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JOURNAL OF NEUROCHEMISTRY, vol. 34, no. 635, June 1980, pages 1351-1361, Raven Press, New York (USA); S.K. GROSS et al.: "Alkali-labile, Sodium borohydride-reducible ganglioside sialic acid residues in brain"

BULLETIN OF MOLECULAR BIOLOGY AND MEDICINE, vol. 3, September 1978, pages 170-172, Idelson Scientific, Napoli (IT); S. SONNINO et al.: "Occurrence in mouse brain of a new ganglioside carrying and alkali labile linkage"

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(56) References cited:
JOURNAL OF NEUROCHEMISTRY, vol. 28, 1977, pages 1133-1136, Pergamon Press, (GB); "Short communication. Formation of ganglioside internal esters by treatment with trichloroacetic acid-phosphotungstic acid reagent"

CARBOHYDRATE RESEARCH, vol. 41, 1975, pages 344-350, Elsevier Scientific Publishing Company, Amsterdam (NL); S.K. GROSS et al.: "Identification of the internal acetal 5-acetamido-2,7-anhydro-3,5-dideoxy-D-glycero-nonulopyranose"

Courier Press, Leamington Spa, England.

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Description

The present invention relates to a method for preparing inner ester derivatives of gangliosides and pharmaceutical compositions containing such derivatives. The pharmaceutical compositions of the present invention are used to treat disorders of the nervous system resulting from accidents or diseases which have in some way damaged the nerve tissue.

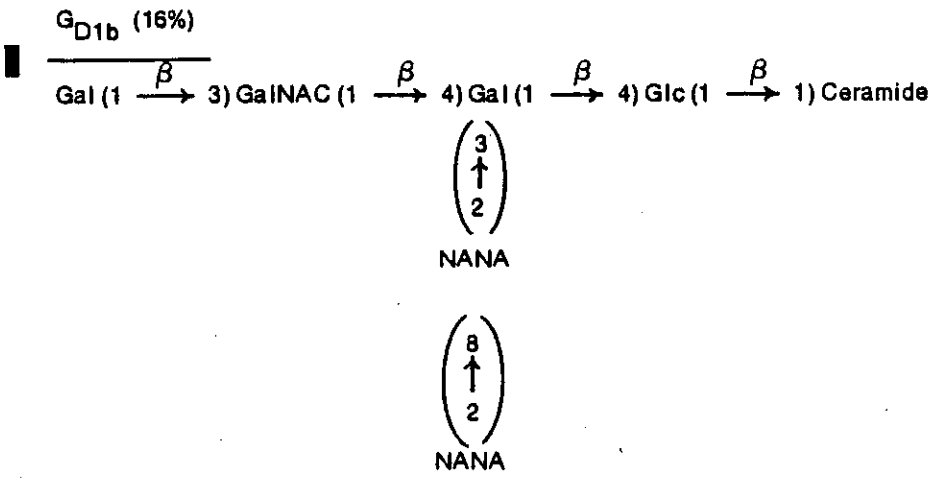
Gangliosides are a group of glycosphingolipids and have a structure containing a carbohydrate portion to which is linked a ceramide and a sialic acid moiety. The carbohydrate portion includes at least one galactose or glucose moiety and at least one N-acetylglucosamine or N-acetylgalactosamine moiety.

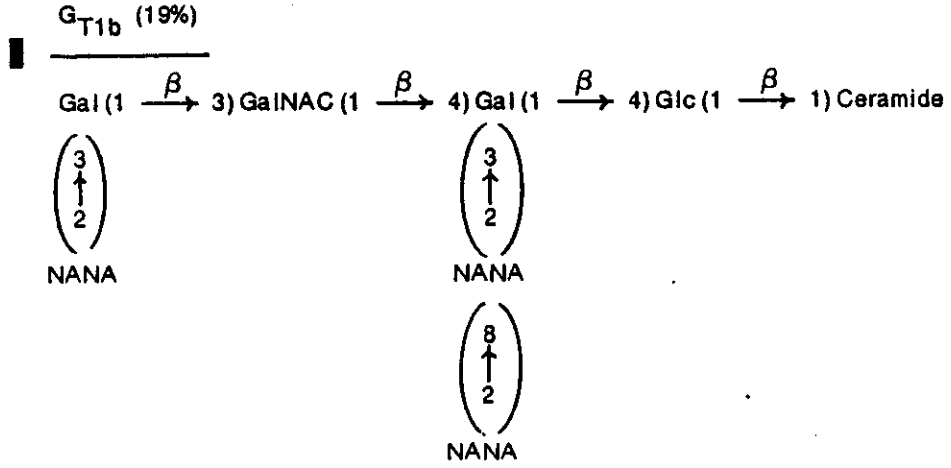
The general structure of a ganglioside can then be represented by the following formula:

one mole of a sialic acid {
- one mole of ceramide
- at least one mole of galactose or glucose
- at least one mole of N-acetylglucosamine or N-acetylgalactosamine

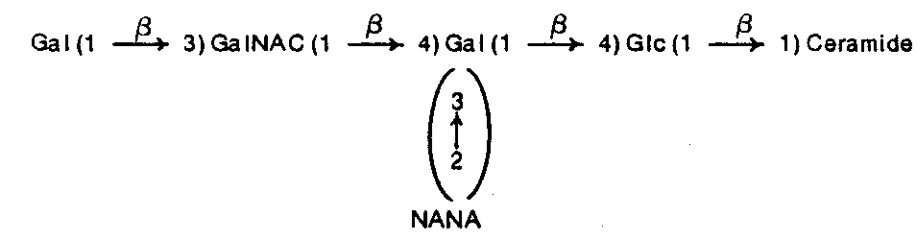
where all of the moieties are linked by a glucosidic bond.

Numerous gangliosides have been identified and have been found to be particularly abundant in nerve tissue, especially in brain tissue. Various studies have shown that the most important of the sialic acids found in gangliosides are N-acetyl-neuraminic acid (NANA) and, to a lesser degree, N-glycolylneuraminic acid. Of the numerous gangliosides which have been identified, the following gangliosides, labeled by their international symbols, have been found to exist in significant amount in ganglioside mixtures extracted from bovine brain tissue:

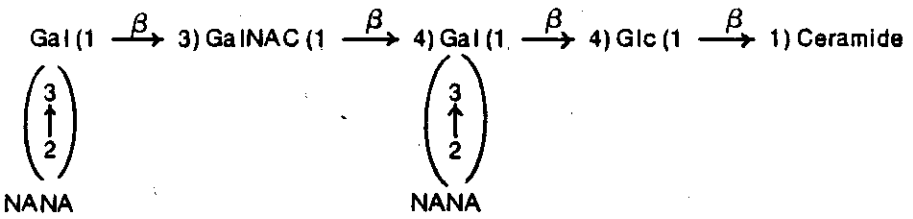




G_{M1} (21%)



G_{D1a} (40%)



where:

Glc represents glucose,

GalNAC represents N-acetylgalactosamine,

Gal represents galactose,

NANA represents N-acetyl-neuraminic acid,

and the percentages in parenthesis indicate the amount of each ganglioside found in the ganglioside mixture extracted from bovine brain tissue.

It is known that gangliosides play an important role in the nervous system and it has recently been demonstrated that gangliosides are useful in the treatment of disorders of the peripheral nervous system and pathologies of the central nervous system—

[Acta psychiat. Scand., *55,* 102, (1977);
Eur. Med. Phys., *13,* 1, (1977);
Ric. Sci. Educ. Perm. *9,* 115, (1978);
Adv. Exp. Biol. *71,* 275, (1976);
Electromyogr. Clin. Neurophysiol., *19,* 353, (1979);
Minerva Medica, *69,* 3277, (1978);
Minerva Stomat., *27,* 177, (1978);
Med. del Lavoro, *68,* 296 (1977);
Brain Res. *197,* 236, (1980)].

The therapeutic action of the gangliosides appears to consist mainly in stimulating sprouting phenomena in the nerve tissue and in activating the membrane enzymes involved in the conduction of nervous stimuli, such as the enzyme (Na^+,K^+)ATPase [Brain Res., *197,* 236, (1980); Leon et al. J. of Neurochem., *37,* 350 (1981)]. Nerve sprouting stimulated by the gangliosides will then encourage regeneration and healing of damaged nerve tissue.

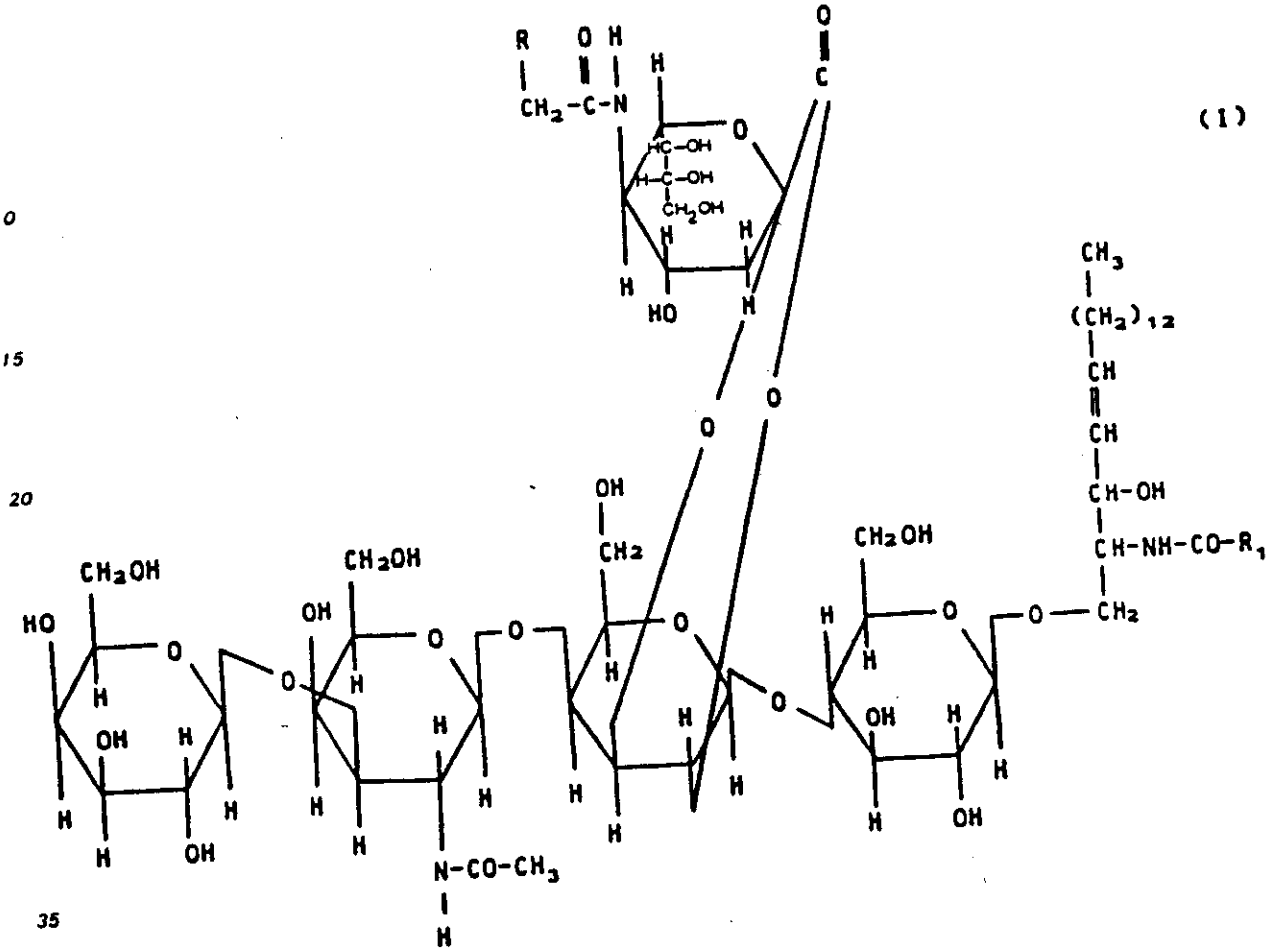
Further studies have been directed to finding compounds which may be even more effective than the gangliosides in treating disorders of the nervous system.

It has now been determined according to the present invention that certain derivatives of gangliosides are more active than the gangliosides themselves in stimulating nerve sprouting and in activating the enzymes of membranes involved in the conduction of the nervous stimulus, such as the enzyme (Na^+,K^+)ATPase. Specifically, it has been found that the inner ester derivatives of gangliosides are particularly active in treating disorders of the nervous system and are more active than the starting parent gangliosides. *In vitro* and *in vivo* testing have shown that the inner ester derivatives are superior to the parent gangliosides in stimulating nerve sprouting and in activating the (Na^+,K^+)ATPase membrane enzyme involved in nerve conduction.

Only some of the possible inner ester derivatives of the gangliosides have thus far been isolated and these only in very small quantities in brain tissue.

The inner esters of gangliosides are formed by the reaction between the carboxyl group of a sialic acid moiety with a hydroxyl group of one of the carbohydrate moieties or another adjoining sialic acid within the same ganglioside molecule [J. of Neurochemistry, *34,* 1351, (1980); Bull. of Molecular Biology and Medicine, *3,* 170, (1978)].

For exemplary purposes, one possible inner ester derivative of a ganglioside could be represented by the following structure:



(I)

wherein:

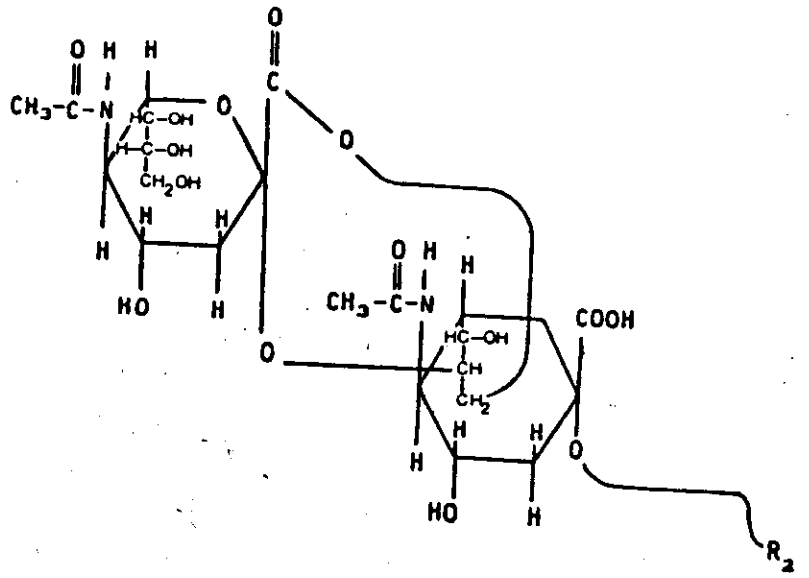
R in the sialic acid moiety is H or OH, and

R_1 in the ceramide group is a fatty acid such as oleic, stearic or linoleic acid.

The inner ester ganglioside derivative (I) is an example of derivative in which the carboxyl group of the sialic acid is ester bonded to a hydroxyl group of one of the carbohydrate moieties, specifically galactose. The formation of the inner ester bond, together with the normal glucosidic bond between the sialic acid and carbohydrate moiety, creates a lactonic ring, typically five or six-membered, characteristic of the structure of the inner ester ganglioside derivatives. While Formula (I) has been shown for exemplary purposes, it is to be noted that other lactonic rings having 5 or more membered ring structures could be formed as the sialic acid carboxyl group ester bonds with the hydroxyl group of a carbohydrate moiety.

As noted above, the inner ester ganglioside derivatives can also be formed when the carboxyl group of a sialic acid ester bonds to an adjoining sialic acid to which it is glucosidically bonded in the starting parent ganglioside.

Such a structure could be represented by the following formula:

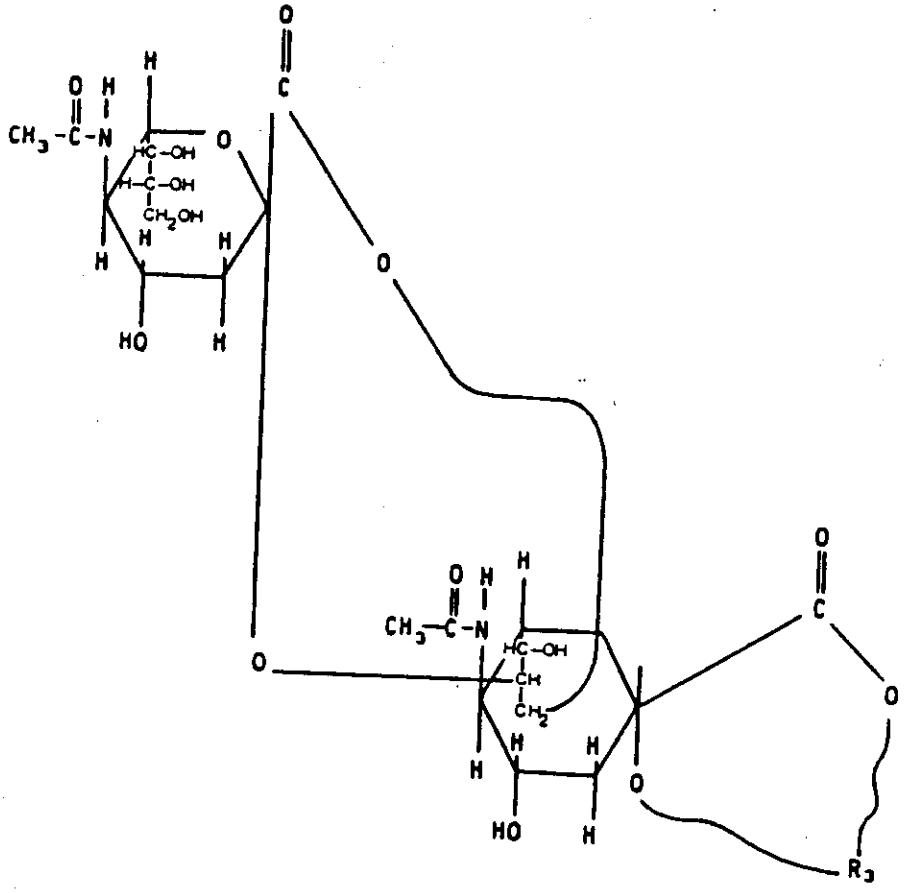


(II)

wherein:

R_2 represents the carbohydrate moiety which is glucosidally linked to the sialic acid moiety.

Another possible inner ester ganglioside derivative could be represented by the following formula:



(III)

wherein:

R_3 represents the carbohydrate moiety to which the adjoining sialic acid is ester bonded.

Formula (III) then represents an inner ester ganglioside derivative in which a sialic acid is ester bonded to an adjoining sialic acid which is itself ester bonded to a carbohydrate moiety.

It is therefore apparent that many variations of the above described derivatives could be formed, so that the inner ester derivatives of gangliosides are generally formed of a carbohydrate portion, at least one ceramide and at least one sialic acid moiety wherein one or more of the sialic acids are ester bonded to a carbohydrate moiety and/or one or more of the sialic acids are ester bonded to an adjoining sialic acid.

Numerous inner ester derivatives of gangliosides are thus possible, of which the above described are shown for exemplary purposes only.

Method of Preparation

Some prior art methods for the preparation of inner ester ganglioside derivatives are known and include the following:

1. The formation of internal esters by simply allowing the gangliosides to stand in an acetic or trichloroacetic acid solution [Sphingolipids, Sphingolipidoses and Allied Disorders, Adv. Exp. Med. Biol. *19*, 95 (1972); J. Neurochem. *28*, 1133, (1977)].

According to this method, it is necessary to operate at a very high ratio of acetic acid to ganglioside and a complete transformation of the gangliosides is not obtained.

For this reason, a final purification step is necessary and is usually performed by an ion exchange resin, such as Sephadex®.

2. The reaction of a water soluble carbodiimide with gangliosides in an aqueous medium [Carbohydr. Res. *41*, 344, (1975)].

By this method, a complete transformation of the gangliosides is also not obtained because the reaction is performed in an aqueous medium. Utilization of this method results in very low yields and a final purification of the inner ester product is necessary.

According to the present invention, a new and improved method for preparing inner ester ganglioside derivatives is described whereby high yields of the derivatives are obtained.

The inner ester ganglioside derivative of the present invention comprises:

(a) a carbohydrate portion, at least one ceramide and at least one acid moiety;

(b) said carbohydrate portion including at least one N-acetylgalactosamine or N-acetylglucosamine moiety and at least one glucose or galactose moiety;

(c) said acid moiety including at least one N-acetyl-neuramic acid or N-glycolylneuraminic acid; and

(d) the carboxyl group of at least one of said acid moieties being ester bonded to a hydroxyl group of one of said carbohydrates or one of said acid moieties to form a lactonic ring.

The method of the present invention comprises reacting under anhydrous conditions in a non aqueous organic solvent, a ganglioside or a mixture of gangliosides or a salt of such ganglioside or mixture of gangliosides with a tertiary nitrogen base, with a lactonization reagent to form at least one lactonic bond in said inner ester ganglioside derivatives. Suitable organic solvents to be used in the present invention include dimethyl sulfoxide (DMSO), dimethylformamide (DMF), sulfolane, tetrahydrofuran, dimethoxyethane and pyridine or mixtures thereof.

Suitable lactonization reagents include carbodiimides soluble in organic solvents such as dicyclohexylcarbodiimide, benzyl-isopropylcarbodiimide and benzylethylcarbodiimide, 2-chloro-1-methyl-pyridinium salts, ethoxyacetylene and Woodward reagent (N-ethyl-5-phenyl-isoxazolium-3'sulfonate).

While the prior art methods of reacting gangliosides with a carbodiimide in an aqueous medium result in very low yields of the inner ester derivatives, it has been found that the process of the present invention which comprises reacting gangliosides in a non-aqueous medium, results in very high, that is, substantially quantitative yields of the desired inner ester derivatives in amounts superior to that possible with the prior art methods.

The starting ganglioside compounds used in the process of the present invention are extracted from the brain tissue of mammals, most preferably from bovines.

The following examples represent methods according to the present invention for preparing inner ester derivatives of gangliosides.

Example 1

A mixture of gangliosides sodium salts is obtained by extraction from bovine brains and 5g of this mixture are dissolved in 50 ml of DMSO. Then, 4g of anhydrous styrene type resin (sulfonic acid) (50—100 mesh), H^+ form) are added to the mixture and the resulting system is stirred for 30 minutes at room temperature. This treatment with an anionic exchange resin converts all of the ganglioside carboxylate groups to —COOH (carboxyl) groups.

Complete conversion of the carboxylate groups is confirmed by an appropriate physical analytical method, such as atomic absorption. The resin is then filtered under suction and the solution is treated with 1.5g of dicyclohexylcarbodiimide and allowed to stand for one hour. The dicyclohexylurea which precipitates is removed by filtration and the remaining solution is treated with 100 ml of acetone causing precipitation of the product inner ester ganglioside derivatives.

The method yields 4.6g of inner ester product (about 90—95% of the theoretical value).

The presence of the inner ester derivatives is confirmed by infrared spectroscopy and by thin layer chromatography.

— IR Spectroscopy: Performed on a KBr pellet, the ester-lactone bond produces a band at 1750 cm^{-1}.

— Thin Layer Chromatography: On silica gel plates, solvent system $CHCl_3$/MeOH/$CaCl_2$ 0.3% (55/45/10, v/v/v), the R_f of the mixture of internal esters ranges between 0.7 and 0.85.

The R_f of the final products exceeds the R_f of the mixture of the starting compounds.

The chromatography results thus show the absence of any starting material. By treatment with a 0.1N solution of Na_2CO_3 at 60°C of 1 hour, the ester bonds are cleaved and the original mixture of starting ganglioside compounds can be obtained.

Example 2

9g of a ganglioside mixture (sodium salt) are dissolved in 80 ml of distilled water and passed through a column filled with 20g of styrene type resin (sulfonic acid) (100—200 mesh triethylammonium form).

This product, rendered anhydrous in high vacuum, is dissolved (with the aid of a sonicator bath) in 200 ml of anhydrous tetrahydrofuran containing 8 ml of triethylamine.

This solution is slowly added to 600 ml of anhydrous tetrahydrofuran (4 hours) containing 40 mM of 2-chloro-1-methyl-pyridinium salt (where the anion could be, for example, iodide, toluene-4-sulfonate and trifluoromethane sulfonate), under continuous stirring and maintaining a constant temperature of 45°C.

This reaction is carried out for 18 hours at 45°C.

The excess reagent is filtered off and the mixture is concentrated in a stream of nitrogen, the residue is redissolved in 90 ml of chloroform/methanol 1/1 and precipitated in 450 ml of acetone. The product is finally dried in high vacuum.

Yield — 7.9g (89.7% of the theoretical value).

— Thin layer chromatography: On silica gel plates, solvent system chloroform/methanol/$CaCl_2$ 0.3% (55/45/10), the R_f of the mixture of inner esters ranges between 0.7 and 0.85.

The R_f of the final products exceeds the R_f of the mixture of the starting compounds.

The chromatography results thus show the absence of any starting material. By treatment with 0.1N solution of Na_2CO_3 at 60°C for one hour, the ester bonds are cleaved and the original ganglioside compounds can be obtained.

— The IR spectrum of the inner or internal esters of the ganglioside mixture, performed on a KBr pellet, shows the typical ester absorption of 1750 cm^{-1}.

Example 3

8g of GM_1 (sodium salt) are dissolved in 80 ml of distilled water and passed through a column filled with 10g of styrene type resin (sulfonic acid) (100—200 mesh triethylammonium form).

This product, rendered anhydrous in high vacuum, is dissolved (with the aid of a sonicator bath) in 200 ml of anhydrous tetrahydrofuran containing 4 ml of triethylamine.

This solution is slowly added to 600 ml of anhydrous tetrahydrofuran (4 hours) containing 20 mM of 2-chloro-1-methyl-pyridinium salt (where the anion could be, for example, iodide, toluene-4-sulfonate, trifluoromethane sulfonate, etc.), under continuous stirring and maintaining a constant temperature of 45°C.

This reaction is carried out for 18 hours at 45°C.

The excess reagent is filtered off and the mixture is concentrated in a stream of nitrogen, the residue is redissolved in 80 ml of chloroform/methanol 1/1 and precipitated in 400 ml of acetone. The product is finally dried in high vacuum.

Yield — 7.0g (88.4% of the theoretical value).

— Thin layer chromatography: On silica gel plates, solvent system chloroform/methanol/$CaCl_2$ 0.3% (55/45/10), the R_f of the final product (0.70) exceeds the R_f of the starting compound.

The chromatography results thus show the absence of any starting material. By treatment with 0.1N solution of Na_2CO_3 at 60°C for one hour, the ester bond is cleaved and the original ganglioside can be obtained.

— The IR spectrum of the inner ester of GM_1, performed on a KBr pellet, shows the typical ester absorption of 1750 cm^{-1}.

Example 4

9g of a ganglioside mixture (sodium salt) are dissolved in 80 ml of distilled water and passed through a column filled with 20g of styrene type resin (sulfonic acid) (100—200 mesh pyridinium form).

This product, rendered anhydrous in high vacuum, is dissolved in 800 ml of anhydrous tetrahydrofuran containing 4.2g (60 mM) of ethoxyacetylene.

This mixture is refluxed for 3 hours, the refluxer is cooled at −10°C and equipped with a drying valve.

After removing the solvents and excess of ethoxyacetylene, the residue is dissolved in 80 ml of chloroform/methanol 1/1 and precipitated in 400 ml of acetone.

Yield — 8.1g (92.0% of the theoretical value).

— Thin layer chromatography: On silica gel plates, solvent system chloroform/methanol/$CaCl_2$ 0.3% (55/45/10), the R_f of the mixture of inner esters ranges between 0.7 and 0.85.

The R_f of the final products exceeds the R_f of the mixture of the starting compounds.

The chromatography results thus show the absence of any starting material. By treatment with 0.1N solution of Na_2CO_3 at 60°C for one hour, the ester bonds are cleaved and the original ganglioside compounds can be obtained.

— The IR spectrum of the inner esters of the ganglioside mixture, performed on a KBr pellet, shows the typical ester absorption of 1750 cm^{-1}.

Example 5

8g of GM_1 (sodium salt) are dissolved in 80 ml of distilled water and passed through a column filled with 10g of styrene type resin (sulfonic acid) (100—200 mesh pyridinium form).

This product, rendered anhydrous in high vacuum, is dissolved in 800 ml of anhydrous tetrahydrofuran and 2.1g (30mM) of ethoxyacetylene.

This mixture is refluxed for 3 hours, the refluxer is cooled at −10°C and equipped with a drying valve.

After removing the solvents and excess of ethoxyacetylene, the residue is dissolved in 80 ml of chloroform/methanol 1/1 and precipitated in 400 ml of acetone.

Yield — 7.2g (91.0% of the theoretical value).

— Thin layer chromatography: On silica gel plates, solvent system chloroform/methanol/$CaCl_2$ 0.3% (55/45/10), the R_f of the final product (0.70) exceeds the R_f (0.65) of the starting compound.

The chromatography results thus show the absence of any starting material. By treatment with 0.1N solution of Na_2CO_3 at 60°C for one hour, the ester bond is cleaved and the original ganglioside can be obtained.

— The IR spectrum of the inner ester of GM_1, performed on a KBr pellet, shows the typical ester absorption of 1750 cm^{-1}.

Example 6

9g of ganglioside mixture (sodium salt) are dissolved in 80 ml of distilled water and passed through a column filled with 20g of styrene type resin (sulfonic acid) (100—200 mesh pyridinium form).

This product, rendered anhydrous in high vacuum and dissolved in 200 ml of anhydrous pyridine, is added to a suspension of 5.52g (10 mM) of the Zwitterionic Woodward reagent [N-ethyl-5-phenylisoxazolium-3'-sulfonate, Woodward et al., J. Am. Chem. Soc. *83*, 1010—1012, (1961)] in 200 ml of anhydrous pyridine. This reaction mixture is stirred for 10 days at room temperature.

After filtration of the excess reagent and complete removal of the solvent, the residue is dissolved in 90 ml of chloroform/methanol 1/1 and precipitated in 450 ml of acetone.

Yield — 7.2g (81.8% of the theoretical value).

— Thin layer chromatography: On silica gel plates, solvent system chloroform/methanol/$CaCl_2$ 0.3% (55/45/10), the R_f of the mixture of inner esters ranges between 0.7 and 0.85.

The R_f of the final products exceeds the R_f of the mixture of the starting compounds.

The chromatography results thus show the absence of any starting material. By treatment with 0.1N solution of Na_2CO_3 at 60°C for one hour, the ester bonds are cleaved and the original ganglioside compounds can be obtained.

— The IR spectrum of the inner esters of the ganglioside mixture, performed on a KBr pellet, shows the typical ester absorption of 1750 cm^{-1}.

Example 7

8g of GM_1 (sodium salt) are dissolved in 80 ml of distilled water and passed through a column filled with 10g of styrene type resin (sulfonic acid) (100—200 mesh pyridinium form).

This product, rendered anhydrous in high vacuum and dissolved in 200 ml of anhydrous pyridine, is added to a suspension of 1.26g (5 mM) of the Zwitterionic Woodward reagent (N-ethyl-5-phenyl-isoxazolium-3'-sulfonate), in 200 ml of anhydrous pyridine. This reaction mixture is stirred for 10 days at room temperature.

After filtration of the excess reagent and complete removal of the solvent, the residue is dissolved in 80 ml of chloroform/methanol 1/1 and precipitated in 400 ml of acetone.

Yield — 6.3g (79.5% of the theoretical value).

— Thin layer chromatography: On silica gel plates, solvent system chloroform/methanol/$CaCl_2$ 0.3% (55/45/10), the R_f of the final product (0.70) exceeds the R_f (0.65) of the starting compound.

The chromatography results thus show the absence of any starting material. By treatment with 0.1N solution of Na_2CO_3 at 60°C for one hour, the ester bond is cleaved and the original ganglioside can be obtained.

— The IR spectrum of the inner ester of GM_1 performed on a KBr pellet, shows the typical ester absorption of 1750 cm^{-1}.

PHARMACOLOGICAL PROPERTIES

While some inner ester derivatives of gangliosides and methods for making such derivatives have been discussed in the prior art, there has been no previous disclosure of biological activity or possible pharmaceutical uses for the inner ester derivatives. However, according to the present invention, it has been found that inner ester ganglioside derivatives have a very high activity in treating disorders of the nervous system and, in fact, have a much higher activity than that of the ganglioside themselves. The inner ester ganglioside derivatives may be used to treat a variety of nerve disorders including peripheral and central nervous system disorders resulting from diseases or accidents. The compounds may also be used in post-operative therapy following surgery which affects the nerves, such as hemorrhoid surgery.

The superior pharmacological properties of the inner ester ganglioside derivatives which are the subject of this invention can be evaluated in comparison with gangliosides by the following tests:

1 — Neurite sprouting in pheocromocitoma cell line (PC_{12}).

2 — Neuronal membrane (Na^+K^+)ATPase activity.

3 — Recovery of the electroretinogramme following dazzling.

1 — Effect of Inner Ester Ganglioside Derivatives on Neurite Sprouting of PC_{12} —

Materials and Methods

Neurite sprouting may be considered as localized neuronal differentiation and the biochemical mechanism by which the ganglioside molecules produce the above effect can be studied by evaluating an *in vitro* cel culture model [PC_{12} cell line derived from a 1A subclone supplied by Dr. P. Calissano (C.N.R. — Laboratorio di Biologia Cellulare — Roma, Italy)] — ["Gangliosides in Neurological and Neuromuscular Function, Development and Repair", Ed. by M. M. Rapport and A. Gorio, Raven Press, New York (1981)].

In this model, the gangliosides or inner ester ganglioside derivatives are added to the PC_{12} culture medium together with the nerve growth factor (NGF), a specific inducer of PC_{12} differentiation to stimulate neurite sprouting. The neurite sprouting stimulated by the gangliosides can then be compared to that stimulated by the inner ester ganglioside derivatives.

Specifically, the cells (100,000/plate) were maintained at 37°C in an Heraeus incubator (5% CO_2 and 95% humidified air) and plated in a Collagen-coated tissue culture, 60 mm Integrid Falcon Plates, in the presence of the following culture medium:

— 85% RPM 1640 (Gibco),
— 10% heat-inactivated horse serum (Gibco),
— 5% fetal calf serum (Gibco),
— 50 U/ml penicillin, and
— 25 mg/ml streptomycin.

The medium was changed every 48 hours.

In such conditions, the cells divide but do not form neurites. Addition of NGF (50 ng/ml) causes the cells to cease proliferation, form neurites and differentiate within 5—10 days. This effect was evaluated by counting the number of cells with neurites beginning on day 5 and then on every alternate day.

The gangliosides and their derivatives (1 mM) were added to the culture medium simultaneously with NGF and their effect was assessed on days 7 and 9 by counting the number of cells containing neurites.

Results

The results of the comparative studies on neurite sprouting are summarized in Table I, hereinafter.

The results obtained show that the inner ester ganglioside derivatives of the present invention have the capability to stimulate neuronal sprouting and proved to be much more active than gangliosides in producing the effect both in seven days, and in nine days.

TABLE I

Effect of ganglioside derivatives on neurite sprouting in PC_{12} cells

Compound	Cell Line	Medium	Concentration	% of the number of cells with neurites	
				day 7	day 9
● Control	PC_{12}	85% RPM 1640, 10% heat-inact. horse serum, 5% fetal calf serum, 50 U/ml penicillin, and 25 mg/ml streptomycin		31	35.7
● Gangliosides	PC_{12}	85% RPM 1640, 10% heat-inact. horse serum, 5% fetal calf serum, 50 U/ml penicillin, and 25 mg/ml streptomycin	1 nM	49.5	62.3
● Inner esters of gangliosides	PC_{12}	85% RPM 1640, 85% RPM 1640, 10% heat-inact. horse serum, 5% fetal calf serum, 50 U/ml penicillin, and 25 mg/ml streptomycin	1 mM	56.3	69.2

2 — Effect of Ganglioside Derivatives on the Neuronal Membrane (Na^+,K^+)ATPase Activity —

The capability of the gangliosides or inner ester ganglioside derivatives to activate the membrane enzyme (Na^+,K^+)ATPase can be evaluated *in vitro* in a neuronal membrane preparation [J. of Neurochem., cited supra].

Materials and Methods

a — Preparation of the Rat Brain Crude Mitochondrial Fraction (P_2 Fraction).

The procedure used for preparing the P_2 fraction was derived from that of Morgan and al, Biochem. Biophys. Acta *241,* 737 (1971). (All operations were performed at 0°—4°C; the x g values are average centrifugal forces). Sprague Dawley (from Charles River) adult male rats (150—175g body weight) were decapitated and their brains rapidly removed and washed in ice-cold isotonic solution. After excision of the cerebellum, the brains were homogenized by 12 up-and-down strokes of a motor-operated Teflon®-glass homogenizer (stated radial clearance, 0.25 mm; 800 r.p.m.) using 4 vol. of the homogenizing solution (0.32M sucrose containing 1 mM potassium phosphate buffer and 0.1 nM bisodium EDTA, pH 7.27).

The homogenate was diluted to 10% (w/V) in the homogenizing solution, filtered through four layers of cheese cloth, and centrifuged at 1000 × g for 15 minutes.

The resulting pellet was washed with the same starting volume of homogenizing solution and centrifuged as above. The combined supernatants were centrifuged at 17,500 × g for 25 minutes (these gravitational conditions were employed in order to have the maximal enrichment of nerve endings in the fraction) and the pellet was washed four times with 9 volumes (each time) of homogenizing solution and centrifuged (17,500 × g for 25 minutes). The final pellet, referred to as the "P_2 fraction" contains, as the major constituents, unruptured mitochondria and nerve endings. The final pellet was homogenously resuspended with an appropriate volume of homogenizing solution with the above Teflon®-glass homogenizer and immediately used for assay. In order to avoid inconsistencies associated with storage, fresh P_2 fractions were always prepared prior to use.

The P_2 fraction preparations had a ganglioside content of 33.9±2.8 (S.D.) nmoles bound NeuAc/mg protein.

b — ATPase Assay.

ATPase activity was measured spectrophotometrically according to Wallick et al [(J. Pharm. Exptl. Therap. *189,* 434, (1974)].

The reaction mixture, unless otherwise stated, consisted of:

— 50 mM sucrose,
— 0.2 mM bisodium EDTA (adjusted to pH 7.4),
— 100 mM NaCl,
— 5 mM $MgCl_2$
— 10 mM KCL,
— 2 mM phospho(enol)pyruvate monopotassium salt (PEP) (adjusted to pH 7.4),
— 3 mM ATP,
— 50 mM TRIS-HCl (pH 7.4),
— 0.33 mM NADH,
— 30 µg/ml pyruvate-kinase (PK), and
— 10 µg/ml lactate-dehydrogenase (LDH),

in a final volume of 3 ml and a final pH of 7.2

The reaction was started by the addition of 50—75 µg (as protein) of P_2 fraction. (Na^+,K^+)ATPase activity was obtained by the difference between total ATPase and Mg^{2+} dependent ATPase measured in the presence of 3×10^{-5} M ouabain. The time required for each individual assay 3—5 minutes.

ATPase activity was expressed as International Units (IU) (µmoles ATP hydrolyzed/mg protein/min).

The activity of the ganglioside derivatives (50 nM) was assayed following incubation with the neuronal membranes at 37°C for two hours.

Results

The results of the comparative studies on ATPase activity are summarized in Table II, hereinafter.

The results obtained show that the inner ester ganglioside derivatives of the present invention have the capability to activate the neuronal membrane enzyme (Na^+,K^+)ATPase and proved to be much more active than gangliosides under the same conditions of molar concentration.

TABLE II

Effect of ganglioside derivatives on the neuronal membrane (Na^+, K^+)ATPase

Compound	Incubation time	Incubation temperature	Concentration (nM)	% increase of (NA^+, K^+)ATPase activity
● Control	120 min	37°C	—	100
● Gangliosides	120 min	37°C	50	142
● Inner esters of gangliosides	120 min	37°C	50	174

3 — Effect of Ganglioside Derivatives on Recovery of the Electroretinogramme Damaged by Dazzling — The capability of gangliosides or inner ester ganglioside derivatives to accelerate the recovery of the retinal electrical activity can be evaluated after physical damage (dazzling) in rabbits.

In this model, gangliosides or inner ester ganglioside derivatives are administered parenterally (intravenously) ["Int. Symposium on the Neurochemistry of the Retina", Athens, August 28—September 1 (1979) (Communication)] — [("Satellite Meeting on Biochemical and Pharmacological Implications of Gangliosides Functions", Cortona, August 28—31 (1975) (Communications)].

Methods and Materials

Male New Zealand rabbits weighing between 1.9 and 2 kg were utilized. ["Int. Symp. on the Neurochemistry of the Retina", Athens, August 28—September 1 (1979)].

Corneal anaesthesia was induced by a local application of 0.4% novesine. The electroretinogramme was registered by applying a corneal electrode (according to Henkes) fixed with a mild suction. The reference electrode consisted of a needle inserted subcutaneously into the frontal area.

The following apparatus was used:

— AC preamplifier 5A22N Tektronics (10 Hz DC filters);
— Neuroaverager 1172 OTE Biomedica analyzer;
— XY plotter L800 Linseis recorder;
— 1273 OTE Biomedica photostimulator.

Photostimulation was carried out with five flashes of 0.2 watts/sec. of 10 sec. of duration and 0.5 Hertz of frequency.

Base time for the registration was 100 msec (pre-set=5).

The animals were kept in a dark room for 30 minutes to adapt to darkness in conditions of constant air, temperature and noise.

The following determinations were performed:

1. Three base controls, one every 15 minutes, were evaluated in all animals. The mean of waves a+b (peak to peak) was calculated.

2. The animals were then subjected to dazzling for 20 minutes by utilizing a Schott Mainz KL150B lamp positioned at a distance of 1 cm from the corneal electrode.

TABLE III

Effect of ganglioside derivatives on the electroretinogramme recovery

Compound	Animals	Dosage i.v.	% ERG recovery in respect to controls		
			20 min.	40 min.	60 min.
● Gangliosides	Rabbit	33 nmole/kg	+5	+9.0	+13.0
● Inner esters of gangliosides	Rabbit	33 nmole/kg	+8	+12.7	+20.5

After this period, the electroretinogramme (ERG) recovery was determined by measuring the amplitude of the ERG at 20, 40 and 60 minutes following the dazzling period. This permitted an assessment of the animals' basal condition to be made.

The animals were subsequently treated with the compounds and 30 minutes later were once again subjected to dazzling and the registration of the ERG took place in identical conditions as mentioned above.

The compounds were administered at 33 nmole/kg by an intravenous injection.

Results

The results of the comparative studies on electroretinogramme recovery are summarized in Table III, hereinabove.

The results thus obtained show that the internal ester derivatives of the present ivnention do increase recovery in electroretinograms and show a larger activity than the gangliosides at all of the examined times.

THERAPEUTIC UTILIZATION

The inner ester ganglioside derivatives of the present invention can be used as drugs for different therapies for treating the nervous system, particularly peripheral nerve disorders and pathologies of the central nervous system. More particularly, the inner ester ganglioside derivatives can be used in the treatment of peripheral nervous system disorders due to traumatic, compressive, degenerative or toxic-infectious causes where the stimulation of nerve regeneration and recovery of neuromuscular function is necessary, and in central nervous system disorders due to traumatic, anoxic, degenerative or toxic-infectious causes where the stimulation of neuronal sprouting is necessary for functional recovery.

These disorders have previously been treated by the use of gangliosides; however, the above described tests show that the inner ester derivatives of gangliosides have much greater activity than that of the gangliosides themselves.

The inner ester ganglioside compounds of the present invention can be utilized as drugs in pharmaceutical preparations administered to humans or animals intramuscularly, subcutaneously or intradermally, by intravenous injection or infusion. The preparations can be solutions of the compounds or a lyophilized powder of the compounds in association with one or more pharmaceutically acceptable carriers or diluents, and contained in buffered media at a suitable pH and isohosmotic with physiological fluids. The dosage administered will depend upon the desired effect and upon the desired administration route. For example (which is not limitative) the dosage can be between 0.05 and 5 mg of active compound per kg of body weight by day with a unitary dosage between 0.05 and 2 mg/kg of body weight.

The therapeutic compositions of the present invention are usually prepared with a mixture of different inner ester ganglioside derivatives but could be prepared containing only one isolated active derivative.

For exemplary purposes only, Table 4, hereinafter, shows some possible pharmaceutical composition preparations which could be prepared in the form of a solution for treating disorders of the nervous system.

The preparations shown in Table IV can be directly administered to animals or humans by any of the above-described routes. Additionally, the compositions may contain from about 2% to about 50% by weight of active substance.

TABLE IV

Examples of pharmaceutical composition solutions for injection

Preparation No. 1 — one ampoule of 2 ml contains:		
active substance	mg	5
sodium chloride	mg	16
citrate buffer pH 6 in distilled pyrogen free water	q.s.a. ml	2
Preparation No. 2 — one ampoule of 2 ml contains:		
active substance	mg	50
sodium chloride	mg	16
citrate buffer pH 6 in distilled pyrogen free water	q.s.a. ml	2
Preparation No. 3 — one vial of 4 ml contains:		
active substance	mg	100
sodium chloride	mg	32
citrate buffer pH 6 in distilled pyrogen free water	q.s.a. ml	4

Again for exemplary purposes, Table V shows some possible pharmaceutical composition systems which could be prepared for treating disorders of the nervous system. The pharmaceutical composition systems shown in Table V are prepared as two vial systems.

A first active substance vial is prepared containing from about 10 to about 90% by weight of a lyophilized powder of the active substance together with a pharmaceutically acceptable excipient, such as glycine and mannitol.

A second solvent vial is prepared containing the desired amount of solvent, such as sodium chloride and a citrate buffer.

Just prior to administration, the contents of the two vials are admixed and the lyophilized active substance powder quickly dissolves to produce an injectable solution.

The pharmaceutical composition system, including a first vial containing the lyophilized powder of the active substance, is the preferred pharmaceutical composition of the present invention since the active substance inner ester ganglioside derivatives are more stable in this state as compared to the pharmaceutical composition solutions *per se*.

TABLE V

Examples of pharmaceutical composition systems

System No. 1 —		
a. one lyophile vial of 2 ml contains:		
active substance	mg	5
glycine	mg	30
b. one solvent ampoule of 2 ml contains:		
sodium chloride	mg	16
citrate buffer in distilled pyrogen free water	q.s.a. ml	2
System No. 2 —		
a. one lyophile vial of 3 ml contains:		
active substance	mg	5
mannitol	mg	40
b. one solvent ampoule of 2 ml contains:		
sodium chloride	mg	16
citrate buffer in distilled pyrogen free water	q.s.a. ml	2
System No. 3 —		
a. one lyophile ampoule of 3 ml contains:		
active substance	mg	50
glycine	mg	25
b. one solvent ampoule of 3 ml contains:		
sodium chloride	mg	24
citrate buffer in distilled pyrogen free water	q.s.a. ml	3

TABLE V (continued)

Examples of pharmaceutical composition systems

System No. 4 —		
a. one lyophile ampoule of 3 ml contains:		
active substance	mg	50
mannitol	mg	20
b. one solvent ampoule of 3 ml contains:		
sodium chloride	mg	24
citrate buffer in distilled pyrogen free water	q.s.a. ml	3
System No. 5 —		
a. one lyophile vial of 5 ml contains:		
active substance	mg	100
glycine	mg	50
b. one solvent ampoule of 4 ml contains:		
sodium chloride	mg	32
citrate buffer pH 6 in distilled pyrogen free water	q.s.a. ml	4
System No. 6 —		
a. one lyophile vial of 5 ml contains:		
active substance	mg	100
mannitol	mg	40
b. one solvent ampoule of 4 ml contains:		
sodium chloride	mg	32
citrate buffer pH 6 in distilled pyrogen free water	q.s.a. ml	4

Claims

1. A method for preparing inner ester ganglioside derivatives which comprises reacting in a non aqueous organic solvent, a ganglioside or a mixture or gangliosides or a salt of such ganglioside or mixture of gangliosides with a tertiary nitrogen base, with a lactonization reagent to form at least one lactonic bond in said inner ester ganglioside derivatives.

2. The method of claim 1, wherein the ganglioside or mixture of gangliosides is extracted from bovine brains.

3. The method of claim 1, wherein said organic solvent is selected from dimethyl sulfoxide, dimethylformamide, sulfolane, tetrahydrofuran, dimethoxyethane and pyridine.

4. A method for preparing inner ester ganglioside derivatives which comprises:

(a) dissolving a ganglioside or mixture of gangliosides or salt thereof, obtained from a mammal in an aprotic solvent;

(b) adding an ion exchange resin to said ganglioside or mixture of said gangliosides or salt thereof whereby the carboxylate groups of said gangliosides are converted to carboxyl groups or tertiary nitrogen salt; and

(c) treating said ganglioside or mixture of gangliosides with a carbodiimide to form at least one lactonic bond in said inner ester ganglioside derivative.

5. The method of claim 1 or 4, wherein said lactonization reagent is selected from carbodiimides soluble in organic solvents, 2-chloro-1-methylpyridinium salts, ethoxyacetylene and N-ethyl-5-phenylisoxazolium-3'-sulfonate.

6. The method of claim 4, wherein the carbodiimide is dicyclohexylcarbodiimide, benzylisopropylcarbodiimide or benzylethylcarbodiimide.

7. The method of claim 1 or 4, wherein said inner ester ganglioside derivative is precipitated with acetone.

8. The method of claim 4, wherein said salt of step (b) of such ganglioside or mixture of gangliosides is a tertiary nitrogen salt prepared by converting the carboxylate groups in said gangliosides to a tertiary nitrogen salt prepared by converting the carboxylate groups in said gangliosides to a tertiary nitrogen salt thereof by means of ion exchange.

9. The method of claim 4, wherein said salt is tertiary nitrogen salt with triethylammonium or pyridinium.

10. The method of any of claims 1 to 9, wherein the reaction with a lactonization reagent results in a complete transformation of said gangliosides.

11. A composition for treating peripheral nervous system disorders due to traumatic, compressive, degenerative or toxic-infectious causes for the stimulation of nerve regeneration and recovery of neuromuscular function or for treating central nervous system disorders due to traumatic, anoxic, degenerative or toxic-infectious causes where the stimulation of neuronal sprouting is necessary for functional recovery which comprises associated to a pharmaceutically acceptable carrier or diluent, an effective amount of at least one inner ester ganglioside derivative comprising:

(a) a carbohydrate portion, at least one ceramide and at least one acid moiety;

(b) said carbohydrate portion including at least one N-acetylgalactosamine or N-acetylglucosamine moiety and at least one glucose or galactose moiety;

(c) said acid moiety including at least one N-acetyl-neuramic acid or N-glycolylneuraminic acid; and

(d) the carboxyl group of at least one of said acide moieties being ester bonded to a hydroxy group of one of said carbohydrates or one of said acid moieties to form a lactonic ring.

12. The composition of claim 11, wherein:

the carbohydrate portion of said ganglioside derivatives has the structure;

(Gal)(GalNAC)(Gal)(Glc)(Ceramide)

where:

Gal is a galactose moiety;

GalNAC is an N-acetylgalactosamine moiety, and

Glc is a glucose moiety.

13. The composition of claim 11 or 12, wherein said ganglioside derivative has at least one N-acetyl-neuramic acid bonded to at least one of said Gal moieties.

14. The composition of claim 11, wherein:

(a) at least one of said ganglioside derivatives has the structure:

(Gal)(GalNAC)(Gal)(Glc)(Ceramide)
|
NANA

wherein:

Gal is a galactose moiety;

GalNAC is an N-acetylgalactosamine moiety,

Glc is a glucose moiety, and

NANA is an N-acetyl-neuraminic acid moiety,

and wherein:

(b) said NANA is ester bonded to said Gal.

15. The composition of claim 11 or 14, wherein said acid moiety consists of N-acetyl-neuraminic acid.

16. The composition of claim 11, wherein the carbohydrate portion consists of galactose, glucose and N-acetylgalactosamine moieties.

17. The composition of claim 11, wherein said ganglioside derivative comprises a mixture of:

(a)

(Gal)(GalNAC)(Gal)(Glc)(Ceramide)
|
NANA

(b) (Gal)(GalNAC)(Gal)(Glc)(Ceramide)
NANA NANA

(c) (Gal)(GalNAC)(Gal)(Glc)(Ceramide)
NANA
NANA

and

(d) (Gal)(GalNAC)Gal)(Glc)(Ceramide)
NANA NANA
NANA

wherein:
Gal represents a galactose moiety,
GalNAC represents an N-acetylgalactosamine moiety,
Glc represents a glucose moiety, and
NANA represents an N-acetyl-neuraminic moiety, and wherein at least one of said NANA moieties is ester bonded to a hydroxy group of a galactose moiety and/or an adjoining NANA moiety to form a lactonic ring.

18. The composition according to any of claims 11 to 17, wherein said ganglioside derivative is a completely transformed product consisting in an inner ester ganglioside derivative wherein the carboxyl group is ester bonded either to a hydroxy group of one of said carbohydrate portions or to one of the acid moieties to form a lactonic ring.

19. The composition according to any of claims 11 to 18, in the form of an injectable solution.

20. The composition according to any of claims 11 to 18, wherein it is supported on a carrier or diluted in a diluent consisting of a mixture of sodium chloride and a citrate buffer.

21. The composition according to any of claims 11 to 20, wherein said ganglioside derivative is present in an amount of from about 2% to about 50% by weight.

22. A pharmaceutical composition system, containing a pharmaceutically acceptable carrier or diluant, which comprises a first container containing a solvent system and a second container containing a solid excipient and a lyophilized power of an effective nervous system treating amount of at least one inner ester ganglioside derivative, said ganglioside derivative comprising:

(a) a carbohydrate portion, at least one ceramide and at least one acid moiety;

(b) said carbohydrate portion including at least one N-acetylgalactosamine or N-acetylglucosamine moiety and at least one glucose or galactose moiety;

(c) said acid moiety including at least one N-acetyl-neuraminic acid or N-glycolylneuraminic acid; and

(d) the carboxyl group of at least one of said acid moieties being ester bonded to a hydroxy group of one of said carbohydrates or one of said acid moieties to form a lactonic ring, whereby the contents of said containers are mixed to form a treatment solution.

23. The pharmaceutical composition system of claim 22, wherein said first container contains sodium chloride and a citrate buffer.

24. The pharmaceutical composition system of claim 22, wherein said solid excipient is glycine.

25. The pharmaceutical composition system of claim 22 or 23, wherein said solid excipient is mannitol.

26. The pharmaceutical composition system of claim 22, wherein said second container contains said ganglioside derivative in an amount of from about 10% to about 90% by weight.

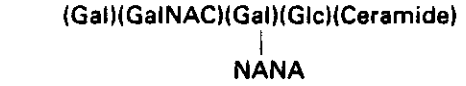
27. Pharmaceutical composition system according to any of claims 22 to 26, wherein:
the carbohydrate portion of said ganglioside derivatives has the structure;

(Gal)GalNAC)(Gal)(Glc)(Ceramide)

where:
Gal is a galactose moiety;
GalNAC is an N-acetylgalactosamine moiety, and
Glc is a glucose moiety.

28. Pharmaceutical composition system according to claim 27: wherein: said ganglioside derivative has at least one N-acetyl-neuraminic acid bonded to at least one of said Gal moieties.

29. The pharmaceutical composition system according to any of claims 22 to 26, wherein:
(a) at least one of said ganglioside derivatives has the structure:



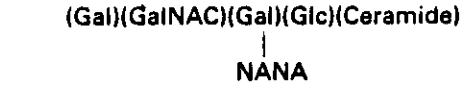
wherein:
Gal is a galactose moiety,
GalNAC is an N-acetylgalactosamine moiety,
Glc is a glucose moiety, and
NANA is an N-acetyl-neuraminic moiety, and wherein:
(b) said NANA is ester bonded to said Gal.

30. The pharmaceutical composition system according to claim 29, wherein said acid moiety consists of N-acetyl-neuraminic acid.

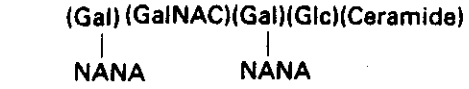
31. The pharmaceutical composition system according to any of claims 22 to 27, wherein the carbohydrate portion consists of galactose, glucose and N-acetylgalactosamine moieties.

32. The pharmaceutical composition system according to any of claims 22 to 27, wherein said ganglioside derivative comprises of mixture of:

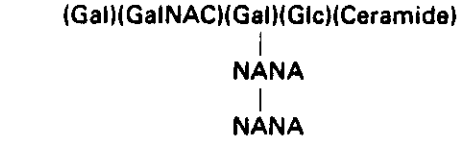
(a)



(b)

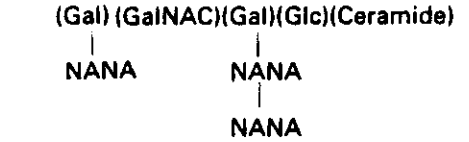


(c)



and

(d)



wherein:
Gal represents a galactose moiety,
GalNAC represents an N-acetylgalactosamine moiety,
Glc represents a glucose moiety, and
NANA represents an N-acetyl-neuraminic moiety, and wherein at least one of said NANA moieties is ester bonded to a hydroxy group of a galactose moiety and/or adjoining NANA moiety to form a lactonic ring.

33. The pharmaceutical composition system according to any one of claims 22 to 32, wherein said ganglioside derivative is a completely transformed product, consisting in an inner ester ganglioside derivative wherein the carboxyl group is ester bonded to a hydroxy group of one of the carbohydrate portions or to one of the acid moieties to form a lactonic ring.

Revendications

1. Procédé de préparation de dérivés de ganglioside à ester interne, dans lequel on fait réagir dans un solvant organique non aqueux un ganglioside ou un mélange de gangliosides ou encore un sel de ganglioside ou de mélange de gangliosides avec une base azotée tertiaire avec un agent de lactonisation afin de produire au moins une liaison lactonique dans lesdits dérivés de ganglioside à ester interne.

2. Procédé selon la revendication 1, dans lequel le ganglioside ou le mélange de gangliosides est extrait de cervelles de bovins.

3. Procédé selon la revendication 1, dans lequel ledit solvant organique est choisi dans le groupe: diméthyl-sulfoxyde, diméthylformamide, sulfolane, tétrahydrofuranne, diméthoxyéthane et pyridine.

4. Procédé de préparation de dérivés de ganglioside à ester interne, dans lequel:

(a) on dissout dans un solvant aprotique un ganglioside ou un mélange de gangliosides ou encore un de leurs sels, ce ganglioside, mélange ou sel étant obtenus à partir d'un mammifère;

(b) on ajoute audit ganglioside, mélange de gangliosides ou sel une résine échangeuse d'ions, de sorte que les groupes carboxylates desdits gangliosides sont transformés en groupes carboxyles ou en sels d'amine tertiaire; et

(c) on traite ledit ganglioside ou mélange de gangliosides par un carbodiimide afin de créer au moins une liaison lactonique dans ledit dérivé de ganglioside à ester interne.

5. Procédé selon la revendication 1 ou 4, dans lequel ledit agent de lactonisation est choisi dans le groupe: carbodiimides solubles dans les solvants organiques, sels de 2-chloro-1-méthylpyridinium, éthoxyacétylène et N-éthyl-5-phénylisoxazolium-3'-sulfonate.

6. Procédé selon la revendication 4, dans lequel de carbodiimide est le dicyclo-hexyl-carbodiimide, le benzyl-isopropylcarbodiimide ou le benzyl-éthylcarbodiimide.

7. Procédé selon la revendication 1 ou 4, dans lequel ledit dérivé de ganglioside à ester interne est précipité à l'aide d'acétone.

8. Procédé selon la revendication 4, dans lequel ledit sel de l'étape (b) dudit ganglioside ou mélange de gangliosides est un sel d'amine tertiaire préparé par transformation des groupes carboxylates desdits gangliosides en un de leurs sels d'amine tertiaire au moyen d'un échange d'ions.

9. Procédé selon la revendication 4, dans lequel ledit sel est un sel d'amine tertiaire formé avec du triéthylammonium ou du pyridinium.

10. Procédé selon une quelconque des revendications 1 à 9, dans lequel la réaction avec un agent de lactonisation conduit à une transformation complète desdits gangliosides.

11. Composition pour le traitement des troubles du système nerveux périphérique dus à des causes traumatiques, compressives, dégénératives ou toxico-infectieuses, pour stimuler la régénération des nerfs et le rétablissement des fonctions neuromusculaires, ou pour le traitement des troubles du système nerveux central dus à des causes traumatiques, anoxiques, dégénératives ou toxico-infectieuses, où la stimulation du développement neuronal est nécessaire pour le rétablissement fonctionnel, ladite composition comprenant, en association avec un support ou diluant pharmaceutiquement acceptable, une quantité efficace d'au moins un dérivé de ganglioside à ester interne renfermant:

(a) une partie hydrocarbonée, au moins un céramide et au moins un reste acide;

(b) ladite partie hydrocarbonée renfermant au moins un reste N-acétylgalactosamine ou N-acétylglucosamine et au moins un reste glucose ou galactose;

(c) ledit reste acide renfermant au moins un acide N-acétylneuraminique ou N-glycolylneuraminique; et

(d) le groupement carboxyle d'au moins un desdits restes acides étant esterifié par un groupement hydroxy d'une desdites parties hydrocarbonées ou d'un desdits restes acides, de manière à former un noyau lactonique.

12. Composition selon la revendication 11, dans laquelle: la partie hydrocarbonée desdits dérivés de ganglioside présente la structure:

(Gal)(GalNAC)(Gal)(Glc)(céramide)

où:

Gal est un reste de galactose;

GalNAC est un reste de N-acétylgalactosamine, et

Glc est un reste de glucose.

13. Composition selon la revendication 11 ou 12, dans laquelle ledit dérivé de ganglioside comporte au moins un acide N-acétyl-neuraminique lié à au moins un desdits restes Gal.

14. Composition selon la revendication 11, dans laquelle:

(a) au moins un desdits dérivé de ganglioside présente la structure:

(Gal)(GalNAC)(Gal)(Glc)(céramide)

où NANA

Gal est un reste de galactose;
GalNAC est un reste de N-acétylgalactosamine;
Glc est un reste de glucose; et
NANA est un reste d'acide N-acétylneuraminique, et dans laquelle:
(b) NANA est un ester lié à Gal.

15. Composition selon la revendication 11 ou 14, dans laquelle ledit reste acide est de l'acide N-acétyl-neuraminique.

16. Composition selon la revendication 11, dans laquelle la partie hydrocarbonée est constituée par des restes de galactose, glucose et N-acétylgalactosamine.

17. Composition selon la revendication 11, dans laquelle ledit dérivé de ganglioside comprend un mélange de:

(a) (Gal)(GalNAC)(Gal)(Glc)(céramide)
|
NANA

(b) (Gal)(GalNAC)(Gal)(Glc)(céramide)
| |
NANA NANA

(c) (Gal)(GalNAC)(Gal)(Glc)(céramide)
|
NANA
|
NANA

et

(d) (Gal)(GalNAC)(Gal)(Glc)(céramide)
| |
NANA NANA
|
NANA

où

Gal représente un reste de galactose;
GalNAC représente un reste de N-acétylgalactosamine;
Glc représente un reste de glucose; et
NANA représente un reste N-acétylneuraminique, et dans laquelle au moins un desdits restes NANA est esterifié par un groupement hydroxy d'un reste de galactose et/ou d'un reste adjacent, de manière à former un noyau lactonique.

18. Composition selon une quelconque des revendications 11 à 17, dans laquelle ledit dérivé de ganglioside est un produit entièrement transformé constitué par un dérivé de ganglioside à ester interne où le groupement carboxyle est esterifié par un groupement hydroxy d'une desdites parties hydrocarbonées, ou de l'un des restes acides, afin de former un noyau lactonique.

19. Composition selon une quelconque des revendications 11 à 18, sous la forme d'une solution injectable.

20. Composition selon une quelconque des revendications 11 à 18, qui est associée à un support ou diluée dans un diluant constitué par un mélange de chlorure de sodium et d'un tampon à base de citrate.

21. Composition selon une quelconque des revendications 11 à 20, dans laquelle le dérivé de ganglioside représente environ 2% à environ 50% en poids.

22. Système pharmaceutique comportant un support ou diluant pharmaceutiquement acceptable et comprenant un premier récipient renfermant un système de solvants, ainsi qu'un second récipient renfermant un excipient solide et une poudre lyophilisée, en quantite suffisante pour le traitement efficace du système nerveux, consistant en au moins un dérivé de ganglioside à ester interne, ledit dérivé de ganglioside comprenant:

(a) une partie hydrocarbonée, au moins un céramide et au moins un reste acide;
(b) ladite partie hydrocarbonée comportant au moins un reste de N-acétylgalactosamine ou N-acétylglucosamine et au moins un reste de glucose ou galactose;
(c) ledit reste acide renfermant au moins un acide N-acétyl-neuraminique ou N-glycolylneuraminique; et
(d) le groupement carboxyl d'au moins un desdits restes acides étant esterifié par un groupement

hydroxy d'une des parties hydrocarbonées ou d'un desdits restes acides, afin de former un noyau lactonique, les contenus respectifs desdits récipients étant destinés à être mélangés pour former une solution de traitement.

23. Système pharmaceutique selon la revendication 22, dans lequel ledit premier récipient renferme du chlorure de sodium et un tampon à base de citrate.

24. Système pharmaceutique selon la revendication 22, dans lequel ledit excipient solide est constitué de glycine.

25. Système pharmaceutique selon la revendication 22 ou 23, dans lequel ledit excipient solide est constitué de mannitol.

26. Système pharmaceutique selon la revendication 22, dans lequel ledit second récipient renferme ledit dérivé de ganglioside dans une proportion d'environ 10% à environ 90% en poids.

27. Système pharmaceutique selon une quelconque des revendications 22 à 26, dans lequel: la partie hydrocarboné desdits dérivés de ganglioside présente la structure:

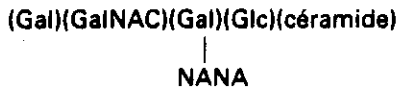
(Gal)(GalNAC)(Gal)(Glc)(céramide)

où

Gal est un reste de galactose;
GalNAC est un reste de N-acétylgalactosamine; et
Glc est un reste de glucose.

28. Système pharmaceutique selon la revendication 27, dans lequel: ledit dérivé de ganglioside comporte au moins un acide N-acétyl-neuraminique lié à au moins un des restes Gal.

29. Système pharmaceutique selon un quelconque des revendications 22 à 26, dans lequel:
(a) au moins un desdits dérivés de ganglioside présente la structure:



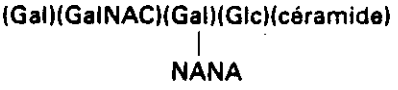
où

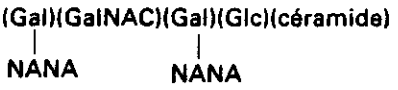
Gal est un reste de galactose;
GalNAC est un reste de N-acétylgalactosamine;
Glc est un reste de glucose; et
NANA est un reste d'acide N-acétylneuraminique, et dans laquelle:
(b) NANA est un ester lié à Gal.

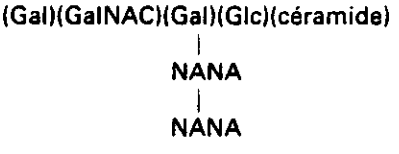
30. Système pharmaceutique selon la revendication 29, dans lequel ledit reste acide est de l'acide N-acétyl-neuraminique.

31. Système pharmaceutique selon une quelconque des revendications 22 à 27, dans lequel ladite partie hydrocarbonée est constituée par des restes de galactose, glucose et N-acétylgalactosamine.

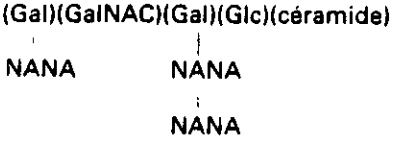
32. Système pharmaceutique selon une quelconque des revendications 22 à 27, dans lequel ledit dérivé de ganglioside comprend un mélange de:

(a) 

(b) 

(c) 

et

(d) 

où

Gal représente un reste de galactose;
GalNAC représente un reste de N-acétylgalactosamine;
Glc représente un reste de glucose; et
NANA représenté un reste N-acétyl-neuraminique, et dans lequel au moins un des restes NANA est estérifié par un groupement hydroxy d'un reste de galactose et/ou d'un reste NANA voisin afin de former un noyau lactonique.

33. Système pharmaceutique selon une quelconque des revendications 22 à 32, dans lequel ledit dérivé de ganglioside est un produit entièrement transformé constitué par un dérivé de ganglioside à ester interne dans lequel le groupment carboxyle est un ester lié à un groupement hydroxy d'une des parties hydrocarbonées ou à un des restes acides, de manière à former un noyau lactonique.

Patentansprüche:

1. Verfahren zum Herstellen von inneren Estergangliosidderivaten, welches das Umsetzen eines Gangliosids oder einer Mischung von Gangliosiden oder eines Salzes eines derartigen Gangliosids oder einer Mischung von Gangliosiden mit einer tertiären Stickstoffbase in einem nicht wässerigen organischen Lösungsmittel mit einem Lactonisierungsreagens unter Bildung zumindest einer Lactonbindung in den genannten inneren Estergangliosidderivaten umfaßt.

2. Verfahren nach Anspruch 1, worin das Gangliosid oder die Mischung von Gangliosiden aus Rindergehirnen extrahiert wird.

3. Verfahren nach Anspruch 1, worin das genannte organische Lösungsmittel ausgewählt wird aus Dimethylsulfoxid, Dimethylformamid, Sulfolan, Tetrahydrofuran, Dimethoxyäthan und Pyridin.

4. Verfahren zum Herstellen von inneren Estergangliosidderivaten, welches umfaßt:

(a) das Lösen eines Gangliosids oder einer Mischung von Gangliosiden oder eines Salzes hievon, erhalten von einem Säugetier, in einem aprotischen Lösungsmittel;

(b) das Zusetzen eines Ionenaustauscherharzes zum genannten Gangliosid oder der Mischung der genannten Ganglioside oder einem Salz hievon, wodurch die Carboxylatgruppen der genannten Ganglioside in Carboxylgruppen oder ein tert.Stickstoffsalz überführt werden; und

(c) das Behandeln des genannten Gangliosids oder der Mischung von Gangliosiden mit einem Carbodiimid unter Bildung zumindest einer Lactonbindung im gennanten inneren Estergangliosidderivat.

5. Verfahren nach Anspruch 1 oder 4, worin das genannte Lactonisierungsmittel ausgewählt wird aus Carbodiimiden, die in organischen Lösungsmitteln löslich sind, 2-Chlor-1-methylpyridiniumsalzen, Äthoxyacetylen und N-Äthyl-5-phenylisoxazolium-3'-sulfonat.

6. Verfahren nach Anspruch 4, worin das Carbodiimid Dicyclohexylcarbodiimid, Benzylisopropylcarbodiimid oder Benzyläthylcarbodiimid ist.

7. Verfahren nach Anspruch 1 oder 4, worin das genannte innere Estergangliosidderivat mit Aceton ausgefällt wird.

8. Verfahren nach Anspruch 4, worin das genannte Salz von Schritt (b) eines derartigen Gangliosids oder einer Mischung von Gangliosiden ein tert.Stickstoffsalz ist, hergestellt durch Überführen der Carboxylatgruppen in den genannten Gangliosiden in ein tert.Stickstoffsalz hievon mittels Ionenaustausch.

9. Verfahren nach Anspurch 4, worin das genannte Salz das tert.Stockstoffsalz mit Triäthylammonium oder Pyridinium ist.

10. Verfahren nach einem der Ansprüche 1 bis 9, worin die Reaktion mit einem Lactionisierungsreagens zu einer vollständigen Überführung der genannten Ganglioside führt.

11. Zusammensetzung zum Behandeln von Störungen des peripheren Nervensystems auf Grund von traumatischen, kompressiven, degenerativen oder toxisch-infektiösen Ursachen für die Stimulierung der Nervenregeneration und Wiedergewinnung von neuromuskulärer Funktion oder zum Behandeln von Störungen des zentralen Nervensystems auf Grund von traumatischen, anoxischen, degenerativen oder toxisch-infektiösen Ursachen, wo die Stimulierung von Neuronenentwicklung für die funktionelle Wiedergewinnung notwendig ist, welche zusammen mit einem pharmazeutisch annehmbaren Träger oder Verdünnungsmittel einen wirksamen Anteil zumindest eines inneren Estergangliosidderivats aufweist, umfassend:

(a) einen Kohlehydratteil, zumindest ein Ceramid und zumindest einen Säureteil;

(b) wobei der Kohlehydratteil zumindest einen N-Acetylgalaktosamin- oder N-Acetylglucosaminteil und zumindest einen Glucose- oder Galaktoseteil umfaßt;

(c) der genannte Säureteil zumindest eine N-Acetylneuraminsäure oder N-Glykolylneuraminsäure umfaßt; und

(d) die Carboxylgruppe zumindest eines der gennanten Säureteile an eine Hydroxygruppe eines der genannten Kohlehydrate oder eines der genannten Säureteile unter Bildung eines Lactonringes estergebunden ist.

12. Zusammensetzung nach Anspruch 11, worin der Kohlehydratteil der genannten Gangliosidderivate die Formel

(Gal)(GalNAC)(Gal)(Glc)(Ceramid)

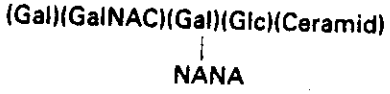
aufweist, worin

Gal ein Galaktoseteil ist,

GalNAC einen N-Acetylgalaktosaminteil bedeutet und
Glc einen Glucoseteil darstellt.

13. Zusammensetzung nach Anspruch 11 oder 12, worin das genannte Gangliosidderivat zumindest eine N-Acetylneuraminsäure gebunden an zumindest einen der genannten Gal-Teile aufweist.

14. Zusammensetzung nach Anspruch 11, worin
(a) zumindest eines der genannten Gangliosidderivate die Formel

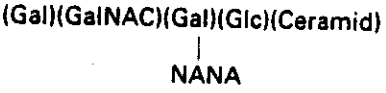


aufweist, worin
Gal ein Galaktoseteil ist,
GalNAC einen N-Acetylgalaktosaminteil bedeutet,
Glc einen Glucoseteil darstellt und
NANA ein N-Acetylneuraminsäureteil ist, und worin
(b) der genannte NANA an Gal estergebunden ist.

15. Zusammensetzung nach Anspruch 11 oder 14, worin der genannte Säureteil aus N-Acetylneuraminsäure besteht.

16. Zusammensetzung nach Anspruch 11, worin der Kohlehydratteil aus Galaktose-, Glucose- und N-Acetylgalaktosaminteilen besteht.

17. Zusammensetzung nach Anspruch 11, worin das genannte Gangliosidderivat eine Mischung von

(a)


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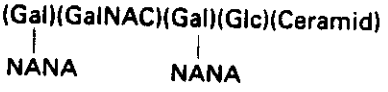

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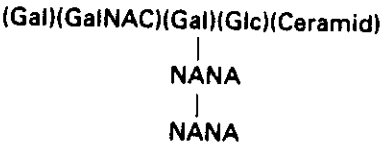

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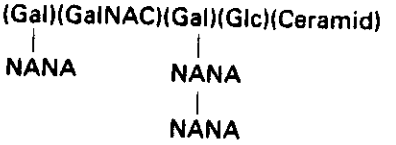


umfaßt, worin
Gal einen Galaktoseteil bedeutet,
GalNAC ein N-Acetylgalaktosaminteil ist,
Glc einen Glucoseteil darstellt, und
NANA einen N-Acetylneuraminsäureteil bedeutet und worin zumindest einer der genannten NANA-Teile an eine Hydroxygruppe eines Galaktoseteils und/oder einen angrenzenden NANA-Teile unter Bildung eines Lactonringes estergebunden ist.

18. Zusammensetzung nach einem der Ansprüche 11 bis 17, worin das genannte Ganglioside ein vollständig umgewandeltes Produkt ist, das aus einem inneren Estergangliosidderivat besteht, worin die Carboxylgruppe entweder an eine Hydroxygruppe eines der genannten Kohlehydratteile oder einen der Säureteile unter Bildung eines Lactonringes estergebunden ist.

19. Zusammensetzung nach einem der Ansprüche 11 bis 18 in Form einer injizierbaren Lösung.

20. Zusammensetzung nach einem der Ansprüche 11 bis 18, die von einem Träger getragen wird oder in einem Verdünnungsmittel bestehend aus einer Mischung von Natriumchlorid und einem Citratpuffer verdünnt ist.

21. Zusammensetzung nach einem der Ansprüche 11 bis 20, worin das genannte Gangliosidderivat in einem Anteil von etwa 2 bis etwa 50 Gew.% vorhanden ist.

22. Pharmazeutisches Zusammensetzungssystem enthaltend einen pharmazeutisch annehmbaren Träger oder ein derartiges Verdünnungsmittel, welches einen ersten Behälter, der ein Lösungsmittelsystem enthält, und einen zweiten Behälter, der einen festen Exzipienten und ein gefriergetrocknetes Pulver eines zum Behandeln des Nervensystems wirksamen Anteils zumindest eines inneren Estergangliosidderivats aufweist, umfaßt, welches Gangliosidderivat umfaßt:

(a) einen Kohlehydratteil, zumindest ein Ceramid und zumindest einen Säureteil;

(b) wobei der genannte Kohlehydratteil zumindest einen N-Acetylgalaktosamin- oder N-Acetylglucosaminteil und zumindest einen Glucose- oder Galaktoseteil umfaßt;

(c) der genannte Säureteil zumindest eine N-Acetylneuraminsäure oder N-Glykolylneuraminsäure umfaßt; und

(d) die Carboxylgruppe zumindest eines der genannten Säureteile an eine Hydroxygruppe eines der genannten Kohlehydrate oder einen der genannten Säureteile unter Bildung eines Lactonringes estergebunden ist, wodurch die Inhalte der genannten Behälter unter Bildung einer Behandlungslösung gemischt werden.

23. Pharmazeutisches Zusammensetzungssystem nach Anspruch 22, worin der genannte erste Behälter Natriumchlorid und einen Citratpuffer enthält.

24. Pharmazeutisches Zusammensetzungssystem nach Anspruch 22, worin der genannte feste Exzipient Glycin ist.

25. Pharmazeutisches Zusammensetzungssystem nach Anspruch 22 oder 23, worin der genannte feste Exzipient Mannit ist.

26. Pharmazeutisches Zusammensetzungssystem nach Anspruch 22, worin der genannte zweite Behälter das genannte Gangliosidderivat in einem Anteil von etwa 10 bis etwa 90 Gew.% enthält.

27. Pharmazeutisches Zusammensetzungssystem nach einem der Ansprüche 22 bis 26, worin der Kohlehydratteil der genannten Gangliosidderivate die Formel

(Gal)(GalNAC)(Gal)(Glc)(Ceramid)

aufweist, worin

Gal ein Galaktoseteil ist;

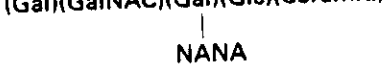
GalNAC einen N-Acetylgalaktosaminteil bedeutet und

Glc einen Glucoseteil darstellt.

28. Pharmazeutisches Zusammensetzungssystem nach Anspruch 27, worin das genannte Gangliosidderivat zumindest eine N-Acetylneuraminsäure an zumindest einen der genannten Gal-Teile gebunden aufweist.

29. Pharmazeutisches Zusammensetzungssystem nach einem der Ansprüche 22 bis 26, worin

(a) zumindest eines der genannten Gangliosidderivate die Formel



aufweist, worin

Gal ein Galaktoseteil ist,

GalNAC einen N-Acetylgalaktosaminteil bedeutet,

Glc einen Glucoseteil darstellt und

NANA ein N-Acetylneuraminsäureteil ist und worin

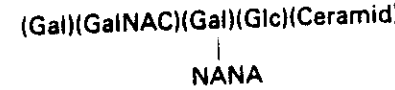
(b) NANA an Gal estergebunden ist.

30. Pharmazeutisches Zusammensetzungssystem nach Anspruch 29, worin der genannte Säureteil aus N-Acetylneuraminsäure besteht.

31. Pharmazeutisches Zusammensetzungssystem nach einem der Ansprüche 22 bis 27, worin der Kohlehydratteil aus Galaktose-, Glucose- und N-Acetylgalaktosaminteilen besteht.

32. Pharmazeutisches Zusammensetzungssystem nach einem der Ansprüche 22 bis 27, worin das genannte Gangliosidderivat eine Mischung von

(a)



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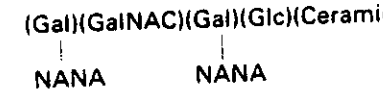

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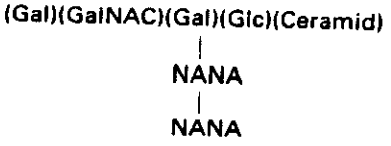

und

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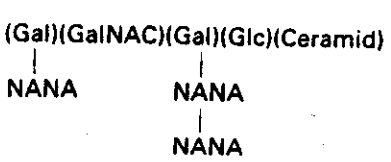

umfaßt, worin
Gal ein Galaktoseteil ist;
GalNAC ein N-Acetylgalaktosaminteil bedeutet;
Glc einen Glucoseteil darstellt, und
NANA ein N-Acetylneuraminsäureteil ist, und worin zumindest einer der genannten NANA-Teil an eine Hydroxygruppe eines Galaktoseteils und/oder einen angrenzenden NANA-Teil unter Bildung eines Lactonringes estergebunden ist,

33. Pharmazeutisches Zusammensetzungssystem nach einem der Ansprüche 22 bis 32, worin das genannte Gangliosidderivat ein vollständig umgewandeltes Produkt ist, das aus einem inneren Estergangsliosidderivat besteht, worin die Carboxylgruppe an eine Hydroxygruppe eines der Kohlenhydratteile oder an einen der Säureteile unter Bildung eines Lactonringes estergebunden ist.

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Associate Director :
Mrs J Needle

OUR REF: 57370/AP/SD YOUR REF:

30th September 1985.

The Comptroller,
The Patent Office,
25 Southampton Buildings,
London WC2A 1AY.

Dear Sir,

European Patent No. 0072722
FIDIA SpA
National Phase of Granted European Patent

We write to request that our address be entered on the Register of Patents as the address for service in the United Kingdom in respect of the above-mentioned European Patent (U.K.) to comply with Rule 30 of the U.K. Patent Rules 1978. Accordingly, we enclose a Declaration of Authorisation on Patents Form 51/77 (in duplicate).

The said European Patent is to be granted on European Patent Application No. 82401385.8.

We should be grateful if you would acknowledge receipt of this letter by returning the duplicate Form 51/77.

Yours faithfully,
PAGE WHITE & FARRER.

Page, White & Farrer

Encls.

72722

PATENTS ACT 1977

PATENTS FORM No. 51/77
(PLEASE FILE IN DUPLICATE)
(Rule 90)

The Comptroller
The Patent Office
25 Southampton Buildings
London, WC2A 1AY

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2. Enter in full, name and address of applicant, proprietor or other person who has authorised agent. The full names of all partners in a firm must be entered.
3. State the application or other proceeding in relation to which the authorisation was made, quoting the application number or patent number as appropriate.

I/We (see note 1) PAGE WHITE & FARRER
5 Plough Place, New Fetter Lane, London EC4A 1HY.
ADP Code No.

declare that I/we have been authorised by (see note 2) FIDIA S.p.A.
Via Ponte Della Fabbrica 3-1, 1-35031 Abano Terme (Padova) Italy

to act as agent in the matter of (see note 3) European Patent (U.K.) No. 0072722

and request that all communications relating thereto be sent to ~~me~~/us at ~~my~~/our address given above.

THE PATENT OFFICE
30 SEP 1985
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Signature Page, White & Farrer

PAGE WHITE & FARRER -
Agents for the Applicant.

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(84) Designated States:
Austria, Federal Republic of Germany, United Kingdom, The Netherlands, Sweden.

(54) Title:
A method for preparing ganglioside derivatives and use thereof in pharmaceutical compositions.

(73) Proprietor:
FIDIA SpA, Via Ponte Della Fabbrica 3-A, I-35031 Abano Terme (Padova), Italy.

(72) Inventors:
Della Valle, Francesco, Via Cerato 14, Padova, Italy.

Romeo, Aurelio, Viale-Ippocrate 93, Rome, Italy.

(51) Classified to:
C07H 19/00

Address for Service:

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