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# A novel ganglioside with a free amino group in bovine brain

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A novel ganglioside which binds cholera-toxin B-subunit was purified from bovine brain by an h.p.l.c. system using an Aquasil column subsequent to Q-Sepharose column chromatography. T.l.c./immunostaining showed that the isolated ganglioside had about 60% of the binding reactivity of the authentic ganglioside $G_{M1}$ for cholera-toxin B-subunit. On h.p.t.l.c., this ganglioside migrated between ganglioside $G_{D1a}$ and $G_{D1b}$, and was found to give positive reactions with ninhydrin and fluorescamine reagents which specifically react with amino groups. The presence of a free amino group was further confirmed by chemical re-N-acetylation. The N-acetylated product had an identical $R_F$ value on h.p.t.l.c. and similar reactivity with cholera-toxin B-subunit as the authentic $G_{M1}$. H.p.t.l.c., t.l.c./immunostaining, negative-ion fast-atom-bombardment (f.a.b.)-m.s., and $^1$H-n.m.r. spectroscopy of the novel ganglioside unequivocally demonstrated that it has the basal structure of $G_{M1}$ with de-N-acetylated neuraminic acid instead of *N*-acetylneuraminic acid. In the present study we report for the first time that a ganglioside derivative containing de-N-acetylated neuraminic acid, de-N-acetylated $G_{M1}$, exists in natural brain tissues.

## INTRODUCTION

It has been well known that particular complex carbohydrates are specifically expressed in early embryos as regional and temporal markers [2–5]. Gangliosides are one of the common complex carbohydrates and have been suggested to play essential roles in cellular phenomena such as cell–cell interaction, differentiation and signal transduction via receptors [6,7]. Gangliosides associated with these phenomena exist as minor components in plasma membrane and, furthermore, some gangliosides have various modifications in their sialic acid residues, such as de-N-acetylation, deamination or O-acetylation [8–10]. De-N-acetylated gangliosides especially have been shown to modulate the phosphorylation of epidermal-growth-factor receptor in a cultured cell line [8]. Because of their putative biological activities, it has been an experimental issue to isolate and characterize those minor gangliosides. Bovine brain is a suitable source for obtaining those gangliosides. It has been reported to contain more than 100 species of gangliosides, including extremely minor components. Indeed, using Q-Sepharose column chromatography, we isolated and characterized several bioactive gangliosides from bovine brain efficiently [11–14].

Cholera toxin consists of two classes of subunits, A and B, which play different roles: the A-subunit penetrates into the plasma membrane after the B-subunit has bound to a receptor on the cell surface; adenylate cyclase is activated [15–18]. Since it was reported that cholera toxin specifically bound to ganglioside $G_{M1}$ (nomenclature of Svennerholm [1]) on plasma membrane [19–22], $G_{M1}$ has been considered as a possible receptor for this toxin. Subsequent studies showed that this toxin bound to several gangliosides with lower binding activity [23–26].

In the present study we have found that a novel ganglioside in bovine brain which strongly bound to cholera-toxin B-subunit had a ninhydrin-positive reaction. The ganglioside has been identified as a $G_{M1}$ analogue containing de-N-acetylated neuraminic acid.

## MATERIALS AND METHODS

### Materials

H.p.t.l.c. plates (silica-gel 60) and plastic t.l.c. plates (POLYGRAM SIL G) were purchased from E. Merck Co. (Darmstadt, Germany) and Macherey-Nagel (Düren, Germany), respectively. The authentic $G_{M1}$ was purified from bovine brain. Horseradish-peroxidase (HRP)-conjugated cholera-toxin B-subunits were all purchased from List Biological Laboratories, (Campbell, CA, U.S.A.). All other chemicals were of the highest purity available.

### T.l.c.

For analytical purposes, high performance t.l.c. (h.p.t.l.c.) plates (silica-gel 60) were used. The solvent systems used in this study were: (i) chloroform/methanol/12 mM $MgCl_2$ (5:4:1, by vol.) and (ii) chloroform/methanol/12 mM $MgCl_2$/aq. $NH_3$ (50:40:7:3, by vol.). Gangliosides were revealed with resorcinol/HCl reagent. For t.l.c./immunostaining, plastic t.l.c. plates were used with the same solvent systems. Quantitative analysis of ganglioside binding to cholera-toxin B-subunit was performed using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

### Isolation of de-N-acetylated $G_{M1}$ from bovine brain

Six monosialoganglioside fractions (fraction nos. 1–6) were obtained from a bovine brain ganglioside mixture (10 g dry wt.) by Q-Sepharose column chromatography as described previously [11]. Fraction no. 3, which was obtained immediately after $G_{M1b}$ (NeuAc) was eluted, contained three cholera-toxin-B-subunit-positive gangliosides. Two of these gangliosides were found to be

Abbreviations used: the nomenclature used for gangliosides is based on the system of Svennerholm [1]; Cer, ceramide; asialo ganglioside $G_{M1}$, Gg4Cer (gangliotetraosylceramide); h.p.t.l.c., high-performance t.l.c.; DMSO, dimethyl sulphoxide; HRP, horseradish peroxidase; f.a.b., fast-atom bombardment.

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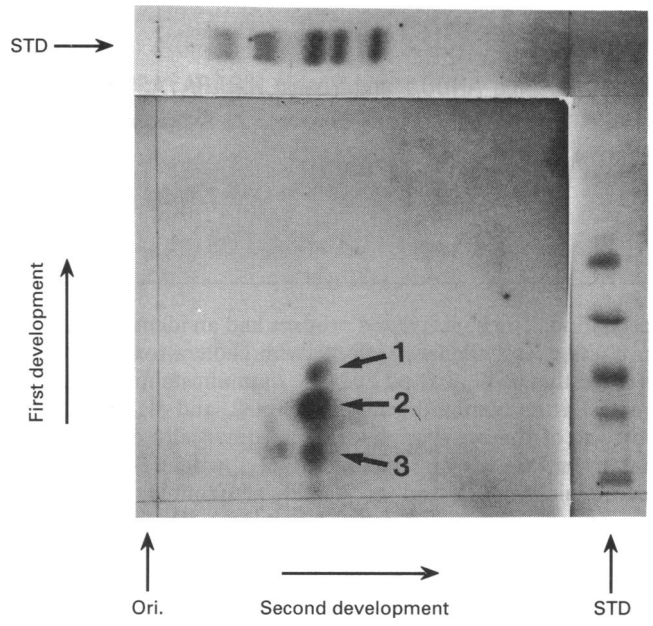


**Figure 1 Detection of gangliosides with binding activity to cholera-toxin B-subunit by t.l.c./immunostaining**

T.l.c./immunostaining was carried out as described in the Materials and methods section. The first development of the t.l.c. plate was performed using solvent system (ii), and the second development, after complete drying of the plate, was done using solvent system (i). Abbreviations: Ori., origin; STD, total gangliosides from bovine brain as standard.

$G_{M1}$ (NeuAc) and $G_{M1}$ (NeuGc). The third unknown ganglioside was purified by h.p.l.c. with a TRI ROTAR-VI h.p.l.c. System (Japan Spectroscopic Co., Tokyo, Japan) using a column of Senshu Pak AQUASIL SS-762N (20 mm × 300 mm; Senshu Scientific Company Ltd., Tokyo, Japan). Gangliosides were eluted with a linear gradient system prepared from a first solvent mixture of chloroform/methanol/water (60:25:1, by vol.) and a final solvent mixture of chloroform/methanol/water (40:20:3, by vol.).

### Negative-ion F.a.b.m.s.

F.a.b.m.s. of the purified ganglioside was performed using JMS-HX110 mass spectrometer/JMA-DA5000 data system (JEOL, Tokyo, Japan). A sample was mixed with triethanolamine or *m*-nitrobenzyl alcohol as a matrix. The ion accelerating voltage was 8.0 kV, and the primary beam for bombardment was 6.0 keV of xenon.

### $^1$H-n.m.r. spectroscopy

The $^1$H-n.m.r. spectrum was obtained with a 400 MHz n.m.r. spectrometer (model XL-400; Varian). A sample (1 mg) was dissolved in 0.5 ml of [$^2H_6$]dimethyl sulphoxide containing tetramethylsilane. The probe temperature was 40 °C.

### N-acetylation of de-N-acetylated $G_{M1}$

A 1 $\mu$g portion of purified de-N-acetylated $G_{M1}$ was dissolved in 100 $\mu$l of a mixture of acetic anhydride/methanol (1:1, v/v) and the reaction was continued for 6 h at room temperature. After the reaction mixture had been evaporated in a nitrogen stream, the product was subjected to mild alkaline hydrolysis in 100 $\mu$l of

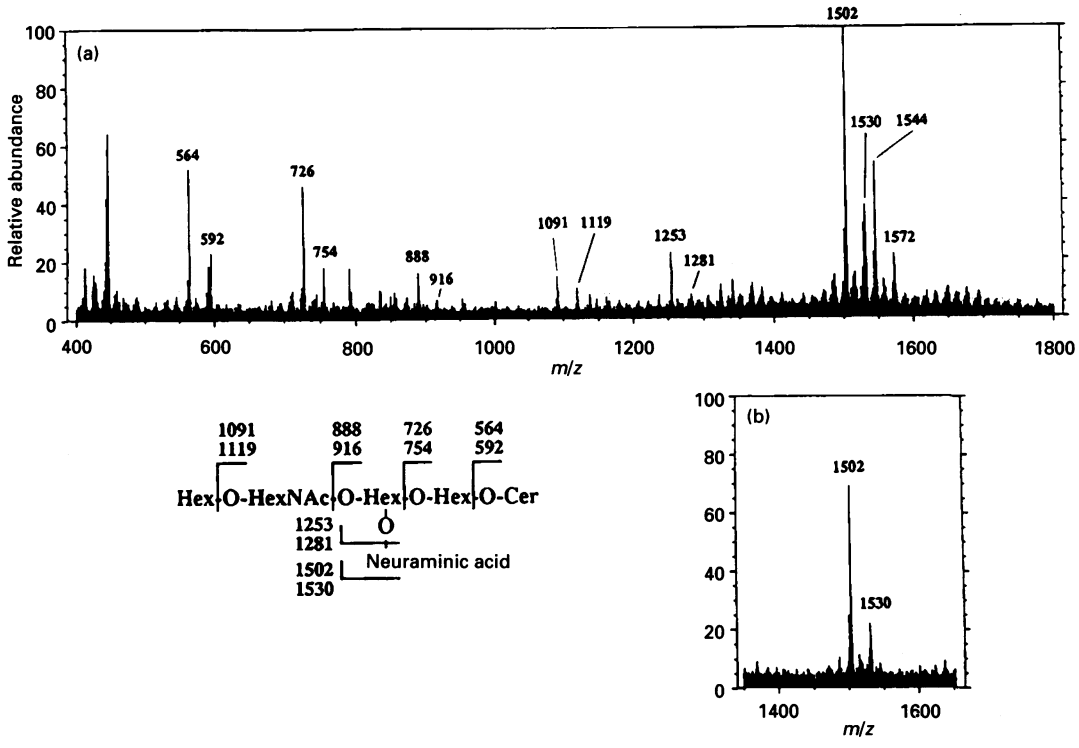


**Figure 2 Negative-ion f.a.b.m.s. of de-N-acetylated ganglioside**

Spectra (**a**) and (**b**) were obtained using triethanolamine and *m*-nitrobenzylalcohol as matrices respectively. The ions at *m/z* 1502 and 1530 correspond to $[M-H]^-$.

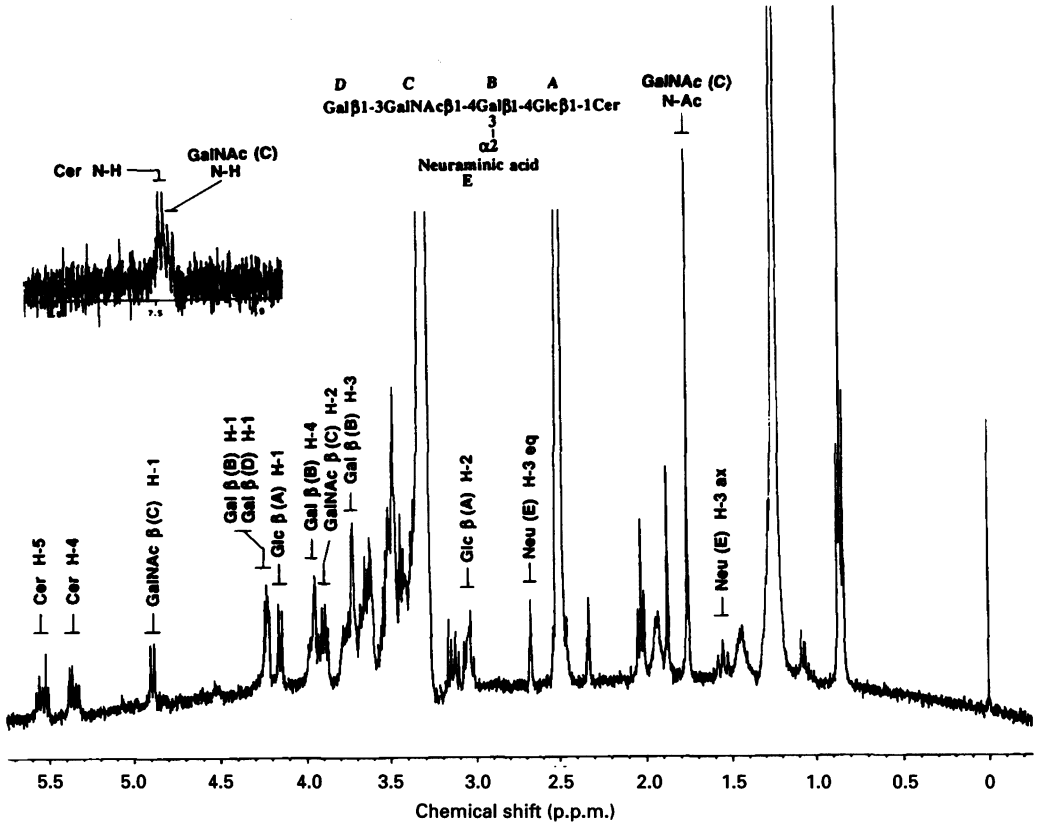


**Figure 3 $^1$H-n.m.r. spectrum of the purified ganglioside**

The spectrum was taken at 40 °C at 400 MHz.

methanol/water (1:1, v/v) containing aq. 15% $NH_3$. After an overnight incubation at room temperature, the reaction mixture was evaporated in a nitrogen stream. The product finally obtained was dissolved in a mixture of chloroform/methanol (2:1, v/v) and then examined for its binding to cholera-toxin B-subunit by t.l.c./immunostaining.

### Immunochemical methods

T.l.c./immunostaining of the purified ganglioside and its chemical re-acetylated derivative was performed using the procedure of Magnani et al. [27] as modified by Higashi et al. [28]. After developing with the solvent systems (i) or (ii), the plastic plate was dried and soaked for 1 h at room temperature in solution A (PBS containing 1% egg albumin and 1% polyvinylpyrrolidone) to block non-specific antibody binding. The plate was then incubated for 2 h at 37 °C in 1:1000-diluted cholera-toxin B-subunit conjugated with HRP (1.0 mg/ml) in solution A. After several washes with PBS, the plate was developed in a substrate solution (0.6 mg/ml 4-chloro-1-naphthol/0.01% $H_2O_2$/200 mM NaCl/Tris/HCl, pH 7.4).

## RESULTS

### Isolation of an unknown ganglioside which binds cholera-toxin B-subunit

Three cholera-toxin-B-subunit-positive gangliosides existed in a monosialoganglioside fraction (fraction no. 3) obtained from Q-Sepharose column chromatography (Figure 1). From their mobilities on t.l.c., two of them were identified as GM1 with N-acetylated (arrow 1) and N-glycolylated (arrow 2) neuraminic acid respectively. The third (arrow 3), which was positive with both ninhydrin and fluorescamine reactions (results not shown), was isolated by h.p.l.c. and identified as de-N-acetylated $G_{M1}$, as described below. We obtained about 2 mg of the ganglioside from 10 g of bovine brain ganglioside mixture.

### Negative-ion f.a.b.m.s. of the purified ganglioside

The negative-ion f.a.b.m.s. spectra of the purified ganglioside are shown in Figure 2. Quasi-molecular ions $[M-H]^-$ were clearly detected at $m/z$ 1502 and 1530 with $m$-nitrobenzylalcohol as a matrix (Figure 2b) while two additional ions at $m/z$ 1544 and 1572 appeared with triethanolamine (Figure 2a). The ions at $m/z$ 1544 and 1572, which correspond to $[M-H]^-$ ions of authentic $G_{M1}$ with N-acetylated neuraminic acid, seem to be pseudo-peaks as reported for de-N-acetylated $G_{M3}$ by Nores et al. [29]. $[M-H]^-$ ions at $m/z$ 1502 and 1530 indicated that the ganglioside is a derivative lacking one acetyl residue from authentic $G_{M1}$ with N-acetylated neuraminic acid. The fragment ions at $m/z$ 1281, 1119, 916, 754, and 592 showed the sugar sequence as follows: Hex-HexNAc-Hex-Hex-Cer. This result therefore suggested that the ganglioside had neuraminic acid instead of *N*-acetylneuraminic acid.

### 400 MHz $^1$H-n.m.r. spectroscopy of the purified ganglioside

$^1$H-n.m.r. spectrum of the purified ganglioside is shown in Figure 3. The chemical shifts for anomeric protons were assigned

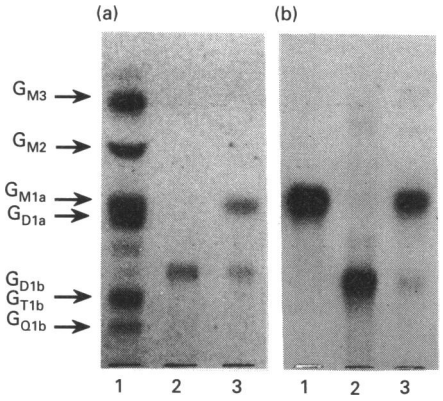


**Figure 4 Binding of N-acetylated ganglioside to cholera-toxin B-subunit**

Chemical N-acetylation and t.l.c./immunostaining were performed as described in the Materials and methods section. (**a**) Resorcinol staining on an h.p.t.l.c. plate; (**b**) t.l.c./immunostaining with cholera-toxin B-subunit on a plastic t.l.c. plate; lane 1, standard ganglioside mixture ($G_{M3}$, $G_{M2}$, $G_{M1}$, $G_{D1a}$, $G_{D1b}$, $G_{T1b}$ and $G_{Q1b}$); lane 2, de-N-acetylated ganglioside from bovine brain; lane 3, N-acetylated derivative. The t.l.c. plate was developed using solvent system (ii).

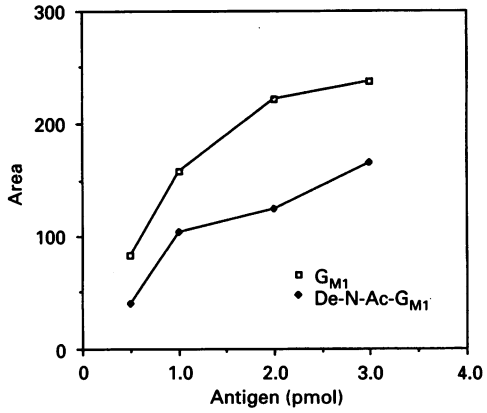


**Figure 5 Reactivity of de-N-acetylated ganglioside (De-N-Ac-$G_{M1}$) to cholera-toxin B-subunit by t.l.c./immunostaining**

T.l.c./immunostaining were performed as described in the Materials and methods section. After the t.l.c. plate had been stained, each positive band was quantified densitometrically with a Personal Densitometer (Molecular Dynamics, U.S.A.).

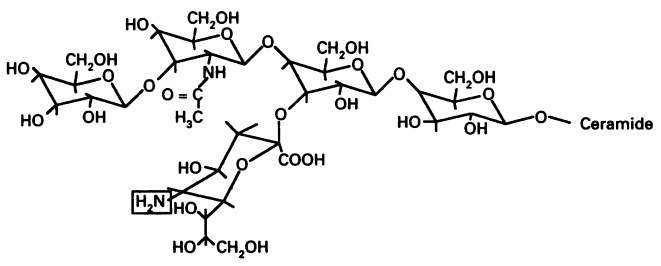


**Figure 6 Structure of de-N-acetylated ganglioside from bovine brain**

The ceramide portion consists of the same composition of authentic $G_{M1}$ (NeuAc) from bovine brain.

according to references reported by Koerner et al. [30] as follows: 4.15 p.p.m. ($J_{1,2}$ 7.7 Hz) for $\beta$-anomeric proton of glucose, 4.22 p.p.m. ($J_{1,2}$ 7.0 Hz) for that of non-reducing terminal galactose, 4.24 p.p.m. ($J_{1,2}$ 8.1 Hz) for that of internal galactose, and 4.89 p.p.m. ($J_{1,2}$ 8.8 Hz) for that of *N*-acetylgalactosamine. Whereas methyl signal at 1.76 p.p.m. exhibited the presence of the acetoamide group bound to *N*-acetylgalactosamine, the signal at 1.89 p.p.m. for that bound to *N*-acetylneuraminic acid was not detected significantly. Furthermore, in the downfield region (7.5–8.0 p.p.m.), the resonance for the amide proton of *N*-acetylneuraminic acid was absent too. These results indicate that the purified ganglioside has a core structure of ganglio-tetraosylceramide ($Gg_4Cer$), and a neuraminic acid instead of an *N*-acetylneuraminic acid.

### N-acetylation of the purified ganglioside

N-Acetylation of the purified ganglioside caused change of its $R_F$ value on h.p.t.l.c. from between $G_{D1a}$ and $G_{D1b}$ to that of $G_{M1}$ with N-acetylated neuraminic acid (Figure 4a). The purified ganglioside, before and after N-acetylation, potently reacted to cholera-toxin B-subunit. (Figure 4b). This result further supports that the purified ganglioside is a $G_{M1}$ analogue, which contains neuraminic acid instead of *N*-acetylneuraminic acid.

### Comparison of the binding reactivity between the purified ganglioside and $G_{M1}$

Figure 5 shows the binding of the purified ganglioside to cholera-toxin B-subunit on t.l.c. The reactivity of the novel ganglioside was about 60% of that of authentic $G_{M1}$.

### Structure of the purified ganglioside from bovine brain

From all results presented above, the structure of the purified ganglioside from bovine brain is proposed as shown in Figure 6. This ganglioside has the core structure of Gal$\beta$1-3GalNAc$\beta$1-4Gal$\beta$1-4Glc$\beta$1-1Cer and a neuraminic acid residue bound to internal galactose with an $\alpha$2-3 linkage. We propose to name the ganglioside 'de-N-acetylated $G_{M1}$'.

## DISCUSSION

It has been well known that cholera-toxin B-subunit potently bound to $G_{M1}$ ganglioside [19–22]. It has also been reported that cholera-toxin B-subunit also bound to $G_{D1b}$ and $G_{T1b}$, but their binding reactivities are less than 10% of that of $G_{M1}$ [23–26]. In this study we found a novel ganglioside from bovine brain, which shows strong binding reactivity to cholera-toxin B-subunit. F.a.b.m.s., $^1$H-n.m.r. and chemical analysis show that the isolated ganglioside is $G_{M1}$ with de-N-acetylated neuraminic acid. These results indicate that the acetoamide residue of *N*-acetylneuraminic acid in $G_{M1}$ is not necessary for the binding with cholera-toxin B-subunit.

Although Hanai et al. [8] detected the presence of de-N-acetylated $G_{M3}$ in a cultured cell line using a monoclonal antibody against chemically synthesized $G_{M3}$ derivative [8], they did not isolate it for further characterization. Here, we isolated and fully characterized de-N-acetylated $G_{M1}$ from bovine brain for the first time. The ganglioside reacted not only with cholera-toxin B-subunit, but also with a mouse monoclonal antibody DM2-1, which was established by immunization with chemically synthesized $G_{M2}$ containing de-N-acetylated neuraminic acid. The DM2-1 also recognized chemically synthesized gangliosides with de-N-acetylated neuraminic acid, e.g. $G_{M3}$ and $G_{M2}$

derivatives (S. Fujita, M. Numata, M. Yamakawa, A. Hino, M. Sugimoto, I. Suda, K. Tomita and T. Ogawa, unpublished work). These immunoreactivities support the conclusion that the novel ganglioside is de-N-acetylated $G_{M1}$.

We also found that a series of de-N-acetylated ganglioside homologues (derivatives of $G_{M3}$, $G_{M2}$ and $G_{D1a}$ in which de-N-acetylated neuraminic acid only linked to the internal Gal residue of core structure) exists in bovine brain (results not shown). The de-N-acetylated $G_{M1}$ could be derived from $G_{M1}$ by a de-N-acetylase as described by Manzi et al. [10], who reported that de-N-acetylated $G_{M3}$ and $G_{D3}$ are derived from intact gangliosides in human melanoma cells. On the other hand, de-N-acetylated $G_{M1}$ in bovine brain could be synthesized from de-N-acetylated $G_{M3}$ via de-N-acetylated $G_{M2}$ by the same enzymes in biosynthetic pathway of $G_{M1}$.

In this study we indicated that de-N-acetylated $G_{M1}$ exists in brain tissues. Since glycosphingolipids and their derivatives with the free amino residue, such as lyso-$G_{M3}$, de-N-acetylated $G_{M3}$ and sphingosine, served as bioactive substances [8,31,32], the novel ganglioside may play significant roles in the nervous system.

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