methods

Purification of gangliosides by liquid-liquid partition chromatography

Tomohisa Kato[*,†] **and Kenichi Hatanaka**[1,§]

Japan Chemical Innovation Institute,* 1-3-5 Jimbocho, Chiyoda-ku, Tokyo 101-0051, Japan; Kaneka Corporation,† 3-2-4, Nakanoshima, Kita-ku, Osaka 530-8288, Japan; and Institute of Industrial Science,§ The University of Tokyo, 4-6-1, Komaba, Meguro-ku, Tokyo 153-8505, Japan

Abstract A liquid-liquid partition chromatographic technique was applied to separate amphiphilic glycolipids. A two-phase solvent system composed of *n*-butanol-*t*-butyl methyl ether-acetonitrile-water at a volume ratio of 3:1:1:5 was found to be suitable for separating the gangliosides from total lipids extracted from rat brain by liquid-liquid partition chromatographic systems, namely centrifugal partition chromatography (CPC) and high-speed counter-current chromatography. GM1 could be separated rapidly by using the upper phase as stationary phase for both systems. Moreover, various kinds of gangliosides (GM1, GD1a, GD1b, GT1b) could be separated individually by using the lower phase as stationary phase by CPC. The sample can be recovered without loss by these systems.—Kato, T. and K. Hatanaka. **Purification of gangliosides by liquid-liquid partition chromatography. *J. Lipid Res.* 2008.** 49: **2474–2478.**

Supplementary key words ganglioside • separation • centrifugal partition chromatograph • counter-current chromatography

Gangliosides are acidic glycolipids widely distributed in most vertebrate tissues and body fluids, and are particularly abundant in the nervous system. Ganglioside is a sphingoglycolipid that contains one or more sialic acid residues (*N*-acetylneuraminic acid, *N*-glycolylneuraminic acid) linked to an oligosaccharide chain. In recent years, their various roles in biological functions, such as cell-cell interaction, cell proliferation and differentiation, cell-substrate interaction, carcinogenesis, bacteria or virus infection, immune response, and inflammation, have been reported (1).

Isolation and purification of gangliosides from complex lipid mixtures are generally accomplished by preparative TLC (2), HPLC using anion-exchange columns such as DEAE-Sephadex (3, 4), Q-Sepharose (5) and MonoQ (6), or by using a strong anion exchanger cartridge (InertSep SAX) (7).

In this research, we employed centrifugal partition chromatography (CPC) to separate and purify pseudo-glycolipids that were obtained by the saccharide primer method (8). CPC and high-speed counter-current chromatography (HSCCC) are both liquid-liquid partition chromatographic techniques that have been widely used for separation and purification of natural products (9–13). The difference between CPC and HSCCC is the component (rotor and tubing, respectively), in which liquid-liquid partition is performed (11). It is thought that because mixing conditions for these systems are not identical, amphiphilic compounds such as glycolipids may be separated differently. These liquid-liquid partition chromatographic techniques have advantages over chromatographic techniques such as normal column chromatography and HPLC using solid support. Because irreversible adsorption does not occur, simple rinsing of the instrument allows full recovery of noneluted compounds. Moreover, pretreatment of samples is unnecessary and purification can be accomplished in a short time. Significantly, liquid-liquid partition chromatographic techniques do not require expensive solid support.

In this paper, we describe the successful separation and purification of gangliosides (from complex lipid mixtures extracted from rat brain) by two liquid-liquid partition chromatographic systems, CPC and HSCCC.

MATERIALS AND METHODS

Materials

All organic solvents were of analytical grade and were purchased from Nacalai Tesque, Kyoto, Japan. Rat brain tissue (Rockland) was purchased from Funakoshi, Tokyo, Japan. The strong anion exchanger cartridge (InertSep SAX) was purchased from GL Science, Tokyo, Japan.

Apparatus

CPC was performed using the CPC240 (Sensyu Scientific, Tokyo, Japan). Total rotor volume of this model is 240 ml, and num-

This work was supported by a grant for Development of Novel Diagnostic and Medical Applications through Elucidation of Sugar Chain Functions from the New Energy and Industrial Technology Development Organization.

Manuscript received 10 June 2008 and in revised form 21 July 2008.

Published, JLR Papers in Press, July 21, 2008.
DOI 10.1194/jlr.D800033-JLR200

[1] To whom correspondence should be addressed.
e-mail: hatanaka@iis.u-tokyo.ac.jp

ber of cells is 2,136 (11). HSCCC was performed by using Easy-PREP320 (Kutuwa Sangyo, Hiroshima, Japan, http://www.kutuwa.com). Total column volume of this model is approximately 320 ml. Polytetrafluoroethylene tubing (1.6 mm inner diameter, 0.4 mm thick) was used as separation column. Centrifugal concentrator used was a Tomy CC-105 system with TU-105 low temperature trap, and GCD-051× (Tomy Seiko Co., Ltd., Tokyo, Japan).

Lipid extraction from tissue

Ten pieces of rat brain tissue were homogenized with 100 ml of chloroform-methanol (2:1,v/v). After sonication for 30 min, the extract was transferred into a new tube. The lipids from the remaining pellet were extracted again with 100 ml of chloroform-2-propanol-H_2O (7:11:2, v/v/v). The first and the second extracts were mixed and evaporated. The resulting pellet was dissolved in 12 ml of chloroform-methanol (2:1, v/v). Crude lipid extract (1 ml) was dried up and dissolved in the 5 ml solvent [upper phase-lower phase (1:1, v/v)] used for separation procedure, respectively, and the total amounts were injected into CPC or HSCCC.

Column procedures

Separation of acidic glycolipids from total lipids extracted from rat brain was performed as described previously (7). The SAX cartridge (500 mg) was placed in the extraction manifold apparatus (Waters, Milford, MA) and washed under vacuum (under 10 kPa) with 10 ml of methanol and 10 ml of distilled water. The anion exchange resin was conditioned by washing with 10 ml of 0.8 M ammonium acetate solution to convert to acetate ion form and then washed with 10 ml of distilled water. After equilibration with 10 ml of chloroform-methanol-H_2O (5:10:1, v/v/v), a collection rack with receiving tubes was placed in the extraction manifold apparatus. The lipid extract was diluted with chloroform-methanol-H_2O (5:10:1, v/v/v) and applied to the cartridge column under vacuum. Then 10 ml of chloroform-methanol-H_2O (5:10:1, v/v/v) was pulled through for washing. The cartridge column was then eluted with 10 ml of chloroform-methanol-4.0 M ammonium acetate (5:10:1, v/v/v) and the eluate (acidic glycolipids) was saved. Eluates were evaporated by centrifugal concentrator.

Separation procedure by CPC

CPC was performed with a two-phase solvent system composed of *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v) for the separation of gangliosides. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and was separated into two phases before use. The column (rotor) was filled with upper/lower stationary phase, and the lower/upper mobile phase was pumped into the inlet of the column at a flow rate of 4 ml/min. The apparatus was rotated at 1,200 rpm. After equilibration, the sample solution was applied through the injection valve. The eluent was collected in glass tubes with a fraction collector, and each fraction was subjected to high-performance TLC (HPTLC) analysis.

Separation procedure by HSCCC

The separation was performed according to the standard procedure used for HSCCC (14). A two-phase solvent system used for separation consisted of *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v). The entire column was filled with the upper stationary phase. Then the column was rotated at 800 rpm, and the lower mobile phase was pumped into the inlet of the column at a flow rate of 2.0 ml/min. After equilibration, the sample solution was applied through the injection valve. The eluent was collected in glass tubes with a fraction collector, and each fraction was subjected to HPTLC analysis. After normal elution, the rotation of the column was stopped, and the column contents were collected by pumping the upper stationary phase into the column.

HPTLC

Amphiphilic glycolipids and pseudo-glycolipids synthesized by using cells were analyzed on an HPTLC plate (Merck, Darmstadt, Germany). The developing solvent used was chloroform-methanol-0.25% aqueous KCl (5:4:1, v/v/v). The separated glycolipids

TABLE 1. Selection of two-phase solvent system

Two-phase Solvent System	Gangliosides	$K_{U/L}$ Acid	$K_{U/L}$ Neutral	$K_{U/L}$ Alkali
			mol/mol	
BuOH-tBME-ACN-H_2O (2:2:1:5)	GM1	>100	1.9	1.26
	GD1a	5.7	<0.01	<0.01
	GD1b	16	<0.01	<0.01
	GT1b	1.3	<0.01	<0.01
BuOH-tBME-ACN-H_2O (3:1:1:5)	GM1	20	5.8	5.3
	GD1a	3.5	0.23	0.21
	GD1b	6.9	0.13	0.11
	GT1b	2.2	0.04	<0.01
tBME-ACN-H_2O (2:2:3)	GM1	0.79	<0.01	<0.01
	GD1a	0.065	<0.01	<0.01
	GD1b	<0.01	<0.01	<0.01
	GT1b	<0.01	<0.01	<0.01
BuOH-EtOH-H_2O (4:1:5)	GM1	28		
	GD1a	3.3		
	GD1b	8.8		
	GT1b	3.2		

$K_{U/L}$, partition coefficient. The solvent mixture was thoroughly equilibrated in a separatory funnel by repeated vigorous shaking and degassing several times, and the two solvent phases were separated. Then 5 ml each of the upper and lower phases was transferred into new tubes, sample solutions (5 μl lipid extract, described in Materials and Methods) were added, and the mixture was shaken vigorously. After the two solvents were separated, samples dissolved in the upper and lower phases were analyzed by high-performance TLC. Acid: 1% acetic acid was added to the lower phase; alkali: 1% NH_4OH was added to the lower phase.

were detected by spraying with resorcinol reagent and heating at 120°C (15, 16).

RESULTS AND DISCUSSION

Selection of two-phase solvent system

Crude lipid extract (12 ml) was prepared from 10 samples of rat brain tissue. The major brain gangliosides are GD1a, GD1b, GT1b, and GM1. To find a suitable two-phase solvent system for separation of gangliosides, the partition coefficient ($K_{U/L}$) of each ganglioside involved in the crude lipid extract was measured by using various types of two-phase solvent systems. The results are summarized in **Table 1**. A two-phase solvent system consisting of *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v) seemed to be suitable for separation of each ganglioside, because the $K_{U/L}$ values of the four types of gangliosides were not the same. When an acid (acetic acid) was added, gangliosides became more soluble in the upper organic phase. On the other hand, gangliosides became more soluble in the lower aqueous phase when a base (NH_4OH) was added. However, adding neither an acid nor a base was effective for separation of gangliosides. Chloroform-methanol-water (5:6:4, v/v/v), which can separate neutral pseudo-glycolipids (8), and *n*-hexane-ethyl acetate-methanol-water (1:1:1:1, v/v/v/v) were tried in various modes for the selection of solvents.

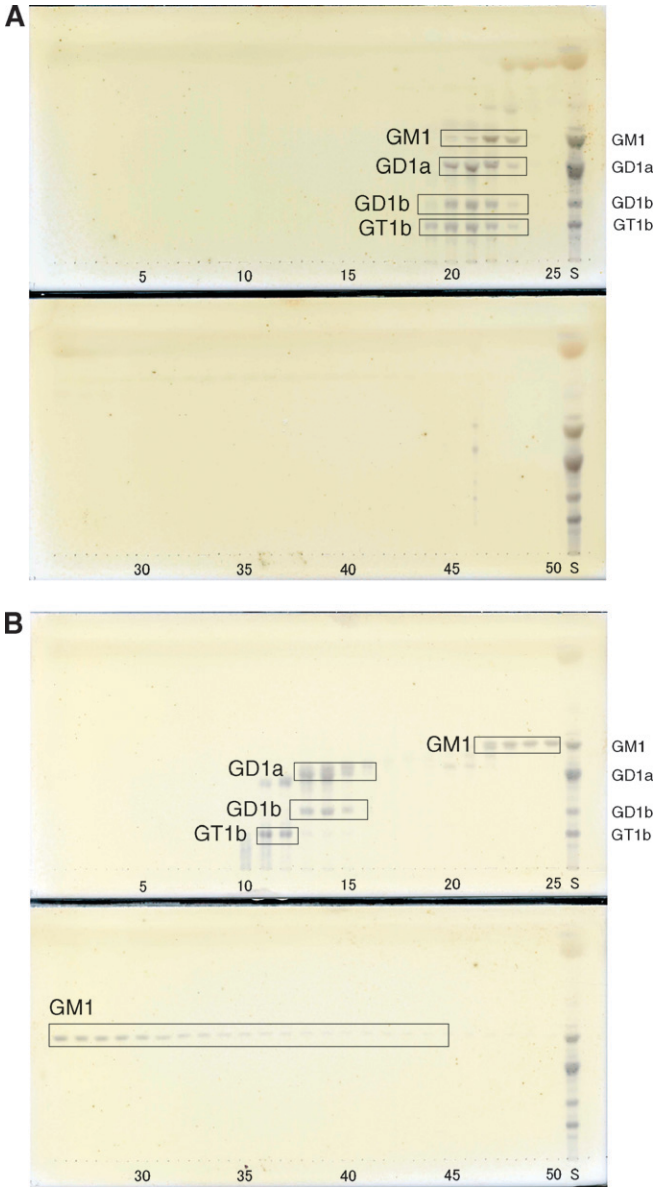

Fig. 1. Elution profile of gangliosides extracted from rat brain separated by centrifugal partition chromatography (CPC). A: Non-treated. B: Treated by strong anion exchanger (SAX) cartridge column. Solvent system: *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 4.0 ml/min; revolution speed: 1,200 rpm; fraction volume: 10 ml. For high-performance TLC (HPTLC) analysis, 50 μl of the fractionated fraction was dried, and then the dried pellets were dissolved in 5 μl chloroform-methanol (2:1, v/v) and applied to the HPTLC plate.

Separation by CPC (lower aqueous phase mobile)

Gangliosides in lipid extracted from rat brain were separated by CPC using a two-phase solvent system composed of *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v) (**Fig. 1**). The column was filled with the upper stationary phase, and the lower phase was used as the mobile phase. At first, nontreated lipid extract was tried for separation by CPC. However, the gangliosides were barely separated (Fig. 1A). The impurity (for example, simple lipid, phospholipid, neutral sphingoglycolipid) may have an inhibitory effect on the separation of gangliosides. Therefore, the lipid extract was purified first by SAX cartridge

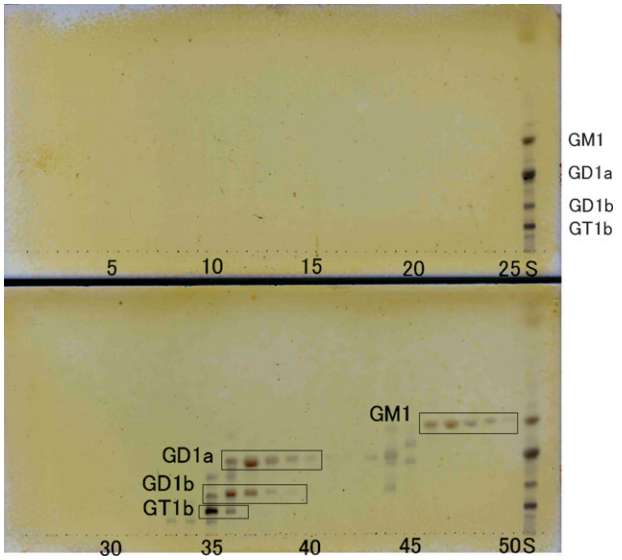

Fig. 2. Elution profile of gangliosides extracted from rat brain after separation by high-speed counter-current chromatography. Solvent system: *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.0 ml/min; revolution speed: 1,000 rpm; fraction volume: 8 ml. For HPTLC analysis, 50 μl of the fractionated fraction was dried, and then the dried pellets were dissolved in 5 μl chloroform-methanol (2:1, v/v) and applied to the HPTLC plate.

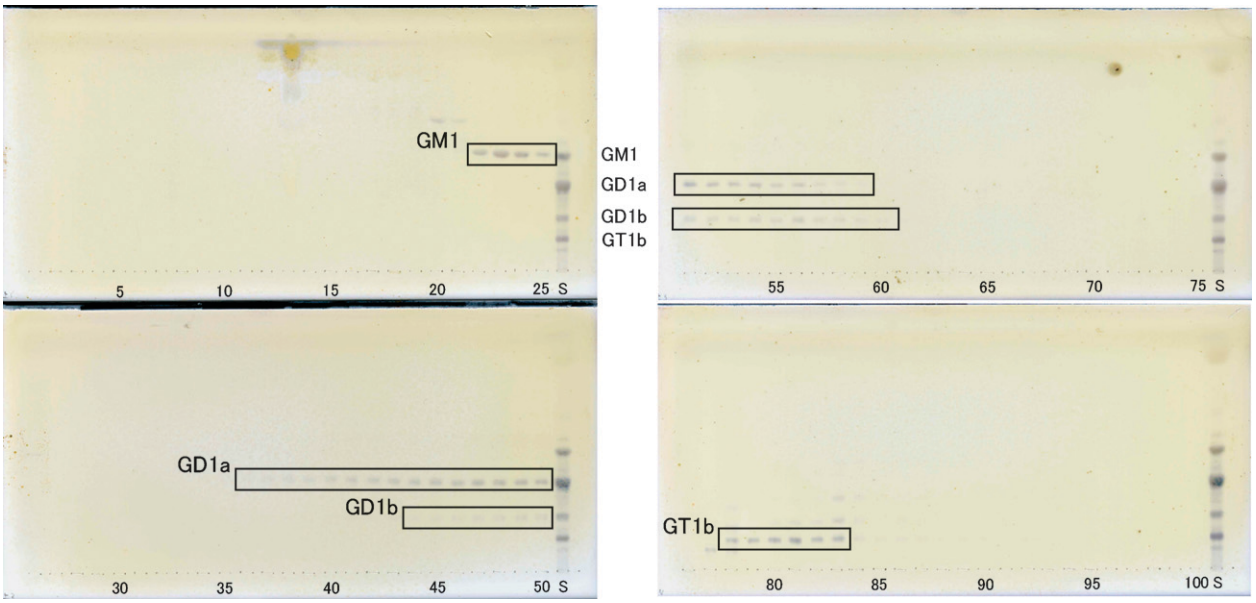

Fig. 3. Elution profile of gangliosides extracted from rat brain separated by CPC. Solvent system: *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v); stationary phase: lower phase; mobile phase: upper phase; flow rate: 4.0 ml/min; revolution speed: 1,200 rpm; fraction volume: 10 ml. After complete injection, gangliosides were eluted first by the mobile phase in ascending mode (fractions 1–75), then by the stationary phase in descending mode (fractions 76–100). For HPTLC analysis, 50 μl of the fractionated fraction was dried, and then the dried pellets were dissolved in 5 μl chloroform-methanol (2:1, v/v) and applied to the HPTLC plate.

column before separation by CPC. As shown in Fig. 1B, GM1 was clearly separated from other gangliosides. The difference between untreated and treated extracts may be due to emulsification of two-phase solvent caused by the neutral compounds, which could be removed by SAX cartridge column treatment.

Separation by HSCCC

Gangliosides in lipid extracted from rat brain were separated by HSCCC using the same two-phase solvent system. Because the suitable K values for HSCCC are less than 1, the lower phase was used as the mobile phase. The ganglioside, lipid extract purified by SAX cartridge column, was separated by HSCCC as shown in **Fig. 2**. The results showed that this two-phase solvent system (mobile phase: lower phase) was particularly effective for the separation of GM1 by HSCCC, or by CPC as described above.

Separation by CPC (upper organic phase mobile)

Gangliosides were separated from nontreated lipid extract by CPC using a two-phase solvent system composed of *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v). Neutral lipids (simple lipid, phospholipid, neutral glycosphingolipid) were dissolved in the upper phase more quickly than the lower phase and are more soluble in the upper phase than gangliosides. Hence, gangliosides were separated from the lipid extract without SAX purification by CPC using the upper phase as the mobile phase. Gangliosides GM1 (fraction number 22–25 in **Fig. 3**), GD1a and GD1b (fraction number 36–59 and 45–60, respectively in Fig. 3), and GT1b (fraction number 78–83 in Fig. 3) were separated. In this mode, because the neutral lipids are removed first, gangliosides may be separated without the influence of the neutral lipids. It is known that minor gangliosides otherwise exist in rat brain (4). Although the spot of ganglioside GQ is detected by HPTLC under one of GT1b, the spot detected at the fraction number 77 is considered to be ganglioside GQ. Gangliosides were separated more effectively by CPC under this condition than previously described (8).

Our new procedure using CPC or HSCCC is particularly suitable for the purification of gangliosides. Significantly, various types of gangliosides (GM1, GD1a, GD1b, GT1b) were separated individually according to their behavior in CPC by using a two-phase solvent system composed of *n*-butanol-*t*-butyl methyl ether-acetonitrile-water (3:1:1:5, v/v/v/v). These techniques allow scaling-up of this procedure for application to industrial production of gangliosides.

REFERENCES

1. Varki, A. 1993. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology.* **3:** 97–130.
2. Van Echten-Deckert, G. 2000. Sphingolipid extraction and analysis by thin-layer chromatography. *In* Methods in Enzymology. Vol. 312. A. H. Merrill, Jr. and Y. A. Hannun, editors. Academic Press Inc., New York. 64–79.
3. Yu, R. K., and R. W. Ledeen. 1972. Gangliosides of human, bovine and rabbit plasma. *J. Lipid Res.* **13:** 680–686.
4. Iwamori, M., and Y. Nagai. 1978. A new chromatographic approach to the resolution of individual gangliosides. Ganglioside mapping. *Biochim. Biophys. Acta.* **528:** 257–267.
5. Hirabayashi, Y., T. Nakao, M. Matsumoto, K. Obata, and S. Ando. 1988. Improved method for large-scale purification of brain gangliosides by Q-sepharose column chromatography. Immunochemical detection of C-series polysialogangliosides in adult bovine brains. *J. Chromatogr.* **445:** 377–384.

6. Mansson, J-E., B. Rosengren, and L. Svennerholm. 1985. Separation of gangliosides by anion-exchange chromatography on MomoQ. *J. Chromatogr.* **332:** 465–472.
7. Kato, T., M. C. Kasuya, and K. Hatanaka. 2008. Rapid separation of gangliosides using strong anion exchanger cartridges. *J. Oleo Sci.* **57:** 397–400.
8. Kato, T., A. Miyagawa, M. C. Z. Kasuya, A. Ito, and K. Hatanaka. 2008. Purification by centrifugal partition chromatography of amphiphilic compounds, glycolipids and pseudo-glycolipids synthesized by using cells. *J. Chromatogr. A.* **1178:** 154–159.
9. Wanasundara, U., and P. Fedec. 2002. Centrifugal partition chromatography (CPC): emerging separation and purification technique for lipid and related compounds. *Food Technol.* **13:** 726–730.
10. Akiyama, T., S. Harada, F. Kojima, N. Kinoshita, M. Hamada, Y. Muraoka, T. Aoyagi, and T. Takeuchi. 1998. Epostatin, new inhibitor of dipeptidyl peptidase II, produced by *Streptomyces* sp. MJ995-OF5. I. Taxonomy of producing strain, fermentation, isolation, physicochemical properties and biological properties. *J. Antibiot. (Tokyo).* **51:** 253–260.
11. Takahashi, Y., and R. Sawa. 2006. Purification of microbial secondary metabolites by high performance centrifugal liquid-liquid partition chromatography. *Bunseki.* **7:** 307–312.
12. Ito, Y. 2003. Technical guidance for high-speed countercurrent chromatography (HSCCC). *FFI Journal.* **208:** 709–724.
13. Ito, Y. 2005. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *J. Chromatogr. A.* **1065:** 145–168.
14. Ito, Y., and H. Oka. 1989. Improved high-speed counter-current chromatograph with three multilayer coils connected in series. *J. Chromatogr.* **475:** 219–227.
15. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta.* **24:** 604–611.
16. Kasuya, M. C. Z., L. X. Wang, Y. C. Lee, M. Mitsuki, H. Nakajima, Y. Miura, T. Sato, K. Hatanaka, S. Yamagata, and T. Yamagata. 2000. Azido glycoside primer: a versatile building block for the biocombinatorial synthesis of glycosphingolipid analogues. *Carbohydr. Res.* **329:** 755–763.