Partial synthesis of ganglioside and lysoganglioside lipoforms as internal standards for MS quantification

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Running title: Non-natural ganglioside and lysoganglioside lipoforms

Abbreviations: CHN analysis, carbon hydrogen nitrogen analysis; DCC, ¹,¹'-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DEAE-, diethylaminoethyl-; GC-FID, gas chromatography with flame ionization detector; GSL, glycosphingolipid; HPTLC, high performance thin-layer chromatography; MAG, myelin-associated glycoprotein; Neu5Ac, N-acetylneuraminic acid; NP, normal phase, RP, reversed phase; RT, room temperature; SCDase, sphingolipid ceramide N-deacylase; VEGF, vascular endothelial growth factor.
Abstract

Within the recent years, ganglioside patterns have been increasingly analyzed by mass spectrometry. However, internals standards for calibration are only available for gangliosides GM1, GM2, and GM3. For this reason, we prepared homologous internal standards bearing non-natural fatty acids of the major mammalian brain gangliosides GM1, GD1a, GD1b, GT1b, and GQ1b, and of the tumor associated gangliosides GM2 and GD2. The fatty acid moieties were incorporated after selective chemical or enzymatic deacylation of bovine brain gangliosides. For modification of the sphingoid bases, we developed a new synthetic method based on olefin cross metathesis. This method was used for the preparation of a lyso-GM1- and a lyso-GM2 standard. The total yield of this method was 8.7 % for the synthesis of d17:1-lyso-GM1 from d20:1/18:0-GM1 \(^1\) in four steps.

The title compounds are currently used as calibration substances for MS quantification and are also suitable for functional studies.

Supplementary key words: brain lipids · chemical synthesis · glycolipids · lipidomics · mass spectrometry · sphingolipids · olefin metathesis

\(^1\)
Introduction

Gangliosides are sialic acid containing glycosphingolipids (GSLs). They can be found in vertebrates and, with a few exceptions, not in invertebrates (1). Gangliosides are especially abundant in neuronal tissues, where their content is one to two orders of magnitude higher than in extraneural tissues (2). In the brain, gangliosides together with other glycosphingolipids are the main glycan carriers (80 % of the total glycan mass in adult rat brain) (3). The main gangliosides in adult mammalian brain are GM1 (Fig. 1), GD1a, GD1b, GT1b, 9-O-Ac-GT1b, and GQ1b (4). They contain mostly C\(_{18}\)- and C\(_{20}\)-sphingosine acylated with stearic acid, which constitutes more than 80 % of the total ganglioside fatty acid content in the nervous system (4). In mammals, C\(_{20}\)-sphingosine-containing gangliosides can only be found in significant amounts in the nervous system (5). In contrast to adult brain, different glycosphingolipid- and ganglioside-series are expressed in the developing nervous system (6).

Subcellularly, the majority of gangliosides is localized in the outer leaflet of the plasma membrane. They are anchored to the membrane by their hydrophobic ceramide part while their hydrophilic carbohydrate part extrudes into the extracellular space. They are part of the cellular glycocalyx and interact with molecules outside the cell (“trans” interaction) and within the same membrane (“cis” interaction) (1). Examples are the “trans”-interaction of ganglioside GM1 with cholera toxin (7), of NeuAca2-3Galβ1-3GalNAc-termini on axonal gangliosides such as GD1a, GT1b, and GM1b with the myelin-associated glycoprotein MAG (8), and the “cis”-interaction of GM3 with the receptors for insulin (9), epidermal growth factor (10), or VEGF (11).

In the past, ganglioside patterns have been largely determined by TLC followed by densitometric quantification (12, 13). Within recent years, ganglioside determinations by mass spectrometric methods have been increasingly applied (14). In addition to ganglioside classes, which are defined by the carbohydrate head group, MS allows also profiling of ganglioside lipoforms (15) with different structure of acyl chain and sphingoid base. Ganglioside quantification is applied to ganglioside pattern investigation (16), in food analyses (17, 18), but also in the analysis of lysosomal storage diseases (19) and in quantitative imaging mass spectrometry (20). Lysogangliosides play a major role in the pathogenesis of gangliosidoses.
Elevated levels of lyso-GM2 and lyso-GA2 are present in the brains of patients with GM2 gangliosidoses (21), and elevated levels of lyso-GM1 and lyso-GA1 are present in patients with GM1 gangliosidosis (22). Both substances are potential biomarkers for these diseases (23) and the lysoganglioside standards prepared in this work can be used for their quantitative analysis.

Quantification of gangliosides by mass spectrometry including quantitative imaging mass spectrometry requires calibration substances. Hereby, calibration is defined as the establishment of a correlation between analyte concentration and mass spectrometer response. Three principle methods can be distinguished: external calibration, standard addition calibration, and internal standard calibration. The internal standard calibration shows the highest accuracy and precision since standard and analyte are measured in the same sample at the same time, and the internal standard acts as a self-correcting system for analyte losses during purification steps and for fluctuations in mass spectrometer response (24). Internal standards are chemically and physically similar, but non-isobaric derivatives of the analyte. Suitable are stable isotope labeled derivatives (type 2 internal standards) or homologous derivatives (type 3) of the analyte. Type 1 internal standards, which are any non-isobaric compounds with similar physical and chemical properties, are less accurate. Usually, the standard is added in a defined amount to the sample at an early stage of sample handling. After purification of the sample, the ratio of the MS response of analyte and standard is measured. The concentration of the analyte can be calculated by the following equation (24):

\[ c_{an} = \frac{I_{an}}{I_{IS}} \cdot c_{IS} = CF_{IS} \cdot I_{an} \]

(I: intensity, an: analyte, IS: internal standard, c: concentration, CFIS: calibration factor)

We report on the preparation of a set of new ganglioside internal standards for the major mammalian brain gangliosides and the tumor-associated gangliosides GM2 and GD2. The title substances were prepared from a mixture of gangliosides from bovine brain, which was separated first into ganglioside classes and then into pure lipoforms. Non-natural chain length in the fatty acid part were introduced by selective chemical
and enzymatic deacylation and reacylation. Furthermore, we developed a new method for the modification of the sphingosine chain length by olefin cross metathesis (Fig. 1). The method was applied for the synthesis of a lyso-GM1- and a lyso-GM2 standard.
Materials and Methods

Materials

All chemicals were of analytical grade or the highest purity available. Water used for buffers and solutions was purified by an ultrapure water system (EASYpure UV/UF D8612, Werner Reinstwassersysteme, Leverkusen, Ger). Solvents used in reactions of oxygen- and moisture sensitive compounds were in anhydrous form. 1-Propanol, methanol, pyridine, and acetic anhydride were degassed before use. The native mixture of bovine brain gangliosides Cronassial®, which consists of 21% of GM1, 40% of GD1a, 16% of GD1b, 19% of GT1b, and 4% of other gangliosides (25), was available in our lab. Sphingolipid ceramide N-deacylase (SCDase) and beta-galactosidase from bovine testes were from Sigma Aldrich (Schnelldorf, Ger). Triton™ X-100, sodium taurodeoxycholate, Grubbs catalyst 2nd gen., Hoveyda-Grubbs cat. 2nd gen., and Stewart-Grubbs catalyst were also from Sigma Aldrich. For NP column chromatography, silica gel 60 (0.040-0.063 mm or 0.015-0.040 mm) from Merck (Darmstadt, Ger) was used. For desalting and RP column chromatography, LiChroprep® RP-18 (0.040-0.063 mm) from Merck was used. For TLC analysis, HPTLC silica gel 60 F254- and HPTLC silica gel 60 RP-18 plates from Merck were used. For anion-exchange chromatography, DEAE Sephadex™ A-25 from Amersham Pharmacia Biotech AB (Uppsala, Swe) was used.

Purification

Desalting was performed by the method of Wiliams and McCluer (26). The usage of polar eluents for column chromatography causes significant contamination of column material in the products. We reduced this contamination to a minimum by the following method. NP silica gel columns of a very small column volume (approx. 1 mL column volume per 5 mg of product) were prepared in filtration columns from Supelco (Bellefonte, USA) using a mobile phase in which the substance is retained on the column. The substance was applied to the column and washed with three column volumes of the same solvent. Then, the substance was eluted by an appropriate solvent of higher polarity.
Analytical procedures

For $^1$H- and $^{13}$C-NMR spectroscopy, Bruker Avance 300, 400, or 500 instruments (Billerica, USA) were used. For exchange of exchangeable protons by deuterium, gangliosides were dissolved in CD$_3$OD and the solvent was removed in a stream of nitrogen. This process was repeated three times before measurement. For EI-MS, a MAT 95 XL sector field instrument (Thermo Finnigan MAT GmbH, Bremen, Ger) or a MAT 90 sector field instrument (Thermo Finnigan MAT GmbH) was used. For ESI-MS and ESI-MS/MS, a nano-ESI-QTOF mass spectrometer (Micromass, Manchester, UK) was used. In general, measurements were conducted in positive ion mode. For interpretation of the ESI-MS/MS spectra, the nomenclature for carbohydrate fragments by Domon and Costello was applied (27). For GC-MS, a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Jpn) equipped with a ZB-5MSi column (l = 30 m, Ø = 0.25 mm, df (film thickness) = 0.25 µm) containing a 5 % phenyl and 95 % dimethylpolysiloxane phase was used. For CHN-microanalysis, a vario EL cube (Elementar Analysensysteme GmbH, Hanau, Ger) was used.

In general, ganglioside yields were determined by dissolving the ganglioside in a defined volume of methanol or water and subsequent sialic acid determination. Ganglioside lipoforms which were quantified by CHN analysis before were used as external standards for the quantification of the synthesized ganglioside derivatives. Photometric sialic acid determination was performed by the resorcinol method of Svennerholm (28) modified by Mietinnen and Takkiluukkainen (29) using an Ultrospec III (Pharmacia Biotech, Uppsala, Swe). Fluorimetric sialic acid determination was performed using the sialic acid assay kit EnzyChrom™ ESLA-100 of BioAssay Systems (Hayward, USA) and a Fluoroscan II (Labsystems Oy, Helsinki, Fin).

Purification of bovine brain gangliosides

To obtain pure natural ganglioside lipoforms as starting material, the ganglioside mixture Cronassial® was purified by anion-exchange chromatography, followed by desalting, NP column chromatography, and RP column chromatography. In the first step, a slightly modified method of Momoi et al. was used (30). Briefly, 5.03 g of ganglioside mixture were applied to a DEAE Sephadex™ A-25 column (2.2 × 22 cm) and
ganglioside classes were eluted by a step gradient of different ammonium acetate concentrations in methanol. After desalting, the ganglioside classes were obtained as colorless solids. GQ1b was already obtained in pure form after these steps. From 5.03 g of starting material 1.28 g of monosialogangliosides, 2.80 g of disialogangliosides, 0.674 g of trisialogangliosides, and 41.5 mg of GQ1b were obtained. Then, proportions of these ganglioside classes were separated by NP column chromatography. The monosialogangliosides were separated isocratically using the solvent system CHCl₃/MeOH/0.2 % CaCl₂ 60:35:8 v/v/v to obtain GM1. The disialogangliosides were separated by a step gradient of CHCl₃/MeOH/2.5 M NH₃ 60:35:6 v/v/v and CHCl₃/MeOH/2.5 M NH₃ 60:35:8 v/v/v to obtain GD1a and GD1b, respectively. Trisialogangliosides were separated isocratically using the solvent system CHCl₃/MeOH/H₂O 50:42:11 v/v/v to obtain GT1b. Proportions of these five gangliosides were separated into their d18:1/18:0- and d20:1/18:0-lipoforms by RP column chromatography using the solvent system MeOH/H₂O in appropriate compositions (90:25 v/v for GM1, 90:49 v/v for GD1a, 90:40 v/v for GD1b, 90:40 v/v for GT1b, and 90:50 v/v for GQ1b). The obtained ganglioside lipoforms were analyzed by ESI-MS, ESI-MS/MS, ¹H-NMR, and CHN analysis (Table 1).

**Chemical preparation of lysogangliosides**

d18:1-Lyso-GM1·NH₃ (7) was prepared from d18:1/18:0-GM1·NH₃ (1) by the method of Sonnino et al. (31). All steps were performed under an argon atmosphere. 20 mg (12.8 µmol) of 1 were dissolved in 9.2 mL of dry Propanol. Then, 2.3 mL of a 1 M potassium hydroxide solution in the same solvent were added and the reaction mixture was heated to 90 °C for 20 h. After cooling to RT, the reaction mixture was brought to pH 9 by adding an aq. ammonium chloride buffer. The solvent was removed in a stream of nitrogen, the crude product was dried in vacuum and subsequently purified by RP column chromatography (1.5 × 3.5 cm, MeOH/H₂O 90:40 v/v). A second purification step was performed by NP column chromatography (2.0 × 22 cm, CHCl₃/MeOH/2.5 M NH₃ 60:40:9 v/v/v). After lyophilization, 7 was obtained as a colorless powder (8.69 mg, 52 %), ESI-MS: 1280.62 [M + H]⁺, 651.80 [M + Na + H]²⁺, 640.81 [M + 2H]²⁺, Rₖ = 0.12 (CHCl₃/MeOH/2.5 M NH₃ 60:40:10 v/v/v). The method was also applied for the
preparation of d18:1-lyso-GD1α·2NH₃ (8) (1.23 mg, 4.8 %) from d18:1/18:0-GD1α·2NH₃ (3), ESI-MS: 1571.75 [M + H]⁺, 797.36 [M + Na + H]²⁺, 794.88 [M + NH₄ + H]²⁺, 786.37 [M + 2H]²⁺, Rᵣ = 0.24 (CHCl₃/MeOH/2.5 M NH₃ 60:44:12 v/v/v).

**Enzymatic preparation of lysogangliosides**

The enzymatic preparation of lysogangliosides was performed by reported methods (32, 33). Triton™ X-100 was used instead of sodium cholate because we observed better conversions. 0.919 mg (491 nmol) of d18:1/18:0-GD1α·2NH₃ (3) were dissolved in 500 µL of 50 mM sodium acetate buffer (pH 6.0), which contained 0.8 % of Triton™ X-100. The mixture was exposed to an ultrasonic bath for 15 s. Then, 3.6 µL (18 mU) of a SCDase solution (in 50 mM sodium acetate buffer, pH 6.0) were added, the aqueous phase was covered with 5 mL of n-decane to remove the fatty acid from the equilibrium, and the mixture was incubated at 37 °C. Every 2 h the n-decane phase was replaced. To obtain a complete conversion of the educt further 36 mU of enzyme were added over a period of 2 d. The reaction was stopped by the addition of a few drops of 2.5 M ammonia. The solvent was removed in a stream of nitrogen, the crude product was dried in vacuum and subsequently purified by NP column chromatography (2.0 × 23 cm, CHCl₃/MeOH/2.5 M NH₃ 60:40:10 v/v/v). After lyophilization, d18:1-lyso-GD1α·2NH₃ (8), was obtained as a colorless powder (0.74 mg, 47 % from two preparations), ESI-MS: m/z 1571.67 [M + H]⁺, 915.46 [Y₂α + 2H]⁺, 816.30 (1.6) [M + K + Na]³⁺, 797.32 [M + Na + H]²⁺, 794.84 [M + NH₄ + H]²⁺, 786.33 [M + 2H]²⁺, Rᵣ = 0.24 (CHCl₃/MeOH/2.5 M NH₃ 60:44:12 v/v/v). The method was also applied for the preparation of d20:1-lyso-GD1b·2NH₃ (9) (1.72 mg, 53 %), from d20:1/18:0-GD1b·2NH₃ (4), ESI-MS: m/z 1599.67 [M + H]⁺, 811.32 [M + Na + H]²⁺, 808.85 [M + NH₄ + H]²⁺, 800.33 [M + 2H]²⁺, Rᵣ = 0.18 (CHCl₃/MeOH/2.5 M NH₃ 60:44:12 v/v/v), d20:1-lyso-GT1b·3NH₃ (10) (1.16 mg, 61 %) from d20:1/18:0-GT1b·3NH₃ (5), ESI-MS: m/z 1890.74 [M + H]⁺, 956.89 [M + Na + H]²⁺, 945.90 [M + 2H]²⁺, Rᵣ = 0.26 (CHCl₃/MeOH/2.5 M NH₃ 50:50:16 v/v/v), and d20:1-lyso-GQ1b·4NH₃ (11) (0.97 mg, 44 %) from d20:1/18:0-GQ1b·4NH₃ (6), ESI-MS: m/z 1113.92 [M + 2Na]²⁺, 1102.93 [M + Na + H]³⁺, 1099.95 [M + NH₄ + H]³⁺, 1091.93 [M + 2H]²⁺, Rᵣ = 0.25 (CHCl₃/MeOH/2.5 M NH₃ 50:50:16 v/v/v).
**Synthesis of 2,5-dioxopyrrolidin-1-yl tetradecanoate (12)**

3.00 g (13.1 mmol) of tetradecanoic acid and 3.24 g of DCC (15.7 mmol) were dissolved in 26.2 mL of dry tetrahydrofuran and stirred at RT for 10 min. Then, 1.81 (15.7 mmol) of N-hydroxysuccinimide in 14.1 mL of the same solvent were added and the reaction mixture was stirred at RT for 22.5 h. TLC analysis (CHCl₃/MeOH 50:1 v/v) revealed a complete conversion of the educt. After this, the reaction mixture was filtrated by suction. The solvent was removed under reduced pressure and the residue was recrystallized from ethanol containing a trace of water (34). The product was obtained as a colorless solid (3.08 g, 72 %), ¹H-NMR (300 MHz, CDCl₃): δ [ppm] = 2.83 (s, 4H, H-3, 4), 2.60 (t, 2H, ³J_H2'-H3' = 7.5 Hz, H-2'), 1.74 (tt, 2H, ³J_H3'-H4' = 7.5 Hz, ³J_H3'-H2' = 7.5 Hz, H-3'), 1.46 – 1.18 (m, 20H), 0.88 (t, 3H, ³J_H14'-H13' = 6.7 Hz, H-14'), ¹³C-NMR (75 MHz, CDCl₃): δ [ppm] = 169.15 (RCON, C-2, 5), 168.68 (RCO₂R', C-1'), 31.90 (CH₂, C-2'), 30.93 (CH₂, C-12'), 29.63-28.77 (8 CH₂), 25.57 (CH₂, C-3, 4), 24.56 (CH₂, C-3'), 22.67 (CH₂, C-13'), 14.09 (CH₃, C-14'), EI-MS: m/z 211.3 [M⁺·C₄H₄NO₃⁻], 129.1 [C₈H₁₇O⁺], 98.2 [C₆H₁₄⁺], 84.1 [C₆H₁₂⁺], CHN analysis: calc.: C, 66.43; H, 9.60; N, 4.30, found: C, 66.03; H, 9.63; N, 4.29. The method was also applied for the synthesis of 2,5-dioxopyrrolidin-1-yl heptadecanoate (13) (0.897 g, 66 %), ¹H NMR (400 MHz, CDCl₃): δ [ppm] = 2.83 (s, 4H, H-3, 4), 2.59 (t, 1H, ³J_H2'-H3' = 7.5 Hz, H-2'), 1.74 (tt, 2H, ³J_H3'-H4' = 7.5 Hz, ³J_H3'-H2' = 7.5 Hz, H-3'), 1.45 – 1.19 (m, 26H), 0.88 (t, 3H, ³J_H17'-H16' = 6.9 Hz, H-17'), ¹³C-NMR (75 MHz, CDCl₃): δ [ppm] = 169.15 (RCON, C-2, 5), 168.68 (RCO₂R', C-1'), 31.90 (CH₂, C-2'), 30.93 (CH₂, C-15'), 29.66-28.78 (11 CH₂), 25.57 (CH₂, C-3, 4), 24.56 (C-3'), 22.67 (CH₂, C-16'), 14.10 (CH₃, C-17'), EI-MS: m/z 367.3 [M⁺], 253.3 [M⁺·C₄H₄NO₃⁻], 98.1 [C₇H₁₄⁺], 84.1 [C₆H₁₂⁺], CHN analysis: calc.: C, 68.63; H, 10.15; N, 3.81, found: C, 68.63; H, 10.14; N, 3.75.

**Reacylation of lysogangliosides**

All steps were performed under an argon atmosphere. 0.98 mg (0.610 µmol) of d18:1-lyso-GD1α·2NH₃ (8) and 7.6 µL (44.7 µmol) of Hünig’s base were dissolved in 62 µL of dry dimethylformamide. Then, 38 µL of the same solvent containing 1.43 mg (3.89 µmol) of 2,5-dioxopyrrolidin-1-yl heptadecanoate (13) were added. The reaction mixture was stirred at 30 °C for 28 h. Then, a few drops of 2.5 M ammonia were added,
the solvent was removed in a stream of nitrogen, the crude product was dried in vacuum and subsequently purified by NP column chromatography (2.0 × 20 cm, CHCl$_3$/MeOH/2.5 M NH$_3$ 60:40:10 v/v/v). After lyophilization, d18:1/17:0-GD1a·2NH$_3$ (16) was obtained as a colorless powder (0.98 mg, 86 %), ESI-MS: m/z 929.51 [M + 2NH$_4$]$^+$, 921.00 [M + NH$_4$ + H]$^+$, 912.48 [M + 2H]$^+$, $R_f = 0.18$ (CHCl$_3$/MeOH/2.5 M NH$_3$ 60:40:10 v/v/v). The method was also applied for the synthesis of d18:1/14:0-GM1·NH$_3$ (14) (6.32 mg, 28 %) from 7, ESI-MS: m/z 1490.72 [M + H]$^+$, 764.83 [M + K + H]$^+$, 754.37 [M + NH$_4$ + H]$^+$, 745.86 [M + 2H]$^+$, $R_f = 0.22$ (CHCl$_3$/MeOH/2.5 M NH$_3$ 60:40:9 v/v/v), d18:1/14:0-GD1a·2NH$_3$ (15) (0.88 mg, 42 %) from 8, ESI-MS: m/z 918.86 [M + K + NH$_4$]$^+$, 910.86 [M + Na + NH$_4$]$^+$, 908.39 [M + 2NH$_4$]$^+$, 899.88 [M + NH$_4$ + H]$^+$, 891.37 [M + 2H]$^+$, $R_f = 0.25$ (CHCl$_3$/MeOH/2.5 M NH$_3$ 60:44:12 v/v/v), d20:1/14:0-GD1b·2NH$_3$ (17) (1.28 mg, 70 %) from 9, ESI-MS: m/z 1809.92 [M + H]$^+$, 1964.44 [M + Na + H]$^+$, 905.45 [M + 2H]$^+$, $R_f = 0.21$ (CHCl$_3$/MeOH/2.5 M NH$_3$ 60:44:12 v/v/v), d20:1/14:0-GT1b·3NH$_3$ (18) (0.40 mg, 34 %) from 10, ESI-MS: m/z 1070.48 [M + K + H]$^+$, 1070.99 [M + Na + NH$_4$]$^+$, 1062.49 [M + Na + H]$^+$, 1051.50 [M + 2H]$^+$, $R_f = 0.29$ (CHCl$_3$/MeOH/2.5 M NH$_3$ 60:50:15 v/v/v), and d20:1/14:0-GQ1b·4NH$_3$ (19) (0.19 mg, 19 %) from 11, ESI-MS: m/z 1227.01 [M + K + Na]$^+$, 1216.02 [M + K + H]$^+$, 1208.03 [M + Na + H]$^+$, 1205.55 [M + NH$_4$ + H]$^+$, 1197.04 [M + 2H]$^+$, $R_f = 0.27$ (CHCl$_3$/MeOH/2.5 M NH$_3$ 50:50:16 v/v/v).

**Enzymatic degalactosylation of ganglioside standards**

1.34 mg (0.889 µmol) of d18:1/14:0-GM1·NH$_3$ (14) were dissolved in 410 µL of 100 mM McIlvaine buffer (pH 4.3), which contained 3.9 mM sodium taurodeoxycholate. Then, 200 µL of buffer containing 3.2 mM Triton™ X-100 and 41 µL of pure buffer were added. The mixture was exposed to an ultrasonic bath for 15 s. Then, 150 µL (134 µM) of beta-galactosidase solution were added. After 1d, TLC analysis (CHCl$_3$/MeOH/2.5 M NH$_3$ 50:40:9 v/v/v) revealed a conversion of approx. 50 %. Additional 150 µL of enzyme solution were added. After a further day, the conversion was complete and the reaction was stopped by addition of a few drops of 2.5 M ammonia. The solvent was removed in a stream of nitrogen and the residue was redissolved in 1 mL of methanol. The enzyme was separated by centrifugation (12100 × g),
which was repeated five times. The supernatant was separated and evaporated in a stream of nitrogen. The crude product was purified by preparative HPTLC (20 × 20 cm glass plate, CHCl₃/MeOH/2.5 M NH₃ 60:40:9 v/v/v). Silica gel was removed by the method described under “Purification”. After lyophilization, d18:1/14:0-GM2·NH₃ (20) was obtained as a colorless powder (1.84 mg, 77 % from two preparations), ESI-MS: m/z 1345.81 [M + NH₄]⁺, 1328.78 [M + H]⁺, 681.49 [M + 2NH₄]²⁺, 664.89 [M + 2H]²⁺, Rₛ = 0.27 (CHCl₃/MeOH/2.5 M NH₃ 60:40:9 v/v/v). The method was also applied for the preparation of d20:1/14:0-GD2·2NH₃ (21) from d20:1/14:0-GD1b·2NH₃ (17). For this conversion, the concentrations of detergents and enzyme were doubled. 21 was obtained as a colorless powder (0.25 mg, 61 %), ESI-MS: m/z 846.43 [M + 2Na]²⁺, 835.44 [M + Na + H]²⁺, 841.47 [M + 2NH₄]²⁺, 832.96 [M + NH₄ + H]²⁺, 824.45 [M + 2H]²⁺, Rₛ = 0.30 (CHCl₃/MeOH/2.5 M aq. NH₃ 60:44:12 v/v/v).

Synthesis of lysoganglioside standards

(E)-Hexacos-13-ene (22)

All steps were performed under an argon atmosphere. 200 mg (1.02 mmol) of 1-tetradecene and 36.7 mg (0.204 mmol, 20 mol%) of tetrafluoro-1,4-benzoquinone were dissolved in 2.8 mL of dry toluene. 6.33 mg (10.1 µmol, 0.99 mol%) of Hoveyda-Grubbs catalyst 2nd gen. [1,3-bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(o-isopropoxyphenylmethylene) ruthenium(II) were added and the reaction mixture was stirred at RT for 3 h. Ethylene was removed by a stream of argon during the reaction. The solvent was removed in a stream of nitrogen and the catalyst was removed by NP column chromatography. The crude product was applied to the column (1.0 × 4.0 cm) in hexane/EtOAc 80:1 v/v. The column was eluted by four column volumes of the same solvent. Product containing fractions were pooled, the solvent was removed in a stream of nitrogen and subsequently dried in vacuum. After recrystallization from 4 °C cold ethanol, 22 was obtained as a colorless solid (58.8 mg, 32 %), ¹H NMR (400 MHz, CDCl₃): δ [ppm] = 5.38 (tt, 2H, ³J_H13-H12 = 3.6 Hz, ⁴J_H13-H11 = 1.4 Hz, H-13, 14), 1.97 (m, 4H, H12, 15), 1.26 (m, 40H), 0.88 (t, 6H, ³J_H1-H2 = 6.8 Hz, H-1, 26), EI-MS: m/z 364.45 [M⁺], 111.15 [C₆H₁₅⁺], 97.10 [C₇H₁₃⁺], 83.10 [C₆H₁₁⁺],
69.00 [C₆H₆⁺], 57.10 [C₄H₇⁺], 55.05 [C₄H₉⁺], GC-MS: tᵣ 14.14 min, 100 %, M⁺ = 364.45, CHN analysis: calc.: C, 85.63; H, 14.37, found: C, 85.21; H, 14.11.

**Synthesis of pentadeca-O-acetyl-GM1 (d20:1/18:0) sialoyl-II²-lactone (23)**

All steps were performed under an argon atmosphere. 30.9 mg (19.4 µmol) of d20:1/18:0-GM1·NH₃ (2) and 0.5 mg (4.09 µmol) of DMAP were suspended in 832 µL of dry pyridine. 413 µL of acetic anhydride were added and the reaction mixture was stirred at RT for 15 h. It took a few hours for the reactants to dissolve completely. The solvent was removed in a stream of nitrogen and the crude product was purified by NP column chromatography (2.0 × 21 cm, toluene/acetone 5:4 v/v). After drying in vacuum, 23 was obtained as a colorless solid (26.3 mg, 62 %), ¹H NMR (400 MHz, CDCl₃): see table 2, ESI-MS: m/z 2220.10 [M + MeOH + H]⁺, 2188.07 [M + H]⁺, 1127.56 [M + MeOH + 2NH₄]²⁺, 1119.05 [M + MeOH + NH₄ + H]³⁺, 1110.54 [M + MeOH + 2H]²⁺, 1111.55 [M + 2NH₄]²⁺, 1103.04 [M + NH₄ + H]²⁺, 1094.53 [M + 2H]²⁺, Rᵣ = 0.23 (toluene/acetone 5:4 v/v).

**Synthesis of O-(tetradeca-O-acetylmnosialogangliotetraosyl)-(1→1)-(2R,3S,4E)-3-O-acetyl-2-octadecanoylamino-5-phenylpent-4-en-1-ol sialoyl-II²-lactone (24)**

All steps were performed under an argon atmosphere. 39.7 mg (18.3 µmol) of a 1:1 mixture of pentadeca-O-acetyl-GM1 (d20:1/18:0) sialoyl-II²-lactone (23) and pentadeca-O-acetyl-GM1 (d18:1/18:0) sialoyl-II²-lactone (M = 2173.41 gmol⁻¹) were dissolved in 477 µL of dry dichloromethane in a 1 mL glass vial. 33.0 mg (183 µmol) of (E)-stilbene and 23.9 mg (28.2 µmol) of Grubbs catalyst 2nd gen. [1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene]dichloro(phenylmethylene)(tricyclohexylphosphine)ruthenium(II) were added and the reaction mixture was stirred at 43 °C for 46 h. TLC monitoring (toluene/acetone 1:1 v/v) revealed a conversion of more than 90 %. The solvent was removed in a stream of nitrogen, the crude product was dried in vacuum and subsequently purified by NP column chromatography (2.0 × 22 cm, toluene/acetone 10:9 v/v). Traces of catalyst were removed by the method described under “Purification”. After drying in vacuum, 24 was obtained as a colorless solid (25.7 mg, 68 %), ¹H NMR (400
MHz, CDCl₃): see table 2, ESI-MS: m/z 2085.94 [M + MeOH + H]+, 2053.90 [M + H]+, 1060.46 [M + MeOH + 2NH₄]²⁺, 1051.95 [M + MeOH + NH₄ + H]²⁺, 1043.44 [M + MeOH + 2H]²⁺, 1044.45 [M + 2NH₄]²⁺, 1035.94 [M + NH₄ + H]²⁺, 1027.43 [M + 2H]²⁺, Rf = 0.15 (toluene/acetone 5:4 v/v).

Synthesis of pentadeca-O-acetyl-GM1 (d17:1/18:0) sialoyl-II²-lactone (25)

All steps were performed under an argon atmosphere. 15.5 mg (7.55 µmol) of 24, 55.0 mg (151 µmol) of (E)-hexacos-13-ene (22), and 3.43 mg (31.7 µmol, 20 mol%) of p-benzoquinone were dissolved in 271 µL of dry dichlormethane in a 1 mL glass vial. Then, 19.8 mg (31.6 µmol, 20 mol%) of Hoveyda-Grubbs catalyst 2nd gen. were added and the reaction mixture was stirred at 40 °C. After 1 d, TLC monitoring (toluene/actone 1:1 v/v) revealed a conversion of approx. 70 %. Further 19.8 mg of catalyst and 3.43 mg of p-benzoquinone were added and the reaction was stirred at 40 °C for another day. After a total of 41 h, the solvent was removed in a stream of nitrogen and the crude product was dried in vacuum. Subsequently, it was purified by NP column chromatography (2.0 × 23 cm, toluene/acetone 10:9 v/v). Traces of catalyst were removed by the method described under “Purification”. After drying in vacuum, 25 was obtained as a colorless solid (9.91 mg, 61 %), ¹H NMR (400 MHz, CDCl₃): see table 2, ESI-MS: m/z 2178.10 [M + MeOH + H]+, 2146.05 [M + H]+, 1106.56 [M + MeOH + 2NH₄]²⁺, 1098.04 [M + MeOH + NH₄ + H]²⁺, 1089.53 [M + MeOH + 2H]²⁺, 1090.54 [M + 2NH₄]²⁺, 1082.02 [M + NH₄ + H]²⁺, 1073.52 [M + 2H]²⁺, Rf = 0.20 (toluene/acetone 5:4 v/v).

Preparation of d17:1-lyso-GM1·NH₃ (26)

In the next step, pentadeca-O-acetyl-GM1 (d17:1/18:0) sialoyl-II²-lactone (25) was deprotected and regioselectively deacylated by the method described under “Chemical preparation of lysogangliosides”. Briefly, 9.40 mg (4.38 µmol) of 25 were applied. The reaction time was 19 h. The crude product was purified by RP column chromatography (0.9 × 2.8 cm, MeOH/H₂O 90:40 v/v). A second purification step was performed by NP column chromatography (2.0 × 19 cm, CHCl₃/MeOH/2.5 M NH₃ 60:40:10 v/v/v). MS analysis revealed that the product contained 4.8 % of the d16:1-lyso-GM1 isomer (see results section),
so the product was purified by isocratic RP column chromatography (1.0 × 22 cm, MeOH/H₂O 90:31 v/v),
again. After lyophilization, 26 was obtained as a colorless powder (1.93 mg, 34 %), ESI-MS: m/z 1266.60
[M + NH₄ + H]²⁺, 633.88 [M + 2H]²⁺, Rᵣ = 0.14 (CHCl₃/MeOH/2.5 M NH₃ 60:40:10 v/v/v).

Preparation of d17:1-lyso-GM2·NH₃ (27)
d17:1-Lyso-GM2·NH₃ (27) was prepared by the method described under “Enzymatic degalactosylation of
ganglioside standards”. Briefly, 1.09 mg (0.849 µmol) of d17:1-lyso-GM1·NH₃ (26) and 314 mU of beta-
galactosidase were applied. The reaction time was 15.5 h. The crude product was purified by NP column
chromatography (2.0 × 19 cm, CHCl₃/MeOH/2.5 M NH₃ 60:40:10 v/v/v). After lyophilization, 27 was
obtained as a colorless powder (0.66 mg, 69 %). ESI-MS: m/z 1126.53 [M + Na]⁺, 1104.54 [M + H]⁺,
563.76 [M + Na + H]²⁺, 561.28 [M + NH₄ + H]³⁺, 552.77 [M + 2H]³⁺, Rᵣ = 0.16 (CHCl₃/MeOH/2.5 M NH₃
60:40:9 v/v/v).

Validation of the lysoganglioside lipoforms as calibrators
Six different concentrations of pure d18:1-lyso-GM1·NH₃ and of pure d18:1-lyso-GM2·NH₃ (0 µM, 5 µM,
10 µM, 15 µM, 20 µM, 25 µM, and 35 µM) in methanol were prepared. The d18:1-lyso-GM1·NH₃ solutions
were spiked each by 26 to a final concentration of 13.4 µM of the internal standard and the d18:1-lyso-
GM2·NH₃ solutions were spiked by 27 to a concentration of 14.0 µM of the internal standard. The solutions
were measured in the full scan mode and the ratio of the peak height of the most intensive peak
([M + K + H]²⁺) was plotted against the concentration of the analyte (see Fig. 8 in the results section).
Results

Purification of bovine brain gangliosides to obtain starting materials

5.03 g of bovine brain gangliosides (Cronassial®) were separated into different ganglioside classes by anion-exchange chromatography. Monosialogangliosides were eluted by an ammonium acetate concentration of 0.05 mol/L. Disialogangliosides and trisialogangliosides were successively eluted by an ammonium acetate concentration of 0.15 mol/L and were separated without overlap. GQ1b was eluted by an ammonium acetate concentration of 0.45 mol/L and was already obtained in pure form after desalting. The recovery of the anion-exchange chromatography was 95 %. In the next step the ganglioside classes were purified by NP column chromatography using different techniques and different solvent systems. The disialogangliosides were separated into GD1a and GD1b. The purified gangliosides were analyzed by HPTLC, ESI-MS and ESI-MS/MS. The NP HPTLC showed only one band for each ganglioside (Fig. 2A). The RP-TLCs showed two distinct bands for each ganglioside (Fig. 2B, given for GQ1b), whereby the upper band corresponds to the d18:1/18:0-lipoform while the lower band corresponds to the d20:1/18:0-lipoform. In the next step the gangliosides were separated into their lipoforms by RP column chromatography using MeOH/H₂O in different compositions as eluent. The purity of the ganglioside lipoforms was demonstrated by RP-TLC and ESI-MS. Their structure was analyzed by ESI-MS/MS and ¹H-NMR. CHN analysis demonstrated a purity of 86-95 % compared to the theoretical value, which is due to residual water content (Table 1). The residual water content was 10-15 % when the gangliosides were dried by lyophilization and around 5 % when the gangliosides were dried in vacuum. An overview about the preparations which were performed in this work is given in figure 3.

Preparation of lysogangliosides

In this work one step preparations of lysogangliosides were applied. d18:1-Lyso-GM1·NH₃ was prepared by alkaline treatment of d18:1/18:0-GM1·NH₃ in a yield of 52 %, which is comparable to the yield of 54 % reported by Sonnino et al. (31). This method turned out to be not suitable for the regioselective deacylation of oligosialogangliosides: d18:1-lyso-GD1a·2NH₃ was obtained in a yield of only 4.8 % due to concomitant
deacetylation of sialic acid groups. Hence, we applied a biocatalyst to this reaction. The enzyme SCDase (EC 3.5.1.69) catalyzes the hydrolysis of the amide bond within the ceramide moiety of various glycosphingolipids, but not ceramides, in a reversible manner (35). We applied the method to d18:1/18:0-GD1a-2NH3 (3), d20:1/18:0-GD1b-2NH3 (4), d20:1/18:0-GT1b-3NH3 (5), and d20:1/18:0-GQ1b-4NH3 (6) (Table 3). Yields were in the range of 44 to 60 %. The ESI-MS spectrum of d18:1-lyso-GD1a showed no impurities of other GD1a lipoforms. By comparing the [M + 2H]2+ peaks it was found that d20:1-lyso-GD1b-2NH3 contained 3.1 % of d18:1-lyso-GD1b-2NH3 due to traces of d18:1/20:0-GD1b-2NH3 in the precursor, which could not be separated by RP column chromatography. Also, d20:1-lyso-GT1b-3NH3 contained 4.7 % of its d18:1-lipoform. For d20:1-lyso-GQ1b-4NH3 no other GQ1b-lipoforms were detected.

Reacylation of lysogangliosides

The lysogangliosides were reacylated by the method of Schwarzmann and Sandhoff (36) using N-hydroxysuccinimide esters of tetradecanoic acid and heptadecanoic acid. The reaction was applied to d18:1-lyso-GM1-NH3 (7), d18:1-lyso-GD1a-2NH3 (8), d20:1-lyso-GD1b-2NH3 (9), d20:1-lyso-GT1b-3NH3 (10), and d20:1-lyso-GQ1b-4NH3 (11). Yields were in the range of 19-86 % (Table 4). The ESI-MS of 14, 15, 16 (Fig. 4), and 19 showed no traces of other ganglioside lipoforms than the desired products. The ESI-MS of d20:1/14:0-GD1b-2NH3 (17) showed an impurity of 3.4 % of d18:1/14:0-GD1b-2NH3 and the ESI-MS of d20:1/14:0-GT1b-3NH3 (18) showed an impurity of 4.8 % of d18:1/14:0-GT1b-3NH3 as it was observed for their precursors.

Preparation of ganglioside standards by enzymatic degalactosylation

For the preparation of gangliosides that do not occur in larger amounts in bovine brain, we applied glycosidase-treatment to ganglioside standards already modified in their lipid part. The commercially available enzyme beta-galactosidase (EC 3.2.1.23) from bovine testes can hydrolyze terminal galactosyl residues in β1-3, β1-4, and β1-6 linkage from saccharides, glycosaminoglycans, glycoproteins, and
glycolipids (37). The reaction was applied to the preparation of d18:1/14:0-GM2·NH$_3$ (20) from d18:1/14:0-GM1·NH$_3$ (14) and d20:1/14:0-GD2·2NH$_3$ (21) from d20:1/14:0-GD1b·2NH$_3$ (16). The preparations had yields of 77% and 61%, respectively (Table 5). The lower yield for the preparation of GD2 derivatives is due to a slight desialylation of GD1b, which occurs at the applied pH of 4.5. This was demonstrated by applying the reaction conditions described to GD1b in the absence of enzyme. The ESI-MS of 20 showed no traces of other GM2 lipoforms than the desired product. The ESI-MS of 21 showed an impurity of 3.2% of d18:1/14:0-GD2·2NH$_3$ as already observed for the precursor.

Modification of the sphingosine chain of gangliosides

The olefin cross metathesis is a suitable reaction for the modification of the sphingosine chain of gangliosides and glycosphingolipids because it tolerates hard nucleophiles and electrophiles and it is performed under neutral conditions. The following synthetic steps were performed for the synthesis of d17:1-lyso-GM1·NH$_3$ (26) from d20:1/18:0-GM1·NH$_3$ (2) (Fig. 5). Our approach includes a stilbenolysis followed by the introduction of a new alkyl chain. We did not choose an ethenolysis because reported yields (tested by the model methyl oleate using standard Ru-based catalysts) are only in the range of 13 to 57% (38) and 13 to 24% (39), respectively. In the recent years, more effective catalysts for ethenolysis have been developed, which give yields up to 95% (40) resp. 80% (41) but only for (Z)-olefins. These catalysts are much more reactive towards (Z)-olefins than towards (E)-olefins because the residues of the olefin are forced into the same direction in the metallacyclobutane formation by bulky ligands (42). A direct reaction of a peracetylated ganglioside lactone with 13-hexacosene is impracticable because of separation problems.

The procedure illustrated in Fig. 5 has three advantages: First, the phenyl group facilitates the chromatographic purification because it is more polar than an alkyl group. Second, the phenyl group serves as an UV-probe. Third, stilbene is not prone to isomerizations because the phenyl group stops the migration of the double bond. In the first step 2 was peracetylated by a modified method of Schwarzmann et al. (43). These reaction conditions led to the formation of lactone 23 in a yield of 62%. In the ESI-MS spectrum only peaks of the product and the methyl ester of the product, which is formed by opening of the lactone
by methanol, which is used as solvent in the measurement, were found. Compound 23 was converted to 24 by a tenfold excess of (E)-stilbene and 14 mol% of Grubbs catalyst 2nd gen. in a yield of 68 %. The 1H-NMR spectrum demonstrated that selectively the E-isomer was formed due to the bulky residues. (E)-Hexacosene (22) was synthesized by an olefin cross metathesis of 1-tetradecene because it is commercially not available. It was observed that only a statistical mixture of constitutional isomers is obtained if no hydride scavenger is used. Presence of 20 mol% of p-benzoquinone leads to an isomeric purity of 98 % as analyzed by GC-MS. By using 20 mol% of tetrafluoro-1,4-benzoquinone an isomeric purity of 100 % was obtained. In the 1H-NMR and 13C-NMR spectra, only one signal for the olefinic protons was observable so we concluded that selectively the E-isomer was formed. Then, 24 was converted to 25 by a twentyfold excess of 22 in a yield of 61 %. Hereby, also selectively the E-isomer was formed. To optimize the conditions for the formation of 25 we tested the more effective hydride scavengers tetrafluoro-1,4-benzoquinone and 2,6-dichloro-1,4-benzoquinone as well as the Stewart-Grubbs catalyst, which contains N-tolyl groups instead of N-mesityl groups in the N-heterocyclic carbene ligand (Fig. 6). This catalyst is reported to be more active than the Hoveyda-Grubbs catalyst 2nd gen. in cross metathesis reactions of sterically challenging olefins (44). The results are given in table 6. Unexpectedly, the more active hydride scavengers tetrafluoro-1,4-benzoquinone and 2,6-dichloro-1,4-benzoquinone suppressed the catalyst activity even when they were added in low amounts. Furthermore, the Stewart-Grubbs catalyst showed no activity towards 24 regardless of the hydride scavenger tested. So the most effective reaction conditions are 20 mol% of Hoveyda-Grubbs catalyst 2nd gen. and 20 mol% of p-benzoquinone. In the last step the protecting groups and also the acyl chain were removed from 25 by using the chemical deacylation method to prepare d17:1-lyso-GM1·NH3 (26). The yield was 34 %. The ESI-MS showed that the product contained 4.8 % of d16:1-lyso-GM1·NH3, which indicates that isomerizations could not be completely suppressed by p-benzoquinone, but to an extent larger than 95 %. Other isomers were not found. The isomer was removed by isocratic RP column chromatography. A proportion of 26 was used for the preparation of d17:1-lyso-GM2·NH3 (27) by the described enzymatic degalactosylation method. The yield was 69 % (Fig. 7).
As a proof of principle, the synthesized lysoganglioside standards 26 and 27 were tested for the quantification of pure natural lysogangliosides (Fig. 8). Good linearity was obtained for both compounds in the applied concentration range. As expected, quantification of pure substances is possible in the full scan mode but for biological samples MS/MS modes have to be applied.
Discussion

Partial synthesis of non-natural ganglioside lipoforms

For the synthesis of internal ganglioside standards, we chose a partial synthesis rather than a total synthesis because of the high effort necessary for the latter. Starting materials were obtained by purification of a ganglioside mixture from bovine brain. It was confirmed by ESI-MS that bovine brain gangliosides mainly contain d18:1/18:0- and d20:1/18:0-lipoforms. Usually, the purity of gangliosides is determined by fluorimetric (45) or photometric (28, 29) sialic acid determination, sphingoid base determination by HPLC (46), GC-FID (30) or densitometry (47). We analyzed the purified ganglioside lipoforms by elemental analysis and used these substances as external standards for fluorimetric and photometric sialic acid determination. The results of the CHN analysis demonstrated that there is residual water content of 10-15% in the gangliosides after lyophilization. By drying in vacuum, it can be reduced to 5%. For modification of the fatty acid acyl chain, we followed a chemical and an enzymatical deacylation approach. The results of the chemical deacylation demonstrated that this method is appropriate for the selective deacylation of GM1, but inappropriate for the preparation of oligosialolysogangliosides. The enzymatic deacylation turned out to be more appropriate: yields were in the range of 44 to 60% for the preparation of lyso-GD1a (8), lyso-GD1b (9), lyso-GT1b (10), and lyso-GQ1b (11). This is comparable to the results of Ando et al. (48), who reported yields of 62% resp. 52% for the preparation of lyso-GM1 and lyso-GM2. Due to the occurrence of traces of d18:1/20:0-lipoforms in the starting material, which could not be separated from the d20:1/18:0-lipoforms by RP column chromatography, the prepared d20:1-lyso-GD1b·2NH₃ (9) and d20:1-lyso-GT1b·3NH₃ (10) contained 3.1% resp. 4.7% of their d18:1-lipoform. Since they do not disturb the mass spectrometric application of the standards, we decided not to remove these minor byproducts. The reacylation of the lysogangliosides was carried out in yields in the range of 19 to 86%. In general, the reaction works accurate for monosialolysogangliosides and disialolysogangliosides with yields up to 86%. For lyso-GT1b and lyso-GQ1b, the yields drops to 34% and 19%, respectively. An optimization of the reaction conditions for these substrates should be performed in the future.
For the enzymatic degalactosylation of d18:1/14:0-GM1·NH$_3$ (7) a yield of 77 % was obtained, which is higher than the yield of 54 % obtained by Larsson et al. (49) for a fluorescence labeled GM1 substrate. For the degradation of d17:1-lyso-GM1·NH$_3$ (26) to d17:1-lyso-GM2·NH$_3$ (27), a comparable yield of 69 % was obtained. The method is also applicable to GD1b derivatives although we observed a slight degradation to GM1 due to the low pH value. Hereby, a sufficient yield of 61 % was obtained for the degradation of d20:1/14:0-GD1b·2NH$_3$ (17) to d20:1/14:0-GD2·2NH$_3$ (21). As far as we know, this method has not been applied to lyso-GM1 and GD1b derivatives in the literature.

**Development of a new synthetic method to modify the sphingosine chain of gangliosides**

We developed a synthetic method based on the olefin cross metathesis for the preparation of gangliosides and lysogangliosides with homogenous and, if required, artificial sphingoid bases. Advantages of this method are a simplified chromatographic separation by introduction of a phenyl group, the opportunity to use gangliosides heterogeneous in their sphingosine part as educts, and the applicability of the method for the synthesis of lysoganglioside standards. A drawback of the method is that isomerizations can only be suppressed in a range of 95 %. A direct modification of lysogangliosides by an olefin metathesis is impracticable because the reaction does not tolerate amino groups.

We are aware of only two methods for the partial synthesis of ganglioside lipoforms as MS-standards in the literature (Fig. 9). The method by Neuenhofer et al. (36, 50) has a total yield of approx. 29 % for the synthesis of, for example, d18:1/17:0-GM1 from d18:1/18:0-GM1. It includes a total deacylation of the ganglioside except the N-acetylgalactosamine residue followed by a regioselective protection of the amino group in the sphingosine part. Subsequently, the remaining amino groups are reacetylated and the protecting group is removed. The enzymatic deacylation (32, 51) followed by chemical reacylation has the highest overall yield of approx. 42 %. The olefin metathesis method for the synthesis of, for example, d17:1/18:0-GM1 from GM1 has a total yield of approx. 23 % if the yield for the removal of the protecting groups is estimated by 90 %. But in contrast to our method the other methods require homologous pure gangliosides as starting materials. Current experiments in our group indicate that the method is applicable to
peracetylated disialogangliosides, but eventually not to trisialogangliosides because they might be too unreactive in olefin metathesis reactions. Furthermore, an application to neutral glycosphingolipids and ceramides should be possible with even better yields because they are more reactive in olefin metathesis reactions.

Conclusion

Analytical and functional studies of ganglioside lipoforms is an emerging area of research (1, 14, 15). We provide a protocol for the preparation of artificial ganglioside and lysoganglioside lipoforms starting from a ganglioside mixture. It relies on selective chemical and enzymatical deacylation steps, reacylation of the sphingosine part and also selective degalactosylation. These methods were used for the preparation of new internal standards for GM1, GM2, GD2, GD1a, GD1b, GT1b, and GQ1b. We also developed a new olefin metathesis method, which was used for the synthesis of a lyso-GM1 and a lyso-GM2 standard. These compounds are suitable as calibrator substances for the mass spectrometric determination of gangliosides and lysogangliosides and can also be applied for functional studies.

Up to know, no method was reported for the modification of the sphingosine part of gangliosides. The method should also be suited for isotope labeling of the sphingosine part of sphingolipids and gangliosides because the phenyl group in intermediates of the metathesis reaction facilitates the chromatographic separation of educts and products. Furthermore, this method may also be used to incorporate fluorescent dyes into the sphingosine part.
Acknowledgments/grant support

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References


Footnotes to text

1This nomenclature designates the sphingosine part of gangliosides by the number of hydroxyl groups (m = mono, d = di, t = tri) and the number of carbon: number of double bonds. The same is applied to the N-linked fatty acid, whose description is separated by a slash. Furthermore, the ganglioside nomenclature recommended by Svennerholm and the IUPAC-IUBMB nomenclature is used.
Figure and table legends

Figure 1: Possible concepts for the synthesis of internal ganglioside standards.

Figure 2: HPTLC analysis of the purified gangliosides. A: NP TLC of GM1, GD1a, GD1b, GT1b, and GQ1b. Solvent system: CHCl₃/MeOH/0.2 % CaCl₂ 50:42:11 v/v/v. B: RP TLC of GQ1b. Solvent system: MeOH/H₂O 90:20 v/v.

Figure 3: Overview about the preparations performed in this work.

Figure 4: ESI-MS of d18:1/17:0-GD1a·2NH₃ (16)

Figure 5: Synthetic approach for the modification of the sphingosine part of gangliosides

Figure 6: Important achiral olefin metathesis catalysts. A: Grubbs catalyst 2nd gen. B: Hoveyda-Grubbs catalyst 2nd gen. C: Stewart-Grubbs catalyst.

Figure 7: ESI-MS of d17:1-lyso-GM2·NH₃ (27)

Figure 8: Quantification of pure lyso-GM1·NH₃ and lyso-GM2·NH₃. A concentration series of pure d18:1-lyso-GM1 and d18:1-lyso-GM2 was quantified by using the internal standards d17:1-lyso-GM1·NH₃ (26) and d17:1-lyso-GM2·NH₃ (27).

Figure 9: Comparison of published methods for the partial synthesis of ganglioside lipoforms to our new method. On the left the method of Neuenhofer et al. is shown. The first three steps can be replaced by
the enzymatic deacylation method, which is shown in the center. The olefin metathesis method developed by us is shown on the right.

**Table 1:** Pure ganglioside lipoforms that were obtained by purification of a bovine brain gangliosides mixture. The results of the CHN analysis are given in column 3. The gangliosides were not hundred percent pure due to residual water content. Compound 2 was dried in vacuum while the other compounds were dried by lyophilization.

**Table 2:** Characteristic $^1$H-NMR chemical shifts of compounds 23, 24, and 25. The olefinic $^3J$ coupling constants are given in brackets.

**Table 3:** Results of the enzymatic deacylation of gangliosides. The required amount of the enzyme SCDase for a conversion larger than 90 % is given in column 2.

**Table 4:** Ganglioside standards that were synthesized by deacylation and reacylation of the fatty acid of gangliosides. The yields of the reacylation step are given in column 3.

**Table 5:** Ganglioside standards that were prepared by enzymatic degalactosylation. The required amount of the enzyme beta-galactosidase for a conversion larger than 90 % is given in column 2.

**Table 6:** Optimization of the reaction conditions for the synthesis of compound 25 from compound 24. The olefin metathesis catalysts tested are given in column 1. They were applied in 20 mol%. (E)-Hexacos-13-ene (22) was applied in tenfold excess. The different hydride scavengers tested are given in column 2. Conversions of 24 to 25, which are given in column 3, were estimated by TLC after 1 d of reaction time.
### TABLE 1

<table>
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<th>Ganglioside</th>
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<th>Purity (%)</th>
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</tr>
<tr>
<td>d20:1/18:0-GT1b·3NH3 (5)</td>
<td>40.4</td>
<td>86.9</td>
</tr>
<tr>
<td>d20:1/18:0-GQ1b·4NH3 (6)</td>
<td>18.8</td>
<td>85.7</td>
</tr>
</tbody>
</table>
### TABLE 2

<table>
<thead>
<tr>
<th>Protons</th>
<th>23</th>
<th>24</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Olefinic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cer, H-5</td>
<td>5.76 ($^3J = 15.1$ Hz)</td>
<td>6.65 ($^3J = 15.9$ Hz)</td>
<td>5.77 ($^3J = 15.2$ Hz)</td>
</tr>
<tr>
<td>Cer, H-4</td>
<td>unresolved</td>
<td>6.09 ($^3J = 15.9$ Hz)</td>
<td>unresolved</td>
</tr>
<tr>
<td><strong>Anomeric</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glc</td>
<td>4.76</td>
<td>4.76</td>
<td>4.76</td>
</tr>
<tr>
<td>Gal (II)</td>
<td>unresolved</td>
<td>unresolved</td>
<td>unresolved</td>
</tr>
<tr>
<td>Gal (IV)</td>
<td>5.04</td>
<td>5.04</td>
<td>5.04</td>
</tr>
<tr>
<td>GalNAc</td>
<td>5.63</td>
<td>5.74</td>
<td>5.64</td>
</tr>
<tr>
<td><strong>Methyl</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkyl chain</td>
<td>0.87</td>
<td>0.87</td>
<td>0.87</td>
</tr>
<tr>
<td>GalNAc</td>
<td>1.87</td>
<td>1.89</td>
<td>1.89</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>1.96</td>
<td>1.96</td>
<td>1.96</td>
</tr>
<tr>
<td>O-Ac</td>
<td>2.01-2.18</td>
<td>2.02-2.18</td>
<td>2.02-2.18</td>
</tr>
</tbody>
</table>

### TABLE 3

<table>
<thead>
<tr>
<th>Lysoganglioside</th>
<th>$n_\text{SCDase}/n_\text{substrate}$</th>
<th>Amount of lyso-ganglioside (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d18:1-lyso-GD1a·2NH$_3$ (8)</td>
<td>110</td>
<td>0.74</td>
<td>47</td>
</tr>
<tr>
<td>d20:1-lyso-GD1b·2NH$_3$ (9)</td>
<td>110</td>
<td>1.72</td>
<td>53</td>
</tr>
<tr>
<td>d20:1-lyso-GT1b·3NH$_3$ (10)</td>
<td>73.6</td>
<td>1.16</td>
<td>60</td>
</tr>
<tr>
<td>d20:1-lyso-GQ1b·4NH$_3$ (11)</td>
<td>74.3</td>
<td>0.97</td>
<td>44</td>
</tr>
<tr>
<td>Ganglioside standard</td>
<td>Amount (mg)</td>
<td>Yield (%)</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>-------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>d18:1/14:0-GM1·NH₃ (14)</td>
<td>6.32</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>d18:1/14:0-GD1a·2NH₃ (15)</td>
<td>0.88</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>d18:1/17:0-GD1a·2NH₃ (16)</td>
<td>0.98</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>d20:1/14:0-GD1b·2NH₃ (17)</td>
<td>1.28</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>d20:1/14:0-GT1b·3NH₃ (18)</td>
<td>0.40</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>d20:1/14:0-GQ1b·4NH₃ (19)</td>
<td>0.19</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Product</th>
<th>n_{\text{beta-galactosidase}}/n_{\text{substrate}}</th>
<th>Amount (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d18:1/14:0-GM2·NH₃ (20)</td>
<td>300</td>
<td>1.84</td>
<td>77</td>
</tr>
<tr>
<td>d20:1/14:0-GD2·2NH₃ (21)</td>
<td>359</td>
<td>0.25</td>
<td>61</td>
</tr>
</tbody>
</table>
### TABLE 6

<table>
<thead>
<tr>
<th>Catalyst (20 mol%)</th>
<th>Hydride scavenger</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoveyda-Grubbs cat. 2nd gen.</td>
<td>$p$-benzoquinone (40 mol%)</td>
<td>30</td>
</tr>
<tr>
<td>Hoveyda-Grubbs cat. 2nd gen.</td>
<td>$p$-benzoquinone (20 mol%)</td>
<td>60</td>
</tr>
<tr>
<td>Hoveyda-Grubbs cat. 2nd gen.</td>
<td>tetrafluoro-1,4-benzoquinone (40 mol%)</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Hoveyda-Grubbs cat. 2nd gen.</td>
<td>tetrafluoro-1,4-benzoquinone (20 mol%)</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Hoveyda-Grubbs cat. 2nd gen.</td>
<td>tetrafluoro-1,4-benzoquinone (1 mol%)</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Hoveyda-Grubbs cat. 2nd gen.</td>
<td>2,6-dichloro-1,4-benzoquinone (20 mol%)</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Hoveyda-Grubbs cat. 2nd gen.</td>
<td>2,6-dichloro-1,4-benzoquinone (5 mol%)</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Hoveyda-Grubbs cat. 2nd gen.</td>
<td>2,6-dichloro-1,4-benzoquinone (1 mol%)</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Stewart-Grubbs cat.</td>
<td>$p$-benzoquinone (20 mol%)</td>
<td>0</td>
</tr>
<tr>
<td>Stewart-Grubbs cat.</td>
<td>tetrafluoro-1,4-benzoquinone (20 mol%)</td>
<td>0</td>
</tr>
<tr>
<td>Stewart-Grubbs cat.</td>
<td>2,6-dichloro-1,4-benzoquinone (20 mol%)</td>
<td>0</td>
</tr>
</tbody>
</table>
Figures

**FIGURE 1**

![Chemical structure of GM1](image)

Modify by olefin metathesis

Modify by deacylation

**FIGURE 2**

![Gel electrophoresis images](image)

GM1  GD1a  GD1b  GT1b  GQ1b

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GM1</td>
</tr>
<tr>
<td>2</td>
<td>GD1a</td>
</tr>
<tr>
<td>3</td>
<td>GD1b</td>
</tr>
<tr>
<td>4</td>
<td>GT1b</td>
</tr>
<tr>
<td>5</td>
<td>GQ1b</td>
</tr>
</tbody>
</table>

**A**

**B**

- d18:1/18:0-GQ1b
- d20:1/18:0-GQ1b

GQ1b
FIGURE 6

A

B

C

FIGURE 7
FIGURE 9

\[
\begin{align*}
\text{d18:1/18:0-GM1} \quad & \xrightarrow{\text{alkaline hydrolysis}} \quad \text{GM1} \\
& \quad \xrightarrow{\text{peracetylation}} \\
\text{deacetyl d18:1-lyso-GM1} \quad & \xrightarrow{\text{1. FmocCl}} \quad \text{pentadeca-O-acetyl-GM1 sialoyl-I-II-lactone} \\
& \quad \xrightarrow{\text{2. Ac}_2\text{O}} \\
& \quad \xrightarrow{\text{SCDase}} \\
\text{N-Fmoc-d18:1-lyso-GM1} \quad & \quad \text{+ exc. stilbene} \\
& \quad \text{Grubbs cat. 2nd gen.} \\
& \quad \text{68 %} \\
& \quad \text{+ NH}_3(\text{l}) \\
& \quad \text{73 %} \\
\text{d18:1-lyso-GM1} \quad & \xrightarrow{\text{reacylation}} \\
& \quad \text{80 %} \\
\text{d18:1/17:0-GM1} \quad & \xrightarrow{\text{alkaline hydrolysis}} \\
& \quad \text{d17:1/18:0-GM1}
\end{align*}
\]

\[O-(\text{tetradeca-O-acetylmnosialogangliotetraosyl})-(1\"1)-(2R,3S,4E)-3-O-acetyl-2-octadecanoylamino-5-phenylpent-4-en-1-ol sialoyl-I-II-lactone\]

\[\text{pentadeca-O-acetyl-GM1 (d17:1/18:0) sialoyl-I-II-lactone}\]

\[\text{Hoveyda-Grubbs cat. 2nd gen.} \quad 61 \%
\]