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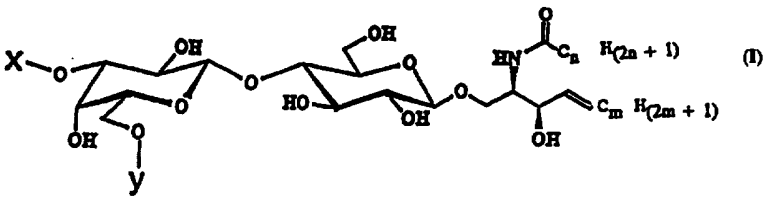
(57) Abstract

Compositions of matter comprising glycosphingolipids useful for suppressing an immune response having formula (I) wherein x is (a) or (b) or H; wherein Y is (c) or H; wherein m is 10 to 20; and wherein n is 1 to 14. Also presented are methods for suppressing an immune response in an animal employing glycosphingolipids as shown above. Synthetic gangliosides having artificial hydrophobic anchors, useful for suppressing an immune response having formula (II) wherein A is a carbohydrate moiety of a ganglioside, n is 5 to 20 and m is 5 to 20. Also presented are methods for suppressing an immune response in an animal and compositions of matter employing synthetic gangliosides having artificial hydrophobic anchors, as shown above. Simplified carbohydrate moiety-gangliosides, useful for suppressing an immune response according to formula (III) wherein B is a ceramide moiety of a ganglioside. Also presented are methods for suppressing an immune response in an animal and compositions of matter employing simplified carbohydrate moiety-ganglioside as shown above.



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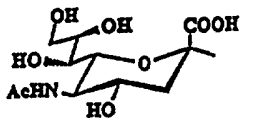

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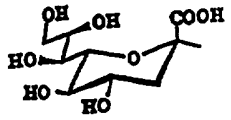

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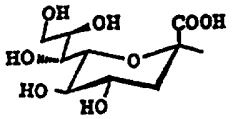

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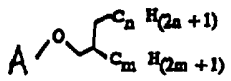

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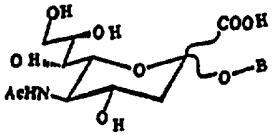

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SYNTHETIC GANGLIOSIDE DERIVATIVES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to synthetic ganglioside derivatives, not found in nature, which are useful as immunosuppressive agents. More particularly, the present invention relates to glycosphingolipids, artificial anchor gangliosides and simplified carbohydrate moiety-gangliosides which are useful pharmaceutical agents for inhibiting an immune response.

2. Description of Related Art

Although the immune response is often seen as beneficial, in certain circumstances the immune response to an antigen can actually be harmful to the animal in which the immune response occurs. An example where the immune response creates a condition wherein the host is subject to serious pathologic sequelae is in such autoimmune diseases as lupus erythematosus, rheumatoid arthritis, diabetes, and Crohn's disease. In autoimmune diseases, the immune response is directed against host tissues, and therefore use of immunosuppressive agents is a treatment approach.

Another, and one of the most important, areas which often requires substantial immunosuppression is tissue transplantation, where the suppression of the immune response is crucial in order to prevent graft rejection by the host (host versus graft reaction, HVG) and graft rejection of the host (graft versus host rejection, GVH). Typically, the tissue which is grafted is allogeneic, where the inhibition of alloreactive T lymphocytes by immunosuppressive agents is essential to the

prevention of allograft rejection. Depending upon the nature of the allograft (i.e. liver, kidney, or bone marrow), the course of immunosuppressive therapy may be relatively brief (months) or may have to be continued indefinitely (years to lifetime). All of the immunosuppressive agents used thus far have significant drawbacks relating either to direct toxicity on other organ systems or to failure to provide "balanced" immunosuppression. The latter problem has two distinct aspects; on one hand inadequate suppression of the immune response can lead to rejection, while on the other hand excessive immunosuppression can allow the development of opportunistic infections and neoplasia. Thus, the need to develop an effective non-toxic immunosuppressive agent which does not cause the above severe complications continues.

At present, multi-drug therapy, including cytotoxic agents, is utilized following organ transplantation. This typically comprises combination therapy, such as treatment with cyclosporin A, azathioprine, and prednisone, the rationale being that each drug acts at a different stage in the immune response and the combination therapy will require lower doses of each individual drug, thus diminishing their dose-related side effects. However, the side effects remain significant while the efficacy of this form of therapy is still not satisfactory. Rejection continues to account for nearly 50% of graft losses in renal transplantation. And, distinguishing rejection from cyclosporin A nephrotoxicity may be difficult.

Another major cause of graft loss is systemic infection, usually by opportunistic infections, which require the tapering or cessation of immunosuppression, which leads to graft loss. Also, with such combination therapy in transplantation, there has been a significant increase in the incidence of lymphomas (Wilkinson, et al., "Transplantation," 47:293-296, 1989). The chronic failure of

immunosuppressive therapy is revealed by the fact that the graft survival rate of 85% at 1 year drops to 67% at 5 years (Kahan, et al., "Am J. Kidney Dis," 5:288-295, 1985) in recipients of cadaveric renal transplants receiving triple therapy. Clearly, then the existing immunosuppressive therapy is inadequate. This has stimulated the search for, and development of, new immunosuppressive drugs, and particularly agents that are not directly toxic to either the immune system or to other organ systems. One approach to overcoming the problems associated with present immunosuppressive drugs is the use of biological agents which are actually produced by the animal. An example of such biological agents are the gangliosides.

Gangliosides are a class of glycosphingolipids. As shown schematically in FIG. 1, gangliosides have a structure containing a carbohydrate moiety linked to a ceramide. The carbohydrate moiety includes a sugar moiety which has at least one monosaccharide and one or more sialic acid moiety(s), i.e. sialic acid groups (N-acetyl or N-glycolyl neuraminic acid). FIG. 2 sets forth the nomenclature which is used to describe the ceramide moiety. The ceramide moiety includes a long chain base (LCB) portion and a fatty acid (FA) portion. The number to the left of the colon indicates the carbon chain length of the fatty acid or long chain base, and the number to the right indicates the degree of unsaturation. The major long chain base structures (to the left of the dash) of normal human brain gangliosides are d18:1 and d20:1, and of extraneural gangliosides, d18:1. The major fatty acid structures (to the right of the dash) are 18:0 and 20:0.

Gangliosides are also classified according to the number of monosaccharides in the carbohydrate moiety and the number of sialic acid groups present in the sialic acid moiety(s); Further classification is dependent upon where and how many sialic acid(s) are bound to the carbohydrate moiety. For example, the international symbol G_{M1a}

designates one of the more common gangliosides which has been extensively studied. The subscript, "M" in the symbol indicates that the ganglioside is a monosialoganglioside and "1" indicates that there are four saccharide units present in the carbohydrate moiety. The subscripts "a", "b" or "c" indicate isomers of the particular ganglioside described which differ in the position of the sialic acid(s). The subscripts "D", "T" and "Q" used as international ganglioside symbols represents gangliosides, trisialongangliosides and tetrasialongangliosides, respectively. The subscripts "2", "3" and "4" represent trisaccharide, disaccharide and monosaccharide gangliosides, respectively. The terminal saccharide is the saccharide which is located at the end of the carbohydrate moiety which is opposite to the end that is attached to the ceramide moiety.

Ten common human brain gangliosides and their biosynthetic pathway are set forth in the Fig. 3. The structure of each ganglioside is set forth using conventional abbreviations for the ceramide, saccharide and sialic acid (SA) groups. Fig. 3 also outlines the biosynthetic pathway of the gangliosides. The biosynthesis of gangliosides is discussed in detail in S. Roseman, *Chem. Phys. Lipids,* 5: 270-297, 1970.

It is well know that gangliosides are functionally important in the nervous system and it has been claimed that gangliosides are useful in the therapy of peripheral nervous system disorders. Numerous gangliosides are derivatives thereof have been used to treat a wide variety of nervous system disorders including cerebral ischemic strokes. For example, see U.S. Pat. Nos. 4,940,694; 4,937,232; and 4,716,223. Gangliosides have also been used to affect the activity of phagocytes (U.S. Pat. No. 4,831,021) and to treat gastrointestinal disease-producing organisms (U.S. Pat. No. 4,762,822).

The use of gangliosides and ganglioside analogues to suppress or to otherwise affect the immune system has not yet been investigated as extensively as their use in neurological disorders.

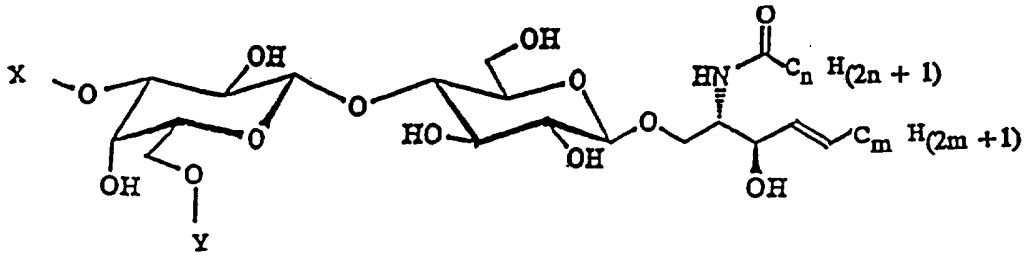
The first report of ganglioside suppression of immune responses *in vivo* was published twenty years ago by Agarwal and Neter, who discovered inhibition by gangliosides of the primary antibody response to bacterial antigens in mice (Agarwal, et al., *J. Immunol.*, 107: 1448-1456, 1971). Recent studies have shown that tumor gangliosides which are shed *in vivo* enhance tumor formation in mice (Ladisch, et al., *J.Clin.Invest.*, 79:1879-1882, 1987), a finding confirmed by other laboratories (Allessandri, et al., *Cancer Res.*47:4243-4347, 1987; Saha, et al., *Int.J.Cancer*, 41:432-435, 1988); indirect evidence (Ladisch, et al., *J.Clin.Invest.*, 79:1879-1882, 1987) suggests that this enhancement occurs by an immunologic mechanism. However, a recent investigation into the *in vivo* immunosuppressive effect of G_{M1} ganglioside or mixed bovine brain gangliosides (mainly G_{M1}), G_{D1a}, G_{D1b}, and GT_{1b}) was conducted by Presti, D. et al., (Presti, D. et al. *J. Neuroimmunology*, 22: 233-239, 1989) The study concluded that there was no evidence of a suppressive effect on humoral or cellular immunity exhibited *in vivo* by the G_{M1} ganglioside or the mixed brain gangliosides.

As noted above, gangliosides are composed of three elements. The role these elements play, however, in the immunosuppressive activity of gangliosides is unknown. Indeed, in the past, the identification of preferred active ganglioside structures has largely been limited to naturally occurring gangliosides. Although naturally occurring gangliosides vary to some extent in the structure of their elements, the available variants do not permit a full exploration of the role the various elements play in immunosuppression.

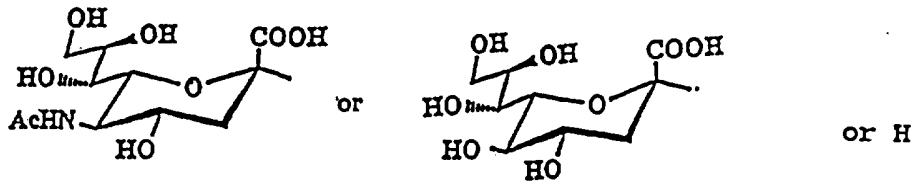
There is, thus, a continuing need to develop chemically synthesized gangliosides, wherein the various elements of naturally occurring gangliosides are replaced with synthetic or artificial moieties.

SUMMARY OF THE INVENTION

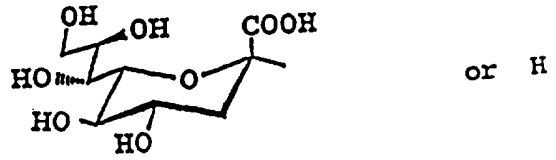
As a first aspect of the present invention, a composition of matter is presented which comprises a glycosphingolipid which has the formula



wherein x is



wherein Y is

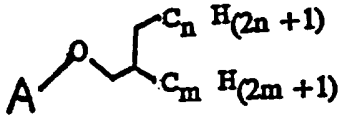


where m is 10 to 20 and wherein n is 1 to 14 and a pharmaceutically acceptable carrier for the glycosphingolipid.

This aspect of the present invention is based on the discovery that glycosphingolipids having shorter synthetic fatty acyl chains are more potent immunosuppressives than their longer fatty acyl chain counterparts. Accordingly, the shorter fatty acyl chain glycosphingolipids as set forth above are thus useful in suppressing an immune response in an animal.

In this regard, the present invention includes methods for suppressing an immune response in an animal via administration of an immune response suppressing effective amount of a glycosphingolipid according to the above formula.

Another aspect of the present invention is a synthetic ganglioside having an artificial hydrophobic anchor according to the formula



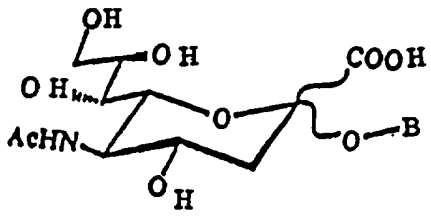
wherein A is a carbohydrate moiety of a ganglioside, n is 5 to 20 and m is 5 to 20.

This aspect of the present invention is based on the discovery in accordance with the present invention that the ceramide moiety of a ganglioside can be replaced with an artificial hydrophobic anchor structure, resulting in a highly immunosuppressive molecule. Synthetic gangliosides having artificial hydrophobic anchors in accordance with the present invention are useful for suppressing an immune response in an animal.

The present invention includes methods for suppressing an immune response in an animal via administration of an immune response suppressing effective amount of a synthetic ganglioside having an artificial hydrophobic anchor according to the above formula.

Also presented in accordance with the present invention are compositions of matter comprising a synthetic ganglioside having an artificial hydrophobic anchor according to the above formula and a pharmaceutically acceptable carrier for the synthetic ganglioside having an artificial hydrophobic anchor.

Yet another aspect of the present invention concerns a simplified carbohydrate moiety ganglioside according to the formula



wherein B is a ceramide moiety of a ganglioside. This aspect of the present invention is based on the discovery in accordance with the present invention that the carbohydrate portions of a ganglioside can be simplified to a sialosyl moiety, resulting in a highly immunosuppressive agent.

Presented in accordance with this aspect of the present invention are methods for suppressing an immune response in an animal via administration of an immune response suppressing effective amount of a simplified carbohydrate moiety-ganglioside according to the above formula.

Also presented in accordance with the present invention are compositions of matter comprising a simplified carbohydrate moiety-ganglioside according to the above formula and a pharmaceutically acceptable carrier for the simplified carbohydrate moiety-ganglioside.

The above discussed and many other features and attendant advantages of the present invention will become apparent as the

invention becomes better understood by reference to the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the structure of a ganglioside.

FIG. 2 depicts the structure and nomenclature of the ceramide portion of a ganglioside.

FIG. 3 depicts the carbohydrate structure and biosynthetic pathway of 10 naturally occurring gangliosides.

FIG. 4 is a graphical representation of the inhibition of the human lymphoproliferative response (^{3}H-thymidine uptake) by G_{M3} N = 1 (●) and lyso G_{M3} (■).

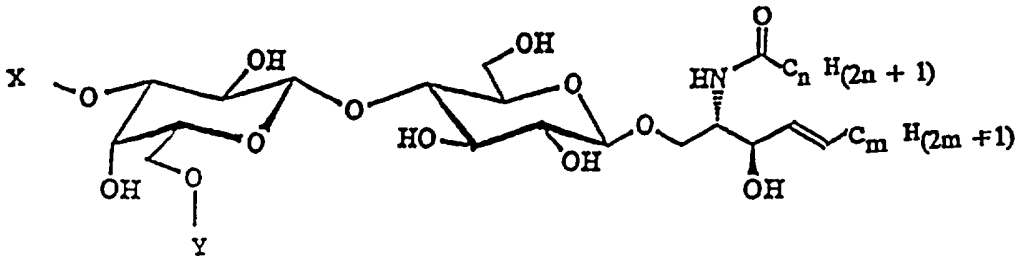
FIG. 5 is a bar graph showing inhibition of the human lymphoproliferative response by G_{M3} n = 1, n = 13, n = 17, and n = 23.

FIG. 6 is a graphical representation of the inhibition of the human lymphoproliferative response (^{3}H-thymidine uptake) by dialkyl G_{M3}.

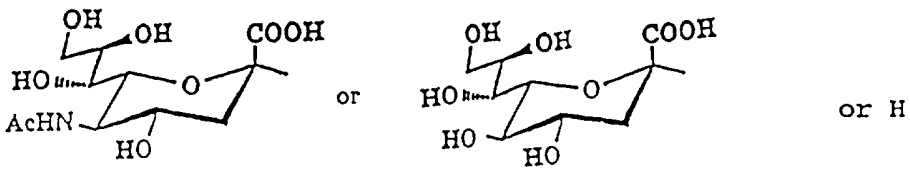
FIG. 7 is a graphical representation of the inhibition of the human lymphoproliferative response (^{3}H-thymidine uptake) by dialkyl G_{M3} (●) and d 18:1 - C18:0 G_{M3} (▲).

DETAILED DESCRIPTION OF THE PRESENT INVENTION

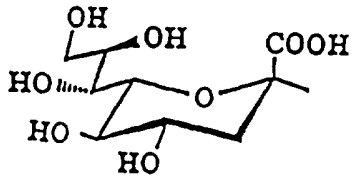
One aspect of the present invention involves a composition of matter comprising a glycosphingolipid having the formula



wherein X is



wherein Y is



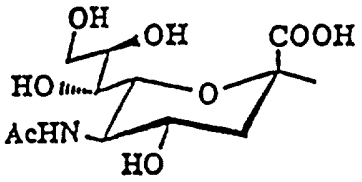
or H

wherein m is 10 to 20 and wherein n is 1 to 14 and a pharmaceutically acceptable carrier for the glycosphingolipid. Another aspect of the present invention is a method for suppressing an immune response in an animal comprising administering an immune response suppressing effective amount of a glycosphingolipid having the above formula to an animal.

These aspects of the present invention are based on the discovery in accordance with the present invention that glycosphingolipids having shorter synthetic fatty acyl chains are more potent immunosuppressors than longer fatty acyl chain-containing glycosphingolipids of identical carbohydrate structure. For instance, a glycosphingolipid according to the above formula wherein n = 1 was found in accordance with the present invention to have greater immunosuppressive activity than glycosphingolipids wherein n is 17 or 23. Other species of glycosphingolipids wherein n is 1 to 14 and wherein m is 10 to 20 are also expected to have higher immunosuppressive activity than their longer fatty acyl chain-containing counterparts. Thus, ceramide moieties with shorter fatty acyl chains, as defined above may be linked to a carbohydrate moiety corresponding to that of any naturally occurring ganglioside.

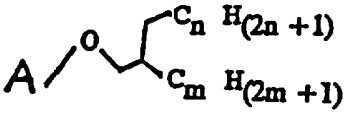
Preferably, in a glycosphingolipid according to the methods and compositions of matter of the present invention, m is 13 and n is from 1 to 5. Most preferably, m is 13 and n is 1. This is also the case with regard to the compositions of matter and methods employing a glycosphingolipid as disclosed in accordance with the instant invention.

In a preferred exemplary glycosphingolipid according to the present invention, X is preferably



and Y is preferably H. Further, when X and Y are as described in this paragraph, m is preferably 13 and n is preferably from 1-5. Most preferably, X and Y are as described in this paragraph, m is 13 and n is 1. Gangliosides containing a synthetic fatty acyl structure in accordance with the present invention are synthesized according to methods well known to those of skill in the art. See, e.g., Murase et al. (1989) Carbohydr. Res. 188, 71-80; KDN analogs are synthesized according to Terada, et al. (1993) J. Carbohydr. Chem. 12, 425-440.

Another aspect of the present invention is a synthetic ganglioside having an artificial hydrophobic anchor according to the formula



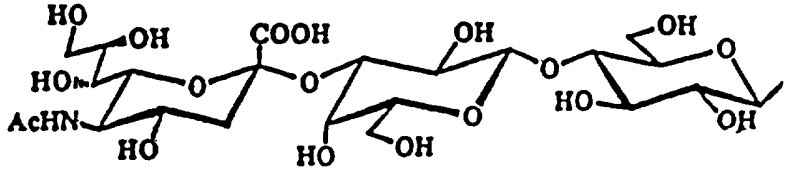
wherein A is a carbohydrate moiety which corresponds to the carbohydrate moiety of a naturally occurring ganglioside, n is 5 to 20 and m is 5 to 20. As used with respect to the present invention, "carbohydrate moiety" includes both the oligosaccharide core, and any attendant sialic acid residues of any naturally occurring ganglioside as shown in FIG. 2. Exemplary carbohydrate moieties from which A may be selected include those shown in FIG. 3. Two other aspects of the

present invention are a composition of matter comprising a synthetic ganglioside having an artificial hydrophobic anchor according to the formula set forth in this paragraph and a pharmaceutically acceptable carrier for the synthetic ganglioside; and a method of suppressing an immune response in an animal comprising the step of administering an immune response suppressing effective amount of a synthetic ganglioside according to the formula set forth in this paragraph.

This aspect of the present invention is based on the discovery in accordance with the present invention that the ceramide moiety of a ganglioside can be replaced with an artificial hydrophobic anchor structure, resulting in an immunosuppressive agent more potent than naturally occurring gangliosides. Other artificial hydrophobic anchor sequences may also be used in accordance with the present invention, including, for example, those containing additional methylene groups between the oxygen atom and the alkane chains as shown in the above formula.

In a synthetic ganglioside having an artificial hydrophobic anchor according to the present invention, n is preferably 13. Further, m is preferably 14. Most preferably n is 13 and m is 14.

In a synthetic ganglioside having an artificial hydrophobic anchor in accordance with the present invention, A is preferably



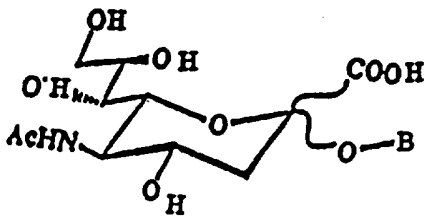
In the most preferred exemplary embodiment of the present invention, A is as shown above, n is 13 and m is 14.

The preferred embodiments of the synthetic gangliosides having an artificial hydrophobic anchor are also the preferred embodiments for its related compositions of matter and methods of suppressing an immune response in an animal.

Synthetic gangliosides having an artificial hydrophobic anchor, in accordance with the present invention, may generally be synthesized according to the methodologies employed with respect to the synthesis of a synthetic ganglioside having an artificial hydrophobic anchor as set forth in the examples.

Carbohydrate moities are synthesized according to the following references: T. Murase, A. Kameyama, K.P.R. Kartha, H. Ishida, M. Kiso, and A. Hasegawa, J. Carbohydr. Chem., 8, 265 (1989). T. Murase, H. Ishida, M. Kiso, and A. Hasegawa, Carbohydr. Res., 188, 71 (1989); A. Hasegawa, T. Murase, K. Adachi, M. Morita, and M. Kiso, J. Carbohydr. Chem., J. Carbohydr. Chem., 9, 181 (1990); A. Hasegawa, T. Murase, M. Morita, H. Ishida, and M. Kiso, J. Carbohydr. Chem., 9, 201 (1990). T. Terada, M. Kiso, and A. Hasegawa, J. Carbohydr. Chem., 12, 425 (1993); T. Terada, M. Kiso, and A. Hasegawa, Carbohydr. Res., 259, 201 (1994); Carbohydrates -- Synthetic Methods adn Applications in Medicinal Chemistry--- pp 243-266 (1992) Eds. by H. Ogura, A. Hasegawa, and T. Suami, Kodansha-VCH; Synthetic Oligosaccharide--- Indispensable Probes for the Life Sciences---Ed. by P. Kovac, ACS Symposium Series 560, American Chemical Society, pp. 184-197 (1994), by A. Hasegawa.

Yet another aspect of the present invention is a simplified carbohydrate-moiety ganglioside according to the formula



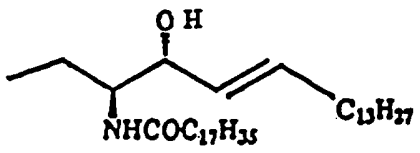
wherein B is a ceramide moiety which corresponds to the ceramide moiety present in a naturally occurring ganglioside. Exemplary ceramide moieties from which B may be selected include, using the nomenclature

of FIG. 2, those with a long chain base of d18:1 or d20:1 in combination with any of the following: C16:0, C18:0, C20:0, C22:0, C24:0 and C24:1. In addition, synthetic ceramide moities, as disclosed herein with respect to the glycosphingolipid aspect of the present invention, are also expected to provide potent immunosuppressors when linked to a sialosyl residue as disclosed above. Examples of these synthetic ceramide groups include C2:0, C10:0 and C14:0.

Two other related aspects of the present invention are a composition of matter comprising a simplified carbohydrate moiety-ganglioside according to the above formula and a pharmaceutically acceptable carrier for the simplified carbohydrate moiety-ganglioside; and a method for suppressing an immune response in an animal comprising the step of administering to the animal an immune response suppressing effective amount of a simplified carbohydrate moiety-ganglioside according to the above formula.

The simplified carbohydrate moiety-ganglioside aspect of the present invention is based on the discovery in accordance with the present invention that the carbohydrate portion of a ganglioside can be simplified to a sialosyl moiety resulting in a highly effective immunosuppressive agent.

In a simplified carbohydrate-moiety ganglioside according to the present invention, B is preferably



This is also the preferred embodiment for the method and composition of matter employing a simplified carbohydrate moiety-ganglioside.

Simplified carbohydrate moiety-gangliosides, in accordance with the present invention, may generally be synthesized according to the methodologies set forth with rspect to the synthesis of a specific simplified carbohydrate moiety-ganglioside as set forth in the examples. Ceramide moieties are generally synthesized according to Ito, et al., J. Carbohyudr., Chem., 6, 117 (1987).

As demonstrated in the examples below, the glycosphingolipids, artificial anchor gangliosides, and simplified carbohydrate moiety-ganglioside and their corresponding compositions of matter are potent immunosuppressive agents and they are useful for treating animals, including humans, where it is desirous to reduce an immune response. It is desirous to reduce an immune response, for example, in order to inhibit rejection of a tissue graft.

In the present invention, the term "suppressive" denotes a lessening of the detrimental effect of the undesirable immune response in the animal receiving therapy. The term "immune response suppressing effective amount" means that the amount of agent used is of sufficient quantity to suppress the cause of disease or symptoms due to the undesirable immune response. The term "animal" also denotes humans.

The dosage ranges for the glycosphingolipids, artificial anchor gangliosides and simplified carbohydrate moiety-gangliosides ("immunosuppressive agents") of the present invention are those large enough to produce the desired effect: the immune response shows some degree of suppression. The dosage should not be so large as to cause adverse side effects. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the animal and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary from less than 1 mg/kg/dose to about 100 mg/kg/dose, preferably

about 5 mg/kg/dose to 10 mg/kg/dose, in one or more dose administrations daily.

The immunosuppressive agents of the present invention can be administered parenterally by single injections or by gradual infusion over time. The immunosuppressive agents can also be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavitarily, or transdermally.

Pharmaceutically acceptable carriers include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol., polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils, intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved by the use of polymers to complex or adsorb the immunosuppressive agents of the present invention. The controlled delivery may be exercised by selecting appropriate macromolecules (for example, polyesters, polyamino carboxymethylcellulose, and protamine sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the immunosuppressive agents of the present invention into

particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly (lactic acid) or ethylene vinylacetate copolymers.

In order to protect the immunosuppressive agents from binding with plasma proteins, it is preferred that the gangliosides be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly (Methymethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nonacapsules or in macroemulsions. Such teachings are disclosed in *Remington's Pharmaceutical Sciences* (16th Ed., A. Oslo, ed., Mack, Easton, PA, 1980).

The immunosuppressive agents of the present invention are well suited for use in targetable drug delivery systems such as synthetic or natural polymers in the form of macromolecular complexes, nanocapsules, microspheres, or beads, and lipid-based systems including oil-in-water emulsions, liposomes, and resealed erythrocytes. Miscelles and mixed micelles are particularly preferred for delivering the immunosuppressive agents of the present invention. These systems are known collectively as colloidal drug delivery systems. Typically such colloidal particles containing the dispersed gangliosides are about 50 nm - 2 μm in diameter. The size of the colloidal particles allows them to be administered intravenously such as by injection, or as an aerosol. Materials used in the preparation of colloidal systems are typically sterilizable via filter sterilization, nontoxic, and biodegradable, for example albumin, ethylcellulose, casein, gelatin, lecithin, phospholipids, and soybean oil. Polymeric colloidal systems are prepared by a process similar to the coacervation of microencapsulation.

Most preferred as a targeted delivery system for the immunosuppressive agents of the present invention are liposomes.

When phospholipids are gently dispersed in aqueous media, they swell, hydrate, and spontaneously form multilamellar concentric bilayer vesicles with layers of aqueous media separating the lipid bilayer. Such systems are usually referred to as multilamellar liposomes or multilamellar vesicles (MLVs) and have diameters ranging from about 100nm to about 4 um. When MLVs are sonicated, small unilamellar vesicles (SUVs) with diameters in the range of from about 20 to about 50 nm are formed, which contain an aqueous solution in the core of the SUV.

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidyserine, and phosphatidylethanolamine. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and are saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine.

In preparing liposomes containing the immunosuppressive agents of the present invention, such variables as the efficiency of ganglioside encapsulation, lability of the ganglioside, homogeneity and size of the resulting population of liposomes, immunosuppressive agent-to-lipid ratio, permeability instability of the preparation, and pharmaceutical acceptability of the formulation should be considered. Szoka, et al., *Annual Review of Biophyusics and Bioengineering*, 9:467, 1980; Deamer, et al., in *Liposomes, Marcel Dekker*, New York, 1983, 27: Hope, et al., *Chem. Phys. Lipids*, 40:89, 1986).

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be further distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelian systems (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves the alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposomes themselves in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization. Alternatively, liposomes may physically localize in capillary beds such as the lung or may be given by site-specific injection.

Another targeted delivery system which can be used with the immunosuppressive agents of the present invention is resealed erythrocytes. When erythrocytes are suspended in a hypotonic medium, swelling occurs and the cell membrane ruptures. As a consequence, pores are formed with diameters of approximately 200-500 A which allow equilibration of the intracellular and extracellular environment. If the ionic strength of this surrounding media is then adjusted to isotonic conditions and the cells incubated at 37°C, the pores will close such that the erythrocyte reseals. This technique can be utilized with the immunosuppressive agents of the present invention to entrap the immunosuppressive agent inside the resealed erythrocyte. The resealed erythrocyte containing the immunosuppressive agent can them be used for targeted delivery.

The targeted delivery system containing the immunosuppressive agents of the present invention may be administered in a variety of ways to a host, particularly a mammalian host, such as intravenously,

intramuscularly, subcutaneously, intra-peritoneally, intravascularly, topically, intracavitarily, transdermally, intranasally, and by inhalation. The concentration of the gangliosides will vary upon the particular application, the nature of the disease, the frequency of administration, or the like. The targeted delivery system-encapsulated ganglioside may be provided in a formulation comprising other compounds as appropriate and an aqueous physiologically accepted medium, for example, saline, phosphate buffered saline, or the like.

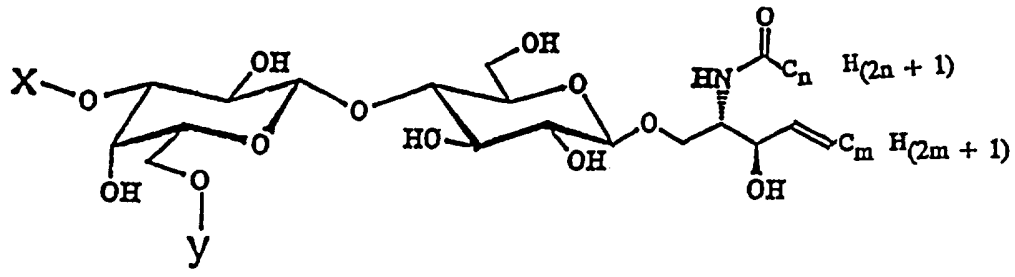
The above disclosure generally describes the present invention. A further understanding can be obtained by reference to the following specific examples which are provided for purposes of illustration and are not intended to be limiting.

EXAMPLES

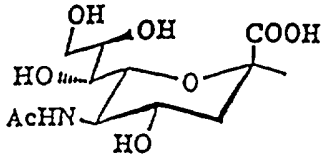
GLYCOSPHINGOLIPIDS WITH REDUCED FATTY ACYL CHAIN LENGTH EXHIBIT ENHANCED IMMUNOSUPPRESSIVE ACTIVITY

In this example, the immunosuppressive activity of glycosphingolipids with reduced fatty acyl chain length were compared to that of their longer fatty acyl chain containing counterparts.

Glycosphingolipids according to the structure



wherein x is



wherein Y is H wherein m is 13 and wherein n is either -1 (a lyso glycosphingolipid, having no fatty acyl portion), 1, 13, 17 or 23, were tested for their immunosuppressive activity. The carbohydrate portion of the studied glycosphingolipids corresponds to that of G_{M3} and, thus, the studied glycosphingolipids are also referred to as G_{M3} n = a number from -1 to 23.

Materials and Methods

Lymphocyte proliferation assay: An assay of the human cellular immune response, lymphoproliferation stimulated by a specific antigen, tetanus toxoid (Ladisch et al., Brochim Briphys. Aota, 1125, 180-88 (1992), has been used to measure the immunosuppressive effects of the synthetic ganglioside derivatives of the present invention. Briefly, normal human peripheral blood mononuclear leukocytes were isolated by Ficoll-hypaque density gradient centrifugation (Boyum, *Scand. J. Clin. Lab. Invest* 21, 77-89 (1968) from whole blood collected in preservative-free heparin (50 U/ml). The cells were washed three times and resuspended in complete HB104 medium. Autologous human plasma was added to a final concentration of 0.5%. Normal human peripheral blood mononuclear leukocytes were cultured in 96-well (A/2) tissue culture clusters (Costar No. 3696).

Synthetic ganglioside derivatives were suspended in medium by brief sonication before addition to the cell cultures. 10 µl synthetic ganglioside derivative solution were added per well, followed by addition of the peripheral blood mononuclear leukocytes (PBMC, 25 µl, $2x10^6$ cells/ml complete medium). After a 3 h preincubation at 37°C, 10 µl of the previously determined optimal concentration of the stimulant of lymphoproliferation, tetanus toxoid (3.5 Lf/ml, Mass.Dept. of Health, Boston, MA) was added. 10 µl of basal medium alone was added to unstimulated control cultures. The complete cultures were incubated at 37°C in 95% air/5% CO_2 for 6 days *Biochem. Biophys. Aota* 1125,

180-88 (1992). As has been previously documented under these conditions *Biochim. Biophys,. Aota* 1125m 180-88 (1992), gangliosides are not toxic to the cells. At the end of the culture period, 0.5μCi [^{3}H]thymidine in 50 μl medium was added to each well. The cultures were incubated for an additional 4.5 h and harvested onto glass fiber filter paper. Cellular [^{3}H]thymidine uptake was quantified by ß-scintillation counting. Mean net [^{3}H]thymidine uptake in stimulated cultures was determined by subtracting the mean cpm of unstimulated cultures. Percent inhibition was calculated by comparing the mean net [^{3}H]thymidine uptake of cultures containing gangliosides with that of cultures without synthetic ganglioside derivatives.

In vivo Assay *of Immunosuppressive Activity*: Footpad injection of the synthetic ganglioside derivatives being studied and of the stimulant of the cellular immune response (allogeneic cells), is subsequently followed by harvest of the popliteal node and assessment of the node size, cell number, specific proliferation response and generation of specific cytotoxicity.

Mice: C3H (H-2^K) and BALB/c (H-2^d) mice are obtained at 6 weeks of age and used in these experiments at 7-12 weeks of age. The animals are murine virus free strains purchased from Charles River, Wilmington, Massachusetts.

Preparation of stimulator cells: Spleens are removed aseptically and immediately placed in murine complete media [RPMI 1640 w/o L-glutamine (Whittaker Bioproducts, Walkersville, Md) supplemented with 10% FCS; 1% of MEM non-essential amino acids (Cellgro), sodium pyruvate, L-glutamine, Penicillin 50U/ml/Streptomycin 50μg/ml and 10mM Hepes Buffer (Whittaker Bioproducts)] then transferred to a 60 x 10 mm petri dish. A sterile single cell suspension is prepared by gently pressing the spleen onto a cell dissociation sieve (Sigma). Mononuclear cells are isolated by Ficoll-Hypaque density gradient separation, followed

by lysis of erythrocytes (ACK lysing buffer pH 7.4). The cells are washed and their viability determined by trypan blue exclusion. Allogeneic (BALB/c) splenocytes are diluted to the appropriate concentration in saline and are injected into the footpad of C3H mice together with the synthetic ganglioside derivatives to be tested.

Preparation of synthetic ganglioside derivatives: Synthetic ganglioside derivatives are aliquoted in HPLC-grade chloroform:methanol (1:1), and dried in glass microvials. The synthetic ganglioside derivatives are then resuspended for injection in 0.9% NaCl, sonicated for 2 min in a Branson water bath sonicator.

Injections: Spleen cells or tumor cells and the synthetic ganglioside derivatives being studied are injected into the left hind footpad in a total volume of 30μl. Cyclosporin A is administered i.p.

Isolation of popliteal lymph nodes: Primed animals are killed by cervical dislocation on day 4, the popliteal lymph node draining the left and right footpads are removed aseptically, trimmed free of excess fat, weighted and placed on ice in tubes containing tissue culture medium. The nodes are then teased with a flat end of a 3 ml syringe to obtain a single cell suspension, which is washed in complete murine media containing 0.1% 2mercaptoethanol (Gibco, NY). The cells are then quantified and their viability determined by trypan blue exclusion. The cell concentration is adjusted to 2 x 10^6 cells/ml.

Cell cultures: 2 x 10^5 lymph node cells in 100μl are cultured for 18 hours in complete medium with 0.5 μC ^{3}H-thymidine incorporation quantified by ß-scintillation counting as a measure of in vivo lymphocyte activation (50).

RESULTS

Role of ceramide structure in determining immunosuppressive activity of glycosphingolipids: The activities of two synthetic glycosphingolipids, G_{M3} n=1 and G_{M3} n=-1 (lyso G_{M3}) were compared at

various concentrations for their inhibition of the human lymophoproliferative response (FIG. 4). Each point in FIG. 4 represents the mean ± SEM of triplicate cultures, control stimulation was 11.5 ± 3.0 x 10^3 CPM. The importance of a fatty acyl structure was evidenced by the higher degree of immunosuppressive activity of the glycosphingolipid wherein n = 1 (ID_{50} = 0.2 nm), compared to that of the glycosphingolipid wherein n = -1 (lyso G_{M3}) (FIG. 4).

Next, the relative immunosuppressive activities of a number of glycosphingolipids were compared: G_{M3} (n = 1, n = 13, n = 17 and n = 23), (FIG. 5). Each bar represents the mean ± SEM [^{3}H] thymidine uptake of cultures exposed to 5μM of the indicated G_{M3} species. Control stimulation = 11.5 ± 3.0 X 10^3 cpm. The tested glycosphingolipids exhibited an immunosuppressive effect which increased as the length of the fatty acyl portion decreased, with n = 1 having the highest immunosuppressive activity.

G_{M3} n = 1 was also tested for immunosuppressive activity *in vivo*. A single dose of G_{M3} n = 1 was found to be almost as immunosuppressive as systemically administered cyclosporin A, a known immunosuppressant (Table 1). The in vivo immunosuppressive activity of G_{M3} n = 1 was also compared to that of mixed human brain gangliosides; G_{M3} n = 1 was found to be much more active than the mixed brain gangliosides.

TABLE 1

Parameter	Control	Cyclosporin A	G_{M3} n=1
lymph node mass, mg[1]			
unstimulated	1.17±0.17	0.99±0.34	1.29±0.24
stimulated	2.77±0.53	1.26±0.22	1.84±0.30
net increase	1.60±0.46	0.27±0.48	0.56±0.36
lymphocytes x 10^7 [2]	2.26	0.30	1.03
[^{3}H]thymidine uptake, cpm[3]	1108±283	201±18	329±18

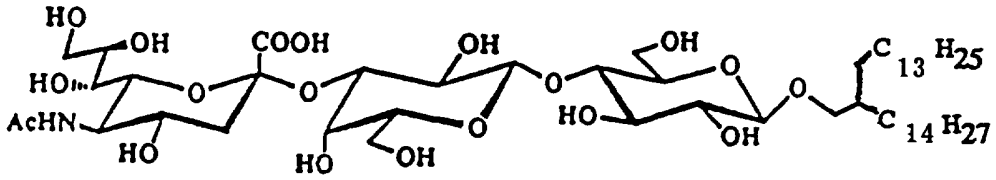
1 Synthetic ganglioside G_{M3} n=1 (10nmol) was coinjected into the left hind footpad of C3H mice together with allogeneic splenocytes (BALB/C, 2.5 x 10^6), which was compared with the systemic administered cyclosporin A (24 mg/kg/dose i.p.x 4 doses). On day 4, the popliteal lymph nodes draining the left (stimulated) and the right footpad (unstimulated) were removed, and the lymph node mass measured. The data represent the mean ± SD of five mice in each group in this representative experiment. The difference in the net increase of popliteal nodes between control and G_{M3}6 n=1 treated groups is statistically significant ($P < 0.01$).

2 The total mononuclear leukocytes recovered from five stimulated popliteal lymph nodes of five mice in each group.

3 The spontaneous lymphoproliferation was measured by cellular [^{3}H]thymidine incorporation at the cell density of 2x10^5 cells/well. The data represent the mean ± SD of three cultures.

IMMUNOSUPPRESSIVE PROPERTIES OF AN ARTIFICIAL ANCHOR GANGLIOSIDE

In this example, a synthetic ganglioside having an artificial hydrophobic anchor having the structure

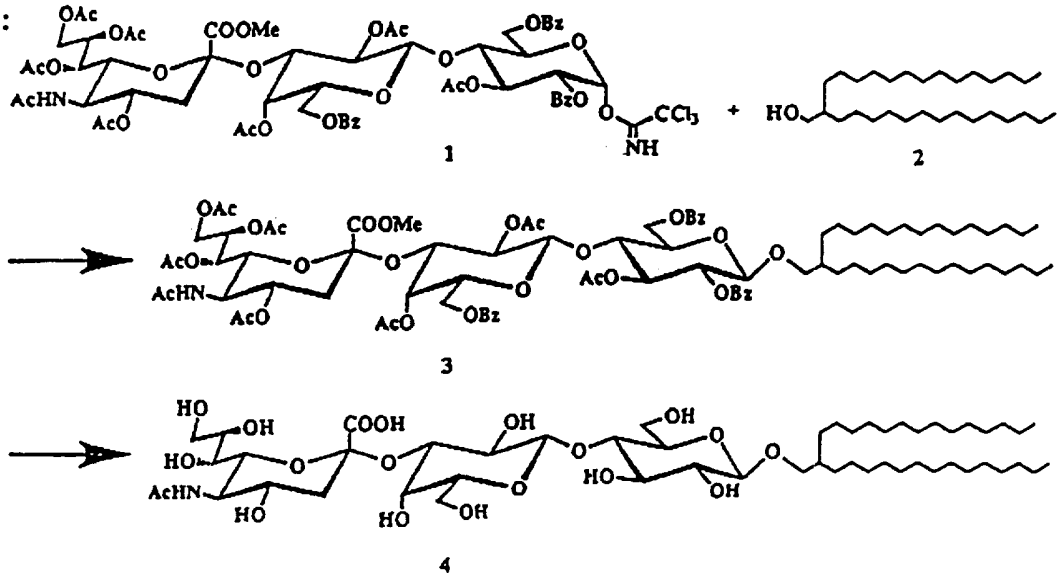


was tested to determine whether it was immunosuppressive. This compound has a carbohydrate portion corresponding to G_{M3} and is also referred to as dialkyl G_{M3}.

Materials and Methods

Synthesis of dialkyl G_{M3}:

Dialkyl G_{M3} was synthesized according to the following general strategy:



A. Synthesis of 2-(Tetradecylhexadecyl) O-(methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→3))-O-(2,4-di-O-acetyl-6-O-benzoyl-ß-D-

galactopyranosyl)-(1→4)-3-O-acetyl-2,6-di-O-benzoyl-ß-D-glucopyranoside (3 from general strategy set forth above).

To a solution of the trichloracetimidate (Murase, et al. Carbohydr. Res.188, 71-80(1989) (1; 150 mg, 0.11 mmol) and 2-tetradecylhe adecyl-1-ol (2; 120 mg, 0.27 mmol) in CH_2C1_2 (3mL) was added molecular sieves 4A, AW 300 (2g), and the mixture was stirred for 30 min., then cooled to 0° C. Boron trifluoride etherate (0.04 mL) was added to this mixture, and this was stirred for 4 h at 0°c and filtered. Dichloromethane (50mL) was added to the filtrate, and this was washed with MNa2C03 and water, dried (Na_2SO_4) and evaporated. Column chromatography of the residue on silica gel (30g) with 3:2 ethyl acetate-hexane gave 3 (0.16 g, 89%) as an amorphous mass.

B. Synthesis of 2-(Tetradecylhexadecyl) *O*-(5-acetamido-3, 5-dideoxy-D-*glycero*-*D*-*galacto*-2-nonulopyranosylonic acid) - (2→3) - O-ß-D-galactopyranosyl-(1→4)-ß-D-glucopyranoside (4 from general strategy).

To a solution of 3 (75 mg, 0.045 mmol) in methanol (5mL) were added 5 drops of 28% sodium methoxide solution in methanol, and the mixture was stirred for 10h at room temperature, and then water (0.5mL) was added. The solution was stirred for another 8h and neutralized with Amberlite IR-120(H^+) resin, then concentrated. Column chromatography (MeOH) of the residue on Sephadex LH-20 (30g) gave 4 (quantitative) as an amorphous mass.

C. Synthesis of 2-Tetradecylhexadecyl-1-01(2 from general strategy)

Compound 2 was obtained as an amorphous mass from 2-tetradecyl-hexadecanoic acid via methyl esterification and subsequent reduction of the methyl ester with $LiAlH_4$.

Assays: *In vivo* and *in vitro* assays of immunosuppression were as described above.

Quantitative and qualitative analysis of dialkyl G_{M3}: Dialkyl G_{M3} was quantified by resorcinol assay Svennerholm, *Biochem.Biophys.* Acta 24, 604-611 (1957) and analyzed by high performance - TLC. The developing solvent system was chloroform/methanol/0.2% $CaCl_2$ $2H_2O$ (60:40:9, by volume), and the glycoconjugates were stained by resorcinol - HC1 Ledeen et al., *Methods Enzymol.* 83, 139-191 (1982).

Results

Immunosuppressive activity of dialkyl G_{M3}: Chemically synthesized dialkyl G_{M3} was assessed for its immunosuppressive activity in a tetanus toxoid-induced human lymphoproliferation assay over a range of glycoconjugate concentrations (0-20 μM). The % inhibition of cellular proliferation by glycoconjugate-treated cultures was calculated in five separate experiments by comparing the mean net [^{3}H]thymidine uptake of triplicate glycoconjugate-treated cultures with control cultures. Each point represents the mean inhibition ± SD in three experiments. Control stimulation was 2.2 ± 0.6 x 10^4 cpm. the ID_{50} for dialkyl G_{M3} was less than 0.3 μM. As shown in Figure 6, dialkyl G_{M3} has marked immunosuppressive activity. The concentration causing 50% inhibition of the antigen-induced human lymphoproliferative response (ID_{50}), was less than 1 μM, and 90% inhibition was observed at <7 μM. When these high degrees of inhibition were compared with those obtained by G_{M3} (d:18:1-C18:0) in parallel experiments, one of the naturally occurring species of G_{M3} which was also obtained by chemical synthesis, the higher degree of inhibition of dialkyl G_{M3} than that of G_{M3} is readily observed. Chemically synthesized dialkyl G_{M3} (●) and d18:1-C18:0 G_{M3} (▲) were assessed for immunosuppressive activity in the tetanus toxoic-induced human lkymphoproliferation assay over a range of ganglioside concentrations (0-10 μM). Each point represents the mean ± SEM of triplicate cultures. Control stimulation was 1.6 ± 0.4 x 10^4 cpm. Dialkyl G_{M3} had an ID_{50} of 0.2μM, and was four-fold more

potent than G_{M3} D18:1-c18:0 (FIG. 7). These results demonstrate that the chemically synthesized dialkyl G_{M3} strongly inhibits the human cellular immune response *in vitro*, as measured by tetanus toxoid-induced lymphoproliferation.

Inhibition of the allogeneic immune response in vivo: To determine the potential significance of the *in vitro* immunosuppressive activity of dialkyl G_{M3} which is shown by Figures 6 and 7, a murine model was used to evaluate the *in vivo* immunosuppressive activity of dialkyl G_{M3}. In this model, the immune response in a local microenvironment directed against allogeneic cells is assessed. Allogeneic (C3H mice) spleen cells were injected into the footpad of BALB/c mice and the draining popliteal lymph nodes were removed from the sacrificed mice four days later. By allogeneic stimulation, a specific immune response developed in the popliteal lymph node (Kroczek et al. *J. Immunol.* 139, 3597-3603 (1987), which was assessed by the increased lymph node mass, lymphocyte number, and *in vitro* lymphoproliferative response. Systemic administration of cyclosporin A has a marked inhibitory effect on the allogeneic immune response (Morris et al. *Transplant Proc.* 22, 1638-1641 (1990). When dialkyl G_{M3} (10 nmol or 11 μg/mouse) was administered together with the allogeneic cells, a marked suppression of the immune response was observed (Table 2). This was evident as assessed by three parameters. First, there is a striking inhibition in the increase of lymph node mass. The net increase of lymph node mass in the mice of the control group stimulated with the allogeneic cells is 1.6 mg, the increase is only 0.45 mg when dialkyl G_{M3} was coinjected with the allogeneic cells, which is very close to that for the systemically administered cyclosporin A (0.3 mg).

These results were confirmed by enumerating the total mononuclear cells recovered from the draining stimulated popliteal

lymph nodes. The lymphocyte (Mononuclear-leukocyte) number is $0.8x10^7$ for the dialkyl G_{M3} treated group (five nodes from five mice), and $0.3 x10^7$ for the cyclosporin A treated group. These numbers are $\leq$ 1/3 of that of the control group (2.3×10^7 cells). Furthermore, the *in vitro* spontaneous proliferative assay by these recovered lymphocytes shows that dialkyl G_{M3}, like cyclosporin A, markedly suppresses the proliferation as measured by [^{3}H] thymidine incorporation under the conditions of three different cell densities (Table 2). For example, under the condition of $2x10^5$ cells, the [^{3}H]thymidine uptake for the group of dialkyl G_{M3} treatment is only 20% that of control group. Together, these results demonstrate substantial *in vivo* immunosuppressive activity of dialkyl G_{M3}.

TABLE 2

PARAMETER	CONTROL	CYCLOSPORIN A	DIALKYL G_{M3}
lymph node mass, mg[4]			
unstimulated	1.17±0.17	0.99±0.34	1.41±0.17
stimulated	2.77±0.53	1.26±0.22	1.86±0.25
net increase	1.60	0.27	0.45
lymphocytes x 10^{7} [5]	2.26	0.30	0.83
[^{3}H] thymidine uptake, cpm [6]			
2x10^5 cells	1108±18	201±18	317±45
1x10^5 cells	372±63	101±4	152±15
0.5x10^5 cells	245±53	63±9	107±29

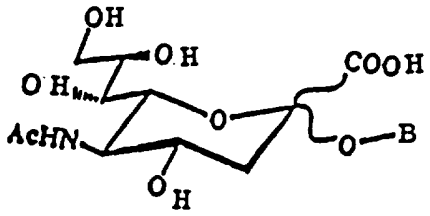
[4] Allogeneic splenocytes (BALB/C, 2.5 x 106) were injected into the left hind footpad of C3H mice. In the group of dialkyl G_{M3} treatment, 11μg of dialkyl G_{M3} was coinjected together with the allogeneic cells, which was compared with the systemic administration of CSA (24 mg/kg/day i.p.x4 days). On day 4, the popliteal lymph nodes draining the left (stimulated) and the right footpad (unstimulated) were removed, and the lymph node mass measured. The data represent the mean ± SD of five mice in each group in this representative experiments. The difference between control and dialkyl G_{M3} (or cyclosporin A) - treated groups is considered statistically significant, the P value is <0.01.

[5] The total mononuclear leukocytes recovered from five stimulated popliteallymph nodes of five mice in each group.

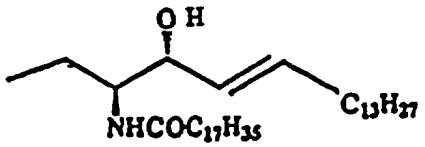
[6] The spontaneous lymphoproliferation was measured by [^{3}H]thymidine incorporation at three different cell density. The data represent the mean ± SEM of three cultures.

A SIMPLIFIED CARBOHYDRATE MOIETY-GANGLIOSIDE IS AN IMMUNOSUPPRESSANT

In this example a simplified carbohydrate moiety-ganglioside having the structure



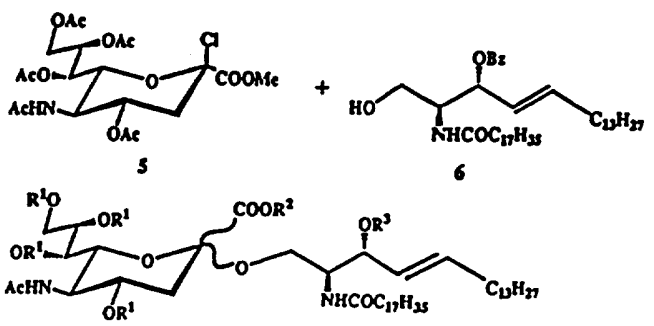
wherein B is



and wherein the stereochemistry indicated by wavy lines is either *α* or ß was tested for its immunosuppressive properties. This simplified carbohydrate moiety-ganglioside is also referred to as G_{M5} (αG_{M5} or βG_{M5}).

Materials and methods

Synthesis: The general synthesis strategy for synthesizing G_{M5} is shown below:



5 6

7 R^1 = Ac, R^2 = Me, R^3 = Bz (Bz = benzoyl)
8 $R^1 = R^2 = R^3$ = H

The bond indicated by a wavy line, indicates that the stereochemistry at that position may be either *α* or ß. The synthesis of each is described below.

A. Synthesis of (2S,3R,4E)-3-0-Benzoyl-1-0-(methyl 5-acetamido-4,7,8,9-tetra-0-acetyl-3,5-dideoxy-D-*glycero*-a-and ß-D-*galacto*-2-nonulopyranosylonate)-2-octadecanamido-4-octadecene-1, 3-diol (7*α* and 7ß).

Condensation of 6 (300 mg,0.45 mmol) and 5 (460 mg, 0.9 mmol) in dichloromethane (5mL) in the presence of Molecular sieves 4A (200mg), 2,4,6-trimethylpyridine (0.16mL) and silver triflate (385mg), overnight at room temperature in the dark, gave 7*α* (133mg, 26%) and 7ß (159 mg, 31%), respectively, after column chromatography (silica gel, 30:1 CH2C12-MeOH).

B. Synthesis of (2S,3R,4E)-1-0-(5-acetamido-3,5-dideoxy-D-*glycero*-*α*-and ß-D-*galacto*-f2-nonulopyranosylonicacid)-2-octadecanamido-4-octadecene-1,3-diol (8a and 8ß). O-Deacylations of 7*α*(300mg) and 7ß(300mg) were performed with a catalytic amount of sodium methoxide in methanol solution. Saponification of the methyl ester group was performed with 0.1M potassium hydroxide (0.43mL) in methanol solution (3mL) for 3h at room temperature, to give 8*α* (αG_{M5}) and 8ß (βG_{M5}) in quantitative yields, respectively.

RESULTS

Immunosuppressive Activity of α and ß G_{M5} In Vitro: The immunosuppressive activity of *α* and ß G_{M5} were determined by the human lymphocyte proliferation assay as described above. Table 3 demonstrates that both *α* and ß G_{M5} are potent immunosuppressive agents. αG_{M5} exhibits 99% inhibition of human lymphoproliferation at both 2.5 and 5.0 nM. ß G_{M5} exhibits slightly less inhibition: 86% at 2.50 μM and 97% at 5.0 μM.

TABLE 3

	[^{3}H] Thymidine Uptake CPMX10^{-3}		Inhibition (%)	
	2.5 μM	5.0 μM	2.5μM	5.0 μM
control	9.8	9.8		
G_{M3}				
d18:1-C14:0	4.6	0.8	53	92
G_{M5} (d18:1-C18:0)				
αsialosyl ceramide	0.1	0.1	99	99
ßsialosyl ceramide	1.4	0.3	86	97
dialkyl G_{M3}	0.7	0.3	93	97

In addition the ID90 of αG_{M5} was determined to be $\approx$ 2.5 μM (FIG. 8).

Immunosuppressive Activity of αG_{M5} In Vivo: The ability of αG_{M5} to inhibit the alloimmune response in draining popliteal lymph nodes in vivo (as described above) was determined (Table 4). αG_{M5} was formed to be highly immunosuppressive *in vivo* as compared to systemically administered cyclosporin A (CSA). αG_{M5} caused a 2/3 reduction in cellular immune response (the increase in lymph mass caused by *in vivo* allostimulation).

TABLE 4

	lymph node mass, mg[7]		
	unstimulated	stimulated	net increase
Control	1.30±0.28	2.93±0.81	1.63
CSA	0.67±0.06	0.85±0.21	0.18
G_{M5} α Sialosyl ceramide	0.82±0.26	1.40±0.22	0.58

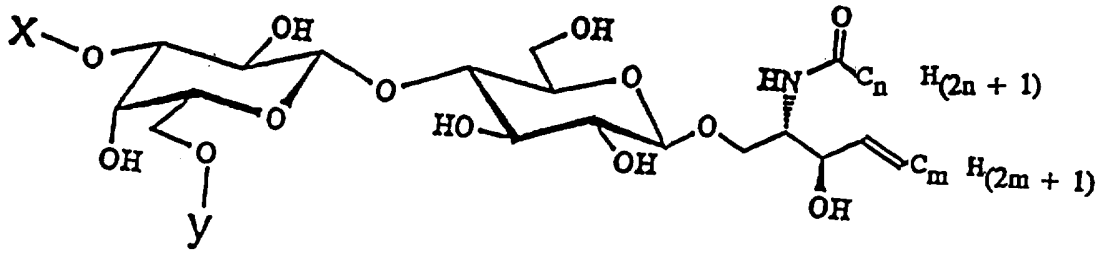
Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

[7] Allogeneic splenocytes (BALB/C, 2.5 x 106) were injected into the left hind footpad of C3H mice. In ganglioside-treated group, 10nmol of each gangliosides were coinjected together with the allogeneic cells, which was compared with the systemic administration of CSA (24 mg/kg/dose i/i.p.x 4 doses). On day 4, the popliteal lymph nodes draining the left (stimulated) and the right footpad (unstimulated) were removed, and the lymph node mass measured. The data represent the mean ±SD of five mice in each group in this representative experiment. The difference between control and ganglioside (or cyclosporinA)-treated groups is statistically significant, the P value is <0.01.

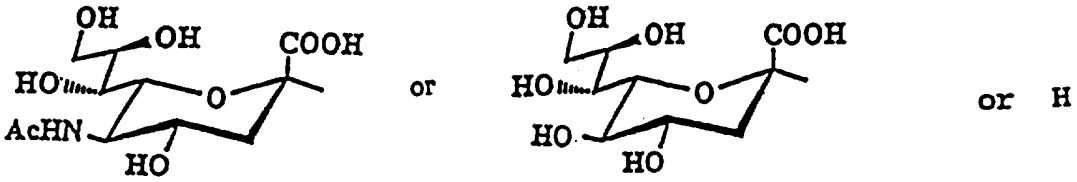
CLAIMS

WHAT IS CLAIMED IS:

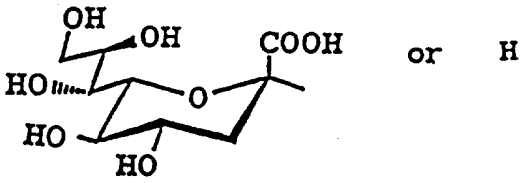
1. A composition of matter comprising a glycosphingolipid having the formula



wherein X is

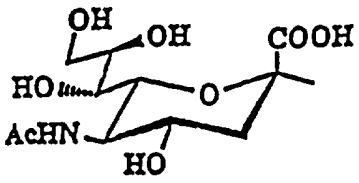


wherein Y is



wherein m is 10 to 20 and wherein n is 1 to 14 and a pharmaceutically acceptable carrier for said glycosphingolipid.

2. A composition of matter according to claim 1 wherein X is



and Y is H.

3. A composition of matter according to claim 2 wherein n is 1 to 5 and m is 13.

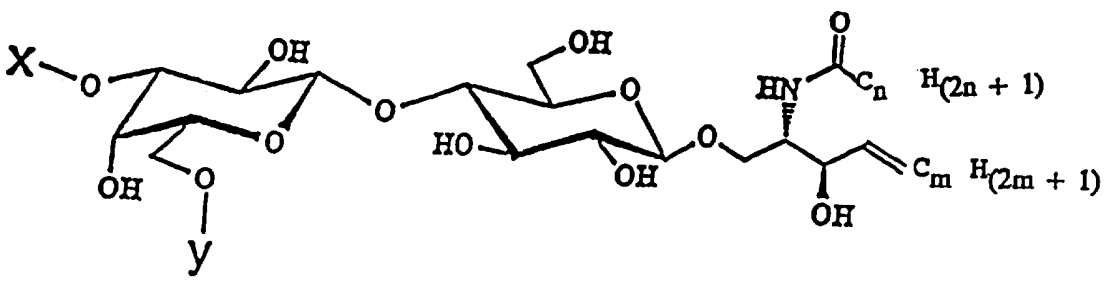
4. A composition of matter according to claim 3 wherein n is 1.

5. A composition of matter according to claim 1 wherein n is 1-5 and m is 13.

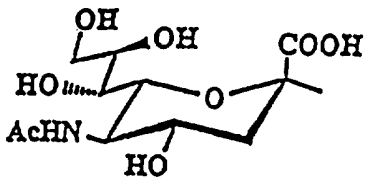
6. A composition of matter according to claim 5 wherein n is 1.

7. A method for suppressing an immune response in an animal comprising the step of

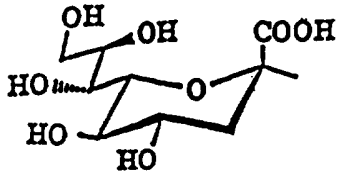
administering to said animal an immune response suppressing effective amount of a glycosphingolipid having the formula



wherein X is

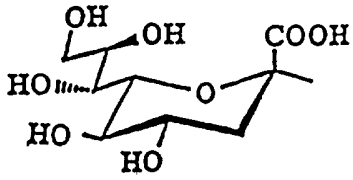


or



or H

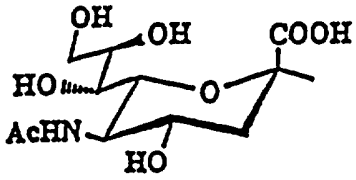
wherein Y is



or H

wherein m is 10 to 20 and wherein n is 1 to 14.

8. A method for suppressing an immune response according to claim 7 wherein X is



and wherein Y is H.

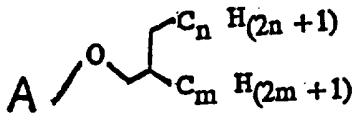
9. A method for suppressing an immune response according to claim 8 wherein n is 1 to 5 and m is 13.

10. A method for suppressing an immune response according to claim 9 wherein n is 1.

11. A method for suppressing an immune response according to claim 7 wherein n is 1-5 and m is 13.

12. A method for suppressing an immune response according to claim 11 wherein n is 1.

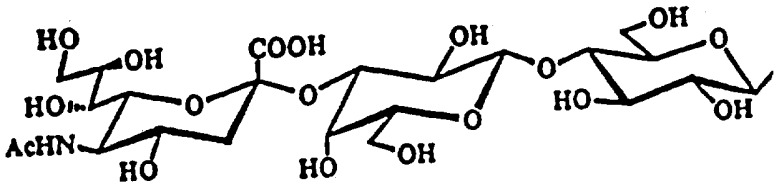
13. A synthetic ganglioside having an artificial hydrophobic anchor according to the formula



wherein A is a carbohydrate moiety of a ganglioside, n is 5 to 20 and m is 5 to 20.

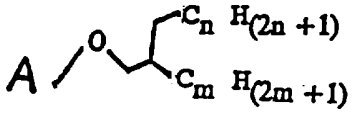
14. A synthetic ganglioside having an artificial hydrophobic anchor according to claim 13 wherein n is 13 and m is 14.

15. A synthetic ganglioside having an artificial hydrophobic anchor according to claim 13 wherein A is



16. A synthetic ganglioside having an artificial hydrophobic anchor according to claim 15 wherein n is 13 and m is 14.

17. A composition of matter comprising a synthetic ganglioside having an artificial hydrophobic anchor according to the formula

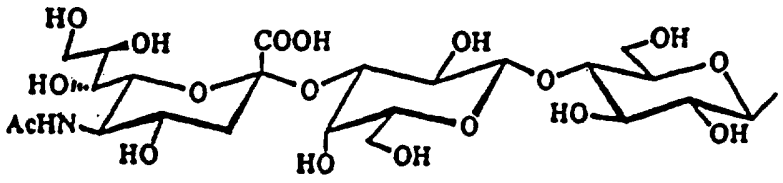


wherein A is a carbohydrate moiety of a ganglioside, n is 5 to 20 and m is 5 to 20

and a pharmaceutically acceptable carrier for said synthetic ganglioside.

18. A composition of matter according to claim 17 wherein n is 13 and m is 14.

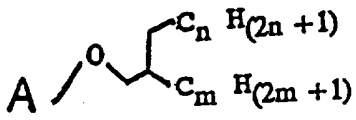
19. A composition of matter according to claim 17 wherein A is



20. A composition of matter according to claim 19 wherein n is 13 and m is 14.

21. A method of suppressing an immune response in an animal comprising the step of

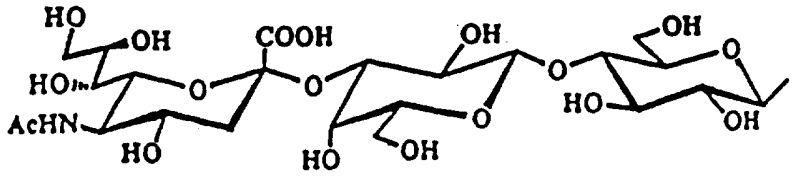
administering an immune response suppressing effective amount of a synthetic ganglioside having an artificial hydrophobic anchor having the structure:



wherein A is a carbohydrate moiety of a ganglioside, n is 5 to 20 and m is 5 to 20.

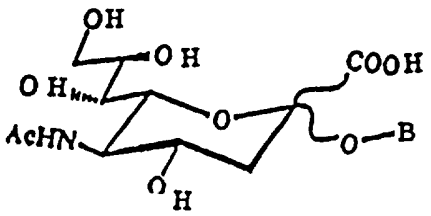
22. A method of suppressing an immune response in an animal according to claim 21 wherein n is 13 and m is 14.

23. A method of suppressing an immune response in an animal according to claim 21 wherein A is



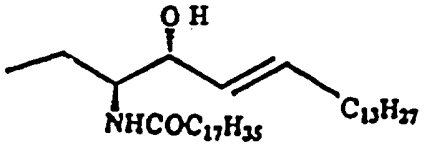
24. A method of suppressing an immune response in an animal according to claim 23 wherein n is 13 and m is 14.

25. A simplified carbohydrate moiety-ganglioside according to the formula

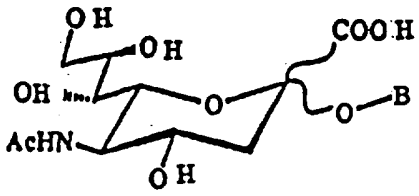


wherein B is a ceramide moiety of a naturally occurring ganglioside.

26. A simplified carbohydrate moiety-ganglioside according to claim 25 wherein B is

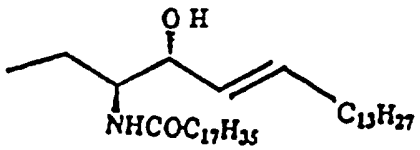


27. A composition of matter comprising a simplified carbohydrate moiety-ganglioside according to the formula



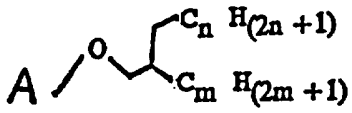
wherein B is a ceramide moiety of a naturally occurring ganglioside and a pharmaceutically acceptable carrier for said simplified carbohydrate moiety-ganglioside.

28. A composition of matter according to claim 27 wherein B is



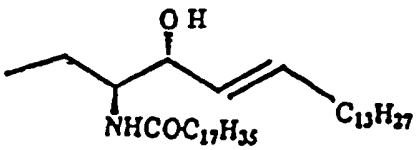
29. A method of suppressing an immune response in an animal comprising the step of

administering to said animal an immune response suppressing effective amount of a simplified carbohydrate moiety-ganglioside according to the formula



wherein B is a ceramide moiety of a naturally occurring ganglioside.

30. A method of suppressing an immune response in an animal according to claim 29 wherein B is



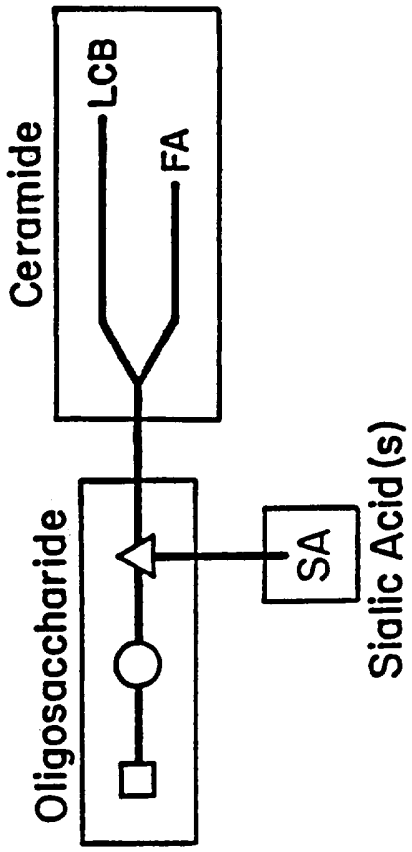

Ceramide
LCB
FA
Oligosaccharide
SA
Sialic Acid(s)

FIG. 1

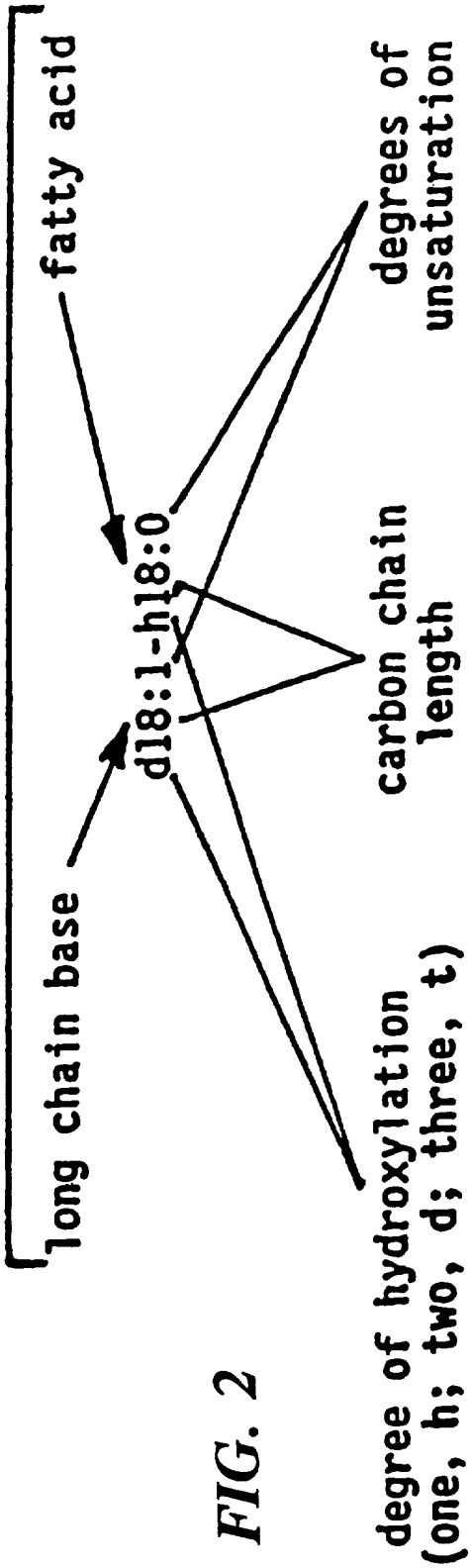
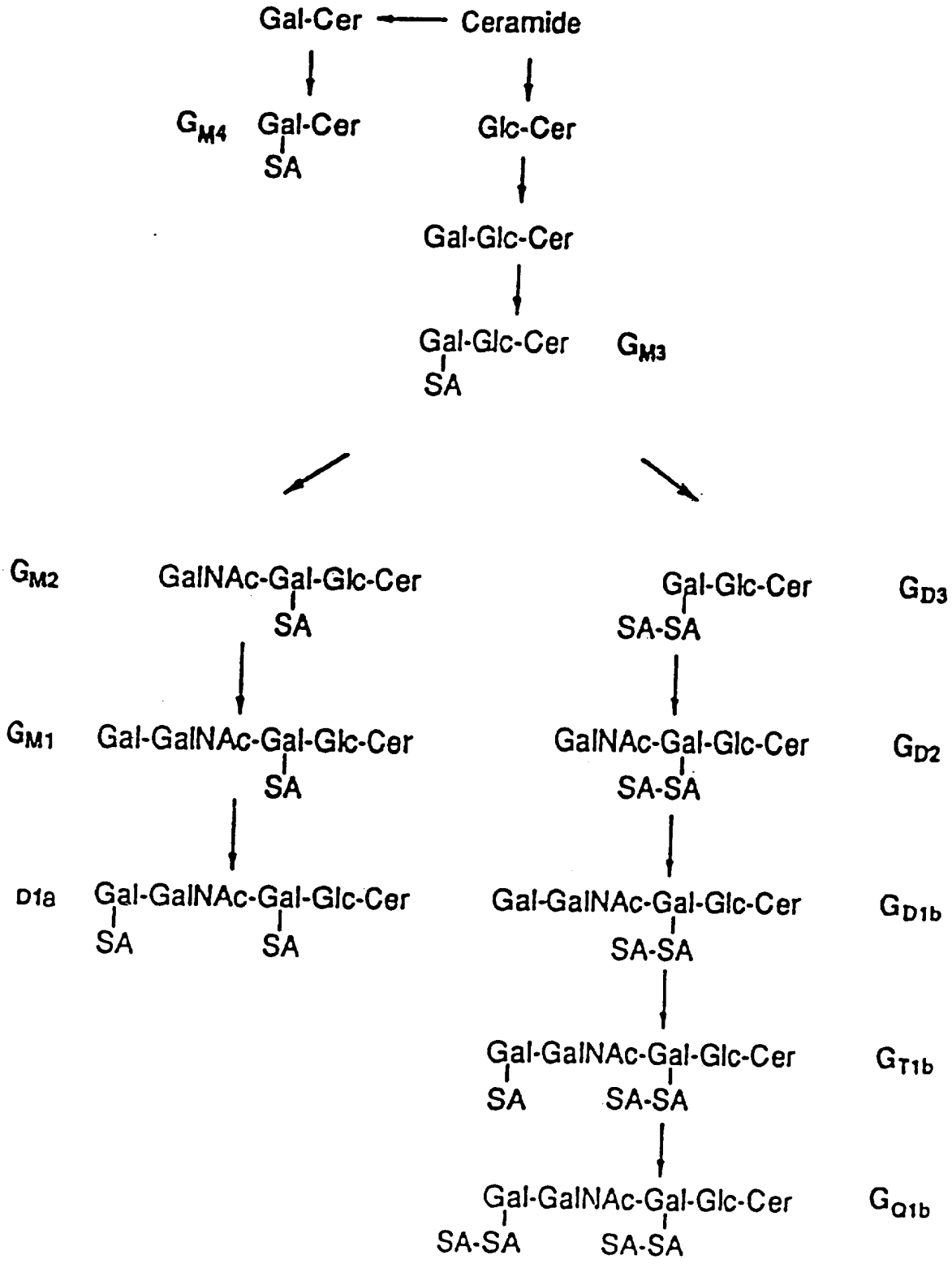

CERAMIDE
fatty acid
d18:1-h18:0
long chain base
degrees of unsaturation
carbon chain length
degree of hydroxylation (one, h; two, d; three, t)

FIG. 2

FIG. 3


Gal-Cer
Ceramide
G_{M4}
Gal-Cer
SA
Glc-Cer
Gal-Glc-Cer
Gal-Glc-Cer
SA
G_{M3}
G_{M2}
GalNAc-Gal-Glc-Cer
SA
Gal-Glc-Cer
SA-SA
G_{D3}
G_{M1}
Gal-GalNAc-Gal-Glc-Cer
SA
GalNAc-Gal-Glc-Cer
SA-SA
G_{D2}
D1a
Gal-GalNAc-Gal-Glc-Cer
SA
SA
Gal-GalNAc-Gal-Glc-Cer
SA-SA
G_{D1b}
Gal-GalNAc-Gal-Glc-Cer
SA
SA-SA
G_{T1b}
Gal-GalNAc-Gal-Glc-Cer
SA-SA
SA-SA
G_{Q1b}

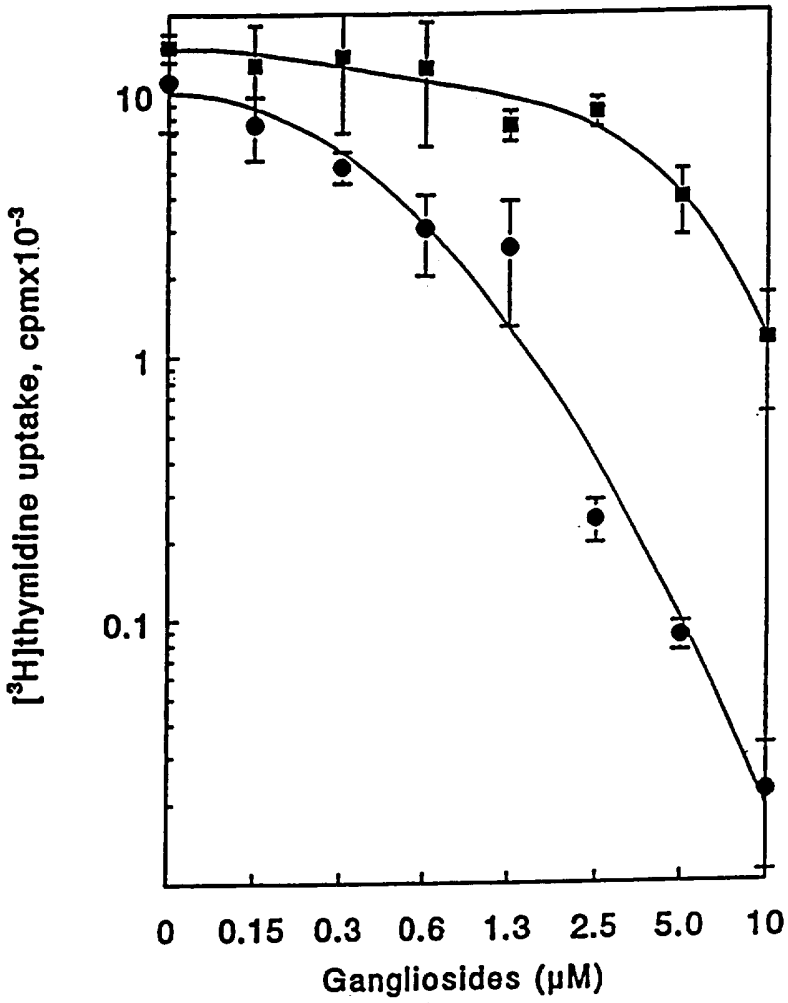

[3H]thymidine uptake, cpmx10-3
10
1
0.1
0
0.15
0.3
0.6
1.3
2.5
5.0
10
Gangliosides (μM)

FIG. 4

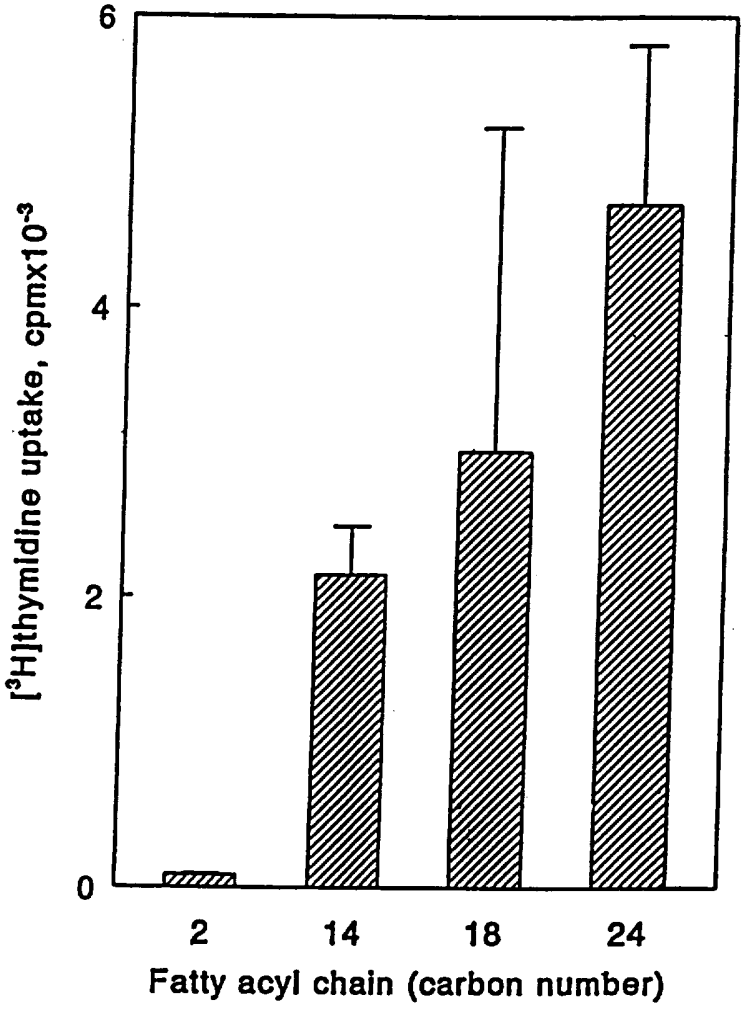

6
4
2
0
[³H]thymidine uptake, cpmx10⁻³
2
14
18
24
Fatty acyl chain (carbon number)

FIG. 5

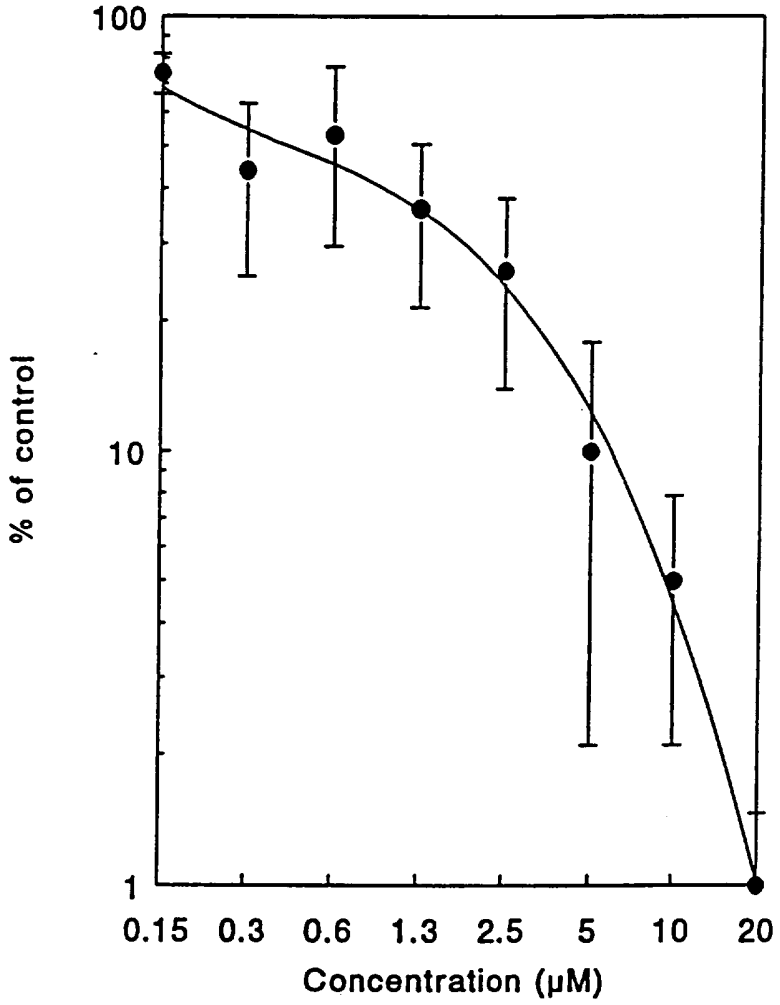


FIG. 6

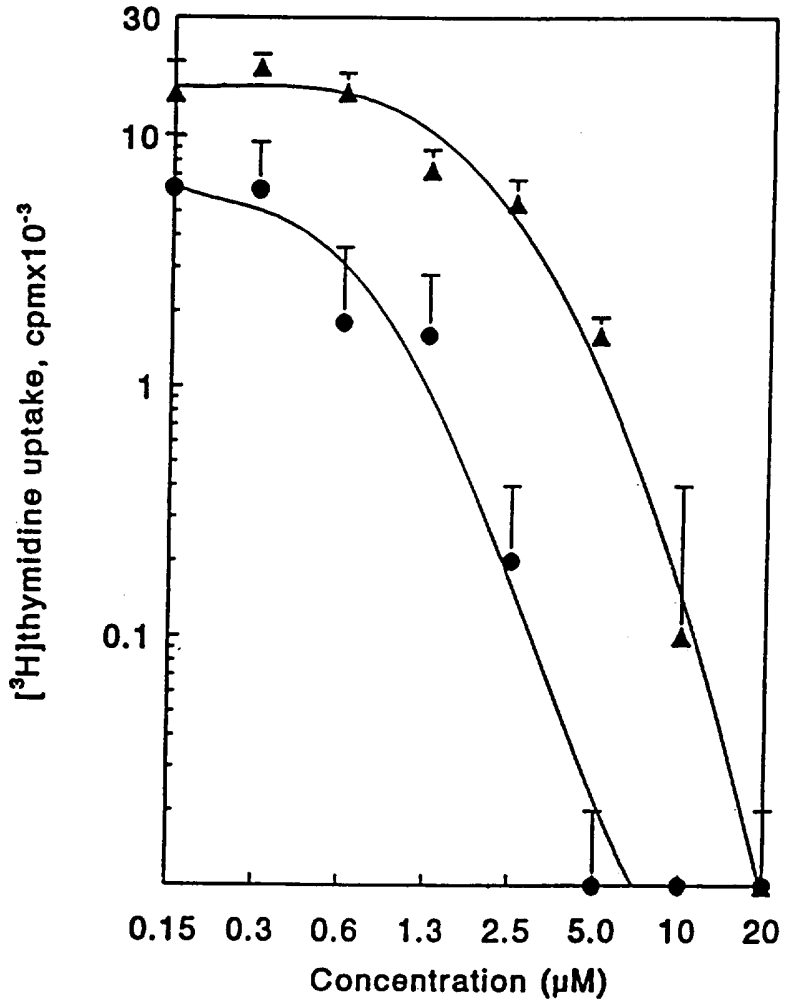

30
10
1
0.1
[3H]thymidine uptake, cpmx10-3
0.15 0.3 0.6 1.3 2.5 5.0 10 20
Concentration (μM)

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11670

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/73; C07H 3/04, 3/06
US CL :536/17.2, 17.9, 53, 55, 55.1; 514/25, 53, 54, 61
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/17.2, 17.9, 53, 55, 55.1; 514/25, 53, 54, 61

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Biochemistry, Volume 32, Number 31, issued 1993, J. P. Slotte et al., "Cholesterol Interacts With Lactosyl and Maltosyl Cerebrosides But Not With Glucosyl or Galactosyl Cerebrosides in Mixed Monolayers", pages 7886-7892, see the Abstract and Chart I.	1 --- 5, 6
X --- Y	Neurochemical Research, Volume 16, Number 11, issued November 1991, M. Pitto et al., "Metabolism of Semisynthetic Single-Chain GM1 Derivatives in Cerebellar Granule Cells in Culture", pages 1187-1192, see the Abstract.	1 --- 5, 6
X --- Y	JP, A, 01-093,562 (SHIONOGI AND COMPANY, LIMITED) 12 April 1989, see page 24, column 1.	1 --- 5, 6

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
07 DECEMBER 1995

Date of mailing of the international search report
30 JAN 1996

Name and mailing address of the ISA/US
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Facsimile No. (703) 305-3230

Authorized officer
KATHLEEN KAHLER FONDA
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11670

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Methods in Enzymology, Volume 242, issued 1994, M. Kiso et al., "Synthesis of Ganglioside GM3 and Analogs Containing Modified Sialic Acids and Ceramides", pages 173-183, see the entire document.	1-6
A	Journal of Carbohydrate Chemistry, Volume 12, Numbers 4-5, issued 1993, T. Terada et al., "Synthetic Studies on Silaoglycoconjugates. 44. Synthesis of KDN-Gangliosides GM4 and GM3", pages 425-440, see the entire article.	1-6
A	Bioscience, Biotechnology, and Biochemistry, Volume 56, Number 3, issued 1992, A. Hasegawa et al., "Synthetic Studies on Sialoglycoconjugates. Part 35. Synthesis of a Ganglioside GM3 Position Isomer, N-Acetylneuraminosyl-alpha(2--> 6)-lactosyl -beta(1--> 1)-ceramide", pages 501-511, see the entire article.	1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11670

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-12

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11670

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS--sarch terms glycosphingolipid#, ?immun?, drug#, pharmaceuti?, medic?, therap?
REGISTRY, HCAOLD, HCAPLUS, USPATFULL--structure search, inventor name search

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species and the claims which correspond to them are as follows:

Group I, claims 1-12, drawn to glycosphingolipid compounds, pharmaceutical compositions containing them, and a method of suppressing an immune response.

Group II, claims 13-24, drawn to synthetic gangliosides, pharmaceutical compositions containing them, and a method of suppressing an immune response.

Group III, claims 25-30, drawn to simplified carbohydrate moiety gangliosides, pharmaceutical compositions containing them, and a method of suppressing an immune response.

The following claims are generic: none.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons. Compounds of each of the three groups differ significantly in chemical structure. It would not be obvious to modify the compounds of a particular group to obtain compounds of either of the other two groups. Furthermore, a showing that compounds of one group were effective for suppressing an immune response would not suggest that compounds of the other groups would have the same properties.