7.5, 12 mL) containing 100mM NaCl, 0.5% Triton X-100, and sialyltransferase (0.3 U); $MnCl_2$ (35 mg, 0.176 mmol) was added to the mixture which was incubated at 37°. The reaction was monitored by t.l.c. on silica gel (7:3 propanol–water). CMP-Neu*p*5Ac was added at the rate of 20 mg/day and the incubation was allowed to continue for 5 days. At the end of the incubation, the mixture was lyophilized and chromatographed on a silica gel column (2 × 10 cm, flash chromatography) which was first eluted with 19:1 propanol–water and then with 7:3 propanol–water to afford a mixture (180 mg) of the sialylated glycoside **4**, *N*-acetylneuraminic acid, and salts. This mixture was evaporated to dryness, redissolved in a small volume of water, and divided

into two portions which were successively applied to a column (4 × 90 cm) of Sephadex G-15, equilibrated and eluted with water, to afford pure **4** (41 mg, 51%), $[\alpha]_D^{20}$ −22° (*c* 0.4, water); ^{1}H-n.m.r. (D_2O) : δ 1.74 (t, 1 H, $J_{3''a,4''} = J_{3''a,3''e}$ 12.5 Hz, H-3″a), 1.87 (s, 3 H, NAc), 2.00 (s, 3 H, NAc), 2.72 (dd, 1 H, $J_{3''e,4''}$ 4.5 Hz, H-3″*e*), 3.83 (s, 3 H, OMe), 4.41 (d, 1 H, $J_{1',2'}$ 7.75 Hz, H-1′), 4.54 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.59 (d, 1 H, J_{gem} 12 Hz, CH_2Ph), 4.78 (d, 1 H, CH_2Ph), 6.98 (m, 2 H, Ph), and 7.29 (m, 2 H, Ph).

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