Monoclonal Antibody A2B5, Which Detects Cell Surface Antigens, Binds to Ganglioside GT3 (II3 (NeuAc)3LacCer) and to Its 9-O-Acetylated Derivative*

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The monoclonal antibody A2B5 recognizes antigens at the surface of neuronal and glial cells but also at the surface of thymus epithelia and pancreatic islet cells. Although these antigens have been characterized as polysialogangliosides, A2B5 also reacts with other unidentified gangliosides. In order to characterize further the epitope of A2B5, two new ganglioside isoforms of chicken brain are identified in this study. One is the ganglioside NeuAcα2-8NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1ceramide (GT3) and the other is a 9-O-acetylated derivative of GT3. This derivative was purified from 10-day embryonic chicken brain. Acetyl groups substituted on sialic acid were removed either by alkali treatment or by incubation with influenza virus C, which contains receptor-degrading enzyme (a neuraminidase 9-O-acetyl esterase). The product of alkali treatment or viral action was detected by the antibody 18B8 which is specific for GT3. The deacetylated product still reacts with A2B5. These data and the results of mild oxidation of the antigen with sodium periodate suggest that the epitope recognized by antibody A2B5 contains the trisialyl structure found in GT3 but does not include the polyalcohol chain of the terminal sialic acid which can be oxidized by periodate or acetylated without modifying the affinity for the antibody. The epitope recognized by A2B5 is different from the epitope recognized by the antibody 18B8 in that 18B8 requires the three sialic acids with an intact and unsubstituted polyalcohol chain. Antibody 18B8 does not bind to 9-O-acetylated GT3 or GT3 oxidized by sodium periodate.

Monoclonal antibody A2B5 was originally prepared against chicken embryo retina cells (1). The antigens recognized by the antibody are localized on the plasma membrane of retina neurons, and antigen expression depends on the stage of neuronal differentiation. The antigens are detected on bipotential rat glial progenitor cells in optic nerve which differentiates into either oligodendrocytes which lose the antigen or type 2 astrocytes which maintain the antigen (2). The neuronal differentiation of an embryonal carcinoma cell line (3) or of cloned human teratoma cells (4) induced by retinoic acid modulates the expression of cell surface A2B5 antigens. The antibody labels Alzheimer's neurofibrillary tangles and fetal human neurons (5) and astrocytes (6). However, the A2B5 antigens are not restricted to nervous tissues as they also occur in human pancreatic islet tumor cells and other tumors derived from cells of the amine precursor uptake and decarboxylation series (7) and in rat insulinoma cells (8, 9). They are also expressed by thymic epithelial cells, which contain thymopoietin and thymosin α1 (10, 11).

The antibody A2B5 detects polysialogangliosides on cell surfaces, and its immunoreactivity is lost by treatment of cells or tissues with neuraminidase (1). However, its specificity is still controversial. Even though one of its ganglioside antigens has been characterized as Gd1α (12) and a recent report provided evidence of specific reactivity of A2B5 with GT3 and Gd1α (13), other reports showed reactivity with other gangliosides such as Gb1α and disialogangliosides (14, 15) or with many gangliosides in neuronal tissue which do not correspond to the major gangliosides of this tissue (15). Since monoclonal antibody A2B5 should be used to obtain new fundamental insight into the tissue distribution and the metabolism of the gangliosides it recognizes, the specificity of this antibody has been reexamined. Two major ganglioside antigens were isolated from embryonic chicken brain and identified in this study; one is the trisialosganglioside GT3 and the other is its 9-O-acetylated derivative. Thus, antibody A2B5 differs from antibody 18B8 which detects synapse-associated antigens (16) and which has been previously described as specific for GT3 (17). The chemical or enzymatic modifications of the gangliosides allowed us to improve the definition of the structure of the epitope recognized by A2B5 and to compare its epitope with that recognized by 18B8.

**EXPERIMENTAL PROCEDURES**

**Materials**

Monoclonal antibody A2B5, an IgM, was produced by hybridoma obtained by fusion of spleen cells from a BALB/c mouse immunized with..
with 14-day chick embryonic retina cells with P3 × 63 Ag 8 mouse mvea cells (1). Hybrioma cells were obtained from American Type Culture Collection (Rockville, MD). Monoclonal antibody 18BS (16), an IgM (κ) directed against ganglioside GT³ (17) was kindly supplied by Dr. M. Nirenberg, National Institute of Mental Health. Affinity-purified goat antibodies to mouse IgM purchased from Biosys (Compiegne, France) were labeled with ¹²⁵I by the IODO-GEN method (17). Gangliosides GT² and GT³, LVB, and GT₁ were purified from human brain. Gangliosides GT² and GT³ were purified from 10-day chick embryonic retina. Purified GT² lactone and GT₂ lactone were the gift of Dr. O. Glinou, Medica Milano, Italy. Influenza C virus (strain Johannesburg 1/66) was provided by the Central Public Health Laboratory, Virus Reference Laboratory, London.

Thin Layer Chromatography

Glycolipids were chromatographed on glass or aluminum-backed high performance thin layer chromatography plates (HPTLC, Silica Gel 60, E. Merck, Darmstadt, West Germany) in chloroform:methanol:0.26% KCІ in H₂O (6:4:1 by volume, solvent A). Gangliosides chromatographed on glass HPTLC plates were visualized with resorcinol reagent.

Immunostaining of Glycolipid Antigens

Ganglioside antigens were detected on thin layer chromatograms by autoradiography as previously described (18). Briefly, glycolipids were chromatographed as described above. The dried chromatogram was soaked for 2 min in a 0.1% solution of polysinebutylmethacrylate (Polysciences, Inc.) in hexane. After drying in air, the chromatogram was sprayed with buffer A (0.05 M Tris, 0.15 M NaCl, pH 7.8, with 1% bovine serum albumin and 0.1% sodium azide) and soaked in the same buffer until all of the silica gel was wet. The plate was then overlaid with monoclonal antibody solution diluted 1:20 with buffer A (60 μl/cm²) and incubated for 1 h at room temperature or overnight at 4 °C. The chromatogram was washed four times in buffer A and overlaid with buffer A containing 2 × 10⁶ cpm/ml ¹²⁵I-labeled goat anti-mouse IgM antibodies. After 1 h at room temperature, the chromatograms were washed as before in cold phosphate-buffered saline (PBS), dried, and exposed to XAR-5 x-ray film (Eastman Kodak).

Solid Phase Radioimmunoassay

The binding of antibody to glycolipid was measured by solid phase radioimmunoassay as previously described (17, 19). glycolipid in 20 μl of ethanol was added to wells of a round bottom polystyrene microtiter plate (Nunc). The solutions were dried by evaporation. The wells were then filled with buffer A. After 30 min, the wells were emptied and washed with buffer A and then to each was added 30 μl of monoclonal antibody solution diluted 1:20 with buffer A. The wells were covered with Parafilm, incubated for 1 h at room temperature, washed three times with buffer A, and then to each was added about 60,000 cpm of ¹²⁵I-labeled goat anti-mouse IgM antibodies (30 μl of buffer A). After 1 h, the wells were washed six times with cold phosphate-buffered saline, cut from the plate, and assayed for ¹²⁵I in a γ scintillation spectrometer.

Isolation of the Ganglioside Antigens

Lipids were extracted from 10–12-day chick embryonic brains (115 g wet weight) with chloroform: methanol:H₂O (4:8:3 by volume) as previously described (17, 20). Total lipid extract was submitted to a Folch partition (21). The combined upper phases were desalted by the same solvent. They were separated into neutral and acidic fractions previously described (17, 20). Total lipid extract was submitted to a 2-step procedure for its ability to bind antibody A2BS as described above.

Alkaline Treatment of Ganglioside Antigen

Aqueous Alkaline Treatment of Ganglioside Antigens Before Their Separation by Chromatography—Gangliosides from 300–500 mg (wet weight) of embryonic chicken brain were incubated in 0.25 ml of 17 mM ammonium hydroxide overnight (22). The ammonium hydroxide was removed rapidly by evaporation in a Speed-vac concentrator. In a control experiment gangliosides were incubated with distilled water in place of ammonium hydroxide and then chromatographed on HPTLC plates.

Alkaline Treatment of Chromatograms by Ammonia Vapors—After chromatography of gangliosides, the chromatograms were dried and placed for 5 h in a tank saturated with ammonia (22). The plates were then removed and put in an air stream overnight to eliminate ammonia. The chromatograms were then immunostained with the antibodies.

Antigens derived from alkaline-labile antigen were detected by two-dimensional thin layer chromatography with an intermediate alkaline treatment with ammonia vapors before the runs, as described previously (23). Briefly, after chromatography of gangliosides in the first dimension, the plates were exposed to ammonia as above. The second chromatographic run was performed in the same solvent, and the chromatograms were immunostained with the antibodies.

Treatment of Ganglioside Antigens with Influenza C Virus (INF-C)

Influenza C virus (strain Johannesburg 1/66) was grown in the amniotic cavity of 8-day-old embryonated chicken eggs. After 48 h of incubation at 33 °C, the amniotic fluids were harvested and kept at 4 °C. Before each experiment with virus, hemagglutination titer was determined at 4 °C in microtitrator plates using 0.5% suspension of Hen red blood cells in PBS as previously described (24). Virus stocks whose hemagglutination titers was 1:250 were used for the following experiments.

After chromatography of glycolipids, the chromatograms were treated with INF-C virus whose receptor-destroying enzyme, which is a 9-O-acetyl esterase (25–28), is inactivated by disopropyl fluorophosphosphate (DFP) (29). Inactivation of esterase was carried out as described previously (29). Briefly, 100 μl of INF-C virus preparation were diluted either in 900 μl of 1% isopropyl alcohol in PBS or in 900 μl of 1 mM DFP in 1% isopropyl alcohol in PBS and incubated for 30 min at 4 °C. The chromatograms were treated with polysinebutylmethacrylate and soaked in buffer A. They were washed twice with PBS and overlaid with treated or untreated virus. One plate was overlaid with amniotic fluid diluted 1:20 with PBS as control of acetyl esterase activity in the fluid. After a 2-h incubation at 37 °C, the plates were washed three times with buffer A and then immunostained with the antibodies.

Mild Oxidation of Ganglioside Antigens with Sodium Periodate

After chromatography of glycolipids, the chromatograms were treated with INF-C virus whose receptor-destroying enzyme, which is a 9-O-acetyl esterase (25–28), is inactivated by disopropyl fluorophosphosphate (DFP) (29). Inactivation of esterase was carried out as described previously (29). Briefly, 100 μl of INF-C virus preparation were diluted either in 900 μl of 1% isopropyl alcohol in PBS or in 900 μl of 1 mM DFP in 1% isopropyl alcohol in PBS and incubated for 30 min at 4 °C. The chromatograms were treated with polysinebutylmethacrylate and soaked in buffer A. They were washed twice with PBS and overlaid with treated or untreated virus. One plate was overlaid with amniotic fluid diluted 1:20 with PBS as control of acetyl esterase activity in the fluid. After a 2-h incubation at 37 °C, the plates were washed three times with buffer A and then immunostained with the antibodies.

RESULTS

Antibody A2BS Binds to Ganglioside GT³ and an Unknown Major Ganglioside Isolated from Chicken Brain—Since it is known that gangliosidic antigens recognized by antibody A2BS are polysialylated (1, 12, 13), the purified trisialoganglioside GT³ isolated from chicken retina was tested by solid phase radioimmunoassay for its ability to bind antibody A2BS. Fig. 1, panel A, shows that antibody A2BS, like anti-
9-O-Acetylated Ganglioside Antigens

FIG. 1. Binding of antibody A2B5 to ganglioside GT3. Binding to ganglioside was measured by solid phase radiinunooassay as described under “Experimental Procedures.” Panel A, the binding of antibody A2B5 (O—O) and 18B8 (C—C) to 3.5 ng of purified GT3 is compared. Panel B, the binding of antibody A2B5 to GT3 (O) and to GM3, Gb3, GD1a, GD1b, GD2, GD3, Gb1, lactone, Gb2, lactone, GT3b, and Gb1b (C) is shown.

Antibody 18B8 specific for GT3 (17), binds to purified GT3. Other purified gangliosides tested for their ability to bind antibody A2B5 included GM1, GM2, GD1a, GD1b, GD3, GT3b, GM, lactone, and GD1b, lactone. None of these gangliosides binds to antibody A2B5 (Fig. 1, panel B). When the antibody A2B5 binding was tested by immunostaining of thin layer chromatograms, no reactivity of the antibody with these gangliosides was observed (data not shown).

Antibody A2B5 binds to other gangliosides isolated from chicken brain, and their accumulation in the brain changes during embryonic development (Fig. 2). One major antigen (antigen I) with a chromatographic mobility similar to that of GT3 is recognized by A2B5 but not by 18B8. In an attempt to identify antigen I, 8- and 10-day embryonic chicken brains which contain a relatively high concentration of the antigen were used for its isolation. In order to protect possible ester linkages in the structure of ganglioside, the purification procedure omitted the alkaline hydrolysis step normally used to eliminate phospholipids. The ganglioside antigen was purified on an anion exchange column and eluted by a 0.12–0.16 M ammonium acetate gradient. The ganglioside was then purified by high performance liquid chromatography, and the column fractions were analyzed by immunostaining thin layer chromatograms with the antibody A2B5. Fig. 3, panel A, shows the elution of antigen I in fractions 70–110 of the silicic acid column. The doublets, which probably differ in their ceramides, could be separated. The antigen eluted in fractions 102–118 was identified as GT3 by using antibody 18B8 (data not shown). When the fractions were analyzed by chemical staining with resorcinol reagent (Fig. 3, panel B), only one ganglioside, which was coincident with the antigen I, was detected in fractions 84. However, immunostaining of chromatograms of the pooled

FIG. 2. Occurrence of ganglioside antigens during development of chicken brain. Total gangliosides from 15 mg wet weight of tissues were chromatographed on HPTLC plates, and the ganglioside antigens were visualized by immunostaining with antibodies A2B5 and 18B8 as described under “Experimental Procedures.” Lanes 1–5, 7–10, 12–17, and 20-day chick embryonic brain, respectively. The migration of standard glycolipids is indicated at the margins.
fractions with antibodies A2B5 and 18B8 detected a weak contamination of antigen I with GrS.

The fractionation of gangliosides is due to the presence of ester linkages which are present in O-acetylated gangliosides or in lactones of gangliosides isolated from different tissues (22, 23, 31-40). The O-acetylated gangliosides are usually more resistant to alkali treatment than the lactones of gangliosides. Purified antigen I was treated with aqueous ammonia as described under "Experimental Procedures." Treated and nontreated with the antibody A2B5. As seen in Fig. 4, lane 2, antibody A2B5 did not detect antigen I treated with concentrated ammonia. This result suggests that antigen I is an alkali-labile ganglioside and probably contains O-acetylated sialic acid residues.

Identification of Alkali-labile Antigen I—In order to identify the alkali-labile antigen, the product of its alkali treatment was analyzed. Purified antigen I was chromatographed on HPTLC plates, and the chromatograms were exposed to ammonia vapors (22). The reactivity of antibodies A2B5 and 18B8 against the treated ganglioside was tested by immunostaining of chromatograms (Fig. 5, panel A). Antibody A2B5 still binds to the product of hydrolysis of the alkali-labile antigen. Antibody 18B8, which did not bind to this antigen, now binds to the hydrolyzed molecule identified as G\(_{\text{T3}}\). To confirm this data, which suggest that antigen I is the alkali-labile G\(_{\text{T3}}\), antigen I was analyzed by two-dimensional thin layer chromatography with treatment with ammonia between runs (23). This method allows identification of one ganglioside and its alkali-labile derivative by comparing their chromatographic mobilities in each dimension. As is shown in Fig. 5, panel B, two additional gangliosides (spots II and X) generated by alkali treatment are recognized by both antibodies.

The antigen II generated by treatment with ammonia after the first dimension has the same chromatographic mobility as G\(_{\text{T3}}\) in the second dimension. These results suggest that antigen II is probably G\(_{\text{T3}}\). Moreover, the new antigen II has the same chromatographic mobility in the first dimension as antigen I, suggesting that antigen II derives from alkali-labile antigen I. These results provide strong evidence that the alkali-labile antigen I can be identified as alkali-labile G\(_{\text{T3}}\).

The antigen X generated by exposure to ammonia is still not identified.

Antigen I Is an Alkali-labile Ganglioside—The alkali lability of gangliosides is due to the presence of ester linkages which are present in O-acetylated gangliosides or in lactones of gangliosides isolated from different tissues (22, 23, 31-40). The O-acetylated gangliosides are usually more resistant to alkali treatment than the lactones of gangliosides. Purified antigen I was treated with aqueous ammonia as described under "Experimental Procedures." Treated and nontreated gangliosides were then analyzed in parallel by thin layer chromatography, and the chromatogram was immunostained with antibody A2B5. As seen in Fig. 4, lane 2, antibody A2B5 did not detect antigen I treated with concentrated ammonia. This result suggests that antigen I is an alkali-labile ganglioside and probably contains O-acetylated sialic acid residues.

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Antigen I Is a Substrate for the Receptor-destroying Enzyme of INF-C—To test the possibility that alkali-labile G\(_{\text{T3}}\) is O-acetylated, the purified ganglioside was treated with influenza C virus whose surface glycoprotein (hemagglutinin) recognizes N-acetyl-9-O-acetyleneuraminic acid (25) and contains the receptor-destroying enzyme identified as a neuraminidate O-acetyl esterase which cleaves preferentially 9-O-acetyl groups from N-acetyl-9-O-acetyleneuraminic acid (26-28). This esterase is inactivated by DFP, a serine esterase inhibitor (29).

Gangliosides were chromatographed on HPTLC plates, and the chromatograms were incubated with INF-C virus or DFP-inactivated INF-C virus as described under "Experimental Procedures." Immunostaining of chromatograms with antibody A2B5 did not detect any modification of the antigen catalyzed by the viral enzyme (Fig. 6, A-C). A new antigen identified as G\(_{\text{T3}}\) by antibody 18B8 is produced by treatment of ganglioside with INF-C virus (Fig. 6, panel B). The new G\(_{\text{T3}}\) has the same chromatographic mobility as antigen I. These results suggest that antigen I is the substrate of influenza C-neuraminidate-9-O-acetyl esterase, and it is identified as 9-O-acetylated G\(_{\text{T3}}\). G\(_{\text{T3}}\) was not formed if the virus was preincubated with DFP (Fig. 6, panel C). No G\(_{\text{T3}}\) was produced in the control (Fig. 6, panel A) showing the absence of esterase activity in the amniotic fluid.

Effect of Mild Oxidation of the Antigens by Sodium Periodate—The role of the polyalcohol chain of G\(_{\text{T3}}\) in the epitope recognized by antibody A2B5 was studied. The ganglioside
antigens were submitted to mild oxidation by sodium periodate, which cleaves, under defined conditions, the polyalcohol exopyranosyl chain of the sialyl residues (30). If the chain is substituted (O-acetylation or glycosidic linkages) oxidation does not occur. In the case of Gr3 only one unsubstituted carbon (9-acetylation or glycosidic linkages) oxidation of the chain, the chromatograms were submitted to ammonia vapors prior to being treated by sodium periodate. (Fig. 7, panel B). Finally, the ganglioside is a substrate of receptor-destroying enzyme of influenza C virus which is a 9-O-acetyl neuraminidase esterase (25–28). The transformation of the antigen by influenza C virus is inhibited by preincubation of the virus with the esterase inhibitor disopropyl fluorophosphate. The product of the enzymatic reaction binds antibody 18B8 and is identified as GT3.

Monoclonal antibody A2B5 detects polysialogangliosides on the surface of many different tissues from various species (1–9). However, the exact epitope recognized by this antibody is not well defined. Previous reports showed the reactivity of the antibody with G_{T23} (13) and G_{Q4c} (12), which is a derivative of GT3 (41) or with many unidentified gangliosides in neuronal tissue (15). The specificity of the antibody A2B5 was reexamined, and this work characterizes the major antigens found in chicken brain. One of these antigens was characterized as G_{T23}. The specificity of binding of antibody A2B5 to G_{T23} was revealed by immunostaining of thin layer chromatograms and solid phase radioimmunoassay using purified G_{T23}. This result agrees with the earlier observations (13). Monoclonal antibody 18B8 which is directed against G_{T23} (17) was used as a control. The binding of both antibodies to the lipid was compared and found to be similar. A second major antigen detected by A2B5 migrates faster than G_{T23} in chromatography. It was isolated from 10-day embryonic chicken brain and characterized as 9-O-acetylated G_{T23} by the following criteria. (i) During purification, which did not include the alkali treatment normally used to hydrolyze the glycerophospholipids, the antigen was eluted from an anion exchange column in the trisialoganglioside fraction by 0.12–0.16 M ammonium acetate. (ii) This antigen is alkali-labile, and the product of the alkali treatment is identified as GT3 by its chromatographic mobility and the binding of antibody 18D8. (iii) Its chromatographic mobility on thin layer chromatography is higher than that of G_{T23} and could be explained by an increased hydrophobicity because of the presence of an O-acetyl group substitution at the hydroxyl position of sialic acid residues. (iv) The ganglioside is a substrate of receptor-destroying enzyme of influenza C virus which is a 9-O-acetyl neuraminidase esterase (25–28). The transformation of the antigen by influenza C virus is inhibited by preincubation of the virus with the esterase inhibitor disopropyl fluorophosphate. The product of the enzymatic reaction binds antibody 18B8 and is identified as GT3.

Monoclonal antibody A2B5 detects polysialogangliosides or to G_{T23} and G_{Q4c}. These results agree with previous reports (12, 13). These data suggest that the antibody A2B5 binding requires the trisialyl structure with α2-8 linkages, found in G_{T23} and its derivative G_{Q4c}. The antibody 18B8, like the antibody A2B5, binds to the trisialyl structure found in G_{T23}. However, these antibodies are different since antibody A2B5 binds to the alkali-labile 9-O-acetylated G_{T23} whereas 18B8 does not. After mild oxidation of G_{T23} with sodium periodate which cleaves the polyalcohol exopyranosyl chain of external sialic acid, A2B5 still binds to the ganglioside, but the binding of antibody 18B8 is abolished. These results suggest that the epitope recognized by antibody A2B5 does not completely abolished after their oxidation with 1 mM periodate (Fig. 7, panels E and F).

These results show the role of the polyalcohol chain, present in antigen G_{T23}, in the structure of the epitope recognized by the antibodies. The binding of antibody A2B5 to oxidized G_{T23} suggests that the binding of the antibody to G_{T23} does not require carbon 9 of the polyclonal chain. This carbon can be also esterified as in 9-O-acetylated G_{T23} the ganglioside is still recognized by the antibody. On the other hand, the binding of antibody 18B8 depends on the presence of carbon 9 which must be unsubstituted as the 18B8 antibody does not bind to oxidized G_{T23} or to 9-O-acetylated G_{T23}.
not include the carbons of the polyalcohol chain in GT3. Carbon 9 of the chain can be removed by oxidation or O-acetylated without modification of the binding of antibody A2B5. In contrast, the terminal carbon of the polyalcohol chain in GT3 must be present and unsubstituted by O-acetyl for the binding of antibody 18B8.

In addition to GT3 and 9-O-acetylated GT3, A2B5 binds to other gangliosides from chicken brain. These antigens were not identified but probably contain the sugar sequence in GT3 or (and) 9-O-acetylated GT3 recognized by the antibody. All the gangliosides recognized by A2B5, especially GT3 and acetylated GT3, decrease during chicken brain development. This suggests that their formation is regulated in the same manner.

Acetylated gangliosides occur in different tissues (31, 32, 34–39). They are developmentally regulated in rabbit cerebrum and cerebellum (22) and have been identified as tumor-specific antigens in some human melanoma cells (37, 38). However, the biological significance of O-acetyl groups is not yet understood. N-acetyl-9-O-acetyleneuraminic acid is the cellular receptor for viruses such as influenza C virus (25). O-Acetylation of N-acetyleneuraminic acid residues in gangliosides increases the possible number of these molecular species leading to a larger diversity in the pattern of cell surface gangliosides. This phenomenon may be involved in the mechanism of cell recognition and influence the turnover and the antigenicity of gangliosides (34). The characterization of gangliosides recognized specifically by the antibody A2B5 is important for the study of the tissue distribution, metabolism, and biological role of these gangliosides.

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9-O-Acetylated Ganglioside Antigens
2803
Monoclonal antibody A2B5, which detects cell surface antigens, binds to ganglioside GT3 (II3 (NeuAc)3LacCer) and to its 9-O-acetylated derivative.

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