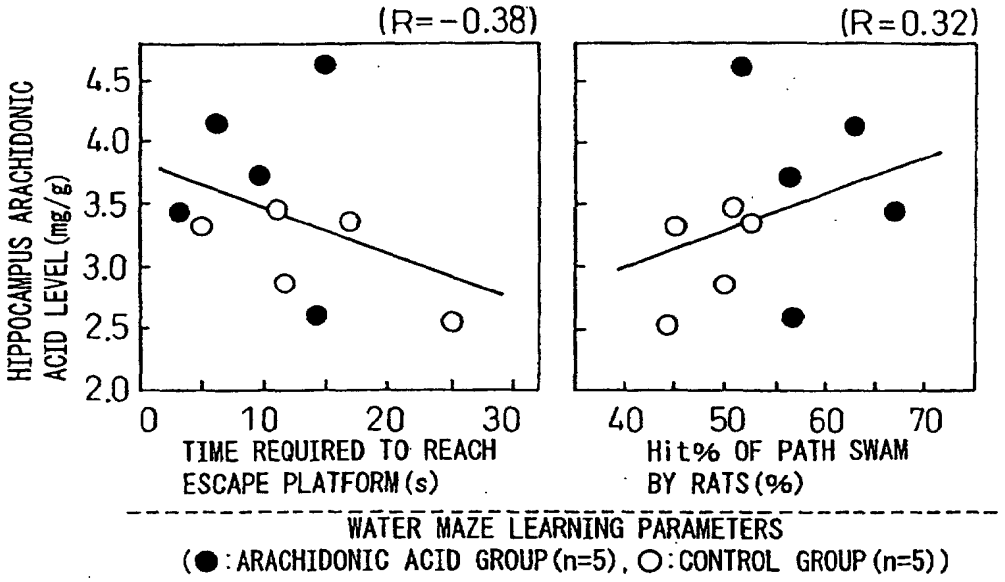
Fig.6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP02/00671

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] A61K31/202, 31/232, A61P25/24, 25/28, A23L1/30, 2/52, A23D9/00

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)
Int.Cl[7] A61K31/202, 31/232, A61P25/24, 25/28, A23L1/30, 2/52, A23D9/00

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LYNCH M. A. et al., Impaired spatial memory in aged rats is associated with alterations in inositol phospholipid metabolism, Neuroreport, 1994, Vol.5, No.12, pages 1493 to 1497	1-32,34
A	WAINWRIGHT P. E. et al., Water maze performance is unaffected in artificially reared rats fed diets supplemented with arachidonic acid and docosahexaenoic acid, J. Nutr., 1999, Vol.129, No.5, pages 1079 to 1089	1-32,34
A	WAINWRIGHT P. E. et al., Arachidonic acid offsets the effects on mouse brain and behavior of a diet with a low (n-6):(n-3) ratio and very high levels of docosahexaenoic acid, J. Nutr., 1997, Vol.127, No.1, pages 184 to 193	1-32,34

☐ 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 but 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	Date of mailing of the international search report
20 March, 2002 (20.03.02)	02 April, 2002 (02.04.02)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP02/00671

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

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

1. [X] Claims Nos.: 33
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 33 pertains to business activities and thus relates to a subject matter which this International Searching Authority is not required, under the provisions of Article 17(2)(a)(i) of the PCT and Rule 39 (iii) of the Regulations under the PCT, to search.

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:

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.:

Remark on Protest
[ ] The additional search fees were accompanied by the applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

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(54) **FATTY ALCOHOLS AND FATTY ACID ESTERS USEFUL FOR TREATMENT OF INFLAMMATION**
FETTALKOHOLE UND FETTSÄUREESTER ZUR BEHANDLUNG VON ENTZÜNDUNGEN
ALCOOLS GRAS ET ESTERS D'ACIDES GRAS UTILES DANS LE TRAITEMENT D'INFLAMMATION

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(56) References cited:
WO-A-01/00139 **WO-A-99/04632**
WO-A-02/083122 **US-A- 3 592 930**
US-A- 5 194 451 **US-B1- 6 210 700**
US-B1- 6 280 755 **US-B1- 6 331 568**
US-B1- 6 365 628

- **SNIPES W ET AL: "Inactivation of lipid-containing viruses by long-chain alcohols." ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. JAN 1977, vol. 11, no. 1, January 1977 (1977-01), pages 98-104, XP009034119 ISSN: 0066-4804**
- **SANDS J ET AL: "EXTREME SENSITIVITY OF ENVELOPED VIRUSES, INCLUDING HERPES SIMPLEX, TO LONG-CHAIN UNSATURATED MONOGLYCERIDES AND ALCOHOLS" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC, US, vol. 15, no. 1, January 1979 (1979-01), pages 67-73, XP009007372 ISSN: 0066-4804**

EP 1 385 500 B1

Description

FIELD OF THE INVENTION

[0001] The present invention relates to anti-inflammatory agents and, more particularly, to fatty alcohols, esters thereof with C_1 - C_6 alkanoic acids or esters of fatty acids with alkanediols or glycerol which are useful in the treatment of immunologically-mediated inflammation.

[0002] **Abbreviations: AA:** adjuvant arthritis: **CFA:** complete Freund's adjuvant; **EAE:** experimental autoimmune encephalomyelitis; **GPSCH:** guinea pig spinal cord homogenate; **IFA:** incomplete Freund's adjuvant; **OA:** oleyl alcohol; **PBS:** phosphate-buffered saline; **SC:** subcutaneously.

BACKGROUND OF THE INVENTION

[0003] Inflammation is commonly divided into three phases: acute inflammation, the immune response and chronic inflammation. Acute inflammation is the initial response to tissue injury and is mediated by the release of histamine, serotonin, bradykinin, prostaglandins and leukotrienes. The immune response, usually preceded by the acute inflammation phase, occurs when immunologically competent cells are activated in response to foreign organisms or antigenic substances liberated during the acute or chronic inflammatory response. The outcome of the immune response for the host may be beneficial, as when it causes invading organisms to be phagocytosed or neutralized. However, the outcome may be deleterious if it leads to chronic inflammation without resolution of the underlying injurious process as it occurs in rheumatoid arthritis.

[0004] The treatment of patients with inflammation envisages the relief of pain, which is the presenting symptom and the major continuing complaint of the patient, as well as the slowing or arrest of the tissue-damaging process.

[0005] Anti-inflammatory agents are usually classified as steroidal or glucocorticoids and nonsteroidal anti-inflammatory agents (NSAIDs). The glucocorticoids are powerful anti-inflammatory agents but the high toxicity associated with chronic corticosteroid therapy inhibits their use except in certain acute inflammatory conditions. Therefore, the nonsteroidal anti-inflammatory drugs have assumed a major role in the treatment of chronic conditions such as rheumatoid arthritis.

[0006] Among the nonsteroidal anti-inflammatory agents are included derivatives of aminoarylcarboxylic acids, arylacetic acids, arylbutyric acids, arylcarboxylic acids, arylpropionic acids, pyrazole, pyrazolone, salicylic acid and some other derivatives of different chemical structure, including specific anti-arthritic/anti-rheumatic agents.

[0007] Some fatty alcohols and esters of fatty acids have been described as solvents or emulsifiers for use in pharmaceutical compositions. For example, cetyl alcohol may be used in pharmaceutical compositions as emulsifying and stiffening agent (The Merck Index, pp. 347-8, # 2037), oleyl alcohol may be used as a carrier for medicaments (The Merck Index, p. 1222, # 6900), and alkyl esters of oleic acid may be used as solvents for medicaments (The Merck Index, p. 6899, # 6898).

[0008] A mixture of higher aliphatic primary alcohols, primarily isolated from beeswax, was described as having moderate anti-inflammatory activity. The composition of such a mixture was not disclosed (Rodriguez et al., 1998).

[0009] Feeding laboratory animals with fish oil rich in the long-chain n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), was described to reduce acute and chronic inflammatory responses, to improve survival to endotoxin and in models of autoimmunity and to prolong the survival of grafted organs, and it was therefore suggested that fish oil supplementation may be clinically useful in acute and chronic inflammatory conditions and following transplantation (Calder, 1998). A pharmaceutical preparation comprising eicosapentaenoic acid and/or stearidonic acid for treatment of schizophrenia is described in WO 98/16216 and US 6,331,568.

[0010] Modified polyunsaturated fatty acids and derivatives thereof have been proposed for pharmaceutical uses. WO 99/27924 and US 6,280,755 describe anti-inflammatory fatty acids uninterrupted by a methylene group for use in topical pharmaceutical and cosmetic compositions. WO 97/38688 and US 6,262,119 describe polyunsaturated fatty acids having 1 or 2 substitutions selected from oxa and thia in position beta or gamma to the acyl group, for treating or ameliorating symptoms of T-cell mediated disease. WO 99/58122 and US 6,365,628 describe saturated fatty acids in which one or more methylene groups are substituted by O, S, SO, SO_2, or Se and alkyl esters thereof, for treatment or prevention of diabetes. US 5,019,383 describes synthetic vaccines comprising a peptide residue coupled to one or more alkyl or alkenyl groups of at least 12 carbon atoms or other lipophilic substance, wherein said alkyl or alkenyl group may be a fatty acid residue coupled to one or more functional groups of a polyfunctional group which is bound to the N-terminal amino group and/or C-terminal carboxy group of the peptide residue.

[0011] There is no description in the literature that isolated fatty alcohols or esters thereof with alkanoic acids may be used themselves as medicaments, and specifically not that they may be involved in immunomodulation of inflammation.

SUMMARY OF THE INVENTION

[0012] It has now been surprisingly found, in accordance with the present invention, that certain long-chain fatty alcohols, esters thereof with C_1 - C_6 alkanoic acids, or certain esters of long-chain fatty acids with alkanediols or glycerol can suppress inflammation in experimental adjuvant arthritis (AA) and experimental autoimmune encephalomyelitis (EAE) models in rats and can prevent graft rejection in mice.

[0013] The present invention thus relates to pharmaceutical compositions for the treatment of inflammation, particularly immunologically-mediated inflammation, comprising as active ingredient an immunomodulator selected from: (a) a saturated or cis-unsaturated C_{10}-C_{18} fatty alcohol.

[0014] In another embodiment, the invention relates to the use of an immunomodulator selected from: (a) a saturated or cis-unsaturated C_{10}- C_{18} fatty alcohol for the preparation of a pharmaceutical composition for the treatment of inflammation, in particular immunologically-mediated inflammation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

Fig. 1 shows the dose response effect of oleyl alcohol (OA) on adjuvant arthritis (AA). Different doses of OA were administered subcutaneously to rats once 14 days before induction of AA.

Fig. 2 is a graph showing the disease profile of Lewis rats with experimental autoimmune encephalomyelitis (EAE) and treated with oleyl alcohol. Oleyl alcohol was administered to the rats 14 days before induction of EAE. Control group was treated with incomplete Freund's adjuvant (IFA).

Fig. 3 is a graph showing the disease profile of Lewis rats with EAE and treated with IFA. IFA was administered to the rats 14 days before induction of EAE. Control group was not treated.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The present invention provides immunomodulators selected from: a saturated or cis-unsaturated C_{10}- C_{18} fatty alcohol.

[0017] According to one preferred embodiment of the invention, the pharmaceutical composition comprises a long-chain saturated or unsaturated C_{10}-C_{18}, preferably C_{16}-C_{18}, most preferably a C_{18}, fatty alcohol.

[0018] Examples of C_{10}-C_{18} saturated fatty alcohols that can be used according to the invention include, but are not limited to, decyl alcohol, lauryl alcohol, myristyl alcohol, stearyl alcohol and preferably cetyl alcohol (also known as palmityl alcohol).

[0019] The unsaturated fatty alcohol according to the invention has preferably one or more double bonds in the cis form and 16-18 carbon atoms and may be, without being limited to, oleyl alcohol (cis-9-octadecenol), linoleyl alcohol (cis-9,12-octadecadienol), γ-linolenyl alcohol (cis-6,9,12-octadecatrienol) and linolenyl alcohol (cis-9,12,15-octadecatrienol). In preferred embodiments, the fatty alcohol used in the compositions of the invention is cetyl, linolenyl or, most preferably, oleyl alcohol.

[0020] The C_{10}-C_{18} fatty acid is preferably a C_{16}-C_{18} most preferably a C_{18} fatty acid. In one embodiment, the C_{10}-C_{18} fatty acid is saturated such as, but without being limited to, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid. In another embodiment, the C_{10}-C_{20} fatty acid is a cis-unsaturated fatty acid such as, but without being limited to, palmitoleic acid (cis-9-hexadecenoic acid), oleic acid (cis-9-octadecenoic acid), cis-vaccenic acid (cis-11-octadecenoic acid), linoleic acid (cis-9,12-octadecadienoic acid), γ-linolenic acid (cis-6,9,12-octadecatrienoic acid), linolenic acid (cis-9,12,15-octadecatrienoic acid) and arachidonic acid (cis-5,8,11,14-eicosatetraenoic acid).

[0021] According to the invention, the alkanediol has 2 to 8, preferably 2 to 4, and more preferably, 2 carbon atoms, and is selected from, but not being limited to, 1,3-propanediol, 1,4-butanediol and, preferably, 1,2-ethylene glycol. An example of such an ester is 1,2-ethylene glycol monooleate.

[0022] According to another embodiment of the invention, the active ingredient of the pharmaceutical composition is a mono- or diester of glycerol with the long-chain fatty acid. In one preferred embodiment, the monoglyceride is glycerol monooleate. The diglycerides contain one free hydroxyl group and the other two hydroxyl groups may be both esterified with 2 molecules of the long-chain fatty acid, e.g. glycerol dioleate, or one of the hydroxyl groups is esterified with one molecule of the long-chain fatty acid and a second hydroxyl group is esterified with a C_1- C_6 alkanoic acid such as acetic acid, propionic acid, butyric acid, valeric acid and caproic acid.

[0023] The immune system, in both its innate and adaptive arms, is involved in regulating inflammation of every type, and inflammation is a key factor in processes such as wound healing, connective tissue re-modeling, angiogenesis, organ regeneration, neuroprotection, as well as in the adaptive immune responses seen in autoimmunity, allergies, graft rejection, and infection (see Cohen, 2000; Schwartz and Cohen, 2000). Therefore, anti-inflammatory agents that modulate

the inflammatory response such as those described here will be useful in a variety of conditions.

[0024] Inflammatory disorders that can be treated with the immunomodulators of the present invention include, but are not limited to, immunologically-mediated chronic or acute inflammatory disorders selected from an autoimmune disease, severe allergies, asthma, graft rejection or for the treatment of chronic degenerative diseases such as Alzheimer's disease, and in neuroprotection, organ regeneration, chronic ulcers of the skin, and schizophrenia.

[0025] Examples of autoimmune diseases that can be treated according to the invention are multiple sclerosis or a human arthritic condition, e.g. rheumatoid arthritis, reactive arthritis with Reiter's syndrome, ankylosing spondylitis and other inflammations of the joints mediated by the immune system. Other autoimmune diseases are contemplated and are presented in the following list in the context of the organ or tissue involved. Thus, according to the invention, the immunologically-mediated inflammatory disorder may be myasthenia gravis, Guillain-Barré syndrome, and other inflammatory diseases of the nervous system; psoriasis, pemphigus vulgaris and other diseases of the skin; systemic lupus erythematosus, glomerulonephritis and other diseases affecting the kidneys; atherosclerosis and other inflammations of the blood vessels; autoimmune hepatitis, inflammatory bowel diseases, e.g. Crohn's disease, pancreatitis, and other conditions of the gastrointestinal system; type 1 diabetes mellitus (insulin-dependent diabetes mellitus or IDDM), autoimmune thyroiditis (Hashimoto's thyroiditis), and other diseases of the endocrine system.

[0026] One of the models used to test the anti-inflammatory activity of the agents according to the invention is adjuvant arthritis (AA), an experimental disease of the joints inducible in some strains of rats by immunizing with *Mycobacterium tuberculosis* in complete Freund's adjuvant (CFA). These animals develop an arthritis whose features are similar to those of rheumatoid arthritis in humans and thus serve as animal models of human arthritic conditions such as rheumatoid arthritis, reactive arthritis in Reiter's syndrome, ankylosing spondylitis and other inflammations of the joints which appear to be mediated by the immune system (Pearson, 1964). Adjuvant arthritis also serves as a model of immune-mediated inflammation in general including cell-mediated autoimmune reactions, graft rejection and allergic reaction. For example, treatments which can suppress rheumatoid arthritis include immunosuppressive agents such as corticosteroids, cyclosporin A (Jaffee et al., 1989; Pollock et al., 1989), azathioprine, and other immunosuppressive agents which are broadly used in the treatment of autoimmune diseases. Therefore, suppression of adjuvant arthritis by a therapeutic agent indicates that the agent is potentially useful as a broad anti-inflammatory agent.

[0027] The pharmaceutical composition provided by the present invention may be in solid, semisolid or liquid form and may further include pharmaceutically acceptable fillers, carriers or diluents, and other inert ingredients and excipients. The composition can be administered by any suitable route such as, but not limited to, oral, topical, or parenteral e.g. by injection through subcutaneous, intravenous, intramuscular, or any other suitable route. Since many of the compounds are oily, they are preferably administered parenterally, more preferably subcutaneously. If given continuously, the compounds of the present invention are each typically administered by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. The dosage will depend of the state of the patient and severity of the disease and will be determined as deemed appropriate by the practitioner.

[0028] For parenteral administration, the compounds may be formulated by mixing each at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. Generally, the formulations are prepared by contacting the compounds of the present invention each uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils can be also useful, as well as liposomes. These preparations can be made by conventional methods known to those skilled in the art, for example as described in "Remington's Pharmaceutical Science", A.R. Gennaro, ed., 17th edition, 1985, Mack Publishing Company, Easton, PA, USA.

[0029] The invention will now be illustrated by the following non-limiting examples.

EXAMPLES

Example 1. Anti-inflammatory effect of oleyl alcohol and other agents-protection against adjuvant arthritis (AA)

[0030] AA was induced by immunizing inbred 8-10-week old Lewis rats (Harlan-Olac Limited, Blackthorn, Oxon, UK), at the base of the tail with 1 mg/0.1 ml of killed *Mycobacterium tuberculosis* (Sigma) in IFA (Sigma) as described (Pearson, 1956). Arthritis of the limbs was noted to develop 12-14 days later and was scored on a scale of 0-16 summing the severity of the inflammation of each of the 4 limbs on a scale of 0-4, as described (Holoshitz et al., 1983). The peak of the arthritis usually was observed around day 26 after immunization.

[0031] Control rats were untreated or treated by injections of saline. A positive control of immunosuppression was obtained by including a group of rats treated with the corticosteroid agent dexamethasone (200 μg) administered intraperitoneally every other day beginning on day 12 after induction.

[0032] The immunomodulator of the invention (100 μl oleyl alcohol, glycerol mono-oleate, linolenyl alcohol or cetyl alcohol) was administered subcutaneously (SC) once 14 days before induction of AA or on day 12 after induction of AA. The percent inhibition of inflammation measured on the day of maximal inflammation was computed as follows:

$$\frac{\text{mean maximal score of test group}}{\text{mean maximal score of control group}} \times 100\%$$

[0033] All four compounds were found to be effective, producing more than 60% inhibition of inflammation whereas oleic acid had no effect. The results are summarized in Table 1.

[0034] Two further experiments showed that 500 μl of oleyl alcohol (100 μl corresponds to about 90 mg oleyl alcohol) suppressed the inflammation by 96% and 91%.

Table 1. Effects of various agents on the inflammation of adjuvant arthritis

Compound Tested	% Inhibition (100 μl)
Glycerol mono-oleate	98%
Oleyl alcohol	78%
Linolenyl alcohol	75%
Cetyl alcohol	66%

Example 2. Protection against AA by different doses of oleyl alcohol

[0035] To study the dose response effect of oleyl alcohol in AA, oleyl alcohol was administered subcutaneously in doses of 10, 50,100 or 500 mg to Lewis rats once 14 days before induction of AA, as described in Example 1 above.

[0036] Fig. 1 shows the dose response effect of oleyl alcohol. It can be seen that increasing doses of oleyl alcohol suppressed the arthritis. On the day of peak disease, day 26, the inflammation was suppressed by 14% (10 μl), 61% (50 μl), 78% (100 μl) and 90% (500 μl).

Example 3. Anti-inflammatory effect of oleyl alcohol and other immunomodulators and protection against EAE in DA rats

[0037] Experimental autoimmune encephalomyelitis (EAE) is an experimental autoimmune disease inducible in some strains of rats by immunization with myelin basic protein (MBP) or proteolipid protein (PLP) in complete Freund's adjuvant (CFA) or with an emulsion of the rat's spinal cord in either CFA or incomplete Freund's adjuvant (IFA). EAE in DA rats is considered as a model of chronic EAE. Within two to three weeks the animals develop cellular infiltration of the myelin sheaths of the central nervous system resulting in demyelination and paralysis. Most of the animals die, but others have milder symptoms, and some animals develop a chronic form of the disease that resembles chronic relapsing and remitting multiple sclerosis (MS) in humans. Therefore, these animals with EAE serve as a model for the human MS autoimmune disease. EAE develops in the animal about 12 days after immunization and is characterized by paralysis of various degrees due to inflammation of the central nervous system. In some strains, like the Lewis rat, the paralysis can last up to 6-7 days and the rats usually recover unless they die during the peak of their acute paralysis. In other strains of rats like the DA rat, the paralysis can be chronic and remitting.

[0038] For the induction and clinical assessment of EAE, spinal cord obtained from DA rats is frozen, thawed and minced thoroughly with a spatula before immunization. Rats are immunized by one subcutaneous injection (just under the skin) into the dorsal base of the tail with 200 μl emulsion prepared from 1:1 IFA (Difco, Detroit, MI, USA) and antigen (volume/weight, i.e. 100 μl IFA/100 mg of whole spinal cord) or from 1:1 CFA (IFA was complemented with 4 mg/ml of *Mycobacterium tuberculosis* strain 37RA) and antigen (volume/weight, i.e. 100 μl CFA/100 mg of whole spinal cord). The emulsion was prepared by titration with a gas-tight glass syringe and a needle, 1.2 mm diameter. Rats are regularly weighed and examined for clinical signs of EAE. A four-graded scale was used to assess clinical severity: 0, no paralysis; 1, tail weakness (hanging); 2, hind limb paralysis; 3, hind and fore limb paralysis; 4, severe total paralysis (Lorentzen et al., 1995).

[0039] Groups of 5 or 7 DA strain female rats, 8-9 week old, are immunized in the hind footpads with 0.1 ml per footpad of IFA containing 100 mg of whole, homogenized DA spinal cord, for a total of 200 mg per rat. On the day of immunization, the rats are treated by SC injection with oleyl alcohol or other agent according to the invention (100 μl) or with paraffin

oil (control). The rats are scored for EAE on a severity scale of 0 - 4 as described above.

Example 4. Anti-inflammatory effect of oleyl alcohol and protection against EAE in Lewis rats

[0040] EAE induced in Lewis rats is considered as a model of acute inflammation in the brain (as opposed to the chronic disease in DA rats).

[0041] For EAE induction, three lyophilized guinea pig spinal cord homogenate (GPSCH) emulsions were prepared as follows: (i) 25 mg of lyophilized GPSCH (GP2) was suspended in 2.5 ml of sterile PBS (Sigma) and incubated for one hour at 37° C; (ii) 54.1 mg of *Mycobacterium tuberculosis* H37Ra (MT, Difco) was suspended in 13.5 ml CFA (Sigma) containing 1mg/ml MT to obtain 5 mg/ml MT; (iii) 2.5 ml CFA (5 mg/ml MT) was added into vial with 2.5 ml of PBS containing 25 mg GPSCH to yield 5 mg/ml GPSCH and 2.5 mg/ml MT. The mixture was transferred into a glass syringe connected to a second glass syringe through a Luer lock bridge. The material was mixed well by transferring from one syringe to another for about 10 minutes until the materiall was well emulsified. The emulsion of GPSCH at a dose of 1 mg/rat and MT at a dose of 0.5 mg/rat in CFA induced EAE in rats (based on previous titration).

[0042] For the treatment, two groups of eight 9-10 weeks old Lewis rats (Harlan, Israel), were treated with the test samples (oleyl alcohol or IFA) 14 days before EAE induction. The group treated with IFA served as the control group. The test samples were injected at a dose of 0.5 ml/kg once, subcutaneously. A third group of 8 rats was not treated and served as non-treated control.

[0043] EAE was induced in rats of all three groups 14 days after injection of the test samples by injection with 0.1 ml of the GPSCH emulsion in CFA into each of the hind leg foot pads (0.2 ml per rat).

[0044] The EAE clinical signs were observed and scored from the 9th day post-EAE induction until the termination of the experiment according to the following five-graded scale to assess clinical severity: 0, normal behavior; 1, weight loss; 2, tail weakness; 3, hind legs hypotonia and weakness; 4, hind legs paralysis; 4, severe total paralysis; 5, impaired respiration and/or convulsions and/or full paralysis or death. All rats having scores of 1 and above were considered sick.

[0045] The calculation of EAE results was carried out as follows:

(i) *Calculation of the incidence of disease*

[0046] The number of sick animals in each group were summed. The incidence of disease and the % activity were calculated as follows:

$$\text{Incidence of disease} = \frac{\text{No. of sick rats in group}}{\text{No. of rats in group}} \times 100\%$$

$$\%\ \text{activity}\ * = 1 - \frac{(\text{disease incidence in treated group})}{\text{disease incidence in control group}} \times 100\%$$

$$* = \text{(according to incidence)}$$

(ii) *Calculation of the mean maximal score (MMS)*

[0047] The maximal score of each rat in the group were summed. The mean maximal score (MMS) and the % activity of the group were calculated as follows:

$$\text{Mean Maximal score} = \frac{\sum \text{Maximal score of each rat}}{\text{No. of rats in the group}}$$

$$\% \text{ activity } * = \left(1 - \frac{\text{MMS of treated group}}{\text{MMS of control group}}\right) \text{X } 100$$

* = (% activity according to MMS)

(iii) *Calculation of the group mean score (GMS)*

[0048] The mean score of each rat during the observation period were summed (score 5 was counted forward). The mean score of the group and its % activity were calculated as follows:

$$\text{Mean score} = \frac{\sum \text{Group score of each rat}}{\text{No. of rats in the group}}$$

$$\% \text{ activity } * = \left(1 - \frac{\text{GMS of treated group}}{\text{GMS of control group}}\right) \text{X } 100$$

* = (% activity according to GMS)

(iv) *Calculation of the mean onset of disease*

[0049] The time of disease onset (days) for each rat in the group were summed. The mean onset of disease for the group was calculated. The time of onset of disease for those rats that did not develop EAE was considered as 25 days (duration of study).

(v) *Calculation of the mean duration of disease*

[0050] The disease duration (days) of each rat in each group were summed. The mean disease duration of the group was calculated. The disease duration of rats that did not develop EAE was considered as zero.

[0051] The evaluation of the clinical manifestations of EAE, i.e. % incidence of disease, MMS, GMS, mean duration and onset of EAE disease is summarized in Table 2. The graphs of the disease profile for each group are presented in Figs. 2 and 3 for treatment with oleyl alcohol and IFA, respectively.

[0052] As shown by the results, no essential difference in incidence of disease (62.5% to 75% incidence) or mean maximum score (1.75 to 2.38 MMS) was observed between the IFA-injected groups and non-treated control group. Oleyl alcohol showed a beneficial effect on all the clinical parameters that were tested. It exhibited 77.1% activity according to group mean score (GMS) and 63% activity according to mean maximum score (MMS) compared to the non-treated control group. The mean onset of disease was 18.6 days in the oleyl alcohol treated group compared to 15.5 days in the non-treated control group. The duration of disease was 2.0 days in the oleyl alcohol treated group compared to 5.13 days in the non-treated control group. The duration of the EAE clinical signs in the tested groups was between 1 and 7 days, except one rat in the group treated with IFA. IFA had minor effect, if any, on the rat EAE. No mortality was observed in the tested groups, except one rat in the non-treated control group.

Table 2: Evaluation EAE clinical results

Group No.	Test Sample	% Incidence	% Activity Incidence	MMS	% Activity MMS	GMS	% Activity GMS	Mean Onset of disease (Day No.)	Disease duration (days)
1	OA	50.0%	33.3%	0.88	63.0%	0.22	77.1%	18.6	2.0
2	IFA	62.5%	16.7%	1.75	26.5%	0.52	45.8%	17.0	3.75
3	NTC	75.0%	NA	2.38	NA	0.96	NA	15.5	5.13
OA-Oleyl alcohol; NTC-non-treated control; NA- Not applicable									

Example 5. Effect of oleyl alcohol on skin allograft survival

[0053] The immune system represents a strong barrier for successful transplantation of organs or tissues between non-genetically identical donor and recipient. Both $CD4^+$ and $CD8^+$ T cells participate in graft rejection.

[0054] Skin graft transplantation is carried out essentially as described before (Birk et al., 1999). Thus, mice are shaved and 1 cm^2 sections of skin are cut from the dorsal side of sacrificed donors and cleaned in PBS. Two patches of dorsal skin, 1 cm^2 each, are cut from the anesthetized recipients (Nembutal 6 mg/ml, 0.25 ml/mouse) in preparation for the allograft. Two donor allografts per recipient are grafted onto the dorsal lesioned patches. Histoacryl (B. Braun Melsungen AG, Melsungen, Germany) is applied around the graft. Nobecutan (ASTR, Astra Tech, Glos G15, UK) is sprayed over the grafts.

[0055] In the experiment, groups of 6 BALB/c female mice, 8-week old, are grafted with 1 cm^2, full thickness skin grafts from C57BL/6 female mice, 8-week old. On the day of grafting, a group of recipient mice is treated either with paraffin oil or SC with 100 µl oleyl alcohol or another immunomodulator according to the invention. The day of rejection is scored. The transplanted skin in the mice treated with the immunomodulator survives longer in comparison with the untreated control mice.

Example 6. Prevention and treatment of SLE

[0056] Systemic lupus erythematosus is an autoimmune disease in which both autoantibodies and immune complexes are involved. In order to test the immunomodulators of the invention, mice with experimental SLE or (NZBxNZW)F1 mice that spontaneously develop autoimmune diseases that closely resemble SLE, can be used.

[0057] In order to induce experimental SLE, BALB/c mice are immunized with the human or murine anti-DNA monoclonal antibody 16/6Id (20 µg/mouse) in CFA in the hind footpads and boosted 3 weeks later with the same amount of the immunizing antibody in PBS. The mice are then tested for autoantibody production and clinical manifestations characteristic of experimental SLE. In order to either prevent induction of experimental SLE or to cure mice afflicted with the disease, mice are given oleyl alcohol or another immunomodulator according to the invention subcutaneously (100 µl per mouse) before or concomitant with the immunization and some weeks after immunization. The number of injections is based on the effect of the tested compound on the disease induction and progression. The animals are regularly weighed and examined for clinical signs of SLE as described, for example, in WO 96/30057.

Example 7. Prevention and treatment of autoimmune thyroiditis

[0058] Experimental autoimmune thyroiditis (EAT) can be induced in a number of animals by immunizing with thyroglobulin in CFA. Both humoral antibodies and T_{DTH} cells directed against the thyrogllobulin develop, resulting in thyroid inflammation. EAT appears to best mimic Hashimoto's thyroiditis.

[0059] EAT is induced as previously described (Rose et al., 1971) by injecting each mouse subcutaneously with thyroglobulin extract obtained from one thyroid gland. The extract is emulsified in IFA (Difco Laboratories, Detroit, Mich.), to which are added 7mg/ml *Mycobacterium tuberculosis,* H37Ra strain (Difco Laboratories). This injection is repeated one week later. Donors of thyroglobulin extract are mice of the C3H/eB strain. 4-5 weeks later, EAT is assayed by removing thyroid glands of recipient mice, fixing them in 10% formalin solution and then in 70% alcohol, and examining microscopic sections stained with hematoxylin and eosin. Microscopic slides are coded and examined without knowledge of their identity. A diagnosis of EAT is made by observing at least one unequivocal focus of infiltration by mononuclear cells. Treatment is performed by injecting SC oleyl alcohol or another immunomodulator (100 µl per animal) before induction of EAT, concomitant with or thereafter (control animals are injected paraffin oil), and the animals are regularly

weighed and examined for clinical signs of EAT by known conventional methods.

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Claims

1. A saturated or cis-unsaturated C_{10}-C_{18} fatty alcohol for use as an immunomodulator in the treatment of immunologically-mediated chronic or acute inflammatory disorders selected from an autoimmune disease, severe allergies, asthma, graft rejection or for the treatment of chronic degenerative diseases such as Alzheimer's disease, and in neuroprotection, organ regeneration, chronic ulcers of the skin, and schizophrenia.

2. The embodiment according to claim 1, wherein the immunomodulator is a saturated C_{10}-C_{18} fatty alcohol such as decyl alcohol, lauryl alcohol, myristyl alcohol, cetyl alcohol and stearyl alcohol.

3. The embodiment according to claim 1, wherein the immunomodulator is a cis-unsaturated C_{16}-C_{18} fatty alcohol such as oleyl alcohol, linoleyl alcohol, γ-linolenyl alcohol and linolenyl alcohol.

4. The embodiment according to claim 1, wherein said autoimmune disease is multiple sclerosis or a human arthritic condition.

5. The embodiment according to claim 4, wherein said human arthritic condition is selected from rheumatoid arthritis, reactive arthritis with Reiter's syndrome, ankylosing spondylitis and other inflammations of the joints mediated by the immune system.

6. The embodiment according to claim 1, wherein said immunologically-mediated inflammatory disorder is myasthenia gravis, Guillain-Barré syndrome, and other inflammatory diseases of the nervous system; psoriasis, pemphigus vulgaris and other diseases of the skin; systemic lupus erythematosus, glomerulonephritis and other diseases affecting the kidneys; atherosclerosis and other inflammations of the blood vessels; autoimmune hepatitis, inflammatory bowel diseases, pancreatitis, and other conditions of the gastrointestinal system; type 1 diabetes mellitus, autoimmune thyroiditis, and other diseases of the endocrine system.

Patentansprüche

1. Gesättigter oder cis-ungesättigter C_{10}-C_{18}-Fettalkohol zur Verwendung als Immunmodulator bei der Behandlung von immunologisch vermittelten chronischen oder akuten entzündlichen Erkrankungen, ausgewählt aus einer Autoimmunerkrankung, schweren Allergien, Asthma, Transplantatabstoßung, oder für die Behandlung chronischer degenerativer Erkrankungen, wie Alzheimer-Krankheit, und bei der Neuroprotektion, der Organregeneration, chronischen Geschwüren der Haut und Schizophrenie.

2. Ausführungsform nach Anspruch 1, wobei der Immunmodulator ein gesättigter C_{10}-C_{18}-Fettalkohol, wie Decylalkohol, Laurylalkohol, Myristylalkohol, Cetylalkohol und Stearylalkohol, ist.

3. Ausführungsform nach Anspruch 1, wobei der Immunmodulator ein cis-ungesättigter C_{16}-C_{18}-Fettalkohol, wie Oleylalkohol, Linoleylalkohol, γ-Linolenylalkohol und Linolenylalkohol, ist.

4. Ausführungsform nach Anspruch 1, wobei die Autoimmunerkrankung Multiple Sklerose oder ein arthritischer Zustand beim Menschen ist.

5. Ausführungsform nach Anspruch 4, wobei der arthritische Zustand beim Menschen aus rheumatoider Arthritis, reaktiver Arthritis mit Reiter-Syndrom, Morbus Bechterew und anderen durch das Immunsystem vermittelten Entzündungen der Gelenke ausgewählt ist.

6. Ausführungsform nach Anspruch 1, wobei es sich bei der immunologisch vermittelten entzündlichen Erkrankung um Myasthenia gravis, Guillain-Barré-Syndrom und andere entzündliche Erkrankungen des Nervensystems; Schuppenflechte, Pemphigus vulgaris und andere Erkrankungen der Haut; systemischen Lupus erythematodes, Glomerulonephritis und andere die Nieren beeinträchtigende Erkrankungen; Atherosklerose und andere Entzündungen der Blutgefäße; autoimmune Hepatitis, entzündliche Darmerkrankungen, Pankreatitis und andere Zustände des Magen-Darm-Systems; Diabetes mellitus Typ 1, Autoimmunthyroiditis; und andere Erkrankungen des endokrinen Systems handelt.

Revendications

1. Alcool gras en C_{10} à C_{18} saturé ou cis-insaturé à utiliser comme immunomodulateur dans le traitement de troubles inflammatoires chroniques ou aigus induits par voie immunologique, choisis parmi une maladie auto-immune, des allergies sévères, l'asthme, le rejet de greffe ou pour le traitement de maladies dégénératives chroniques telles que la maladie d'Alzheimer et dans la neuroprotection, la régénérescence des organes, les ulcères chroniques cutanés et la schizophrénie.

2. Mode de réalisation selon la revendication 1, dans lequel l'immunomodulateur est un alcool gras en C_{10} à C_{18} saturé tel que l'alcool décylique, l'alcool laurylique, l'alcool myristylique, l'alcool cétylique et l'alcool stéarylique.

3. Mode de réalisation selon la revendication 1, dans lequel l'immunomodulateur est un alcool gras en C_{16} à C_{18} cis-insaturé tel que l'alcool oléylique, l'alcool linoleylique, l'alcool γ-linolénylique et l'alcool linolénylique.

4. Mode de réalisation selon la revendication 1, dans lequel ladite maladie auto-immune est la sclérose en plaques ou une affection arthritique humaine.

5. Mode de réalisation selon la revendication 4, dans lequel ladite affection arthritique humaine est choisie parmi l'arthrite rhumatoïde, l'arthrite réactive avec le syndrome de Reiter, la spondylite ankylosante et autres inflammations des articulations induites par le système immunitaire.

6. Mode de réalisation selon la revendication 1, dans lequel ledit trouble inflammatoire induit par voie immunologique est la myasthénie gravis, le syndrome de Guillain-Barré et autres maladies inflammatoires du système nerveux ; le psoriasis, le pemphigus vulgaire et autres maladies cutanées ; le lupus érythémateux systémique, la glomérulonéphrite et autres maladies rénales ; l'athérosclérose et autres inflammations des vaisseaux sanguins ; l'hépatite auto-immune, les maladies intestinales inflammatoires, la pancréatite et autres affections du système gastro-intestinal ; le diabète sucré de type 1, la thyroïdie auto-immune et autres maladies du système endocrinien.

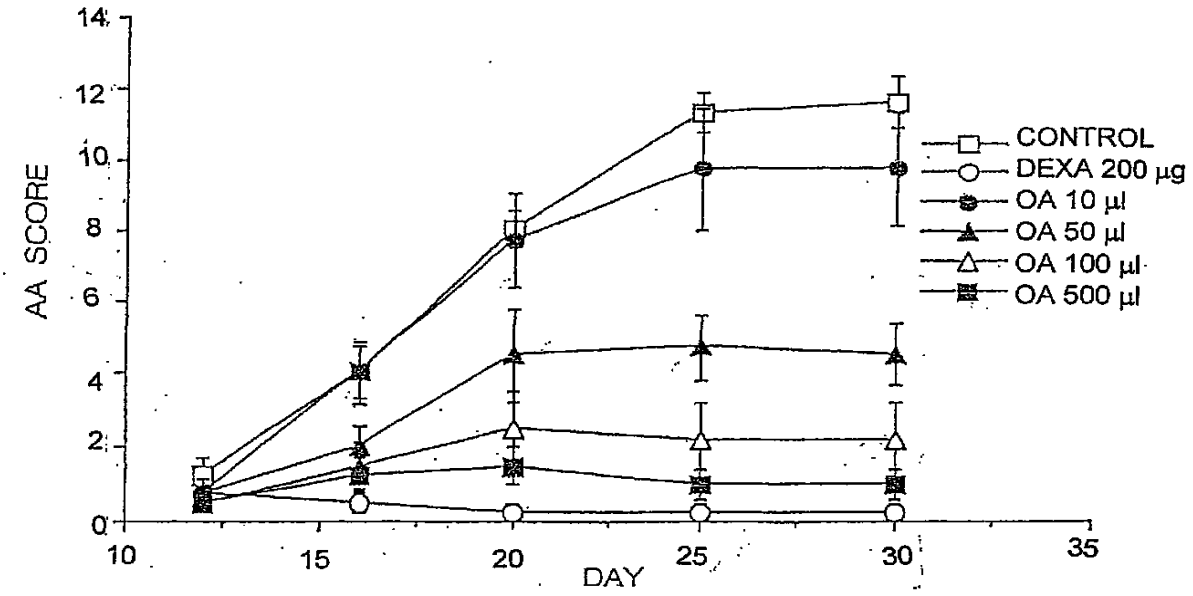

Fig. 1

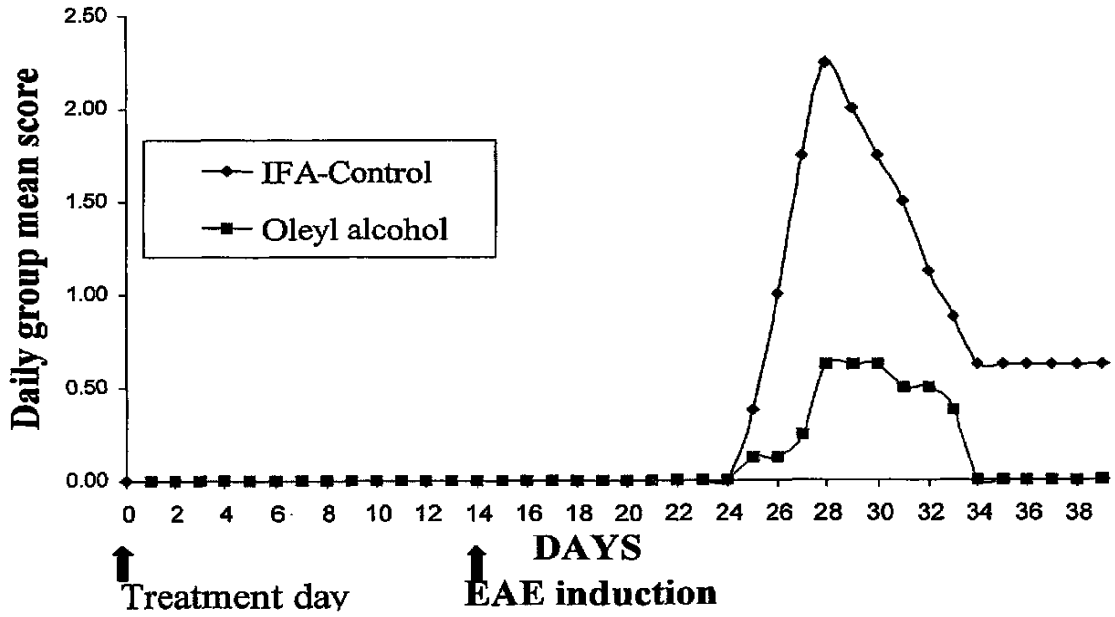

Fig. 2

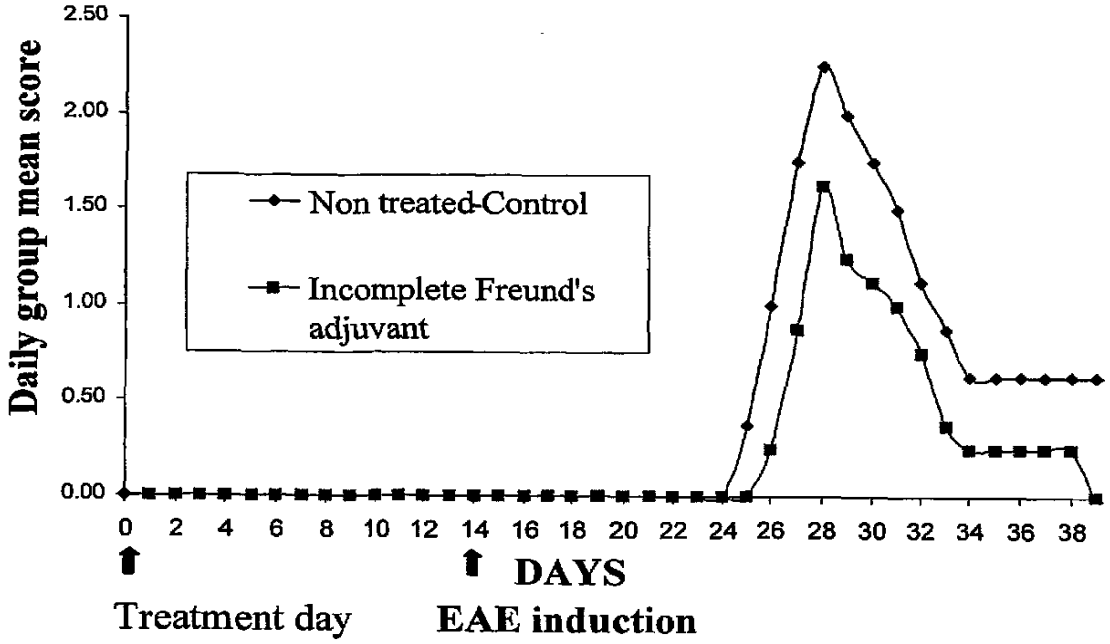

Fig. 3

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WO 02/083122 A1

(54) Title: FATTY ALCOHOLS AND FATTY ACID ESTERS USEFUL FOR TREATMENT OF INFLAMMATION

(57) Abstract: Immunomodulators selected from: (a) a saturated or cis-unsaturated C_{10} - C_{20} fatty alcohol or an ester thereof with a C_1 - C_6 alkanoic acid; (b) a monoester of a C_2 - C_8 alkanediol or of Glycerol with a saturated or cis-unsaturated C_{10} - C_{20} fatty acid; and (c) a diester of glycerol with a saturated or cis-unsaturated C_{10}- C_{20} fatty acid, are useful for treatment of inflammation, particularly immunologically-mediated inflammation such as it occurs in autoimmune diseases.

FATTY ALCOHOLS AND FATTY ACID ESTERS USEFUL FOR TREATMENT OF INFLAMMATION

FIELD OF THE INVENTION

The present invention relates to anti-inflammatory agents and, more particularly, to fatty alcohols, esters thereof with $C_1 - C_6$ alkanoic acids or esters of fatty acids with alkanediols or glycerol which are useful in the treatment of immunologically-mediated inflammation.

Abbreviations: **AA:** adjuvant arthritis: **CFA:** complete Freund's adjuvant; **EAE:** experimental autoimmune encephalomyelitis; **GPSCH:** guinea pig spinal cord homogenate; **IFA:** incomplete Freund's adjuvant; **OA:** oleyl alcohol; **PBS:** phosphate-buffered saline; **SC:** subcutaneously.

BACKGROUND OF THE INVENTION

Inflammation is commonly divided into three phases: acute inflammation, the immune response and chronic inflammation. Acute inflammation is the initial response to tissue injury and is mediated by the release of histamine, serotonin, bradykinin, prostaglandins and leukotrienes. The immune response, usually preceded by the acute inflammation phase, occurs when immunologically competent cells are activated in response to foreign organisms or antigenic substances liberated during the acute or chronic inflammatory response. The outcome of the immune response for the host may be beneficial, as when it causes invading organisms to be phagocytosed or neutralized. However, the outcome may be deleterious if it leads to chronic inflammation without resolution of the underlying injurious process as it occurs in rheumatoid arthritis.

The treatment of patients with inflammation envisages the relief of pain, which is the presenting symptom and the major continuing complaint of the patient, as well as the slowing or arrest of the tissue-damaging process.

Anti-inflammatory agents are usually classified as steroidal or glucocorticoids and nonsteroidal anti-inflammatory agents (NSAIDs). The glucocorticoids are powerful anti-inflammatory agents but the high toxicity associated with chronic corticosteroid therapy inhibits their use except in certain acute inflammatory conditions. Therefore, the nonsteroidal anti-inflammatory drugs have assumed a major role in the treatment of chronic conditions such as rheumatoid arthritis.

Among the nonsteroidal anti-inflammatory agents are included derivatives of aminoarylcarboxylic acids, arylacetic acids, arylbutyric acids, arylcarboxylic acids, arylpropionic acids, pyrazole, pyrazolone, salicylic acid and some other derivatives of different chemical structure, including specific anti-arthritic/anti-rheumatic agents.

Some fatty alcohols and esters of fatty acids have been described as solvents or emulsifiers for use in pharmaceutical compositions. For example, cetyl alcohol may be used in pharmaceutical compositions as emulsifying and stiffening agent (The Merck Index, pp. 347-8, # 2037), oleyl alcohol may be used as a carrier for medicaments (The Merck Index, p. 1222, # 6900), and alkyl esters of oleic acid may be used as solvents for medicaments (The Merck Index, p. 6899, # 6898).

A mixture of higher aliphatic primary alcohols, primarily isolated from beeswax, was described as having moderate anti-inflammatory activity. The composition of such a mixture was not disclosed (Rodriguez et al., 1998).

Feeding laboratory animals with fish oil rich in the long-chain n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), was described to reduce acute and chronic inflammatory responses, to improve survival to endotoxin and in models of autoimmunity and to prolong the survival of grafted organs, and it was therefore suggested that fish oil supplementation may be clinically useful in acute and chronic inflammatory conditions and following transplantation (Calder, 1998). A

pharmaceutical preparation comprising eicosapentaenoic acid and/or stearidonic acid for treatment of schizophrenia is described in WO 98/16216 and US 6,331,568.

Modified polyunsaturated fatty acids and derivatives thereof have been proposed for pharmaceutical uses. WO 99/27924 and US 6,280,755 describe anti-inflammatory fatty acids uninterrupted by a methylene group for use in topical pharmaceutical and cosmetic compositions. WO 97/38688 and US 6,262,119 describe polyunsaturated fatty acids having 1 or 2 substitutions selected from oxa and thia in position beta or gamma to the acyl group, for treating or ameliorating symptoms of T-cell mediated disease. WO 99/58122 and US 6,365,628 describe saturated fatty acids in which one or more methylene groups are substituted by O, S, SO, SO_2, or Se and alkyl esters thereof, for treatment or prevention of diabetes. US 5,019,383 describes synthetic vaccines comprising a peptide residue coupled to one or more alkyl or alkenyl groups of at least 12 carbon atoms or other lipophilic substance, wherein said alkyl or alkenyl group may be a fatty acid residue coupled to one or more functional groups of a polyfunctional group which is bound to the N-terminal amino group and/or C-terminal carboxy group of the peptide residue.

There is no description in the literature that isolated fatty alcohols or esters thereof with alkanoic acids may be used themselves as medicaments, and specifically not that they may be involved in immunomodulation of inflammation.

SUMMARY OF THE INVENTION

It has now been surprisingly found, in accordance with the present invention, that certain long-chain fatty alcohols, esters thereof with $C_1 - C_6$ alkanoic acids, or certain esters of long-chain fatty acids with alkanediols or glycerol can suppress inflammation in experimental adjuvant arthritis (AA) and experimental autoimmune encephalomyelitis (EAE) models in rats and can prevent graft rejection in mice.

The present invention thus relates to pharmaceutical compositions for the treatment of inflammation, particularly immunologically-mediated inflammation, comprising as active ingredient an immunomodulator selected from: (a) a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol or an ester thereof with a $C_1 - C_6$ alkanoic

acid; (b) a monoester of a $C_2 - C_8$ alkanediol or of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid; and (c) a diester of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid.

In another embodiment, the invention relates to the use of an immunomodulator selected from: (a) a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol or an ester thereof with a $C_1 - C_6$ alkanoic acid; (b) a monoester of a $C_2 - C_8$ alkanediol or of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid; and (c) a diester of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid, for the preparation of a pharmaceutical composition for the treatment of inflammation, in particular immunologically-mediated inflammation.

In still another embodiment, the invention relates to a method for the treatment of inflammatory disorders, in particular immunologically-mediated inflammation, which comprises administering to an individual in need thereof an effective amount of an agent selected from an immunomodulator selected from: (a) a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol or an ester thereof with a $C_1 - C_6$ alkanoic acid; (b) a monoester of a $C_2 - C_8$ alkanediol or of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid; and (c) a diester of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the dose response effect of oleyl alcohol (OA) on adjuvant arthritis (AA). Different doses of OA were administered subcutaneously to rats once 14 days before induction of AA.

Fig. 2 is a graph showing the disease profile of Lewis rats with experimental autoimmune encephalomyelitis (EAE) and treated with oleyl alcohol. Oleyl alcohol was administered to the rats 14 days before induction of EAE. Control group was treated with incomplete Freund's adjuvant (IFA).

Fig. 3 is a graph showing the disease profile of Lewis rats with EAE and treated with IFA. IFA was administered to the rats 14 days before induction of EAE. Control group was not treated.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides immunomodulators selected from: (a) a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol or an ester thereof with a $C_1 - C_6$ alkanoic acid; (b) a monoester of a $C_2 - C_8$ alkanediol or of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid; and (c) a diester of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid.

According to one preferred embodiment of the invention, the pharmaceutical composition comprises a long-chain saturated or unsaturated C_{10}-C_{20}, preferably C_{16}-C_{20}, most preferably a C_{18}, fatty alcohol.

Examples of C_{10}-C_{20} saturated fatty alcohols that can be used according to the invention include, but are not limited to, decyl alcohol, lauryl alcohol, myristyl alcohol, stearyl alcohol and preferably cetyl alcohol (also known as palmityl alcohol).

The unsaturated fatty alcohol according to the invention has preferably one or more double bonds in the cis form and 16-18 carbon atoms and may be, without being limited to, oleyl alcohol (cis-9-octadecenol), linoleyl alcohol (cis-9,12-octadecadienol), γ-linolenyl alcohol (cis-6,9,12-octadecatrienol) and linolenyl alcohol (cis-9,12,15-octadecatrienol). In preferred embodiments, the fatty alcohol used in the compositions of the invention is cetyl, linolenyl or, most preferably, oleyl alcohol.

In another embodiment, the pharmaceutical composition of the invention comprises an ester of a fatty alcohol as defined above with a $C_1 - C_6$ alkanoic acid such as acetic acid, propionic acid, butyric acid, valeric acid and caproic acid.

In a further embodiment, the pharmaceutical composition of the invention comprises an ester of a saturated or cis-unsaturated C_{10}-C_{20} fatty acid with an alcohol selected from a C_2-C_8 alkanediol or glycerol, said ester being a monoester with said C_2-C_8 alkanediol or glycerol or a diester with glycerol.

The C_{10}-C_{20} fatty acid is preferably a C_{16}-C_{20}, most preferably a C_{18} fatty acid. In one embodiment, the C_{10}-C_{20} fatty acid is saturated such as, but without being

limited to, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid. In another embodiment, the C_{10}-C_{20} fatty acid is a cis-unsaturated fatty acid such as, but without being limited to, palmitoleic acid (cis-9-hexadecenoic acid), oleic acid (cis-9-octadecenoic acid), cis-vaccenic acid (cis-11-octadecenoic acid), linoleic acid (cis-9,12-octadecadienoic acid), γ-linolenic acid (cis-6,9,12-octadecatrienoic acid), linolenic acid (cis-9,12,15-octadecatrienoic acid) and arachidonic acid (cis-5,8,11,14-eicosatetraenoic acid).

According to the invention, the alkanediol has 2 to 8, preferably 2 to 4, and more preferably, 2 carbon atoms, and is selected from, but not being limited to, 1,3-propanediol, 1,4-butanediol and, preferably, 1,2-ethylene glycol. An example of such an ester is 1,2-ethylene glycol monooleate.

According to another embodiment of the invention, the active ingredient of the pharmaceutical composition is a mono- or diester of glycerol with the long-chain fatty acid. In one preferred embodiment, the monoglyceride is glycerol monooleate. The diglycerides contain one free hydroxyl group and the other two hydroxyl groups may be both esterified with 2 molecules of the long-chain fatty acid, e.g. glycerol dioleate, or one of the hydroxyl groups is esterified with one molecule of the long-chain fatty acid and a second hydroxyl group is esterified with a $C_1 - C_6$ alkanoic acid such as acetic acid, propionic acid, butyric acid, valeric acid and caproic acid.

The immune system, in both its innate and adaptive arms, is involved in regulating inflammation of every type, and inflammation is a key factor in processes such as wound healing, connective tissue re-modeling, angiogenesis, organ regeneration, neuroprotection, as well as in the adaptive immune responses seen in autoimmunity, allergies, graft rejection, and infection (see Cohen, 2000; Schwartz and Cohen, 2000). Therefore, anti-inflammatory agents that modulate the inflammatory response such as those described here will be useful in a variety of conditions.

Inflammatory disorders that can be treated with the immunomodulators of the present invention include, but are not limited to, immunologically-mediated chronic or acute inflammatory disorders selected from an autoimmune disease, severe

allergies, asthma, graft rejection or for the treatment of chronic degenerative diseases such as Alzheimer's disease, and in neuroprotection, organ regeneration, chronic ulcers of the skin, and schizophrenia.

Examples of autoimmune diseases that can be treated according to the invention are multiple sclerosis or a human arthritic condition, e.g. rheumatoid arthritis, reactive arthritis with Reiter's syndrome, ankylosing spondylitis and other inflammations of the joints mediated by the immune system. Other autoimmune diseases are contemplated and are presented in the following list in the context of the organ or tissue involved. Thus, according to the invention, the immunologically-mediated inflammatory disorder may be myasthenia gravis, Guillain-Barré syndrome, and other inflammatory diseases of the nervous system; psoriasis, pemphigus vulgaris and other diseases of the skin; systemic lupus erythematosus, glomerulonephritis and other diseases affecting the kidneys; atherosclerosis and other inflammations of the blood vessels; autoimmune hepatitis, inflammatory bowel diseases, e.g. Crohn's disease, pancreatitis, and other conditions of the gastrointestinal system; type 1 diabetes mellitus (insulin-dependent diabetes mellitus or IDDM), autoimmune thyroiditis (Hashimoto's thyroiditis), and other diseases of the endocrine system.

One of the models used to test the anti-inflammatory activity of the agents according to the invention is adjuvant arthritis (AA), an experimental disease of the joints inducible in some strains of rats by immunizing with *Mycobacterium tuberculosis* in complete Freund's adjuvant (CFA). These animals develop an arthritis whose features are similar to those of rheumatoid arthritis in humans and thus serve as animal models of human arthritic conditions such as rheumatoid arthritis, reactive arthritis in Reiter's syndrome, ankylosing spondylitis and other inflammations of the joints which appear to be mediated by the immune system (Pearson, 1964). Adjuvant arthritis also serves as a model of immune-mediated inflammation in general including cell-mediated autoimmune reactions, graft rejection and allergic reaction. For example, treatments which can suppress rheumatoid arthritis include immunosuppressive agents such as corticosteroids,

cyclosporin A (Jaffee et al., 1989; Pollock et al., 1989), azathioprine, and other immunosuppressive agents which are broadly used in the treatment of autoimmune diseases. Therefore, suppression of adjuvant arthritis by a therapeutic agent indicates that the agent is potentially useful as a broad anti-inflammatory agent.

The pharmaceutical composition provided by the present invention may be in solid, semisolid or liquid form and may further include pharmaceutically acceptable fillers, carriers or diluents, and other inert ingredients and excipients. The composition can be administered by any suitable route such as, but not limited to, oral, topical, or parenteral e.g. by injection through subcutaneous, intravenous, intramuscular, or any other suitable route. Since many of the compounds are oily, they are preferably administered parenterally, more preferably subcutaneously. If given continuously, the compounds of the present invention are each typically administered by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. The dosage will depend of the state of the patient and severity of the disease and will be determined as deemed appropriate by the practitioner.

For parenteral administration, the compounds may be formulated by mixing each at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. Generally, the formulations are prepared by contacting the compounds of the present invention each uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils can be also useful, as well as liposomes. These preparations can be made by conventional methods known to those skilled in the art, for example as described in "Remington's

Pharmaceutical Science", A.R. Gennaro, ed., 17th edition, 1985, Mack Publishing Company, Easton, PA, USA.

The invention will now be illustrated by the following non-limiting examples.

EXAMPLES

Example 1. Anti-inflammatory effect of oleyl alcohol and other agents-protection against adjuvant arthritis (AA)

AA was induced by immunizing inbred 8-10-week old Lewis rats (Harlan-Olac Limited, Blackthorn, Oxon, UK), at the base of the tail with 1 mg/0.1 ml of killed *Mycobacterium tuberculosis* (Sigma) in IFA (Sigma) as described (Pearson, 1956). Arthritis of the limbs was noted to develop 12-14 days later and was scored on a scale of 0-16 summing the severity of the inflammation of each of the 4 limbs on a scale of 0-4, as described (Holoshitz et al., 1983). The peak of the arthritis usually was observed around day 26 after immunization.

Control rats were untreated or treated by injections of saline. A positive control of immunosuppression was obtained by including a group of rats treated with the corticosteroid agent dexamethasone (200 μg) administered intraperitoneally every other day beginning on day 12 after induction.

The immunomodulator of the invention (100 μl oleyl alcohol, glycerol mono-oleate, linolenyl alcohol or cetyl alcohol) was administered subcutaneously (SC) once 14 days before induction of AA or on day 12 after induction of AA. The percent inhibition of inflammation measured on the day of maximal inflammation was computed as follows:

$$\frac{\text{mean maximal score of test group}}{\text{mean maximal score of control group}} \times 100\%$$

All four compounds were found to be effective, producing more than 60% inhibition of inflammation whereas oleic acid had no effect. The results are summarized in Table 1.

Two further experiments showed that 500 µl of oleyl alcohol (100 µl corresponds to about 90 mg oleyl alcohol) suppressed the inflammation by 96% and 91%.

Table 1. Effects of various agents on the inflammation of adjuvant arthritis

Compound Tested	% Inhibition (100 µl)
Glycerol mono-oleate	98%
Oleyl alcohol	78%
Linolenyl alcohol	75%
Cetyl alcohol	66%

Example 2. Protection against AA by different doses of oleyl alcohol

To study the dose response effect of oleyl alcohol in AA, oleyl alcohol was administered subcutaneously in doses of 10, 50, 100 or 500 mg to Lewis rats once 14 days before induction of AA, as described in Example 1 above.

Fig. 1 shows the dose response effect of oleyl alcohol. It can be seen that increasing doses of oleyl alcohol suppressed the arthritis. On the day of peak disease, day 26, the inflammation was suppressed by 14% (10 µl), 61% (50 µl), 78% (100 µl) and 90% (500 µl).

Example 3. Anti-inflammatory effect of oleyl alcohol and other immunomodulators and protection against EAE in DA rats

Experimental autoimmune encephalomyelitis (EAE) is an experimental autoimmune disease inducible in some strains of rats by immunization with myelin basic protein (MBP) or proteolipid protein (PLP) in complete Freund's adjuvant (CFA) or with an emulsion of the rat's spinal cord in either CFA or incomplete Freund's adjuvant (IFA). EAE in DA rats is considered as a model of chronic EAE. Within two to three weeks the animals develop cellular infiltration of the myelin sheaths of the central nervous system resulting in demyelination and paralysis. Most

of the animals die, but others have milder symptoms, and some animals develop a chronic form of the disease that resembles chronic relapsing and remitting multiple sclerosis (MS) in humans. Therefore, these animals with EAE serve as a model for the human MS autoimmune disease. EAE develops in the animal about 12 days after immunization and is characterized by paralysis of various degrees due to inflammation of the central nervous system. In some strains, like the Lewis rat, the paralysis can last up to 6-7 days and the rats usually recover unless they die during the peak of their acute paralysis. In other strains of rats like the DA rat, the paralysis can be chronic and remitting.

For the induction and clinical assessment of EAE, spinal cord obtained from DA rats is frozen, thawed and minced thoroughly with a spatula before immunization. Rats are immunized by one subcutaneous injection (just under the skin) into the dorsal base of the tail with 200 µl emulsion prepared from 1:1 IFA (Difco, Detroit, MI, USA) and antigen (volume/weight, i.e. 100 µl IFA/100 mg of whole spinal cord) or from 1:1 CFA (IFA was complemented with 4 mg/ml of *Mycobacterium tuberculosis* strain 37RA) and antigen (volume/weight, i.e. 100 µl CFA/100 mg of whole spinal cord). The emulsion was prepared by titration with a gas-tight glass syringe and a needle, 1.2 mm diameter. Rats are regularly weighed and examined for clinical signs of EAE. A four-graded scale was used to assess clinical severity: 0, no paralysis; 1, tail weakness (hanging); 2, hind limb paralysis; 3, hind and fore limb paralysis; 4, severe total paralysis (Lorentzen et al., 1995).

Groups of 5 or 7 DA strain female rats, 8-9 week old, are immunized in the hind footpads with 0.1 ml per footpad of IFA containing 100 mg of whole, homogenized DA spinal cord, for a total of 200 mg per rat. On the day of immunization, the rats are treated by SC injection with oleyl alcohol or other agent according to the invention (100 µl) or with paraffin oil (control). The rats are scored for EAE on a severity scale of 0 - 4 as described above.

Example 4. Anti-inflammatory effect of oleyl alcohol and protection against EAE in Lewis rats

EAE induced in Lewis rats is considered as a model of acute inflammation in the brain (as opposed to the chronic disease in DA rats).

For EAE induction, three lyophilized guinea pig spinal cord homogenate (GPSCH) emulsions were prepared as follows: (i) 25 mg of lyophilized GPSCH (GP2) was suspended in 2.5 ml of sterile PBS (Sigma) and incubated for one hour at 37° C; (ii) 54.1 mg of *Mycobacterium tuberculosis* H37Ra (MT, Difco) was suspended in 13.5 ml CFA (Sigma) containing 1mg/ml MT to obtain 5 mg/ml MT; (iii) 2.5 ml CFA (5 mg/ml MT) was added into vial with 2.5 ml of PBS containing 25 mg GPSCH to yield 5 mg/ml GPSCH and 2.5 mg/ml MT. The mixture was transferred into a glass syringe connected to a second glass syringe through a Luer lock bridge. The material was mixed well by transferring from one syringe to another for about 10 minutes until the materiall was well emulsified. The emulsion of GPSCH at a dose of 1 mg/rat and MT at a dose of 0.5 mg/rat in CFA induced EAE in rats (based on previous titration).

For the treatment, two groups of eight 9-10 weeks old Lewis rats (Harlan, Israel), were treated with the test samples (oleyl alcohol or IFA) 14 days before EAE induction. The group treated with IFA served as the control group. The test samples were injected at a dose of 0.5 ml/kg once, subcutaneously. A third group of 8 rats was not treated and served as non-treated control.

EAE was induced in rats of all three groups 14 days after injection of the test samples by injection with 0.1 ml of the GPSCH emulsion in CFA into each of the hind leg foot pads (0.2 ml per rat).

The EAE clinical signs were observed and scored from the 9th day post-EAE induction until the termination of the experiment according to the following five-graded scale to assess clinical severity: 0, normal behavior; 1, weight loss; 2, tail weakness; 3, hind legs hypotonia and weakness; 4, hind legs paralysis; 4, severe total paralysis; 5, impaired respiration and/or convulsions and/or full paralysis or death. All rats having scores of 1 and above were considered sick.

The calculation of EAE results was carried out as follows:

(i) *Calculation of the incidence of disease*

The number of sick animals in each group were summed. The incidence of disease and the % activity were calculated as follows:

$$\text{Incidence of disease} = \frac{\text{No. of sick rats in group}}{\text{No. of rats in group}} \times 100\%$$

$$\%\ \text{activity}\ * = 1 - \frac{\text{(disease incidence in treated group)}}{\text{disease incidence in control group}} \times 100\%$$

* = (according to incidence)

(ii) *Calculation of the mean maximal score (MMS)*

The maximal score of each rat in the group were summed. The mean maximal score (MMS) and the % activity of the group were calculated as follows:

$$\text{Mean Maximal score} = \frac{\sum \text{Maximal score of each rat}}{\text{No. of rats in the group}}$$

$$\%\ \text{activity}\ * = \left(1 - \frac{\text{MMS of treated group}}{\text{MMS of control group}}\right) \text{X}\, 100$$

* = (% activity according to MMS)

(iii) *Calculation of the group mean score (GMS)*

The mean score of each rat during the observation period were summed (score 5 was counted forward). The mean score of the group and its % activity were calculated as follows:

$$\text{Mean score} = \frac{\sum \text{Group score of each rat}}{\text{No. of rats in the group}}$$

$$\%\ \text{activity}\ * = \left(1 - \frac{\text{GMS of treated group}}{\text{GMS of control group}}\right) \text{X}\, 100$$

* = (% activity according to GMS)

(iv) *Calculation of the mean onset of disease*

The time of disease onset (days) for each rat in the group were summed. The mean onset of disease for the group was calculated. The time of onset of disease for those rats that did not develop EAE was considered as 25 days (duration of study).

(v) *Calculation of the mean duration of disease*

The disease duration (days) of each rat in each group were summed. The mean disease duration of the group was calculated. The disease duration of rats that did not develop EAE was considered as zero.

The evaluation of the clinical manifestations of EAE, i.e. % incidence of disease, MMS, GMS, mean duration and onset of EAE disease is summarized in Table 2. The graphs of the disease profile for each group are presented in Figs. 2 and 3 for treatment with oleyl alcohol and IFA, respectively.

As shown by the results, no essential difference in incidence of disease (62.5% to 75% incidence) or mean maximum score (1.75 to 2.38 MMS) was observed between the IFA-injected groups and non-treated control group. Oleyl alcohol showed a beneficial effect on all the clinical parameters that were tested. It exhibited 77.1% activity according to group mean score (GMS) and 63% activity according to mean maximum score (MMS) compared to the non-treated control group. The mean onset of disease was 18.6 days in the oleyl alcohol treated group compared to 15.5 days in the non-treated control group. The duration of disease was 2.0 days in the oleyl alcohol treated group compared to 5.13 days in the non-treated control group. The duration of the EAE clinical signs in the tested groups was between 1 and 7 days, except one rat in the group treated with IFA. IFA had minor effect, if any, on the rat EAE. No mortality was observed in the tested groups, except one rat in the non-treated control group.

Table 2: Evaluation EAE clinical results

Group No.	Test Sample	% Incid-ence	% Activity Incidence	MMS	% Activity MMS	GMS	% Activity GMS	Mean Onset of disease (Day No.)	Disease duration (days)
1	OA	50.0%	33.3%	0.88	63.0%	0.22	77.1%	18.6	2.0
2	IFA	62.5%	16.7%	1.75	26.5%	0.52	45.8%	17.0	3.75
3	NTC	75.0%	NA	2.38	NA	0.96	NA	15.5	5.13

OA-Oleyl alcohol; NTC-non-treated control; NA- Not applicable

Example 5. Effect of oleyl alcohol on skin allograft survival

The immune system represents a strong barrier for successful transplantation of organs or tissues between non-genetically identical donor and recipient. Both $CD4^+$ and $CD8^+$ T cells participate in graft rejection.

Skin graft transplantation is carried out essentially as described before (Birk et al., 1999). Thus, mice are shaved and 1 cm^2 sections of skin are cut from the dorsal side of sacrificed donors and cleaned in PBS. Two patches of dorsal skin, 1 cm^2 each, are cut from the anesthetized recipients (Nembutal 6 mg/ml, 0.25 ml/mouse) in preparation for the allograft. Two donor allografts per recipient are grafted onto the dorsal lesioned patches. Histoacryl (B. Braun Melsungen AG, Melsungen, Germany) is applied around the graft. Nobecutan (ASTR, Astra Tech, Glos G15, UK) is sprayed over the grafts.

In the experiment, groups of 6 BALB/c female mice, 8-week old, are grafted with 1 cm^2, full thickness skin grafts from C57BL/6 female mice, 8-week old. On the day of grafting, a group of recipient mice is treated either with paraffin oil or SC with 100 µl oleyl alcohol or another immunomodulator according to the invention. The day of rejection is scored. The transplanted skin in the mice treated with the immunomodulator survives longer in comparison with the untreated control mice.

Example 6. Prevention and treatment of SLE

Systemic lupus erythematosus is an autoimmune disease in which both autoantibodies and immune complexes are involved. In order to test the

immunomodulators of the invention, mice with experimental SLE or (NZBxNZW)F1 mice that spontaneously develop autoimmune diseases that closely resemble SLE, can be used.

In order to induce experimental SLE, BALB/c mice are immunized with the human or murine anti-DNA monoclonal antibody 16/6Id (20 μg/mouse) in CFA in the hind footpads and boosted 3 weeks later with the same amount of the immunizing antibody in PBS. The mice are then tested for autoantibody production and clinical manifestations characteristic of experimental SLE. In order to either prevent induction of experimental SLE or to cure mice afflicted with the disease, mice are given oleyl alcohol or another immunomodulator according to the invention subcutaneously (100 μl per mouse) before or concomitant with the immunization and some weeks after immunization. The number of injections is based on the effect of the tested compound on the disease induction and progression. The animals are regularly weighed and examined for clinical signs of SLE as described, for example, in WO 96/30057.

Example 7. Prevention and treatment of autoimmune thyroiditis

Experimental autoimmune thyroiditis (EAT) can be induced in a number of animals by immunizing with thyroglobulin in CFA. Both humoral antibodies and T_{DTH} cells directed against the thyrogllobulin develop, resulting in thyroid inflammation. EAT appears to best mimic Hashimoto's thyroiditis.

EAT is induced as previously described (Rose et al., 1971) by injecting each mouse subcutaneously with thyroglobulin extract obtained from one thyroid gland. The extract is emulsified in IFA (Difco Laboratories, Detroit, Mich.), to which are added 7mg/ml *Mycobacterium tuberculosis,* H37Ra strain (Difco Laboratories). This injection is repeated one week later. Donors of thyroglobulin extract are mice of the C3H/eB strain. 4-5 weeks later, EAT is assayed by removing thyroid glands of recipient mice, fixing them in 10% formalin solution and then in 70% alcohol, and examining microscopic sections stained with hematoxylin and eosin. Microscopic slides are coded and examined without knowledge of their identity. A diagnosis of

EAT is made by observing at least one unequivocal focus of infiltration by mononuclear cells. Treatment is performed by injecting SC oleyl alcohol or another immunomodulator (100 µl per animal) before induction of EAT, concomitant with or thereafter (control animals are injected paraffin oil), and the animals are regularly weighed and examined for clinical signs of EAT by known conventional methods.

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CLAIMS:

1. A pharmaceutical composition for treatment of inflammation, particularly immunologically-mediated inflammation, comprising as active ingredient an immunomodulator selected from: (a) a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol or an ester thereof with a $C_1 - C_6$ alkanoic acid; (b) a monoester of a $C_2 - C_8$ alkanediol or of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid; and (c) a diester of glycerol with a saturated or cis-unsaturated $C_{10}- C_{20}$ fatty acid.

2. The pharmaceutical composition according to claim 1, wherein the active ingredient is a saturated C_{10}-C_{20} fatty alcohol.

3. The pharmaceutical composition according to claim 2, wherein the saturated C_{10}-C_{20} fatty alcohol is selected from decyl alcohol, lauryl alcohol, myristyl alcohol, cetyl alcohol and stearyl alcohol.

4. The pharmaceutical composition according to claim 1, wherein the active ingredient is a cis-unsaturated C_{16}-C_{18} fatty alcohol.

5. The pharmaceutical composition according to claim 4, wherein the cis-unsaturated C_{16}-C_{18} fatty alcohol is selected from oleyl alcohol, linoleyl alcohol, γ-linolenyl alcohol and linolenyl alcohol.

6. The pharmaceutical composition according to claim 1, wherein the active ingredient is an ester of a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol with a $C_1 - C_6$ alkanoic acid.

7. The pharmaceutical composition according to claim 1, wherein the active ingredient is a monoester of a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid with a $C_2 - C_8$ alkanediol.

8. The pharmaceutical composition according to claim 7, wherein said alkanediol is selected from 1,2-ethylene glycol, 1,3-propanediol and 1,4-butanediol.

9. The pharmaceutical composition according to claim 1, wherein the active ingredient is a monoester of a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid with glycerol.

10. The pharmaceutical composition according to claim 1, wherein the active ingredient is a diester of a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid with glycerol.

11. The pharmaceutical composition according to any one of claims 1 and 7 to 10, wherein said fatty acid is a saturated C_{10}-C_{20} fatty acid.

12. The pharmaceutical composition according to claim 11, wherein said saturated fatty acid is selected from capric acid, lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid.

13. The pharmaceutical composition according to any one of claims 1 and 7 to 10, wherein said fatty acid is a cis-unsaturated $C_{10} - C_{20}$ fatty acid.

14. The pharmaceutical composition according to claim 13, wherein said cis-unsaturated C_{10}-C_{20} fatty acid is selected from palmitoleic acid, oleic acid, cis-vaccenic acid, linoleic acid, γ-linolenic acid, linolenic acid, and arachidonic acid.

15. The pharmaceutical composition according to claim 14, wherein said active ingredient is glycerol monooleate.

16. The pharmaceutical composition according to claim 14, wherein said active ingredient is glycerol dioleate.

17. A pharmaceutical composition according to any one of claims 1 to 16 for the treatment of immunologically-mediated chronic or acute inflammatory disorders selected from an autoimmune disease, severe allergies, asthma, graft rejection or for the treatment of chronic degenerative diseases such as Alzheimer's disease, and in neuroprotection, organ regeneration, chronic ulcers of the skin, and schizophrenia.

18. The pharmaceutical composition according to claim 17, wherein said autoimmune disease is multiple sclerosis or a human arthritic condition.

19. The pharmaceutical composition according to claim 18, wherein said human arthritic condition is selected from rheumatoid arthritis, reactive arthritis with Reiter's syndrome, ankylosing spondylitis and other inflammations of the joints mediated by the immune system.

20. The pharmaceutical composition according to claim 17, wherein said immunologically-mediated inflammatory disorder is myasthenia gravis, Guillain-Barré syndrome, and other inflammatory diseases of the nervous system; psoriasis, pemphigus vulgaris and other diseases of the skin; systemic lupus erythematosus, glomerulonephritis and other diseases affecting the kidneys; atherosclerosis and other inflammations of the blood vessels; autoimmune hepatitis, inflammatory bowel diseases, pancreatitis, and other conditions of the gastrointestinal system; type 1 diabetes mellitus, autoimmune thyroiditis, and other diseases of the endocrine system.

21. Use of an immunomodulator selected from: (a) a saturated or cis-unsaturated C_{10} – C_{20} fatty alcohol or an ester thereof with a C_1 – C_6 alkanoic acid; (b) a monoester of a C_2 – C_8 alkanediol or of glycerol with a saturated or cis-unsaturated C_{10} – C_{20} fatty acid; and (c) a diester of glycerol with a saturated or cis-unsaturated C_{10}– C_{20}

fatty acid, for the preparation of a pharmaceutical composition for the treatment of inflammation, particularly immunologically-mediated inflammation.

22. The use according to claim 21, wherein said immunomodulator is a saturated C_{10}-C_{2O} fatty alcohol.

23. The use according to claim 22, wherein said saturated C_{10}-C_{2O} fatty alcohol is selected from decyl alcohol, lauryl alcohol, myristyl alcohol, cetyl alcohol and stearyl alcohol.

24. The use according to claim 21, wherein said immunomodulator is a cis-unsaturated C_{16}-C_{18} fatty alcohol.

25. The use according to claim 24, wherein the cis-unsaturated C_{16}-C_{18} fatty alcohol is selected from oleyl alcohol, linoleyl alcohol, γ-linolenyl alcohol and linolenyl alcohol.

26. The use according to claim 21, wherein the immunomodulator is an ester of a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol with a $C_1 - C_6$ alkanoic acid.

27. The use according to claim 21, wherein said immunomodulator is a monoester of a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid with a $C_2 - C_8$ alkanediol.

28. The use according to claim 27, wherein said alkanediol is selected from 1,2-ethylene glycol, 1,3-propanediol and 1,4-butanediol.

29. The use according to claim 21, wherein said immunomodulator is a monoester of glycerol with a saturated or cis-unsaturated C_{10}-C_{20} fatty acid.

30. The use according to claim 21, wherein said immunomodulator is a diester of glycerol with a saturated or cis-unsaturated C_{10}-C_{20} fatty acid.

31. The use according to any one of claims 21 and 27 to 30, wherein said fatty acid is a saturated C_{10}-C_{20} fatty acid.

32. The use according to claim 31, wherein said saturated C_{10}-C_{20} fatty acid is selected from capric acid, lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid.

33. The use according to any one of claims 21 and 27 to 30, wherein said fatty acid is a cis-unsaturated C_{10}-C_{20} fatty acid.

34. The use according to claim 33, wherein said cis-unsaturated C_{10}-C_{20} fatty acid is selected from palmitoleic acid, oleic acid, cis-vaccenic acid, linoleic acid, γ-linolenic acid, linolenic acid, and arachidonic acid.

35. The use according to claim 34, wherein said innunomodulator is glyceryl monooleate.

36. The use according to claim 34, wherein said immunomodulator is glyceryl dioleate.

37. The use according to any one of claims 21 to 36, wherein said pharmaceutical composition is for the treatment of immunologically-mediated chronic or acute inflammatory disorders selected from an autoimmune disease, severe allergies, asthma, graft rejection or for the treatment of chronic degenerative diseases such as Alzheimer's disease, and in neuroprotection, organ regeneration, chronic ulcers of the skin, and schizophrenia.

38. The use according to claim 37, wherein said autoimmune disease is multiple sclerosis or a human arthritic condition.

39. The use according to claim 38, wherein said human arthritic condition is selected from rheumatoid arthritis, reactive arthritis with Reiter's syndrome, ankylosing spondylitis and other inflammations of the joints mediated by the immune system.

40. The use according to claim 37, wherein said immunologically-mediated inflammatory disorder is myasthenia gravis, Guillain Barré syndrome, and other inflammatory diseases of the nervous system; psoriasis, pemphigus vulgaris and other diseases of the skin; systemic lupus erythematosus, glomerulonephritis and other diseases affecting the kidneys; atherosclerosis and other inflammations of the blood vessels, autoimmune hepatitis, inflammatory bowel diseases, pancreatitis, and other conditions of the gastrointestinal system; type 1 diabetes mellitus, thyroiditis, and other diseases of the endocrine system.

41. A method for the treatment of inflammation, particularly immunologically-mediated inflammation, which comprises administering to a patient in need an effective amount of an immunomodulator selected from: (a) a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol or an ester thereof with a $C_1 - C_6$ alkanoic acid; (b) a monoester of a $C_2 - C_8$ alkanediol or of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid; and (c) a diester of glycerol with a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid.

42. A method according to claim 41, wherein said immunomodulator is a saturated C_{10}-C_{12} fatty alcohol.

43. The method according to claim 42, wherein said saturated C_{10}-C_{20} fatty alcohol is selected from decyl alcohol, lauryl alcohol, myristyl alcohol, cetyl alcohol and stearyl alcohol.

44. The method according to claim 41, wherein said immunomodulator is a cis-unsaturated C_{16}-C_{18} fatty alcohol.

45. The method according to claim 44, wherein the cis-unsaturated C_{16}-C_{18} fatty alcohol is selected from oleyl alcohol, linoleyl alcohol, γ-linolenyl alcohol and linolenyl alcohol.

46. The method according to claim 41, wherein the immunomodulator is an ester of a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty alcohol with a $C_1 - C_6$ alkanoic acid.

47. The method according to claim 41, wherein said immunomodulator is a monoester of a saturated or cis-unsaturated $C_{10} - C_{20}$ fatty acid with a $C_2 - C_8$ alkanediol.

48. The method according to claim 47, wherein said alkanediol is selected from 1,2-ethylene glycol, 1,3-propanediol and 1,4-butanediol.

49. The method according to claim 41, wherein said immunomodulator is a monoester of glycerol with a saturated or cis-unsaturated C_{10}-C_{20} fatty acid.

50. The method according to claim 41, wherein said immunomodulator is a diester of glycerol with a saturated or cis-unsaturated C_{10}-C_{20} fatty acid.

51. The method according to any one of claims 41 and 47 to 50, wherein said fatty acid is a saturated C_{10}-C_{20} fatty acid.

52. The method according to claim 51, wherein said saturated C_{10}-C_{20} fatty acid is selected from capric acid, lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid.

53. The method according to any one of claims 41 and 47 to 50, wherein said fatty acid is a cis-unsaturated C_{10}-C_{20} fatty acid.

54. The method according to claim 53, wherein said cis-unsaturated C_{10}-C_{20} fatty acid is selected from palmitoleic acid, oleic acid, cis-vaccenic acid, linoleic acid, γ-linolenic acid, linolenic acid, and arachidonic acid.

55. The method according to claim 54, wherein said immunomodulator is glyceryl monooleate.

56. The method according to claim 54, wherein said immunomodulator is glyceryl dioleate.

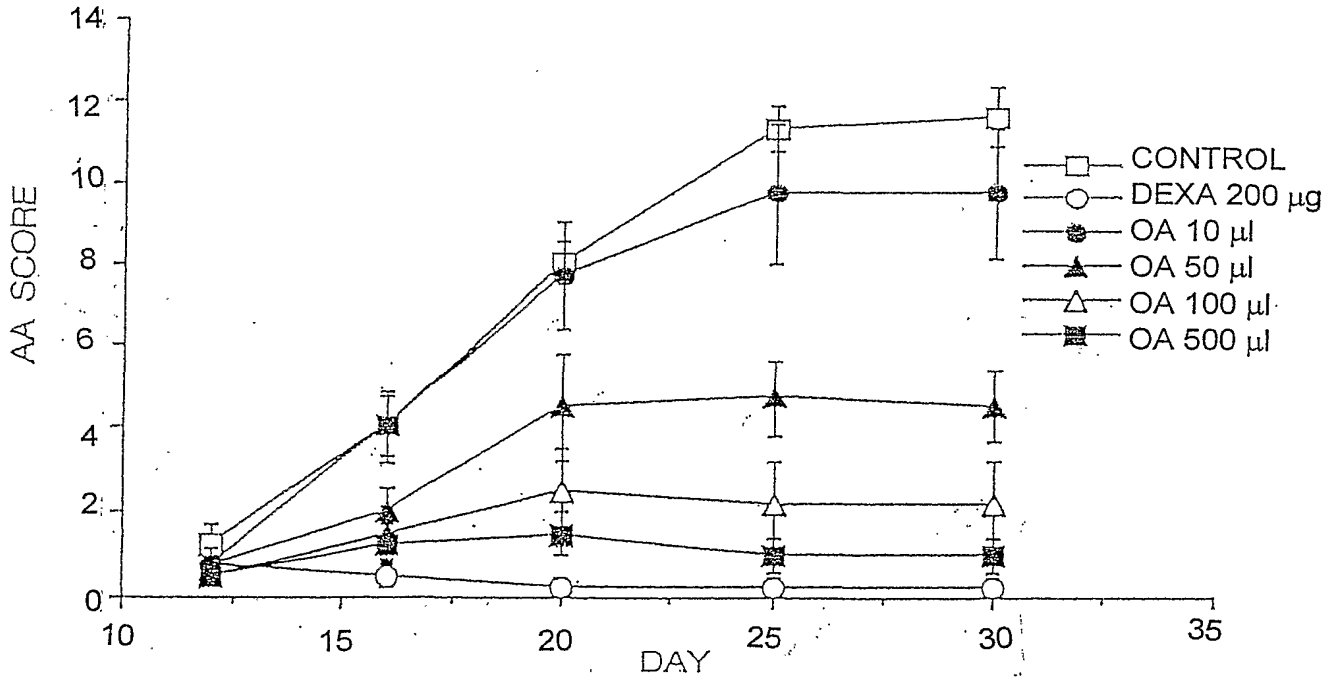
57. A method according to any one of claims 41 to 56 for the treatment of immunologically-mediated inflammatory disorders selected from an autoimmune disease, severe allergies, asthma, graft rejection or for the treatment of chronic degenerative diseases such as Alzheimer's disease, and in neuroprotection, organ regeneration, chronic ulcers of the skin, and schizophrenia.

58. The method according to claim 57, wherein said autoimmune disease is multiple sclerosis or a human arthritic condition.

59. The method according to claim 58, wherein said human arthritic condition is selected from rheumatoid arthritis, reactive arthritis with Reiter's syndrome, ankylosing spondylitis and other inflammations of the joints mediated by the immune system.

60. The method according to claim 57, wherein said immunologically-mediated inflammatory disorder is myasthenia gravis, Guillain Barré syndrome, and other

inflammatory diseases of the nervous system; psoriasis, pemphigus vulgaris and other diseases of the skin; systemic lupus erythematosus, glomerulonephritis and other diseases affecting the kidneys; atherosclerosis and other inflammations of the blood vessels, autoimmune hepatitis, inflammatory bowel diseases, pancreatitis, and other conditions of the gastrointestinal system; type 1 diabetes mellitus, thyroiditis, and other diseases of the endocrine system.


AA SCORE
DAY
CONTROL
DEXA 200 µg
OA 10 µl
OA 50 µl
OA 100 µl
OA 500 µl
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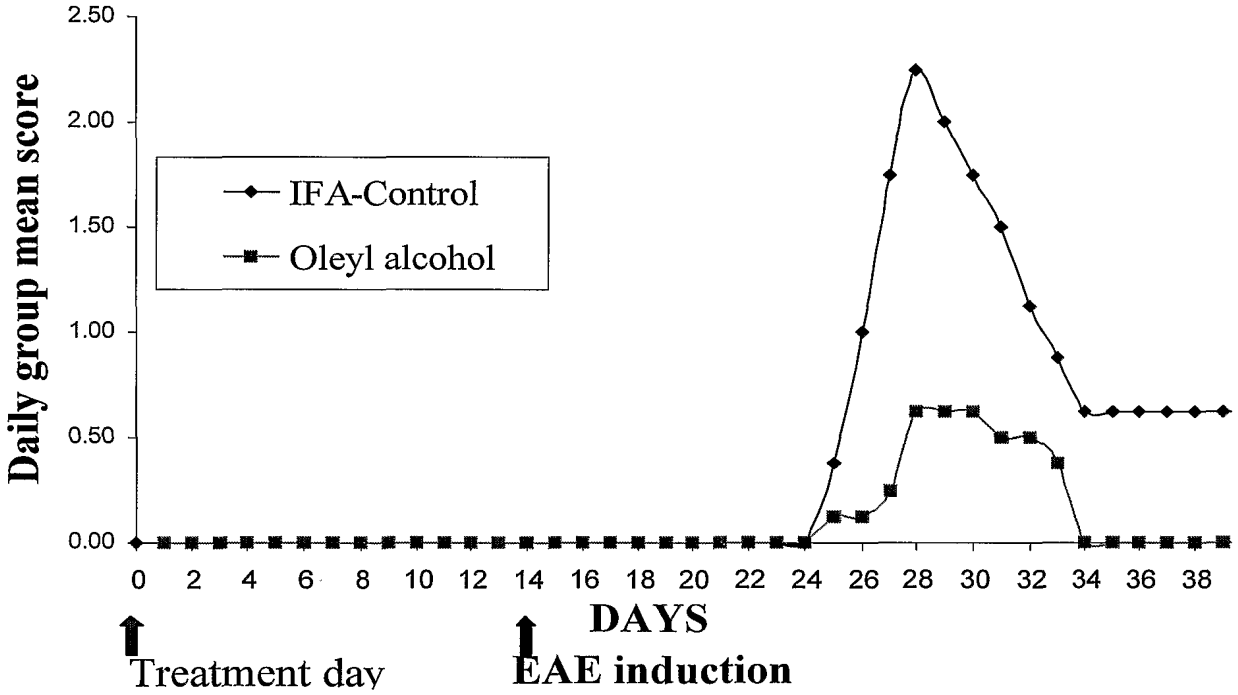
2
4
6
8
10
12
14
10
15
20
25
30
35

Fig. 1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2.00
1.50
1.00
0.50
0.00
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Oleyl alcohol
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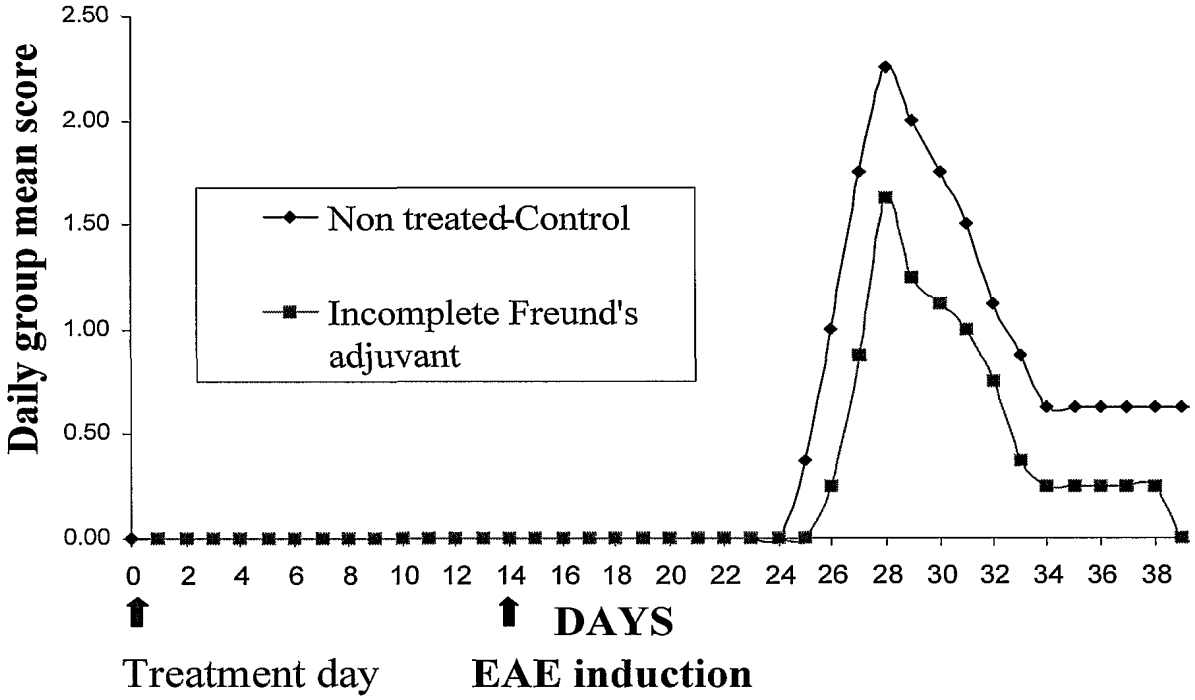
DAYS
Treatment day
EAE induction

Fig. 2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0.00
Non treated-Control
Incomplete Freund's adjuvant
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
DAYS
Treatment day
EAE induction

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL02/00294

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 31/22, 31/225, 31/20

US CL :514/ 546, 547, 558, 560, 825, 863, 866, 879, 886, 925, 928

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. : 514/ 546, 547, 558, 560, 825, 863, 866, 879, 886, 925, 928

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)

cas-online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,210,700 B1 (VALENTE et al.) 03 April 2001, see the entire document.	1-12, 14-16, 21-36, 41-56
A,P	US 6,280,755 B1 (BERGER et al.) 28 August 2001, see the entire document.	1-12, 14-16, 21-36, 41-56
A,P	US 6,331,568 B1 (HORROBIN) 18 December 2001, see the entire document.	1-12, 14-16, 21-36, 41-56
A,P	US 6,365,628 B1 (BERGE) 02 April 2002, see the entire document.	1-12, 14-16, 21-36, 41-56

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search 08 AUGUST 2002	Date of mailing of the international search report 11 SEP 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Paige Brudzer for KEVIN E. WEDDINGTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL02/00294

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.: 13, 17-20, 37-40 and 57-60
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:

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.:

Remark on Protest

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

☐ No protest accompanied the payment of additional search fees.

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Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/092540 A1

(54) **Title:** PRODUCTION AND USE OF A POLAR LIPID-RICH FRACTION CONTAINING OMEGA-3 AND/OR OMEGA-6 HIGHLY UNSATRUATED FATTY ACIDS FROM MICROBES, GENETICALLY MODIFIED PLANT SEEDS AND MARINE ORGANISMS

(57) **Abstract:** The production and use, and in particular, the extraction, separation, synthesis and recovery of polar lipid-rich fractions containing eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA(n-3) or DPA(n-6)), arachidonic acid (ARA), and eicosatetraneonoic acid (C20:4n-3) from microorganisms, genetically modified seeds and marine organisms (including fish and squid) and their use in human food applications, animal feed, pharmaceutical applications and cosmetic applications.

PRODUCTION AND USE OF A POLAR LIPID-RICH FRACTION CONTAINING OMEGA-3 AND/OR OMEGA-6 HIGHLY UNSATURATED FATTY ACIDS FROM MICROBES, GENETICALLY MODIFIED PLANT SEEDS AND MARINE ORGANISMS

FIELD OF THE INVENTION

The present invention relates to the fields of production and use, and in particular, the extraction, separation, synthesis and recovery of polar lipid-rich fractions containing eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA(n-3) or DPA(n-6)), arachidonic acid (ARA), and eicosatetraenoic acid (C20:4n-3) from microorganisms, genetically modified seeds and marine organisms (including fish and squid) and their use in human food applications, animal feed, pharmaceutical applications and cosmetic applications.

BACKGROUND OF THE INVENTION

Highly unsaturated fatty acids of the omega-6 and omega-3 series represent a special class of bioactive lipids in that they are important structurally in membranes in the body, but also participate directly and indirectly in communication between cells through the eicosanoid pathways and by their influence of these fatty acids on gene expression. Six of these fatty acids, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA(n-3) or DPA(n-6)), arachidonic acid (ARA), and eicosatetraenoic acid (C20:4n-3) have been shown to be effective in preventing/treating cardiovascular disease, inflammatory disease, immune function imbalances, fertility and some of these fatty acids (ARA, DHA DPA(n-6) are important structurally in the brain and nervous system. Recent evidence indicates that some highly unsaturated fatty acids may be more bioavailable when supplied in a phospholipid form than in a triglyceride form. EPA, DHA, and ARA have historically been supplied to the nutritional supplement markets in the form of oil extracted from algae or fish. However recent evidence indicates that some polyunsaturated fatty acids may be more bioavailable in a phospholipid form rather than in a triglyceride form. This may be because of the bipolar nature of phospholipids, making them readily solubilizable in the gut and available for digestion and uptake. This same bipolar property of phospholipids additionally would make these fatty acids more functional in topical applications such as creams and lotions or more soluble in aqueous-based applications such as beverages because of there ability

to participate in emulsification processes. We propose that there may be important advantages in supplying these omega-3 and omega-6 HUFAs in the form of phospholipids and improved processes for recovering polar lipids enriched in these fatty acids are also needed.

Examples of polar lipids include phospholipids (e.g. phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidyl-glycerol, diphosphatidylglycerols), cephalins, sphingolipids (sphingomyelins and glycosphingolipids), and glycoglycerolipids. Phospholipids are composed of the following major structural units: fatty acids, glycerol, phosphoric acid, and amino alcohols. They are generally considered to be structural lipids, playing important roles in the structure of the membranes of plants, microbes and animals. Because of their chemical structure, polar lipids exhibit a bipolar nature, exhibiting solubility or partial solubility in both polar and non-polar solvents. The term polar lipid within the present description is not limited to natural polar lipids but also includes chemically modified polar lipids. Although the term oil has various meanings, as used herein, it will refer to the triacylglycerol fraction.

One of the important characteristics of polar lipids, and especially phospholipids, is that they commonly contain polyunsaturated fatty acids (PUFAs: fatty acids with 2 or more unsaturated bonds). In many plant, microbial and animal systems, they are especially enriched in the highly unsaturated fatty acids (HUFAs: fatty acids with 4 or more unsaturated bonds) of the omega-3 and omega-6 series. Although these highly unsaturated fatty acids are considered unstable in triacylglycerol form, they exhibit enhanced stability when incorporated in phospholipids.

The primary sources of commercial PUFA-rich phospholipids are soybeans and canola seeds. These biomaterials do not contain any appreciable amounts of highly unsaturated fatty acids unless they have been genetically modified. The phospholipids (commonly called lecithins) are routinely recovered from these oilseeds as a by-product of the vegetable oil extraction process. For example, in the production of soybean or canola oil, the beans (seeds) are first heat-treated and then crushed, ground, and/or flaked, followed by extraction with a non-polar solvent such as hexane. Hexane removes the triacylglycerol-rich fraction from the seeds together with a varying amount of polar lipids (lecithins). The extracted oil is then de-gummed (lecithin removal) either physically or chemically as a part of the normal oil refining process and the precipitated lecithins

recovered. This process however has two disadvantages: (1) the seeds must be heat-treated before extraction with hexane, increasing the processing cost, and increasing undesirable oxidation reactions and denaturing the protein fraction, thereby decreasing its value as a by-product; and (2) the use of the non-polar solvents such as hexane also presents toxicity and flammability problems that must be dealt with.

The crude lecithin extracted in the "de-gumming" process can contain up to about 33% oil (triacylglycerols) along with sterols and glucosides. One preferred method for separating this oil from the crude lecithin is by extraction with acetone. The oil (triacylglycerols) is soluble in acetone and the lecithin is not. The acetone solution is separated from the precipitate (lecithin) by centrifugation and the precipitate dried under first a fluidized bed drier and then a vacuum drying oven to recover the residual acetone as the product is dried. Drying temperatures of 50-70°C are commonly used. The resulting dried lecithins contain approximately 2-4% by weight of oil (triacylglycerols). Process temperatures above 70°C can lead to thermal decomposition of the phospholipids. However, even at temperatures below 70°C the presence of acetone leads to the formation of products that can impair the organoleptic quality of the phospholipids. These by-products can impart musty odors to the product and also a pungent aftertaste.

What is needed is an improved process for effectively recovering polar lipids and phospholipids rich in omega-3 and omega-6 HUFAs from biomaterials that enables the use of these fatty acid in food, nutritional supplement, pharmaceutical and cosmetic applications. Furthermore the fractions are needed as an ingredient in feed for companion animals and in aquaculture.

SUMMARY OF THE INVENTION

In accordance with the present invention, an improved process is provided for recovering polar lipids enriched in omega-3 and/or omega-6 HUFAs from native biomaterials such as seeds and microorganisms and the use thereof.

In one embodiment of the present invention, a method is provided for providing a human, animal or aquaculture organism diet supplement enriched with at least one of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (n-3)(DPAn-3), eicosatetraenoic acid (n-3), docosapentaenoic acid (n-6)(DPAn-6) or arachidonic acid (ARA). The method includes the steps of producing a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic

acid and ARA from genetically modified seeds or marine animals; and providing the polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA in a form consumable or usable by humans or animals. Preferably, the animal is a companion animal.

In another embodiment of the present invention, a method is provided for treating a deficiency in at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The method includes the steps of producing a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and providing the polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA to treat the deficiency. The deficiency can result in an inflammatory condition, an immune system imbalance, a cardiovascular disease, a developmental deficit related to nervous system development, a woman's health condition or an infant's health condition.

In another embodiment of the present invention, a method is provided for treating a chronic inflammatory disease state of the lung. The method includes the steps of producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; blending the phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one of EPA-, GLA- or SDA-rich oils; and producing an aerosol comprising the blend for the treatment of the disease states. The chronic inflammatory disease state of the lung can result in chronic obstructive pulmonary disease (COPD), asthma or cystic fibrosis.

In another embodiment of the present invention, a method is provided for the treatment of skin lesions, induced burn, UV-irradiation or other skin disorders. The method includes the steps of producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; blending the phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one EPA-, GLA- or SDA-rich oil; and producing a lotion or cream for the treatment of the skin disorders.

In another embodiment of the present invention, a method is provided for treating cachexia and severe fat malabsorption. The method includes the steps of producing a purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA; blending the purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one other purified phospholipid; blending the purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one DHA, EPA, GLA- or SDA-rich oil; and producing a liquid or dried dietetic product for the treatment of the disease states. The cachexia or severe fat malabsorption can be a result of cancer or Crohn's disease. Preferably, the at least one other purified phospholipid is obtained from the group consisting of soy, rape seed, evening primrose, safflower, sunflower, canola, peanut, egg and mixtures thereof.

In another embodiment of the present invention, a method is provided for the treatment of *H. pylori*-infection of gastrointestinal tract. The method includes the steps of producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; blending the phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one EPA-, GLA- or SDA-rich oil; and producing a fat emulsion or a dietetic product for the treatment of the disease.

In another embodiment of the present invention, a method is provided for providing a fat blend enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The method includes the steps of extracting a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and mixing the polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with another oil. Preferably, the another oil is selected from the group consisting of fish oil, microbial oil, vegetable oil, GLA-containing oil, SDA-containing oil or mixtures thereof.

In another embodiment of the present invention, a method is provided for providing a blend of polar lipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The method includes the steps of extracting a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6),

eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and mixing the polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with another polar lipid. Preferably, the another polar lipid is selected from the group consisting of soy polar lipids, rapeseed polar lipids, sunflower polar lipids, safflower polar lipids, canola polar lipids, peanut polar lipids or egg yolk polar lipids and mixtures thereof.

In another embodiment of the present invention, a fat blend is provided enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The fat blend includes a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and another oil. Preferably, the another oil is selected from the group consisting of fish oil, microbial oil, vegetable oil, GLA-containing oil, SDA-containing oil and mixtures thereof.

In another embodiment of the present invention, a method is provided for producing a blend of polar lipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The method includes the steps of extracting an EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched polar lipid-rich fraction from microbes, seeds or marine animals; and mixing the EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched polar lipid-rich fraction with another polar lipid. Preferably, the another polar lipid is selected from the group consisting of soy polar lipids, rapeseed polar lipids, sunflower polar lipids, safflower polar lipids, canola polar lipids, peanut polar lipids or egg yolk polar lipids and mixtures thereof.

In another embodiment of the present invention, purified phospholipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA derived from polar lipid-rich fraction extracted from genetically modified seeds or marine animals are provided. Preferably, the EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched phospholipid-fraction is in a form consumable or usable by humans or animals.

In another embodiment of the present invention, a method is provided for providing a human, animal or aquaculture organism diet supplement enriched with at least one of eicosapentaenoic acid (EPA), docosapentaenoic acid (n-3)(DPAn-3), eicosatetraenoic acid (n-3) or docosapentaenoic acid (n-6)(DPAn-6). The method includes the steps of producing a polar lipid-rich fraction enriched with at least one of

EPA, DPA(n-3), DPA(n-6) and eicosatetraenoic acid from microbes, genetically modified seeds or marine animals; and providing the polar lipid-rich fraction enriched with at least one of EPA, DPA(n-3), DPA(n-6) and eicosatetraenoic acid in a form consumable or usable by humans or animals.

The polar lipid-rich fraction of the methods or products of can be provided as an ingredient of dietetic, pharmaceutical and cosmetic applications.

The fat blend of the methods or products of the present invention can be provided as an ingredient of dietetic, pharmaceutical and cosmetic applications.

The blend of polar lipids of the methods or products of the present invention can be provided as an ingredient of dietetic, pharmaceutical and cosmetic applications.

The purified phospholipids of the methods or products of the present invention can be provided as an ingredient of dietetic, pharmaceutical and cosmetic applications.

Preferably, the marine animals of the methods and products of the present invention are fish, squid, mollusks or shrimp. Preferably, the marine animals are fish or fish eggs from the group including salmon, tuna, haddock, sardines, mackerel, or menhaden.

Preferably, the microbes of the methods and products of the present invention are selected from fungi, microalgae, protozoa or bacteria. More preferably, microbes are selected from the Stramenopiles, Thraustochytriales, Chrysophyceae, Xanthophyceae, Bacillariophyceae, Dinophyceae, Phaeophyceae, Rhodophyceae, Chlorophyceae, Euglenophyceae, Cryptophyceae, Oomycetes, Chytridomycetes, or Zygomycetes. More preferably, the microbes are selected from the group of genera consisting of Mortierella, Mucor, Phycomyces, Rhizopus, Pythium, Ochromonas, Nitzschia, Phaeodactylum, Skeletonema, Fucus, Laminaria, Platymonas, Achyla, Phytophera, Schizochytrium, Thraustochytrium, or Crypthecodinium.

Preferably, the EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid, ARA or mixture thereof employed in the methods and products of the present invention makes up at least two weight percent of the total fatty acids of the polar lipid fraction.

Preferably, the EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid, ARA or mixture thereof employed in the methods and products at the present invention makes up at least five weight percent of the total fatty acids of the polar lipid fraction.

Preferably, the plant seeds or microbes employed in the methods and products to the present invention have been genetically modified to increase their n-3 or n-6 HUFA content.

Preferably, the seeds or microbes used in the methods and products of the present invention have been genetically modified to increase the production of at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA.

Preferably, the seeds employed in the methods and products of the present invention are selected from the group consisting of canola, rapeseed, linseed, flaxseed, sunflower, safflower, peanuts, soybeans or corn. Preferably, the polar lipid-rich fraction is extracted from the seeds and microbes using alcohol.

In an alternative embodiment of the present invention, the polar lipid-rich fraction is derived as a by-product (e.g., by de-gumming) of oil extraction from the seeds using hexane and other non-polar solvents.

Preferably, the polar lipid-rich fraction used in the methods and products of the present invention is extracted from the seeds and microbes by use of gravity or centrifugal extraction technology.

DETAILED DESCRIPTION OF THE INVENTION

Because of their bipolar nature, polar lipids (including phospholipids) are of significant commercial interest as wetting and emulsifying agents. These properties may also help make HUFAs in the phospholipids more bioavailable, in addition to enhancing their stability. These properties make phospholipids ideal forms of ingredients for use in nutritional supplements, food, infant formula, pharmaceutical, and cosmetic applications. Dietary benefits of phospholipids include both improved absorption and improved incorporation. Phospholipids also have a broad range of functionality in the body in that they are important cell membrane constituents, they are good emulsifiers, they can act as intestinal surfactants, serve as a choline source and as a source of HUFAs.

EPA, DHA, and ARA are normally produced for the nutritional supplement market and food uses through the extraction (by cooking) of fish (EPA and DHA) such as menhaden, tuna or salmon, or hexane extraction of fungal biomass (ARA) from the genus Mortierella. The phospholipids in both processes are removed in a later degumming step that produces a waste material comprising a complex mixture of neutral lipids, sterols,

glucosides and phospholipids. This material is normally sold to the domestic animal feed industry to dispose of it.

Besides fish and fungal biomass, there are microbial sources of DHA and DPA(n-6). For the present invention, useful microbes can be selected from fungi, microalgae, protozoa or bacteria. These organisms can be selected from the groups including the Stramenopiles, Thraustochytriales, Chrysophyceae, Xanthophyceae, Bacillariophyceae, Dinophyceae, Phaeophyceae, Rhodophyceae, Chlorophyceae, Euglenophyceae, Cryptophyceae, Oomycetes, Chytridomycetes, or Zygomycetes. Useful microbes can also be selected from the group of genera consisting of Mortierella, Mucor, Phycomyces, Rhizopus, Pythium, Ochromonas, Nitzschia, Phaeodactylum, Skeletonema, Fucus, Platymonas, Achyla, Phytophera, Schizochytrium, Thraustochytrium, or Crypthecodinium. Microorganisms are good sources of phospholipids because they can be grown in culture in a manner that optimizes phospholipid production and minimizes triglyceride (oil) production. On the other hand the methods used in this invention allow both oil and phospholipids to be recovered separately in forms that can be used directly in food, feed, nutritional supplements, cosmetic or pharmaceutical application.

DHA, EPA and ARA phospholipids can be recovered from fish, microalgae, or fungi through the degumming process described above. However as noted this produces a complex material containing many other compounds including neutral lipids, sterols, glucosides, etc

A preferred embodiment of the present invention is to use alcohol and centrifugation to recover the omega-3 and/or omega-6 HUFA-rich phospholipids. Preferred methods for this recovery are described in the following references, which are incorporated by reference herein in their entirety:

i. PCT Application Serial No. PCT/US01/12047, entitled "Method for the Fractionation of Oil and Polar Lipid-Containing Native Raw Materials" filed April 12, 2001;

ii. PCT Application Serial No. PCT/US01/12049, entitled "Method For The Fractionation Of Oil And Polar Lipid-Containing Native Raw Materials Using Water-Soluble Organic Solvent And Centrifugation" filed April 12, 2001.

Once the omega-3 and/omega-6 rich phospholipid fractions have been extracted by these preferred processes, they can be used directly as ingredients or they can be purified further and even separated into phospholipid classes by well-known techniques

such as different forms of chromatography, molecular distillation, and special refining techniques. Polar lipid groups phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and sphingolipids. The phospholipid rich polar lipids or the purified phospholipid rich fractions can also be mixed with another lipid or oil such as fish lipids, microbial lipids, vegetable lipids, GLA-containing lipids, SDA-containing lipids and mixtures thereof, or be mixed with another phospholipid fraction (lecithin) such as soy or egg yolk lecithin, sunflower lecithin, peanut lecithin or mixtures thereof prior to use as a nutritional supplement, feed or food ingredient. These mixtures of phospholipids can also be incorporated into creams or lotions for topical applications (e.g. treating of skin conditions) or skin lesions induced by burns, UV-irradiation or other skin damaging processes. The mixtures can also be processed to produce a liquid or spray-dried dietetic product or fat emulsion for treating cachexia and severe fat malabsorption or for treatment of *H. pylori* infection of the gastrointestinal tract or incorporated into aerosol sprays for treating chronic inflammatory disease states of the lung (e.g., COPD, asthma, cystic fibrosis).

Advantages of the present invention including providing omega-3 and/or omega-6 HUFAs in a more bioactive and functional form (phospholipid) than the triglyceride form and include a better process (e.g., a) no need for heat treatment; b) no use of toxic solvents (like hexane) and c) no artifacts and off-flavors due to the use of acetone) for recovering these phospholipids from oilseeds and microbes. The EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and/or ARA content of the total fatty acids of the polar lipid fractions and comprise at least two weight percent of the total fatty acids of the polar lipid fraction, more preferably greater than 5 weight percent, more preferably greater than 10 weight percent, and most preferably greater than 20 weight percent of total fatty acids in the polar lipid fraction.

EXAMPLE

Example 1

Phospholipids were extracted from salmon, salmon roe, Black tiger prawns, squid, Schizochytrium sp, and the total fatty acid content of the phospholipids was determined by gas chromatography. The results are presented in Table 1. As can be observed the phospholipid fraction of these biomaterials can be used to deliver omega-3 and/or omega-

6 HUFAs and in this form these bioactive fatty acids should be more stable, more bioavailable, and more functional.

Table 1. Total fatty acid content of phospholipids extracted from 4 types marine animal products and forma microbial source.

		Salmon	Salmon roe	Tiger Prawn	Squid	Schizochy-trium
COMPOUND		PL's	PL's	PL's	PL's	PL's
		% TFA	% TFA	%TFA	% TFA	% TFA
MYRISTATE	C14:0	2.99	4.58	1.14	1.87	2.66
MYRISTOLEATE	C14:1	0,00	0,00	0.05	0.03	0,00
PALMITATE	C16:0	26.48	29.661	25.44	30.37	28.25
PALMITOLEATE	C16:1	2.86	4.38	1.55	0.23	0.62
STEARATE	C18:0	5.74	6.19	13.59	4.41	1.75
OLEATE	C18:1	9.74	13.36	13.33	1.86	6.99
LINOLEATE	C18:2n6	3.14	1.05	12.51	0.32	2.46
GAMMA LINOLENATE	C18:3n6	0.00	0.00	0.00	0.00	1.09
ARACHIDATE	C20:0	0.00	0.00	0.74	0.13	0,00
LINOLENATE	C18:3n3	0.49	0.22	0.54	0.05	0.00
OCTADECATETRAENO-ATE	C18:4	0.50	0.33	0.08	0.07	0.00
EICOSENOATE-11	C20:1	0,75	1.07	0.85	5.54	0.00
EICOSADIENOATE-11,14	C20:2	0,00	0.00	0.56	0.34	0,00
BEHENATE	C22:0	0,00	0,00	1.35	0.08	0,00
EICOSATRIENOATE	C20:3n3	1.01	3.58	0.07	0.42	1.61
ARACHIDONATE	C20:4 n6	0,89	0.56	5.54	1.70	1.18
ERUCATE	C22:1	0,21	0,00	0.04	1.41	0.00
EICOSAPENTAENOATE	C20:5n3	9.50	9.39	8.47	14.71	5.11
LIGNOCERATE	C24:0	0,00	0,00	0.56	0.00	0.00
NERVONATE	C24:1	1.61	1.65	0.73	0.82	0,00
DOCOSAPENTAENO-ATE n-6	C22:5n6	0,00	0,00	0.45	0.22	17.68
DOCOSAPENTAENO-ATE n-3	C22:5n3	3.16	4.40	0.55	0.44	0,00
DOCOSAHEXAENOATE	C22:6n3	30.92	19.50	11.86	35.54	30.61
		100,00	100,00	100.00	100,00	100,00

The present invention, in various embodiments, includes components, methods, processes, systems and/or apparatus substantially as depicted and described herein, including various embodiments, subcombinations, and subsets thereof. Those of skill in the art will understand how to make and use the present invention after understanding the present disclosure. The present invention, in various embodiments, includes providing devices and processes in the absence of items not depicted and/or described herein or in various embodiments hereof, including in the absence of such items as may have been

used in previous devices or processes, *e.g.*, for improving performance, achieving ease and/or reducing cost of implementation.

The foregoing discussion of the invention has been presented for purposes of illustration and description. The foregoing is not intended to limit the invention to the form or forms disclosed herein. Although the description of the invention has included description of one or more embodiments and certain variations and modifications, other variations and modifications are within the scope of the invention, *e.g.*, as may be within the skill and knowledge of those in the art, after understanding the present disclosure. It is intended to obtain rights which include alternative embodiments to the extent permitted, including alternate, interchangeable and/or equivalent structures, functions, ranges or steps to those Claimed, whether or not such alternate, interchangeable and/or equivalent structures, functions, ranges or steps are disclosed herein, and without intending to publicly dedicate any patentable subject matter.

We claim:

1. A method for providing a human, animal or aquaculture organism diet supplement enriched with at least one of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (n-3)(DPAn-3), eicosatetraenoic acid (n-3), docosapentaenoic acid (n-6)(DPAn-6) or arachidonic acid (ARA) comprising the steps:

(a) producing a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from genetically modified seeds or marine animals; and

(b) providing said polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA in a form consumable or usable by humans or animals.

2. The method of Claim 1, wherein the animal is a companion animal.

3. A method for treating a deficiency in at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising the steps:

(a) producing a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and

(b) providing said polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA to treat said deficiency.

4. The method of Claim 3, wherein said deficiency results in an inflammatory condition, an immune system imbalance, a cardiovascular disease, a developmental deficit related to nervous system development, a woman's health condition or an infant's health condition.

5. A method for treating a chronic inflammatory disease state of the lung comprising the steps:

(a) producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals;

(b) blending said phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one of EPA-, GLA- or SDA-rich oils; and

(c) producing an aerosol comprising the blend of step (b) for the treatment of said disease states.

6. The method of claim 5, wherein the chronic inflammatory disease state of the lung is COPD, asthma or cystic fibrosis.

7. A method for the treatment of skin lesions, induced burn, UV-irradiation or other skin disorders comprising the steps:

(a) producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals;

(b) blending said phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one EPA-, GLA- or SDA-rich oil; and

(c) producing a lotion or cream for the treatment of said skin disorders.

8. A method for treating cachexia or fat malabsorption comprising the steps:

(a) producing a purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA;

(b) blending said purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one other purified phospholipid;

(c) blending said purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one DHA, EPA, GLA- or SDA-rich oil; and

(d) producing a liquid or dried dietetic product for the treatment of said disease states.

9. The method of Claim 8, wherein the cachexia or fat malabsorption is a result of cancer or Crohn's disease.

10. The method of Claim 8, wherein the at least one other purified phospholipid is obtained from the group consisting of soy, rape seed, evening primrose, safflower, sunflower, canola, peanut, egg and mixtures thereof.

11. A method for the treatment of *H. pylori*-infection of gastrointestinal tract comprising the steps:

(a) producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals;

(b) blending said phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one EPA-, GLA- or SDA-rich oil; and

(c) producing a fat emulsion or a dietetic product for the treatment of said disease.

12. A method for providing a fat blend enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising the steps:

(a) extracting a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and

(b) mixing said polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with another oil.

13. The method of Claim 12, wherein said another oil is selected from the group consisting of fish oil, microbial oil, vegetable oil, GLA-containing oil, SDA-containing oil or mixtures thereof.

14. A method for providing a blend of polar lipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising the steps:

(a) extracting a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and

(b) mixing said polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with another polar lipid.

15. The method of Claim 14, wherein said another polar lipid is selected from the group consisting of soy polar lipids, rape seed polar lipids, sunflower polar lipids, safflower polar lipids, canola polar lipids, peanut polar lipids or egg yolk polar lipids and mixtures thereof.

16. A fat blend enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising:

(a) a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and

(b) another oil.

17. The oil of Claim 16, wherein said another oil is selected from the group consisting of fish oil, microbial oil, vegetable oil, GLA-containing oil, SDA-containing oil and mixtures thereof.

18. A blend of polar lipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising the steps:

(a) extracting a EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched polar lipid-rich fraction from microbes, seeds or marine animals; and

(b) mixing said EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched polar lipid-rich fraction with another polar lipid.

19. The method of Claim 18, wherein said another polar lipid is selected from the group consisting of soy polar lipids, rape seed polar lipids, sunflower polar lipids, safflower polar lipids, canola polar lipids, peanut polar lipids or egg yolk polar lipids and mixtures thereof.

20. Purified phospholipids enriched with at least one EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA derived from polar lipid-rich fraction extracted from genetically modified seeds or marine animals.

21. The purified phospholipids of Claim 20, wherein said EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched phospholipid-fraction is in a form consumable or usable by humans or animals.

22. A method for providing a human, animal or aquaculture organism diet supplement enriched with at least one of eicosapentaenoic acid (EPA), docosapentaenoic acid (n-3)(DPAn-3), eicosatetraenoic acid (n-3) or docosapentaenoic acid (n-6)(DPAn-6) comprising the steps:

(a) producing a polar lipid-rich fraction enriched with at least one of EPA, DPA(n-3), DPA(n-6) and eicosatetraenoic acid from microbes, genetically modified seeds or marine animals; and

(b) providing the polar lipid-rich fraction enriched with at least one of EPA, DPA(n-3), DPA(n-6) and eicosatetraenoic acid in a form consumable or usable by humans or animals.

23. A polar lipid-rich fraction of the method or product of any preceding Claim as an ingredient of dietetic, pharmaceutical or cosmetic applications.

24. A fat blend of the method or product of any preceding Claim as an ingredient of dietetic, pharmaceutical or cosmetic applications.

25. A blend of polar lipids of the method or product of any preceding Claim as an ingredient of dietetic, pharmaceutical or cosmetic applications.

26. Purified phospholipids of the method or product of any preceding Claim as an ingredient of dietetic, pharmaceutical or cosmetic applications.

27. The method or product of any preceding Claim, wherein said marine animals are fish, squid, mollusks or shrimp.

28. The method or product of any preceding Claim, wherein said marine animals are fish or fish eggs from the group including salmon, tuna, haddock, sardines, mackerel, or menhaden.

29. The method or product of any preceding Claim, wherein said microbes are selected from fungi, microalgae, protozoa or bacteria.

30. The method or product of any preceding Claim, wherein said microbes are selected from the Stramenopiles, Thraustochytriales, Chrysophyceae, Xanthophyceae, Bacillariophyceae, Dinophyceae, Phaeophyceae, Rhodophyceae, Chlorophyceae, Euglenophyceae, Cryptophyceae, Oomycetes, Chytridomycetes, or Zygomycetes.

31. The method or product of any preceding Claim, wherein said microbes are selected from the group of genera consisting of Mortierella, Mucor, Phycomyces, Rhizopus, Pythium, Ochromonas, Nitzschia, Phaeodactylum, Skeletonema, Fucus, Laminaria, Platymonas, Achyla, Phytophera, Schizochytrium, Thraustochytrium, or Crypthecodinium.

32. The method of or product of any preceding Claim, wherein EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid, ARA or mixture thereof comprises at least two weight percent of the total fatty acids of the polar lipid fraction.

33. The method or product of any preceding Claim, wherein EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid, ARA or mixture thereof comprises at least five weight percent of the total fatty acids of the polar lipid fraction.

34. The method or product of any preceding Claim, wherein said plant seeds or microbes have been genetically modified to increase their n-3 or n-6 HUFA content.

35. The method or product of any preceding Claim, wherein said seeds or microbes have been genetically modified to increase the production of at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA.

36. The method or product of any preceding Claim, wherein said seeds are selected from the group consisting of canola, rape seed, linseed, flaxseed, sunflower, safflower, peanuts, soybeans or corn.

37. The method or product of any preceding Claim, wherein said polar lipid-rich fraction is extracted from said seeds and microbes using alcohol.

38. The method or product of any preceding Claim, wherein said polar lipid-rich fraction is derived as a by-product of oil extraction from said seeds using hexane or other non-polar solvent.

39. The method or product of any preceding Claim, wherein said polar lipid-rich fraction is extracted from said seeds and microbes by use of gravity or centrifugal extraction technology.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/15454

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07C 1/100
US CL :554/8, 20, 224; 514/821, 824; 424/450
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. : 554/8, 20, 224; 514/821, 824; 424/450

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)

CAPLUS, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,539,133 A (KOHN et al.) 23 July 1996. see entire document.	1-39
Y	US 4,816,271 A (SCAFFIDI) 28 March 1989. SEE ENTIRE DOCUMENT.	1-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
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(54) Title: PRODUCTION AND USE OF A POLAR LIPID-RICH FRACTION CONTAINING OMEGA-3 AND/OR OMEGA-6 HIGHLY UNSATRUATED FATTY ACIDS FROM MICROBES, GENETICALLY MODIFIED PLANT SEEDS AND MARINE ORGANISMS

(57) Abstract: The production and use, and in particular, the extraction, separation, synthesis and recovery of polar lipid-rich fractions containing eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA(n-3) or DPA(n-6)), arachidonic acid (ARA), and eicosatetraneonoic acid (C20:4n-3) from microorganisms, genetically modified seeds and marine organisms (including fish and squid) and their use in human food applications, animal feed, pharmaceutical applications and cosmetic applications.

PRODUCTION AND USE OF A POLAR LIPID-RICH FRACTION CONTAINING OMEGA-3 AND/OR OMEGA-6 HIGHLY UNSATURATED FATTY ACIDS FROM MICROBES, GENETICALLY MODIFIED PLANT SEEDS AND MARINE ORGANISMS

FIELD OF THE INVENTION

The present invention relates to the fields of production and use, and in particular, the extraction, separation, synthesis and recovery of polar lipid-rich fractions containing eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA(n-3) or DPA(n-6)), arachidonic acid (ARA), and eicosatetraenoic acid (C20:4n-3) from microorganisms, genetically modified seeds and marine organisms (including fish and squid) and their use in human food applications, animal feed, pharmaceutical applications and cosmetic applications.

BACKGROUND OF THE INVENTION

Highly unsaturated fatty acids of the omega-6 and omega-3 series represent a special class of bioactive lipids in that they are important structurally in membranes in the body, but also participate directly and indirectly in communication between cells through the eicosanoid pathways and by their influence of these fatty acids on gene expression. Six of these fatty acids, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA(n-3) or DPA(n-6)), arachidonic acid (ARA), and eicosatetraenoic acid (C20:4n-3) have been shown to be effective in preventing/treating cardiovascular disease, inflammatory disease, immune function imbalances, fertility and some of these fatty acids (ARA, DHA DPA(n-6) are important structurally in the brain and nervous system. Recent evidence indicates that some highly unsaturated fatty acids may be more bioavailable when supplied in a phospholipid form than in a triglyceride form. EPA, DHA, and ARA have historically been supplied to the nutritional supplement markets in the form of oil extracted from algae or fish. However recent evidence indicates that some polyunsaturated fatty acids may be more bioavailable in a phospholipid form rather than in a triglyceride form. This may be because of the bipolar nature of phospholipids, making them readily solubilizable in the gut and available for digestion and uptake. This same bipolar property of phospholipids additionally would make these fatty acids more functional in topical applications such as creams and lotions or more soluble in aqueous-based applications such as beverages because of there ability

to participate in emulsification processes. We propose that there may be important advantages in supplying these omega-3 and omega-6 HUFAs in the form of phospholipids and improved processes for recovering polar lipids enriched in these fatty acids are also needed.

Examples of polar lipids include phospholipids (e.g. phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidyl-glycerol, diphosphatidylglycerols), cephalins, sphingolipids (sphingomyelins and glycosphingolipids), and glycoglycerolipids. Phospholipids are composed of the following major structural units: fatty acids, glycerol, phosphoric acid, and amino alcohols. They are generally considered to be structural lipids, playing important roles in the structure of the membranes of plants, microbes and animals. Because of their chemical structure, polar lipids exhibit a bipolar nature, exhibiting solubility or partial solubility in both polar and non-polar solvents. The term polar lipid within the present description is not limited to natural polar lipids but also includes chemically modified polar lipids. Although the term oil has various meanings, as used herein, it will refer to the triacylglycerol fraction.

One of the important characteristics of polar lipids, and especially phospholipids, is that they commonly contain polyunsaturated fatty acids (PUFAs: fatty acids with 2 or more unsaturated bonds). In many plant, microbial and animal systems, they are especially enriched in the highly unsaturated fatty acids (HUFAs: fatty acids with 4 or more unsaturated bonds) of the omega-3 and omega-6 series. Although these highly unsaturated fatty acids are considered unstable in triacylglycerol form, they exhibit enhanced stability when incorporated in phospholipids.

The primary sources of commercial PUFA-rich phospholipids are soybeans and canola seeds. These biomaterials do not contain any appreciable amounts of highly unsaturated fatty acids unless they have been genetically modified. The phospholipids (commonly called lecithins) are routinely recovered from these oilseeds as a by-product of the vegetable oil extraction process. For example, in the production of soybean or canola oil, the beans (seeds) are first heat-treated and then crushed, ground, and/or flaked, followed by extraction with a non-polar solvent such as hexane. Hexane removes the triacylglycerol-rich fraction from the seeds together with a varying amount of polar lipids (lecithins). The extracted oil is then de-gummed (lecithin removal) either physically or chemically as a part of the normal oil refining process and the precipitated lecithins

recovered. This process however has two disadvantages: (1) the seeds must be heat-treated before extraction with hexane, increasing the processing cost, and increasing undesirable oxidation reactions and denaturing the protein fraction, thereby decreasing its value as a by-product; and (2) the use of the non-polar solvents such as hexane also presents toxicity and flammability problems that must be dealt with.

The crude lecithin extracted in the "de-gumming" process can contain up to about 33% oil (triacylglycerols) along with sterols and glucosides. One preferred method for separating this oil from the crude lecithin is by extraction with acetone. The oil (triacylglycerols) is soluble in acetone and the lecithin is not. The acetone solution is separated from the precipitate (lecithin) by centrifugation and the precipitate dried under first a fluidized bed drier and then a vacuum drying oven to recover the residual acetone as the product is dried. Drying temperatures of 50-70°C are commonly used. The resulting dried lecithins contain approximately 2-4% by weight of oil (triacylglycerols). Process temperatures above 70°C can lead to thermal decomposition of the phospholipids. However, even at temperatures below 70°C the presence of acetone leads to the formation of products that can impair the organoleptic quality of the phospholipids. These by-products can impart musty odors to the product and also a pungent aftertaste.

What is needed is an improved process for effectively recovering polar lipids and phospholipids rich in omega-3 and omega-6 HUFAs from biomaterials that enables the use of these fatty acid in food, nutritional supplement, pharmaceutical and cosmetic applications. Furthermore the fractions are needed as an ingredient in feed for companion animals and in aquaculture.

SUMMARY OF THE INVENTION

In accordance with the present invention, an improved process is provided for recovering polar lipids enriched in omega-3 and/or omega-6 HUFAs from native biomaterials such as seeds and microorganisms and the use thereof.

In one embodiment of the present invention, a method is provided for providing a human, animal or aquaculture organism diet supplement enriched with at least one of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (n-3)(DPAn-3), eicosatetraenoic acid (n-3), docosapentaenoic acid (n-6)(DPAn-6) or arachidonic acid (ARA). The method includes the steps of producing a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic

acid and ARA from genetically modified seeds or marine animals; and providing the polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA in a form consumable or usable by humans or animals. Preferably, the animal is a companion animal.

In another embodiment of the present invention, a method is provided for treating a deficiency in at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The method includes the steps of producing a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and providing the polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA to treat the deficiency. The deficiency can result in an inflammatory condition, an immune system imbalance, a cardiovascular disease, a developmental deficit related to nervous system development, a woman's health condition or an infant's health condition.

In another embodiment of the present invention, a method is provided for treating a chronic inflammatory disease state of the lung. The method includes the steps of producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; blending the phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one of EPA-, GLA- or SDA-rich oils; and producing an aerosol comprising the blend for the treatment of the disease states. The chronic inflammatory disease state of the lung can result in chronic obstructive pulmonary disease (COPD), asthma or cystic fibrosis.

In another embodiment of the present invention, a method is provided for the treatment of skin lesions, induced burn, UV-irradiation or other skin disorders. The method includes the steps of producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; blending the phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one EPA-, GLA- or SDA-rich oil; and producing a lotion or cream for the treatment of the skin disorders.

In another embodiment of the present invention, a method is provided for treating cachexia and severe fat malabsorption. The method includes the steps of producing a purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA; blending the purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one other purified phospholipid; blending the purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one DHA, EPA, GLA- or SDA-rich oil; and producing a liquid or dried dietetic product for the treatment of the disease states. The cachexia or severe fat malabsorption can be a result of cancer or Crohn's disease. Preferably, the at least one other purified phospholipid is obtained from the group consisting of soy, rape seed, evening primrose, safflower, sunflower, canola, peanut, egg and mixtures thereof.

In another embodiment of the present invention, a method is provided for the treatment of *H. pylori*-infection of gastrointestinal tract. The method includes the steps of producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; blending the phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one EPA-, GLA- or SDA-rich oil; and producing a fat emulsion or a dietetic product for the treatment of the disease.

In another embodiment of the present invention, a method is provided for providing a fat blend enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The method includes the steps of extracting a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and mixing the polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with another oil. Preferably, the another oil is selected from the group consisting of fish oil, microbial oil, vegetable oil, GLA-containing oil, SDA-containing oil or mixtures thereof.

In another embodiment of the present invention, a method is provided for providing a blend of polar lipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The method includes the steps of extracting a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6),

eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and mixing the polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with another polar lipid. Preferably, the another polar lipid is selected from the group consisting of soy polar lipids, rapeseed polar lipids, sunflower polar lipids, safflower polar lipids, canola polar lipids, peanut polar lipids or egg yolk polar lipids and mixtures thereof.

In another embodiment of the present invention, a fat blend is provided enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The fat blend includes a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and another oil. Preferably, the another oil is selected from the group consisting of fish oil, microbial oil, vegetable oil, GLA-containing oil, SDA-containing oil and mixtures thereof.

In another embodiment of the present invention, a method is provided for producing a blend of polar lipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA. The method includes the steps of extracting an EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched polar lipid-rich fraction from microbes, seeds or marine animals; and mixing the EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched polar lipid-rich fraction with another polar lipid. Preferably, the another polar lipid is selected from the group consisting of soy polar lipids, rapeseed polar lipids, sunflower polar lipids, safflower polar lipids, canola polar lipids, peanut polar lipids or egg yolk polar lipids and mixtures thereof.

In another embodiment of the present invention, purified phospholipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA derived from polar lipid-rich fraction extracted from genetically modified seeds or marine animals are provided. Preferably, the EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched phospholipid-fraction is in a form consumable or usable by humans or animals.

In another embodiment of the present invention, a method is provided for providing a human, animal or aquaculture organism diet supplement enriched with at least one of eicosapentaenoic acid (EPA), docosapentaenoic acid (n-3)(DPAn-3), eicosatetraenoic acid (n-3) or docosapentaenoic acid (n-6)(DPAn-6). The method includes the steps of producing a polar lipid-rich fraction enriched with at least one of

EPA, DPA(n-3), DPA(n-6) and eicosatetraenoic acid from microbes, genetically modified seeds or marine animals; and providing the polar lipid-rich fraction enriched with at least one of EPA, DPA(n-3), DPA(n-6) and eicosatetraenoic acid in a form consumable or usable by humans or animals.

The polar lipid-rich fraction of the methods or products of can be provided as an ingredient of dietetic, pharmaceutical and cosmetic applications.

The fat blend of the methods or products of the present invention can be provided as an ingredient of dietetic, pharmaceutical and cosmetic applications.

The blend of polar lipids of the methods or products of the present invention can be provided as an ingredient of dietetic, pharmaceutical and cosmetic applications.

The purified phospholipids of the methods or products of the present invention can be provided as an ingredient of dietetic, pharmaceutical and cosmetic applications.

Preferably, the marine animals of the methods and products of the present invention are fish, squid, mollusks or shrimp. Preferably, the marine animals are fish or fish eggs from the group including salmon, tuna, haddock, sardines, mackerel, or menhaden.

Preferably, the microbes of the methods and products of the present invention are selected from fungi, microalgae, protozoa or bacteria. More preferably, microbes are selected from the Stramenopiles, Thraustochytriales, Chrysophyceae, Xanthophyceae, Bacillariophyceae, Dinophyceae, Phaeophyceae, Rhodophyceae, Chlorophyceae, Euglenophyceae, Cryptophyceae, Oomycetes, Chytridomycetes, or Zygomycetes. More preferably, the microbes are selected from the group of genera consisting of Mortierella, Mucor, Phycomyces, Rhizopus, Pythium, Ochromonas, Nitzschia, Phaeodactylum, Skeletonema, Fucus, Laminaria, Platymonas, Achyla, Phytophera, Schizochytrium, Thraustochytrium, or Crypthecodinium.

Preferably, the EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid, ARA or mixture thereof employed in the methods and products of the present invention makes up at least two weight percent of the total fatty acids of the polar lipid fraction.

Preferably, the EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid, ARA or mixture thereof employed in the methods and products at the present invention makes up at least five weight percent of the total fatty acids of the polar lipid fraction.

Preferably, the plant seeds or microbes employed in the methods and products to the present invention have been genetically modified to increase their n-3 or n-6 HUFA content.

Preferably, the seeds or microbes used in the methods and products of the present invention have been genetically modified to increase the production of at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA.

Preferably, the seeds employed in the methods and products of the present invention are selected from the group consisting of canola, rapeseed, linseed, flaxseed, sunflower, safflower, peanuts, soybeans or corn. Preferably, the polar lipid-rich fraction is extracted from the seeds and microbes using alcohol.

In an alternative embodiment of the present invention, the polar lipid-rich fraction is derived as a by-product (e.g., by de-gumming) of oil extraction from the seeds using hexane and other non-polar solvents.

Preferably, the polar lipid-rich fraction used in the methods and products of the present invention is extracted from the seeds and microbes by use of gravity or centrifugal extraction technology.

DETAILED DESCRIPTION OF THE INVENTION

Because of their bipolar nature, polar lipids (including phospholipids) are of significant commercial interest as wetting and emulsifying agents. These properties may also help make HUFAs in the phospholipids more bioavailable, in addition to enhancing their stability. These properties make phospholipids ideal forms of ingredients for use in nutritional supplements, food, infant formula, pharmaceutical, and cosmetic applications. Dietary benefits of phospholipids include both improved absorption and improved incorporation. Phospholipids also have a broad range of functionality in the body in that they are important cell membrane constituents, they are good emulsifiers, they can act as intestinal surfactants, serve as a choline source and as a source of HUFAs.

EPA, DHA, and ARA are normally produced for the nutritional supplement market and food uses through the extraction (by cooking) of fish (EPA and DHA) such as menhaden, tuna or salmon, or hexane extraction of fungal biomass (ARA) from the genus Mortierella. The phospholipids in both processes are removed in a later degumming step that produces a waste material comprising a complex mixture of neutral lipids, sterols,

glucosides and phospholipids. This material is normally sold to the domestic animal feed industry to dispose of it.

Besides fish and fungal biomass, there are microbial sources of DHA and DPA(n-6). For the present invention, useful microbes can be selected from fungi, microalgae, protozoa or bacteria. These organisms can be selected from the groups including the Stramenopiles, Thraustochytriales, Chrysophyceae, Xanthophyceae, Bacillariophyceae, Dinophyceae, Phaeophyceae, Rhodophyceae, Chlorophyceae, Euglenophyceae, Cryptophyceae, Oomycetes, Chytridomycetes, or Zygomycetes. Useful microbes can also be selected from the group of genera consisting of Mortierella, Mucor, Phycomyces, Rhizopus, Pythium, Ochromonas, Nitzschia, Phaeodactylum, Skeletonema, Fucus, Platymonas, Achyla, Phytophera, Schizochytrium, Thraustochytrium, or Crypthecodinium. Microorganisms are good sources of phospholipids because they can be grown in culture in a manner that optimizes phospholipid production and minimizes triglyceride (oil) production. On the other hand the methods used in this invention allow both oil and phospholipids to be recovered separately in forms that can be used directly in food, feed, nutritional supplements, cosmetic or pharmaceutical application.

DHA, EPA and ARA phospholipids can be recovered from fish, microalgae, or fungi through the degumming process described above. However as noted this produces a complex material containing many other compounds including neutral lipids, sterols, glucosides, etc

A preferred embodiment of the present invention is to use alcohol and centrifugation to recover the omega-3 and/or omega-6 HUFA-rich phospholipids. Preferred methods for this recovery are described in the following references, which are incorporated by reference herein in their entirety:

i. PCT Application Serial No. PCT/US01/12047, entitled "Method for the Fractionation of Oil and Polar Lipid-Containing Native Raw Materials" filed April 12, 2001;

ii. PCT Application Serial No. PCT/US01/12049, entitled "Method For The Fractionation Of Oil And Polar Lipid-Containing Native Raw Materials Using Water-Soluble Organic Solvent And Centrifugation" filed April 12, 2001.

Once the omega-3 and/omega-6 rich phospholipid fractions have been extracted by these preferred processes, they can be used directly as ingredients or they can be purified further and even separated into phospholipid classes by well-known techniques

such as different forms of chromatography, molecular distillation, and special refining techniques. Polar lipid groups phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and sphingolipids. The phospholipid rich polar lipids or the purified phospholipid rich fractions can also be mixed with another lipid or oil such as fish lipids, microbial lipids, vegetable lipids, GLA-containing lipids, SDA-containing lipids and mixtures thereof, or be mixed with another phospholipid fraction (lecithin) such as soy or egg yolk lecithin, sunflower lecithin, peanut lecithin or mixtures thereof prior to use as a nutritional supplement, feed or food ingredient. These mixtures of phospholipids can also be incorporated into creams or lotions for topical applications (e.g. treating of skin conditions) or skin lesions induced by burns, UV-irradiation or other skin damaging processes. The mixtures can also be processed to produce a liquid or spray-dried dietetic product or fat emulsion for treating cachexia and severe fat malabsorption or for treatment of *H. pylori* infection of the gastrointestinal tract or incorporated into aerosol sprays for treating chronic inflammatory disease states of the lung (e.g., COPD, asthma, cystic fibrosis).

Advantages of the present invention including providing omega-3 and/or omega-6 HUFAs in a more bioactive and functional form (phospholipid) than the triglyceride form and include a better process (e.g., a) no need for heat treatment; b) no use of toxic solvents (like hexane) and c) no artifacts and off-flavors due to the use of acetone) for recovering these phospholipids from oilseeds and microbes. The EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and/or ARA content of the total fatty acids of the polar lipid fractions and comprise at least two weight percent of the total fatty acids of the polar lipid fraction, more preferably greater than 5 weight percent, more preferably greater than 10 weight percent, and most preferably greater than 20 weight percent of total fatty acids in the polar lipid fraction.

EXAMPLE

Example 1

Phospholipids were extracted from salmon, salmon roe, Black tiger prawns, squid, Schizochytrium sp, and the total fatty acid content of the phospholipids was determined by gas chromatography. The results are presented in Table 1. As can be observed the phospholipid fraction of these biomaterials can be used to deliver omega-3 and/or omega-

6 HUFAs and in this form these bioactive fatty acids should be more stable, more bioavailable, and more functional.

Table 1. Total fatty acid content of phospholipids extracted from 4 types marine animal products and forma microbial source.

		Salmon	**Salmon roe**	**Tiger Prawn**	**Squid**	**Schizochy-trium**
COMPOUND		**PL's**	**PL's**	**PL's**	**PL's**	**PL's**
		% TFA	% TFA	%TFA	% TFA	% TFA
MYRISTATE	C14:0	2.99	4.58	1.14	1.87	2.66
MYRISTOLEATE	C14:1	0,00	0,00	0.05	0.03	0,00
PALMITATE	C16:0	26.48	29.661	25.44	30.37	28.25
PALMITOLEATE	C16:1	2.86	4.38	1.55	0.23	0.62
STEARATE	C18:0	5.74	6.19	13.59	4.41	1.75
OLEATE	C18:1	9.74	13.36	13.33	1.86	6.99
LINOLEATE	C18:2n6	3.14	1.05	12.51	0.32	2.46
GAMMA LINOLENATE	C18:3n6	0.00	0.00	0.00	0.00	1.09
ARACHIDATE	C20:0	0.00	0.00	0.74	0.13	0,00
LINOLENATE	C18:3n3	0.49	0.22	0.54	0.05	0.00
OCTADECATETRAENO-ATE	C18:4	0.50	0.33	0.08	0.07	0.00
EICOSENOATE-11	C20:1	0,75	1.07	0.85	5.54	0.00
EICOSADIENOATE-11,14	C20:2	0,00	0.00	0.56	0.34	0,00
BEHENATE	C22:0	0,00	0,00	1.35	0.08	0,00
EICOSATRIENOATE	C20:3n3	1.01	3.58	0.07	0.42	1.61
ARACHIDONATE	C20:4 n6	0,89	0.56	5.54	1.70	1.18
ERUCATE	C22:1	0,21	0,00	0.04	1.41	0.00
EICOSAPENTAENOATE	C20:5n3	9.50	9.39	8.47	14.71	5.11
LIGNOCERATE	C24:0	0,00	0,00	0.56	0.00	0.00
NERVONATE	C24:1	1.61	1.65	0.73	0.82	0,00
DOCOSAPENTAENO-ATE n-6	C22:5n6	0,00	0,00	0.45	0.22	17.68
DOCOSAPENTAENO-ATE n-3	C22:5n3	3.16	4.40	0.55	0.44	0,00
DOCOSAHEXAENOATE	C22:6n3	30.92	19.50	11.86	35.54	30.61
		100,00	100,00	100.00	100,00	100,00

The present invention, in various embodiments, includes components, methods, processes, systems and/or apparatus substantially as depicted and described herein, including various embodiments, subcombinations, and subsets thereof. Those of skill in the art will understand how to make and use the present invention after understanding the present disclosure. The present invention, in various embodiments, includes providing devices and processes in the absence of items not depicted and/or described herein or in various embodiments hereof, including in the absence of such items as may have been

used in previous devices or processes, *e.g.*, for improving performance, achieving ease and/or reducing cost of implementation.

The foregoing discussion of the invention has been presented for purposes of illustration and description. The foregoing is not intended to limit the invention to the form or forms disclosed herein. Although the description of the invention has included description of one or more embodiments and certain variations and modifications, other variations and modifications are within the scope of the invention, *e.g.*, as may be within the skill and knowledge of those in the art, after understanding the present disclosure. It is intended to obtain rights which include alternative embodiments to the extent permitted, including alternate, interchangeable and/or equivalent structures, functions, ranges or steps to those Claimed, whether or not such alternate, interchangeable and/or equivalent structures, functions, ranges or steps are disclosed herein, and without intending to publicly dedicate any patentable subject matter.

We claim:

1. A method for providing a human, animal or aquaculture organism diet supplement enriched with at least one of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (n-3)(DPAn-3), eicosatetraenoic acid (n-3), docosapentaenoic acid (n-6)(DPAn-6) or arachidonic acid (ARA) comprising the steps:

(a) producing a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from genetically modified seeds or marine animals; and

(b) providing said polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA in a form consumable or usable by humans or animals.

2. The method of Claim 1, wherein the animal is a companion animal.

3. A method for treating a deficiency in at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising the steps:

(a) producing a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and

(b) providing said polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA to treat said deficiency.

4. The method of Claim 3, wherein said deficiency results in an inflammatory condition, an immune system imbalance, a cardiovascular disease, a developmental deficit related to nervous system development, a woman's health condition or an infant's health condition.

5. A method for treating a chronic inflammatory disease state of the lung comprising the steps:

(a) producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals;

(b) blending said phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one of EPA-, GLA- or SDA-rich oils; and

(c) producing an aerosol comprising the blend of step (b) for the treatment of said disease states.

6. The method of claim 5, wherein the chronic inflammatory disease state of the lung is COPD, asthma or cystic fibrosis.

7. A method for the treatment of skin lesions, induced burn, UV-irradiation or other skin disorders comprising the steps:

(a) producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals;

(b) blending said phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one EPA-, GLA- or SDA-rich oil; and

(c) producing a lotion or cream for the treatment of said skin disorders.

8. A method for treating cachexia or fat malabsorption comprising the steps:

(a) producing a purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA;

(b) blending said purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one other purified phospholipid;

(c) blending said purified phospholipid enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one DHA, EPA, GLA- or SDA-rich oil; and

(d) producing a liquid or dried dietetic product for the treatment of said disease states.

9. The method of Claim 8, wherein the cachexia or fat malabsorption is a result of cancer or Crohn's disease.

10. The method of Claim 8, wherein the at least one other purified phospholipid is obtained from the group consisting of soy, rape seed, evening primrose, safflower, sunflower, canola, peanut, egg and mixtures thereof.

11. A method for the treatment of *H. pylori*-infection of gastrointestinal tract comprising the steps:

(a) producing a purified phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals;

(b) blending said phospholipid fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with at least one EPA-, GLA- or SDA-rich oil; and

(c) producing a fat emulsion or a dietetic product for the treatment of said disease.

12. A method for providing a fat blend enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising the steps:

(a) extracting a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and

(b) mixing said polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with another oil.

13. The method of Claim 12, wherein said another oil is selected from the group consisting of fish oil, microbial oil, vegetable oil, GLA-containing oil, SDA-containing oil or mixtures thereof.

14. A method for providing a blend of polar lipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising the steps:

(a) extracting a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and

(b) mixing said polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA with another polar lipid.

15. The method of Claim 14, wherein said another polar lipid is selected from the group consisting of soy polar lipids, rape seed polar lipids, sunflower polar lipids, safflower polar lipids, canola polar lipids, peanut polar lipids or egg yolk polar lipids and mixtures thereof.

16. A fat blend enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising:

(a) a polar lipid-rich fraction enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid and ARA from microbes, genetically modified seeds or marine animals; and

(b) another oil.

17. The oil of Claim 16, wherein said another oil is selected from the group consisting of fish oil, microbial oil, vegetable oil, GLA-containing oil, SDA-containing oil and mixtures thereof.

18. A blend of polar lipids enriched with at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA comprising the steps:

(a) extracting a EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched polar lipid-rich fraction from microbes, seeds or marine animals; and

(b) mixing said EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched polar lipid-rich fraction with another polar lipid.

19. The method of Claim 18, wherein said another polar lipid is selected from the group consisting of soy polar lipids, rape seed polar lipids, sunflower polar lipids, safflower polar lipids, canola polar lipids, peanut polar lipids or egg yolk polar lipids and mixtures thereof.

20. Purified phospholipids enriched with at least one EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA derived from polar lipid-rich fraction extracted from genetically modified seeds or marine animals.

21. The purified phospholipids of Claim 20, wherein said EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA-enriched phospholipid-fraction is in a form consumable or usable by humans or animals.

22. A method for providing a human, animal or aquaculture organism diet supplement enriched with at least one of eicosapentaenoic acid (EPA), docosapentaenoic acid (n-3)(DPAn-3), eicosatetraenoic acid (n-3) or docosapentaenoic acid (n-6)(DPAn-6) comprising the steps:

(a) producing a polar lipid-rich fraction enriched with at least one of EPA, DPA(n-3), DPA(n-6) and eicosatetraenoic acid from microbes, genetically modified seeds or marine animals; and

(b) providing the polar lipid-rich fraction enriched with at least one of EPA, DPA(n-3), DPA(n-6) and eicosatetraenoic acid in a form consumable or usable by humans or animals.

23. A polar lipid-rich fraction of the method or product of any preceding Claim as an ingredient of dietetic, pharmaceutical or cosmetic applications.

24. A fat blend of the method or product of any preceding Claim as an ingredient of dietetic, pharmaceutical or cosmetic applications.

25. A blend of polar lipids of the method or product of any preceding Claim as an ingredient of dietetic, pharmaceutical or cosmetic applications.

26. Purified phospholipids of the method or product of any preceding Claim as an ingredient of dietetic, pharmaceutical or cosmetic applications.

27. The method or product of any preceding Claim, wherein said marine animals are fish, squid, mollusks or shrimp.

28. The method or product of any preceding Claim, wherein said marine animals are fish or fish eggs from the group including salmon, tuna, haddock, sardines, mackerel, or menhaden.

29. The method or product of any preceding Claim, wherein said microbes are selected from fungi, microalgae, protozoa or bacteria.

30. The method or product of any preceding Claim, wherein said microbes are selected from the Stramenopiles, Thraustochytriales, Chrysophyceae, Xanthophyceae, Bacillariophyceae, Dinophyceae, Phaeophyceae, Rhodophyceae, Chlorophyceae, Euglenophyceae, Cryptophyceae, Oomycetes, Chytridomycetes, or Zygomycetes.

31. The method or product of any preceding Claim, wherein said microbes are selected from the group of genera consisting of Mortierella, Mucor, Phycomyces, Rhizopus, Pythium, Ochromonas, Nitzschia, Phaeodactylum, Skeletonema, Fucus, Laminaria, Platymonas, Achyla, Phytophera, Schizochytrium, Thraustochytrium, or Crypthecodinium.

32. The method of or product of any preceding Claim, wherein EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid, ARA or mixture thereof comprises at least two weight percent of the total fatty acids of the polar lipid fraction.

33. The method or product of any preceding Claim, wherein EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid, ARA or mixture thereof comprises at least five weight percent of the total fatty acids of the polar lipid fraction.

34. The method or product of any preceding Claim, wherein said plant seeds or microbes have been genetically modified to increase their n-3 or n-6 HUFA content.

35. The method or product of any preceding Claim, wherein said seeds or microbes have been genetically modified to increase the production of at least one of EPA, DHA, DPA(n-3), DPA(n-6), eicosatetraenoic acid or ARA.

36. The method or product of any preceding Claim, wherein said seeds are selected from the group consisting of canola, rape seed, linseed, flaxseed, sunflower, safflower, peanuts, soybeans or corn.

37. The method or product of any preceding Claim, wherein said polar lipid-rich fraction is extracted from said seeds and microbes using alcohol.

38. The method or product of any preceding Claim, wherein said polar lipid-rich fraction is derived as a by-product of oil extraction from said seeds using hexane or other non-polar solvent.

39. The method or product of any preceding Claim, wherein said polar lipid-rich fraction is extracted from said seeds and microbes by use of gravity or centrifugal extraction technology.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/15454

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07C 1/100
US CL :554/8, 20, 224; 514/821, 824; 424/450
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. : 554/8, 20, 224; 514/821, 824; 424/450

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)

CAPLUS, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,539,133 A (KOHN et al.) 23 July 1996. see entire document.	1-39
Y	US 4,816,271 A (SCAFFIDI) 28 March 1989. SEE ENTIRE DOCUMENT.	1-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

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(54) KRILL EXTRACTS FOR PREVENTION AND/OR TREATMENT OF CARDIOVASCULAR DISEASES

KRILLEXTRAKTE ZUR PRÄVENTION UND/ODER BEHANDLUNG VON HERZ-KREISLAUF-ERKRANKUNGEN

EXTRAITS À BASE DE KRILL POUR LA PREVENTION ET/OU LE TRAITEMENT DES MALADIES CARDIOVASCULAIRES

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WO-A-00/23546

- **DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; 1985, IAROSCHKIN A P ET AL: "Technochemical characterisitcs of canned Natural Antartic shrimp meat and its nutritive value" Database accession no. nlm4002685**
- **IKEDA I: "EFFECTS OF LONG-TERM FEEDING OF MARINE OILS WITH DIFFERENT POSITIONAL DISTRIBUTION OF EICOSAPENTAENOIC AND DOCOSAHEXAENOIC ACIDS ON LIPID METABOLISM, EICOSANOID PRODUCTION, AND PLATELET AGGREGATION IN HYPERCHOLESTEROLEMIC RATS" LIPIDS, vol. 33, no. 9, 1998, pages 897-904, XP009068910**

EP 1 406 641 B1

Description

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention relates to multi-therapeutic extracts derived from krill and/or marine, which can prevent and/or treat several diseases.

Description of Prior Art

[0002] Krill is the common name for small, shrimp-like crustaceans, however not shrimp, that swarm in dense shoals, especially in Antarctic waters. It is one of the most important food source for fish, some kind of birds and especially for baleen whales as being an important source of protein. Krill is also a good source of omega-3 fatty acid, which are well known for their health benefits.

[0003] It is known in the art to use krill and/or marine enzymes for the treatment of a great variety of diseases in human and animals such as infections, inflammations, cancers, HIV/AIDS, pain, polyps, warts, hemorrhoids, plaque, wrinkles, thin hairs, allergic itch, anti-adhesion, eye disease, acne, cystic fibrosis and immune disorders including autoimmune disease and cancer.

[0004] It is also known in the art that krill and/or marine oil may be used for the treatment of autoimmune murine lupus and other autoimmune diseases and can also be used for treating cardiovascular diseases.

[0005] However, the krill and/or marine oil used for these treatments has only conserved its omega-3 fatty acids as active ingredients, which is a very small part of all the active ingredients of the krill and/or marine itself. This fact reduces the potential of the krill and/or marine oil as a treatment for these diseases.

[0006] There is an increasing demand for treatments using products derived from a natural source, therefore, it would be highly desirable to be provided with a krill and/or marine extract having an enhanced potential for prevention and/or treatment and/or management of disease.

SUMMARY OF THE INVENTION

[0007] In accordance with the present invention there is provided a composition for use in a method of prevention, therapy and/or treatment of several disease, the method comprising the administration of a therapeutically effective amount of krill oil to a patient.

[0008] In a preferred embodiment of the present invention the krill and oil is obtained from a process comprising the steps of:

(a) placing krill material in a ketone solvent, preferably acetone to achieve extraction of the soluble lipid fraction from the marine and/or aquatic animal material;

(b) separating the liquid and solid contents;

(c) recovering a first lipid rich fraction from the liquid contents by evaporation of the solvent present in the liquid contents;

(d) placing the solid contents in an organic solvent selected from the group of solvents consisting of alcohol, preferably ethanol, isopropanol or t-butanol and esters of acetic acid, preferably ethyl acetate to achieve extraction of the remaining soluble lipid fraction from the marine and/or aquatic material;

(e) separating the liquid and solid contents;

(f) recovering a second lipid rich fraction by evaporation of the solvent from the liquid contents; and

(g) recovering the solid contents.

[0009] In a preferred embodiment of the present invention, the krill and oil comprises Eicosapentanoic acid, Docosahexanoic acid, Phosphatidylcholine, Phosphatidylinositol, Phosphatidylserine, Phosphatidylethanolamine, Sphingomyelin, a-tocopherol, all-trans retinol, Astaxanthin and flavonoid.

[0010] In another embodiment of the present invention, the krill oil comprises Eicosapentanoic acid, Docosahexanoic

acid, Linolenic acid, Alpha-linolenic acid, Linoleic acid, Arachidonic acid, Oleic acid, palmitic acid, palmitoleic acid, stearic acid, nervonic acid, Phosphatidylcholine, Phosphatidylinositol, Phosphatidylserine, Phosphatidylethanolamine, Sphingomyelin, Cholesterol, Triglycerides, Monoglycerides, a-tocopherol, all-trans retinol, Astaxanthin, Canthaxanthin, β-carotene, flavonoid, Zinc, Selenium, sodium, potassium and calcium.

[0011] In another embodiment of the present invention, the krill oil comprises Eicosapentanoic acid, Docosahexanoic acid, Linolenic acid, Alpha-linolenic acid, Linoleic acid, Arachidonic acid, Oleic acid, palmitic acid, palmitoleic acid, stearic acid, Phosphatidylcholine, Phosphatidylinositol, Phosphatidylserine, Phosphatidylethanolamine, Sphingomyelin, Cholesterol, Triglycerides, Monoglycerides, a-tocopherol, all-trans retinol, Astaxanthin, Canthaxanthin, β-carotene, Zinc and Selenium.

[0012] The diseases that can be treated and/or prevented by the method of the present invention are cardiovascular diseases.

[0013] In accordance with the present invention there is also provided a composition for the treatment and/or prevention and/or therapy of the previously mentioned diseases, the composition comprising a therapeutically effective amount of krill oil in association with a pharmaceutically acceptable carrier.

[0014] In accordance with the present invention, it is further provided the use of krill oil for the treatment and/or prevention and/or therapy of the previously mentioned diseases.

[0015] In accordance with the present invention, it is also provided the use of krill oil for the manufacture of a medicament for the treatment and/or prevention and/or therapy of the previously mentioned diseases.

DETAILED DESCRIPTION OF THE INVENTION

[0016] In accordance with the present invention, there is provided krill extract for prevention and/or treatment and/or therapy of several diseases.

[0017] A multi-therapeutic oil extract free of enzyme is derived from krill, found in any marine environment around the world, for example, the Antarctic ocean (euphasia superba), the Pacific ocean (euphasia pacifica), the Atlantic ocean, the Indian ocean, in particular coastal regions of Mauritius Island and/or Reunion Island of Madagascar, Canadian West Coast, Japanese Coast, St-Lawrence Gulf and Fundy Bay, and this oil extract is a free fatty acid lipid fraction.

[0018] The extraction process can be described as the following:

(a) Placing aquatic krill in a ketone solvent, preferably acetone, to achieve the extraction of grease from the krill and/or marine;

(b) Separating the liquid and the solid phases;

(c) Recovering a lipid rich fraction from the liquid phase obtained at step (b) by evaporation of the solvent present in the liquid phase;

(d) Placing the solid phase in an organic solvent, which can be alcohol, preferably ethanol, isopropanol or t-butanol, or esters of acetic acid, preferably ethyl acetate. This in order to extract the remaining soluble lipid fraction from the solid phase;

(e) Separating the liquid and the solid phases; and

(f) Recovering a lipid rich fraction from the liquid phase obtained at step (e) by evaporation of the solvent present in the liquid phase.

[0019] The active components of the enzyme-free krill and/or marine oil extract are:

lipids

[0020]

i) Omega-3:

i. Eicosapentanoic acid: >8g/100g

ii. Docosahexanoic acid: >2g/100g

iii. Linolenic acid: >0.10g/100g

iv. Alpha-linolenic acid: >0.3g/100g

[0021] In the preferred embodiment of the present invention, the Omega-3 are found in more than 30g/100g.

ii) Omega-6: i. Linoleic acid: >0.9g/100g

ii. Arachidonic acid: <0.45g/100g, preferably < 0.6g/100g

iii) Omega-9: i. Oleic acid: >5g/100g

iv) palmitic acid: >10g/100g

v) palmitoleic acid: 0.08g/100g

vi) stearic acid: > 0.5g/100g

Phospholipids

[0022]

Phosphatidylcholine:>4.5g/100g

Phosphatidylinositol: >107mg/100g

Phosphatidylserine: >75 mg/100g

Phosphatidylethanolamine: >0.5g/100g

Sphingomyelin: >107mg/100g

Neutral lipids

[0023]

Cholesterol: <3g/100g

Triglycerides: <55g/100g

Monoglycerides: >0.5g/100g

[0024] In another embodiment of the present invention, the neutral lipids of the krill and/or marine extract also comprises:

Diglycerides: >0.5g/100g

Antioxydants

[0025]

α-tocopherol (vitamin E): >1.0 IU/100g

all-trans retinol (vitamin A): >1500 IU/100g

β-carotene: > 3000 μg/100 ml

Pigments

[0026]

Astaxanthin: >20 mg/100g

Canthaxanthin: > 2 mg/100g

Metals

[0027]

Zinc: >0.1 mg/100g

Selenium: >0.1 mg/100g

[0028] In another embodiment of the present invention, the krill and/or marine extract also comprises:

Flavonoids: >0.5mg/100g

Sodium: < 500mg/100g

Calcium: >0.1 mg/100g

Potassium: >50mg/100g

Aluminum: < 8.5mg/100g

Protein: > 4g/100g

Moisture and volatile matter: <0.8%

[0029] After characterization of the krill oil extract, it was determined that the extract contains less than 25 ppm of solvent residue from the extraction process.
The oil has the following stability indexes:

Peroxide value: < 0.1 (mEq/kg)

Oil Stability index: < 0.1 after 50 hours at 97.8°C

Saponification index: 70-180

Iodine value:60-130%

[0030] The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

Example 1

Cardiovascular disease prevention and/or treatment

[0031] Krill oil has been shown to decrease cholesterol *in vivo.* It also inhibits platelet adhesion and plaque formation and reduces vascular endothelial inflammation in a patient. It can offer hypertension prophylaxis. It prevents oxidation of low-density lipoprotein. It may have an inhibitory effect on the secretion of VLDL due to increased intracellular degradation of apo B-100. It also offers a post-myocardial infarction prophylaxis because of its ability to decrease CIII apolipoprotein B, to decrease CIII non-apolipoprotein B lipoproteins and to increase antithrombin III levels. Krill and/or marine oil is suitable for prophylactic usage against cardiovascular disease in human where cardiovascular disease relates to coronary artery disease, hyperlipidemia, hypertension, ischemic disease (relating to angina, myocardial inf-

arction, cerebral ischemia, shock without clinical or laboratory evidence of ischemia, arrhythmia)

[0032] To evaluate the effects of krill oil on the course of arteriosclerotic coronary artery disease and hyperlipidemia, a study was performed (prospective clinical trial, statistical significance $p<0.05$) with patients with known hyperlipidemia.

[0033] A group of 13 patients took krill oil concentrate gelules. Both fish oil and krill oil contained equal amounts of omega-3 fatty acids. Recommended dosage is of 1 to 6 capsules per day, each capsule containing 800 mg of oil. In this study, each patient took 6 capsules per day.

[0034] The patients were tested for LDL, HDL, Triglycerides, vital signs, CBC, SGOT/SGPT, γ-GT, ALP, Urea, Creatine, Glucose, K^+, Na^+, Ca^{2+} and total indirect bilirubin cholesterol before treatment and also at 2 months.

[0035] Table 1 is showing the results obtained from the previously described tests:

Table 1

Paired Samples Test								
Paired Differences								
Parameter tested	Mean	SD.	Std. Error Mean	95% Confidence Interval of the Difference		t-vatue	df	Sig. (2-tailed)
				Lower	Upper			
Cholesterol	.4954	.55800	.15476	.1582	.8326	3.201	12	.008
Triglycerides	.3538	.54543	.15127	.0242	.6834	2.339	12	.037
Triglycerides	.3538	.54543	.15127	.0242	.6834	2.339	12	.037
HDL	-.2108	.29859	.08281	-.3912	-.0303	-2.545	12	.026
HDL	-.2108	.29859	.08281	-.3912	-.0303	-2.545	12	.026
LDL	.2846	.47333	.13128	-.0014	.5706	2.168	12	.051
LDL	.2846	.47333	.13128	-.0014	.5706	2.168	12	.051
Chol / HDL	.3600	.53446	.14823	.0370	.6830	2.429	12	.032
Chol / HDL	.3600	.53446	.14823	.0370	.6830	2.429	12	.032

[0036] From the above, it was shown that a daily uptake of 1 to 4.8 g of krill extract was providing to the patients a cholesterol decrease in the range of 15%, a triglycerides decrease in the range of 15%, a HDL increase in the range of 8%, a LDL decrease in the range of 13% and a Cholesterol/HDL ratio decrease of 14%.

[0037] This shows that an uptake of krill extract has a beneficial effect on patient suffering from hyperlipidemia, which is known to be the primary causative factor of atherosclerosis.

[0038] The following example 2 to 11 are for reference purposes only.

Example 2

Arthritis treatment

[0039] Krill and/or marine oil offers symptomatic relief for Arthritis where arthritis relates to adult arthritis, Still's disease, polyarticular or pauciarticular juvenile rheumatoid arthritis, rheumatoid arthritis, osteoarthritis because it has been shown that it provides a clinical improvement in decreasing the number of tender joints and of analgesics consumed daily by decreasing the production of Interleukin-8 and Interleukin-1 in human patients. Patients with a bleeding tendency or severe psychiatric disease were excluded from the study.

[0040] To evaluate the effects of krill and/or marine oil supplementation on the clinical course of osteoarthritis, a study was performed (prospective clinical trial, statistical significance $p<0.05$) with patients diagnosed with and treated for osteoarthritis which is Active class I, II or III and having treatment with NSAIDs and/or analgesics for at least 3 months before enrollment.

[0041] A group of 13 patients took krill and/or marine oil concentrate capsules at a daily rate of 6 capsules of 800mg krill oil per capsule. The recommended dosage varies between 1 and 4.8 grams of pure krill extract per day. Patients were asked to follow a normal healthy diet consisting of 20% fat (less than 10% animal fat), 40% protein and 40% carbohydrates.

[0042] The inclusion criteria for the study are being aged between 50 and 65 years, both genders being admissible,

having a clinical diagnosis of primary osteoarthritis (mild to moderate) 6 to 12 months prior to study enrollment including pain and stiffness, radiographic conformation of illness prior to enrollment. It also include evidence of measurable symptoms of OA for at least 3 months prior to study enrollment requiring the use of acetaminophen, anti-inflammatory agents or opioid analgesics. Patients were asked to stop the use of all "pain-killers" the week prior to initiation of the trial for wash-out purposes.

[0043] The Exclusion criteria were a severe osteoarthritis, unavoidable sustained use of NSAID's, aspirin or other medicines for anti-inflammatory use, use of topical analgesics within 4 weeks of randomization visit, steroid injection into either knee within past 3 months, initiation of physical therapy or muscle conditioning within 3 months, seafood allergies, use of anticoagulants or salicylates, alcohol consumption exceeding 3 mixed drinks per day, concurrent medical/arthritic disease that could confound or interfere with the evaluation of pain, prior surgery (including arthroscopy) of either knee, a known "secondary" cause of osteoarthritis.

[0044] Evaluation was based on daily dose of NSAIDs and/or analgesics and/or SAARDs, number of painful joints, number or swollen joints, duration of morning stiffness, visual analog scale (0-100) WOMACscale and SF36. Preliminary results have been obtained after 2 months. The number of NSAIDs and/or analgesics and/or SAARDs

Table 2

	Frequency	%	Valid %	Cumulative %
No change	3	23.1	23.1	23.1
Pain relief	10	76.9	76.9	100.0
Total	13	100.0	100.0	

[0045] This shows that ten out of 13 (76.9%) people reported a significant pain relief and improvement of flexibility of large joints (lower back, knees, shoulders)

Example 3

Skin Cancer Prophylaxis

[0046] Krill and/or marine oil has been shown to be a skin cancer prophylactic because of its retinol anti-carcinogenic effect, Astaxanthin anti-carcinogenic effect and its phopholipid anti-carcinogenic effect.

[0047] To evaluate the photoprotective potential of krill and/or marine oil against UVB-induced skin cancer, a study was performed on nude mice, preferably on C57BL6 Nude Congenic Mice - B6NU-T (heterozygotes) because of their proven susceptibility to skin cancer.

[0048] Groups were formed as follows: 48 fish oil: 16 with oral supplementation (po) 16 with local application, 16 with po and local application; 48 krill and/or marine oil: 16 with po, 16 with local application, 16 with po and local application. In order to establish efficacy of krill and/or marine oil for the prevention of skin cancer, the test was conducted as a randomized blind controlled trial (statistical significance $p<0.05$). Half of the mice have been treated orally or topically or both with oil containing 100% by weight krill and/or marine oil and the other half have been treated the same way with fish oil.

[0049] Nutrition was fat-free chow for the first week and was modified accordingly with the assigned group as described below for the following 2-20 weeks in the quantity of 1 ml of oil per day.

[0050] The mice were divided in six groups as follows:

Group A: fat-free chow with supplementation of fish oil (20% of total calories)

Group B: fat-free chow (100% of calories) + local application of fish oil 2 times per day

Group C: fat free chow with supplementation of fish oil (20% of total calories) + local application of soy oil 2 times per day

Group D: fat-free chow with supplementation of krill and/or marine oil (20% of total calories)

Group E: fat free chow (100% of calories) + local application of krill and/or marine oil 2 times per day

Group F: fat-free chow with supplementation or krill and/or marine oil (20% of total calories) + local application of

krill and/or marine oil 2 times per day

[0051] The mice had been submitted to UVB radiation using a fluorescent test lamp, emission spectrum 270-400 nm during weeks 2-20. The essay were performed during 30 minutes of UVB exposure per day and the test lamp was at a distance of 30 cm from the mice. At the end of the 20 weeks, or when malignant tumors had formed, mice were anesthetized with ether and sacrificed. Skin was examined blind by pathologists for signs of carcinogenesis.

[0052] The following tables (Tables 3-8) are showing the results obtained about the incidence of cancer when ultra-violet radiations are administered to mice's skin during 5 weeks.

Table 3

Krill extract Oral uptake					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Benign	14	87.5	87.5	87.5
	Cancer	2	12.5	12.5	100.0
	Total	16	100.0	100.0	

Table 4

Control Oral uptake					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Benign	14	87.5	87.5	87.5
	Cancer	2	12.5	12.5	100.0
	Total	16	100.0	100.0	

Table 5

Krill extract topical uptake					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	BENIGN	16	100.0	100.0	100.0

Table 6

Control topical uptake					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	BENIGN	5	31.3	31.3	31.3
	Cancer	11	68.8	68.8	100.0
	Total	16	100.0	100.0	

Table 7

Krill extract topical and oral uptake					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	BENIGN	16	100.0	100.0	100.0

Table 8

Control topical and oral uptake					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	BENIGN	10	62.5	62.5	62.5
	Cancer	6	37.5	37.5	100.0
	Total	16	100.0	100.0	

[0053] The results obtained shows that both oral and topical use of krill oil is effective for the protection of the skin against the harmful effects fo UVB radiation induced skin cancer.

Example 4

Transdermal transport in therapeutic applications

[0054] Krill and/or marine oil enhances transdermal transportation as a substrate for dermatological topical therapeutic applications. It may be used in dermatological treatments via creams, ointments, gels, lotions and oils. It may also be used in various therapeutic applications such as relating to anesthesic, corticosteroids, anti-inflammatory, antibiotic and ketolytic functions.

[0055] To evaluate the efficacy of krill and/or marine oil as a substrate for topical treatments and the speed of transdermal absorption of krill and/or marine alone or as a substrate, a study was performed as a randomized blind controlled trial on C57BL6 nude Congenic Mice - B6NUT (heterozygotes).

[0056] The results appearing in tables 5 and 6 are showing that topical treatment with krill oil faciliate the absorption of retinol and other antioxydants through the dermis which in turn result in significant photoprotective potential which in turn results in 100% protection from UVB induced skin cancer. In contrast, fish oil application with all-trans retinol resulted in 68.8% incidence of cancer.

Example 5

Transdermal Transport for dermatological topical cosmetic applications

[0057] Krill and/or marine oil can be used to enhance transdermal transportation as a substrate for dermatological topical cosmetic applications where cosmetic applications relate, to skin hydration, anti-wrinkle, keratolytics, peeling and mask via creams, ointments, gels, lotions or oils.

[0058] To evaluate the effects of Krill and/or marine oil in aging and facial wrinkles, a study was conducted as a prospective clinical trial on patients concerned about facial dryness and wrinkles. Those patients had no prognosis severely limited by other dermatological or non-dermatological condition, bleeding tendency or severe psychiatric disease.

[0059] 13 Healthy caucasian women with facial dryness or wrinkles have been included in this study. Women have been asked to take 6 capsules a day, each capsule containing 800 mg of krill extract. The recommended daily dosage is of about 1 to 4.8 g of krill extract.

[0060] Table 9 shows results obtained on skin hydration following the method previously described.

Table 9

Changes in skin hydration				
	Frequency	**%**	**Valid %**	**Cumulative %**
No change	4	30.8	30.8	30.8
Hydration	9	69.2	69.2	100.0
Total	13	100.0	100.0	

[0061] The results of the pilot study after 2 months indicate that nine out of 13 (69.2%) people reported a significant improvement of the hydration, texture and elasticity of the skin (face, hands and arms) in human patients.

[0062] Moreover, these results are also indicative that krill extract is useful for anti-wrinkle treatment. The mechanism

of all-trans retinol, which is included in the krill oil, as an anti-wrinkle works as follows:

- Regeneration and distinctive anti-inflammatory effects
- Improve blood irrigation
- Increases the epidermis regeneration by increasing the rate of cell division and turnover
- Accelerates the differentiation of keratin
- Regenerates the collagen
- Allows cells in the top layer of the skin, which are always being replaces, to mature more normally than untreated sun-damaged cells
- Reduces the activation of enzymes that break down the proteins collagen and elastin that provide structural support for the skin.

The results obtained with krill extract administered on a patent's skin show that the krill extract is having an anti-wrinkle effect by increasing the hydration and the mechanism above described.

Example 6

Premenstrual syndrome

[0063] Table 10 shows results obtained from the use of krill oil to reduce the pain and mood changes associated with premenstrual syndrome in women. Krill oil extract was administered to 7 women during 2 months. The women were taking 6 capsules of krill extract per day, each capsule containing 800 mg of krill oil. A recommended daily intake of krill oil is of about 1 to 4.8 grams. All participants were advised to continue with their usual nutrition habits and to refrain from initiating any restrictions in their diet. No serious side effects were reported.

[0064] All women enrolled reported noticeable emotional and/or physical discomfort 7 to 10 days prior to menstruation. A self-assessment visual analogue scale validated for the assessment of the premenstrual syndrome, ranging from 0 (no symptoms) to 10 (unbearable) was used as a primary outcome in order to evaluate the effect of krill extract on premenstrual discomfort.

[0065] Data analysis has been reported on 60% of the women participating in the study who have completed a two months regimen. The majority of the women (73.3%) showed a clinically significant reduction in both emotional and physical distress prior to menstruation (see Table 10).

Table 10

Frequency distribution of the effect of krill extract on premenstrual syndrome symptomatology			
PMS symptoms	**Frequency %**	**Valid %**	**Cumulative %**
No change	26.7	26.7	26.7
Positive	73.3	73.3	100.0
Total	100.0	100.0	

Example 7

Diabetes

[0066] 8 human patients were taking krill extract at the dosage of 6 capsules a day, each capsule containing 800 mg of krill extract, during 2 months. A recommended daily intake of krill oil is of about 1 to 4.8 grams. The Table 11 is showing the variation in the glucose tested for the patients after 2 months.

Table 11

Variation in glucose in patients							
Paired Differences							
Parameter tested	Mean	SD.	Std. Error Mean	95% Confidence Interval of the Difference	t-value	df	Sig. (2-tailed)
Glucose	.5778	.60369	.20123	.1137 - 1.0418	2.871	8	.021

[0067] A blood glucose decrease of 20% was obtained for the patients taking krill extract, which shows that an uptake of krill extract is controlling blood glucose content and therefore controlling diabetes in human patients.

[0068] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

Claims

1. A composition comprising krill oil in association with a pharmaceutically acceptable carrier, for use in a method for decreasing cholesterol in a patient,
wherein said krill oil is administered to the patient in a quantity in a range of 1 to 4.8 grams per day,
wherein said krill oil is obtainable from a process comprising the steps of:

   a) placing krill in a ketone solvent to achieve extraction of the soluble lipid fraction from said krill ;
   b) separating the liquid and solid phases;
   c) recovering a first lipid rich fraction from the liquid phase obtained at step (b) by evaporation of the solvent present in the liquid phase;
   d) placing the solid phase in an organic solvent selected from the group consisting of alcohol and esters of acetic acid to achieve extraction of the remaining soluble lipid fraction from the solid phase ;
   e) separating the liquid and solid phases; and
   f) recovering a second lipid rich fraction from the liquid phase obtained at step (e) by evaporation of the solvent present in the liquid phase.

2. The composition according to claim 1, wherein the ketone solvent used in step (a) of the process is acetone.

3. The composition according to claim 1 or 2, wherein the organic solvent used in step (d) of the process is selected from the group consisting of ethanol, isopropanol and t-butanol.

4. The composition according to claim 1 or 2, wherein the organic solvent used in step (d) of the process is ethyl acetate.

5. The composition according to anyone of claims 1-4, wherein said quantity is 4.8 grams per day.

6. The composition according to anyone of claims 1-5, wherein said krill oil comprises Eicosapentanoic acid, Docosahexanoic acid, Phosphatidylcholine, Phosphatidylinositol, Phosphatidylserine, Phosphatidylethanolamine, Sphingomyelin, α-tocopherol, all-trans retinol, Astaxanthin and flavonoid.

7. The composition according to anyone of claims 1-5, wherein said krill oil comprises Eicosapentanoic acid, Docosahexanoic acid, Linolenic acid, Alpha-linolenic acid, Linoleic acid, Arachidonic acid, oleic acid, palmitic acid, palmitoleic acid, stearic acid, nervonic acid, Phosphatidylcholine, Phosphatidylinositol, Phosphatidylserine, Phosphatidylethanolamine, Sphingomyelin, cholesterol, triglycerides, monoglycerides, α-tocopherol, all-trans retinol, Astaxanthin, canthaxanthin, β-carotene flavonoid, zinc, selenium, sodium, potassium and calcium.

8. The composition according to anyone claims 1-7, wherein said composition is administered orally.

9. The composition according to anyone claims 1-8, wherein said patient is a patient suffering from hyperlipidemia.

Patentansprüche

1. Zusammensetzung, aufweisend Krillöl, zusammen mit einem pharmazeutisch akzeptablen Träger, zur Verwendung in einem Verfahren zur Verringerung von Cholesterin bei einem Patienten, wobei das Krillöl dem Patienten in einer Menge in einem Bereich von 1 bis 4,8 Gramm pro Tag verabreicht wird,
wobei das Krillöl erhältlich ist aus einem Verfahren, aufweisend die Schritte:

   a) Einbringen von Krill in ein Ketonlösungsmittel, um die Extraktion der löslichen Lipidfraktion aus dem Krill zu erreichen,
   b) Trennen der flüssigen und festen Phasen,
   c) Wiedergewinnen einer ersten lipidreichen Fraktion aus der flüssigen Phase, die in Schritt b) erhalten wurde, durch Verdampfen des in der flüssigen Phase vorhandenen Lösungsmittels,
   d) Einbringen der festen Phase in ein organisches Lösungsmittel, ausgewählt aus der Gruppe bestehend aus Alkohol und Estern von Essigsäure, um Extraktion der verbleibenden löslichen Lipidfraktion aus der festen Phase zu erreichen,
   e) Trennen der flüssigen und festen Phasen, und
   f) Wiedergewinnen einer zweiten lipidreichen Fraktion aus der flüssigen Phase, die in Schritt (e) erhalten wurde, durch Verdampfen des in der flüssigen Phase vorhandenen Lösungsmittels.

2. Zusammensetzung gemäß Anspruch 1, wobei das in Schritt (a) des Verfahrens verwendete Ketonlösungsmittel Aceton ist.

3. Zusammensetzung gemäß Anspruch 1 oder 2, wobei das in Schritt (d) des Verfahrens verwendete organische Lösungsmittel ausgewählt wird aus der Gruppe bestehend aus Ethanol, Isopropanol und t-Butanol.

4. Zusammensetzung gemäß Anspruch 1 oder 2, wobei das in Schritt (d) des Verfahrens verwendete organische Lösungsmittel Ethylacetat ist.

5. Zusammensetzung gemäß irgendeinem der Ansprüche 1-4, wobei die Menge 4,8 Gramm pro Tag ist.

6. Zusammensetzung gemäß irgendeinem der Ansprüche 1-5, wobei das Krillöl Eicosapentansäure, Docosahexansäure, Phosphatidylcholin, Phosphatidylinositol, Phosphatidylserin, Phosphatidylethanolamin, Sphingomyelin, α-Tocopherol, all-trans-Retinol, Astaxanthin und Flavonoid aufweist.

7. Zusammensetzung gemäß irgendeinem der Ansprüche 1-5, wobei das Krillöl Eicosapentansäure, Docosahexansäure, Linolensäure, alpha-Linolensäure, Linolsäure, Arachidonsäure, Oleinsäure, Palmitinsäure, Palmitoleinsäure, Stearinsäure, Nervonsäure, Phosphatidylcholin, Phosphatidylinositol, Phosphatidylserin, Phosphatidylethanolamin, Sphingomyelin, Cholesterin, Triglyceride, Monoglyceride, α-Tocopherol, all-trans-Retinol, Astaxanthin, Canthaxanthin, β-Karotin, Flavonoid, Zink, Selen, Natrium, Kalium und Kalzium aufweist.

8. Zusammensetzung gemäß irgendeinem der Ansprüche 1-7, wobei die Zusammensetzung oral verabreicht wird.

9. Zusammensetzung gemäß irgendeinem der Ansprüche 1-8, wobei der Patient ein Patient ist, der an Hyperlipidämie leidet.

Revendications

1. Composition comprenant de l'huile de krill en association avec un véhicule pharmaceutiquement acceptable, pour une utilisation dans une méthode pour réduire le taux de cholestérol chez un patient, **caractérisée en ce que** ladite huile de krill est administrée au patient dans une quantité de 1 à 4,8 grammes par jour,
ladite huile de krill étant susceptible d'être obtenue par un procédé comprenant les étapes de :

   a) mettre du krill dans un solvant cétonique pour réaliser une extraction de la fraction lipidique soluble dudit krill;
   b) séparer la phase liquide de la phase solide;

c) récupérer une première fraction riche en lipides de la phase liquide obtenue à l'étape (b) par évaporation du solvant présent dans la phase liquide;
d) mettre la phase solide dans un solvant organique choisi dans le groupe constitué d'alcool et d'esters d'acide acétique pour réaliser l'extraction de la fraction lipidique soluble restante de la phase solide;
e) séparer la phase liquide de la phase solide; et
f) récupérer une seconde fraction riche en lipides de la phase liquide obtenue à l'étape(e)par évaporation du solvant présent dans la phase liquide.

2. Composition selon la revendication 1, **caractérisée en ce que** le solvant cétonique utilisé à l'étape (a) du procédé est l'acétone.

3. Composition selon la revendication 1 ou 2, **caractérisée en ce que** le solvant organique utilisé à l'étape (d) du procédé est choisi dans le groupe constitué de l'éthanol, l'isopropanol et le t-butanol.

4. Composition selon la revendication 1 ou 2, **caractérisée en ce que** le solvant organique utilisé à l'étape (d) du procédé est l'acétate d'éthyle.

5. Composition selon l'une quelconque des revendications 1 à 4, **caractérisée en ce que** ladite quantité est de 4.8 grammes par jour.

6. Composition selon l'une quelconque des revendications 1 à 5, **caractérisée en ce que** ladite huile de krill comprend de l'acide eicosapentaénoïque, de l'acide docosahexaénoïque, de la phosphatidylcoline, du phosphatidylinositol, de la phosphatidylsérine, de la phosphatidyléthanolamine, de la sphingomyéline, de l'α-tocophérol, du tout-trans rétinol, de l'astaxanthine et de la flavonoïde.

7. Composition selon l'une quelconque des revendications 1 à 5, **caractérisée en ce que** ladite huile de krill comprend de l'acide eicosapentaénoïque, de l'acide docosahexaénoïque, de l'acide linolénique, de l'acide α-linolénique, de l'acide linoléique, de l'acide arachidonique, de l'acide oléique, de l'acide palmitique, de l'acide palmitoléique, de l'acide stéarique, de l'acide nervonique, de la phosphatidylcholine, du phosphatidylinositol, de la phosphatidylsérine, de la phosphatidyléthanolamine, de la sphingomyéline, du cholestérol, des triglycérides, des monoglycérides, de l'α-tocophérole, du tout-trans rétinol, de l'astaxanthine, de la cantaxanthine, du ß-carotène, de la flavonoïde, du zinc, du sélénium, du sodium, du potassium et du calcium.

8. Composition selon l'une quelconque des revendications 1 à 7, **caractérisée en ce que** ladite composition est administrée oralement.

9. Composition selon l'une quelconque des revendications 1 à 8, **caractérisée en ce que** ledit patient est un patient souffrant d'hyperlipidémie.

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(54) Title: COMPOSITION WITH EFFECTS OF DECLINE PREVENTION, IMPROVEMENT OR ENHANCEMENT OF NORMAL RESPONSES OF COGNITIVE ABILITIES OF A HEALTHY PERSON

(57) Abstract: Arachidonic acid and/or compounds containing arachidonic acid as a constituent fatty acid are used to obtain compounds which prevent decline of, improve or enhance normal responses of cognitive abilities of a healthy person, and which are highly suitable for pharmaceuticals as well as food products, with minimal side effects.

WO 2004/028529 A1

DESCRIPTION

COMPOSITION WITH EFFECTS OF DECLINE PREVENTION, IMPROVEMENT OR ENHANCEMENT OF NORMAL RESPONSES OF COGNITIVE ABILITIES OF A HEALTHY PERSON

FIELD OF THE INVENTION

The present invention provides foods and beverages with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, containing, as active ingredients, arachidonic acid and/or compounds with arachidonic acid as a constituent fatty acid, and a process for their production. More specifically, the invention provides foods and beverages with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, awareness level and/or discriminatory ability with respect to events selected from the group consisting of auditory stimuli, visual stimuli, gustatory stimuli, olfactory stimuli and somatosensory stimuli, which contain, as active ingredients, one or more species selected from the group consisting of arachidonic acid, arachidonic acid alcohol esters and triglycerides, phospholipids or glycolipids containing arachidonic acid as part or all of the constituent fatty acid, as well as a process for their production.

BACKGROUND ART

Cognition is the process of selecting information from the external environment and clearly identifying it, and more specifically, of transmitting external stimuli such as visual, auditory, olfactory, gustatory and somatosensory stimuli to the brain through sensory organs and processing and precisely identifying them by coordinated functioning of multiple regions of the brain.

Cognitive abilities consist of the two factors of information processing speed (speed of transforming external stimuli) and the amount of information processing resources (the level of resource allocation for information processing on external stimuli), and the effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person according to the present invention are therefore understood to refer to decline prevention, improvement or enhancement of normal functions such as attention, memory, perception, language, calculation and the like, while being distinct from improvement of impaired function.

Various therapeutic agents have been researched and developed in relation to impaired cognitive function. Unfortunately, however, no effective compounds have yet been found that prevent decline of, improve or enhance normal responses of cognitive abilities of a healthy person. That is, although methods are known for determining cognitive function impairment and evaluating improving effects (see, for example, Nishimura, T., Takeda, M., "Diagnostic imaging of Alzheimer's dementia", pp.27-36, Medical View Publications (2/10/2001)) are known, such methods do not allow evaluation of decline prevention, improvement or enhancement of normal responses. A need has therefore existed for an effective means of objectively evaluating normal responses of cognitive abilities of a healthy person in order to objectively develop and research drug agents for decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person.

Attention has recently become focused on event related potentialss of brain as a physiological index to serve as an objective evaluation of cognitive ability. It is possible to record the electrical activity exhibited by neurons through electrodes attached on the human scalp. Background brain waves are generally known

as continuous electrical activity which varies depending on the arousal level. A transient minute action potential is also present which varies in relation to a given specifiable event. This action potential is currently referred to as the "event related potentialss of brain", and is interpreted as the potential induced by internal or external stimuli. The event related potentialss of brain, a minute potential variation of about 0.1 μV to several tens of μV, is composed of a series of positive waves and negative waves. The positive wave appearing near 300 msec for the stimulus event among this series of potential variation is associated with cognitive function and is termed P300. The time to appearance of P300 for a stimulus event is called the P300 latency and represents information processing speed (speed of transforming external stimuli), while the height of the peak from the baseline is called the P300 amplitude and represents the amount of information processing resources (the level of resource allocation for information processing on external stimuli) (see, for example, Mitamura, S., "Current Therapy", Vol.18, No.4, pp.618-621, Life Medicom (2/10/2001)). It has thus become possible to objectively measure human cognitive response by measuring the event related potentialss of brain.

External stimuli are transmitted through sensory organs to the brain, and the brain is the tissue like adipose mass, about 1/3 of the white matter and 1/4 of the gray matter consists of phospholipids. Because most of the polyunsaturated fatty acids of the phospholipids constituting the cell membranes of the brain are arachidonic acid and docosahexaenoic acid, it has been suggested that these polyunsaturated fatty acids may play some role in enhancing learning and memory abilities and preventing or improving senile dementia. Arachidonic acid and docosahexaenoic acid cannot be synthesized *de novo* in animals and therefore must be ingested from food

either directly or indirectly (as linoleic acid precursor of arachidonic acid or α-linoleic acid precursor of docosahexaenoic acid). Attention has thus been focused on enhancing effects on learning and memory and preventive and improving effects on senile dementia by externally supplying arachidonic acid and docosahexaenoic acid. Docosahexaenoic acid is found abundantly in fish oil sources and a great deal of research has been conducted on its effects of brain function improvement, while inventions have been disclosed in regard to its use in learning ability reinforcers, memory reinforcers, dementia preventing agents, dementia treatment agents, anti-dementia agents or functional foods with brain function-improving effects (see, for example, Japanese Unexamined Patent Publication No. 7-82146, Japanese Unexamined Patent Publication No. 5-117147 and Japanese Unexamined Patent Publication No. 2-49723). Furthermore, arachidonic acid and/or compounds containing arachidonic acid as a constituent fatty acid have recently been shown by the present inventors, to improve age-related decline in learning ability, based on results of administering arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid to aged animals subjected to a Morris water maze test, as described in Japanese Unexamined Patent Publication No. 2003-48831, which was published on February 21, 2003, entitled "Composition Having Effects Of Preventing Or Ameliorating Conditions Or Diseases Caused By Brain Hypofunction". However, this particular invention is directed toward decline in brain function and discloses nothing regarding effects on normal responses of cognitive abilities of a healthy person.

Some attempts have been made to determine the improving effects of several compounds on cognitive response based on event related potentialss of brain. Miyanaga, K. examined the pharmacological action of DHA on brain function, measuring event related potentialsss

of brain before and after orally administering capsules containing 2400 mg DHA to 26 healthy subjects, and discovered that the P300 latency was significantly shortened and the P300 amplitude was significantly augmented (see Miyanaga, K., "Shoku no Kagaku" [Food Science] pp.84-96, Korin Books (1999)). However, due to a lack of comparative results with placebo samples, no correlation could be established between blood DHA levels and P300 results. In a prolonged administration test with daily administration of capsules containing 900 mg DHA to 97 healthy geriatric subjects for 6 months, no change in P300 was observed, thus leaving uncertainty about the efficacy of DHA.

Therefore, while docosahexaenoic acid is considered to exhibit an improving effect on learning ability, the fact that no effective results were found in terms of the event related potentials of brain as an index of cognitive ability means that it remains unclear whether or not arachidonic acid and/or compounds with arachidonic acid as a constituent fatty acid are effective for decline prevention, improvement and enhancement of normal responses of cognitive abilities of a healthy person.

DISCLOSURE OF THE INVENTION

Problems to be Solved by the Invention

Consequently, it has been ardently desired to develop compounds which prevent decline of, improve or enhance normal responses of information processing speed (speed of transforming external stimuli) and the amount of information processing resources (the level of resource allocation for information processing on external stimuli), as the two factors of cognitive abilities, and which are highly suitable for pharmaceuticals as well as food products, with minimal side effects.

Means for Solving the Problems

The present invention therefore provides foods and beverages with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, containing, as active ingredients, arachidonic acid and/or compounds with arachidonic acid as a constituent fatty acid, and a process for their production. More specifically, the invention provides foods and beverages with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, awareness level and/or discriminatory ability with respect to events (auditory stimuli, visual stimuli, gustatory stimuli, olfactory stimuli, somatosensory stimuli), which containes as active ingredients one or more species selected from the group consisting of arachidonic acid, arachidonic acid alcohol esters and triglycerides, phospholipids or glycolipids comprising arachidonic acid as part or all of the constituent fatty acid, as well as a process for their production.

As a result of diligent research for the purpose of elucidating the effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person by arachidonic acid and/or compounds comprising arachidonic acid as a constituent fatty acid, the present inventors surprisingly discovered an effect of arachidonic acid and/or compounds comprising arachidonic acid as a constituent fatty acid, based on evaluation using the event related potentials of brain as an index.

The present inventors also succeeded in achieving microbial industrial production of triglycerides containing at least 10 wt% arachidonic acid, and elucidated the effects of those triglycerides in effects tests according to the invention.

The present inventors further succeeded in achieving enzymatic production of oils and fats containing triglycerides with medium-chain fatty acids bound at the

1,3-positions and arachidonic acid bound at the 2-position, and elucidated the effects of those triglycerides in effects tests according to the invention.

The present invention therefore provides foods and beverages with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, containing as active ingredients arachidonic acid and/or compounds with arachidonic acid as a constituent fatty acid, and a process for their production. More specifically, the invention provides foods and beverages with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, awareness level and/or discriminatory ability with respect to events (auditory stimuli, visual stimuli, gustatory stimuli, olfactory stimuli, somatosensory stimuli), which containes as active ingredients one or more species selected from the group consisting of arachidonic acid, arachidonic acid alcohol esters and triglycerides, phospholipids or glycolipids comprising arachidonic acid as part or all of the constituent fatty acid, as well as a process for their production.

According to the invention it is possible to provide foods and beverages having effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person which containes as active ingredients arachidonic acid and/or compounds with arachidonic acid as a constituent fatty acid, and a process for their production, thus the invention is particularly useful in modern society.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a bar graph showing changes in arachidonic acid levels in serum phospholipids before and after ingestion of arachidonic acid-containing edible oil capsules and olive oil-containing capsules (placebo

capsules).

Fig. 2 is a line graph showing changes in P300 latency before and after ingestion of arachidonic acid-containing edible oil capsules and olive oil-containing capsules (placebo capsules).

Fig. 3 is a line graph showing changes in P300 amplitude before and after ingestion of arachidonic acid-containing edible oil capsules and olive oil-containing capsules (placebo capsules).

Fig. 4 is a pair of dot graphs showing correlation between P300 (P300 latency and amplitude) and arachidonic acid levels in serum phospholipids.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to foods and beverages with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, comprising as active ingredients arachidonic acid and/or compounds with arachidonic acid as a constituent fatty acid, and to a process for their production.

The compositions of the invention have effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, or stated differently, they have effects of decline prevention, improvement or enhancement of normal responses of awareness level of a healthy person and have effects of decline prevention, improvement or enhancement of normal responses of discriminatory ability of a healthy person with respect to events (auditory stimuli, visual stimuli, gustatory stimuli, olfactory stimuli, somatosensory stimuli), and are able to exhibit their effects in foods or beverages, pharmaceuticals, quasi drugs and the like. The effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person are also effective as foods or beverages, pharmaceuticals and

quasi drugs designed for the purpose of decline prevention, improvement or enhancement of normal functions such as attention, memory, perception, language, calculation and the like, and as foods or beverages, healthy foods, functional foods, special health care foods, infant foods, geriatric foods, etc. designed for the purpose of maintaining or enhancing concentration, maintaining attention, clearing the head or stimulating the mind, rejuvenation and the like.

The active ingredients according to the invention may be, instead of free arachidonic acid, any compounds having arachidonic acid as a constituent fatty acid. Compounds having arachidonic acid as a constituent fatty acid include arachidonic acid salts such as calcium and sodium salts, or arachidonic acid alcohol esters such as arachidonic methyl ester, arachidonic acid ethyl ester and the like. Triglycerides, diglycerides, monoglycerides, phospholipids or glycolipids containing arachidonic acid as part or all of the constituent fatty acid may also be used.

For application to a food product, the arachidonic acid is preferably in the form of triglycerides or phospholipids, and especially triglycerides. While virtually no natural sources of arachidonic acid-containing triglycerides (or triglycerides including triglycerides with arachidonic acid as part or all of the constituent fatty acid) are known, the present inventors have succeeded in enabling industrial use of triglycerides containing arachidonic acid as a constituent fatty acid, and have been the first to elucidate the effect of the active ingredient of the invention in humans by prolonged administration to humans and analysis of event related potentials of brain allowing objective evaluation of cognitive abilities, and to demonstrate that it has an effect of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person.

According to the present invention, therefore, there may be used triglycerides which include triglycerides wherein part or all of the constituent fatty acids are arachidonic acid as the active ingredient of the invention (arachidonic acid-containing triglycerides). For application to food products, the arachidonic acid-containing triglycerides are preferably in the form oils or fats (triglycerides) wherein the proportion of arachidonic acid is at least 20 wt% (W/W), preferably at least 30 wt% and more preferably at least 40 wt% of the total fatty acids of the triglycerides. According to the invention, therefore, there may be used any arachidonic acid-containing oils or fats (triglycerides) obtained by culturing microbes capable of producing the same.

As examples of microbes capable of producing arachidonic acid-containing oils or fats (triglycerides) there may be mentioned microbes belonging to the genus *Mortierella*, *Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*, *Cladosporium*, *Mucor*, *Fusarium*, *Aspergillus*, *Rhodotorula*, *Entomophthora*, *Echinosporangium* or *Saprolegnia*. As microbes belonging to the genus *Mortierella* subgenus *Mortierella* there may be mentioned *Mortierella elongata*, *Mortierella exigua*, *Mortierella hygrophila*, *Mortierella alpina*, and the like. Specifically, there may be mentioned strains such as *Mortierella elongata* IFO8570, *Mortierella exigua* IFO8571, *Mortierella hygrophila* IFO5941, *Mortierella alpina* IFO8568, ATCC16266, ATCC32221, ATCC42430, CBS219.35, CBS224.37, CBS250.53, CBS343.66, CBS527.72, CBS529.72, CBS608.70, CBS754.68, etc.

These strains are all available without restriction from the Institute for Fermentation, Osaka (IFO), the American Type Culture Collection (ATCC) and the Centralbureau voor Schimmelcultures (CBS). In addition, there may be used strain *Mortierella elongata* SAM0219 (FERM P-8703) (FERM BP-1239) separated from soil by the research group of the present inventors.

For culturing of the strains used for the invention, the spores, hypha or cells of a seed culture solution obtained by preculturing are inoculated to liquid or solid medium for main culturing. In the case of a liquid medium, the carbon source used may be any commonly used one such as glucose, fructose, xylose, saccharose, maltose, solubilized starch, molasses, glycerol or mannitol, although there is no limitation to these. As nitrogen sources there may be used organic nitrogen sources, including natural nitrogen sources such as peptone, yeast extract, malt extract, meat extract, casamino acid, corn steep liquor, soybean protein, defatted soybean, cottonseed meal or the like, as well as urea, and inorganic nitrogen sources such as sodium nitrate, ammonium nitrate and ammonium sulfate. If necessary, trace nutrient sources including inorganic salts such as phosphoric acid salts, magnesium sulfate, iron sulfate and copper sulfate or vitamins may also be used. These medium components are not particularly restricted so long as they are at concentrations which do not inhibit growth of the microbes. In practice, the carbon source will generally be added in a total amount of 0.1-40 wt% (W/V) and preferably 1-25 wt% (W/V). The initial nitrogen source addition may be at 0.1-10 wt% (W/V) and preferably 0.1-6 wt% (W/V), with further addition of the nitrogen source during culturing if desired.

Oils or fats (triglycerides) containing at least 45 wt% (W/W) arachidonic acid may be prepared as the active ingredient for the invention by controlling the carbon source concentration in the medium. The culturing produces cells in the growth phase up to day 2-4 of culturing, and cells in the oil/fat accumulating phase after day 2-4 of culturing. The initial carbon source concentration is 1-8 wt% and preferably 1-4 wt%, and the carbon source is successively added only during the initial periods of the cell growth phase and oil/fat

accumulating phase, up to a total successive carbon source addition of 2-20 wt% and preferably 5-15 wt%. The successive addition of the carbon source during the initial periods of the cell growth phase and oil/fat accumulating phase is based on the initial nitrogen source concentration, for a carbon source concentration of zero in the medium from the 7th day of culturing, preferably from the 6th day of culturing and more preferably from the 4th day of culturing, to obtain oils or fats (triglycerides) comprising at least 45 wt% arachidonic acid, as an active ingredient according to the invention.

The culturing temperature for the arachidonic acid-producing microbe will differ depending on the microbe, but may be 5-40°C and preferably 20-30°C, or the cells may be cultured at 20-30°C for growth and the culturing continued at 5-20°C to produce unsaturated fatty acids. Such temperature management can also be used to increase the proportion of polyunsaturated fatty acids among the produced fatty acids. The pH of the medium may be 4-10 and preferably 5-9, and the culturing method may be submerged culturing, shake culturing, or stationary culturing. The culturing will usually be conducted for 2-30 days, preferably for 5-20 days and more preferably for 5-15 days.

As a means for increasing the proportion of arachidonic acid in the arachidonic acid-containing oils or fats (triglycerides) other than controlling the carbon source concentration in the medium, the arachidonic acid-containing oils or fats may be selectively hydrolyzed to obtain arachidonic acid-rich oils or fats. Lipases used for selective hydrolysis are not specific to triglyceride positions and, as the hydrolytic activity is lower in proportion to the number of double bonds, fatty acid ester bonds other than those of polyunsaturated fatty acids are hydrolyzed. Also, transesterification occurs between the produced PUFA triglycerides, resulting in

triglycerides with increased polyunsaturated fatty acids (see, for example, Enhancement of Arachidonic Acid: Selective Hydrolysis of a Single-Cell Oil from *Mortierella* with *Candida cylindracea* lipase", J. Am. Oil Chem. Soc., 72, pp.1323-1327, AOCS Press (1998)). Thus, arachidonic acid-rich oils or fats (triglycerides) obtained by selective hydrolysis of arachidonic acid-containing oils or fats may be used as active ingredients according to the invention. A higher proportion of arachidonic acid is preferred with respect to the total fatty acids in arachidonic acid-containing oils or fats (triglycerides) of the invention in order to minimize the effects of the other fatty acids, but there is no restriction to a high proportion and, in practice, the absolute amount of arachidonic acid can pose a problem for application to food products, although oils or fats (triglycerides) containing arachidonic acid at 10 wt% or more may be used in practice.

As triglycerides comprising arachidonic acid as part or all of the constituent fatty acid according to the invention there may be used triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position. There may also be used oils or fats (triglycerides) comprising at least 5 mole percent, preferably at least 10 mole percent, more preferably at least 20 mole percent and most preferably at least 30 mole percent of triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position. The medium-chain fatty acids bound at the 1,3-positions of the triglycerides may be selected from among fatty acids of 6 to 12 carbons. As examples of fatty acids of 6 to 12 carbons there may be mentioned caprylic acid and capric acid, with 1,3-capryloyl-2-arachidonoyl-glycerol (hereinafter referred to as "8A8") being preferred.

Triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-

position are most suitable as oils or fats (triglycerides) for infants or elderly persons. Ingested oils or fats (triglycerides) are usually hydrolyzed by pancreatic lipases upon entering the small intestine, and since pancreatic lipases are 1,3-position specific type, the 1,3-positions of the triglycerides are cleaved to produce two free fatty acid molecules while simultaneously producing one 2-monoacylglyceride (MG) molecule. Because 2-MG has very high bile acid solubility and is highly absorbable, 2-position fatty acids are generally considered to be better absorbed. Also, 2-MG exhibits surfactant action when dissolved in bile acids, and thus functions to increase the absorption of the free fatty acids. The free fatty acids and 2-MG then form bile acid complex micelles with cholesterol or phospholipids and are taken up by intestinal epithelial cells, resulting in resynthesis of triacylglycerols and finally release into the lymph as chylomicrons. However, the fatty acid properties of the pancreatic lipases are highest for saturated fatty acids, while arachidonic acid is cleaved less efficiently. An additional problem is that pancreatic lipase activity is lower in infants and the elderly, such that the ideal oils or fats (triglycerides) are triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position.

One specific production process for triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position is a production process whereby lipase is allowed to act only on 1,3-ester bonds of triglycerides in the presence of arachidonic- containing oils or fats (triglycerides) and medium-chain fatty acids.

The oil or fat (triglycerides) used as the starting material consists of triglycerides with arachidonic acid as a constituent fatty acid, and when the proportion of arachidonic acid is high with respect to the total fatty

acids of the triglycerides, a temperature of 30-50°C and preferably 40-50°C is employed which is higher than the ordinary enzyme reaction temperature of 20-30°C, in order to prevent reduction in reaction yield due to increase in unreacted oil or fat (the triglyceride starting material and the triglycerides wherein only one of the 1,3-position fatty acids is a medium-chain fatty acid).

As lipases which act specifically on the 1,3-position ester bonds of triglycerides there may be mentioned those produced by microbes belonging to the genus *Rhizopus*, *Rhizomucor*, *Aspergillus* or the like, as well as pig pancreatic lipases. Such lipases may be commercially available ones. Examples thereof include *Rhizopus delemar* lipase (Talipase, product of Tanabe Seiyaku), *Rhizomucor miehei* lipase (Ribozyme IM by Novo Nordisk Co., Ltd.) and *Aspergillus niger* lipase (Lipase A, product of Amano Enzyme Co., Ltd.), with no particular limitation to these enzymes, as any 1,3-position specific type lipases may be used.

The form in which the lipase is used is preferably as an immobilized lipase on an immobilizing support, in order to confer heat stability to the enzyme as the reaction temperature is 30°C or higher and preferably 40°C or higher for increased reaction efficiency. Highly porous resins are used as immobilizing supports, and there may be mentioned ion-exchange resin supports with pores of at least about 100 angstroms, such as Dowex MARATHON WBA (trademark of Dow Chemical).

A 0.5 to 20 times weight of aqueous solution of the 1,3-position specific type lipase with respect to the immobilizing support is suspended therein, and a 2 to 5 times amount of cold acetone (for example, -80°C) with respect to the suspension is slowly added while stirring to form a precipitate. The precipitate is dried under reduced pressure to prepare the immobilized enzyme. As a simpler method, a 0.05 to 0.4 times amount of the 1,3-position specific type lipase with respect to the

immobilizing support is dissolved in a minimal amount of water, and then the solution is mixed with the immobilizing support while stirring and the mixture is dried under reduced pressure to prepare the immobilized enzyme. This procedure results in immobilization of about 90% of the lipase on the support but with absolutely no exhibited transesterification activity, and therefore the immobilized enzyme may be activated and provided for production most efficiently by pretreatment in the substrate (raw material oil or fat and medium-chain fatty acids) containing 1 to 10 wt% (W/V) water, and preferably in the substrate containing 1 to 3 wt% water.

The amount of water added to the reaction system is extremely important depending on the type of enzyme, since transesterification will not proceed in the absence of water, while an excess of water causes hydrolysis and a reduced glyceride yield (with diglycerides and monoglycerides being produced upon hydrolysis). In such cases, however, the immobilized enzyme activated by pretreatment may be used to lessen the importance of the amount of water added to the reaction system, allowing transesterification reaction to occur efficiently even in a system containing no water. Moreover, the type of enzyme agent may also be selected to allow omission of pretreatment.

Thus, by using a heat-resistant immobilized enzyme and increasing the enzyme reaction temperature, it is possible to efficiently produce triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position without reduction in reaction efficiency, even in the case of arachidonic acid-containing oils or fats (triglycerides) with low reactivity for 1,3-position specific type lipases.

The process for production of foods and beverages with effects of decline prevention, improvement or

enhancement of normal responses of cognitive abilities of a healthy person employs arachidonic acid and/or a compound with arachidonic acid as a constituent fatty acid, either alone or in admixture with a food or beverage material containing essentially no, or only a trace amount of, arachidonic acid. Here, "trace amount" means that arachidonic acid is present in the food or beverage material but in an amount which does not reach the daily intake of arachidonic acid according to the invention, as described hereunder, if the food composition is ingested by an individual.

Oils and fats (triglycerides) containing arachidonic acid as part or all of the constituent fatty acid have unlimited possibilities for use as raw materials and additives in foods, beverages, pharmaceuticals and quasi drugs. Furthermore, there are no limitations on the purpose or amounts of their use.

As examples of food compositions there may be mentioned common food products as well as functional foods, nutritional supplements, modified milk for immature infants, modified milk for infants, infant food products, maternal foods or geriatric foods. As examples of food products containing oils or fats there may be mentioned natural foods which originally contain oils and fats, such as meat, fish and nuts, food products with oils and fats added during preparation, such as soups, food products using oils or fats as heating media, such as donuts, oil or fat food products such as butter, processed food products with oils or fats added during processing, such as cookies, or food products sprayed or coated with oils or fats during final processing, such as hard biscuits. They may also be added to agricultural foods, fermented foods, livestock foods, marine foods or beverages which contain no oils or fats. They may also be in the form of functional food products, pharmaceuticals or quasi drugs, and for example, in the form of enteral nutrients, powders, granules, lozenges,

oral solutions, suspensions, emulsions, syrups or the like.

The composition of the invention may also contain, in addition to the active ingredient of the invention, various carriers and additives commonly used in foods and beverages, pharmaceuticals or quasi drugs. In particular it preferably contains antioxidants in order to prevent oxidation of the active ingredient of the invention. As antioxidants there may be mentioned natural antioxidants such as tocopherols, flavone derivatives, phyllodulcin, kojic acid, gallic acid derivatives, catechins, fukiic acid, gossypol, pyrazine derivatives, sesamol, guaiaol, guaiac acid, p-coumaric acid, nordihydroguaiatic acid, sterols, terpenes, nucleic acid bases, carotenoids, lignans and the like, and synthetic antioxidants such as ascorbic palmitic ester, ascorbic stearic ester, butylhydroxyanisole (BHA), butylhydroxytoluene (BHT), mono-t-butylhydroquinone (TBHQ) and 4-hydroxymethyl-2,6-di-t-butylphenol (HMBP). As tocopherols there may be mentioned α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, ε-tocopherol, ξ-tocopherol, η-tocopherol and tocopherol esters (acetic acid tocopherol, etc.). As examples of carotenoids there may be mentioned β-carotene, cantaxanthin, astaxanthin and the like.

As supports to be used in the composition of the invention in addition to the active ingredient of the invention, there may be mentioned various types of carrier supports, extenders, diluents, fillers, dispersing agents, excipients, binder solvents (for example, water, ethanol or vegetable oils), solubilizing aids, buffers, dissolution accelerators, gelling agents, suspending agents, wheat flour, rice flour, starch, corn starch, polysaccharides, milk protein, collagen, rice oil, lecithin and the like. As examples of additives there may be mentioned vitamins, sweeteners, organic acids, coloring agents, flavorings, dehumidifying agents,

fibers, electrolytes, minerals, nutrients, antioxidants, preservatives, aromatic agents, humidifiers, natural edible extracts, vegetable extracts and the like, although there is no limitation to these.

The principal medicinal component of the arachidonic acid or the compound with arachidonic acid as a constituent fatty acid is arachidonic acid. The daily intake of arachidonic acid from food is reported to be 0.14 g in the Kanto region, Japan and 0.19-0.20 g in the Kansai region, Japan (see "Shishitsu Eiyogaku" [Lipid Nutrition] 4, ed. by the Japan Society for Lipid Nutrition Editing Committee, pp.73-82(1995) ISN 1343-4594 CODEN:SHEIFG), and therefore arachidonic acid must be ingested in a corresponding amount or greater. Thus, the daily intake of arachidonic acid or a compound with arachidonic acid as a constituent fatty acid according to the invention for an adult (60 kg body weight, for example) is 0.001-20 g, preferably 0.01-10 g, more preferably 0.05-5 g and most preferably 0.1-2 g, in terms of arachidonic acid.

When the active ingredient of the invention is actually used in a food or beverage product, the absolute amount of arachidonic acid in the food product is also important. However, since the absolute amount in the food or beverage also varies depending on the amount of ingestion of the food or beverage, the food product preferably contains the triglycerides including triglycerides with arachidonic acid as part or all of the constituent fatty acid, in an amount of at least 0.0003 wt%, preferably at least 0.003 wt% and more preferably at least 0.03 wt% in terms of arachidonic acid. When triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position are added to a food or beverage, the amount of the triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position is at least 0.001 wt%, preferably at least 0.01

wt% and more preferably at least 0.1 wt%.

When the composition of the invention is used as a pharmaceutical product, it may be produced by a common method for drug manufacturing, for example, a method described the Japanese Pharmacopoeia, or a method based thereon.

When the composition of the invention is used as a pharmaceutical product, the content of the active ingredient in the composition is not particularly restricted so long as the object of the invention is achieved, and the mixing proportion may be as deemed appropriate.

When the composition of the invention is used as a pharmaceutical product, it is preferably administered in unit dosages, with oral administration being particularly preferred. The doses of the composition of the invention will differ depending on the age, body weight and symptoms of the patient and the number of times administered, and for example, the arachidonic acid and/or compound with arachidonic acid as a constituent fatty acid according to the invention may usually be administered at about 0.001-20 g, preferably about 0.01-10 g, more preferably about 0.05-5 g and most preferably about 0.1-2 g in terms of arachidonic acid per day for an adult (approximately 60 kg), in divided doses of 1 to 3 times a day.

The major fatty acids of brain phospholipid membranes are arachidonic acid and docosahexaenoic acid, and therefore the composition of the invention is preferably a combination of arachidonic acid and docosahexaenoic acid in consideration of maintaining balance. Generally speaking, arachidonic acid (n-6 unsaturated fatty acid) and docosahexaenoic acid (n-3 unsaturated fatty acid) are biosynthesized by the same enzyme from linoleic acid and α-linolenic acid, respectively. When arachidonic acid is administered alone, therefore, biosynthesis of docosahexaenoic acid is

inhibited. Conversely, when docosahexaenoic acid is administered alone, biosynthesis of arachidonic acid is inhibited. In order to avoid such unbalances, it is preferred for arachidonic acid and docosahexaenoic acid to be consumed in combination. Furthermore, as the proportion of eicosapentaenoic acid is very low in brain phospholipid membranes, the combination preferably contains virtually no eicosapentaenoic acid. It therefore preferably contains no eicosapentaenoic acid, or only up to 1%. The composition more preferably contains virtually no eicosapentaenoic acid with the arachidonic acid and docosahexaenoic acid. The combination of arachidonic acid and docosahexaenoic acid also preferably has an arachidonic acid/docosahexaenoic acid ratio (by weight) in the range of 0.1-15 and preferably in the range of 0.25-10. Most preferred are foods and beverages containing eicosapentaenoic acid at no more than 1/5 (by weight) of the arachidonic acid.

The food compositions of the invention for foods or beverages, healthy foods, functional foods, special health care foods, infant foods, geriatric foods and the like include those which are marketed using packaging containers and/or food composition merchandising tools (for example, pamphlets or the like) which state or otherwise indicate that the compositions and/or components in the composition have effects of decline prevention, improvement or enhancement of cognitive abilities, or stated differently, that they have effects of decline prevention, improvement or enhancement of normal responses of awareness level of a healthy person, effects of decline prevention, improvement or enhancement of normal responses of discriminatory ability of a healthy person with respect to events (auditory stimuli, visual stimuli, gustatory stimuli, olfactory stimuli, somatosensory stimuli) and effects of decline prevention, improvement or enhancement of normal functions such as attention, memory, perception, language, calculation and

the like, as well as having effects of maintaining or enhancing concentration, maintaining attention, clearing the head or stimulating the mind, rejuvenation and the like.

EXAMPLES

The present invention will now be explained in greater detail through the following examples, with the understanding that these examples in no way limit the invention.

Example 1

Production process for triglycerides with arachidonic acid as a constituent fatty acid

Mortierella alpina was used as an arachidonic acid-producing strain. A 6 kL portion of medium containing 1.8% glucose, 3.1% defatted soybean flour, 0.1% soybean oil, 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2 \cdot 2H_2O$ and 0.05% $MgCl_2 \cdot 6H_2O$ was prepared in a 10 kL culturing vessel, and the initial pH was adjusted to 6.0. After seeding 30 L of the culture solution, submerged culturing was conducted for 8 days under conditions of 26°C temperature, 360 m^3/h aeration and 200 kPa interior pressure. The spinning was adjusted to maintain a dissolved oxygen concentration of 10-15 ppm. Also, the glucose concentration of the medium was adjusted by feeding to be in the range of 1-2.5% up to the 4th day, and 0.5-1% thereafter (where the percentages are based on weight (W/V)). After completion of the culturing, followed by filtering and drying, the cells containing triglycerides with arachidonic acid as a constituent fatty acid were collected, the oil/fat was obtained from the obtained cells by hexane extraction, and the edible oil/fat was subjected to a purification step (degumming, deacidification, steam distilation, decoloration) to obtain 150 kg of arachidonic acid-containing triglycerides (with arachidonic acid bound at any

positions of the triglycerides). The obtained oil/fat (triglyceride) was methylesterified and the resulting fatty acid methyl ester was analyzed by gas chromatography, which revealed an arachidonic acid proportion of 40.84% of the total fatty acids. The palmitic acid, stearic acid, oleic acid, linoleic acid, γ-linolenic acid and dihomo-γ-linolenic acid contents were 11.63%, 7.45%, 7.73%, 9.14%, 2.23% and 3.27%, respectively. The arachidonic acid-containing oil/fat (triglyceride) was ethylesterified and 99% arachidonic acid ethyl ester was separated and purified from the fatty acid ethyl ester mixture comprising 40% arachidonic acid ethyl ester, by ordinary high performance liquid chromatography.

Example 2

Production of triglycerides including at least 5% of triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position (8A8)

After suspending 100 g of an ion-exchange resin support (Dowex MARATHON WBA™: Dow Chemical) in 80 ml of 12.5% aqueous *Rhizopus delemar* lipase solution (Talipase powder, product of Tanabe Seiyaku), the suspension was dried under reduced pressure to obtain the immobilized lipase.

Next, 80 g of the triglyceride containing 40 wt% arachidonic acid obtained in Example 1 (TGA40S), 160 g of caprylic acid, 12 g of the aforementioned immobilized lipase and 4.8 ml of water were reacted at 30°C for 48 hours while stirring (130 rpm). After completion of the reaction, the reaction mixture was removed to obtain the activated immobilized lipase.

A 10 g portion of the immobilized enzyme (*Rhizopus delemar* lipase, support: Dowex MARATHON WBA™) was packed into a jacketed glass column (1.8 x 12.5 cm, 31.8 ml

volume), and a mixed oil containing the TGA40S obtained in Example 1 and caprylic acid in a proportion of 1:2 was passed through the column at a fixed flow rate (4 ml/h) for continuous reaction to obtain 400 g of reacted oil/fat. The column temperature was 40-41°C. The unreacted caprylic acid and free fatty acids were removed from the reacted oil/fat by molecular distillation, and the edible oil/fat was subjected to a purification step (degumming, deacidification, steam distillation, decoloration) to obtain 8A8-containing oil/fat (triglycerides). The 8A8 content of the obtained 8A8-containing oil/fat (triglycerides) was 31.6% as determined by gas chromatography and high performance liquid chromatography. (The proportions of 8P8, 8O8, 8L8, 8G8 and 8D8 were 0.6, 7.9, 15.1, 5.2 and 4.8%, respectively. The fatty acids P, O, L, G and D bound at the triglyceride 2-position represent palmitic acid, oleic acid, linoleic acid, γ-linolenic acid and dihomo-γ-linolenic acid, respectively, 8P8 is 1,3-capryloyl-2-palmitoleyl-glycerol, 8O8 is 1,3-capryloyl-2-oleoyl-glycerol, 8L8 is 1,3-capryloyl-2-linoleoyl-glycerol, 8G8 is 1,3-capryloyl-2-γ-linolenoyl-glycerol and 8D8 is 1,3-capryloyl-2-dihomo-γ-linolenoyl-glycerol.) Separation and purification by high performance liquid chromatography according to an established protocol yielded 96 mole percent 8A8 from the obtained 8A8-containing oil/fat (triglycerides).

Example 3

Production of test capsules

Water was added to 100 parts by weight of gelatin and 35 parts by weight of food grade glycerin for dissolution at 50-60°C to prepare a gelatin coating with a viscosity of 2000 cp. Next, 0.05 wt% vitamin E oil was mixed with the arachidonic acid-containing oil/fat (triglycerides) obtained in Example 1 to prepare Filling

Content 1. Separately, 0.05 wt% vitamin E oil was mixed with oil/fat (triglycerides) containing 32 mole percent of the 8A8 obtained in Example 2 to prepare Filling Content 2. Filling Content 1 was used for molding and drying of capsules by a common method to manufacture soft capsules containing 200 mg of filling per capsule (arachidonic acid-containing edible oil capsules) while Filling Content 2 was also used for molding and drying of capsules by a common method to manufacture soft capsules containing 200 mg of filling per capsule (8A8-containing edible oil capsules). Soft capsules containing olive oil as the filling content were also manufactured as placebo capsules for a human test.

Example 4

Arachidonic acid-containing edible oil capsule ingestion test to determine effects on cognitive responses in healthy individuals

The event related potentials of brain (ERP) was measured by audible (or auditory discrimination) tasks according to the evoked potential measuring guidelines established by the Evoked Potential Examination Standards Committee of the Japan Society of Clinical Neurophysiology. Specifically, an auditory oddball paradigm was utilized for the ERP measurement and both ears of the test subject were stimulated by two different pure tones at frequencies of 1000 Hz and 2000 Hz were through headphones at ratio of 1:4 and in random order, and the subject was instructed to press a provided button when the 2000 Hz tone was heard and to count the number of times heard. The intensity of the tone was 90 dB, the duration was 100 msec and the inter stimulus interval was random between 1000-3000 msec. The approximate number of stimulations in the actual test was 200 per trial and the duration of the test was about 10 minutes. The test was conducted in 2 trials and the event related potentialss of brain were measured. Electroencephalographies (EEGs)

were recorded by using Ag-AgCl electrodes, of which resistance is no greater than 5 kΩ, placed at three points along the mid-line of the scalp (Fz, Cz and Pz, according as international 10-20 displacements), and reference electrodes on both earlobes.

The maximum positive potential between 250-600 msec from the start of the low-frequency auditory simulation to be perceived (a 2000 Hz tone in this experiment) was identified as the intrinsic component P300 which varies in evaluation of selective attention or cognitive response, the time from the start of stimulation was recorded as the P300 latency (stimulation transmission speed) and the height of the potential from the baseline was recorded as the P300 amplitude (the amount of information processing resources).

The human test for the invention was conducted with due consideration to adhering to the spirit of the Helsinki Declaration.

Upon explanation for consent to test participation, 12 consenting healthy individuals (taking no medication, with no abnormal blood test results and with no infarctions as determined by cranial CT scan) were divided into two groups A and B (A: n=7, B: n=5). During a one month period, Group A was administered three of the arachidonic acid-containing edible oil capsules prepared in Example 3 (80 mg/capsule arachidonic acid) for a daily arachidonic acid intake of 240 mg, while Group B was administered three placebo capsules. The event related potentialss of brain were measured before and after capsule ingestion, and the P300 latency and amplitude were analyzed. The participants in Groups A and B were then taken off the capsules for a washout period of one month. After the washout period, Group A was administered the placebo capsules and Group B was administered the arachidonic acid-containing edible oil capsules for one month, and the event related potentialss of brain were measured in the same manner before and

after capsule ingestion (double blind, crossover test).

Blood was taken at the time of event related potentials of brain measurement, and the total lipids were extracted from the serum of each participant by the Folch method. The lipids were fractionated by thin-layer chromatography, the phospholipid fraction was collected, the water was removed by azeotropic distillation with ethanol, and analysis was performed by gas chromatography upon conversion to fatty acid methyl ester with 10% HCl-methanol, to determine the arachidonic acid content of the serum phospholipids.

Fig. 1 shows changes in arachidonic acid contents in serum phospholipids before and after capsule ingestion. The arachidonic acid contents of the serum phospholipids of subjects ingesting the arachidonic acid-containing edible oil capsules were significantly increased after ingestion of the arachidonic acid-containing edible oil capsules, whereas the arachidonic acid contents of the serum phospholipids of subjects ingesting the placebo capsules were unchanged before and after ingestion of the placebo capsules.

Fig. 2 and Fig. 3 show changes in P300 latency and amplitude before and after capsule ingestion. The P300 latency was significantly shortened by 12.3 msec and the P300 amplitude was significantly increased by 1.9 μV with ingestion of the arachidonic acid-containing edible oil capsules as compared to the placebo capsules. A normal shortening in P300 latency of 1.8 msec/year and decrease in P300 amplitude of 0.2 μV/year are known (Goodin DS et al. 1978), and therefore the results of this test indicate an average rejuvenation for cognitive responses of the subjects of 6.8 years based on P300 latency and 9.5 years based on P300 amplitude.

Next, the correlation between P300 (P300 latency and P300 amplitude) and serum arachidonic acid level (phospholipid) was determined by a first-order curve

based on the least square method using total of 48 data obtained by measuring each of 12 subjects 4 times (Fig. 4). For P300 latency, a significant correlation (coefficient of correlation R = -0.27) was found between the arachidonic acid levels, with shorter latencies resulting from increased arachidonic acid levels. For P300 amplitude as well, a significant correlation (coefficient of correlation R = -0.49) was found between the arachidonic acid levels, with larger P300 amplitudes resulting from increased arachidonic acid levels. This constitutes the first demonstration of improvement of cognitive response by ingestion of arachidonic acid-containing edible oil, and the first proof of arachidonic acid as the cause of the effect.

Example 5

8A8-containing edible oil capsule ingestion test to determine effects on cognitive responses in healthy individuals

Upon explanation for consent to test participation in the same manner as Example 4, 16 consenting healthy individuals (taking no medication, with no abnormal blood test results and with no infarctions as determined by cranial CT scan) were divided into two groups A and B (n=8 for each group). During a one month period, Group A was administered three of the 8A8-containing edible oil capsules prepared in Example 3 (72 mg/capsule arachidonic acid) while Group B was administered three placebo capsules, and upon measuring the event related potentialss of brain and recording the latency and amplitude before and after capsule ingestion (double-blind test), the P300 latency was found to be significantly shortened by 16.3 msec and the P300 amplitude significantly increased by 2.4 μV due to ingestion of the 8A8-containing edible oil capsules. These results indicate an average rejuvenation for cognitive responses of the subjects of 9.1 years based on

P300 latency and 12.0 years based on P300 amplitude.

Example 6

Preparation of capsules containing oil/fat (triglycerides) with arachidonic acid as a constituent fatty acid

Water was added to 100 parts by weight of gelatin and 35 parts by weight of food grade glycerin for dissolution at 50-60°C to prepare a gelatin coating with a viscosity of 2000 cp. Next, 50 wt% of the arachidonic acid-containing oil/fat (triglycerides) obtained in Example 1 was mixed with 50 wt% fish oil (tuna oil comprising eicosapentaenoic acid and docosahexaenoic acid in proportions of 5.1% and 26.5%, respectively, of the total fatty acids), and this was combined with 0.05 wt% vitamin E oil to prepare Filling Content 3. Separately, 80 wt% of the arachidonic acid-containing oil/fat (triglycerides) was mixed with 20 wt% fish oil (tuna oil comprising eicosapentaenoic acid and docosahexaenoic acid in proportions of 5.1% and 26.5%, respectively, of the total fatty acids), and this was combined with 0.05 wt% vitamin E oil to prepare Filling Content 4. The 99% arachidonic acid ethyl ester obtained in Example 1 was also combined with 0.05 wt% vitamin E oil to obtain Filling Content 5. Filling Contents 3-5 were used for molding and drying of capsules by a common method to manufacture soft capsules containing 200 mg of filling per capsule.

Example 7

Use in oil infusion solutions

After combining 400 g of the oil/fat (triglycerides) containing 96% 8A8 obtained in Example 2, 48 g of purified egg yolk lecithin, 20 g of oleic acid, 100 g of glycerin and 40 ml of 0.1 N caustic soda and dispersing the mixture with a homogenizer, distilled water for injection was added to 4 liters. This was emulsified

with a high-pressure spray-type emulsifier to prepare a lipid emulsion. The lipid emulsion was dispensed into plastic bags in portions of 200 ml and subjected to high-pressure steam sterilization treatment at 121°C for 20 minutes to prepare oil infusion solutions.

Example 8 Use in juice

A 2 g portion of β-cyclodextrin was added to 20 ml of a 20% aqueous ethanol solution, and then 100 mg of the arachidonic acid-containing triglycerides obtained in Example 1 (comprising 0.05% vitamin E) was added thereto while stirring with a stirrer, and the mixture was incubated at 50°C for 2 hours. After cooling to room temperature (approximately 1 hour), incubation was continued at 4°C for 10 hours under continuous stirring. The produced precipitate was recovered by centrifugal separation, and after washing with n-hexane, it was lyophilized to obtain 1.8 g of a cyclodextrin clathrated compound containing arachidonic acid-containing triglycerides. A 1 g portion of this powder was uniformly mixed with 10 L of juice to prepare juice containing arachidonic acid-containing triglycerides.

CLAIMS

1. A composition with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, containing arachidonic acid and/or a compound with arachidonic acid as a constituent fatty acid.

2. A composition according to claim 1, wherein the compound with arachidonic acid as a constituent fatty acid is an arachidonic acid alcohol ester or a triglyceride, phospholipid or glycolipid containing arachidonic acid as part or all of the constituent fatty acid.

3. A composition according to claim 2, wherein the triglyceride containing arachidonic acid as part or all of the constituent fatty acid is a triglyceride having medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position.

4. A composition according to claim 3, wherein the medium-chain fatty acids are selected from among fatty acids of 6 to 12 carbons.

5. A composition according to claim 4, wherein the medium-chain fatty acids are selected from among fatty acids of 8 carbons.

6. A composition with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, which contains triglycerides including triglycerides containing arachidonic acid as part or all of the constituent fatty acid.

7. A composition according to claim 6, characterized in that the arachidonic acid content of the triglycerides including triglycerides containing arachidonic acid as part or all of the constituent fatty acid is at least 10 wt% of the total fatty acid in the triglycerides.

8. A composition according to claim 6 or 7, wherein the triglycerides including triglycerides

containing arachidonic acid as part or all of the constituent fatty acid are extracted from microbes belonging to the genus *Mortierella*.

9. A composition according to any one of claims 6 to 8, wherein the triglycerides including triglycerides containing arachidonic acid as part or all of the constituent fatty acid are triglycerides containing no eicosapentaenoic acid or containing no more than 1% eicosapentaenoic acid.

10. A composition with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, which contains triglycerides including at least 5 mole percent of triglycerides with medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position.

11. A composition according to claim 10, wherein the medium-chain fatty acids are selected from among fatty acids of 6 to 12 carbons.

12. A composition according to claim 11, wherein the medium-chain fatty acids are selected from among fatty acids of 8 carbons.

13. A composition according to any one of claims 1 to 12 with effects of decline prevention, improvement or enhancement of the normal responses of a healthy person of processing speed or response speed with respect to events selected from the group consisting of auditory stimuli, visual stimuli, olfactory stimuli, gustatory stimuli and somatosensory stimuli, as a cognitive ability.

14. A composition according to any one of claims 1 to 12 with effects of decline prevention, improvement or enhancement of the normal response of concentration of a healthy person with respect to events selected from the group consisting of auditory stimuli, visual stimuli, olfactory stimuli, gustatory stimuli and somatosensory stimuli, as a cognitive ability.

15. A composition according to any one of claims 1 to 12 with effects of decline prevention, improvement or enhancement of the normal response of awareness level of a healthy person, as a cognitive ability.

16. A composition according to any one of claims 1 to 12 with effects of decline prevention, improvement or enhancement of the normal response of discriminatory ability of a healthy person with respect to events selected from the group consisting of auditory stimuli, visual stimuli, olfactory stimuli, gustatory stimuli and somatosensory stimuli, as a cognitive ability.

17. A composition according to any one of claims 1 to 12 with an effect of shortening P300 latency of the event related potentials of brain (P300), as a response index of cognitive ability.

18. A composition according to any one of claims 1 to 12 with an effect of augmenting the P300 amplitude of the event related potentials of brain (P300), as a response index of cognitive ability.

19. A composition according to any one of claims 1 to 18, wherein the composition is a food composition or pharmaceutical composition.

20. A food composition containing arachidonic acid and/or a compound with arachidonic acid as a constituent fatty acid, in an amount such that the daily ingestion for an adult is 0.001-20 g in terms of arachidonic acid.

21. A food composition according to claim 20, wherein the compound with arachidonic acid as a constituent fatty acid is an arachidonic acid alcohol ester or a triglyceride, phospholipid or glycolipid comprising arachidonic acid as part or all of the constituent fatty acid.

22. A food composition according to claim 21, wherein the triglyceride containing arachidonic acid as part or all of the constituent fatty acid is a triglyceride having medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-

position.

23. A food composition according to claim 22, wherein the medium-chain fatty acids are selected from among fatty acids of 6 to 12 carbons.

24. A food composition according to claim 23, wherein the medium-chain fatty acids are selected from among fatty acids of 8 carbons.

25. A food composition characterized in that the composition contains at least 0.001 wt% of triglycerides having medium-chain fatty acids bound at the 1,3-positions and arachidonic acid bound at the 2-position.

26. A food composition according to claim 25, wherein the medium-chain fatty acids are selected from among fatty acids of 6 to 12 carbons.

27. A food composition according to claim 26, wherein the medium-chain fatty acids are selected from among fatty acids of 8 carbons.

28. A composition according to any one of claims 20 to 27, wherein the food composition is a functional food, nutritional supplement food, special health care food or geriatric food.

29. A composition according to any one of claims 1 to 28, which further comprises docosahexaenoic acid and/or a compound with docosahexaenoic acid as a constituent fatty acid.

30. A composition according to claim 29, wherein the compound with docosahexaenoic acid as a constituent fatty acid is a docosahexaenoic acid alcohol ester or a triglyceride, phospholipid or glycolipid comprising docosahexaenoic acid as part or all of the constituent fatty acid.

31. A composition according to claim 29 or 30, characterized in that the arachidonic acid/docosahexaenoic acid ratio (by weight) in a combination of the arachidonic acid and docosahexaenoic acid is in the range of 0.1-15.

32. A composition according to any one of claims 1

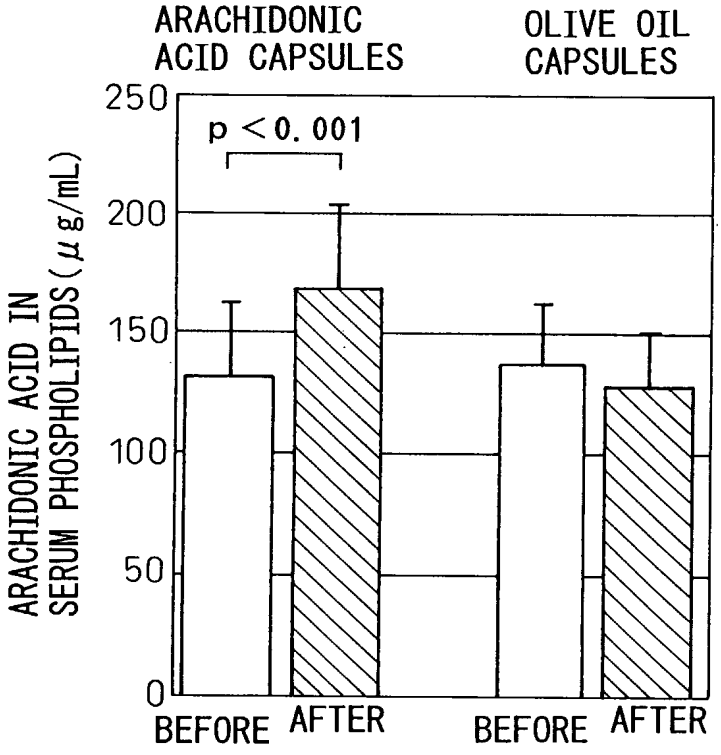
to 31, characterized in that eicosapentaenoic acid is also present in the composition in an amount not exceeding 1/5 of the arachidonic acid in the composition.

33. A process for production of a food composition with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, characterized by combining arachidonic acid and/or a compound with arachidonic acid as a constituent fatty acid, either alone or with a food material containing either essentially no or only a trace amount of arachidonic acid.

34. A method for marketing a composition with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person containing arachidonic acid and/or a compound with arachidonic acid as a constituent fatty acid, the method for marketing a composition with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person being characterized by using a packaging container and/or merchandising tool which indicates that the composition and/or components in the composition have effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person.

35. A composition with effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person, characterized by being a composition containing arachidonic acid and/or a compound with arachidonic acid as a constituent fatty acid which is marketed using a packaging container and/or merchandising tool for the composition indicating that the composition and/or components in the composition have effects of decline prevention, improvement or enhancement of normal responses of cognitive abilities of a healthy person.

Fig. 1



(N = 12, paired t − test)

Fig.2

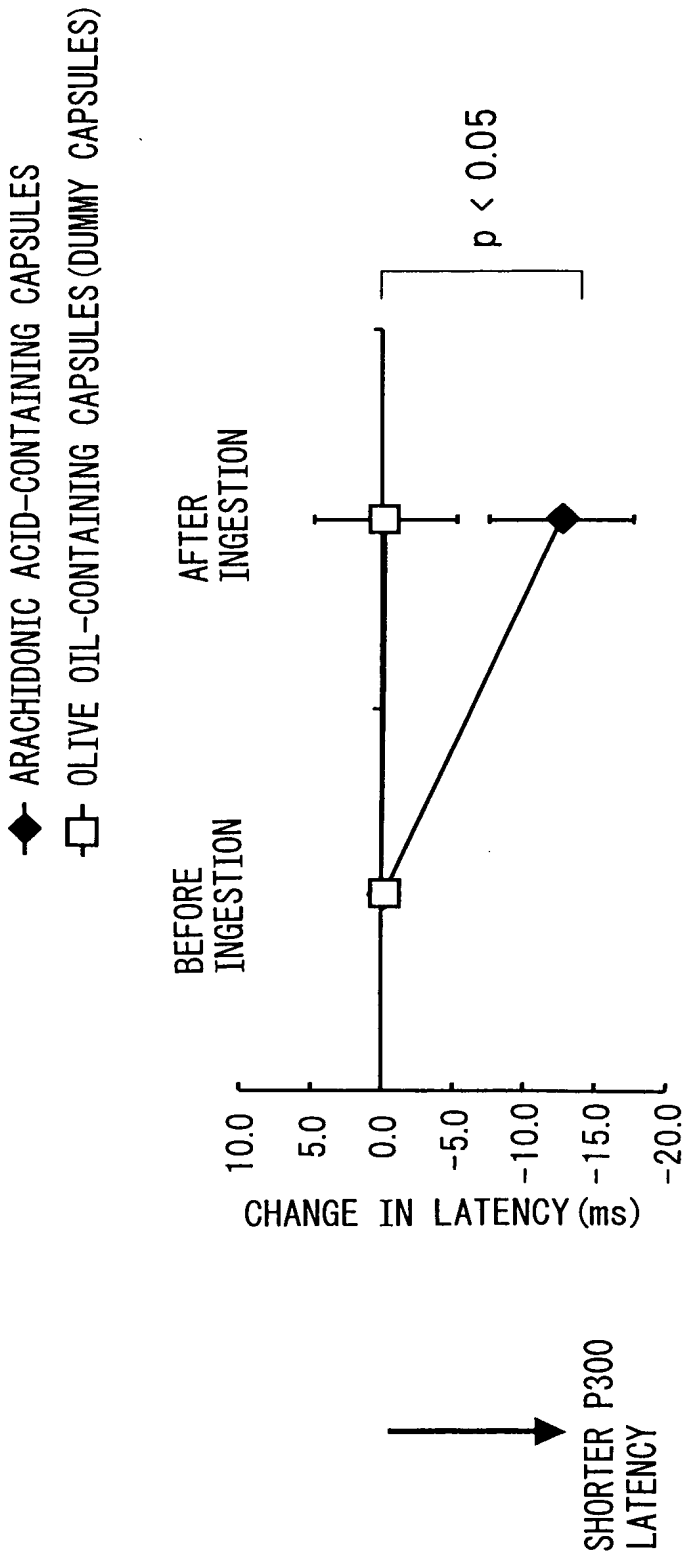

ARACHIDONIC ACID-CONTAINING CAPSULES
OLIVE OIL-CONTAINING CAPSULES(DUMMY CAPSULES)
p < 0.05
AFTER INGESTION
BEFORE INGESTION
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
CHANGE IN LATENCY(ms)
SHORTER P300 LATENCY

Fig.3

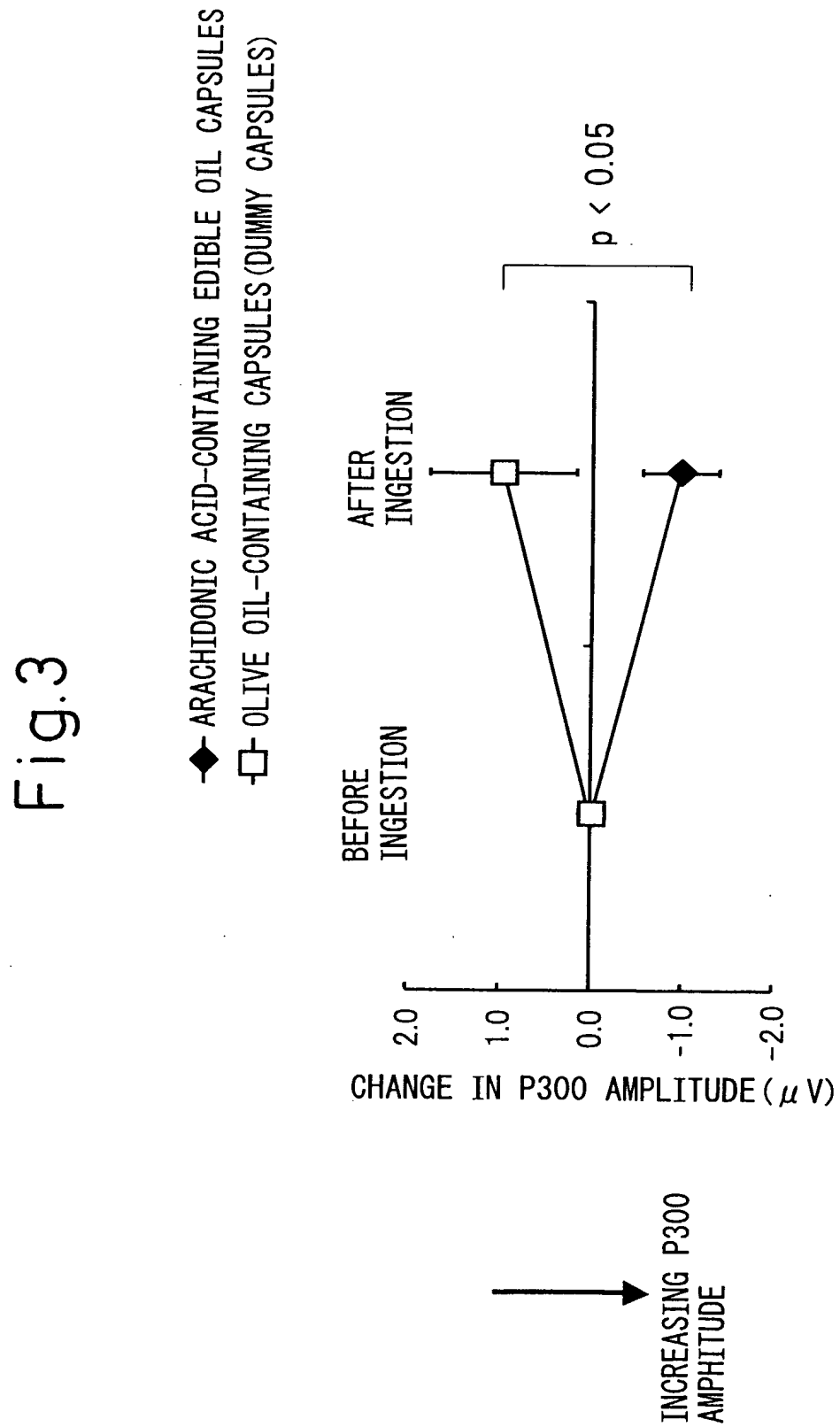
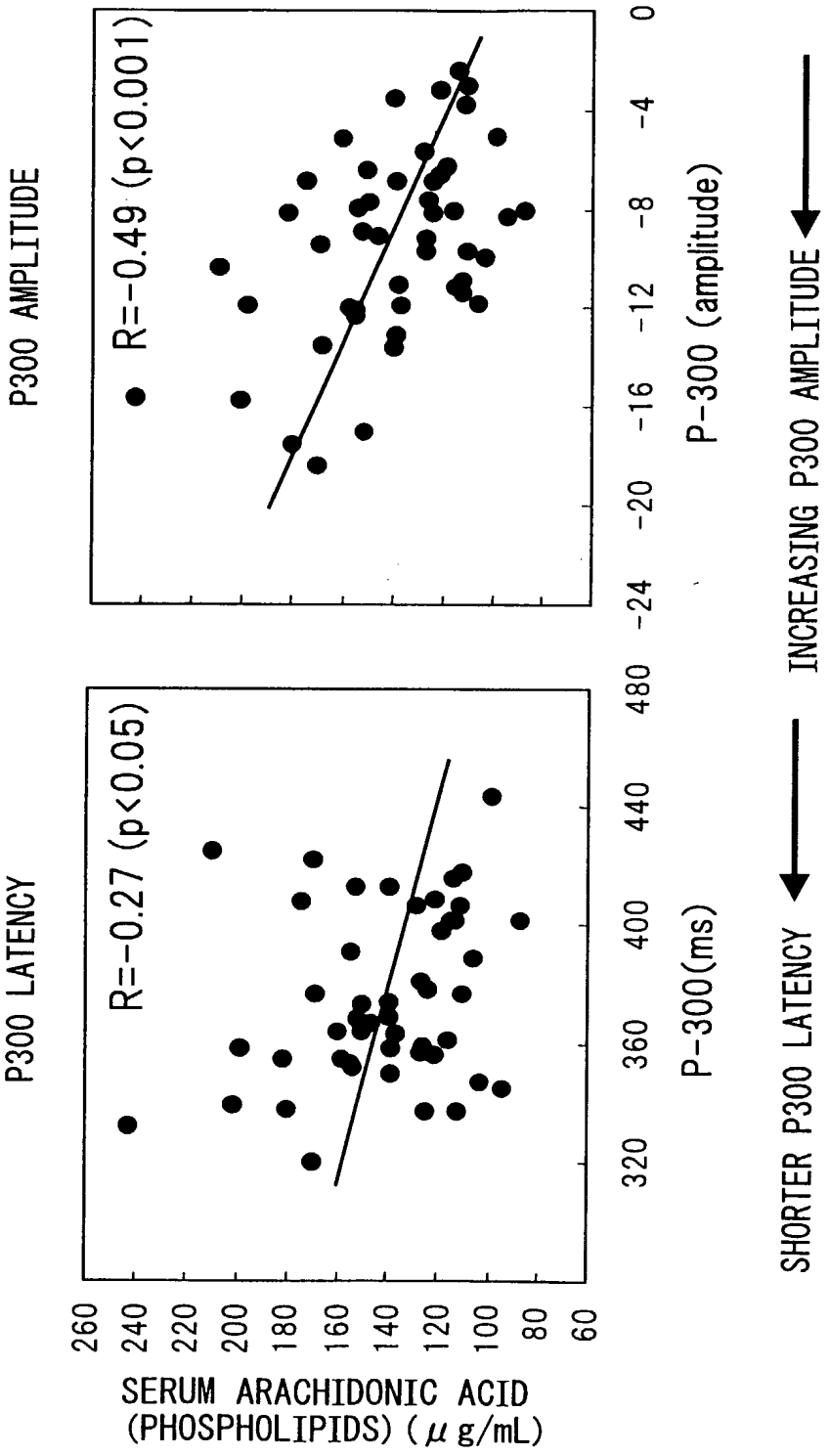

ARACHIDONIC ACID-CONTAINING EDIBLE OIL CAPSULES
OLIVE OIL-CONTAINING CAPSULES(DUMMY CAPSULES)
p < 0.05
AFTER INGESTION
BEFORE INGESTION
2.0
1.0
0.0
-1.0
-2.0
CHANGE IN P300 AMPLITUDE(μV)
INCREASING P300 AMPHITUDE

Fig.4


P300 AMPLITUDE
R=-0.49 (p<0.001)
0
-4
-8
-12
-16
-20
-24
P-300 (amplitude)
INCREASING P300 AMPLITUDE
P300 LATENCY
R=-0.27 (p<0.05)
480
440
400
360
320
P-300(ms)
SHORTER P300 LATENCY
260
240
220
200
180
160
140
120
100
80
60
SERUM ARACHIDONIC ACID
(PHOSPHOLIPIDS) (μg/mL)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP 03/12107

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/202 A61P25/28

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)
IPC 7 A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE, PAJ, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 080 787 A (DOHNALEK MARGARET H ET AL) 27 June 2000 (2000-06-27)	1,2,6, 13-21, 29-35
Y	column 13, line 39-48; claims 1-7,16,17,25	1-35
X	WO 96 10922 A (MILUPA AG ;KOHN GERHARD (DE); SAWATZKI GUENTHER (DE); SCHWEIKHARDT) 18 April 1996 (1996-04-18)	1,2,6, 13-21, 29-35
Y	page 4, line 5 -page 5, line 34; claims 1-4	1-35
X	US 6 034 130 A (BERTHOLET RAYMOND ET AL) 7 March 2000 (2000-03-07)	1-7,9-35
Y	column 2, line 18-61; claims 1,8; example 1; table 3	1-35

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2004	03/02/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Greif, G

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 239 022 A (NESTLE SA) 11 September 2002 (2002-09-11) claims 1-3,10-14	1,2,6, 33-35
X	WO 96 21037 A (MARTEK BIOSCIENCES CORP ;KYLE DAVID J (US)) 11 July 1996 (1996-07-11)	1,2,6-9, 13-21, 33-35
Y	page 4, line 1 -page 5, line 14; claims 1-34	1-35
X	US 4 668 704 A (TARNAWSKI ANDRZEJ S ET AL) 26 May 1987 (1987-05-26) column 2, line 6-52	1,6, 33-35
X	KOLETZKO B ET AL: "Polyunsaturated fatty acids in human milk and their role in early infant development." JOURNAL OF MAMMARY GLAND BIOLOGY AND NEOPLASIA. UNITED STATES JUL 1999, vol. 4, no. 3, July 1999 (1999-07), pages 269-284, XP009024376 ISSN: 1083-3021	1-3,6,7, 10, 13-22, 25,29, 30,33-35
Y	page 270, right-hand column, paragraph 1; table 1	1-35
Y	CARLSON S E: "Docosahexaenoic acid and arachidonic acid in infant development." SEMINARS IN NEONATOLOGY: SN. ENGLAND OCT 2001, vol. 6, no. 5, October 2001 (2001-10), pages 437-449, XP008024374 ISSN: 1084-2756 abstract p. 438, "Effects of essential fatty acid deficiency on brain composition and function"	1-35
Y	AUESTAD NANCY ET AL: "Visual, cognitive, and language assessments at 39 months: a follow-up study of children fed formulas containing long-chain polyunsaturated fatty acids to 1 year of age." PEDIATRICS. UNITED STATES SEP 2003, vol. 112, no. 3 Pt 1, September 2003 (2003-09), pages e177-e183, XP009024347 ISSN: 1098-4275 abstract	1-35

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WILLATTS P ET AL: "Effect of long-chain polyunsaturated fatty acids in infant formula on problem solving at 10 months of age" LANCET, XX, XX, vol. 352, no. 9129, 29 August 1998 (1998-08-29), pages 688-691, XP004265590 ISSN: 0140-6736 page 690, right-hand column, line 1-7; table 1 ---	1-35
Y	LUCAS A A ET AL: "Efficacy and safety of long-chain polyunsaturated fatty acid supplementation of infant-formula milk: a randomised trial" LANCET, XX, XX, vol. 354, no. 9194, 4 December 1999 (1999-12-04), pages 1948-1954, XP004262925 ISSN: 0140-6736 page 1948, right-hand column, paragraphs 1,2 -----	1-35

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/JP 03/12107

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(54) AGGREGATES OF EYEBALLS OF CRUSTACEANS, USAGE, FOODS COATAINING THE SAME, AND PRODUCTION PROCESSES

(57) [Problems] The present invention provides a method for efficiently utilizing crustaceans, particularly, krill as resources. More particularly, the present invention provides a method for efficiently recovering and utilizing lipophilic effective substances such as carotenoids, highly-unsaturated fatty acids, and phospholipids which are components thereof.

[Means for Solving the Problems] There are provided an aggregate of eyeballs of a crustacean; use of an aggregate of eyeballs of a crustacean as a supply source of a carotenoid, highly-unsaturated fatty acids and/or phospholipids; a food or a nutritional hearth supplement for furnishing a carotenoid, highly-unsaturated fatty acids/or phospholipids, which contains an aggregate of eyeballs of a crustacean as it is or in a pulverized state; and a process for producing an aggregate of eyeballs of a crustacean containing reducing a water content of the crustacean to facilitate the separation of the eyeballs and separating the eyeballs from a fish body by physical impact, followed by recovering them. The crustacean is preferably krill or mysid.

EP 1 743 531 A1

Description

Technical Field

[0001] The present invention relates to a composition abundantly containing, for example, carotenoids, highly-unsaturated fatty acids and/or phospholipids, which can be utilized in the fields of food, health food and feed.

Background Art

[0002] Astaxanthin is a member of the carotenoid family and is known to have a characteristic red color. The pigment thereof is used not only as a food colorant, but also as a pigment for fish feed and, further, in recent years, its high antioxidative property is attracted and utilized in a health food and the like. As for matters containing natural astaxanthin, salmon's muscles are well known. Besides, natural astaxanthin is contained in many of other animals and plants, and krill is one of them.

[0003] In crustaceans such as krill, carotenoids, highly-unsaturated fatty acids, phospholipids and the like are contained, and utilization thereof have been conventionally aimed for. However, contents thereof in the crustaceans are not always high in comparison with other raw materials.

[0004] Carotenoids, highly-unsaturated fatty acids, phospholipids and the like are essential components for humans, animals, fish and shellfish, and those originated from various types of raw materials are used. As for commercial supply sources thereof, those which contain them in high concentrations and are easily purified and the like are preferred.

[0005] For example, in the case of the krill, a dried product thereof, namely, krill meal is used as a supply source of extraction of carotenoids. The carotenoids content of fresh krill (water content: about 80%) is from 30 to 40 ppm, but a dried product such as krill meal (water content: about 10%) contains more than 200 ppm of carotenoids. When lipids in the krill meal are extracted by using conventional extraction methods (lipid content is about 10% dry basis), the extracted oil contains about 2000ppm of carotenoids.

[0006] On the other hand, natural carotenoids available in the market are mainly obtained from raw materials including yeasts such as *Pfaffia rhodozyma,* the green algae such as *Haematococcus pluvialis* and *Paracoccus carotinifaciens,* and the carotenoids concentration of the products thereof are from 0.5 to 5%. In the case of krill, the extracted oil thereof contains about 2000ppm, namely, 0.2%, and further concentration process is necessary for market use. Therefore, the production cost for concentration process increases and it cannot be mentioned to be a competitive raw material.

[0007] It has been already reported in 1970s that the concentration of astaxanthin in eyeballs of krill is high (see Non-patent Documents 1 and 2). The present applicant has also reported that the astaxanthin is contained in krill in a high concentration, and particularly, about fourth part of astaxanthin in whole krill is concentrated in eyeballs thereof (see Non-patent Document 3).

[0008] There is no conventional art of using the eyeballs of the crustaceans for specific applications, and as shown in Patent Document 1, a specific type of crustaceans is used and eyeballs thereof are removed when they become obstacles for a specific object.

Non-patent Document 1: Shoku no Kagaku (Science of Food), 44, 91-103, 1978
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Non-patent Document 3: Nippon Suisan Kaisha, Ltd., Central Research Laboratory, Report No. 11, pp. 18-27, 1976
Patent Document 1: JP-A-2000-166516

Disclosure of the Invention

Problems that the Invention is to Solve

[0009] The object of the present invention is to provide a method for efficiently utilizing crustaceans, particularly, krill as resources. More particularly, the object of the present invention is to provide a method for efficiently recovering and utilizing lipophilic effective substances such as carotenoids, highly-unsaturated fatty acids, and phospholipids which are components thereof.

Means for Solving the Problems

[0010] An object of the invention is an aggregate of eyeballs of a crustacean, particularly krill or mysid.

[0011] Another object of the invention is method of using an aggregate of eyeballs of crustacean, particularly krill or mysid, as a supply source of carotenoids, highly-unsaturated fatty acids and/or phospholipids.

[0012] Still another object of the invention is a food or a nutritional supplement for furnishingcarotenoids, highly-unsaturated fatty acids/or phospholipids, said food or said nutritional supplement comprising an aggregate of eyeballs

of crustacean, particularly krill or mysid, as it is or in a pulverized state.

[0013] Even still another object of the invention is process for producing an aggregate of eyeballs of crustacean, particularly krill or mysid, comprising: reducing a water content of the crustacean; and releasing the eyeballs from fish bodies by physical impact, followed by recovering them. For example, the water contents thereof can be reduced by heating, and after drying is performed such that the water contents thereof are reduced to 15% or less, the eyeballs can efficiently be recovered by using an oscillating sieve.

[0014] Even still further another object of the invention is a method for producing a highly-pure aggregate of eyeballs of a crustacean by removing foreign matters other than the eyeballs with a wind grading machine.

Advantage of the Invention

[0015] According to the invention, eyeballs of crustaceans, which are foreign matters when they are merely mixed in whole crustaceans, are converted into useful resources by allowing them into an aggregate of solely eyeballs. This aggregate of the eyeballs can be used as an efficient supply source of carotenoids, highly-unsaturated fatty acids, phospholipids or the like, and can be added to a food, a nutritional supplement or the like as it is or in a pulverized state.

[0016] By utilizing a property of the eyeballs such that they are released by reducing the water contents of the crustaceans by the method according to the invention, namely, heating or the like, the eyeballs being small in size can efficiently be recovered.

[0017] Further, fish bodies of the crustaceans remaining after the recovery of the eyeballs can be utilized as krill meal or the like as usual, since they have only been subjected to heating.

Best Mode for Carrying Out the Invention

[0018] As for crustaceans to be used in the present invention, any type of crustacean can be used, so long as it contains effective components such as carotenoids, highly-unsaturated fatty acids, phospholipids and the like. The preferable examples thereof include krill or mysid in which contents of carotenoids, particularly, astaxanthin is high. Although other shrimps and crabs can be used, when bodies thereof are large in size, eyeballs thereof are sometimes unduly hard, and in that case, the eyeballs are crushed and then used. Further, since effective components are varied in accordance with types of crustaceans, types of crustaceans are selected in accordance with the purposes.

[0019] The term "eyeball of a crustacean" as used herein means a portion connected with the head portion of the crustacean by eye-stalk and is intended to indicate solely an eyeball or eyeball containing eye-stalk.

[0020] The term "aggregate of eyeballs" as used herein does not refer to a unit of one, two, or 100 eyeballs but refers to a unit of kg such they are collected to an extent which can industrially be utilized. As for the aggregate of eyeballs, when it is used as a supply source of carotenoids, highly-unsaturated fatty acids and/or phospholipids, those in which the ratio of the eyeballs to the entire weight is concentrated to 20 to 100% can be used. Further, when it is used in a nutritional food or the like as it is, those in which the ratio of the eyeballs is concentrated to from 80 to 100% are preferable. The aggregate in which the ratio of the eyeballs is about 20% can be obtained only by performing drying to facilitate release of the eyeballs, releasing the eyeballs by physical impact, and then recovering the eyeballs. By further subjecting it to wind grading mashine, grading utilizing inclination or the like, an aggregate in which the ratio of the eyeballs is from 20 to 100% can be obtained.

[0021] Examples of main effective components contained in the eyeballs of crustaceans include carotenoids, highly-unsaturated fatty acids and phospholipids. Examples of carotenoids include astaxanthin, astaxanthin monoester, and astaxanthin diester; examples of highly-unsaturated fatty acids include eicosapentaenoic acid, docosahexaenoic acid, and linolenic acid; and examples of phospholipids include phosphatidyl choline. Since these components are physiologically active in humans, animals, fish and shellfish or the like or essential nutrients for them, they are utilized in a food, a feed, a supplement or the like.

[0022] The aggregate of eyeballs of crustaceans according to the invention can either be used as a food, a feed, or a supplement as it is or be used after being adding thereto in a pulverized form. For example, the eyeballs obtained from heated krill are raw materials as they are such that they only have a taste of dried shrimp, have no strong taste, have a light texture at eating, are fine granular without being crushed and have versatility. Such eyeballs as described above can be added to any foods, so long as the foods are fond of such a shrimp flavor. The eyeballs are also suitable for feeds for fish and shellfish, feeds for livestock, and feeds for growing poultry. Further, they can be utilized as supplements in the form of capsules or tablets.

[0023] The aggregate of eyeballs according to the invention can also be used as a supply source from which astaxanthin or the like is extracted. In this case, it can be extracted far more efficiently in comparison with the case in which it is extracted from whole bodies of crustaceans. The eyeballs are crushed and are subsequently subjected to extraction by using extraction solvents and extraction conditions appropriate for products to be extracted. For example, when the eyeballs of krill are subjected to extraction by using hexane, a lipid in which the concentration of astaxanthin is 4% or

more can be obtained.

[0024] A process for producing an aggregate of eyeballs according to the invention utilizes a property in which reduction of water contents of the crustaceans facilitates release of the eyeballs of crustaceans from other portions. According to the process, the aggregate of eyeballs can be recovered without using any specific apparatus or reagent. This is due to the fact that reduction of the water contents of the crustaceans such as krill or mysid decreases strength of eye-stalks supporting eyeballs and releases eyeballs with weak physical impact.

[0025] As for methods of reducing the water content, drying by heating is most efficient. For example, fresh crustaceans are subjected to a primary heating by means of, for example, boiling, steaming, microwave-heating. Joule heating, or induction heating to raise a product temperature to a boiling point of water or less. Although a given temperature is necessary to denaturalize protease inherent in the krill and to suppress decomposition of protein during processing, unduly high temperature may cause deterioration of lipids, or depletion of useful materials such as astaxanthin. By similar reasons, this primary heating is preferably performed in a short period of time. Further, it is necessary to pay attention not to break eyeballs at the time of heating. Subsequently, drying is performed by hot wind or the like. Heating is preferably performed such that a temperature of a subject (eyeballs or the like) does not come to be more than 100°C. Specifically, drying is preferably performed with hot wind of 120°C or less and a net conveyor type dryer or a fluid bed dryer which can continuously perform drying is used. It is necessary to pay attention as much as possible not to break the eyeballs at the time of drying.

[0026] As described above, since the eyeballs of the crustaceans subjected to heating and drying can be released with weak physical impact, the eyeballs are released with such a degree of vibration as not break fish bodies of the crustaceans, and then collected by a sieve with an appropriate mesh size.

[0027] Drying is performed to such an extent that the water content thereof come to be 15% or less. When drying is not sufficiently performed, yield is deteriorated.

[0028] Other drying methods than heating, such as freeze-drying, wind-drying and the like may be used.

[0029] Eyeballs, which are released by heating and physical impact and then recovered, contain not only eyeballs but also foreign matters such as antennas and maxillipeds. In order to obtain a highly pure aggregate of eyeballs, it is necessary to remove these foreign matters by wind classification, inclination grading or the like. Since the degree of purity of the aggregate of eyeballs is varied depending on the purposes of applications, it is conducted in accordance with such purposes. Since the foreign matters are fundamentally derived from crustaceans, there are many cases in which the foreign matters do not give any problem depending on applications. When it is required to strictly eliminate the foreign matters, remaining foreign matters are removed by subjecting the aggregate of eyeballs to a color sorter machine or the like.

[0030] Since individual krill weight varies widely, as approximate numbers, the total weight of random sampled 100 dried krill was 11 g and then the weight of collected 100 eyeballs of dried krill arbitrarily was 0.1 g. Since one individual krill has two eyeballs, yield of eyeballs of the krill is theoretically about 1.8% of the weight of the krill. When fresh krill is dried, the weight thereof becomes one fifth. Therefore, on calculation, about 3.6 kg of eyeballs of dried krill can theoretically be obtained from one ton of fresh krill.

[0031] Hereinafter, examples of the invention are described. However, the invention should not be limited thereto in any way.

Example 1

Method

[0032] Fresh krill were subjected to a primary heating by steaming to raise a product temperature to a boiling point of water or less (about 90°C), and thereafter, immediately subjected to drying. The drying was performed by using a net conveyor type dryer which can be continuously operated with hot wind drying at 120°C or less. Attention was paid as much as possible at the time of drying not to break the eyeballs. Further, as the drying was proceeded (water content was decreased), the eyeballs came to be easily released from fish bodies of the krill and such release sometimes occurred during drying. These separated eyeballs in process were collected by a cyclone separator installed at dryer exhaust outlet, a moving scraper installed at a lower portion of the net conveyor or the like.

[0033] After being subjected to drying, the krill was subjected to a sieve. The sieve used was an oscillating sieve (Type 403, Dalton Co., Ltd.; screen wire diameter: 1.2 mm; opening (square): 6.0 mm). The foreign matters other than eyeballs, which passed through the mesh, were separated with a wind grading machine.

Results

[0034] After drying, approximately 100% of the eyeballs of the krill were released from fish bodies by physical impact. In separated fish bodies, eyeballs such as black spots were not found by a naked eye observation. Matters passed

through the mesh were eyeballs and foreign matters such as antennas and maxillipeds. At this stage, a ratio of eyeballs increased to about 20 to 30%. Since the ratio of the eyeballs in the weight of the dried krill is about 1.8%, it can be mentioned that it was concentrated by from about 10 to 15 times.

[0035] Since the mixed foreign matters such as antennas and maxillipeds are lighter than eyeballs in weight, they were able to be separated by the wind grading machine. Finally, 16 g of aggregate containing substantially only eyeballs was obtained from 2450 g of dried krill. Since the ratio of the eyeballs in the dried whole krill is theoretically about 1.8%, the theoretical yield of the eyeballs was 36%.

[0036] By examining conditions and adjusting functions of the wind grading machine such that the mesh size of the sieve was set to be from 2.5 to 3.0 mm through which eyeballs just passed, the aggregate containing substantially only eyeballs was able to be recovered with a recovery rate of about 50%. Further, by the separation with an inclined conveyor-belt machinery (an apparatus for separating a spherical body (eyeball) from non-spherical body by transporting on an inclined conveyor-belt machinery), an aggregate containing substantially only eyeballs was able to be recovered with a theoretical yield of 80% or more.

[0037] The obtained aggregate of krill eyeballs was subjected to chemical component analysis. The results are shown below.

water content: 1.5% (measured by infrared moisture meter)

lipid content: 8.5% (measured by Brigh-Dyer method)

total carotenoid: 2990 ppm (After krill eyeballs are dissolved in acetone, absorbance at 478 nm was measured.)

lipid composition: shown in Table 1 (Lipid classes were measured by using TLC-FLD.)

fatty acid composition: shown in Table 2 (Total lipid was methylated by BF_3 methanol method, and then measured by GC.)

Table 1

Components	Content (%)
Wax	2.2±0.5
Triacylglycerol	15.4±0.9
Free fatty acid	1.7±0.2
Sterol	5.8±0.3
Phospholipid	74.9±1.1

Table 2

FA species	Content (%)
C14:0	5.6
C16:0	19.2
C16:1n-7	2.1
C16:2n-4	1.5
C18:0	1.6
C18:1n-9	6.3
C18:1n-7	5.7
C18:2n-6	1.7
C18:3n-3	3.4
C18:4n-3	4.5
C20:4n-6	0.6
C20:4n-3	0.5
C20:5n-3	18.4
C22:5n-3	0.4
C22:6n-3	22.3

(continued)

FA species	Content (%)
Others	6.2

Example 2

[0038] A quick frozen boiled krill on a boat was thawed, heated and dried, and then eyeballs of the krill and a fish body without eyeballs were obtained by a method according to the present invention and were subjected to astaxanthin extraction. A freeze-dried boiled krill was used as reference.

[0039] 600 mg of each of crushed samples was weighed and was extracted three times by using 50 times its volume of hexane, and then a hexane-soluble lipids containing astaxanthin was obtained. After thus-obtained lipids were concentrated and weighted. Subsequently, it was dissolved in acetone, and then absorbance at 478 nm was measured to determine astaxanthin concentration.

[0040] As shown in Table 3, the concentration of astaxanthin in the lipids of the eyeballs of krill was considerably high in comparison with that of the whole body, and only by performing a hexane extraction, the lipids in which the astaxanthin concentration was about 4% was able to be obtained. When astaxanthin is extracted from krill, by extracting astaxanthin not from the whole body but from collected eyeballs, a lipid containing astaxanthin in a high concentration can efficiently be obtained by a small amount of solvent.

[0041] The reason why the content of astaxanthin in the heated and dried product of the krill is lower than that of the freeze-dried product of the krill as a reference, is that astaxanthin is possibly depleted by heating. In this Example, in order to sufficiently perform drying, heating-drying was performed for 7 hours at 80°C. In this regard, it is preferable to select a heating temperature and a heating period of time allowing such depletion of astaxanthin to be minimal. However, even when this depletion is taken into consideration, it is confirmed that the method for extracting astaxanthin in accordance with the method of the invention is sufficiently useful.

Table 3

	Lipid content (%)	Astaxanthin content (ppm)	Astaxanthin concentration in lipid (%)
Eyeballs of krill	4.61	1863	4.04
Fish body of krill (eyeballs removed)	12.19	37	0.03
Heated and dried product of krill* (whole body of krill)	12.05	70	0.06
Freeze-dried product of krill (whole body of krill)	12.57	129	0.10
(* : Data of heated and dried product of krill is a calculated value from data of eyeballs and fish body.)			

Example 3

[0042] Four parts of microcrystalline cellulose and one part of magnesium stearate were mixed to one part of the lipids containing astaxanthin extracted from the eyeballs according to Example 2. Then, the resultant mixture was subjected to tablet-making, to thereby produce tablets.

Example 4

[0043] To the fat containing astaxanthin extracted from the eyeballs according to Example 2, 1% each of vitamin C and vitamin E were added. Then, the resultant mixture was filled in dark-brown gelatin-made soft capsules in accordance with an ordinary method, to thereby produce capsules.

Example 5

[0044] Five percent of lipids containing astaxanthin extracted from the eyeballs according to Example 2, 9% of microcrystalline cellulose, 75% of sucrose, 1% of magnesium stearate and 10% of ascorbic acid were mixed with one another.

Then, tablets were produced from the resultant mixture by an ordinary method.

Industrial Applicability

[0045] An aggregate of eyeballs of a crustacean according to the present invention can be used as a supply source of effective components such as a carotenoid, highly-unsaturated fatty acids or phospholipids. It can be used to a food, feed, nutritional supplement and the like as it is or in a pulverized state, and it can also be used as a raw material for extracting the carotenoid, the highly-unsaturated fatty acids, the phospholipids or the like. The eyeballs of the crustacean, which have conventionally been treated as rather obstacles and impulities, can be utilized in a useful manner.

Claims

1. An aggregate of eyeballs of a crustacean.

2. The aggregate of eyeballs according to claim 1, wherein the crustacean is krill or mysid.

3. A method of using an aggregate of eyeballs of a crustacean as a supply source of carotenoids, highly-unsaturated fatty acids and/or phospholipids.

4. The method of using an aggregate of eyeballs of a crustacean according to claim 3, wherein the aggregate of eyeballs of a crustacean is subjected to a solvent extraction to thereby obtain a carotenoid-containing lipid.

5. The method of using an aggregate of eyeballs of a crustacean according to claim 3 or 4, wherein the crustacean is krill or mysid.

6. A food or a nutritional supplement for furnishing carotenoids, highly-unsaturated fatty acids/or phospholipids, said food or said nutritional supplement comprising an aggregate of eyeballs of a crustacean as it is or in a pulverized state.

7. The food or the nutritional supplement according to claim 6, wherein the crustacean is krill or mysid.

8. A process for producing an aggregate of eyeballs of a crustacean comprising:

   reducing a water content of the crustacean; and
   releasing the eyeballs from a fish body by physical impact, followed by recovering them.

9. The process for producing an aggregate of eyeballs of a crustacean according to claim 8, wherein the water content is reduced by heating.

10. A process for producing an aggregate of eyeballs of a crustacean comprising:

    drying the crustacean by reducing a water content thereof to 15% or less by heating; and
    subjecting the crustacean to vibrate on a sieve to thereby recover the eyeballs.

11. A process for producing a highly-pure aggregate of eyeballs of a crustacean comprising:

    drying the crustacean by reducing a water content thereof to 15% or less by heating;
    subjecting the crustacean to vibrate on a sieve, followed by recover the eyeballs; and
    removing a foreign matter other than the eyeballs with a wind grading machine or an inclined grading machine.

12. The process for producing a highly-pure aggregate of eyeballs of a crustacean according to any one of claims 8 to 11, wherein the crustacean is krill or mysid.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] A23L1/33, A23K1/16, A23L1/30, A61K31/122, 31/202, 31/683, 35/56, A61P3/02

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)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	JP 56-48129 B (Director General of National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries), 13 November, 1981 (13.11.81), (Family: none)	1,2,8-12 3-7
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Y	Toyosuke IMAKI, "Okiami", Shoku no Kagaku, 15 April, 1973 (15.04.73), Vol.11, pages 74 to 86	3-7

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 May, 2005 (10.05.05)	07 June, 2005 (07.06.05)
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International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Toru HIDAKA et al., Shokuhin Tenkabutsu Jiten, Kabushiki Kaisha Shokuhin Kagaku Shinbunsha, 01 October, 2001 (01.10.01), page 56	3-7

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REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- JP 2000166516 A **[0008]**

Non-patent literature cited in the description

- *Shoku no Kagaku,* 1978, vol. 44, 91-103 **[0008]**
- *Shoku no Kagaku,* 1973, vol. 11, 74-86 **[0008]**
- *Nippon Suisan Kaisha, Ltd., Central Research Laboratory,* 1976, vol. 11, 18-27 **[0008]**

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(54) **Title:** USE OF TRIGLYCERIDE OILS CONTAINING GAMMA-LINOLENIC ACID RESIDUES AND LINOLEIC ACID RESIDUES FOR THE TREATMENT OF NEURODEGENERATIVE DISEASE

(57) **Abstract:** A method is provided for treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a triglyceride oil containing both γ-linolenic acid and linoleic acid residues as triglyceride ester, the ratio of γ-linolenic acid to linoleic acid residues at the sn-2 position of the triglyceride being at least 0.8; the amount of γ -linolenic acid residues at the sn-2 position being at least 18 %, wherein the oil is administered at a dose sufficient to maintain or elevate TGF- β1 levels in the patient at a therapeutic level. Preferably the method is that wherein the therapeutic level is such as to produce a TGF- β1/TNF-α ratio of at least 0.5 in blood of a patient, after 18 months of daily dosing. Preferre oils are Borage or Mucor oils having at least 35 % of the sn-2 position fatty acid residues as γ-linolenic acid.

WO 2004/100943 A1

TREATMENT OF NEURODEGENERATIVE CONDITIONS.

The present invention relates to a method for treating neurodegenerative conditions, particularly those in which increase in transforming growth factor β (TGF-β) is beneficial, particularly TGF-β1. More particularly the present invention provides treatment for conditions such as multiple sclerosis and the degenerative sequelae associated with head trauma, stroke and intracranial bleeds, whereby neuronal function is improved or restored from an impaired condition. Further provided are novel use of known and novel compounds comprising unsaturated fatty acid moieties for the manufacture of medicaments capable of effectively treating such conditions, more particularly being capable of achieving previously unattained levels of success with regard to recovery of neurological function.

It is well reported in the literature that essential fatty acids (EFAs) of the n-3 and n-6 unsaturation pattern have beneficial effect in a wide variety of human physiological disorders. WO 02/02105 (Laxdale Limited) describes their beneficial use for an extremely wide range of diseases and as a general nutritional supplement. Harbige (1998) Proc. Nut. Soc. 57, 555-562 reviewed the supplementation of diet with n-3 and n-6 acids in autoimmune disease states, and particularly noted evidence of benefit of γ-linolenic (GLA) and/or linoleic acid (LA) rich oils, such as borage oil, in reducing clinically important signs and symptoms of rheumatoid arthritis.

Two studies on multiple sclerosis (MS) patients are noted that indicate that relapse and severity of the disease might be reduced by treatment with oils containing n-6 acid moieties (Miller et al (1973) and Bates et al (1978)), but a further study failed to confirm this effect (Paty et al (1978). These papers report that supplementation of human patients with about 20g/day of linoleic acid (18:2n-6) affected duration and severity of relapses of multiple sclerosis such that relapses were less frequent, less severe and of shorter duration than controls. Bates noted that a mixture of linoleic acid and γ-linolenic acid had been suggested back in 1957 to be possibly more efficacious in treating inflammation and autoimmune diseases and set out to investigate this in the trial. However, it was found that where this combination

was tried, at 3g oil per day (Naudicelle Evening Primrose oil) patients who had relapses became more ill on the trial oil than on the control.

Meta analysis of these linoleic acid studies by others (Dworkin et al (1984)) demonstrated reduced relapse rate and severity with a decrease in the degree of long-term progression of the disease in patients with mild multiple sclerosis. Later open studies of patients with multiple sclerosis suggest that low fat diet and/or manipulation of dietary n-3 and n-6 fatty acids may be beneficial (Swank & Grimsgaard (1988); Harbige et al (1990).

Although the aetiology of MS remains unknown, strong evidence suggests the presence of autoimmune mechanisms in the disease pathogenesis [Martino & Hartung 1999]. Studies have shown that MS patients have a much higher number of neuro-antigen e.g. myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) autoreactive T-cells which are in an increased state of activation compared with healthy controls [Fredrikson et al 1994, Kerlero de Rosbo et al 1993, 1997, Chou et al 1992, Ota et al 1990, Burns et al 1999, Zhang et al 1994, Tejada-Simon et al 2001]. The actual processes of axonal damage e.g. chronic inflammation, demyelination and astrogliosis in MS is complex but white matter inflammation and demyelination are considered to determine disease severity, whilst recent studies suggested that axonal damage in MS begins in the early stages of the disease and contributes to disability (De Stefano et al, 2001).

Experimental autoimmune encephalomyelitis (EAE) is the most frequently used animal model for immune mediated effects of MS. Studies in the guinea-pig have shown that linoleic acid partially suppresses the incidence and severity of EAE (Meade et al (1978)). Using γ-linolenic acid-rich oils from fungal or plant sources, complete protection was demonstrated in both rats and mice (Harbige et al (1995), 1997b). These investigations demonstrated disease modifying effects of linoleic acid and γ-linolenic acid on clinical and histopathological manifestations of EAE. Depending on dose, γ-linolenic acid was fully protective in acute rat EAE whereas linoleic acid had dose-dependent action on the clinical severity but did not abolish it.

Despite these experimental findings, it is recognised that the human disease, multiple sclerosis, is highly complex and can be conversely exacerbated and ameliorated by the activity of T-cells and other immune response factors. It is thought that the n-6 fatty acids promote autoimmune and inflammatory disease based upon results obtained with linoleic acid only. TGF-β and PGE_2 production has been shown to be increased non-specifically in γ-linolenic acid fed mice *ex vivo*; but whilst TGF-β has been reported to protect in acute and relapsing EAE ((Racke et al (1993); Santambrogio et al (1993)), PG inhibitors such as indomethacin augment, and thus worsen, the disease (Ovadia & Paterson (1982)).

Cytokines are implicated in the pathogenesis of MS, with many studies showing an increase in myelinotoxic inflammatory cytokines (TNF-α, IL-1β and IFN-γ) coinciding with the relapse phase of the disease. Conversely, levels of the anti-inflammatory and immunosuppressive cytokine transforming growth factor-beta1 (TGF-β1) appear to be reduced during a phase of relapse and increase as the patient enters remission. Thus the balance between biologically active TGF-β1 and the pro-inflammatory TNF-α, IL-1β and IFN-γ appears to be dysregulated during MS relapse-remission.

During natural recovery phase from EAE, TGF-β-secreting T-cells inhibit EAE effector cells, TGF-β is expressed in the CNS and, in oral-tolerance-induced protection in EAE, TGF-β and PGE_2 are expressed in the brain (Karpus & Swanborg (1991); Khoury et al (1992)). Harbige ((1998) concluded that dietary γ-linolenic acid effects on EAE are mediated through Th_3-like mechanisms involving TGF-β and possibly through superoxide dismutase antioxidant activity.

It has been suggested to use, *inter alia*, γ-linolenic acid and linoleic acid rich Borage oil as a means to provide immuno-suppression in multiple sclerosis (US 4,058,594). The dose suggested is 2.4 grams of oil per day and no actual evidence of efficacy is provided.

Borage oil (typically 23% γ-linolenic acid and 37% linoleic acid per 100% fatty acid content) has been shown to significantly reduce clinically important signs

and symptoms of autoimmune disease associated with active rheumatoid arthritis (Leventhal et al (1993)). Borage oil and fungal oil (see Figure 1) have been shown to be effective in the EAE animal model use to identify MS candidates, whilst never having been shown to be significantly effective in the human disease. High levels of linoleic rich oil containing low levels of γ-linolenic acid (EPO: linoleic acid:γ-linolenic acid 7:1) partially suppressed the incidence and severity of EAE in rat (Mertin & Stackpoole, 1978) whereas the Naudicelle study referred to above led to worsening of patients. In spite of the use of Borage oil and other GLA/LA containing oils such as Evening Primrose oil by multiple sclerosis sufferers over the past 30 years or so, the vast majority of patients fail to recover from the disease, showing no significant improvement, with the underlying disease continuing to progress to death.

Other more dramatic immunosuppressant treatments, including T cell depleters and modulators such as cyclophosphamide, are also shown to be effective in the EAE model, but where these are employed in the human multiple sclerosis disease symptoms improve, but the underlying disease continues to progress. T-cells indeed produce beneficial cytokines, such as TGF-β1, as well as deleterious ones in man. David Baker of Institute of Neurology, UK summed up the disparity between what is effective in the EAE and in MS with a paper entitled '*Everything stops EAE, nothing stops MS*' at the 10th May 2004UK MS Frontiers meeting of the UK MS Society.

It is clear that immunosuppression alone cannot cure MS. This is almost certainly due to a fundamental underlying metabolic disorder in MS patients that leads to membrane abnormality, cytokine dysregulation and subsequent immune attack and lesioning. Although patients go into remission in relapse-remitting disease, the underlying demyelination proceeds.

The 'gold standard' treatment for MS remains interferon, such as with β-Avonex ®, Rebif ® and other interferon preparations. This gold standard treatment only addresses needs of some, eg 30%, of the patients and even in these symptom improvement is restricted to reduced severity of relapses. Whilst symptoms may be reduced in a proportion of patients, the disease tends to progress to further disability and death due to underlying degeneration.

The present inventors have now surprisingly determined that with compliance to a 'high dose' treatment with triglyceride oil containing γ-linolenic acid with suitable accompanying fatty acid content, remarkable levels of improvement in almost all symptoms of MS can be achieved, way surpassing that provided by the present gold standard treatment. Such success is particularly surprising in the light of the prior use of other γ-linolenic acid containing preparations without such significant success.

Over an 18-month period, patients taking high dose selected borage oil showed significant ($p<0.001$) and marked improvements in EDSS score, a reduced rate of relapse, symptomatic relief of muscle spasticity and painful sensory symptoms, and improved objective measures of cognitive functions. Low dose borage oil was without effect.

Patients taking high dose borage oil maintained their level of peripheral blood mononuclear cell production (PBMC) of TGF-β1 during the trial period, their pro-inflammatory cytokines TNF-α and IL-1β were significantly and markedly (<70%) reduced and they either maintained or increased the PBMC membrane long chain omega-6 fatty acids dihomo-γ-linolenic acid (DHLA) and arachidonic acid (AA) in contrast to patients taking placebo who demonstrated loss of these fatty acids over the course of the trial period.

This whilst immuno-suppression would be expected to reduce active lesioning and neurodegeneration, the present treatment has apparently targeted maintenance of key membrane lipid components that are otherwise specifically lost in MS, suggesting a correction of a metabolic defect not otherwise effectively treated by current therapies. The fact that the low dose (5 grams/day) had no effect on this supports such determination.

Particularly the inventors have determined that a triglyceride oil comprising triglycerides of both γ-linolenic acid and linoleic acid with specific positional distribution within the triglyceride molecules, preferably with oleic acid, can provide significant decreasing EDSS score in multiple sclerosis patients over a number of months and years, a result that is unattainable with any of the currently administered therapies.

γ-Linolenic acid (18:3n-6, GLA) is known to be rapidly converted to longer-chain omega-6 polyunsaturated fatty acids dihomo-γ-linolenic acid and arachidonic acid *in vivo* (Phylactos et al 1994, Harbige et al 1995, 2000). Therefore to increase the level of membrane long chain omega-6 fatty acids in MS the inventors have reviewed results obtained with several GLA-containing oils:- both fungal (from Mucor javanicus) and plant (Borago officianalis), Evening primrose Oenothera spp. or Blackcurrant Ribes spp) as well as a synthetic tri-GLA oil as GLA delivery systems in an *in vivo* experimental animal model of MS known as chronic relapsing experimental autoimmune encephalomyelitis (CREAE).

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune inflammatory disease of the CNS, with or without demyelination, inducible in rodents and other mammalian species. Induction of EAE in rats however (using guinea pig basic protein), does ***not*** produce histological features of demyelination (Brosnan et al 1988) but induces an acute mono-phasic disease pattern, unlike MS which is characterised by CNS demyelination and is clinically relapsing-remitting. Chronic relapsing and demyelinating EAE models (CREAE), which are characterised by demyelination and relapse phases, are therefore currently the animal models of choice for MS research (Fazakerley 1997). With the demonstration that myelin oligodendrocyte glycoprotein (MOG) is an important neuroantigenic target in MS (Genain et al 1999) and the demonstration of far greater responses of peripheral blood auto-reactive lymphocytes to this neuroantigen, compared with MBP, in MS (Kerlero de Rosbo et al 1993, 1997) MOG induced CREAE has become the animal model of choice with features closely resembling those observed in MS (Fazakerely et al 1997, Genain et al 1999, Amor et al 1994).

Based on the results of these experiments two key selection criteria were adopted for selection improved lipid compounds for achieving the current aims. Evidence from CREAE and rat EAE feeding studies indicates that an enriched blackcurrant seed oil (72 % w/w 18:3n-6, GLA) did not protect against EAE (see Table 3). Importantly blackcurrant seed oil has a low sn-2 GLA with most of the GLA in the sn-1 and sn-3 positions (Lawson and Hughes 1988). Furthermore a structured

triacylgcerol containing three GLA's (TG-GLA) provided protective effects similar to that of the borage oil used in CREAE (Table 2). This would also be consistent with the sn-2 GLA being important i.e. the outer pair sn-1 and sn-3 GLA being enzymatically removed *in vivo* and probably undergo oxidation leaving the sn-2 GLA only. This selective hydrolysis arises from the known ability of specific lipases to remove the sn-1 and sn-3 fatty acids from triacylgycerol molecules but an apparent protection of the sn-2 position *in vivo* (Lawson and Hughes 1988, Kyle 1990).

The inventors' review of this data also indicates that the ratio of linoleic acid (LA) to γ-linolenic acid (GLA) residues may be a key efficacy feature of oils containing LA and GLA in the CREAE model of MS (Table 1). Table I shows the compositional analysis and efficacy in CREAE of fungal oil, borage oil, evening primrose oil and safflower oil. The most effective treatment in reducing the incidence of CREAE was fungal oil with an LA : GLA ratio of 0.85. Borage oil was also very effective with an LA : GLA ratio of 1.5. Furthermore, experiments with a structured triglyceride containing GLA at sn-1, sn-2 and sn-3 (TG-GLA) demonstrated GLA to be an active component. Moreover TG-GLA was also effective at a lower dose level than the borage oil (see Table 2).

Different Borage seed oil's also appear to vary in their level of sn-2 GLA e.g. 10 % sn-2 GLA (Liu et al 2000) and 40% sn-2 (Lawson and Hughes 1988) which is consistent with our unpublished observations of sn-2 GLA (range 38 – 46 %) and possibly the failure of some borage oils to produce fully protective effects in CREAE, although other factors such as antioxidant composition may also be important (unpublished). Borage oils having as much as 60% sn-2 GLA have been reported (Huang and Mills (1996) γ-Linolenic acid: metabolism and Its Roles in Nutrition and Mediceine: Chapter 6) and noted to be effective at getting GLA into lymph.

Table 3 of EP 0520624 (Efamol Holdings) compares the triglyceride content of Evening Primrose and Borage Oils, the former being taught to be more therapeutically effective than the latter for a variety of GLA responsive disorders. This document indicates Borage oil to have twenty seven different trigyceride components, only 20% of which have sn-2 GLA. Page 3, lines 40-42 notes that

biological testing has shown that equal amounts of GLA may indeed have very different effects when that GLA is supplied as different oil sources. Crucially, it then directs the reader to one particular fraction present in Evening Primrose Oil (EPO), but not Borage Oil, as being responsible for the former's superior effect in raising PGE1 (see EP 0520624 Chart page 4 and Table 2) and thus anti-inflammatory effect: that fraction being di-linoeoyl-mono-gamma-linolenyl-glycerol (DLMG) which it states to be 18 to 19% of the total triglyceride in EPO. Page 6 clearly teaches that the position of the GLA, in sn-1, 2 or 3, is not important to this effect.

Dines et al (1994) Proceedings of the Physiological Society, Aberdeen Meeting 14-16 September 1994 report on studies treatment of diabetic neuropathy neuronal damage with γ-linolenic acid containing oils of the type advocated by EP 0520624 and again note that Borage Oil was not very effective in treating this neurodegeneration whereas Evening primrose oil was. The paper concludes that Boage Oil contains other constituents that interfere with GLA activity.

In contrast to this prior art, the present inventors used a borage oil that was selected with the highest sn-2 GLA for trial purposes (>40%) – compared with lower amounts in other samples that were available at the time. Blackcurrant seed oil, which at the time was available in relative large quantities was not considered optimal because of it's low sn-2 GLA content.

A further selection criterion was that the level of total long chain monoenoic fatty acids be kept below 5%. There was a significant level of erucic acid (22:1n-9) i.e. 1.4 - 2.38 % of the total fatty acids, and other long chain monoenoic fatty acids i.e. 24:1n-9 (nervonic acid) and 20:1n-9 (gadoleic acid) in different borage seed oil (*Borago officinalis*) samples from different sources (Table 4).

Additionally because of potential impact on absorption, metabolism and immune functions of vitamin E (Harbige 1996, 2003), the trial oil contained only natural levels of vitamin E (0.05 mg/g) with no additionally added vitamin E as is routinely the case with commercial borage seed oils (e.g. 1 mg/g).

It is believed that such selected oils have an immunosuppressant effect, but significantly also have and a metabolic supplementation effect that has benefit in

reducing immune attack on lesions whilst creating the conditions necessary for their repair, something that has not been achieved with any medication previously provided for MS.

In a first aspect of the present invention there is provided a method of treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of an oil containing both γ-linolenic acid and linoleic acid residues as triglyceride ester, the ratio of γ-linolenic acid to linoleic acid residues at the sn-2 position of the triglyceride being at least 0.8; the amount of γ-linolenic acid residues at the sn-2 position being at least 18%, wherein the oil is administered at a dose sufficient to maintain or elevate TGF-β levels in the patient to therapeutic levels.

By therapeutic levels is meant levels at least consistent with healthy subjects. Preferably the dose is such as to produce a TGF-β1/TNF-α ratio in blood of a patient, after 18 months of daily dosing, of 0.4 to 3.0, at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably the dose is such as to produce a TGF-β1/IL-1β ratio in blood of a patient, after 18 months of daily dosing, of at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably said levels are produced after 12 months and more preferably after 6 months.

Typically the amount of oil administered daily will be between 3 and 30 grams, orally dosed, still more preferably between 5 and 20 grams and most preferably between 7 and 18 grams, typically 15 grams.

Most preferably, in addition to the γ-linolenic acid and linoleic acid fatty acid residues, the oil includes an esterified fatty acid that is non-structural, ie. that is metabolised to produce energy, such as oleic acid residues. By residue is meant the moiety that remains after the fatty acid carboxyl group esterifies to one of the hydroxy groups of the glycerol molecule.

Most preferably the oil administered is an oil source from Borage oil or a fungal oil eg. eg from Mucor javanicus,

Typical Borage oil and fungal oil compositions are illustrated in Table 1 wherein 18:2n-6 and 18:3n-6 represent linoleic and γ-linolenic acid residue by percent respectively.

Typically Borage oils contain from 20 to 25% γ-linolenic acid residues as percentage of fatty acid residues in the oil and from 35 to 40% linoleic acid residues. Preferred Borage oils are those in which the amount of esterified γ-linolenic acid at the sn-2 position is at least 35% of fatty acid residues at that position, more preferably greater than 39% and still more preferably greater than 40%. Most preferred oils are over 41%, such as 42 to 44% sn-2 GLA, whilst ideally they will be over 45%. As stated by Huang et al above, 60% sn-2 GLA Borage Oils have been produced and should be available for selection. The sn-1 and sn-3 position residues are preferably linoleic, oleic and γ-linolenic acid residues, with preferred oils having relatively high oleic acid content in at least one, if not both, of these positions, eg, in excess of 12%, more preferably in excess of 14%.

A typical Borage oil suitable for use in the use of the present invention has fatty acid distribution as follows

Sn-1: 14% 18:1 (Oleic), 54% 18:2n-6 (linoleic) and 4% 18:3n-6 (γ-linolenic)

Sn-2: 14% 18:1 (Oleic), 42% 18:2n-6 (linoleic) and 40% 18:3n-6 (γ-linolenic)

Sn-3: 19% 18:1 (Oliec), 18% 18:2n-6 (linoleic) and 30% 18:3n-6 (γ-linolenic)

Where a fungal oil is use, such as from Mucor species, the total amount of γ-linolenic acid residues may be lower than for Borage oil as long as the sn-2 γ-linolenic acid: linoleic acid ratio is at least 0.8, more preferably greater than 1. This is because fungal oils tend to have more 'metabolic' directed oleic acid residues than linoleic acid residues. Thus preferred fungal oils are those in which the amount of esterified γ-linolenic acid at the sn-2 position is at least 18% of fatty acid residues at that position, more preferably at least 20% and most preferably at least 22%. Preferred fungal oils have in excess of 45% of the sn-2 fatty acid residues as oleic acid residues, more preferably in excess of 50%.

Sn-1: 25% 18:1 (Oleic), 5% 18:2n-6 (linoleic) and 13% 18:3n-6 (γ-linolenic)
Sn-2: 54% 18:1 (Oleic), 19% 18:2n-6 (linoleic) and 20% 18:3n-6 (γ-linolenic)
Sn-3: 40% 18:1 (Oliec), 3% 18:2n-6 (linoleic) and 20% 18:3n-6 (γ-linolenic)

It will be realised by those skilled in the art that such oils will need to be sourced by testing for the percentage of said fatty acids at each position as an average over the many triglycerides in a give oil mix. Such is well within the skill of those in the art, such as eg. Mylnefiled Research Services Ltd, Lipid Analysis Unit, Mylnefiled, Inverghowrie, Dundee DD2, 5DA, Scotland UK. The applicants have managed to source a number of such oils meeting the criteria set out above, the highest sn-2 figures ofabout 46% being found for example in New Zealand sourced oils in 2003: this of course may vary from year to year. It is important, however, given the lack of response with the low dose (5g/day) Borage Oil that a patients efforts to recover are not undermined by the equivalent of underdosing by provision of a lower sn-2 % GLA Borage Oil than the daily dose requires.

A further NMR method for analysis of such oils in a selection process is provided in the methods section below. It will however be realised that should all oils available at a given tiem be below the 35% sn-2 GLA figure, and preferaby if they are below 40 or 45%, supplementation with a synthetic triglyceride or tryglyceride mix will be possible. A numebr of suitable lipids are known in the art and may for example be isolated or combined mixtures of LGL, OGO, OGL, LGO or other components known to be present in Borage Oil (see Table 3 of EP 0520624). Even TriGLA might be added (FR 2,617,161 (1988)), although it is preferred for the present purposes to keep the sn-1 and sn-2 position GLA levels from getting too high as that would un the risk of overly pro-inflammatory effect due to overflow into systemic GLA and thus DHGLA and then Arachidonic pools. Synthesis of OGO is for example taught in Y.-S. Huang, X. Lin, P. R. Redden and D. F. Horrobin, *J. Am. Oil Chem.Soc.*, **72**,625-631(1995)*In vitro* Hydrolysis of Natural and Synthetic γ-Linolenic Acid-Containing Triacylglycerols by Pancreatic Lipase and K. Osada, K. Takahashi, M. Hatano and M. Hosokawa, *Nippon Suisan*

Gakkaishi., **57**, 119-125 (1991). Chem. Abs. 115:278299 Molecular Species of Enzymically-synthesized Polyunsaturated Fatty acid-rich Triglycerides.

For treatment regimes where high amounts of any of these high Sn-2 GLA oils are administered it is recommended that the amount of potentially toxic long chain monoenoic fatty acids, such as erucic acid (22:1n-9) and other long chain monoenoic fatty acids i.e. 24:1n-9 (nervonic acid) and 20:1n-9 (gadoleic acid), are as low as possible, preferably lower than 5% of fatty acid residues, more preferably less than 3% and more preferably less than 2%.

Another feature of a preferred oil is low or zero added vitamin E such that only natural levels of vitamin E (0.05 mg/g) is provided.

Further aspects of the present invention provide use of triglyceride oils as described above for the manufacture of a medicament for the treatment of neurodegenerative disease, more specifically for the arresting of underlying neurodegeneration and the restoration of neuronal function. Particularly such medicaments are for the normalising of neuronal membrane composition, the restoration of healthy TGF-β1/TNFα ratios and the ratios of TGF-β1 with other cytokines, the arresting of neurodegeneration in multiple sclerosis and the restoration, in part or completely, of neuronal function such as measured, eg. By MRI or CAT scan or by EDSS score. Such use will include treatment of cerebral impairment after stroke, head trauma and intracranial bleeding.

Also provided are selected triglyceride oils having particular efficacy in treating multiple sclerosis and effecting beneficial changes in ratio of cyctokines *in vivo,* these oils eing those set out as preferred for the method described above.

The oils for use in the present invention may be administered by any of the conventional vehicles known in pharmacy. Most conveniently they are administered as neat oils or in admixture with foodstuffs, in the form of capsules containing such oils, or in enterically coated forms. Other forms will occur to those skilled in the art as delivery technology advances.

It will be realised by those skilled in the art that other beneficial agents may be combined with the oils for use in the present invention. These might be ion channel blcokers , eg. sodium channel blockers, interferons, T-cell depleters, steroids or other palliative agents. It will further be realsied that where the immune and inflammatory responses are being modulated, such combinations will need to be made carefully, given the complex nature of these systems. However, given the delayed response to the present oils, shorter acting agents might be beneficial in the first months of treatment before the TGF-β1 levels are normalised, as long as the additional treatment does not impede this normalization process.

The present invention will now be described by way of Example only by reference to the following non-limiting Tables, Examples and Figures. Further embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

TABLES

Table 1: Shows the compositional % Total fatty acid content of various triglyceride oils and protective effect in EAE.

Table 2: Shows the parameters of the three treatment groups in high sn-2 GLA Borage Oil trial

Table 3: Shows the effect of various forms of GAL on EAE incidence and clinical score in SJL mice.

Table 4: Shows the failure of enriched Blackcurrent oil, a high GLA, but low sn-2-GLA, plant oil, to match fungal and Borage oils in EAE.

Table 5: Shows the results analyses of four batches of trial Borage Oil particularly with respect of monoenes.

Table 6: Shows an analysis of a non-trial oil particularly with respect to monoenes.

FIGURES

Figure 1: Shows peripheral blood mononuclear cell cytokine production in placebo and trail oil treated human MS patients at 18 months.

Figure 2: Shows the effect of placebo and low dose (5g/day) high sn-2 GLA Borage oil on human MS patient EDSS score as compared to high dose (15g/day) displayed as a histogram.

Figure 3: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient EDSS displayed as a graph.

Figure 4: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient Mean Relapse rate (%) as a histogram

Figure 5: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient Mean Relapse rate (%) as a graph.

Figure 6: Shows the effect of linoleic acid:γ-linolenic acid ratio of oils as compared to their protective effect on mice CREAE.

METHODS

Positional Analysis of Gamma-Linolenic acid (GLA) in Borage oil Samples by Quantitative-^{13}C-NMR

Analytical methodologies for the determination of fatty acid composition and positional distribution in triacylglycerols generally require hydrolysis of the triacylglycerols by enzymes or chemical processes and subsequent analysis of the

mono-and diacylglycerol components by chromatographic techniques. These methods are destructive and do not allow the recovery of the original samples. The hydrolysis procedure usually gives rise to some acyl migrations, resulting in substantial errors of the positional distribution.

There are several properties of ^{13}C nuclear magnetic resonance (NMR) that make its application to positional analysis useful. First, the chemical shift is sensitive to the molecular structure, thereby giving rise to a spectrum where each nucleus is identified by a peak at a specific frequency. The resolution of the nuclei in each environment is determined by the linewidth and the chemical shift differences between adjacent peaks . Second, the area under the peak, arising from each nucleus, is proportional to the number of nuclei in that environment because all ^{13}C exhibit the same absorption . Therefore, the chemical shift and the integrated area of each peak can be used for both qualitative and quantitative measurements of each nucleus. Thirdly, the preparation of the sample for this application is simple. Finally, NMR is a non-destructive technique that allows the sample to be recovered for other purposes. ^{13}C NMR methods are normally based on analysing the cluster of signals for the carbonyl carbons. Two clusters of signals are normally observed corresponding to acids in the sn 1,3- and 2-positions. They are usually readily distinquished since the two environments give rise to a separation of about 0.4 ppm. Within each of these two clusters , there must be separate signals for each acid or groups of acids. This criterion is most easily met when the acids have carbon-carbon double bonds (i.e. are unsaturated) close to the carboxyl group e.g. n = 4, 5 or 6. The carbonyl carbon signals from acids of the same double bond group (e.g. EPA and AA) will not normally be distinquished. Such methods seemed to be valuable for analysing GLA-containing triacylglycerols and this proved to be the case .

References:

M. M. Bergana and T. W. Lee, *J. Am. Oil Chem. Soc.*, **73**, 551-556 (1996)

G. Vlahov, *Magn. Reson. Chem.*, **36**, 359-362 (1998)

Experimental

Materials/sample-preparation

Monoacidtriacylglycerols were purchased from Sigma Chemicals and Nu-Chek-Prep Inc:

Tripalmitin	(Tri-16:0)
Tristearin	(Tri-18:0)
Triolein	(Tri-18:1n-9)
Trilinolein	(Tri-18:2n-6)
Trigammalinolenin	(Tri-18:3n-6)
Trieicosenoin	(Tri-20:1n-9)
Trierucin	(Tri-22:1n-9)
Trinervonin	(Tri-24:1n-9)

Approx 180 mg of lipid in 700 uL of deuteriochloroform was used throughout the study.

^{13}C-NMR-data

The proton-decoupled ^{13}C NMR data with supressed NOE were collected at 21°C in a 5-mm broadband probe on a Jeol 500 MHz spectrometer operating at 125.728 MHz. Waltz decoupling was the chosen mode of decoupling and was gated on only during the 14.89s acquisition time. The relaxation delay was set at 30 secs and the pulse angle was 90 °.The spectral window used was ca. 35 ppm (from 173.5 to 172.6 ppm) with a 170 ppm offset. The spectra were internally referenced to $CDCl_3$ at 77.0 ppm . Typically, the approximate number of scans collected for adequate signal-to-noise ranged from 300 to 1200 scans , depending on the complexity of the mixture. The total acquisition time for the experiments ranged between 1-4 h (Borage oil 1272 scans / 4h). Data points 65,536.

CALCULATIONS

The GLA carbonyl signals at the 2- and 1,3 –positions were well separated from all over carbonyl peaks in the spectra of the triacylglycerols. This allowed the ratio of 2-GLA / 1,3-GLA to be determined with reliability and precision in all cases. The method was validated by analysing a test mixture containing 8 triacylglycerols, including trigammalinolenin, of known proportions similar to those present in borage oil. Gross compositions had previously been determined by GLC. Two methods of calculation were employed. The first was a self-contained NMR method calculating the 2-GLA percentage as follows:

$$\frac{\text{Integral of sn-2 peak for GLA} \times 3 \times 100}{\text{Total of all sn-1,2 and 3 integrals}}$$

The second used the ratio of 2-GLA / 1,3 GLA integrals from the NMR and gross GLA composition as determined by GLC as follows:

$$\frac{\text{Integral of sn-2 peak for GLA} \times \%\ \text{GLA from GC analysis} \times 3}{\text{Total integrals for GLA at sn-1,2 and 3}}$$

Results from both computations were in agreement. We regard the composite NMR-GLC method as more precise since it uses the parameters from each method that can be measured with most accuracy. GLA is a major component of borage oil and therefore the 2- /1,3 –GLA ratio can be determined by NMR with precision as the results show. GLC is generally better at determining the composition of even minor fatty acids than NMR but cannot give positional information.

Summary

Details of the experimental data are given in the tables and spectra. It is summarised below. Capsules D and B are sample capsules of provided in the clinical trial reported below.

1. 8-TG-Test-mixture

sn-2	GLA	found	22.2%
		actual	22.6%
		error	1.8%

(n)=2

2.Capsule-D

Gross GLA	found	21.4%	(n = 3)	by GLC
sn-2 GLA	found	42.5%	(n = 2)	by NMR
	error			+/- 1%

3.Capsule-B

Gross GLA	found	21.1%	(n = 3)	by GLC
sn-2 GLA	found	40.8%	(n = 2)	by NMR
	error			+/- 1%

In these borage oil samples the GLA content in the sn-2 position is 41-42% i.e. almost double (1.95 x) that of the gross composition. A probable typical sample reported in the literature shows an enrichment of 1.8 times gross..

The NMR method can be used to give reliable analytical data for GLA at the sn-2

position. Indeed, it is particularly suited to borage oil because of the non-interference of other fatty acids present. Interestingly results from the NMR method are reported to agree with those from the older derivatisation-chromatographic ones. Our previous estimate (40%) derived from these older methods also agrees with the NMR results.

TREATMENT EXAMPLES.

Twenty-eight active relapsing-remitting (two relapses in the preceding 18 months) multiple sclerosis patients (ages ranging from 18 to 65 yrs) were entered into a double-blind placebo controlled trial to investigate the effects of encapsulated borage oil on clinical activity and laboratory parameters over 18 months. This oil was of high Sn-2 γ-linolenic (GLA) content (>40% GLA at Sn-2) with low monene (eg. erusic acid) content and had no added Vitamin E, a known immunomodulator.

Patients were recruited from neurology out-patient clinics at two inner city hospitals; hospital informed consent was obtained on first (baseline) visit. Exclusion criteria include any form of steroid or immunosuppressive drug treatment, pregnancy, hyperlipidemia, regular use of aspirin or related drugs and vitamin or fatty acid supplementation within the previous three months.

Only patients meeting all the following criteria were included in the trial: (a) able to provide informed consent prior to treatment, with the full understanding that consent may be withdrawn at any time without prejudice; (b) male or female out-patients aged 18 to 60 years inclusive; (c) have confirmed diagnosis of clinically definite relapsing MS; (d) have had at least three documented clinical relapses in the past two years; (e) have a baseline Expanded Disability Scoring Scale (EDSS) score of 0.0-5.5 inclusive, provided they have well documented exacerbations; and (f) healthy, apart from the MS-related symptoms, as confirmed by the medical history, physical examination and clinical chemistry, urine and haematological tests.

Patients were randomly allocated by the Pharmacy Department to one of three groups each containing 12 patients:

- One clinical group (n=12) to receive placebo (5 g of Polyethylene Glycol 400)
- Second clinical group (n=12) to receive low-dose (5 g) refined *Borage officinalis*

- Third clinical group (n=12) to receive high-dose (15 g) refined *Borage officinalis*

Supplementation was in the form of one gram oil capsules daily (5/day for low dose, 15/day high dose) for 18 months duration. *Borage officinalis* oil and omega-6 polyunsaturated fatty acids are food ingredients that are generally recognised as safe for human consumption (GRAS). There are no classification or labelling requirements under EC regulations. Clinical assessment included: Extended Disability Scale Scores (EDSS) and clinical relapse record. Venous blood (50 mls) was obtained for laboratory studies on the 1st, 3rd, 6th, 12th, 15th, and 18th month of supplementation.

The following biochemical and immunological parameters were investigated on each visit for comparison with pre-treatment data and between group data:

- Stimulated and unstimulated *ex vivo* peripheral blood mononuclear cell cytokine production: TGF-β1, IFN-γ, TNF-α, IL-1β, IL-6 and IFN-β, which are implicated in the pathogenesis of MS. Cytokine and related gene expression.
- Soluble adhesion molecules in serum particularly ICAM-1 and VCAM-1
- Peripheral blood mononuclear cell membrane fatty acids and plasma phospholipid fatty acid composition.

Results are shown in Tables 1 and 2 and Figures 1 to 5.

The primary outcome parameter was the number of clinical relapses between baseline (Month 0) and the end of treatment (Month 18). Secondary outcome parameters included: the time to first clinical relapse; severity of relapses, as assessed by EDSS score and the use of steroid treatment; and changes in EDSS at Month 3, 6, 9, 12, and 18 compared to baseline and defined as at least 1.0 point increase in the EDSS that is sustained for 3 months or at least 1.5 point increase on the EDSS from the baseline EDSS that is sustained for 3 months. As this trial did not receive external funding, it was not possible for financial reasons to evaluate MS diseases activity with magnetic resonance imaging. 1 of 3

Eleven patients were in the placebo group, seven patients had been taking low-dose Borage oil, and ten patients had been taking high-dose Borage oil. The study

drug was well-tolerated, and there were no serious adverse events during the 18-month trial.

RESULTS

Two patients had developed diarrhoea, both of whom were later confirmed to have been taking high-dose Borage oil. The diarrhoea was mild in one patient, but was moderately severe in the second patient, who later discontinued the study drug. The code was not broken and the diarrhoea had stopped after the discontinuation of the drug, but reappeared upon re-challenge. Therefore, this patient was withdrawn from the trial. The remaining patients who were treated with high-dose Borage oil showed excellent clinical improvement on all primary and secondary outcome criteria. For example, their mean EDSS score after 6 months of treatment had improved from baseline EDSS (Figure 1). More importantly, the mean number of clinical relapses had significantly reduced after 6 months of treatment when compared to the number of relapses in the placebo group (Figure 2). In contrast, patients who had been receiving low-dose Borage oil did not show any clinical improvement when compared to the placebo group. In addition to its beneficial effect on MS disease activity, high dose Borage oil provided some symptomatic relief of muscle spasticity (stiffness) and painful sensory symptoms, and also improved cognitive functions.

As can be seen for the figures below, relapse rate after 9, 12 and 18 months was down to zero in the high dose group. The increase seen at 15 months was due to a patient dropping out of this group.

The following are three brief case histories to illustrate the therapeutic benefits of high dose high sn-2 GLA Borage oil. The first two are from the trial while the third is a post trial patient for whom MRI studies were obtained.

Patient 1 (Treatment):

The first patient was a 48 year old woman who had had a clinically active, relapsing remitting MS for 9 years. She had originally worked as a full-time administrator at the local Health Authority, but she was unable to perform her duties

because of her severe MS. Therefore, she later worked as a part-time secretary, but still had difficulties in mobilization because of muscles stiffness and sensory disturbances. She was also experiencing severe clinical relapses at an average of one relapse every nine months. Most of these relapses had resulted in hospital admissions for steroid therapy. In view of her active MS, she was recruited into the Borage oil trial. There were no adverse events relating to the study, and after taking the medication for four months, she experienced good improvement in her walking and sensory symptoms.

About nine months after therapy, she was well enough to start full-time employment. In addition, she remained relapse-free for the 18-month duration of the clinical trial. Following the conclusion of the trial, the treatment code revealed that she was taking high-dose Borage oil.

Patient 2 (Control):

The second case was a 46-year old woman who also had a clinically active relapsing remitting MS for 8 years. She had originally worked as a shop assistant, but became unemployed after MS was diagnosed.

Her symptoms included difficulty with mobilisation and painful sensory symptoms in both legs. She had experienced three clinical relapses in the two years preceding the clinical trial, and had been admitted to hospital twice for steroid therapy. Consequently, she was recruited into the Borage oil trial, but her walking continued to deteriorate. Six months into the trial, she need to use a walking stick and also received treatment with Baclofen to reduce low limb spasticity. Approximately ten months after starting the Borage oil trial, she was admitted to hospital because of severe clinical relapse, which was treated with steroids. She later developed bladder disturbances and began to use a wheelchair for long journeys. The treatment code was broken after the conclusion of the 18-month trial, and she was found to have been taking placebo. Since then, she started using a walking frame for journeys exceeding 50 yards.

Patient 3: Treatment (additional to trial)

The third case was a 26 year-old man who was diagnosed with definite MS in April 2001. His symptoms had started in 1999 when he complained of diffuse, intractable pain affecting various parts of his body, particularly the left side of the chest and abdomen. This was followed by intermittent numbness in the hands and feet, associated with fluctuating weakness. There were also distressing bladder symptoms in the form of urinary frequency and urgency. The diagnosis of MS in 2001 was based on his relapsing remitting symptoms, and was confirmed by positive cerebrospinal fluid analysis and magnetic resonance imaging (MRI) of the brain, which showed multiple white matter abnormalities in both cerebral hemispheres. Symptoms did not respond to various pharmaceutical therapies.

In April 2003, oral supplementation with the present high dose Borage oil was commenced. The patient reported dramatic improvement in his symptoms within three months of starting this oral supplementation. His painful sensory symptoms disappeared completely. He reported no numbness or weakness since May 2003, and noticed significant improvement in his bladder control. The oral supplementation caused no adverse events. A repeat brain MRI was undertaken to verify the reported improvement in Mr N's symptoms. The repeat MRI showed a reduction in the size and distribution of the white matter abnormalities.

TABLE 1

Compositional (% Total FAs) Characteristics of Various Oils and their Protective Effects in EAE

Treatment	18:2n-6	18:3n-6	18:2n-6/18:3n-6	18:1n-9	INCIDENCE OF EAE
FGO	17	20	0.6	35	0/10
BOO	37	24	1.5	15	3/10
EPO	71	9.4	7.5	9	7/10
SAF	66	-	-	17	9/10
Controls	-	-	-	-	9/10

FGO, Fungal Oil; BOO, Borage Oil; EPO, Evening Primrose Oil, SAF, Safflower Oil.

TABLE 2

Treatment Groups- Borage oil-MS trial

Group		Female	Male	Mean Relapse Rate (in past two years)	Mean Base EDSS	Number
	Placebo	7	4	2.6	3.9	11
	Low Dose	5	2	2.9	3.5	7
	High Dose	8	2	3.4	2.8	10
Total		20	8	2.9	3.4	28

Table 3. Molecular Species Comparison of Triacylglycerol-GLA (TG-GLA), Ethyl-Ester-GLA (EE-GLA) and *Borago Officinalis* Oil-GLA (BOR-GLA) in MOG-induced CREAE in SJL Mice

Treatment	No. with EAE	Mean Clinical Score
Control	10/11	3.3±1.3
EE-GLA[a]	5/6	3.0±0.8
TG-GLA[a]	3/6	1.0±1.3[c]
BOR-GLA[b]	3/6	1.0±1.2[c]

[a] Animals given 100 µl of test lipid; [b]250µl BOR-GLA given. Significance of difference compared with controls, [c] $p < 0.05$

27

Table 4. Effect of enriched black-currant seed oil (73% GLA) on the incidence of EAE

	% Incidence of EAE (Days after immunisation)							
	13		17		21			
Controls (n=10)	60		90		10			
Blackcurrant (n=10)	10		80		70			

Note: Blackcurrant oil delays the incidence but does not provide full protection. Animals were fed 7 days after sensitization (immunisation).

TABLE 5: ANALYSIS REPORTS ON TRIAL BORAGE OIL (% Total Fatty Acids)

	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE4
Fatty Acids %				
16:0	13.29	13.47	12.86	13.11
16:1n7	0.21	0.22	0.21	0.21
18:0	3.50	3.47	3.54	3.50
18:1n9	16.22	16.22	16.33	16.22
18:1n7	0.64	0.66	0.65	0.64
18:2n6	38.00	38.01	38.25	37.96
18:3n6	22.59	22.66	22.69	22.56
18:3n3	0.18	0.18	0.17	0.19
20:0	0.20	0.18	0.20	0.21
20:1n9	2.96	2.88	3.06	3.06
22:1n9	1.55	1.41	1.50	1.58
24:1n9	0.60	0.63	0.52	0.71

TABLE 6. ANALYSIS OF A *NON*-TRIAL BORAGE OIL (% Total Fatty Acids)

Fatty Acids	% Total Fatty Acids
16:0	11.07
16:1n-7	0.17
18:0	3.70
18:1n-9	16.37
18:1n-7	0.66
18:2n-6	37.71
18:3n-6	21.89
18:3n-3	0.17
20:0	0.25
20:1n-9	3.79 ↑
22:1n-9	2.38 ↑ (high)
24:1n-9	1.47 ↑

CLAIMS.

1. A method of treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a triglyceride oil containing both γ-linolenic acid and linoleic acid residues as triglyceride ester, the ratio of γ-linolenic acid to linoleic acid residues at the sn-2 position of the triglyceride being at least 0.8; the amount of γ-linolenic acid residues at the sn-2 position being at least 18%, wherein the oil is administered at a dose sufficient to maintain or elevate TGF-β1 levels in the patient at a therapeutic level.

2. A method as claimed in Claim 1 wherein the therapeutic level is such as to produce a TGF-β1/TNF-α ratio of at least 0.5 in blood of a patient, after 18 months of daily dosing.

3. A method as claimed in Claim 2 wherein the ratio is at least 0.75.

4. A method as claimed in Claim 2 wherein the ratio is at least 1.

5. A method as claimed in Claim 1 wherein the amount of oil administered is between 3 and 30 grams per day.

6. A method as claimed in Claim 1 wherein the oil is administered orally.

7. A method as claimed in Claim 1 wherein the dose is sufficient to administer at least 1 gram of γ-linolenic acid residues, as residues in the sn-2 position, excluding other γ-linolenic acid content of the oil.

8. A method as claimed in any one of the preceding claims wherein the amount of γ-linolenic acid in the sn-2 position in the dose of oil is sufficient to administer at least 2 grams of said sn-2 γ-linolenic acid.

9. A method as claimed in any one of the preceding claims wherein the dose is between 8 and 20 grams.

10. A method as claimed in any one of the preceding claims wherein in addition to the γ-linolenic acid and linoleic acid fatty acid residues, the triglyceride includes an esterified fatty acid that is non-structural.

11. A method as claimed in claim 10 wherein the triglyceride contains oleic acid residues.

12. A method as claimed in claim 1 wherein the oil is that obtained from a fungus or a plant selected from the group consisting of Mucor and Borago species.

13. A method as claimed in Claim 12 wherein the fungus or plant is selected from Mucor javanicus and Borago officianalis.

14. A method as claimed in Claim 1 wherein the oil is a Borago oil in which the percentage of esterified γ-linolenic acid at the sn-2 position is at least 35% of fatty acid residues at that position

15. A method as claimed in Claim 14 wherein the percentage of esterified γ-linolenic acid at the sn-2 position is at least 39% of fatty acid residues at that position.

16. A method as claimed in Claim 14 wherein the percentage of esterified γ-linolenic acid at the sn-2 position is at least 45% of fatty acid residues at that position

17. A method as claimed in any one of the preceding claims wherein the fatty acid residues in the sn-1 and sn-3 position include linoleic, oleic and γ-linolenic acid residues.

18. A method as claimed in any one of the preceding claims wherein the triglyceride oil has an oleic acid content in one or both of the sn-1 and sn-3 positions of in excess of 12%.

19. A method as claimed in Claim 1 wherein the oil is Mucor oil and, the total percentage of esterified γ-linolenic acid residues at the sn-2 position is at least 20% of fatty acid residues at that position.

20. A method as claimed in Claim 19 wherein the triglyceride oil has in excess of 45% of the sn-2 fatty acid residues as oleic acid residues.

21. A| method as claimed in Claim 19 wherein the triglyceride oil has in excess of 50% of the sn-2 fatty acids as oleic acid residues.

22. A method as claimed in any one of the preceding claims wherein the triglyceride oil contains less than 5% monoenoic fatty acid residues as % total fatty acid residues.

23. A method as claimed in Clam 22 wherein the triglyderide oil contains less than 5% in total erucic acid (22:1n-9), 24:1n-9 (nervonic acid) and 20:1n-9 (gadoleic acid) as a percentage of total fatty acid residues .

24. A method as claimed in Claim 22 or 23 wherein the amount of said acid is between 1% and 5% of fatty acid residues in the oil.

25. A method as claimed in any one of the preceding claims wherein the oil has no added vitamin E.

26. A method as claimed in any one of the preceding claims wherein the amount of Vitamin E is between 0 and 0.1mg/g.

27. A method as claimed in any one of the preceding claims wherein the neurodegenerative disease is arrested or neuronal function is restored.

28. A method as claimed in any one of the preceding claims wherein treatment is for multiple sclerosis or the degenerative sequelae associated with head trauma, stroke and intracranial bleeds.

29. A method as claimed in claim 28 wherein the treatment repairs lesions.

30. A method as claimed in Claim 1 or 28 wherein the treatment uses a dose sufficient to relieve muscle spasticity and/or pain.

31. A method as claimed in Claim 1 or 28 wherein the dosage is sufficient to improve cognitive function.

32. A method as claimed in Claim 1 or 28 wherein the dosage is sufficient to eliminate relapses.

33. A method as claimed in Claim 1 or 28 wherein the dosage is sufficient to improve the patients EDSS score by at least 1 unit over a period of 1 years treatment.

34. A method as claimed in Claim 1 or Claim 28 wherein the dosage is sufficient to restore EDSS of a patient with EDSS above 2.5 to below 2 over a period of 1 years treatment.

35. Use of an oil as described in any one of Claims 1 to 34 for the manufacture of a medicament for the treatment of neurodegenerative disease.

36. A pharmaceutical composition for the treatment of neurodegenerative disease comprising a Borago or Mucor species triglyceride oil as described in any one of Claims 14 to 26.

Fig.1.

Peripheral Blood Mononuclear Cell Cytokine Production in Placebo and Oil Treated Multiple Sclerosis Patients at 18 Months

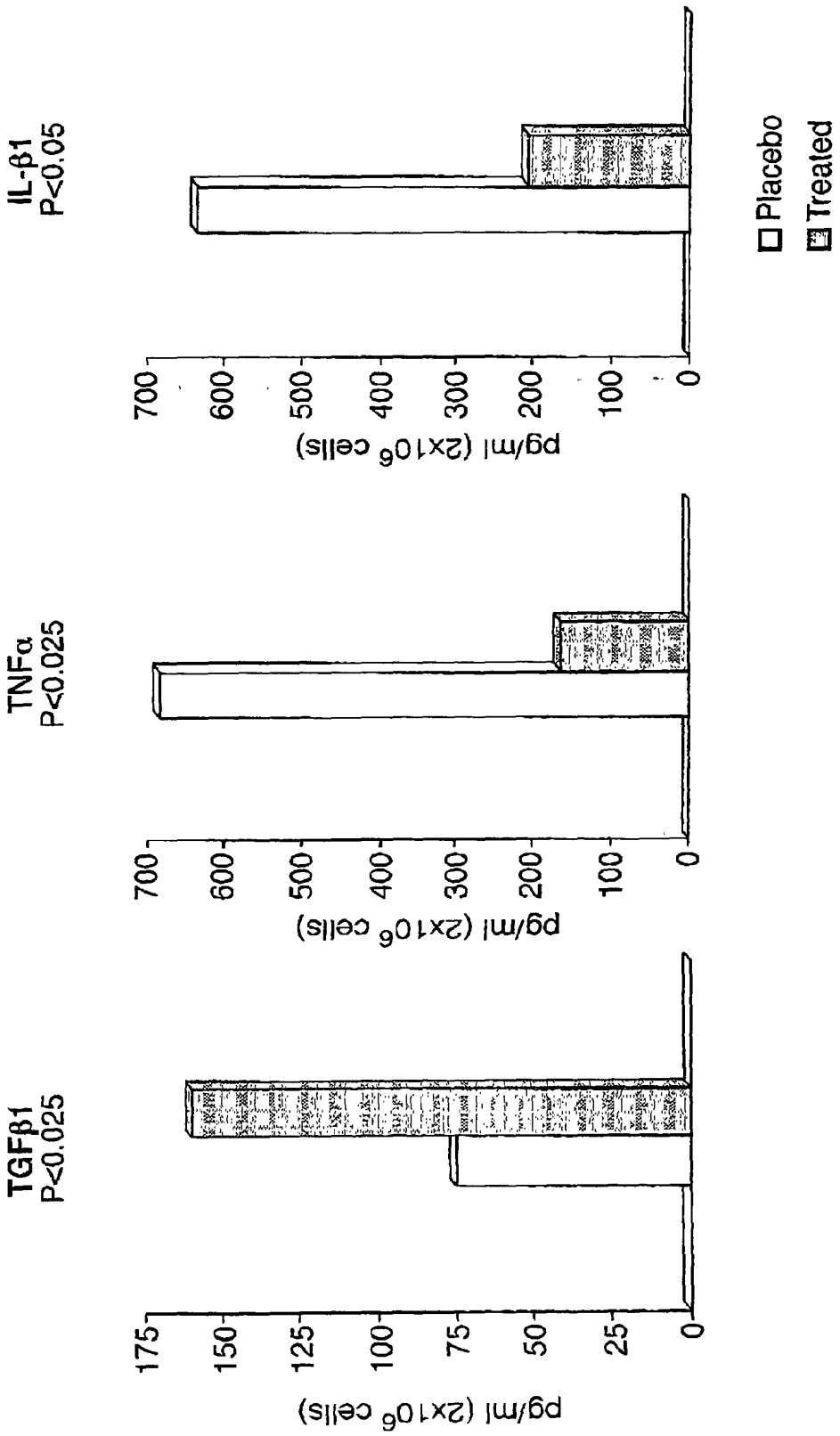
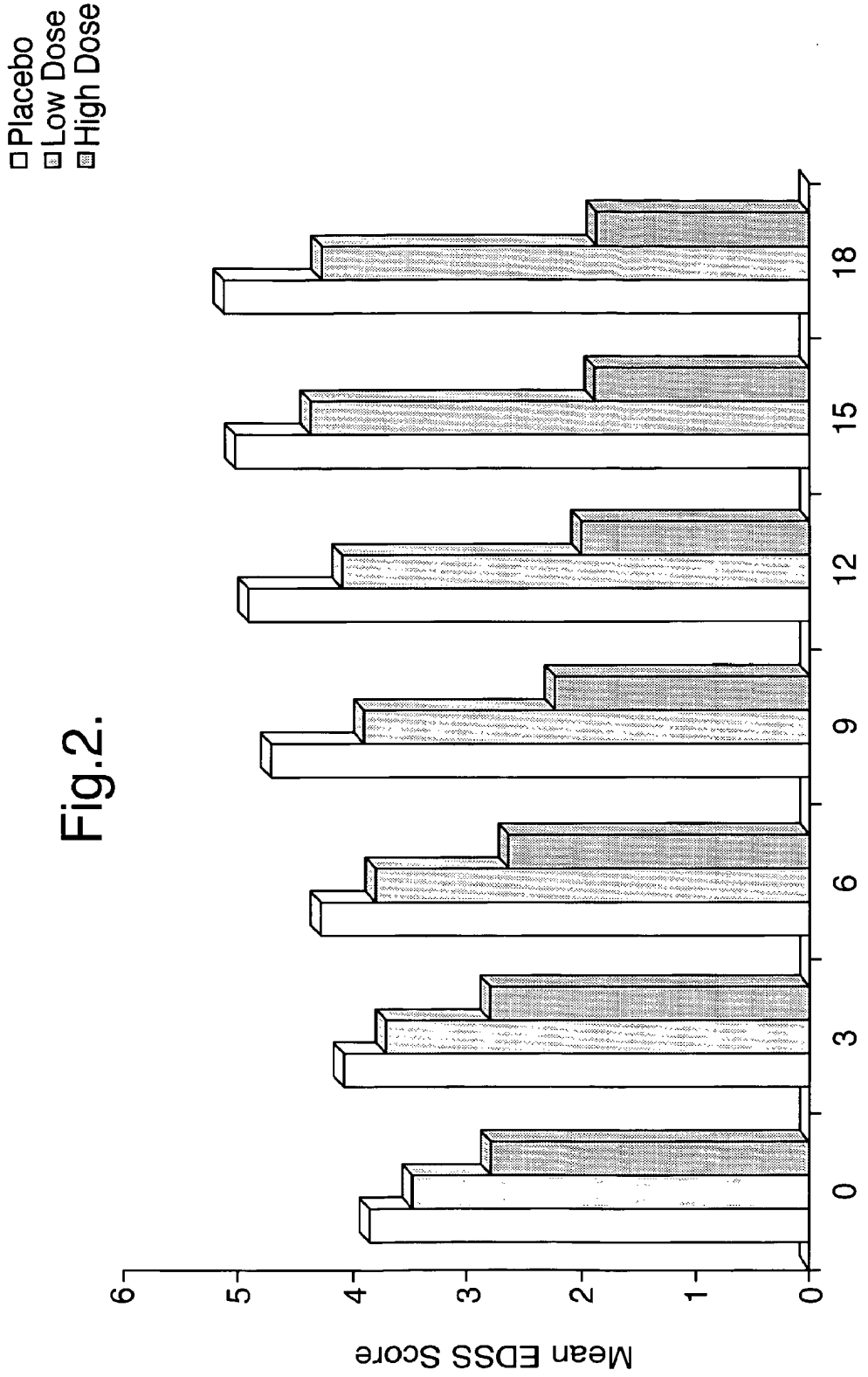


Fig.2.


Placebo
Low Dose
High Dose
Mean EDSS Score
0
1
2
3
4
5
6
0
3
6
9
12
15
18

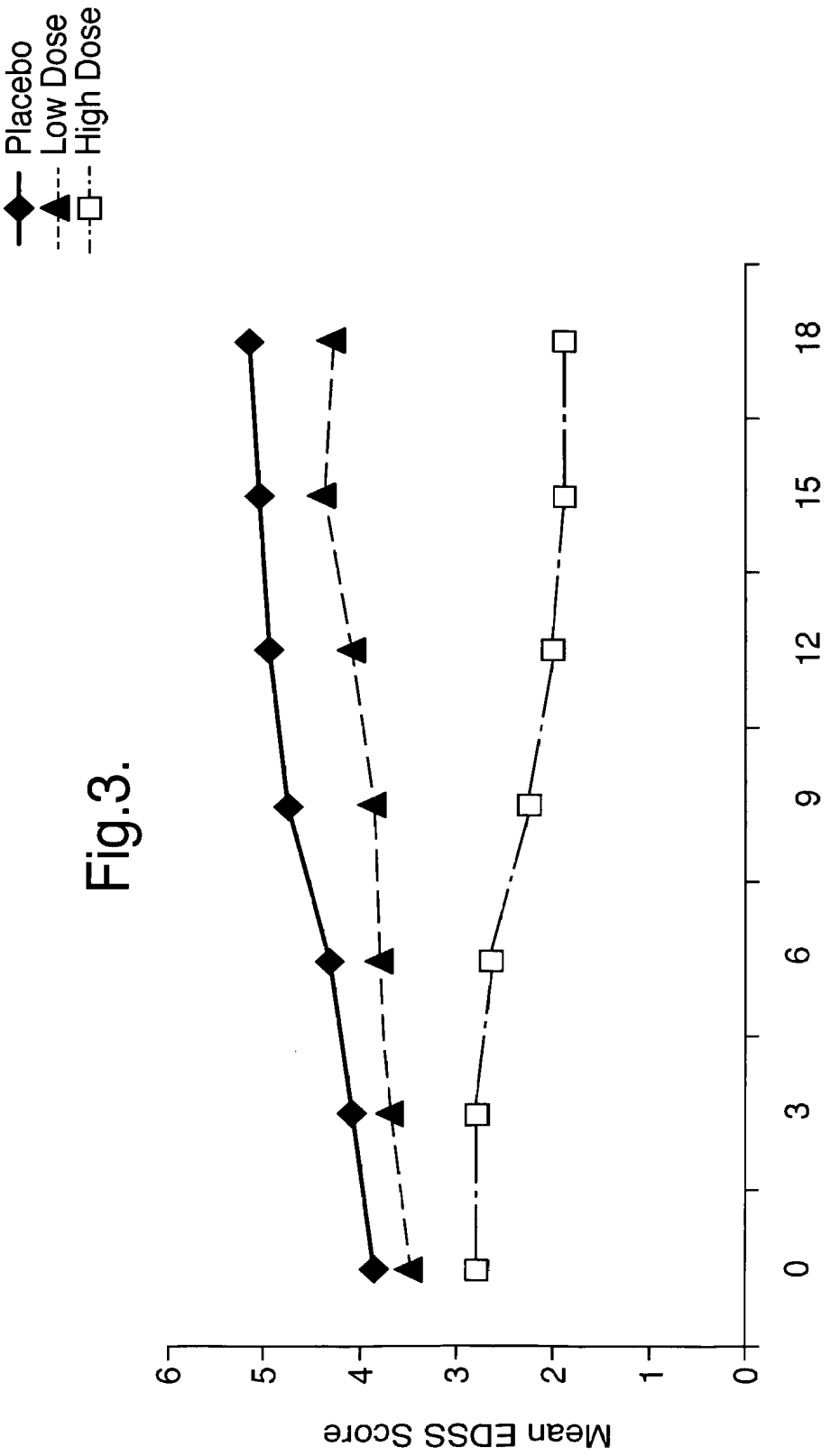

Placebo
Low Dose
High Dose
Mean EDSS Score
0
1
2
3
4
5
6
0
3
6
9
12
15
18

Fig.3.

Fig.4.

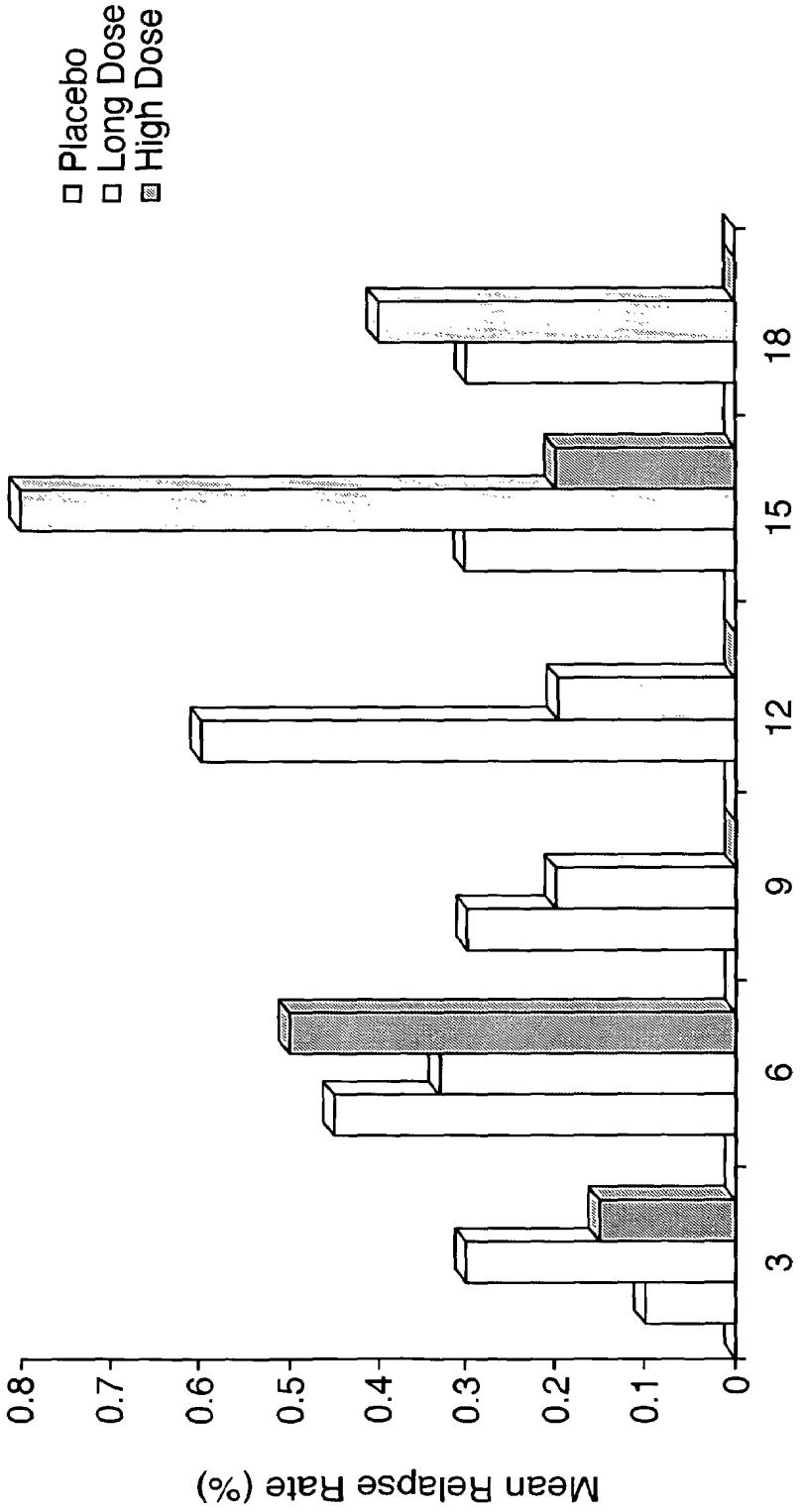

Placebo
Long Dose
High Dose
Mean Relapse Rate (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
3
6
9
12
15
18

Fig.5.

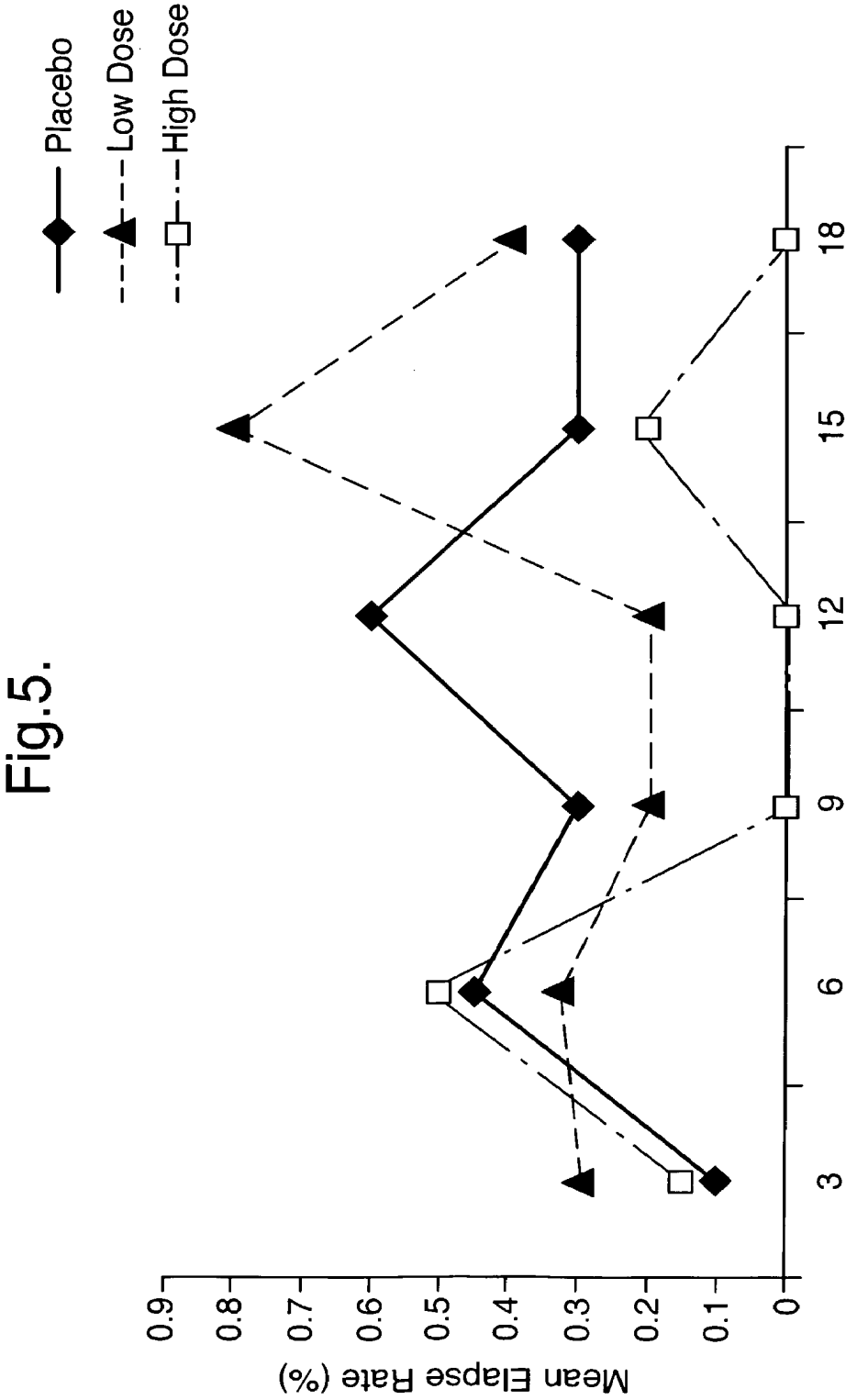

Placebo
Low Dose
High Dose
Mean Elapse Rate (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
3
6
9
12
15
18

Fig.6.

Effect of Ratio of Linoleic Acid to Gamma-Linolenic Acid on Acute Phase of SCH Induced CREAE in Biozzi AB/H Mice

18:2n-6/18:3n-6	Incidence of EAE
0.5	0/10
1.5	4/10
7.5	7/10
Controls	10/10

INTERNATIONAL SEARCH REPORT

national Application No
T/GB2004/002089

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/231 A61K31/232 A61K35/78 A61K35/84 A61P25/28

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)
IPC 7 A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 826 877 A (CARMICHAEL HUGH ET AL) 2 May 1989 (1989-05-02) column 1, line 6 - line 8 column 4, line 58 - line 62 column 5, line 5 - line 28	1-36
Y	US 4 058 594 A (WILLIAMS JOHN) 15 November 1977 (1977-11-15) cited in the application the whole document column 2, line 25 - column 3, line 8	1-36

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in 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 but 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	Date of mailing of the international search report
14 September 2004	30/09/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Albrecht, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAWSON L D ET AL: "TRIACYLGLYCEROL STRUCTURE OF PLANT AND FUNGAL OILS CONTAINING GAMMA LINOLENIC ACID" LIPIDS, vol. 23, no. 4, 1988, pages 313-317, XP009035770 ISSN: 0024-4201 cited in the application table 2	1-36
Y	HARBIGE L S ET AL: "Prevention of experimental autoimmune encephalomyelitis in Lewis rats by a novel fungal source of gamma-linolenic acid" BRITISH JOURNAL OF NUTRITION, vol. 74, no. 5, 1995, pages 701-715, XP009035788 ISSN: 0007-1145 cited in the application page 712-714, chapter "DISCUSSION"	1-36
Y	HOY CARL-ERIK ET AL: "Absorption of gamma-linolenic acid from borage, evening primrose, and black currant seed oils: Fatty acid profiles, triacylglycerol structures, and clearance rates of chylomicrons in the rat" GAMMA-LINOLENIC ACID: METABOLISM AND ITS ROLES IN NUTRITION AND MEDICINE AOCS PRESS {A}, CHAMPAIGN, ILLINOIS, USA, 1996, pages 54-65, XP009035802 & FIRST INTERNATIONAL SYMPOSIUM OF GLA; SAN ANTONIO, TEXAS, USA; MAY 7-11, 1995 ISSN: 0-935315-68-3 cited in the application page 56-57, chapter "Oils" page 58; figure 6.2	1-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2004/002089

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. [X] Claims Nos.: 1-34
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-34 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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 III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

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.:

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

Information on patent family members

national Application No
T/GB2004/002089

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4826877 A	02-05-1989	AT 84414 T	15-01-1993
		AU 586286 B2	06-07-1989
		AU 6340786 A	09-04-1987
		CA 1279824 C	05-02-1991
		DE 3687501 D1	25-02-1993
		DE 3687501 T2	19-05-1993
		EP 0218460 A2	15-04-1987
		ES 2043601 T3	01-01-1994
		GR 3007043 T3	30-07-1993
		HK 129393 A	03-12-1993
		IE 59445 B1	23-02-1994
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		ZA 8607386 A	28-10-1987
US 4058594 A	15-11-1977	GB 1506563 A	05-04-1978
		US 3993775 A	23-11-1976

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— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** USE OF TRIGLYCERIDE OILS CONTAINING GAMMA-LINOLENIC ACID RESIDUES AND LINOLEIC ACID RESIDUES FOR THE TREATMENT OF NEURODEGENERATIVE DISEASE

(57) **Abstract:** A method is provided for treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a triglyceride oil containing both γ-linolenic acid and linoleic acid residues as triglyceride ester, the ratio of γ-linolenic acid to linoleic acid residues at the sn-2 position of the triglyceride being at least 0.8; the amount of γ -linolenic acid residues at the sn-2 position being at least 18 %, wherein the oil is administered at a dose sufficient to maintain or elevate TGF- β1 levels in the patient at a therapeutic level. Preferably the method is that wherein the therapeutic level is such as to produce a TGF- β1/TNF-α ratio of at least 0.5 in blood of a patient, after 18 months of daily dosing. Preferre oils are Borage or Mucor oils having at least 35 % of the sn-2 position fatty acid residues as γ-linolenic acid.

WO 2004/100943 A1

TREATMENT OF NEURODEGENERATIVE CONDITIONS.

The present invention relates to a method for treating neurodegenerative conditions, particularly those in which increase in transforming growth factor β (TGF-β) is beneficial, particularly TGF-β1. More particularly the present invention provides treatment for conditions such as multiple sclerosis and the degenerative sequelae associated with head trauma, stroke and intracranial bleeds, whereby neuronal function is improved or restored from an impaired condition. Further provided are novel use of known and novel compounds comprising unsaturated fatty acid moieties for the manufacture of medicaments capable of effectively treating such conditions, more particularly being capable of achieving previously unattained levels of success with regard to recovery of neurological function.

It is well reported in the literature that essential fatty acids (EFAs) of the n-3 and n-6 unsaturation pattern have beneficial effect in a wide variety of human physiological disorders. WO 02/02105 (Laxdale Limited) describes their beneficial use for an extremely wide range of diseases and as a general nutritional supplement. Harbige (1998) Proc. Nut. Soc. 57, 555-562 reviewed the supplementation of diet with n-3 and n-6 acids in autoimmune disease states, and particularly noted evidence of benefit of γ-linolenic (GLA) and/or linoleic acid (LA) rich oils, such as borage oil, in reducing clinically important signs and symptoms of rheumatoid arthritis.

Two studies on multiple sclerosis (MS) patients are noted that indicate that relapse and severity of the disease might be reduced by treatment with oils containing n-6 acid moieties (Miller et al (1973) and Bates et al (1978)), but a further study failed to confirm this effect (Paty et al (1978). These papers report that supplementation of human patients with about 20g/day of linoleic acid (18:2n-6) affected duration and severity of relapses of multiple sclerosis such that relapses were less frequent, less severe and of shorter duration than controls. Bates noted that a mixture of linoleic acid and γ-linolenic acid had been suggested back in 1957 to be possibly more efficacious in treating inflammation and autoimmune diseases and set out to investigate this in the trial. However, it was found that where this combination

was tried, at 3g oil per day (Naudicelle Evening Primrose oil) patients who had relapses became more ill on the trial oil than on the control.

Meta analysis of these linoleic acid studies by others (Dworkin et al (1984)) demonstrated reduced relapse rate and severity with a decrease in the degree of long-term progression of the disease in patients with mild multiple sclerosis. Later open studies of patients with multiple sclerosis suggest that low fat diet and/or manipulation of dietary n-3 and n-6 fatty acids may be beneficial (Swank & Grimsgaard (1988); Harbige et al (1990).

Although the aetiology of MS remains unknown, strong evidence suggests the presence of autoimmune mechanisms in the disease pathogenesis [Martino & Hartung 1999]. Studies have shown that MS patients have a much higher number of neuro-antigen e.g. myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) autoreactive T-cells which are in an increased state of activation compared with healthy controls [Fredrikson et al 1994, Kerlero de Rosbo et al 1993, 1997, Chou et al 1992, Ota et al 1990, Burns et al 1999, Zhang et al 1994, Tejada-Simon et al 2001]. The actual processes of axonal damage e.g. chronic inflammation, demyelination and astrogliosis in MS is complex but white matter inflammation and demyelination are considered to determine disease severity, whilst recent studies suggested that axonal damage in MS begins in the early stages of the disease and contributes to disability (De Stefano et al, 2001).

Experimental autoimmune encephalomyelitis (EAE) is the most frequently used animal model for immune mediated effects of MS. Studies in the guinea-pig have shown that linoleic acid partially suppresses the incidence and severity of EAE (Meade et al (1978)). Using γ-linolenic acid-rich oils from fungal or plant sources, complete protection was demonstrated in both rats and mice (Harbige et al (1995), 1997b). These investigations demonstrated disease modifying effects of linoleic acid and γ-linolenic acid on clinical and histopathological manifestations of EAE. Depending on dose, γ-linolenic acid was fully protective in acute rat EAE whereas linoleic acid had dose-dependent action on the clinical severity but did not abolish it.

Despite these experimental findings, it is recognised that the human disease, multiple sclerosis, is highly complex and can be conversely exacerbated and ameliorated by the activity of T-cells and other immune response factors. It is thought that the n-6 fatty acids promote autoimmune and inflammatory disease based upon results obtained with linoleic acid only. TGF-β and PGE_2 production has been shown to be increased non-specifically in γ-linolenic acid fed mice *ex vivo*; but whilst TGF-β has been reported to protect in acute and relapsing EAE ((Racke et al (1993); Santambrogio et al (1993)), PG inhibitors such as indomethacin augment, and thus worsen, the disease (Ovadia & Paterson (1982)).

Cytokines are implicated in the pathogenesis of MS, with many studies showing an increase in myelinotoxic inflammatory cytokines (TNF-α, IL-1β and IFN-γ) coinciding with the relapse phase of the disease. Conversely, levels of the anti-inflammatory and immunosuppressive cytokine transforming growth factor-beta1 (TGF-β1) appear to be reduced during a phase of relapse and increase as the patient enters remission. Thus the balance between biologically active TGF-β1 and the pro-inflammatory TNF-α, IL-1β and IFN-γ appears to be dysregulated during MS relapse-remission.

During natural recovery phase from EAE, TGF-β-secreting T-cells inhibit EAE effector cells, TGF-β is expressed in the CNS and, in oral-tolerance-induced protection in EAE, TGF-β and PGE_2 are expressed in the brain (Karpus & Swanborg (1991); Khoury et al (1992)). Harbige ((1998) concluded that dietary γ-linolenic acid effects on EAE are mediated through Th_3-like mechanisms involving TGF-β and possibly through superoxide dismutase antioxidant activity.

It has been suggested to use, *inter alia*, γ-linolenic acid and linoleic acid rich Borage oil as a means to provide immuno-suppression in multiple sclerosis (US 4,058,594). The dose suggested is 2.4 grams of oil per day and no actual evidence of efficacy is provided.

Borage oil (typically 23% γ-linolenic acid and 37% linoleic acid per 100% fatty acid content) has been shown to significantly reduce clinically important signs

and symptoms of autoimmune disease associated with active rheumatoid arthritis (Leventhal et al (1993)). Borage oil and fungal oil (see Figure 1) have been shown to be effective in the EAE animal model use to identify MS candidates, whilst never having been shown to be significantly effective in the human disease. High levels of linoleic rich oil containing low levels of γ-linolenic acid (EPO: linoleic acid:γ-linolenic acid 7:1) partially suppressed the incidence and severity of EAE in rat (Mertin & Stackpoole, 1978) whereas the Naudicelle study referred to above led to worsening of patients. In spite of the use of Borage oil and other GLA/LA containing oils such as Evening Primrose oil by multiple sclerosis sufferers over the past 30 years or so, the vast majority of patients fail to recover from the disease, showing no significant improvement, with the underlying disease continuing to progress to death.

Other more dramatic immunosuppressant treatments, including T cell depleters and modulators such as cyclophosphamide, are also shown to be effective in the EAE model, but where these are employed in the human multiple sclerosis disease symptoms improve, but the underlying disease continues to progress. T-cells indeed produce beneficial cytokines, such as TGF-β1, as well as deleterious ones in man. David Baker of Institute of Neurology, UK summed up the disparity between what is effective in the EAE and in MS with a paper entitled '*Everything stops EAE, nothing stops MS*' at the 10th May 2004UK MS Frontiers meeting of the UK MS Society.

It is clear that immunosuppression alone cannot cure MS. This is almost certainly due to a fundamental underlying metabolic disorder in MS patients that leads to membrane abnormality, cytokine dysregulation and subsequent immune attack and lesioning. Although patients go into remission in relapse-remitting disease, the underlying demyelination proceeds.

The 'gold standard' treatment for MS remains interferon, such as with β-Avonex ®, Rebif ® and other interferon preparations. This gold standard treatment only addresses needs of some, eg 30%, of the patients and even in these symptom improvement is restricted to reduced severity of relapses. Whilst symptoms may be reduced in a proportion of patients, the disease tends to progress to further disability and death due to underlying degeneration.

The present inventors have now surprisingly determined that with compliance to a 'high dose' treatment with triglyceride oil containing γ-linolenic acid with suitable accompanying fatty acid content, remarkable levels of improvement in almost all symptoms of MS can be achieved, way surpassing that provided by the present gold standard treatment. Such success is particularly surprising in the light of the prior use of other γ-linolenic acid containing preparations without such significant success.

Over an 18-month period, patients taking high dose selected borage oil showed significant ($p<0.001$) and marked improvements in EDSS score, a reduced rate of relapse, symptomatic relief of muscle spasticity and painful sensory symptoms, and improved objective measures of cognitive functions. Low dose borage oil was without effect.

Patients taking high dose borage oil maintained their level of peripheral blood mononuclear cell production (PBMC) of TGF-β1 during the trial period, their pro-inflammatory cytokines TNF-α and IL-1β were significantly and markedly (<70%) reduced and they either maintained or increased the PBMC membrane long chain omega-6 fatty acids dihomo-γ-linolenic acid (DHLA) and arachidonic acid (AA) in contrast to patients taking placebo who demonstrated loss of these fatty acids over the course of the trial period.

This whilst immuno-suppression would be expected to reduce active lesioning and neurodegeneration, the present treatment has apparently targeted maintenance of key membrane lipid components that are otherwise specifically lost in MS, suggesting a correction of a metabolic defect not otherwise effectively treated by current therapies. The fact that the low dose (5 grams/day) had no effect on this supports such determination.

Particularly the inventors have determined that a triglyceride oil comprising triglycerides of both γ-linolenic acid and linoleic acid with specific positional distribution within the triglyceride molecules, preferably with oleic acid, can provide significant decreasing EDSS score in multiple sclerosis patients over a number of months and years, a result that is unattainable with any of the currently administered therapies.

γ-Linolenic acid (18:3n-6, GLA) is known to be rapidly converted to longer-chain omega-6 polyunsaturated fatty acids dihomo-γ-linolenic acid and arachidonic acid *in vivo* (Phylactos et al 1994, Harbige et al 1995, 2000). Therefore to increase the level of membrane long chain omega-6 fatty acids in MS the inventors have reviewed results obtained with several GLA-containing oils:- both fungal (from Mucor javanicus) and plant (Borago officianalis), Evening primrose Oenothera spp. or Blackcurrant Ribes spp) as well as a synthetic tri-GLA oil as GLA delivery systems in an *in vivo* experimental animal model of MS known as chronic relapsing experimental autoimmune encephalomyelitis (CREAE).

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune inflammatory disease of the CNS, with or without demyelination, inducible in rodents and other mammalian species. Induction of EAE in rats however (using guinea pig basic protein), does ***not*** produce histological features of demyelination (Brosnan et al 1988) but induces an acute mono-phasic disease pattern, unlike MS which is characterised by CNS demyelination and is clinically relapsing-remitting. Chronic relapsing and demyelinating EAE models (CREAE), which are characterised by demyelination and relapse phases, are therefore currently the animal models of choice for MS research (Fazakerley 1997). With the demonstration that myelin oligodendrocyte glycoprotein (MOG) is an important neuroantigenic target in MS (Genain et al 1999) and the demonstration of far greater responses of peripheral blood auto-reactive lymphocytes to this neuroantigen, compared with MBP, in MS (Kerlero de Rosbo et al 1993, 1997) MOG induced CREAE has become the animal model of choice with features closely resembling those observed in MS (Fazakerely et al 1997, Genain et al 1999, Amor et al 1994).

Based on the results of these experiments two key selection criteria were adopted for selection improved lipid compounds for achieving the current aims. Evidence from CREAE and rat EAE feeding studies indicates that an enriched blackcurrant seed oil (72 % w/w 18:3n-6, GLA) did not protect against EAE (see Table 3). Importantly blackcurrant seed oil has a low sn-2 GLA with most of the GLA in the sn-1 and sn-3 positions (Lawson and Hughes 1988). Furthermore a structured

triacylgcerol containing three GLA's (TG-GLA) provided protective effects similar to that of the borage oil used in CREAE (Table 2). This would also be consistent with the sn-2 GLA being important i.e. the outer pair sn-1 and sn-3 GLA being enzymatically removed *in vivo* and probably undergo oxidation leaving the sn-2 GLA only. This selective hydrolysis arises from the known ability of specific lipases to remove the sn-1 and sn-3 fatty acids from triacylgycerol molecules but an apparent protection of the sn-2 position *in vivo* (Lawson and Hughes 1988, Kyle 1990).

The inventors' review of this data also indicates that the ratio of linoleic acid (LA) to γ-linolenic acid (GLA) residues may be a key efficacy feature of oils containing LA and GLA in the CREAE model of MS (Table 1). Table I shows the compositional analysis and efficacy in CREAE of fungal oil, borage oil, evening primrose oil and safflower oil. The most effective treatment in reducing the incidence of CREAE was fungal oil with an LA : GLA ratio of 0.85. Borage oil was also very effective with an LA : GLA ratio of 1.5. Furthermore, experiments with a structured triglyceride containing GLA at sn-1, sn-2 and sn-3 (TG-GLA) demonstrated GLA to be an active component. Moreover TG-GLA was also effective at a lower dose level than the borage oil (see Table 2).

Different Borage seed oil's also appear to vary in their level of sn-2 GLA e.g. 10 % sn-2 GLA (Liu et al 2000) and 40% sn-2 (Lawson and Hughes 1988) which is consistent with our unpublished observations of sn-2 GLA (range 38 – 46 %) and possibly the failure of some borage oils to produce fully protective effects in CREAE, although other factors such as antioxidant composition may also be important (unpublished). Borage oils having as much as 60% sn-2 GLA have been reported (Huang and Mills (1996) γ-Linolenic acid: metabolism and Its Roles in Nutrition and Mediceine: Chapter 6) and noted to be effective at getting GLA into lymph.

Table 3 of EP 0520624 (Efamol Holdings) compares the triglyceride content of Evening Primrose and Borage Oils, the former being taught to be more therapeutically effective than the latter for a variety of GLA responsive disorders. This document indicates Borage oil to have twenty seven different trigyceride components, only 20% of which have sn-2 GLA. Page 3, lines 40-42 notes that

biological testing has shown that equal amounts of GLA may indeed have very different effects when that GLA is supplied as different oil sources. Crucially, it then directs the reader to one particular fraction present in Evening Primrose Oil (EPO), but not Borage Oil, as being responsible for the former's superior effect in raising PGE1 (see EP 0520624 Chart page 4 and Table 2) and thus anti-inflammatory effect: that fraction being di-linoeoyl-mono-gamma-linolenyl-glycerol (DLMG) which it states to be 18 to 19% of the total triglyceride in EPO. Page 6 clearly teaches that the position of the GLA, in sn-1, 2 or 3, is not important to this effect.

Dines et al (1994) Proceedings of the Physiological Society, Aberdeen Meeting 14-16 September 1994 report on studies treatment of diabetic neuropathy neuronal damage with γ-linolenic acid containing oils of the type advocated by EP 0520624 and again note that Borage Oil was not very effective in treating this neurodegeneration whereas Evening primrose oil was. The paper concludes that Boage Oil contains other constituents that interfere with GLA activity.

In contrast to this prior art, the present inventors used a borage oil that was selected with the highest sn-2 GLA for trial purposes (>40%) – compared with lower amounts in other samples that were available at the time. Blackcurrant seed oil, which at the time was available in relative large quantities was not considered optimal because of it's low sn-2 GLA content.

A further selection criterion was that the level of total long chain monoenoic fatty acids be kept below 5%. There was a significant level of erucic acid (22:1n-9) i.e. 1.4 - 2.38 % of the total fatty acids, and other long chain monoenoic fatty acids i.e. 24:1n-9 (nervonic acid) and 20:1n-9 (gadoleic acid) in different borage seed oil (*Borago officinalis*) samples from different sources (Table 4).

Additionally because of potential impact on absorption, metabolism and immune functions of vitamin E (Harbige 1996, 2003), the trial oil contained only natural levels of vitamin E (0.05 mg/g) with no additionally added vitamin E as is routinely the case with commercial borage seed oils (e.g. 1 mg/g).

It is believed that such selected oils have an immunosuppressant effect, but significantly also have and a metabolic supplementation effect that has benefit in

reducing immune attack on lesions whilst creating the conditions necessary for their repair, something that has not been achieved with any medication previously provided for MS.

In a first aspect of the present invention there is provided a method of treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of an oil containing both γ-linolenic acid and linoleic acid residues as triglyceride ester, the ratio of γ-linolenic acid to linoleic acid residues at the sn-2 position of the triglyceride being at least 0.8; the amount of γ-linolenic acid residues at the sn-2 position being at least 18%, wherein the oil is administered at a dose sufficient to maintain or elevate TGF-β levels in the patient to therapeutic levels.

By therapeutic levels is meant levels at least consistent with healthy subjects. Preferably the dose is such as to produce a TGF-β1/TNF-α ratio in blood of a patient, after 18 months of daily dosing, of 0.4 to 3.0, at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably the dose is such as to produce a TGF-β1/IL-1β ratio in blood of a patient, after 18 months of daily dosing, of at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably said levels are produced after 12 months and more preferably after 6 months.

Typically the amount of oil administered daily will be between 3 and 30 grams, orally dosed, still more preferably between 5 and 20 grams and most preferably between 7 and 18 grams, typically 15 grams.

Most preferably, in addition to the γ-linolenic acid and linoleic acid fatty acid residues, the oil includes an esterified fatty acid that is non-structural, ie. that is metabolised to produce energy, such as oleic acid residues. By residue is meant the moiety that remains after the fatty acid carboxyl group esterifies to one of the hydroxy groups of the glycerol molecule.

Most preferably the oil administered is an oil source from Borage oil or a fungal oil eg. eg from Mucor javanicus,

Typical Borage oil and fungal oil compositions are illustrated in Table 1 wherein 18:2n-6 and 18:3n-6 represent linoleic and γ-linolenic acid residue by percent respectively.

Typically Borage oils contain from 20 to 25% γ-linolenic acid residues as percentage of fatty acid residues in the oil and from 35 to 40% linoleic acid residues. Preferred Borage oils are those in which the amount of esterified γ-linolenic acid at the sn-2 position is at least 35% of fatty acid residues at that position, more preferably greater than 39% and still more preferably greater than 40%. Most preferred oils are over 41%, such as 42 to 44% sn-2 GLA, whilst ideally they will be over 45%. As stated by Huang et al above, 60% sn-2 GLA Borage Oils have been produced and should be available for selection. The sn-1 and sn-3 position residues are preferably linoleic, oleic and γ-linolenic acid residues, with preferred oils having relatively high oleic acid content in at least one, if not both, of these positions, eg, in excess of 12%, more preferably in excess of 14%.

A typical Borage oil suitable for use in the use of the present invention has fatty acid distribution as follows

Sn-1: 14% 18:1 (Oleic), 54% 18:2n-6 (linoleic) and 4% 18:3n-6 (γ-linolenic)

Sn-2: 14% 18:1 (Oleic), 42% 18:2n-6 (linoleic) and 40% 18:3n-6 (γ-linolenic)

Sn-3: 19% 18:1 (Oliec), 18% 18:2n-6 (linoleic) and 30% 18:3n-6 (γ-linolenic)

Where a fungal oil is use, such as from Mucor species, the total amount of γ-linolenic acid residues may be lower than for Borage oil as long as the sn-2 γ-linolenic acid: linoleic acid ratio is at least 0.8, more preferably greater than 1. This is because fungal oils tend to have more 'metabolic' directed oleic acid residues than linoleic acid residues. Thus preferred fungal oils are those in which the amount of esterified γ-linolenic acid at the sn-2 position is at least 18% of fatty acid residues at that position, more preferably at least 20% and most preferably at least 22%. Preferred fungal oils have in excess of 45% of the sn-2 fatty acid residues as oleic acid residues, more preferably in excess of 50%.

Sn-1: 25% 18:1 (Oleic), 5% 18:2n-6 (linoleic) and 13% 18:3n-6 (γ-linolenic)
Sn-2: 54% 18:1 (Oleic), 19% 18:2n-6 (linoleic) and 20% 18:3n-6 (γ-linolenic)
Sn-3: 40% 18:1 (Oliec), 3% 18:2n-6 (linoleic) and 20% 18:3n-6 (γ-linolenic)

It will be realised by those skilled in the art that such oils will need to be sourced by testing for the percentage of said fatty acids at each position as an average over the many triglycerides in a give oil mix. Such is well within the skill of those in the art, such as eg. Mylnefiled Research Services Ltd, Lipid Analysis Unit, Mylnefiled, Inverghowrie, Dundee DD2, 5DA, Scotland UK. The applicants have managed to source a number of such oils meeting the criteria set out above, the highest sn-2 figures ofabout 46% being found for example in New Zealand sourced oils in 2003: this of course may vary from year to year. It is important, however, given the lack of response with the low dose (5g/day) Borage Oil that a patients efforts to recover are not undermined by the equivalent of underdosing by provision of a lower sn-2 % GLA Borage Oil than the daily dose requires.

A further NMR method for analysis of such oils in a selection process is provided in the methods section below. It will however be realised that should all oils available at a given tiem be below the 35% sn-2 GLA figure, and preferaby if they are below 40 or 45%, supplementation with a synthetic triglyceride or tryglyceride mix will be possible. A numebr of suitable lipids are known in the art and may for example be isolated or combined mixtures of LGL, OGO, OGL, LGO or other components known to be present in Borage Oil (see Table 3 of EP 0520624). Even TriGLA might be added (FR 2,617,161 (1988)), although it is preferred for the present purposes to keep the sn-1 and sn-2 position GLA levels from getting too high as that would un the risk of overly pro-inflammatory effect due to overflow into systemic GLA and thus DHGLA and then Arachidonic pools. Synthesis of OGO is for example taught in Y.-S. Huang, X. Lin, P. R. Redden and D. F. Horrobin, *J. Am. Oil Chem.Soc.*,72,625-631(1995)*In vitro* Hydrolysis of Natural and Synthetic γ-Linolenic Acid-Containing Triacylglycerols by Pancreatic Lipase and K. Osada, K. Takahashi, M. Hatano and M. Hosokawa, *Nippon Suisan*

Gakkaishi., **57**, 119-125 (1991). Chem. Abs. 115:278299 Molecular Species of Enzymically-synthesized Polyunsaturated Fatty acid-rich Triglycerides.

For treatment regimes where high amounts of any of these high Sn-2 GLA oils are administered it is recommended that the amount of potentially toxic long chain monoenoic fatty acids, such as erucic acid (22:1n-9) and other long chain monoenoic fatty acids i.e. 24:1n-9 (nervonic acid) and 20:1n-9 (gadoleic acid), are as low as possible, preferably lower than 5% of fatty acid residues, more preferably less than 3% and more preferably less than 2%.

Another feature of a preferred oil is low or zero added vitamin E such that only natural levels of vitamin E (0.05 mg/g) is provided.

Further aspects of the present invention provide use of triglyceride oils as described above for the manufacture of a medicament for the treatment of neurodegenerative disease, more specifically for the arresting of underlying neurodegeneration and the restoration of neuronal function. Particularly such medicaments are for the normalising of neuronal membrane composition, the restoration of healthy TGF-β1/TNFα ratios and the ratios of TGF-β1 with other cytokines, the arresting of neurodegeneration in multiple sclerosis and the restoration, in part or completely, of neuronal function such as measured, eg. By MRI or CAT scan or by EDSS score. Such use will include treatment of cerebral impairment after stroke, head trauma and intracranial bleeding.

Also provided are selected triglyceride oils having particular efficacy in treating multiple sclerosis and effecting beneficial changes in ratio of cyctokines *in vivo,* these oils eing those set out as preferred for the method described above.

The oils for use in the present invention may be administered by any of the conventional vehicles known in pharmacy. Most conveniently they are administered as neat oils or in admixture with foodstuffs, in the form of capsules containing such oils, or in enterically coated forms. Other forms will occur to those skilled in the art as delivery technology advances.

It will be realised by those skilled in the art that other beneficial agents may be combined with the oils for use in the present invention. These might be ion channel blcokers , eg. sodium channel blockers, interferons, T-cell depleters, steroids or other palliative agents. It will further be realsied that where the immune and inflammatory responses are being modulated, such combinations will need to be made carefully, given the complex nature of these systems. However, given the delayed response to the present oils, shorter acting agents might be beneficial in the first months of treatment before the TGF-β1 levels are normalised, as long as the additional treatment does not impede this normalization process.

The present invention will now be described by way of Example only by reference to the following non-limiting Tables, Examples and Figures. Further embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

TABLES

Table 1: Shows the compositional % Total fatty acid content of various triglyceride oils and protective effect in EAE.

Table 2: Shows the parameters of the three treatment groups in high sn-2 GLA Borage Oil trial

Table 3: Shows the effect of various forms of GAL on EAE incidence and clinical score in SJL mice.

Table 4: Shows the failure of enriched Blackcurrent oil, a high GLA, but low sn-2-GLA, plant oil, to match fungal and Borage oils in EAE.

Table 5: Shows the results analyses of four batches of trial Borage Oil particularly with respect of monoenes.

Table 6: Shows an analysis of a non-trial oil particularly with respect to monoenes.

FIGURES

Figure 1: Shows peripheral blood mononuclear cell cytokine production in placebo and trail oil treated human MS patients at 18 months.

Figure 2: Shows the effect of placebo and low dose (5g/day) high sn-2 GLA Borage oil on human MS patient EDSS score as compared to high dose (15g/day) displayed as a histogram.

Figure 3: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient EDSS displayed as a graph.

Figure 4: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient Mean Relapse rate (%) as a histogram

Figure 5: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient Mean Relapse rate (%) as a graph.

Figure 6: Shows the effect of linoleic acid:γ-linolenic acid ratio of oils as compared to their protective effect on mice CREAE.

METHODS

Positional Analysis of Gamma-Linolenic acid (GLA) in Borage oil Samples by Quantitative-^{13}C-NMR

Analytical methodologies for the determination of fatty acid composition and positional distribution in triacylglycerols generally require hydrolysis of the triacylglycerols by enzymes or chemical processes and subsequent analysis of the

mono-and diacylglycerol components by chromatographic techniques. These methods are destructive and do not allow the recovery of the original samples. The hydrolysis procedure usually gives rise to some acyl migrations, resulting in substantial errors of the positional distribution.

There are several properties of ^{13}C nuclear magnetic resonance (NMR) that make its application to positional analysis useful. First, the chemical shift is sensitive to the molecular structure, thereby giving rise to a spectrum where each nucleus is identified by a peak at a specific frequency. The resolution of the nuclei in each environment is determined by the linewidth and the chemical shift differences between adjacent peaks . Second, the area under the peak, arising from each nucleus, is proportional to the number of nuclei in that environment because all ^{13}C exhibit the same absorption . Therefore, the chemical shift and the integrated area of each peak can be used for both qualitative and quantitative measurements of each nucleus. Thirdly, the preparation of the sample for this application is simple. Finally, NMR is a non-destructive technique that allows the sample to be recovered for other purposes. ^{13}C NMR methods are normally based on analysing the cluster of signals for the carbonyl carbons. Two clusters of signals are normally observed corresponding to acids in the sn 1,3- and 2-positions. They are usually readily distinquished since the two environments give rise to a separation of about 0.4 ppm. Within each of these two clusters , there must be separate signals for each acid or groups of acids. This criterion is most easily met when the acids have carbon-carbon double bonds (i.e. are unsaturated) close to the carboxyl group e.g. n = 4, 5 or 6. The carbonyl carbon signals from acids of the same double bond group (e.g. EPA and AA) will not normally be distinquished. Such methods seemed to be valuable for analysing GLA-containing triacylglycerols and this proved to be the case .

References:

M. M. Bergana and T. W. Lee, *J. Am. Oil Chem. Soc.*, **73**, 551-556 (1996)

G. Vlahov, *Magn. Reson. Chem.*, **36**, 359-362 (1998)

Experimental

Materials/sample-preparation

Monoacidtriacylglycerols were purchased from Sigma Chemicals and Nu-Chek-Prep Inc:

Tripalmitin	(Tri-16:0)
Tristearin	(Tri-18:0)
Triolein	(Tri-18:1n-9)
Trilinolein	(Tri-18:2n-6)
Trigammalinolenin	(Tri-18:3n-6)
Trieicosenoin	(Tri-20:1n-9)
Trierucin	(Tri-22:1n-9)
Trinervonin	(Tri-24:1n-9)

Approx 180 mg of lipid in 700 uL of deuteriochloroform was used throughout the study.

^{13}C-NMR-data

The proton-decoupled ^{13}C NMR data with supressed NOE were collected at 21°C in a 5-mm broadband probe on a Jeol 500 MHz spectrometer operating at 125.728 MHz. Waltz decoupling was the chosen mode of decoupling and was gated on only during the 14.89s acquisition time. The relaxation delay was set at 30 secs and the pulse angle was 90 °.The spectral window used was ca. 35 ppm (from 173.5 to 172.6 ppm) with a 170 ppm offset. The spectra were internally referenced to $CDCl_3$ at 77.0 ppm . Typically, the approximate number of scans collected for adequate signal-to-noise ranged from 300 to 1200 scans , depending on the complexity of the mixture. The total acquisition time for the experiments ranged between 1-4 h (Borage oil 1272 scans / 4h). Data points 65,536.

CALCULATIONS

The GLA carbonyl signals at the 2- and 1,3 –positions were well separated from all over carbonyl peaks in the spectra of the triacylglycerols. This allowed the ratio of 2-GLA / 1,3-GLA to be determined with reliability and precision in all cases. The method was validated by analysing a test mixture containing 8 triacylglycerols, including trigammalinolenin, of known proportions similar to those present in borage oil. Gross compositions had previously been determined by GLC. Two methods of calculation were employed. The first was a self-contained NMR method calculating the 2-GLA percentage as follows:

$$\frac{\text{Integral of sn-2 peak for GLA} \times 3 \times 100}{\text{Total of all sn-1,2 and 3 integrals}}$$

The second used the ratio of 2-GLA / 1,3 GLA integrals from the NMR and gross GLA composition as determined by GLC as follows:

$$\frac{\text{Integral of sn-2 peak for GLA} \times \%\ \text{GLA from GC analysis} \times 3}{\text{Total integrals for GLA at sn-1,2 and 3}}$$

Results from both computations were in agreement. We regard the composite NMR-GLC method as more precise since it uses the parameters from each method that can be measured with most accuracy. GLA is a major component of borage oil and therefore the 2- /1,3 –GLA ratio can be determined by NMR with precision as the results show. GLC is generally better at determining the composition of even minor fatty acids than NMR but cannot give positional information.

Summary

Details of the experimental data are given in the tables and spectra. It is summarised below. Capsules D and B are sample capsules of provided in the clinical trial reported below.

1. 8-TG-Test-mixture

sn-2	GLA	found	22.2%
		actual	22.6%
		error	1.8%

(n)=2

2.Capsule-D

Gross GLA	found	21.4%	(n = 3)	by GLC
sn-2 GLA	found	42.5%	(n = 2)	by NMR
	error			+/- 1%

3.Capsule-B

Gross GLA	found	21.1%	(n = 3)	by GLC
sn-2 GLA	found	40.8%	(n = 2)	by NMR
	error			+/- 1%

In these borage oil samples the GLA content in the sn-2 position is 41-42% i.e. almost double (1.95 x) that of the gross composition. A probable typical sample reported in the literature shows an enrichment of 1.8 times gross..

The NMR method can be used to give reliable analytical data for GLA at the sn-2

position. Indeed, it is particularly suited to borage oil because of the non-interference of other fatty acids present. Interestingly results from the NMR method are reported to agree with those from the older derivatisation-chromatographic ones. Our previous estimate (40%) derived from these older methods also agrees with the NMR results.

TREATMENT EXAMPLES.

Twenty-eight active relapsing-remitting (two relapses in the preceding 18 months) multiple sclerosis patients (ages ranging from 18 to 65 yrs) were entered into a double-blind placebo controlled trial to investigate the effects of encapsulated borage oil on clinical activity and laboratory parameters over 18 months. This oil was of high Sn-2 γ-linolenic (GLA) content (>40% GLA at Sn-2) with low monene (eg. erusic acid) content and had no added Vitamin E, a known immunomodulator.

Patients were recruited from neurology out-patient clinics at two inner city hospitals; hospital informed consent was obtained on first (baseline) visit. Exclusion criteria include any form of steroid or immunosuppressive drug treatment, pregnancy, hyperlipidemia, regular use of aspirin or related drugs and vitamin or fatty acid supplementation within the previous three months.

Only patients meeting all the following criteria were included in the trial: (a) able to provide informed consent prior to treatment, with the full understanding that consent may be withdrawn at any time without prejudice; (b) male or female out-patients aged 18 to 60 years inclusive; (c) have confirmed diagnosis of clinically definite relapsing MS; (d) have had at least three documented clinical relapses in the past two years; (e) have a baseline Expanded Disability Scoring Scale (EDSS) score of 0.0-5.5 inclusive, provided they have well documented exacerbations; and (f) healthy, apart from the MS-related symptoms, as confirmed by the medical history, physical examination and clinical chemistry, urine and haematological tests.

Patients were randomly allocated by the Pharmacy Department to one of three groups each containing 12 patients:

- One clinical group (n=12) to receive placebo (5 g of Polyethylene Glycol 400)
- Second clinical group (n=12) to receive low-dose (5 g) refined *Borage officinalis*

- Third clinical group (n=12) to receive high-dose (15 g) refined *Borage officinalis*

Supplementation was in the form of one gram oil capsules daily (5/day for low dose, 15/day high dose) for 18 months duration. *Borage officinalis* oil and omega-6 polyunsaturated fatty acids are food ingredients that are generally recognised as safe for human consumption (GRAS). There are no classification or labelling requirements under EC regulations. Clinical assessment included: Extended Disability Scale Scores (EDSS) and clinical relapse record. Venous blood (50 mls) was obtained for laboratory studies on the 1st, 3rd, 6th, 12th, 15th, and 18th month of supplementation.

The following biochemical and immunological parameters were investigated on each visit for comparison with pre-treatment data and between group data:

- Stimulated and unstimulated *ex vivo* peripheral blood mononuclear cell cytokine production: TGF-β1, IFN-γ, TNF-α, IL-1β, IL-6 and IFN-β, which are implicated in the pathogenesis of MS. Cytokine and related gene expression.
- Soluble adhesion molecules in serum particularly ICAM-1 and VCAM-1
- Peripheral blood mononuclear cell membrane fatty acids and plasma phospholipid fatty acid composition.

Results are shown in Tables 1 and 2 and Figures 1 to 5.

The primary outcome parameter was the number of clinical relapses between baseline (Month 0) and the end of treatment (Month 18). Secondary outcome parameters included: the time to first clinical relapse; severity of relapses, as assessed by EDSS score and the use of steroid treatment; and changes in EDSS at Month 3, 6, 9, 12, and 18 compared to baseline and defined as at least 1.0 point increase in the EDSS that is sustained for 3 months or at least 1.5 point increase on the EDSS from the baseline EDSS that is sustained for 3 months. As this trial did not receive external funding, it was not possible for financial reasons to evaluate MS diseases activity with magnetic resonance imaging. 1 of 3

Eleven patients were in the placebo group, seven patients had been taking low-dose Borage oil, and ten patients had been taking high-dose Borage oil. The study

drug was well-tolerated, and there were no serious adverse events during the 18-month trial.

RESULTS

Two patients had developed diarrhoea, both of whom were later confirmed to have been taking high-dose Borage oil. The diarrhoea was mild in one patient, but was moderately severe in the second patient, who later discontinued the study drug. The code was not broken and the diarrhoea had stopped after the discontinuation of the drug, but reappeared upon re-challenge. Therefore, this patient was withdrawn from the trial. The remaining patients who were treated with high-dose Borage oil showed excellent clinical improvement on all primary and secondary outcome criteria. For example, their mean EDSS score after 6 months of treatment had improved from baseline EDSS (Figure 1). More importantly, the mean number of clinical relapses had significantly reduced after 6 months of treatment when compared to the number of relapses in the placebo group (Figure 2). In contrast, patients who had been receiving low-dose Borage oil did not show any clinical improvement when compared to the placebo group. In addition to its beneficial effect on MS disease activity, high dose Borage oil provided some symptomatic relief of muscle spasticity (stiffness) and painful sensory symptoms, and also improved cognitive functions.

As can be seen for the figures below, relapse rate after 9, 12 and 18 months was down to zero in the high dose group. The increase seen at 15 months was due to a patient dropping out of this group.

The following are three brief case histories to illustrate the therapeutic benefits of high dose high sn-2 GLA Borage oil. The first two are from the trial while the third is a post trial patient for whom MRI studies were obtained.

Patient 1 (Treatment):

The first patient was a 48 year old woman who had had a clinically active, relapsing remitting MS for 9 years. She had originally worked as a full-time administrator at the local Health Authority, but she was unable to perform her duties

because of her severe MS. Therefore, she later worked as a part-time secretary, but still had difficulties in mobilization because of muscles stiffness and sensory disturbances. She was also experiencing severe clinical relapses at an average of one relapse every nine months. Most of these relapses had resulted in hospital admissions for steroid therapy. In view of her active MS, she was recruited into the Borage oil trial. There were no adverse events relating to the study, and after taking the medication for four months, she experienced good improvement in her walking and sensory symptoms.

About nine months after therapy, she was well enough to start full-time employment. In addition, she remained relapse-free for the 18-month duration of the clinical trial. Following the conclusion of the trial, the treatment code revealed that she was taking high-dose Borage oil.

Patient 2 (Control):

The second case was a 46-year old woman who also had a clinically active relapsing remitting MS for 8 years. She had originally worked as a shop assistant, but became unemployed after MS was diagnosed.

Her symptoms included difficulty with mobilisation and painful sensory symptoms in both legs. She had experienced three clinical relapses in the two years preceding the clinical trial, and had been admitted to hospital twice for steroid therapy. Consequently, she was recruited into the Borage oil trial, but her walking continued to deteriorate. Six months into the trial, she need to use a walking stick and also received treatment with Baclofen to reduce low limb spasticity. Approximately ten months after starting the Borage oil trial, she was admitted to hospital because of severe clinical relapse, which was treated with steroids. She later developed bladder disturbances and began to use a wheelchair for long journeys. The treatment code was broken after the conclusion of the 18-month trial, and she was found to have been taking placebo. Since then, she started using a walking frame for journeys exceeding 50 yards.

Patient 3: Treatment (additional to trial)

The third case was a 26 year-old man who was diagnosed with definite MS in April 2001. His symptoms had started in 1999 when he complained of diffuse, intractable pain affecting various parts of his body, particularly the left side of the chest and abdomen. This was followed by intermittent numbness in the hands and feet, associated with fluctuating weakness. There were also distressing bladder symptoms in the form of urinary frequency and urgency. The diagnosis of MS in 2001 was based on his relapsing remitting symptoms, and was confirmed by positive cerebrospinal fluid analysis and magnetic resonance imaging (MRI) of the brain, which showed multiple white matter abnormalities in both cerebral hemispheres. Symptoms did not respond to various pharmaceutical therapies.

In April 2003, oral supplementation with the present high dose Borage oil was commenced. The patient reported dramatic improvement in his symptoms within three months of starting this oral supplementation. His painful sensory symptoms disappeared completely. He reported no numbness or weakness since May 2003, and noticed significant improvement in his bladder control. The oral supplementation caused no adverse events. A repeat brain MRI was undertaken to verify the reported improvement in Mr N's symptoms. The repeat MRI showed a reduction in the size and distribution of the white matter abnormalities.

TABLE 1

Compositional (% Total FAs) Characteristics of Various Oils and their Protective Effects in EAE

Treatment	18:2n-6	18:3n-6	18:2n-6/18:3n-6	18:1n-9	INCIDENCE OF EAE
FGO	17	20	0.6	35	0/10
BOO	37	24	1.5	15	3/10
EPO	71	9.4	7.5	9	7/10
SAF	66	-	-	17	9/10
Controls	-	-	-	-	9/10

FGO, Fungal Oil; BOO, Borage Oil; EPO, Evening Primrose Oil, SAF, Safflower Oil.

TABLE 2

Treatment Groups- Borage oil-MS trial

Group		Female	Male	Mean Relapse Rate (in past two years)	Mean Base EDSS	Number
	Placebo	7	4	2.6	3.9	11
	Low Dose	5	2	2.9	3.5	7
	High Dose	8	2	3.4	2.8	10
Total		20	8	2.9	3.4	28

Table 3. Molecular Species Comparison of Triacylglycerol-GLA (TG-GLA), Ethyl-Ester-GLA (EE-GLA) and *Borago Officinalis* Oil-GLA (BOR-GLA) in MOG-induced CREAE in SJL Mice

Treatment	No. with EAE	Mean Clinical Score
Control	10/11	3.3±1.3
EE-GLA[a]	5/6	3.0±0.8
TG-GLA[a]	3/6	1.0±1.3[c]
BOR-GLA[b]	3/6	1.0±1.2[c]

[a] Animals given 100 µl of test lipid; [b]250µl BOR-GLA given. Significance of difference compared with controls, [c] $p < 0.05$

27

Table 4. Effect of enriched black-currant seed oil (73% GLA) on the incidence of EAE

	% Incidence of EAE (Days after immunisation)							
	13		17		21			
Controls (n=10)	60		90		10			
Blackcurrant (n=10)	10		80		70			

Note: Blackcurrant oil delays the incidence but does not provide full protection. Animals were fed 7 days after sensitization (immunisation).

TABLE 5: ANALYSIS REPORTS ON TRIAL BORAGE OIL (% Total Fatty Acids)

	SAMPLE 1		SAMPLE 2		SAMPLE 3		SAMPLE4	
Fatty Acids %								
16:0	13.29		13.47		12.86		13.11	
16:1n7	0.21		0.22		0.21		0.21	
18:0	3.50		3.47		3.54		3.50	
18:1n9	16.22		16.22		16.33		16.22	
18:1n7	0.64		0.66		0.65		0.64	
18:2n6	38.00		38.01		38.25		37.96	
18:3n6	22.59		22.66		22.69		22.56	
18:3n3	0.18		0.18		0.17		0.19	
20:0	0.20		0.18		0.20		0.21	
20:1n9	2.96		2.88		3.06		3.06	
22:1n9	1.55		1.41		1.50		1.58	
24:1n9	0.60		0.63		0.52		0.71	

TABLE 6. ANALYSIS OF A *NON*-TRIAL BORAGE OIL (% Total Fatty Acids)

Fatty Acids	% Total Fatty Acids
16:0	11.07
16:1n-7	0.17
18:0	3.70
18:1n-9	16.37
18:1n-7	0.66
18:2n-6	37.71
18:3n-6	21.89
18:3n-3	0.17
20:0	0.25
20:1n-9	3.79 ↑
22:1n-9	2.38 ↑ (high)
24:1n-9	1.47 ↑

CLAIMS.

1. A method of treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a triglyceride oil containing both γ-linolenic acid and linoleic acid residues as triglyceride ester, the ratio of γ-linolenic acid to linoleic acid residues at the sn-2 position of the triglyceride being at least 0.8; the amount of γ-linolenic acid residues at the sn-2 position being at least 18%, wherein the oil is administered at a dose sufficient to maintain or elevate TGF-β1 levels in the patient at a therapeutic level.

2. A method as claimed in Claim 1 wherein the therapeutic level is such as to produce a TGF-β1/TNF-α ratio of at least 0.5 in blood of a patient, after 18 months of daily dosing.

3. A method as claimed in Claim 2 wherein the ratio is at least 0.75.

4. A method as claimed in Claim 2 wherein the ratio is at least 1.

5. A method as claimed in Claim 1 wherein the amount of oil administered is between 3 and 30 grams per day.

6. A method as claimed in Claim 1 wherein the oil is administered orally.

7. A method as claimed in Claim 1 wherein the dose is sufficient to administer at least 1 gram of γ-linolenic acid residues, as residues in the sn-2 position, excluding other γ-linolenic acid content of the oil.

8. A method as claimed in any one of the preceding claims wherein the amount of γ-linolenic acid in the sn-2 position in the dose of oil is sufficient to administer at least 2 grams of said sn-2 γ-linolenic acid.

9. A method as claimed in any one of the preceding claims wherein the dose is between 8 and 20 grams.

10. A method as claimed in any one of the preceding claims wherein in addition to the γ-linolenic acid and linoleic acid fatty acid residues, the triglyceride includes an esterified fatty acid that is non-structural.

11. A method as claimed in claim 10 wherein the triglyceride contains oleic acid residues.

12. A method as claimed in claim 1 wherein the oil is that obtained from a fungus or a plant selected from the group consisting of Mucor and Borago species.

13. A method as claimed in Claim 12 wherein the fungus or plant is selected from Mucor javanicus and Borago officianalis.

14. A method as claimed in Claim 1 wherein the oil is a Borago oil in which the percentage of esterified γ-linolenic acid at the sn-2 position is at least 35% of fatty acid residues at that position

15. A method as claimed in Claim 14 wherein the percentage of esterified γ-linolenic acid at the sn-2 position is at least 39% of fatty acid residues at that position.

16. A method as claimed in Claim 14 wherein the percentage of esterified γ-linolenic acid at the sn-2 position is at least 45% of fatty acid residues at that position

17. A method as claimed in any one of the preceding claims wherein the fatty acid residues in the sn-1 and sn-3 position include linoleic, oleic and γ-linolenic acid residues.

18. A method as claimed in any one of the preceding claims wherein the triglyceride oil has an oleic acid content in one or both of the sn-1 and sn-3 positions of in excess of 12%.

19. A method as claimed in Claim 1 wherein the oil is Mucor oil and, the total percentage of esterified γ-linolenic acid residues at the sn-2 position is at least 20% of fatty acid residues at that position.

20. A method as claimed in Claim 19 wherein the triglyceride oil has in excess of 45% of the sn-2 fatty acid residues as oleic acid residues.

21. A| method as claimed in Claim 19 wherein the triglyceride oil has in excess of 50% of the sn-2 fatty acids as oleic acid residues.

22. A method as claimed in any one of the preceding claims wherein the triglyceride oil contains less than 5% monoenoic fatty acid residues as % total fatty acid residues.

23. A method as claimed in Clam 22 wherein the triglyderide oil contains less than 5% in total erucic acid (22:1n-9), 24:1n-9 (nervonic acid) and 20:1n-9 (gadoleic acid) as a percentage of total fatty acid residues .

24. A method as claimed in Claim 22 or 23 wherein the amount of said acid is between 1% and 5% of fatty acid residues in the oil.

25. A method as claimed in any one of the preceding claims wherein the oil has no added vitamin E.

26. A method as claimed in any one of the preceding claims wherein the amount of Vitamin E is between 0 and 0.1mg/g.

27. A method as claimed in any one of the preceding claims wherein the neurodegenerative disease is arrested or neuronal function is restored.

28. A method as claimed in any one of the preceding claims wherein treatment is for multiple sclerosis or the degenerative sequelae associated with head trauma, stroke and intracranial bleeds.

29. A method as claimed in claim 28 wherein the treatment repairs lesions.

30. A method as claimed in Claim 1 or 28 wherein the treatment uses a dose sufficient to relieve muscle spasticity and/or pain.

31. A method as claimed in Claim 1 or 28 wherein the dosage is sufficient to improve cognitive function.

32. A method as claimed in Claim 1 or 28 wherein the dosage is sufficient to eliminate relapses.

33. A method as claimed in Claim 1 or 28 wherein the dosage is sufficient to improve the patients EDSS score by at least 1 unit over a period of 1 years treatment.

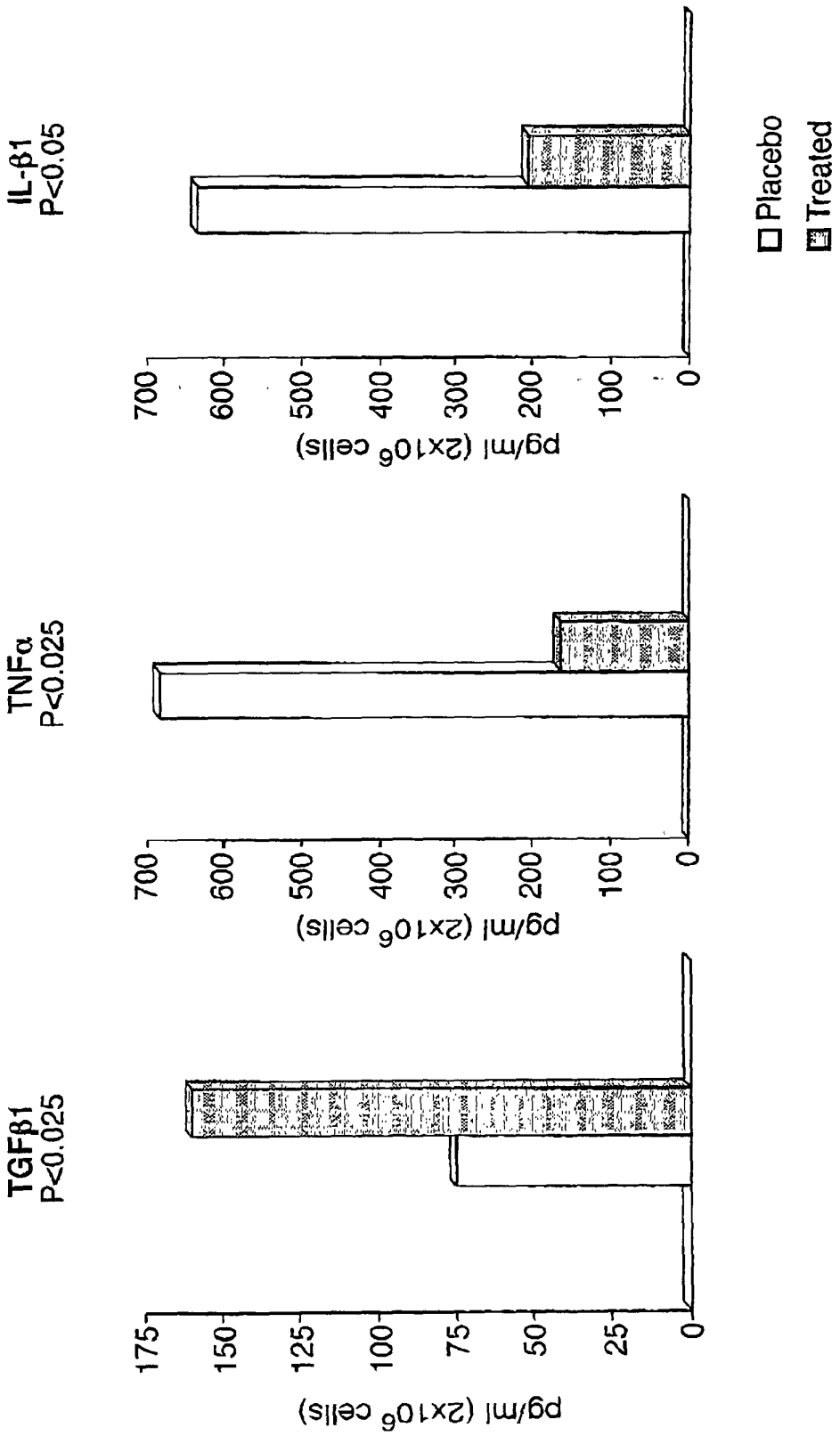
34. A method as claimed in Claim 1 or Claim 28 wherein the dosage is sufficient to restore EDSS of a patient with EDSS above 2.5 to below 2 over a period of 1 years treatment.

35. Use of an oil as described in any one of Claims 1 to 34 for the manufacture of a medicament for the treatment of neurodegenerative disease.

36. A pharmaceutical composition for the treatment of neurodegenerative disease comprising a Borago or Mucor species triglyceride oil as described in any one of Claims 14 to 26.

Fig.1.

Peripheral Blood Mononuclear Cell Cytokine Production in Placebo and Oil Treated Multiple Sclerosis Patients at 18 Months



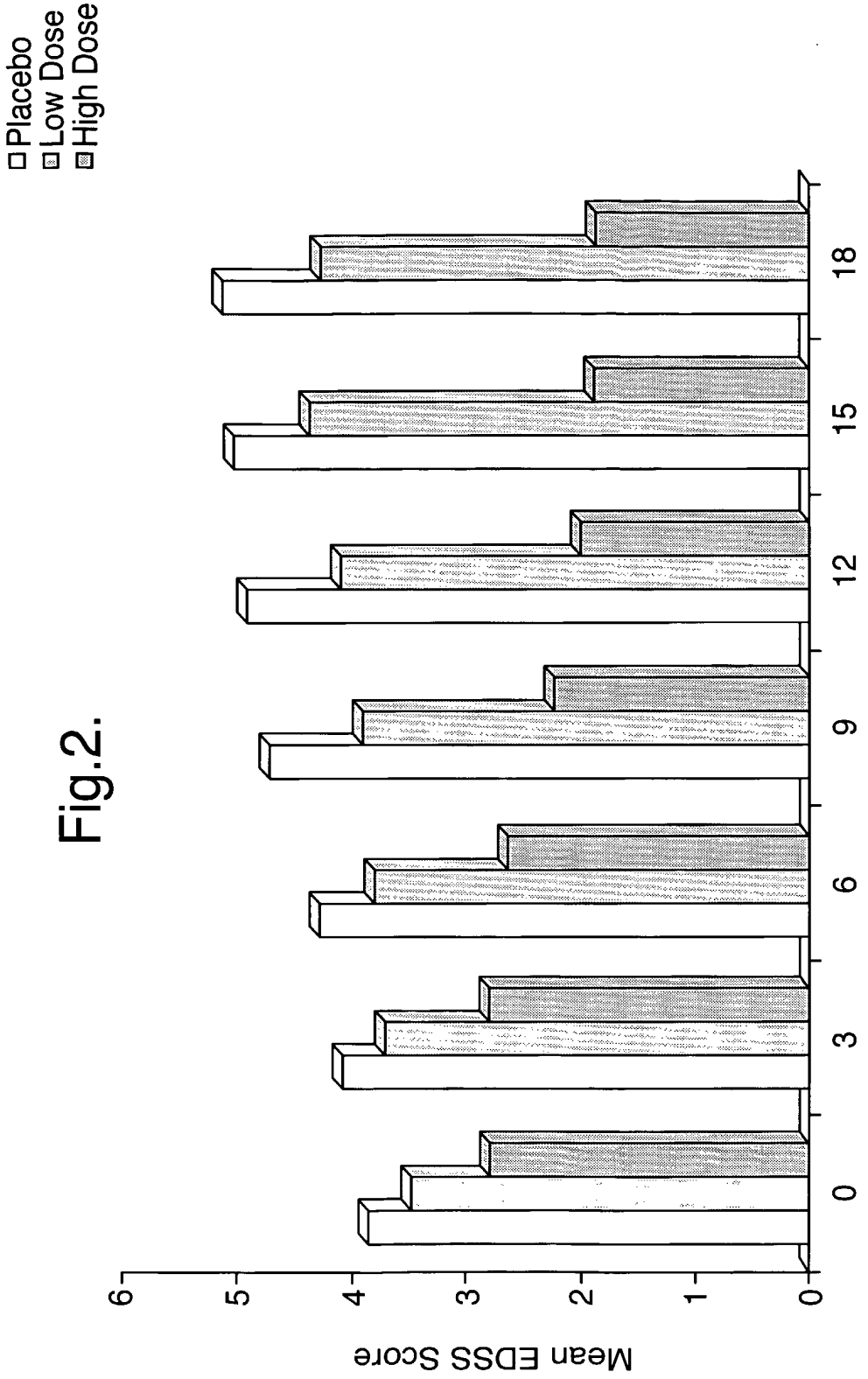

Placebo
Low Dose
High Dose
Mean EDSS Score
0
1
2
3
4
5
6
0
3
6
9
12
15
18

Fig.2.

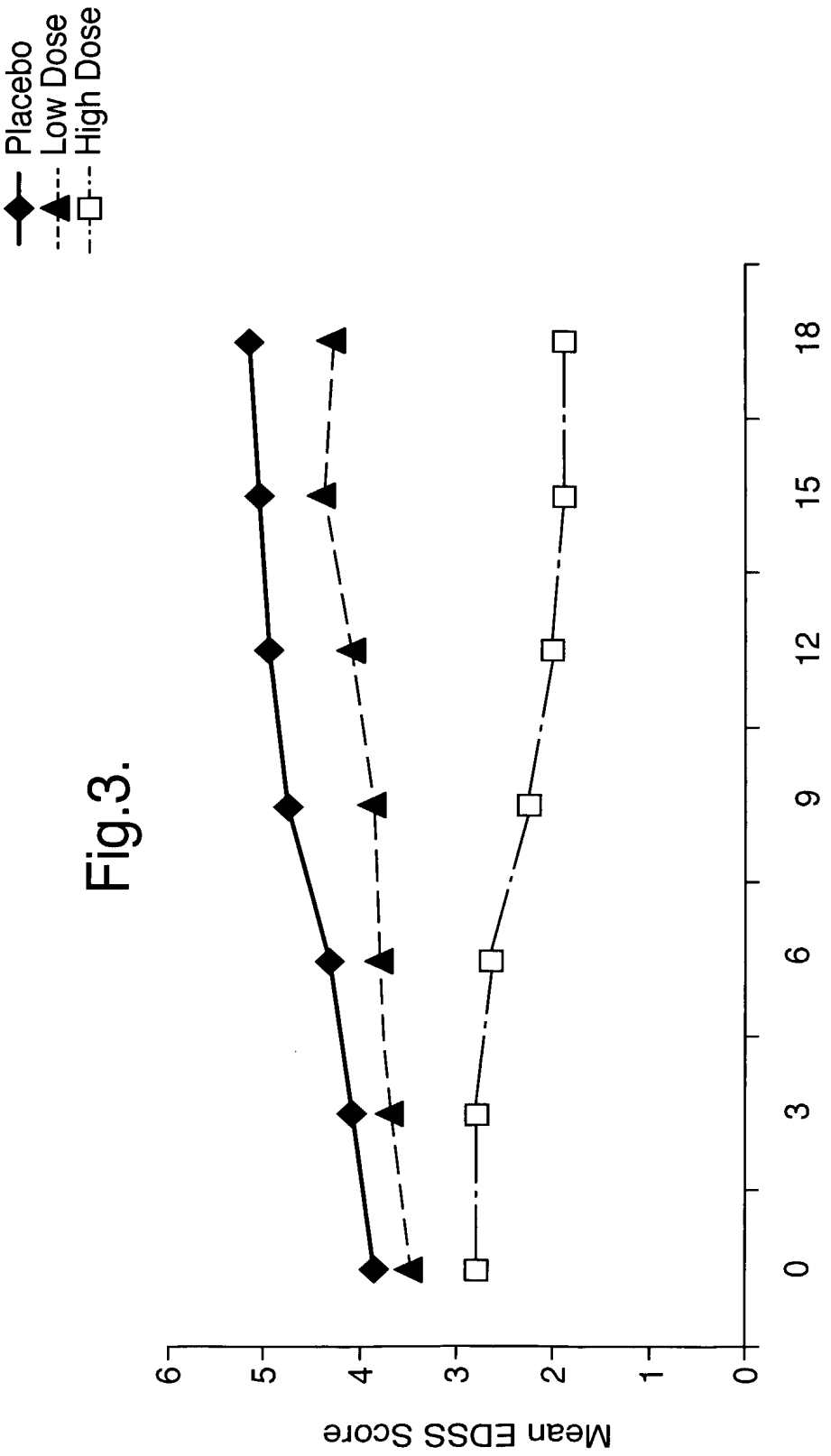

Placebo
Low Dose
High Dose
Mean EDSS Score
0
1
2
3
4
5
6
0
3
6
9
12
15
18

Fig.3.

Fig.4.

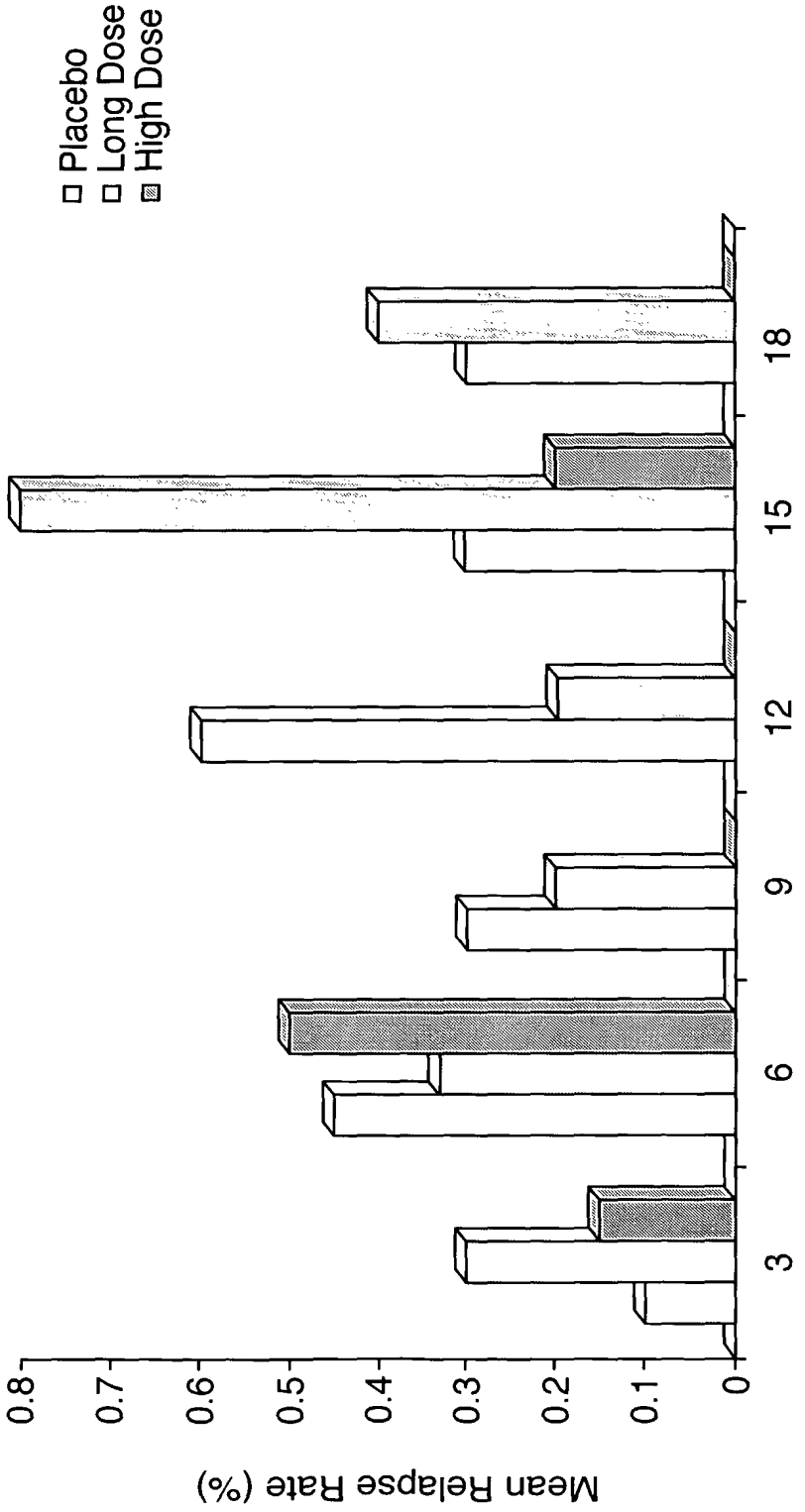

Placebo
Long Dose
High Dose
Mean Relapse Rate (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
3
6
9
12
15
18

Fig.5.

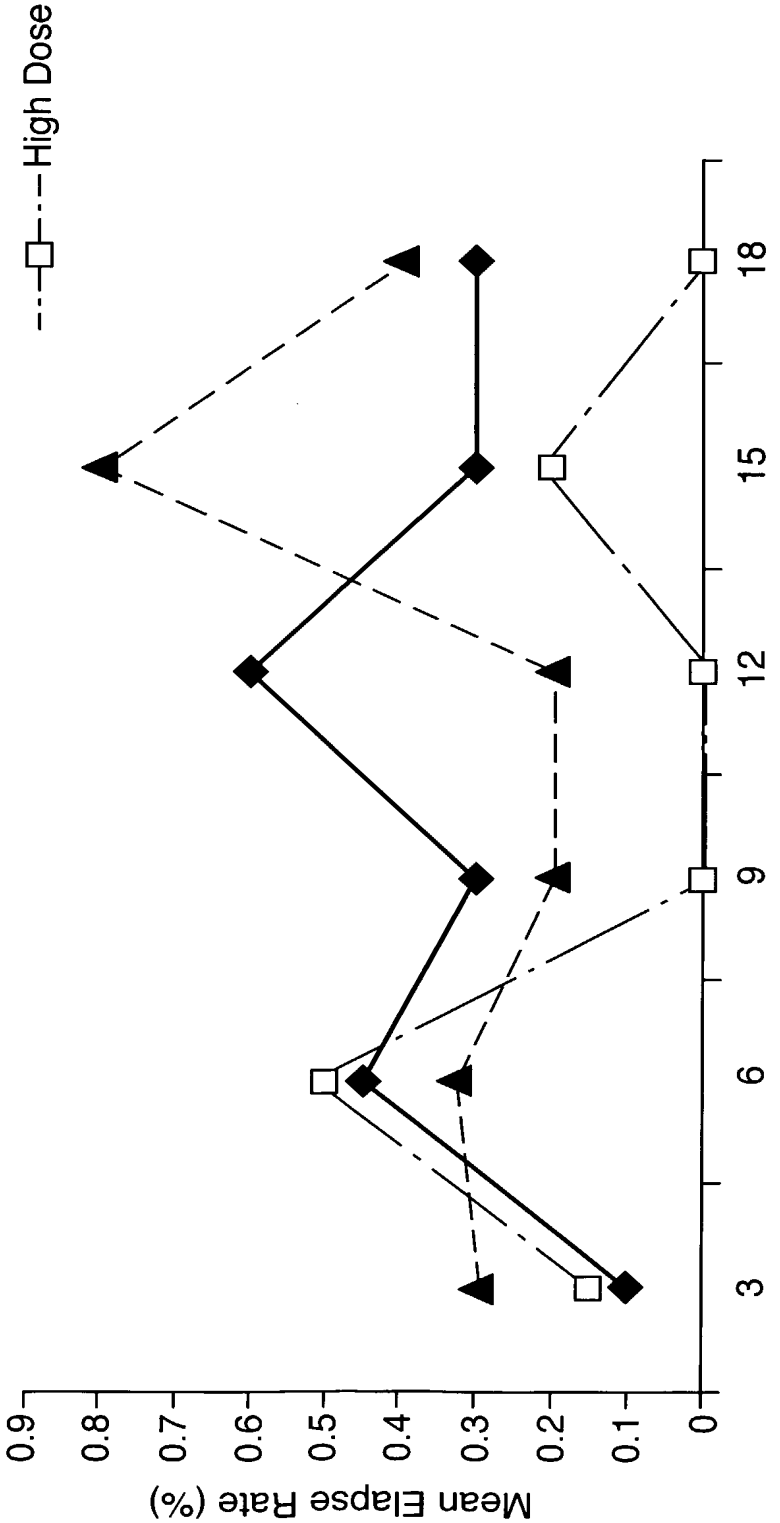

Placebo
Low Dose
High Dose
Mean Elapse Rate (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
3
6
9
12
15
18

Fig.6.

Effect of Ratio of Linoleic Acid to Gamma-Linolenic Acid on Acute Phase of SCH Induced CREAE in Biozzi AB/H Mice

18:2n-6/18:3n-6	Incidence of EAE
0.5	0/10
1.5	4/10
7.5	7/10
Controls	10/10

INTERNATIONAL SEARCH REPORT

national Application No
.T/GB2004/002089

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/231 A61K31/232 A61K35/78 A61K35/84 A61P25/28

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)
IPC 7 A61K A61P

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

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

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 826 877 A (CARMICHAEL HUGH ET AL) 2 May 1989 (1989-05-02) column 1, line 6 - line 8 column 4, line 58 - line 62 column 5, line 5 - line 28	1-36
Y	US 4 058 594 A (WILLIAMS JOHN) 15 November 1977 (1977-11-15) cited in the application the whole document column 2, line 25 - column 3, line 8	1-36

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in 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 but 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

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"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	Date of mailing of the international search report
14 September 2004	30/09/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Albrecht, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAWSON L D ET AL: "TRIACYLGLYCEROL STRUCTURE OF PLANT AND FUNGAL OILS CONTAINING GAMMA LINOLENIC ACID" LIPIDS, vol. 23, no. 4, 1988, pages 313-317, XP009035770 ISSN: 0024-4201 cited in the application table 2	1-36
Y	HARBIGE L S ET AL: "Prevention of experimental autoimmune encephalomyelitis in Lewis rats by a novel fungal source of gamma-linolenic acid" BRITISH JOURNAL OF NUTRITION, vol. 74, no. 5, 1995, pages 701-715, XP009035788 ISSN: 0007-1145 cited in the application page 712-714, chapter "DISCUSSION"	1-36
Y	HOY CARL-ERIK ET AL: "Absorption of gamma-linolenic acid from borage, evening primrose, and black currant seed oils: Fatty acid profiles, triacylglycerol structures, and clearance rates of chylomicrons in the rat" GAMMA-LINOLENIC ACID: METABOLISM AND ITS ROLES IN NUTRITION AND MEDICINE AOCS PRESS {A}, CHAMPAIGN, ILLINOIS, USA, 1996, pages 54-65, XP009035802 & FIRST INTERNATIONAL SYMPOSIUM OF GLA; SAN ANTONIO, TEXAS, USA; MAY 7-11, 1995 ISSN: 0-935315-68-3 cited in the application page 56-57, chapter "Oils" page 58; figure 6.2	1-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2004/002089

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. [X] Claims Nos.: 1-34
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-34 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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 III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

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.:

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

Information on patent family members

national Application No
T/GB2004/002089

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4826877 A	02-05-1989	AT 84414 T	15-01-1993
		AU 586286 B2	06-07-1989
		AU 6340786 A	09-04-1987
		CA 1279824 C	05-02-1991
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		DE 3687501 T2	19-05-1993
		EP 0218460 A2	15-04-1987
		ES 2043601 T3	01-01-1994
		GR 3007043 T3	30-07-1993
		HK 129393 A	03-12-1993
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		SG 115193 G	21-01-1994
		ZA 8607386 A	28-10-1987
US 4058594 A	15-11-1977	GB 1506563 A	05-04-1978
		US 3993775 A	23-11-1976

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(19) World Intellectual Property Organization
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(10) International Publication Number
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(72) Inventors; and

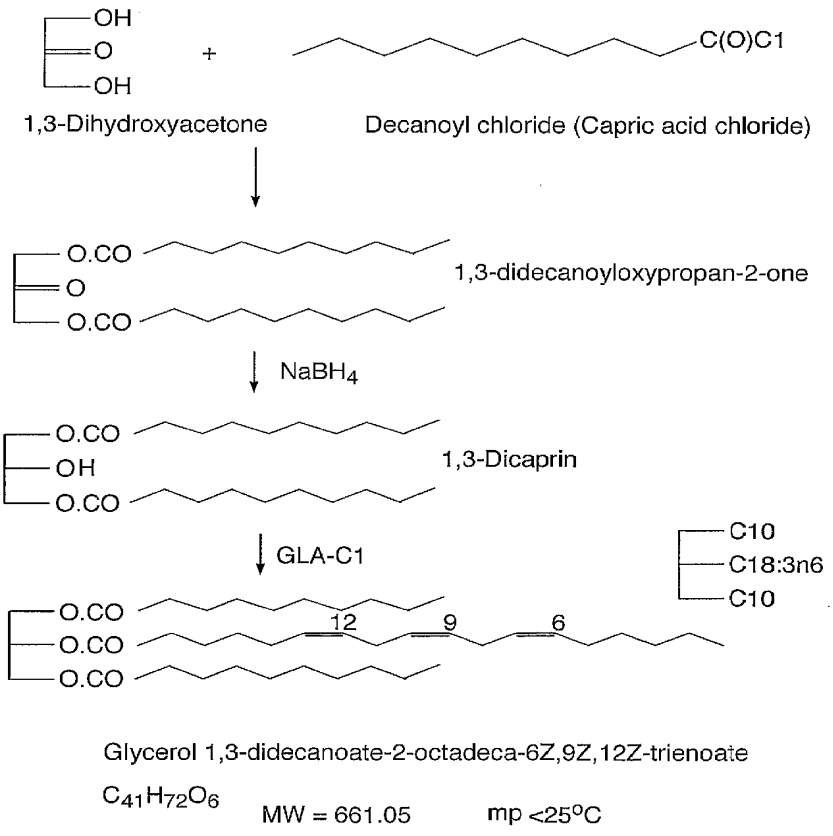
(75) Inventors/Applicants *(for US only)*: **HARBIGE, Laurence, S.** [GB/GB]; The University of Greenwich, Old Royal Naval College, Park Row, Greenwich, London SE10 9LS (GB). **LEACH, Michael, J.** [GB/GB]; The University of Greenwich, Old Royal Naval College, Park Row, Greenwich, London SE10 9LS (GB). **SHARIEF, Mohammed** [GB/GB]; Dept. of Clinical Neurosciences, Guy's, King's & St Thoma's School of Medicine, Hodgkin Building, Guy's Hospital, London SE1 9RT (GB). **BARRACLOUGH, Paul** [GB/GB]; Synnovation Ltd, Unit 8, Nonsuch Industrial Estate, Kiln Lane, Epsom, Surrey KT17 1DH (GB).

(74) Agent: **DOLAN, Anthony, Patrick**; BTG International Limited, 10 Fleet Place, Limeburner Lane, London EC4M 7SB (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,

[Continued on next page]

(54) Title: TREATMENT OF NEURODEGENERATIVE CONDITIONS



Glycerol 1,3-didecanoate-2-octadeca-6Z,9Z,12Z-trienoate

$C_{41}H_{72}O_6$ MW = 661.05 mp <25°C

(57) Abstract: A method is provided for treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a lipid glyceride comprising a glycerol moiety and a fatty acid moiety, the fatty acid moiety being selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid characterised in that the selected fatty acid moiety is attached to the glycerol moiety at its sn-2 position. Preferably the method is that wherein the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF-β1 levels in the patient to therapeutic levels.

WO 2005/018632 A1

KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TREATMENT OF NEURODEGENERATIVE CONDITIONS.

The present invention relates to a method for treating neurodegenerative conditions, particularly those in which increase in transforming growth factor β (TGF-β) is beneficial, particularly TGF-β1. More particularly the present invention provides treatment for neurodegenerative conditions, particularly those such as demyelinating diseases, such as multiple sclerosis, Alzheimer's and Parkinsons diseases and the degenerative sequelae associated with head trauma, stroke and intracranial bleeds, whereby neuronal function may be improved or restored from an impaired condition, eg. by remyeleination.

Further provided are novel use of known and novel compounds comprising unsaturated fatty acid moieties for the manufacture of medicaments capable of effectively treating such conditions, more particularly being capable of achieving previously unattained levels of success with regard to recovery of neurological function.

The inventor's copending unpublished patent application PCT/GB04/002089, incorporated herein by reference, relates to the use of plant and fungal oils for the treatment of neurodegenerative diseases. These oils have high percentages of the essential fatty acid γ-linolenic acid (GLA) at the sn-2 position of their lipids, typically being over 40% of the sn-2 fatty acid total of the oil.

It is well reported in the literature that essential fatty acids (EFAs) of the n-3 and n-6 unsaturation pattern have beneficial effect in a wide variety of human physiological disorders, including autoimmune diasese (WO 02/02105). Harbige (1998) Proc. Nut. Soc. 57, 555-562 reviewed the supplementation of diet with n-3 and n-6 acids in autoimmune disease states, and particularly noted evidence of benefit of γ-linolenic (GLA) and/or linoleic acid (LA) rich oils.

Bates et al noted that lipid oils comprising a mixture of linoleic acid and γ-linolenic acid residues had been suggested back in 1957 to be possibly more efficacious in treating inflammation and autoimmune diseases, but found that at 3g oil per day (Naudicelle Evening Primrose oil 7:1 LA:GLA) patients who had relapses became more ill on the trial oil than on the control.

Although the aetiology of MS remains unknown studies have shown that MS patients have higher than normal neuro-antigen autoreactive T-cells levels. These T-cells react *inter alia* to myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) and are in an increased state of activation compared with healthy controls. The actual processes of axonal damage e.g. chronic inflammation, demyelination and astrogliosis in MS is complex but white matter inflammation and demyelination are considered to determine disease severity, whilst recent studies suggested that axonal damage in MS begins in the early stages of the disease and contributes to disability (De Stefano et al, 2001).

Experimental autoimmune encephalomyelitis (EAE) is the most frequently used animal model for immune mediated effects of MS. Studies in the guinea-pig have shown that linoleic acid partially suppresses the incidence and severity of EAE (Meade et al (1978)). (Harbige et al (1995), 1997b) demonstrated disease modifying effects of linoleic acid and γ-linolenic acid on clinical and histopathological manifestations of EAE. Depending on dose, γ-linolenic acid was fully protective in acute rat EAE whereas linoleic acid had dose-dependent action on the clinical severity but did not abolish it.

Despite these experimental findings, it is recognised that the human disease, multiple sclerosis, is highly complex and can be conversely exacerbated and ameliorated by the activity of T-cells and other immune response factors. It is thought that the n-6 fatty acids promote autoimmune and inflammatory disease based upon results obtained with linoleic acid only. TGF-β1 and PGE_2 production has been shown to be increased non-specifically in γ-linolenic acid fed mice *ex vivo*. TGF-β1 has been reported to protect in acute and relapsing EAE ((Racke et al (1993); Santambrogio et al (1993)), and PG inhibitors such as indomethacin augment, and thus worsen, the disease (Ovadia & Paterson (1982)).

Cytokines are implicated in the pathogenesis of MS, with many studies showing an increase in myelinotoxic inflammatory cytokines (TNF-α, IL-1β and IFN-γ) coinciding with the relapse phase of the disease. Conversely, levels of the anti-inflammatory and immunosuppressive cytokine transforming growth factor-beta1

(TGF-β1) appear to be reduced during a phase of relapse and increase as the patient enters remission. Thus the balance between biologically active TGF-β1 and the pro-inflammatory TNF-α, IL-1β and IFN-γ appears to be dysregulated during MS relapse-remission.

During natural recovery phase from EAE, TGF-β1-secreting T-cells inhibit EAE effector cells, TGF-β1 is expressed in the CNS and, in oral-tolerance-induced protection in EAE, TGF-β and PGE_2 are expressed in the brain (Karpus & Swanborg (1991); Khoury et al (1992)). Harbige ((1998) concluded that dietary γ-linolenic acid effects on EAE are mediated through Th_3-like mechanisms involving TGF-β1 and possibly through superoxide dismutase antioxidant activity.

Borage oil (typically 20% to 23% γ-linolenic acid and 34 to 40% linoleic acid per 100% fatty acid content) and Mucor javanicus fungal oil (see Figure 1) have been shown to be effective in the EAE animal model used to identify MS candidates, whilst never having been shown to be significantly effective in the human disease. High levels of linoleic rich oil containing low levels of γ-linolenic acid (EPO: linoleic acid:γ-linolenic acid 7:1) partially suppressed the incidence and severity of EAE in rat (Mertin & Stackpoole, 1978) whereas the Bates' Naudicelle study referred to above led to worsening of patients. In spite of the use of Borage oil and other GLA/LA containing oils such as Evening Primrose oil by multiple sclerosis sufferers over the past 30 years or so, the vast majority of patients fail to recover from the disease, showing no significant improvement, with the underlying disease continuing to progress to death.

It has been suggested to use, *inter alia*, γ-linolenic acid and linoleic acid rich Borage oil as a means to provide immuno-suppression in multiple sclerosis (US 4,058,594). Critially, the dose suggested is 2.4 grams of oil per day and no actual evidence of efficacy is provided. This is much lower than the low 5g/day dose found to be ineffective *in vivo* in man in the PCT/GB04/002089 study.

Other more dramatic immunosuppressant treatments, including T cell depleters and modulators such as cyclophosphamide, are also shown to be effective in

the EAE model, but where these are employed in the human multiple sclerosis disease symptoms improve, but the underlying disease continues to progress. T-cells indeed produce beneficial cytokines, such as TGF-β1, as well as deleterious ones in man. David Baker of Institute of Neurology, UK summed up the disparity between what is effective in the EAE and in MS with a paper entitled '*Everything stops EAE, nothing stops MS*' at the 10th May 2004 UK MS Frontiers meeting of the UK MS Society.

It is clear that immunosuppression alone cannot cure MS. This is almost certainly due to a fundamental underlying metabolic disorder in MS patients, in addition to the autoimmune disease, that leads to membrane abnormality, cytokine dysregulation and subsequent immune attack and lesioning. Although patients go into remission in relapse-remitting disease, the underlying demyelination proceeds.

The 'gold standard' treatment for MS remains interferon, such as with β-Avonex ®, Rebif ® and other interferon preparations. This gold standard treatment only addresses needs of some, eg 30%, of the patients and even in these symptom improvement is restricted to reduced severity of relapses. Whilst symptoms may be reduced in a proportion of patients, the disease tends to progress to further disability and death due to underlying degeneration.

In their as yet unpublished PCT/GB04/002089 study the present inventors have surprisingly determined that with compliance to a 'high dose' treatment with triglyceride oil containing high levels of sn-2 γ-linolenic acid (>40% of residues at the sn-2 being of γ-linolenic acid) with suitable accompanying fatty acid content, remarkable levels of improvement in almost all symptoms of MS can be achieved, way surpassing that provided by the current gold standard treatment. Such success is particularly surprising in the light of the prior use of other γ-linolenic acid containing preparations without success, such as the Naudicelle study.

The PCT/GB04/002089 study shows that over an 18-month period, patients taking high dose (15g/day) selected high sn-2 γ-linolenic acid borage oil showed significant ($p<0.001$) and marked improvements in EDSS score, a reduced rate of relapse, symptomatic relief of muscle spasticity and painful sensory symptoms, and improved objective measures of cognitive functions. Low doses of 5g/day of this

borage oil were without effect.

Patients taking the highest dose of this borage oil maintained their level of peripheral blood mononuclear cell production (PBMC) of TGF-β1 during the trial period, their pro-inflammatory cytokines TNF-α and IL-1β were significantly and markedly (<70%) reduced and they either maintained or increased the PBMC membrane long chain omega-6 fatty acids dihomo-γ-linolenic acid (DHLA) and arachidonic acid (AA) in contrast to patients taking placebo who demonstrated loss of these fatty acids over the course of the trial period.

This whilst immuno-suppression would be expected to reduce increase of active lesioning and neurodegeneration, the high sn-2 GLA oil treatment apparently targeted maintenance and/or increase of key membrane lipid components that are otherwise specifically lost in MS, being consistent with a correction of a metabolic defect not otherwise effectively treated by current therapies. The fact that the low dose (5 grams/day) had no effect on this supports such determination.

γ-Linolenic acid (18:3n-6, or GLA) is known to be rapidly converted to longer-chain omega-6 polyunsaturated fatty acids dihomo-γ-linolenic acid and arachidonic acid *in vivo* (Phylactos et al 1994, Harbige et al 1995, 2000). Therefore to determine how to increase the level of membrane long chain omega-6 fatty acids in MS the inventors have reviewed their results obtained with several GLA-containing oils:- both fungal (from Mucor javanicus) and plant (Borago officianalis), Evening primrose Oenothera spp. or Blackcurrant Ribes spp) as well as a synthetic tri-GLA oil as GLA delivery systems in an *in vivo* experimental animal model of MS known as chronic relapsing experimental autoimmune encephalomyelitis (CREAE).

Induction of EAE in rats does ***not*** produce histological features of demyelination (Brosnan et al 1988) but induces an acute mono-phasic disease pattern, unlike MS which is characterised by CNS demyelination and is in the majority of cases clinically relapsing-remitting. Chronic relapsing and demyelinating EAE models (CREAE) however are characterised by demyelination and relapse phases. With the demonstration that myelin oligodendrocyte glycoprotein (MOG) is an important neuroantigenic target in MS (Genain et al 1999) and the demonstration of

far greater responses of peripheral blood auto-reactive lymphocytes to this neuroantigen, compared with MBP, in MS (Kerlero de Rosbo et al 1993, 1997) MOG induced CREAE has become the animal model of choice with features closely resembling those observed in MS (Fazakerely et al 1997, Genain et al 1999, Amor et al 1994).

Evidence from the inventor's CREAE and rat EAE feeding studies indicates that an enriched blackcurrant seed oil (72 % w/w 18:3n-6, GLA) did not protect against EAE (see Table 3). Importantly blackcurrant seed oil has a low sn-2 GLA with most of the GLA in the sn-1 and sn-3 positions (Lawson and Hughes 1988). Furthermore a structured triacylgcerol containing three GLA moieties (TG-GLA) provided protective effects similar to that of the borage oil used in CREAE (Table 2). This would also be consistent with the sn-2 GLA being important i.e. the outer pair sn-1 and sn-3 GLA being enzymatically removed *in vivo* and probably undergoing oxidation leaving the sn-2 GLA only. This selective hydrolysis arises from the known ability of specific lipases to remove the sn-1 and sn-3 fatty acids from triacylgycerol molecules but an apparent protection of the sn-2 position *in vivo* (Lawson and Hughes 1988, Kyle 1990).

This review has led the inventors to postulate that glycerides having sn-2-γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid residues will be superior in correcting MS metabolism even to the high sn-2-γ-linolenic acid Borage oil of their earlier trial. This would allow lower doses of lipid to be taken and/or possibly decrease the time of treatment which would result in beneficial effect.

Table 3 of EP 0520624 (Efamol Holdings) compares the triglyceride content of Evening Primrose and Borage Oils, the former being taught to be more therapeutically effective than the latter for a variety of GLA responsive disorders. This document indicates Borage oil to have twenty seven different trigyceride components, only 20% of which have sn-2 GLA. Page 3, lines 40-42 notes that biological testing has shown that equal amounts of GLA may indeed have very different effects when that GLA is supplied as different oil sources. Crucially, it then directs the reader to one particular fraction present in Evening Primrose Oil (EPO),

but not Borage Oil, as being responsible for the former's superior effect in raising PGE1 (see EP 0520624 Chart page 4 and Table 2) and thus anti-inflammatory effect: that fraction being identified as di-linoeoyl-mono-gamma-linolenyl-glycerol (DLMG) which it states to be 18 to 19% of the total triglyceride in EPO. Crictically, page 6 clearly teaches that the position of the GLA, in sn-1, 2 or 3, is not important to this effect.

Dines et al (1994) Proceedings of the Physiological Society, Aberdeen Meeting 14-16 September 1994 report on studies of treatment of diabetic neuropathy neuronal damage with γ-linolenic acid containing oils of the type advocated by EP 0520624 and again note that Borage Oil was not very effective in treating this neurodegeneration whereas Evening primrose oil was. The paper concludes that Borage Oil contains other constituents that interfere with GLA activity.

The present inventors now set out, in view of their results for high sn-2-γ-linolenic acid Borage Oil, to demonstrate that it is indeed the presence of an sn-2-γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid residue in a glyceride, particularly a triglyceride, that gives it efficacy in treating EAE, CREAE and the human disease MS.

In a first aspect the present invention provides a method of treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group of residues consisting of residues of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid.

Particularly advantageously treated neurodegenerative diseases are those involving demyelination. The present method specifically arrests underlying neurodegeneration and restores neuronal function. Particularly the method normalises neuronal membrane composition, and restores healthy PBMC spontaneuosly released TGF-β1/TNFα ratios and the ratios of TGF-β1 with other PBMC released cytokines. Most advantageously the method arrests neurodegeneration in multiple sclerosis of

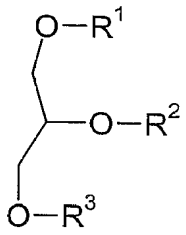
all types but particularly relapsing remitting, primary progressive and chronic progressive MS and the restoration, in part or completely, of neuronal function such as measured, eg. By MRI or CAT scan or by EDSS score. Such method may also be used in treatment of cerebral impairment after stroke, head trauma and intracranial bleeding where there is demyelination or neuronal damage. Further application is provided in treating other chronic demyelination such as in Alzheimer's and Parkinson's disease.

Preferably the the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF-β levels in the patient to therapeutic levels. By therapeutic levels is meant levels at least consistent with healthy subjects. Preferably the dose is such as to produce a TGF-β1/TNF-α ratio spontaneously released from peripheral blood mononuclear cells (PBMCs) isolated from blood of a patient, after 18 months of daily dosing, of 0.4 to 3.0, at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably the dose is such as to produce a TGF-β1/IL-1β ratio in blood of a patient, after 18 months of daily dosing, of at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably said levels are produced after 12 months and more preferably after 6 months.

Typically the amount of lipid administered daily will be between 0.5 and 30 grams, orally dosed, still more preferably between 1 and 20 grams and most preferably between 1 and 18 grams, typically 3 to 5 grams.

Where the sn-2 moiety is that of a γ-linolenic acid residue, the dose may be toward the higher end of these ranges, particuarly where the sn-1 and sn-3 moieties are relatively inert, eg. being metabolically utilised acids such as saturated fatty acids. Where the sn-2 moiety is that of a dihomo-γ-linolenic acid residue, the dose may be less, whilst where the sn-2 moiety is that of an aracidonic acid residue, efficacy is higher, but dosing should be more cautious, due to possibilities of unwanted side effects at higher levels.

More preferably the method is characterised in that the lipid is a monoglyceride, diglyceride or triglyceride containing the at least one sn-2 γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety of general Formula I below:



Formula I

wherein R^1 and R^3 are independently selected from hydrogen and acyl groups, and R^2 is selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid residues.

For the purpose of the present invention acyl groups are defined as comprising at least one carbonyl group on the end of an optionally substituted hydrocarbyl chain selected from alkyl and alkenyl chains, the carbonyl group being directly attached by its carbon to the oxygen of the glycerol residue shown in Formula 1.

Preferred acyl groups R^1 and R^3 are saturated fatty acid moities of formula -$CO-(CH_2)_n-CH_3$, wherein n is an integer selected from 1 to 22, more preferably being 4 to 16, still more preferably being from 5 to 12, most preferably being from 6 to 10. Particularly preferred acyl groups are those of caprylic and capric acids, particularly being 1,3-dicaprylic or 1,3-dicapric glycerols having the γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety at the sn-2 position..

Preferred glycerides for use in the invention are triglycerides.

US 4701469 describes some potential triglycerides for nutraceutical use that the present inventors have determined may be used in the method of the invention, although it only specifically describes 1,3-dioctanyl triglycerides wherein the sn-2 acid is of an EFA, only 1,3-dioctanoyl eicosapenta glycerol is described as having been prepared. These are said to useful in *inter alia* immunomodulation, but although a number of diseases are specified, use in immunosuppresion in neurodegeneration and MS are not listed.

Whilst most preferred groups R^1 to R^3 for inclusion in the compound of formula I are simple saturated fatty acids or naturally occurring fatty acids with structural or metabolic function, such as medium chain or long chain fatty acids, there are other possibilities. Particularly preferred fatty acids are those that are utilised primarily by the metabolism for producing energy. Where fatty acids are structural, that is utilised in membranes, they are conveniently such as γ-linolenic acid, linoleic acid, dihomo-γ-linolenic acid and arachidonic acid residues. By residue is meant the moiety that remains after the fatty acid carboxyl group esterifies to one of the hydroxy groups of the glycerol molecule.

Other preferred acids for sn-1 and sn-3 are selected from fatty acids that are metabolised in the human to yield energy as opposed to a fatty acid that is primarily directed to the structural membrane pool: such preferred acids include oleic acid and palmitic acid.

Where the sn-1 and sn-3 fatty acid chain (R^1 and R^3) is unsaturated it may also be that of other essential fatty acids, such as the n-3 acids such as stearidonic acid, eicosapentanoic acid and docosahyexanoic acid. Where the fatty acid is optionally substituted these will preferably be by hydroxy, oxo, carboxyl, alkyl, alkenyl and alkoxy groups. The hydrocarbyl chain is preferably one of from 1 to 30 carbon atoms in length, more preferably from 4 to 28 carbon atoms in length, still more preferably 4 to 24 carbon atoms in length. Most preferably the hydrocarbyl chain is that of a fatty acid, more particularly a mono or polyunsaturated fatty acid.

Many of the preferred lipids for use in the method of the invention are known and may be prepared by chemical process known in the art. For example, many are commercially available, such as trigamma-linolenin, known as TLG, but herein referred to as GGG, reflecting the identity of groups $R^1R^2R^3$ where G represents γ-linolenic acid residues.

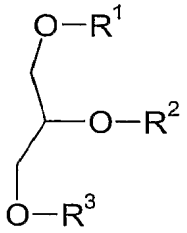
GGG is commercially available from Nu-Check-Prep Inc. EP 0300844 describes its synthesis using a base-catalysed trans-esterification of triacetin with methyl gamma linolenate to give a mixture containing 80% GGG, unreacted methyl γ-linolenate and 10% mono- and di-glycerides.

Triarachidin is known and small quantities can be obtained commercially eg. from Sigma AAA has been synthesised from arachidonic acid by using immobilised lipase patented for angiogenisis-enhancing activity US 4888324.

However, whilst the tri and di-γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid di or triglycerides may be used, the present inventors prefer the use of the mono-γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid sn-2 ester triglycerides because they administer less of the immunomodulatory and proinflammatory fatty acids γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid whilst retaining the enhanced activity that the sn-2 γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety provides with regard to the desired membrane normalising and disease modifying effect.

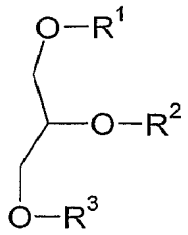
Novel lipids which are preferred are accessible by processes and methods set out in the Examples herein. Most preferred lipids are those where there is just a single γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety esterified to the glycerol at sn-2, with the flanking sn-1 and sn-3 acids being unsaturated medium chain or long chain acids.

Thus a further aspect of the present invention provides novel lipids disclosed herein including compounds of formula II



wherein R^1 and R^3 are the same and are $-C(O)(CH_2)_nCH_3$ wherein n is selected from 4 to 14, more preferably 6 to 10 and most preferably 7, 8 or 9 and R^2 is selected from γ-linolenyl, dihomo-γ-linolenyl and arachidonyl.

A further aspect of the present invention provides a method for synthesis of a compound of general formula III



wherein R^1 and R^3 are the same and are $-C(O)(CH_2)_nCH_3$ wherein n is selected from 4 to 14, more preferably 6 to 10 and most preferably 7, 8 or 9 and R^2 is γ-linolenyl residue, dihomo-γ-linolenyl residue or arachidonyl residue

comprising

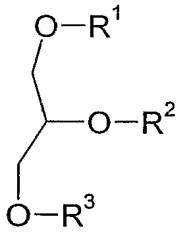
reacting 1,3-dihydroxyacetone with a compound of formula $X\text{-}C(O)(CH_2)_nCH_3$ wherein X is selected from Cl, Br and I,

to give the corresponding 1,3-di-($C(O)(CH_2)_nCH_3$) 2-keto compound

reducing the keto group to the corresponding 1,3-di-($C(O)(CH_2)_nCH_3$) 2-ol

and reacting that with γ-linolenyl chloride or dihomo-γ-linolenyl chloride or arachidonyl chloride.

A still further aspect of the present invention provides a method for synthesis of a compound of general formula IV



wherein R^1 to R^3 are the same and selected from γ-linolenyl residue, dihomo-γ-linolenyl residue or arachidonyl residue

comprising reacting the corresponding γ-linolenyl chloride, dihomo-γ-linolenyl chloride or arachidonyl chloride with glycerol.

Synthesis of some of these compounds is described below and schemes shown in the figures below.

For example, a single-step esterification of glycerol using GLA and a coupling agent, such as DCCI/DMAP (1.1-Dicylcohexylcarbodiimide/ 4-dimethylaminopyridine) coupling reagents may be carried out. This method gives a good yield but generates impurities that, unless removed, make the final oil cloudy. This may be circumvented by using a coupling agent such as EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) which gives rise to water-soluble by-products that are easier to remove. Jpn. Kokai Tokkyo Koho JP 05310638 A2 22Nov 1993 Heisei, 6pp. describes the preparation of tri-α-linolenin (LnLnLn where Ln is linoleic acid) using DCCI, and analogous but different reaction.

A alternative approach provides a two-step sequence that utilises reaction of GLA-Cl (prepared from γ-linolenic acid and oxalyl chloride) and glycerol in dichloromethane/pyridine with good yields at scale-up to 250 g purified by column chromatography. Jpn. Kokai Tokkyo Koho JP 04328199 A2 17 Nov 1992 Heisei, 5pp. (Japan) Concentration of a-linolenic acid triglyceride by flash chromatography. Ando, Yukiki, Watanebe, Yoichi, Takagi, Yoshiaki (Nisshin Oil Mills Ltd, Japan) describes a related but different technique for purification of tri-α-linolenin (LnLnLn).

Comparative example tricaprin (glycerol tridecanate) is a known compound commercially available from Sigma. It has been prepared by reaction of methyl decanoate and sodium glyceroxide with subsequent purification of the crude product by column chromatography (see E. S. Lutton and A. J. Fehl, *Lipids*, **5**, 90-99 (1970))

An alternative method involves the acid-catalysed reaction of glycerol with decanoic acid followed by four crystallisations (see L. H. Jenson and A. J. Mabis, *Acta Cryst.*, **21**, 770 (1966)).

The applicant further provides an improved process which allows glycerol to react with more than 3 equivalents of decanoyl chloride and purified the tricaprin product by recrystallisation.

Further aspects of the present invention provide use of triglyceride oils as described above for the manufacture of a medicament for the treatment of neurodegenerative diseases as set out for the method of the invention. Particularly preferred medicaments are for the arresting and reversing of neurodegeneration in multiple sclerosis of all types but particularly relapsing remitting, primary progressive and chronic progressive and the restoration, in part or completely, of neuronal integrity function such as measured, eg. By MRI or CAT scan or by EDSS score. Other TGF-β1 responsive diseases may be treated as set out previously.

The lipids for use in the present invention may be administered by any of the conventional vehicles known in pharmacy. Most conveniently they are administered as neat oils or in admixture with foodstuffs, in the form of capsules containing such oils, or in enterically coated forms. Other forms will occur to those skilled in the art but Remington Pharmaceutical Sciences 19th Edition.

It will be realised by those skilled in the art that other beneficial agents may be combined with the lipids for use in the present invention or otherwise form part of a treatment regime with the lipids. These might be ion channel blockers, eg. sodium channel blockers, interferons (α, β, or γ), T-cell depleters, steroids or other palliative agents. It will further be realsied that where the immune and inflammatory responses are being modulated, such combinations will need to be made carefully, given the complex nature of these systems. However, given the delayed response to the present oils, shorter acting agents might be beneficial in the first months of treatment before the TGF-β1 levels are normalised, as long as the additional treatment does not impede this normalization process.

The synthesis of structured lipids for use in the present invention is described below together with synthesis of comparative examples. Some of these lipids are novel while others are known but have not been used for the treatment of the invention.

The present invention will now be described by way of Example only by reference to the following non-limiting Tables, Examples and Figures. Further

embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

TABLES

Table 1: Shows the compositional % Total fatty acid content of various triglyceride oils and protective effect in EAE.

Table 2: Shows the parameters of the three treatment groups in high sn-2 GLA Borage Oil trial described in PCT/GB04/002089.

Table 3: Shows the effect of various forms of GLA on EAE incidence and clinical score in SJL mice: lower score indicating improved therapeutic effect.

Table 4: Shows the failure of enriched Blackcurrent oil, a high GLA, but low sn-2-GLA, plant oil, to match fungal and Borage oils in EAE.

FIGURES

Figure 1: Shows spontaneous peripheral blood mononuclear cell cytokine production in placebo and high sn-2 γ-linolenic acid, PCT/GB04/002089 trial oil treated human MS patients at 18 months.

Figure 2: Shows the effect of placebo and low dose (5g/day) high sn-2 GLA Borage oil on human MS patient EDSS score as compared to high dose (15g/day) displayed as a histogram with months treatment on the x axis.

Figure 3: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient Mean Relapse rate (%) as histogram with months on x axis.

Figure 4: Shows the reaction scheme for synthesis of a single fatty acid triacylglyceride for use in the method and use of this invention.

Figure 5: Shows the reaction scheme for synthesis of control compound tricaprin.

Figure 6: Shows the reaction scheme for synthesis of CGC, a mixed fatty acid triacylglyceride of the invention.

Figure 7: Shows the reaction scheme for synthesis of C-DHGLA-C, a mixed fatty acid triacylglyceride of the invention.

Figure 8: Shows the reaction scheme for synthesis of control compound GCG, 1,3-dicapryl, 2-γ-linolenic acid.

Figure 9: Shows the reaction scheme for synthesis of C-AA-C, a mixed fatty acid traiacylglyceride of the invention.

Figure 10 to 19 show the results of EAE studies in SJL and C57BL mice as set out in the examples below. (DHLA=DHGLA: A=AA)

EXAMPLES

High sn-2 Borage oil (PCT/GB04/002089) trial.

Twenty-eight active relapsing-remitting (two relapses in the preceding 18 months) multiple sclerosis patients (ages ranging from 18 to 65 yrs) were entered into a double-blind placebo controlled trial to investigate the effects of encapsulated borage oil on clinical activity and laboratory parameters over 18 months. This oil was of high sn-2 γ-linolenic (GLA) content (>40% of sn-2 residues being γ-linolenic acid) with low monene (eg. erusic acid) content and had no added Vitamin E, a known immunomodulator.

Patients were recruited from neurology out-patient clinics at two inner city hospitals; hospital informed consent was obtained on first (baseline) visit. Exclusion criteria include any form of steroid or immunosuppressive drug treatment, pregnancy, hyperlipidemia, regular use of aspirin or related drugs and vitamin or fatty acid supplementation within the previous three months.

Only patients meeting all the following criteria were included in the trial: (a) able to provide informed consent prior to treatment, with the full understanding that consent may be withdrawn at any time without prejudice; (b) male or female out-patients aged 18 to 60 years inclusive; (c) have confirmed diagnosis of clinically definite relapsing MS; (d) have had at least three documented clinical relapses in the past two years; (e) have a baseline Expanded Disability Scoring Scale (EDSS) score of 0.0-5.5 inclusive, provided they have well documented exacerbations; and (f) healthy, apart from the MS-related symptoms, as confirmed by the medical history, physical examination and clinical chemistry, urine and haematological tests.

Patients were randomly allocated by the Pharmacy Department to one of three groups each containing 12 patients:

- One clinical group (n=12) to receive placebo (5 g of Polyethylene Glycol 400)
- Second clinical group (n=12) to receive low-dose (5 g) refined *Borage officinalis*
- Third clinical group (n=12) to receive high-dose (15 g) refined *Borage officinalis*

Supplementation was in the form of one gram oil capsules daily (5/day for low dose, 15/day high dose) for 18 months duration. *Borage officinalis* oil and omega-6 polyunsaturated fatty acids are food ingredients that are generally recognised as safe for human consumption (GRAS). There are no classification or labelling requirements under EC regulations. Clinical assessment included: Extended Disability Scale Scores (EDSS) and clinical relapse record. Venous blood (50 mls) was obtained for laboratory studies on the 1^{st}, 3^{rd}, 6^{th}, 12^{th}, 15^{th}, and 18^{th} month of supplementation.

The following biochemical and immunological parameters were investigated on each visit for comparison with pre-treatment data and between group data:

- Stimulated and unstimulated *ex vivo* peripheral blood mononuclear cell cytokine production: changes in TGF-β1, IFN-γ, TNF-α, IL-1β, IL-6 and IFN-β, which are implicated in the pathogenesis of MS. Cytokine and related gene expression.
- Soluble adhesion molecules in serum particularly ICAM-1 and VCAM-1
- Peripheral blood mononuclear cell membrane fatty acids and plasma phospholipid fatty acid composition.

Results are shown in Tables 1 and 2 and Figures 1 to 5.

The primary outcome parameter was the number of clinical relapses between baseline (Month 0) and the end of treatment (Month 18). Secondary outcome parameters included: the time to first clinical relapse; severity of relapses, as assessed by EDSS score and the use of steroid treatment; and changes in EDSS at Month 3, 6, 9, 12, and 18 compared to baseline and defined as at least 1.0 point increase in the EDSS that is sustained for 3 months or at least 1.5 point increase on the EDSS from the baseline EDSS that is sustained for 3 months.

Eleven patients were in the placebo group, seven patients had been taking low-dose Borage oil, and ten patients had been taking high-dose Borage oil. The study drug was well-tolerated, and there were no serious adverse events during the 18-month trial.

Isolation and Culture of PBMC

Heparinised whole blood was diluted with an equal volume of Hanks' balanced salt solution (Sigma, UK) and the resulting diluted blood layered onto Lymphoprep (Nycomed, Oslo, Norway). Following density centrifugation at 800g for 30 minutes the PBMC were removed from the interface and diluted in Hanks' solution. The cells were then washed twice by centrifugation for 10 minutes at 250g. The resulting final pellet was then resuspended in culture medium consisting of RPMI-1640 medium (Sigma, UK) supplemented with 2mM L-glutamine, 100U

penicillin and 100μg streptomycin (Sigma, UK) and 10% autologous plasma. $2x10^6$ per ml PBMC, >95% viable as judged by trypan blue exclusion, were added to tissue culture tubes (Bibby Sterilin Ltd, Stone, UK) and incubated for 24h at 37°C with 5% CO_2. The concentration of antigen, cell density and time of culture were all determined in previous kinetic experiments to determine maximum cytokine production (data not shown). Routine cytospin preparations were also prepared for subsequent differential counts. Following incubation the cells were removed from culture by centrifugation at 250g for 10 minutes, the resulting supernatants were then removed, aliquoted and stored at -70°C.

Preparation of Plasma Samples

10ml of heparinised blood was spun at 250g for 10 minutes. The resulting plasma layer was then removed, aliquoted and stored at -70°C.

Detection of Pro-inflammatory Cytokines

TNF-α, IL-1β and IFN-γ in cell culture supernatants and plasma were detected using commercially available paired antibodies enabling cytokine detection in an ELISA format (R&D systems Ltd, Abingdon, UK). The sensitivities for the TNF-α and IFN-γ ELISAs were 15.6-1000pg/ml and 3.9-250pg/ml for IL-1β.

Detection of Biologically Active TGF-β1

Biologically active TGF-β1 in cell culture supernatants and plasma were detected using the commercially available E_{max} ELISA system with a sensitivity of 15.6-1000pg/ml (Promega, Southampton, UK).

Statistical Analysis

Differences in cytokine production were compared using Student's *t*-test and Mann-Whitney *U*-test and were considered significant when p values were less than 0.05.

RESULTS

Two patients had developed diarrhoea, both of whom were later confirmed to have been taking high-dose Borage oil. The diarrhoea was mild in one patient, but was moderately severe in the second patient, who later discontinued the study drug. The code was not broken and the diarrhoea had stopped after the discontinuation of the drug, but reappeared upon re-challenge. Therefore, this patient was withdrawn from the trial. The remaining patients who were treated with high-dose Borage oil showed excellent clinical improvement on all primary and secondary outcome criteria. For example, their mean EDSS score after 6 months of treatment had improved from baseline EDSS (Figure 1). More importantly, the mean number of clinical relapses had significantly reduced after 6 months of treatment when compared to the number of relapses in the placebo group (Figure 2). In contrast, patients who had been receiving low-dose Borage oil did not show any clinical improvement when compared to the placebo group. In addition to its beneficial effect on MS disease activity, high dose Borage oil provided some symptomatic relief of muscle spasticity (stiffness) and painful sensory symptoms, and also improved cognitive functions.

As can be seen for the figures below, relapse rate after 9, 12 and 18 months was down to zero in the high dose group. The increase seen at 15 months was due to the patient dropping out of this group.

The following are three brief case histories to illustrate the therapeutic benefits of high dose high sn-2 GLA Borage oil. The first two are from the trial while the third is a post trial patient for whom MRI studies were obtained.

Patient 1 (Treatment):

The first patient was a 48 year old woman who had had a clinically active, relapsing remitting MS for 9 years. She had originally worked as a full-time administrator at the local Health Authority, but she was unable to perform her duties

because of her severe MS. Therefore, she later worked as a part-time secretary, but still had difficulties in mobilization because of muscles stiffness and sensory disturbances. She was also experiencing severe clinical relapses at an average of one relapse every nine months. Most of these relapses had resulted in hospital admissions for steroid therapy. In view of her active MS, she was recruited into the Borage oil trial. There were no adverse events relating to the study, and after taking the medication for four months, she experienced good improvement in her walking and sensory symptoms.

About nine months after therapy, she was well enough to start full-time employment. In addition, she remained relapse-free for the 18-month duration of the clinical trial. Following the conclusion of the trial, the treatment code revealed that she was taking high-dose Borage oil.

Patient 2 (Control):

The second case was a 46-year old woman who also had a clinically active relapsing remitting MS for 8 years. She had originally worked as a shop assistant, but became unemployed after MS was diagnosed.

Her symptoms included difficulty with mobilisation and painful sensory symptoms in both legs. She had experienced three clinical relapses in the two years preceding the clinical trial, and had been admitted to hospital twice for steroid therapy. Consequently, she was recruited into the Borage oil trial, but her walking continued to deteriorate. Six months into the trial, she need to use a walking stick and also received treatment with Baclofen to reduce low limb spasticity. Approximately ten months after starting the Borage oil trial, she was admitted to hospital because of severe clinical relapse, which was treated with steroids. She later developed bladder disturbances and began to use a wheelchair for long journeys. The treatment code was broken after the conclusion of the 18-month trial, and she was found to have been taking placebo. Since then, she started using a walking frame for journeys exceeding 50 yards.

Patient 3: Treatment (additional to trial)

The third case was a 26 year-old man who was diagnosed with definite MS in April 2001. His symptoms had started in 1999 when he complained of diffuse, intractable pain affecting various parts of his body, particularly the left side of the chest and abdomen. This was followed by intermittent numbness in the hands and feet, associated with fluctuating weakness. There were also distressing bladder symptoms in the form of urinary frequency and urgency. The diagnosis of MS in 2001 was based on his relapsing remitting symptoms, and was confirmed by positive cerebrospinal fluid analysis and magnetic resonance imaging (MRI) of the brain, which showed multiple white matter abnormalities in both cerebral hemispheres. Symptoms did not respond to various pharmaceutical therapies.

In April 2003, oral supplementation with the present high dose Borage oil was commenced. The patient reported dramatic improvement in his symptoms within three months of starting this oral supplementation. His painful sensory symptoms disappeared completely. He reported no numbness or weakness since May 2003, and noticed significant improvement in his bladder control. The oral supplementation caused no adverse events. A repeat brain MRI was undertaken to verify the reported improvement in Mr N's symptoms. The repeat MRI showed a reduction in the size and distribution of the white matter abnormalities.

EXAMPLES; Structured sn-2 lipids

In all the examples below higher purity is obtained by use of higher purity starting material γ-linolenic, dihomo-γ-linolenic or arachidonic acid, such as is available eg from Sigma Aldrich. GLA 95 indicates 95% pure γ-linolenic acid.

Synthesis Example 1: synthesis of Trigammalinolenin

1) Acid chloride method

2.0 g (7.2 mmol, 3.1 equiv) GLA95 (95% pure γ-linolenic acid) was dissolved in 10 ml DCM. 1.01g (0.71 ml, 8.0 mmol, 3.4 equiv) oxalyl chloride in 5

ml DCM added dropwise over 2-3 min under nitrogen. Stirred at RT overnight. Reaction mixture concentrated in vacuo to remove DCM and excess oxalyl chloride. This acid chloride was then added dropwise over 2-3 min to a stirred mixture of 215 mg (2.3 mmol, 1 equiv) of glycerol, 0.58 ml (3.1 equiv) pyridine and 10 ml DCM under nitrogen. The mixture was stirred at RT overnight. The pyridine hydrochloride formed was then filtered off and washed with DCM. The solution was washed 1 x 4 ml water, 0.1N HCl, 5% sodium bicarbonate and 5% NaCl. Dried over magnesium sulphate, filtered and concentrated in vacuo to a yellow oil. This oil was purified on a silica column using 10% ether in hexane as eluting solvent. A clear colourless oil was obtained, a sample of which was trans-esterified and subsequently analysed by GC. The product contained 96.3% GLA

2) DCCI method

2.19 g GLA95 (3.15 equiv), 230 mg (1 equiv) glycerol, 153 mg DMAP (0.5 equiv) were stirred in 10 ml DCM under nitrogen. 1.85 g DCCI (3.6 equiv) in 5 ml DCM was added. The reaction mixture was stirred at RT under nitrogen overnight. The DCU formed was filtered and washed with DCM. DCM washed 1 x 5mls N HCl, water, 5% sodium bicarbonate and water. Dried over magnesium sulphate, filtered and concentrated in vacuo to an oil. This oil was then purified on a silica column using 10% ether in hexane as eluting solvent. 1.47 g (67%) of a slightly cloudy oil was obtained. A sample of this product was trans-esterified and subjected to GC analysis. The product contained 95.8% GLA.

Scale-up

20 g (0.072 mol, 3.1 equiv) of GLA95 (gamma linolenic acid, 95%) was dissolved in 100 ml DCM. 13.7g (9.3 ml, 0.11 mol, 4.78 equiv) oxalyl chloride was added over 3-4 min under nitrogen. The reaction mixture was stirred under nitrogen overnight. It was then concentrated in vacuo to remove DCM and excess oxalyl chloride. This oil was then added dropwise over ca 5 min to a stirred mixture of

2.14g (0.023 mol, 1 equiv) of glycerol, 100 ml DCM and 5.8 ml (5.68 g, 0.072 mol, 3.1 equiv) of pyridine under nitrogen. 85 mg (0.7 mmol, 0.03 equiv) of DMAP (4-dimethylaminopyridine) catalyst was added.. The mixture was stirred at RT overnight. Pyridine hydrochloride was filtered off and washed with DCM. The DCM solution was washed 1x 25 ml: water, 10% sodium bicarbonate, 0.1N HCl, 5% NaCl. (Emulsions formed during this process, especially at first). The DCM was dried over magnesium sulphate, filtered and concentrated in vacuo to a brown oil (~21 g).

The oil was purified on a silica column using 5% ether in hexane at first and then 10%. 15.6g (77% yield) of a clear oil was obtained. By tlc this material contained a small amount of free GLA. (This material was repurified at a later date)

Large Scale-up

The above reaction was repeated on 10 times scale. Thus, 200 g of GLA95, 1L DCM, 137 g of oxalyl chloride, and 21.4 g of glycerol were used. On the addition of the acid chloride the reaction mixture was cooled in a cold water bath and the temperature kept below 35°C. 250g of a brown oil were produced. This was initially purified on a 500 gram silica column. The oil was dissolved in 200 ml hexane and applied to the column. The column was eluted at first with hexane, then 5% ether in hexane and then 10%. Fractions were collected and analysed by tlc eventually yielding two batches of oils. The first A (66 g) contained a small amount of front running impurity and a little GLA (slower running than TGL), the second fraction B (99g) was clear of front running impurity and contained a little GLA.

The large scale reaction was repeated using 169 g of GLA and gave two fractions as above. This time there was 85g of 'A' fraction and 54g of 'B' fraction. Both batches of 'A' were combined and re-purified on a 500g silica column. The 'B' fractions were treated in a similar manner (15g of material from the small-scale reaction were also added to this batch).

Some fractions from the above were again re-purified to eventually give 259 grams of oil. The oil was pumped down on a rotary evaporator under high vacuum to constant weight – 256g. This represents an overall yield of 65%.

Analysis of product.

GC

A small sample was trans-esterified and subjected to GC analysis:

The GLA content was 97.1%. The main impurity was linoleic acid – 1.91%.

Note: The original GLA95 that was used for the synthesis contained 96.2% GLA and 2.42% linoleic acid.

HPLC

An HPLC method was developed using a reversed phase column (Hypersil C18 4.6 x 100 mm), eluting with 80/20 acetonitrile/THF. Detection was by UV at 210 nm. This showed the product to be a mixture of three components. The main peak (93.6%) was the required product. A slower running impurity (representing 5.0% of the product) was probably a **GGLl** triglyceride (Ll = linoleic acid). A second impurity was slightly faster running and represented 1.4% of the product.

Note: Absorption at 210 nm varies considerably between triglycerides of differing fatty acid content. For example trigammalinolenin has a UV absorbtion 5-6 times greater than that of trilinolenin

Summary

254 g of glycerol tri-6,9,12-linolenate (gamma linolenic acid triglyceride, trigammalinolenin, **GGG**) was prepared from 96.2 % GLA by a two-step acid chloride route. It is a clear, pale yellow oil and was stored under nitrogen in the freezer. The GLA content was 97.1 % and no C20:1, C22:1, or C24:1 acids were detected). The HPLC purity was 93.6 %.

Synthesis of higher purity **GGG** would is readily achievable using GLA 98 (98% γ-linolenic acid: Scotia) or higher starting material.

Comparative lipid 1: synthesis Tricaprin (Glycerol tridecanoate)

Small Scale

Glycerol (3.0 g, 0.0325 mol, 1 eq) pyridine (8.1 ml, 0.10 mol, 3.1 eq) and dichloromethane (100 ml) were stirred at room temperature under nitrogen. Decanoyl chloride (21 ml, 19.25 g, 0.10 mol, 3.1 equiv) was then added dropwise over 5 min, with external cooling in a water bath to keep the temperature at 30-35 °C. When the addition was complete 4-dimethylaminopyridine (DMAP (0.12 g, 1 mmol, 0.03 eq) was added and the mixture stirred under nitrogen at room temperature overnight. The precipitated pyridine hydrochloride was removed by filtration and washed with dichloromethane. The combined washing and filtrate was then washed with aqueous solutions (20 ml) of 5% sodium chloride, 5% sodium bicarbonate, 0.1N hydrochloric acid, and 5% sodium chloride. The dichloromethane layer was then dried over $MgSO_4$ and the solvent removed *in vacuo*. The residual oil crystallised on standing. This material was recrystallised from isopropanol (40 ml) to give 15.6 g (86% yield) of a waxy white solid.

Analysis

GC – 99.8% pure

HPLC

(C18 4.6 x 100 mm, ACN/THF 85/15 1 ml/min, λ 210 nm) – 94.9% pure

Large Scale

The above was repeated on 15 times the scale. Glycerol (45.0 g, 0.49 mol, 1 eq), pyridine (121.5 ml, 1.50 mol, 3.1 eq) and dichloromethane (1.5 L) were stirred at room temperature under nitrogen. Decanoyl chloride (315 ml, 288.8 g, 1.50 mol, 3.1 equiv) was then added dropwise over 15 min, with external cooling in a water bath to keep the temperature at 30-35 °C. When the addition was complete 4-dimethylaminopyridine (DMAP (1.8 g, 15 mmol, 0.03 eq)

was added and the mixture stirred under nitrogen at room temperature overnight. The precipitated pyridine hydrochloride was removed by filtration and washed with dichloromethane. The combined washing and filtrate was then washed with aqueous solutions (300 ml) of 5% sodium chloride, 5% sodium bicarbonate, 0.1N hydrochloric acid, and 5% sodium chloride. The dichloromethane layer was then dried over $MgSO_4$ and the solvent removed *in vacuo*. The residual oil crystallised on standing. This material was recrystallised from isopropanol (400 ml) to give 228 g (86% yield) of a waxy white solid.

Analysis

GC – 99.8% pure

HPLC

(C18 4.6 x 100 mm, ACN/THF 85/15 1 ml/min, λ 210 nm) – 94.9% pure

A further batch was made and combined with the small-scale batch above and recrystallised from isopropanol to give 44 g of product. The above batches were combined (268 g) and reanalysed:

GC

99.9% pure

HPLC

97.9%

Summary

263 g of glycerol tridecanoate (tricaprin, CCC) was been prepared from decanoyl chloride (98 %) by a one-step process (scheme given below). It is a white, low-melting solid and was stored under nitrogen in the freezer. The **C** content was 99.9 % of fatty acid content and the HPLC purity was 97.9 %.

Synthesis Example 2: 1,3-Dicaprin 2-gammalinolenoate (Glycerol 1,3-didecanoate 2-octadecatri(6-Z,9-Z,12-Z)enoate or CGC)

This triglyceride is novel. Unlike CGC, its isomer CLnC (Ln = α-linolenic acid), has been identified (see K. Long et al *Biotechnol. Lett.*,**20**, 369-372 (1998). and H. Mu, P. Kalo et al, *Eur. J. Lipid Sci. Technol.*, **102**, 202-211(2000). as a component of coconut oil. In addition, CLxC (Lx = a linolenic acid of unspecified double bond position) has been described (see J. Gresti et al. *J. Dairy Sci.*, **76**, 1850-1869 (1993)),

The two intermediates used in the synthesis of **CGC** are known (see L. El Kihel et al *Arzneim -Forsch./Drug Res.*, **46**, 1040-1044 (1996) and US 4178299. The last step described below is novel and the first two stages are also inventive since they are more suitable for large scale production than those previously reported.

CGC was prepared by reaction of 1,3-Dicaprin with GLA-chloride in dichloromethane-pyridine. 1,3-Dicaprin was prepared by sodium borohydride reduction of 1,3-didecanoyloxypropan-2-one, which was in turn prepared by reaction of decanoyl chloride with 1,3-dihydroxyacetone. The intermediate 1,3-dicaprin must be handled with care since it can undergo acyl migration on exposure to acids , bases and heat. An older method of making 1,3-dicaprin has been described (see A. P. J. Mank et al *Chem. Physics Lipids*, **16**, 107-114 (1976).

A versatile, flexible synthesis of 1,3-diglycerides and triglycerides. by catalysed addition of decanoic acid to a glycidol ester (from epichlorohydrin) is less attractive because of more severe reaction conditions and acyl migration problems. The final product, **CGC**, was purified by careful column chromatography on silica which removed by-products.

Small Scale

1,3-didecanoyloxypropan-2-one

Decanoyl chloride (40.0 ml, 36.8 g, 0.19 mol, 1.98 equiv) was added dropwise over 10-15 min to a stirred suspension of 1,3-dihydroxyacetone dimer (8.68 g, 0.048 mol, 1.0 equiv), pyridine (15.6 ml, 0.19 mol), 4-dimethylaminopyridine (0.18 g, 0.0014 mol, 0.03 equiv) and dichloromethane (DCM, 150 ml) at room temperature

under nitrogen. The temperature of the reaction mixture was kept below 30°C by cooling in a cold water bath. The reaction mixture was stirred at RT under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 25ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and concentrated in vacuo to a yellowish semi-solid. This was then crystallised from methanol (150 ml) to give a white solid. The yield was 28.2 g (73%).

1,3-Dicaprin

The above ketone (28.2 g, 0.071 mol) was dissolved in tetrahydrofuran (THF, 200 ml). Water (10 ml) was then added, the solution cooled to 5°C, and sodium borohydride (5.38 g, 0.14 mol) added portionwise below 10°C. The reaction mixture was stirred at RT for 1h and then concentrated in vacuo to remove THF. The residue was partitioned between ethyl acetate and 5% sodium chloride solution. The aqueous phase was re-extracted with ethyl acetate and the combined extracts dried over $MgSO_4$ and concentrated in vacuo to a waxy solid. This was crystallised twice from hexane to give 11.2g (40%) of a white solid. (99%+ pure by HPLC)

*1,3-Dicaprin 2-gammalinolenoate (**CGC**)*

Gamma-linolenic acid (GLA95, 8.34 g, 0.03 mol) was dissolved in dichloromethane (DCM, 60 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (3.9 ml, 5.67 g, 0.044 mol) added dropwise over 5 mins. The mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 15 min (ice /water cooling) to a stirred solution of 1,3-dicaprin (11.2g, 0.028 mol), DCM (50 ml), pyridine (2.42 ml, 2.37 g, 0.03 mol) and 4-dimethylaminopyridine (0.10 g, 0.0008 mol, 0.03 equiv) at 10-15°C . The temperature was maintained by ice-water cooling. The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed

with DCM. The combined washing and filtrate was washed with 1 x 20ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and the solvent removed in vacuo.The residual brown oil was purified by column chromatography on silica. Elution with hexane and then with 5% ether/hexane gave 10.3g (56%) of a colourless oil. The structure was confirmed by ^{13}C NMR and GLC. Purity determined by HPLC.

Large Scale

1,3-didecanoyloxypropan-2-one

Decanoyl chloride (272 ml, 250 g, 1.3 mol, 2 equiv) was added dropwise over 10-15 min to a stirred suspension of 1,3-dihydroxyacetone dimer (59.1 g, 0.65 mol, 1.0 equiv), pyridine (106 ml, 103.7g 1.3 mol), 4-dimethylaminopyridine (2.38 g, 0.02 mol, 0.03 equiv) and dichloromethane (DCM, 750ml) at room temperature under nitrogen. The temperature of the reaction mixture was kept below 30°C by cooling in a cold water bath. The reaction mixture was stirred at RT under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 150ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and concentrated in vacuo to a yellowish semi-solid. This was then crystallised from methanol (500ml) to give a white solid. The yield was 158 g (60%).

1,3-Dicaprin

The above ketone (158 g, 0.40 mol) was dissolved in tetrahydrofuran (THF, 2.25 L). Water (50 ml) was then added, the solution cooled to 5°C, and sodium borohydride (5.66 g, 1.5eq) added portionwise below 10°C. The reaction mixture was monitored by HPLC (C18, eluted with ACN at 1ml/min λ210nm) (Note: only about 4.5g of the borohydride was in fact added, as all SM had reacted).The reaction mixture was stirred at RT for 1h and then concentrated in vacuo to remove THF. The residue was partitioned between ethyl acetate and 5% sodium chloride solution. The aqueous phase was re-extracted with ethyl acetate and the combined extracts dried

over $MgSO_4$ and concentrated in vacuo to a waxy solid. This was crystallised twice from hexane to give 96g (60%) of a white solid. (98% pure by HPLC)

*1,3-Dicaprin 2-gammalinolenoate (**CGC**)*

Gamma-linolenic acid (GLA95, 120.2g, 0.43mol) was dissolved in dichloromethane (DCM, 750 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (55.7 ml, 82.3 g, 0.65 mol, 1.5eq) added dropwise at 15-20°C over 15 mins. The mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 30-40 min at 10-15°C (ice /water cooling) to a stirred solution of 1,3-dicaprin (164.7g, 0.41 mol), DCM (650 ml), pyridine (33.3 ml, 32.5 g, 0.41 mol) and 4-dimethylaminopyridine (1.50 g, 0.012 mol, 0.03 equiv) at 10-15°C .The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed with DCM. The combined washing and filtrate was washed with 1 x 150 ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and the solvent removed in vacuo to a brown oil (275g).

The scale of the above three reactions was the largest on which each was carried out. The borohydride reduction produced, in addition to 1,3-dicaprin, a by-product in variable yield. The presence of this by-product greatly affected the yield of the isolated pure 1,3-dicaprin; the by-product could only be removed by two crystallisations of the crude product. Since the final product, **CGC**, is purified by column chromatography, it is imperative that the 1,3-dicaprin used for the final step is as pure as possible!

From the above reactions about 440g of crude CGC was produced as a brown oil. This was purified on a series of silica columns using hexane followed by 2-3% ether/hexane. The purification required 7 or 8 columns, using 3-4 kilos of silica, 25-30 litres of solvent (recycling solvent kept this figure low – in practice over 100 litres were used)

The resulting product, a clear almost colourless oil, (264grams) was 96.4% pure by HPLC (C18 4.6 x 100mm, eluted with 85/15 ACN/THF at 1ml/min. UV detection λ210nm). GC indicated a ratio of 66.1/33.9 C/G. NMR analysis indicated the product to have the correct CGC structure and be of at least 95% purity: δ_c (500 MHz, $CDCl_3$) 172.65 (2-GLA carbonyl), 173.25 (1,3- capric carbonyl). Ratio of signals 2.04:1. No signal at 173.0 indicating absence of 1.3-GLA. Trace signal at 172.79 could be oleic acid impurity in GLA or 2-capric acid.

Summary

264 g of glycerol 1,3-didecanoate-2-gammalinolenoate (1,3-dicaprin-2-GLA, CGC) has been prepared from decanoyl chloride (98 %) by a three-step process (scheme given below). It is an almost colourless oil (slight yellow tinge) and was stored under nitrogen in the freezer. The HPLC purity was 96.4 %.

Synthesis Example 3

1,3-Didecanoate-2-dihomo-γ-linolenoate (Glycerol 1,3-didecanoate2- eicosa-(8Z,11Z,14Z)-trienoate or C(DHLA)C

This triglyceride appears to be novel - no reference to it has been found.

DHLA (3.93g, 12.8 mmol, 1 eq) was dissolved in dichloromethane (DCM, 20 ml) and stirred at room temperature under a nitrogen atmosphere. Oxalyl chloride (1.69 ml, 2.46 g, 19.4 mmol, 1.5 eq) was added dropwise over 1-2 min, and left stirring at room temperature overnight. The resulting solution was concentrated *in vacuo* to remove DCM and excess oxalyl chloride. The residual oily acid chloride (DHLA-Cl) was then added dropwise over 5 min at 25°C to a stirred mixture of 1,3-dicaprin (4.91 g, 12.2 mmol, 0.95 eq), pyridine (0.98 ml, 0.96 g 12.1 mmol, 0.95 eq) and 4-dimethylam inopyridine (DMAP, 8 mg, 0.07 mmol, 0.03 eq). The reaction temperature rose to 32 °C during the addition. The reaction was stirred at 30-35°C and monitored by HPLC. The reaction was stopped after 1.5h. The precipitated pyridine hydrochloride was filtered off and washed with DCM. The combined filtrate and washings were then

washed with 1 x 10 ml portions of 5% NaCl, 5% $NaHCO_3$, 0.1N HCl, 5% NaCl. The solution was then dried over $MgSO_4$ and concentrated *in vacuo* to give the crude product as a yellow-orange oil (8.9 g, 86% purity by HPLC). This oil was chromatographed on silica gel (250 g). Elution with hexane and diethyl ether-hexane (2-6%) gave a purified product as a pale yellow oil. Treatment of a hexane solution with decolourising charcoal and removal of the solvent *in vacuo* gave **C(DHLA)C** as a clear colourless oil (6.48g, 98.9% purity by HPLC).

Synthesis Example 4

Triarachidin (Glycerol trieicosotetra5-Z,8-Z,11-Z,14Z-eneoate) or AAA

Arachidonic acid (50.9 g, 0.17 mol, 3 eq) was dissolved in dichloromethane (DCM, 175 ml) and stirred at room temperature under a nitrogen atmosphere. Oxalyl Chloride (21.9 ml, 31.9 g, 0.25 mol, 4.4 eq) was then added to the stirred solution over 5 min and the temperature increased by 4 °C. The resulting yellow-green mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (A-Cl) was then added dropwise over 15 min to a pre-warmed (25 °C) stirred mixture of glycerol (5.11 g, 0.055 mol, 1 eq), pyridine (13.5 ml, 13.2 g, 0.17 mol, 3 eq) and 4-dimethylamino pyridine (DMAP, 0.20 g, 0.002 mol, 0.03 eq). The temperature of the reaction mixture rose to 42 °C during the addition and a gentle reflux was observed. The mixture was stirred at 30-40 °C and monitored by HPLC. After 2 h, no further product formation was observed. The precipitated pyridine hydrochloride was filtered off and washed with DCM. The combined filtrate and washings were then washed with 1 x 50 ml portions of 5% NaCl, 5% $NaHCO_3$, 0.1N HCl, 5% NaCl. The solution was then dried over $MgSO_4$ and concentrated *in vacuo* to give the crude product as a yellow-orange oil (57 g). This oil was purified by column chromatography on silica gel (*ca.* 600 g). Elution with hexane and diethyl ether(2-4%)-hexane gave 22.8 g of the product as an oil. A second batch (17.8 g) was produced from 39.8 g of arachidonic acid, The two batches were combined and residual solvents removed

under vacuo to give 40.5 g (43%) of a mobile pale yellow oil. HPLC purity 84.8% GLC analysis 94.3% AA (arachidonic acid).

Comparative Lipid 2

1,3-Di(octadeca-6Z,9Z,12Z-enoyloxy)propan-2-one

(1,3-Di(γ-linolenoyloxy)propan-2one ,**GonG**) Stage 1 intermediate for **GCG**

Gamma-linolenic acid (GLA95, 197g, 0.71 mol, 2.2 equiv) was dissolved in dichloromethane (DCM, 600 ml) contained in a 2L 3 necked flask. The resulting solution was stirred at RT under nitrogen. Oxalyl chloride (93 ml, 136 g, 1.07 mol, 3.3eq) was added dropwise at 15-20°C over 15 min. The brown mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 20 min at 25°C to a stirred mixture of 1,3-dihydroxyacetone dimer (28.99 g, 0.32 mol, 1.0 equiv), pyridine (52 ml, 50.9 g 0.64 mol, 2.0 equiv), 4-dimethylaminopyridine (2.36 g, 0.02 mol, 0.06 equiv) and dichloromethane (DCM, 600 ml) at room temperature under nitrogen. The temperature of the reaction mixture was allowed to rise to 40°C and the mixture was stirred for a further 2 h under nitrogen (monitored by HPLC). The pyridine hydrochloride that formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 150 ml portions of 5% NaCl, 5% $NaHCO_3$, 0.1N HCl, 5% NaCl. The solution was then dried over $MgSO_4$ and concentrated *in vacuo* to give *ca.* 200 g of a yellow oil. This material was partially purified by column chromatography on silica (600 g). Elution with hexane and then ether-hexane mixtures (2–15%) gave 42 g of a pale yellow oil. This oil was chromatographed again on silica (600 g) and eluted with hexane and then 1-10% ether-hexane to give the product (95.9% purity) as a pale yellow oil. The yield was 42 g (17%).

1,3-Di(octadeca-6Z,9Z,12Z-enoyloxy)propan-2-ol

(1,3-Di(γ-linolenoyloxy)propan-2-ol or 1,3-Di-gamma-linolenin **GolG**) Stage 2 intermediate for **GCG**

13-Di(γ-linolenoyloxy)propan-2-one (**GonG**, 25.5 g, 0.04 mol, 1 eq) was dissolved in tetrahydrofuran (THF, 375 ml) and water (12.7 ml). The solution was vigorously stirred at –20°C, care was taken to keep the reaction temperature below –15°C. Sodium borohydride (790 mg, 0.02 mol, 1.25 eq) was added portionwise to the stirred solution over 3 mins. The reaction mixture was stirred for a further 10 mins at –20°C and hexane (380 ml) then added. The still cold mixture was then washed with water (2 x 200 ml), dried over $MgSO_4$ and concentrated *in vacuo* to give the title compound as a brown oil (27.8g) (82.6 % purity by HPLC, less than 1% migrated material). Another batch was prepared and combined with the first to give 50 g of crude product. This material was purified by column chromatography on silica gel (400 g). Elution with hexane and diethyl ether-hexane mixture (5-20%) gave 36.1 g of the product as a pale oil (91.5 % purity).

(N.B. Care should be taken not to leave the compound on the silica overnight as it appears to undergo a migration reaction, giving **GGol**)

<u>1,3-Di-γ-linolenin 2-decanoate (Glycerol 1,3-dioctadeca-(6Z,9Z,12Z)-trienoate 2-decanoate or **GCG**)</u>

Decanoyl chloride (13.5 ml, 12.4 g, 0.065 mol, 1.1 eq) was added to a stirred solution of 1,3-di-γ-linolenin (36.1 g, 0.059 mol, 1eq), dry pyridine (5.7 ml, 5.6 g, 0.07 mol, 1.1eq), 4-dimethylaminopyridine (0.2 g, 0.002 mol, 0.03 eq) and dichloromethane (DCM, 150 ml) over *ca.* 10 mins. The temperature was maintained at 17 °C –23 °C during addition. The reaction was then stirred at 30-35 °C and monitored by HPLC. A further 1-2 ml of decanoyl chloride was added after 1 h, 1.5 h and 2 h. Further addition appeared to increase the conversion to product as determined by HPLC. After 3 h the reaction mixture was filtered and the filtrate washed with DCM. The combined filtrate and washings were then washed with 1 x 50 ml portions of 5% NaCl, 5% $NaHCO_3$, 0.1N HCl, 5% NaCl. The DCM extract was then dried over $MgSO_4$ and concentrated *in vacuo* to give the crude product as a pale yellow oil; (purity 90% by HPLC). The oil was purified by column chromatography on silica gel (600 g). Elution with hexane and diethyl ether-hexane

(1.5-2.5 then 3.5%) gave the product (**GCG.**) as a clear oil; (35.5 g 96.1% purity by HPLC). Another 7.5 g of pure lipid was obtained by further chromatography on some of the fractions containing only a small amount of impurity.

Synthesis Example 5

1,3-Dicaprin 2-arachidonate (Glycerol 1,3-didecanoate 2-eicosatetra-(5-Z,8-Z,11-Z,14-Z)enoate or **CAC**)

This triglyceride is known. **CAC** has been identified as a constituent of lymph lipids following administration of safflower oil to rats. WO 03 013,497 describing an arachidonic acid containing triglyceride (produced by culturing Mortierella alpina) useful for diseases caused by brain hypofunction, but specifically for cognition enhancement. The two intermediates used in the synthesis of **CAC** are known.

The synthesis of CAC from 1,3-dicaprin, and the purification of this are all novel.

Here **CAC** was prepared by reaction of 1,3-Dicaprin with arachidonyl chloride in dichloromethane-pyridine. 1,3-Dicaprin was prepared by sodium borohydride reduction of 1,3-didecanoyloxypropan-2-one, which was in turn prepared by reaction of decanoyl chloride with 1,3-dihydroxyacetone. The intermediate 1,3-dicaprin must be handled with care since it can undergo acyl migration on exposure to acids , bases and heat. An older method [6] of making 1,3-dicaprin, by catalysed addition of decanoic acid to a glycidol ester (from epichlorohydrin) was deemed less attractive because of more severe reaction conditions and acyl migration problems. The final product, **CAC**, was purified by careful column chromatography on silica which removed by-products.

1,3-Dicaprin 2-arachidonate (CAC)

Arachidonic acid (AA96, 8.34 g, 0.03 mol) was dissolved in dichloromethane (DCM, 60 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (3.9 ml, 5.67 g, 0.044 mol) added dropwise over 5 mins. The mixture was

stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 15 min (ice /water cooling) to a stirred solution of 1,3-dicaprin (11.2g, 0.028 mol), DCM (50 ml), pyridine (2.42 ml, 2.37 g, 0.03 mol) and 4-dimethylaminopyridine (0.10 g, 0.0008 mol, 0.03 equiv) at 10-15°C . The temperature was maintained by ice-water cooling. The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed with DCM. The combined washing and filtrate was washed with 1 x 20ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and the solvent removed in vacuo.The residual brown oil was purified by column chromatography on silica. Elution with hexane and then with 5% ether/hexane gave 10.3g (56%) of a colourless oil. The structure was confirmed by ^{13}C NMR and GLC. Purity determined by HPLC.

Large Scale

1,3-didecanoyloxypropan-2-one

Decanoyl chloride (272 ml, 250 g, 1.3 mol, 2 equiv) was added dropwise over 10-15 min to a stirred suspension of 1,3-dihydroxyacetone dimer (59.1 g, 0.65 mol, 1.0 equiv), pyridine (106 ml, 103.7g 1.3 mol), 4-dimethylaminopyridine (2.38 g, 0.02 mol, 0.03 equiv) and dichloromethane (DCM, 750ml) at room temperature under nitrogen. The temperature of the reaction mixture was kept below 30°C by cooling in a cold water bath. The reaction mixture was stirred at RT under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 150ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and concentrated in vacuo to a yellowish semi-solid. This was then crystallised from methanol (500ml) to give a white solid. The yield was 158 g (60%).

1,3-Dicaprin

The above ketone (158 g, 0.40 mol) was dissolved in tetrahydrofuran (THF, 2.25 L). Water (50 ml) was then added, the solution cooled to 5°C, and sodium borohydride (5.66 g, 1.5eq) added portionwise below 10°C. The reaction mixture was monitored by HPLC (C18, eluted with ACN at 1ml/min λ210nm) (Note: only about 4.5g of the borohydride was in fact added, as all SM had reacted).The reaction mixture was stirred at RT for 1h and then concentrated in vacuo to remove THF. The residue was partitioned between ethyl acetate and 5% sodium chloride solution. The aqueous phase was re-extracted with ethyl acetate and the combined extracts dried over $MgSO_4$ and concentrated in vacuo to a waxy solid. This was crystallised twice from hexane to give 96g (60%) of a white solid. (98% pure by HPLC)

1,3-Dicaprin 2-arachidonate (**CAC**)

Arachidonic acid (AA96, 78.8 g, 0.26 mol) was dissolved in dichloromethane (DCM, 425 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (33.9 ml, 49.4 g, 0.39 mol, 1.5eq) added dropwise at 15-20°C over 15 mins. The mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 30-40 min at 10-15°C (ice /water cooling) to a stirred solution of 1,3-dicaprin (94.2 g, 0.24 mol), DCM (450 ml), pyridine (19.1 ml, 18.6 g, 0.24 mol) and 4-dimethylaminopyridine (1.72 1.50 g, 0.014 mol, 0.06 equiv) at 10-15°C .The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed with DCM. The combined washing and filtrate was washed with 1 x 150 ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and the solvent removed in vacuo to a brown oil (171 g).

The scale of the above three reactions was the largest on which each was carried out. The borohydride reduction produced, in addition to 1,3-dicaprin, a by-product in variable yield. The presence of this by-product greatly affected the yield of the isolated pure 1,3-dicaprin; the by-product could only be removed by two

crystallisations of the crude product. Since the final product, **CAC**, is purified by column chromatography, it is imperative that the 1,3-dicaprin used for the final step is as pure as possible!

412 g of crude **CAC** was produced as a brown oil from the above reactions. This material was purified on a series of silica columns using hexane followed by 1-3% ether/hexane. The purification required 7 or 8 columns, using 3-4 kilos of silica, and 100 litres of solvent.

The resulting product, a clear very pale yellow oil, (295grams) was 95.8% pure by HPLC (C18 4.6 x 100mm, eluted with 85/15 ACN/THF at 1ml/min. UV detection λ210nm). GC indicated a ratio of 66.3/32.1 C/A (1.6% impurity carried through from the 5% impurity in A).

Summary

295 g of glycerol 1,3-didecanoate-2-arachidonate (1,3-dicaprin-2-AA, **CAC**) has been prepared from decanoyl chloride (98 %) and Arachidonic acid (95%) by a three-step process (scheme given below). It is a very pale yellow oil and was stored under nitrogen in the freezer. The HPLC purity is 95.8 %.

Synthesis Example 7

1,3-Dioleoin 2-gammalinolenoate (Glycerol 1,3-dioctadeca-9Z-enoate 2-octadecatri(6-Z,9-Z,12-Z)enoate or **OGO**)

This triglyceride is known: a carbon-14 labelled version has been prepared by normal chemical synthesis and the normal unlabelled form by biochemical synthesis using lipases. **OGO** is not a major component of borage oil but its isomer **OOG** is (9%). The two intermediates used in the synthesis of **CGC** are known. The last step is novel.

The use of, the synthesis of from 1,3-dioleoin, and the purification of CGC are all believed novel. In general triglycerides CXC are preferred over OXO on patent and cost of goods grounds.

OGO was here prepared by reaction of 1,3-Doleoin with GLA-chloride in dichloromethane-pyridine. 1,3-Diolein was prepared by sodium borohydride reduction of 1,3-dioleoylpropan-2-one, which was in turn prepared by reaction of oleoyl chloride with 1,3-dihydroxyacetone. The intermediate 1,3-dioleolin must be handled with care since it can undergo acyl migration on exposure to acids , bases and heat. Older methods [7,8] of making 1,3-dioleoin, via mono-tritylglycerols or glycidyl esters was deemed less attractive because of more steps and acyl migration problems. The final product, **OGO**, was purified by careful column chromatography on silica which removed by-products.

Small Scale

1,3-dioleoylpropan-2-one

155.1g Oleic acid (155.1 g, 0.55 mol, 1.0 equiv, Croda 094 RV05192) was dissolved in dichloromethane (DCM, 500 ml). The solution was stirred at room temperature (RT) under nitrogen and 104.4g (1.5eq 71mls) oxalyl chloride (104.4 g, 71.8 ml, 0.82 mol, 1.5 equiv) was added dropwise at 15-20°C over about 20 mins. The reaction mixture was stirred overnight at RT. The excess oxalyl chloride and DCM were removed in vacuo and the residual oily acid chloride was added dropwise over 15-20 min to a stirred suspension of 1,3-dihydroxyacetone dimer (22.5g, 0.24 mol of monomer), pyridine (40.4 ml), 4-dimethylaminopyridine (1.83g) and dichloromethane (DCM, 500ml) at room temperature under nitrogen. The temperature of the reaction mixture was kept below 20°C by cooling in an ice/water bath. The reaction mixture was stirred at RT under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 150ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and concentrated in vacuo to an orange/brown semi-solid. This was triturated in methanol and stored in the 'fridge overnight. The solid deposited (90% pure by HPLC) was then crystallised from diisopropyl ether (DIPE) and methanol to give

51.3g of an off white solid which was 95% pure by HPLC. Further crystallisation from DIPE/methanol yielded 41g (27%) of a 98% pure product.

1,3-Diolein

The above ketone (32.8 g, 0.053 mol) was dissolved in tetrahydrofuran (THF, 250 ml). Water (10 ml) was then added, the solution cooled to 5°C, and sodium borohydride added portionwise below 10°C. The reaction was followed by HPLC (C18, ACN/THF 90/10 at 2mls/min ,λ210nm) and after all the starting ketone had reacted the addition of the borohydride was stopped (830mg ,0.022 mol added). The mixture was then concentrated in vacuo to remove THF. The residue was partitioned between ethyl acetate and water. The aqueous phase was re-extracted with ethyl acetate and the combined extracts dried over $MgSO_4$ and concentrated in vacuo to an oil (~33g) which solidified on cooling.The product (68% pure by HPLC) was crystallised from 100ml hexane at –20°C (in the freezer) overnight This product (92% pure 21.1g) was recrystallised from hexane (50ml) to give 18.28g (56%yield) of a product 97.5% pure by HPLC.

1,3-Diolein 2-gammalinolenoate (**O-G-O**)

γ-Linolenic acid (GLA95, 41.2 g, 0.15 mol, 1.1 equiv) was dissolved in dichloromethane (DCM, 250 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (19.1 ml, 28.2 g, 0.22 mol, 1.65 equiv) added dropwise over 5 mins. The mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 15 min (ice /water cooling) to a stirred solution of 1,3-diolein (83.5g, 0.13 mol), DCM (250 ml), pyridine (10.9 ml, 10.6 g, 0.14 mol) and 4-dimethylaminopyridine (0.49 g, 0.004 mol, 0.15 equiv) at 10-15°C . The temperature was maintained by ice-water cooling. The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed with DCM. The combined washing and filtrate was washed with 1 x 80ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$

and the solvent removed in vacuo.The residual brown oil was purified by column chromatography on silica. Elution with hexane and then with 5% ether/hexane gave 63.6g (54%) of a colourless oil. Purity determined by HPLC.

Summary

64 g of glycerol 1,3-oleoate-2-gammalinolenoate (1,3-dioleate-2-GLA, OGO) was prepared from oleoyl chloride (98 %) by a three-step process (scheme given below). It was an almost colourless oil (slight yellow tinge) and is being stored under nitrogen in the freezer. The HPLC purity was 89.4 %.

^{13}C NMR Data for Structured lipids

GGG δ_C (125.7 MHz, $CDCl_3$) 172.69 (1C, C-2 carbonyl), 173.09 (2C, C-1, C-3 carbonyls)

CGC δ_C (125.7 MHz, $CDCl_3$) 172.76 (1C, C-2 carbonyl), 173.17 (2C, C-1, C-3 carbonyls)

CAC δ_C (125.7 MHz, $CDCl_3$) 172.65 (1C, C-2 carbonyl), 173.28 (2C, C-1, C-3 carbonyls)

C(DHLA)C δ_C (125.7 MHz, $CDCl_3$) 172.83 (1C, C-2 carbonyl), 173.30 (2C, C-1, C-3 carbonyls)

GCG δ_C (125.7 MHz, $CDCl_3$) 172.91 (1C, C-2 carbonyl), 173.11 (2C, C-1, C-3 carbonyls)

OGO δ_C (125.7 MHz, $CDCl_3$) 172.69 (1C, C-2 carbonyl), 173.25 (2C, C-1, C-3 carbonyls)

AAA δ_C (125.7 MHz, $CDCl_3$) 172.66 (1C, C-2 carbonyl), 173.04 (2C, C-1, C-3

carbonyls)

CCC δ_C (125.7 MHz, $CDCl_3$) 172.81 (1C, C-2 carbonyl), 173.21 (2C, C-1, C-3 carbonyls)

Experimental Procedure

The proton-decoupled ^{13}C NMR spectra with suppressed NOE were collected at 21 °C in a 5-mm broadband probe on a Joel 500 MHz spectrometer operating at 125.728 MHz. Waltz decoupling was the chosen mode of decoupling and was gated on only during the 14.89s acquisition time. The relaxation delay was set at 30 secs and the pulse angle was 90°. The spectral window used was ca.35 ppm (from 173.5 to 172.6 ppm) with a 170 ppm offset. The spectra were internally referenced to $CDCl_3$ at 77.0 ppm. Typically, the approximate number of scans collected for adequate signal-to-noise ranged from 300 to 1200 scans depending on the concentration and purity of the sample. The total acquisition time for the experiments ranged between 2-8h e.g 1272 scans; data points 65,536. Concentrated solutions up to 20% w/v were employed when possible to reduce the acquisition time The chemical shifts quoted vary with the concentration of the solution.

BIOLOGICAL STUDIES.

Chronic Relapsing Experimental Autoimmune Encephalomyelitis (CREAE) Studies .

Induction and Clinical Assessment of EAE

CREAE was induced in C57B1/6 and SJL mice. Animals were injected subcutaneously with 100 μg of the neuroantigen peptide MOG 35-55 (amino acid sequence MEVGWYRSPFSRVVHLYRNGK Genemed Synthesis, Inc) or 1 mg of mouse spinal cord homogenate (SCH), in phosphate buffered saline (PBS), emulsified by sonication for 10 min at room temperature, in incomplete Freund's adjuvant (DIFCO, Detroit, USA) supplemented with 480 μg of *mycobacteria tuberculosis* and 60μg of *Mycobacteria butyricium* (DIFCO, Detroit, USA) on days 0 and 7 as

described previously (Morris-Downes, MM., et al 2002). In addition to optimise the disease mice also received 200 ng (intraperitoneally) of *Bordetella pertussis* toxin dissolved in PBS administered 1hr and 24 hrs after immunization with the MOG neuroantigen and for SCH days 0, 1, 7 and 8.

Animals were weighed from day 5 onwards and examined daily for clinical neurological signs by two experienced investigators and graded according to a previously validated grading scheme (Morris-Downes, MM. et al 2002 and others): 0 = normal; 1 = limp tail and feet; 2 = impaired righting reflex; 3 = partial hind limb paralysis; 4 = complete hindlimb paralysis; 5 = moribund; 6 = death. Animals exhibiting clinical signs of a lesser severity grade than typically observed were scored as 0.5 less than the indicated grade.

Reference

Morris-Downes, MM., et al (2002). Pathological and regulatory effects of anti-myelin antibodies in experimental allergic encephalomyelitis in mice. ***J. Neuroimmunol.*** 125. 114-124.

The mean group EAE score was compared for each test group compared to a respective control group by non-parametric statistical analysis (Mann Whitney U Test).

All MOG-CREAE studies comprised a treatment control group (**C-C-C** or **saline** as selected from the above study). Each structured lipid was tested at 3 dose levels, all treaments being orally administered for 2 weeks from day 7 after inoculation. All treatment groups will contained 10 animals. On completion of studies (day 21), brain and spinal cord were be removed and half of the samples were processed for signs of CNS perivascular mononuclear leucocyte-infiltrated sites and demyelination.

Studies were as follows:

Study 2: Spinal cord homogenate(SCH) EAE in SJL mice.

EAE Induction: 1mg SCH day 0 +day 7 sc. 200ng Pertussis toxin day 0,1, 7 & 8 ip.10mice/group. Mice were treated from day 7 to 21 with CCC or CGC.

Study 3: SCH EAE in SJL mice: Treatment was from PSD 7 to 21, both days inclusive.

Study 4: MOG EAE in C57BL mice: Treatment was from PSD 7 to 21, both days inclusive.

Study 5: SCH EAE in SJL mice: Treatment was from PSD 5 to 18, both days inclusive.

Study 6: MOG EAE in C57BL mice: Treatment was from Days 5 to 21 inclusive except C-DHLA-C group where treatment was from days 5 to 15 inclusive. Animals were culled on PSD 25. [Five animals from an untreated group, 3 animals from control CCC treatment group, 5 animals from GGG 150ul treatment group and 2 animals from GGG 350ul treatment group were sampled for histological analysis on PSD 20].

Study 7: SCH EAE in SJL mice
Treatment was from Days 6 to 20 inclusive.

Study 2 – Spinal cord homogenate (SCH) in SJL mice :-tested

CGC *(50/150/350ul);* ***CCC*** *(350ul).*

GGG*.(50/350ul)*

[Severe disease observed]

Study 3 – SCH/SJL mice:- tested

CCC *(50/150/350ul)*

***CGC** (25/50/150/350ul)*

***GGG** (50/150/350ul)*

OGO. (25/50/150/350ul)

[Severe disease observed]

Study 4 – MOG/ C57BL mice:- tested

***CCC** (50/150/350ul)*

***CGC** (25/50/150/350ul)*

***GGG** (50/150/350ul)*

OGO. (25/50/150/350ul)

Study 6 – MOG/C57BL mice:- tested

***CCC** (150ul)*

***C-DHLA-C** (50ul)*

***CAC** (50/350ul)*

***AAA** (50/150ul)*

***GCG** (50ul)*

***CGC** (50ul)*

***GGG**.(150/350ul)*

[Pathology: CCC; GGG]

Histological examination of the submitted samples of brain and spinal cord showed lesions typical of experimental allergic encephalomyelitis.

Localised and diffuse lesions were characterised by gliosis, myelin vacuolation, axonal degeneration and perivascular cuffing with lymphocytes, macrophages and neutrophils.

Spinal cord lesions were mostly located in subpial white matter and brain lesions mostly occurred in the cerebellar white matter. Lesions were more severe in the spinal cords than in the brains and whereas all animals with brain lesions had lesions in the spinal cord, not all animals with cord lesions had lesions in the brain.

Variation in the severity of changes between individual mice is summarised using a semi-quantitative five point grading system.

Untreated mice had histological scores of 3-4 which correlated with EAE scores of 1.5-3. One mouse showed little pathological change with a zero score. In the GGG treated mice, the majority showed no abnormalities. Two mice from this group had histological scores of 2 and 3 respectively which correlated with EAE severity. scores of 1 and 1.5

The results of the four studies are shown in Figures 11 to 20 below

These show that the compounds G-G-G, A-A-A, C-G-C, C-DHGLA-C, and C-A-C are all capable of reducing severity of CREAE whereas compounds G-C-G and C-C-C failed to treat the condition. Compound O-G-O is believed to work if the dose is adjusted.

As cautioned in the description, the arachidnoic acid compounds are effective, but lead to death of some animals. Surviving animals had much reduced disease. It is believed that the dose of these compounds may be reduced still further to provide survival with satisfactory treatment.

Some of the studies show a bell shaped response curve for compounds C-G-C and G-G-G, suggesting that very high doses are not optimal, as set out above. Such dosing can be conveniently determined by those skilled art, eg. By dose escalatio and monitoring TGF-β1/TNF-α spontaneously release ratio changes from PBMCs.

Given the PCT/GB04/002089 high sn-2 γ-linolenic acid results, the lack of efficacy of low sn-2 black-current oil and G-C-G in CREAE and the low dose efficacy of C-G-C and C-DHGLA-C in Figure 20, it can be seen that sn-2 -γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid lipids provide a novel treatment for MS that far exceeds any current therapy outcome in that lesions are repaired and difficult symptoms are resolved: decreasing EDSS over a period of years being so far unachieved in other treatments.

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TABLE 1

Compositional (% Total FAs) Characteristics of Various Oils and their Protective Effects in EAE

Treatment	18:2n-6	18:3n-6	18:2n-6/18:3n-6	18:1n-9	INCIDENCE OF EAE
FGO	17	20	0.6	35	0/10
BOO	37	24	1.5	15	3/10
EPO	71	9.4	7.5	9	7/10
SAF	66	-	-	17	9/10
Controls	-	-	-	-	9/10

FGO, Fungal Oil; BOO, Borage Oil; EPO, Evening Primrose Oil, SAF, Safflower Oil.

TABLE 2

Treatment Groups- PCT/GB04/002089 Borage oil-MS trial

		Female	Male	Mean Relapse Rate (in past two years)	Mean Base EDSS	Number
Group	Placebo	7	4	2.6	3.9	11
	Low Dose	5	2	2.9	3.5	7
	High Dose	8	2	3.4	2.8	10
Total		20	8	2.9	3.4	28

Table 3. Molecular Species Comparison of Triacylglycerol-GLA (TG-GLA), Ethyl-Ester-GLA (EE-GLA) and PCT/GB04/002089 *Borago Officinalis* Oil-GLA (BOR-GLA) in MOG-induced CREAE in SJL Mice

Treatment	No. with EAE	Mean Clinical Score
Control	10/11	3.3±1.3
EE-GLA[a]	5/6	3.0±0.8
TG-GLA[a]	3/6	1.0±1.3[c]
BOR-GLA[b]	3/6	1.0±1.2[c]

[a] Animals given 100 µl of test lipid; [b]250µl BOR-GLA given. Significance of difference compared with controls, [c] $p < 0.05$

Table 4. Effect of enriched black-currant seed oil (73% GLA) on the incidence of EAE

	% Incidence of EAE (Days after immunisation)		
	13	17	21
Controls (n=10)	60	90	10
Blackcurrant (n=10)	10	80	70

Note: Blackcurrant oil delays the incidence but does not provide full protection. Animals were fed 7 days after sensitization (immunisation).

CLAIMS.

1. A method of treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid

2. A method as claimed in Claim 1 wherein the neurodegenerative disease involves demyelination.

3. A method as claimed in Claim 1 wherein the treatment specifically arrests underlying neurodegeneration and restores neuronal function.

4. A method as claimed in Claim 1 which normalises neuronal membrane composition with respect to γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid lipid content.

5. A method as claimed in Claim 1 which restores healthy TGF-β1/TNFα ratios as measured from spontaneous release from peripheral blood mononuclear cell release.

6. A method as claimed in Claim 1 wherein the disease is multiple sclerosis.

7. A method as claimed in Claim 1 wherein the disease is relapsing remitting multiple sclerosis, primary progressive multiple sclerosis or chronic progressive multiple sclerosis.

8. A method as claimed in Claim 1 wherein the disease is multiple sclerosis and the treatment restores, in part or completely, neuronal function or neuronal integrity as measured by one or more of MRI scan, CAT scan or by EDSS score.

9. A method as claimed in Claim 1 wherein the treatment is of cerebral impairment after stroke, head trauma and intracranial bleeding, Alzheimer's disease or Parkinson's disease where there is demyelination or neuronal damage.

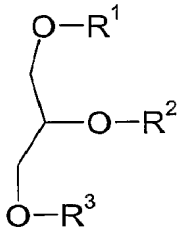
10. A method as claimed in Claim1 wherein the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF-β1 levels in the patient to therapeutic levels.

11. A method as claimed in Claim 1 wherein the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF-β1 levels in the patient to a TGF-β1/TNF-α ratio released spontaneously from peripheral blood mononuclear cells isolated from the blood of a patient, after 18 months of daily dosing, of 0.4 to 3.0, at least 0.5, more preferably at least 0.75 and most preferably at least 1.

12. A method as claimed in Claim11 wherein the dose is such as to produce a TGF-β1/IL-1β ratio in PBMCs isolated from blood of a patient, after 18 months of daily dosing, of at least of at least 0.75.

13. A method as claimed in Claim 1 wherein the amount of lipid administered is between 0.5 and 30 grams, typically 3 to 5 grams, per day.

14. A method as claimed in Claim 1 wherein the lipid is a monoglyceride, diglyceride or triglyceride containing the at least one sn-2 γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety, the lipid being of of general Formula I



Formula I

wherein R^1 and R^3 are independently selected from hydrogen and acyl groups, and R^2 is selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid residues having their carbonyl carbon attached to the oxygen of the glycerol moiety.

15. A method as claimed in Claim 1 wherein R^1 and R^3 are saturated fatty acid moieties of formula $-CO-(CH_2)_n-CH_3$, wherein n is an integer selected from 1 to 22,

16. A method as claimed in Claim 15 wherein R^1 and R^3 are the same and n is an integer of from 5 to 12.

17. A method as claimed in Claim 16 wherein n is an integer of from 6 to 10.

18. A method as claimed in Claim 14 wherein R^1 and R^3 are selected from the group consisting of essential fatty acids or physiologically acceptable fatty acids metabolisable by the human body.

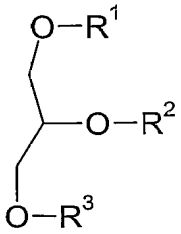
19. A method as claimed in Claim 14 wherein R^1, R^2 and R^3 are all the same and are selected from the group consisting of of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid residues.

20. A pharmaceutial composition characterised in that it comprises a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid.

20. A pharmaceutial composition for treating neurodegeneration characterised in that it comprises a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid.

21. A pharmaceutical composition for treating demyelinating disease comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid.

22. A lipid of formula II



wherein R^1 and R^3 are the same and are $-C(O)(CH_2)_nCH_3$ wherein n is selected from 4 to 14, more preferably 6 to 10 and most preferably 7, 8 or 9 and R^2 is selected from γ-linolenyl, dihomo-γ-linolenyl and arachidonyl residues.

23. Use of a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid, for the manufacture of a medicament for the treatment of neurodegenerative disease

24. Use as claimed in Claim 23 wherein the degenerative disease is a demyelinating disease.

25. Use as claimed in Claim 23 wherein the disease is multiple sclerosis.

26. Use as claimed in Claim 23 wherein the medicament normalises neuronal membrane composition with respect to lipid γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid levels.

27. Use as claimed in Claim 23 wherein the medicament restores TGF-β1/TNFα ratios spontaneously released from peripheral blood mononuclear cells of a patient to healthy levels.

28. Use as claimed in Claim 23 wherein treatment is for multiple sclerosis or the degenerative sequelae associated with head trauma, stroke and intracranial bleeds or neuronal damage caused by Alzheimer's or Parkinson's disease.

29. Use as claimed in Claim 23 wherein the medicament repairs CNS lesions.

30. Use as claimed in Claim 23 wherein the medicament relieves muscle spasticity and/or pain.

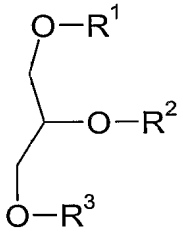
31. Use as claimed in Claim 23 wherein the medicament eliminates relapses.

32. Use as claimed in Claim 23 wherein the medicament improves EDSS score by at least 1 unit over a period of 1 years treatment.

33. Use as claimed in Claim 23 wherein the medicament is sufficient to restore EDSS of a patient with EDSS above 2.5 to below 2 over a period of 1 years treatment.

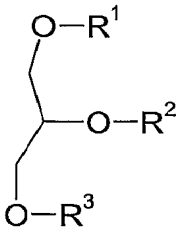
34. Use as claimed in Claim 23 wherein there is improvement in bladder control.

35. A method for synthesis of a compound of general formula III



wherein R^1 and R^3 are the same and are $-C(O)(CH_2)_nCH_3$ wherein n is selected from 4 to 14, more preferably 6 to 10 and most preferably 7, 8 or 9 and R^2 is γ-linolenyl residue, dihomo-γ-linolenyl residue or arachidonyl residue
comprising
reacting 1,3-dihydroxyacetone with a compound of formula $X\text{-}C(O)(CH_2)_nCH_3$
wherein X is selected from Cl, Br and I,
to give the corresponding 1,3-di-($C(O)(CH_2)_nCH_3$) 2-keto compound
reducing the keto group to the corresponding 1,3-di-($C(O)(CH_2)_nCH_3$) 2-ol
and reacting that with γ-linolenyl halide or dihomo-γ-linolenyl halide or arachidonyl halide, wherein halide is chloride, bromide or iodide.

36. A method for synthesis of a compound of general formula IV



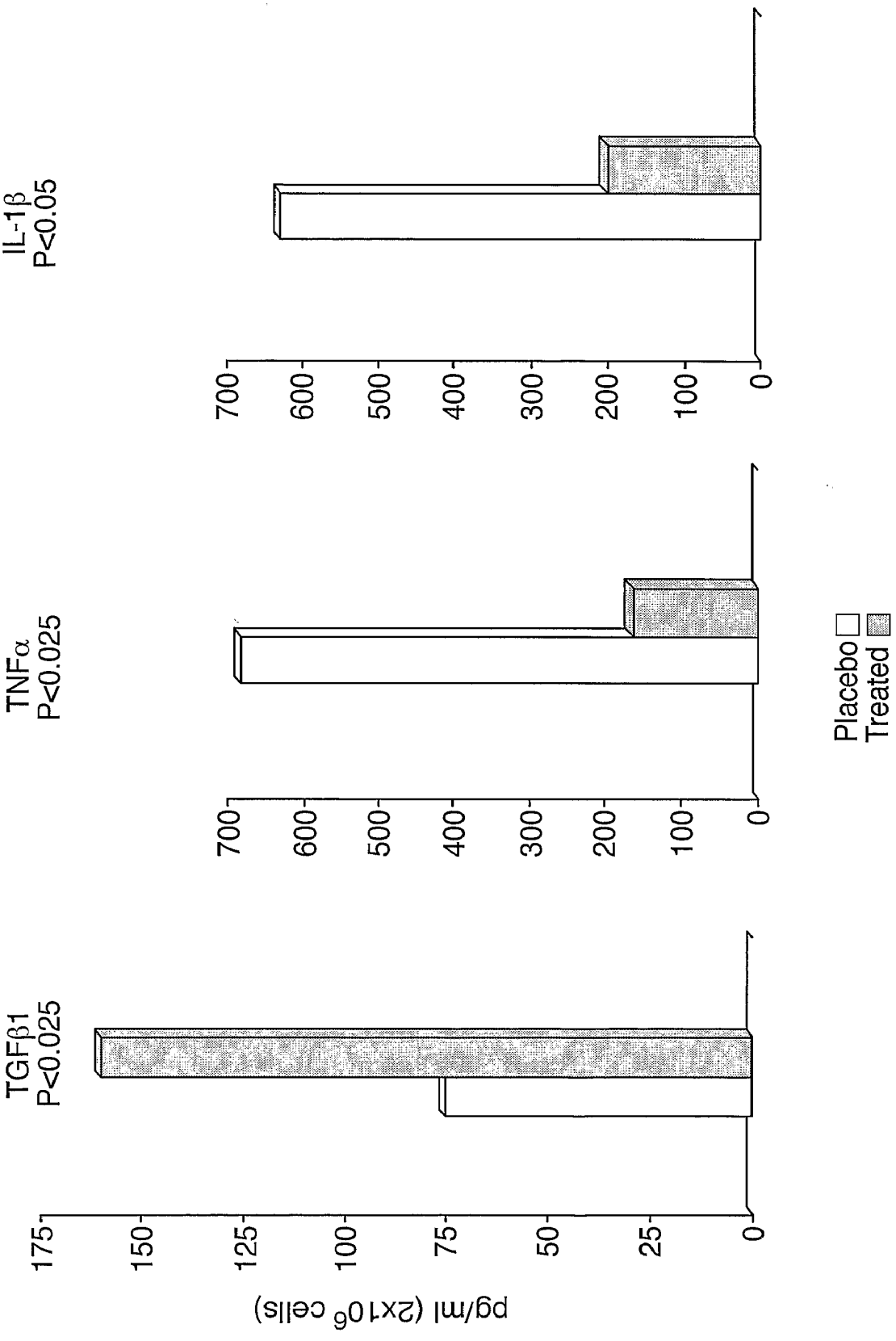
wherein R^1 to R^3 are the same and selected from γ-linolenyl residue, dihomo-γ-linolenyl residue or arachidonyl residue

comprising reacting the corresponding γ-linolenyl halide, dihomo-γ-linolenyl halide or arachidonyl halide, wherein halide is chloride, bromide or iodide, with glycerol

37. A lipid selected from the group consisting of
Glycerol 1,3-didecanoate- 2-octadecatri(6-Z,9-Z,12-Z)enoate
Glycerol 1,3-didecanoate-2- eicosa-(8Z,11Z,14Z)-trienoate
Glycerol trieicosotetra5-Z,8-Z,11-Z,14Z-eneoate

38. A lipid as claimed in Claim 37 for use in therapy.

Fig.1.



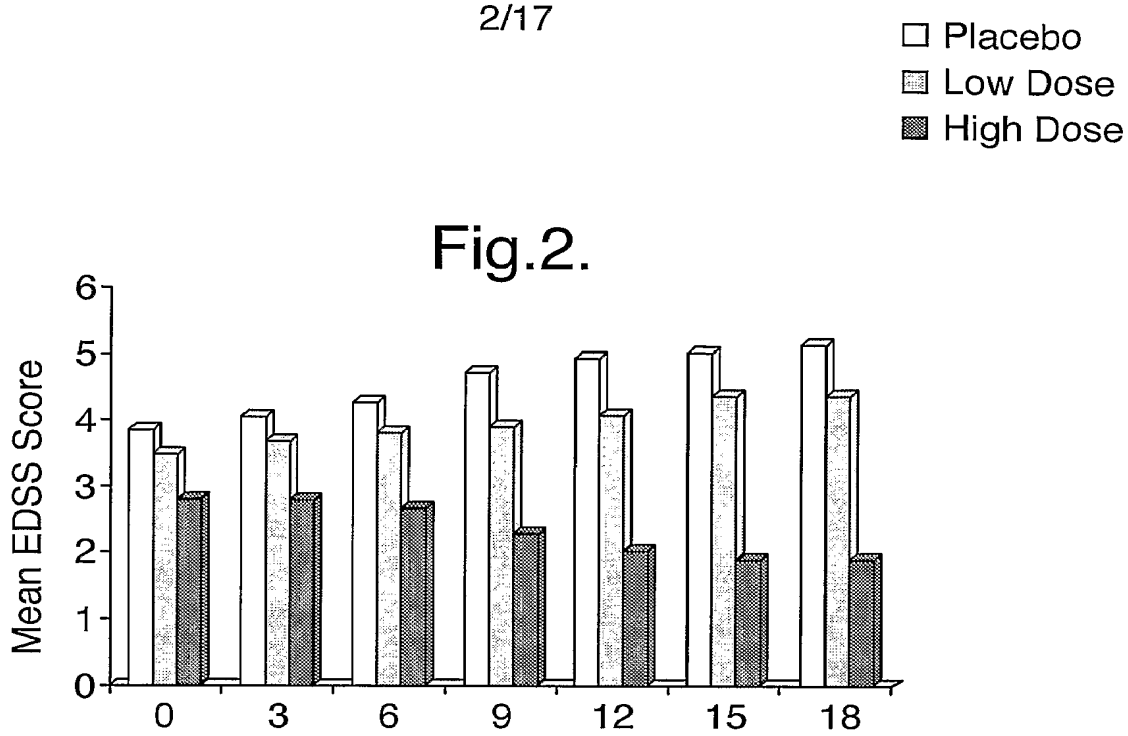


Fig.2.

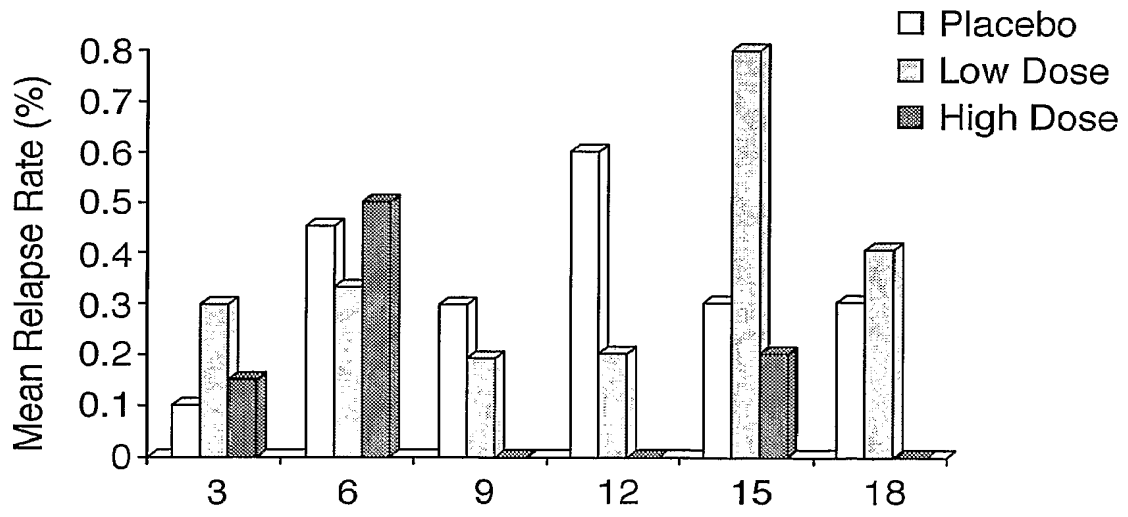
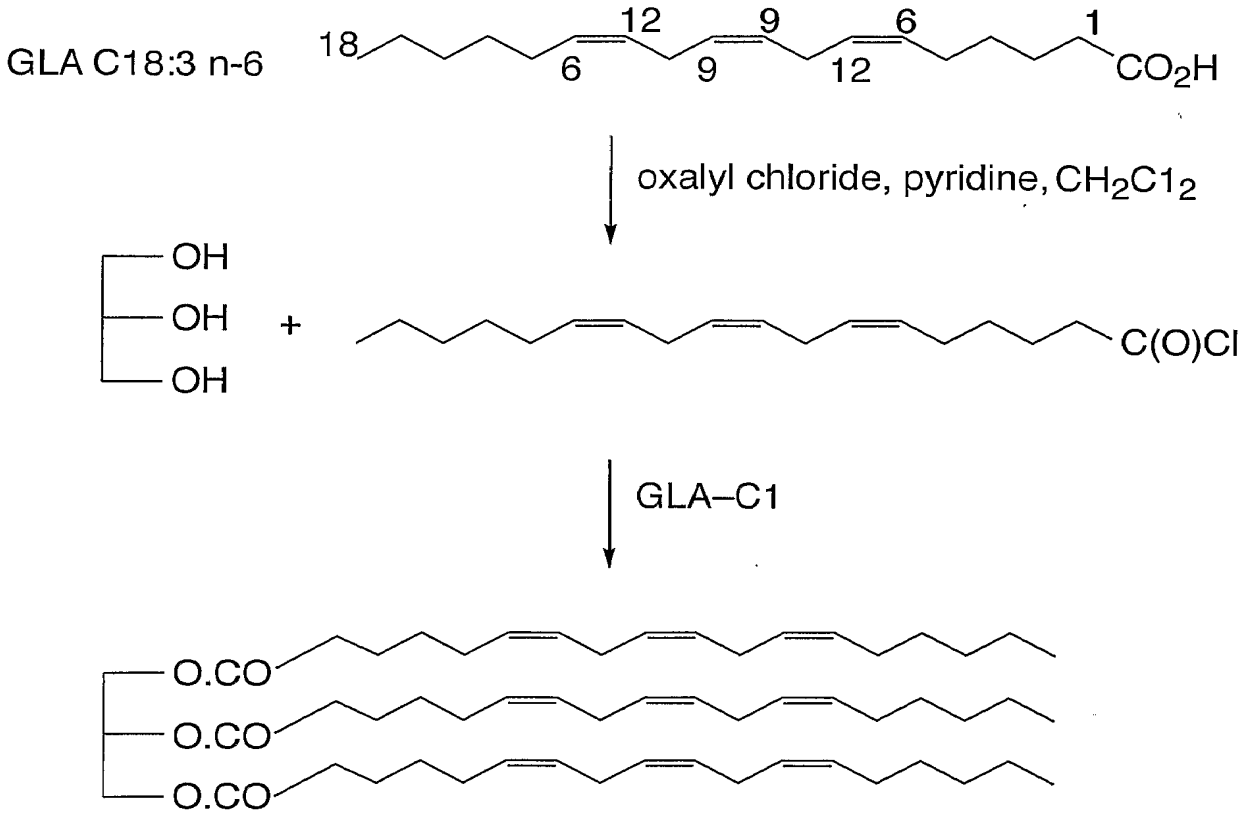


Fig.3.

Fig.4.



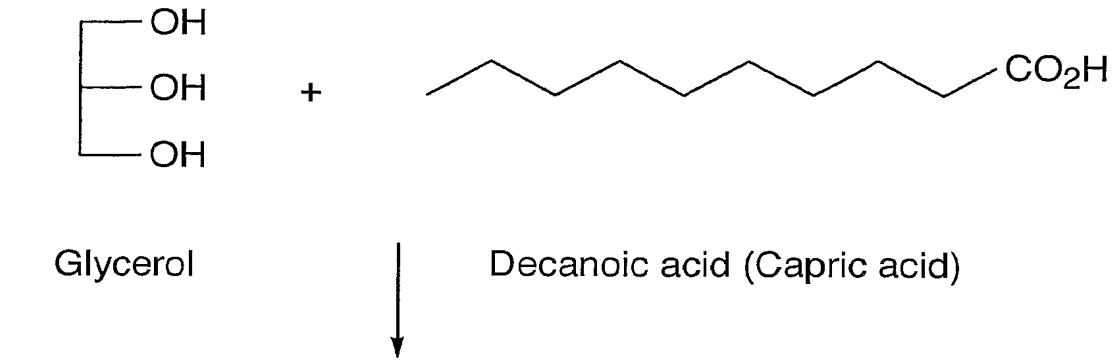
2

Glycerol tris-(6-Z,9-Z,12-Z-octadecatrienoate) $C_{57}H_{92}O_6$

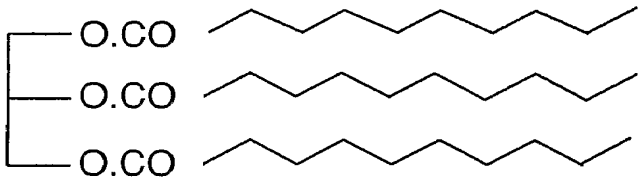
Tri-gamma-linolenin MW = 873.4

Numbering systems: black for biologists; blue for chemists

Fig.5.



Glycerol Decanoic acid (Capric acid)



4

Glycerol tridecanoate $C_{33}H_{62}O_6$

MW=554.85 mp 32°C Exhibits polymorphism

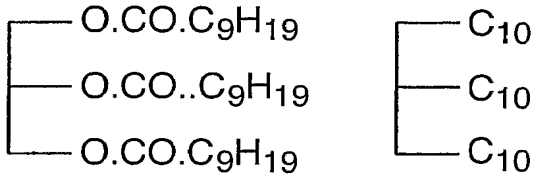
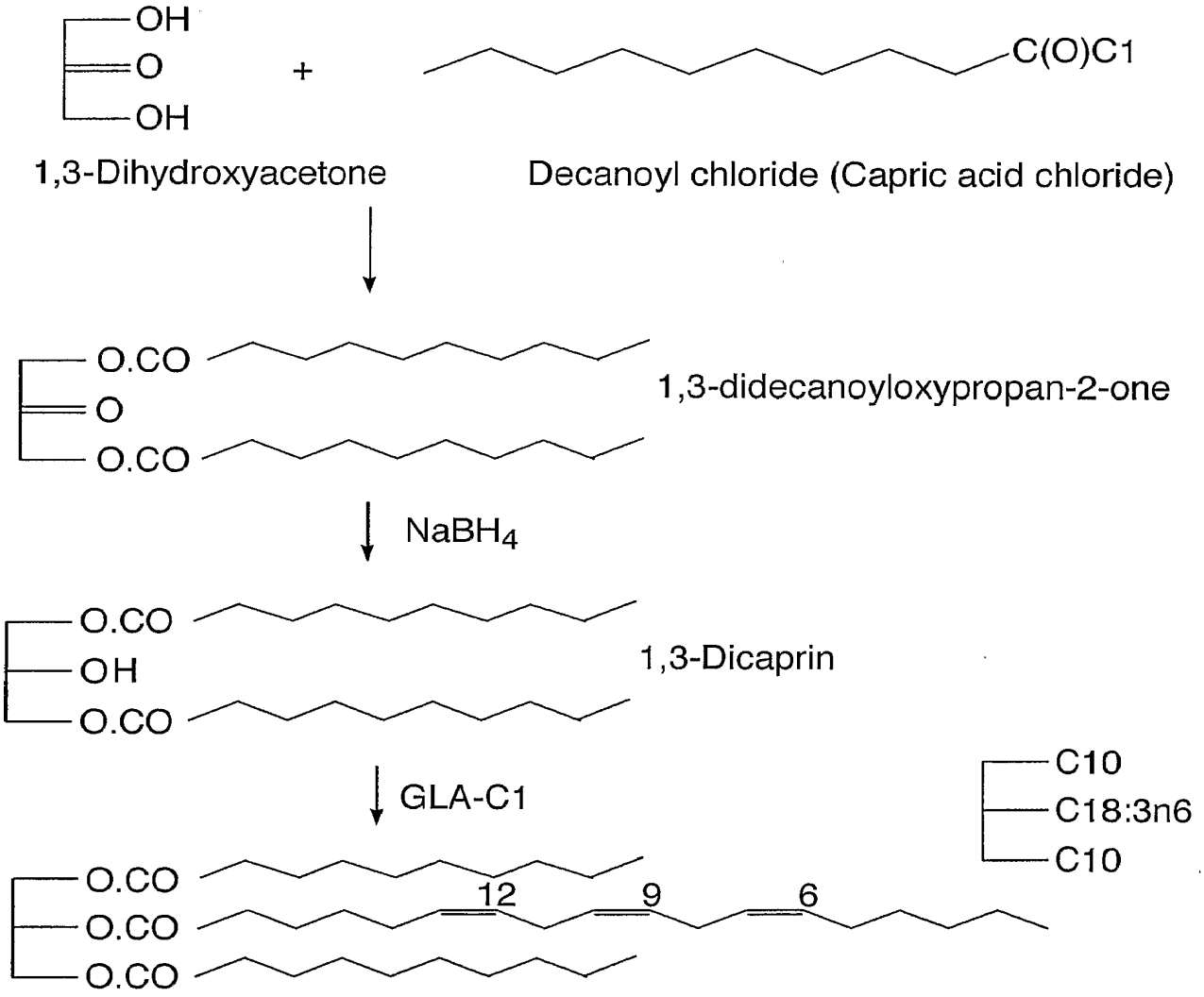


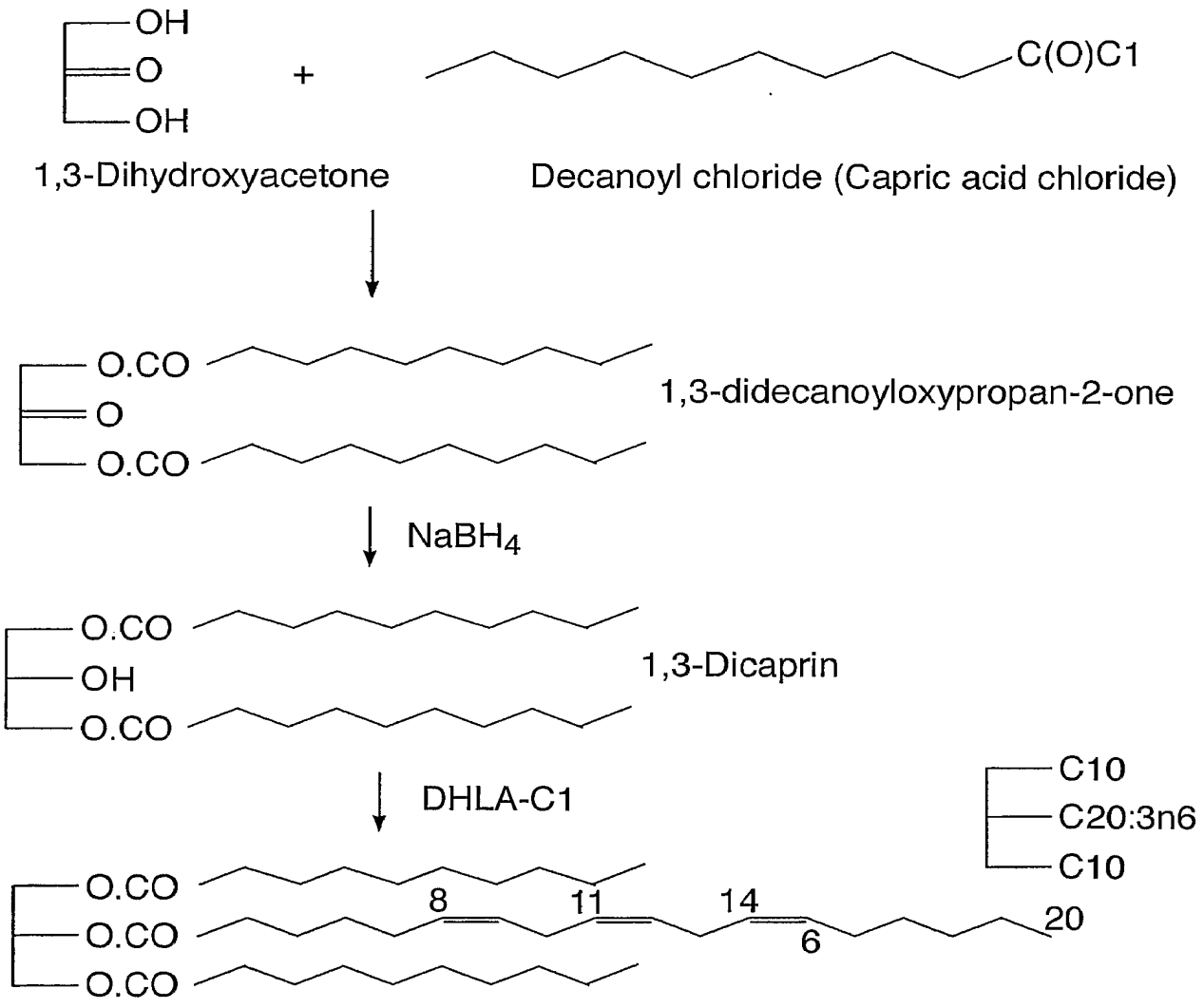
Fig.6.



Glycerol 1,3-didecanoate-2-octadeca-6Z,9Z,12Z-trienoate

$C_{41}H_{72}O_6$ MW = 661.05 mp <25°C

Fig.7.

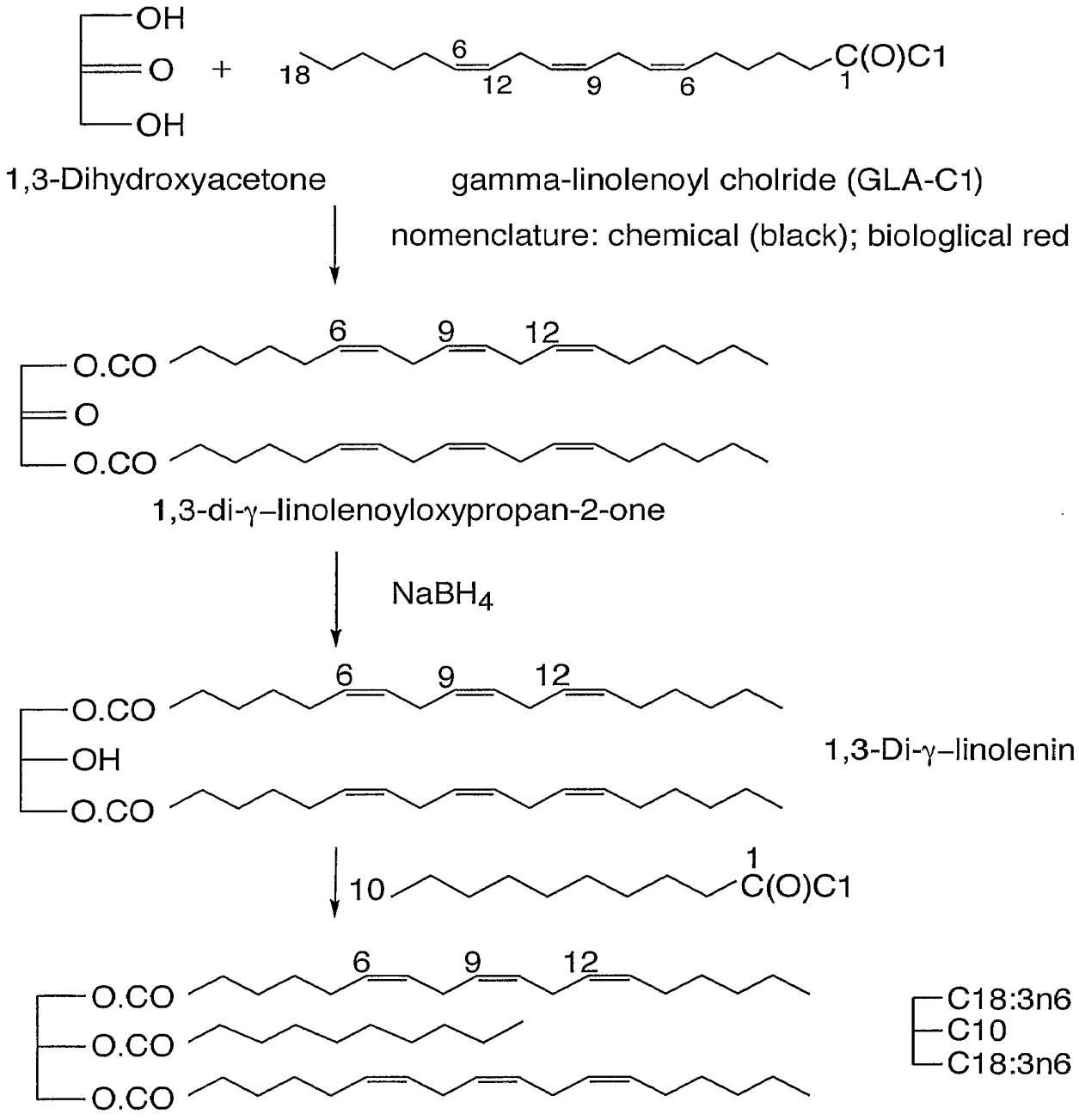


Glycerol 1,3-didecanoate-2-eicosa-8Z,11Z,14Z-trienoate

$C_{43}H_{76}O_6$

MW = 689.1 mp <25°C

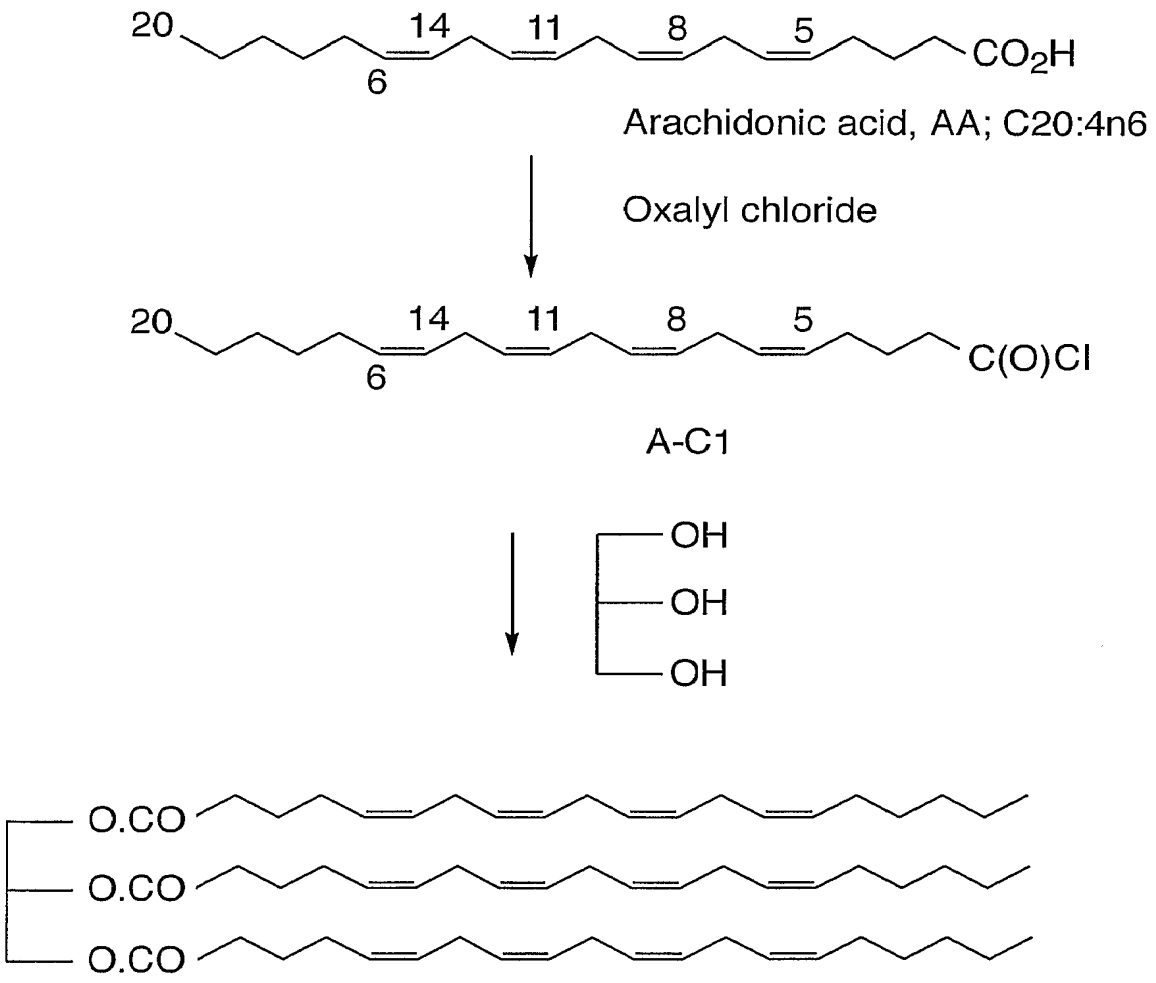
Fig.8.



Glycerol 1,3-dioctadeca-6Z,9Z,12Z-trienoate-2-decanoate

$C_{49}H_{82}O_6$ MW = 766.1 mp <25°C

Fig.9.



Glycerol 1,2,3-tri(5Z,8Z,11Z,14Z-eicosatetraenoate) $C_{63}H_{98}O_6$

M. Wt. 951.5

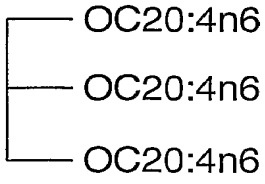
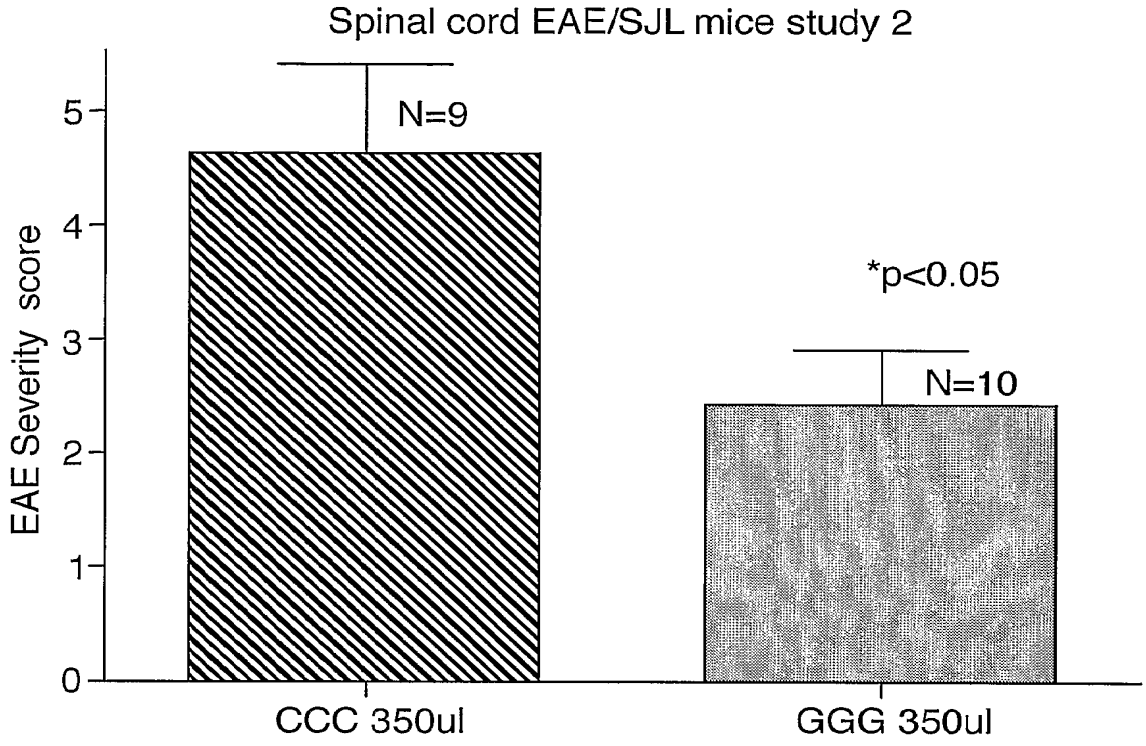


Fig.10.


Spinal cord EAE/SJL mice study 2
EAE Severity score
0
1
2
3
4
5
N=9
*p<0.05
N=10
CCC 350ul
GGG 350ul

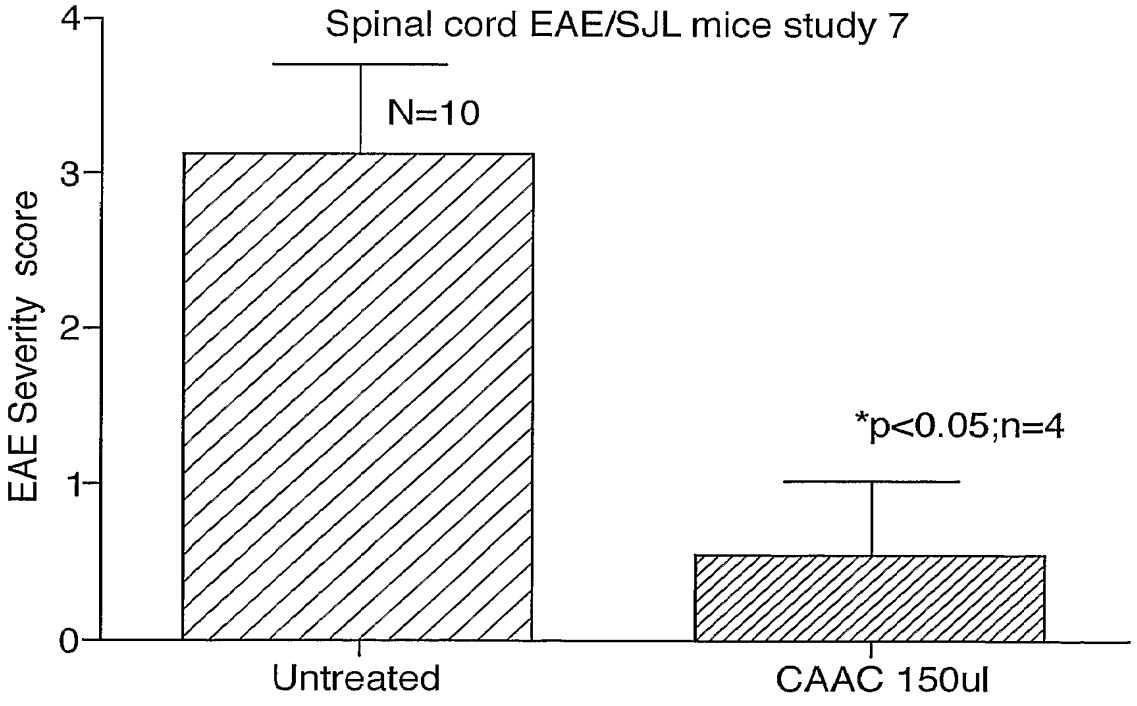
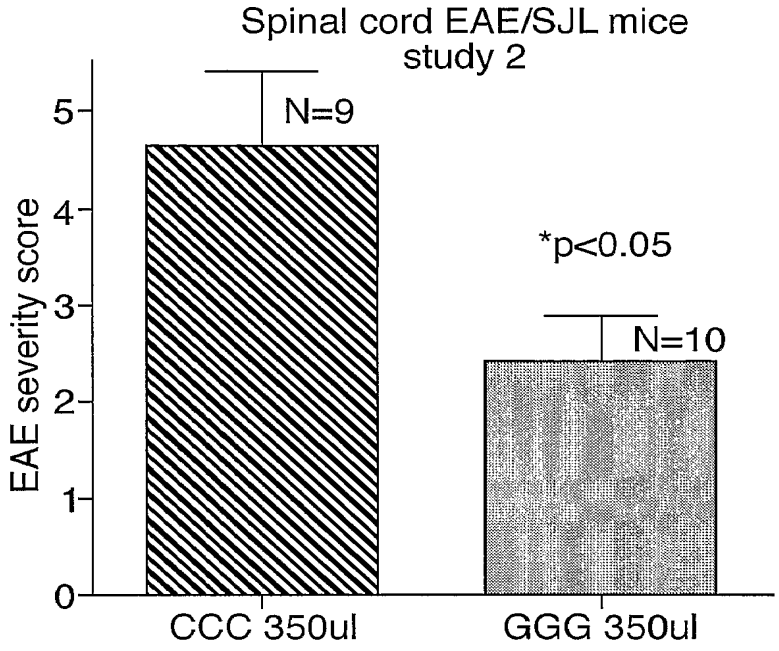
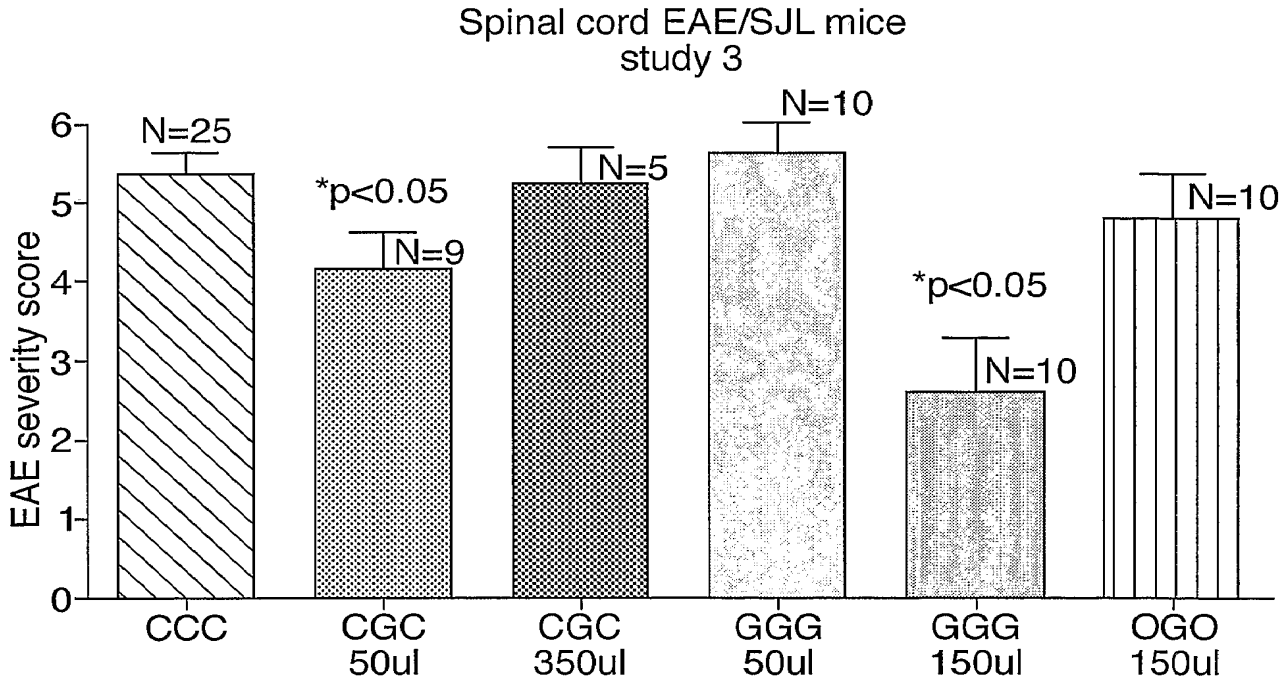

Spinal cord EAE/SJL mice study 7
EAE Severity score
0
1
2
3
4
N=10
*p<0.05;n=4
Untreated
CAAC 150ul

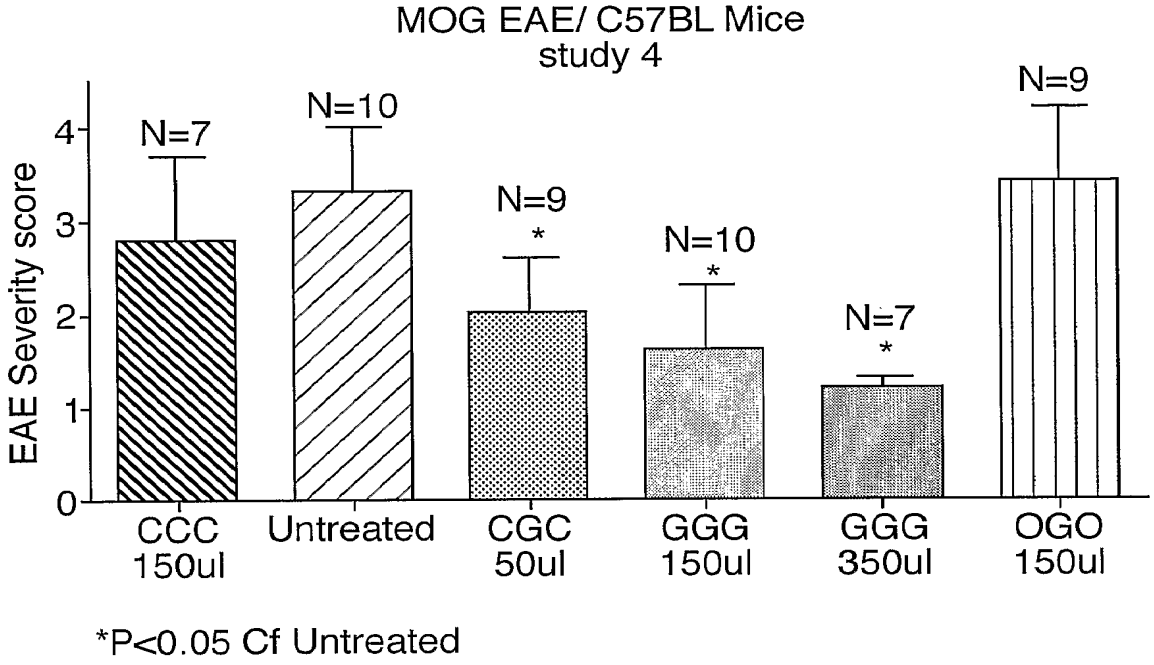
Fig.11.





CCC pooled from 50/150/350ul doses
CGC 25/50/350ul
GGG 50/150/350ul
OGO 25/50/150/350ul

Fig.12.



*P<0.05 Cf Untreated

Fig.13.

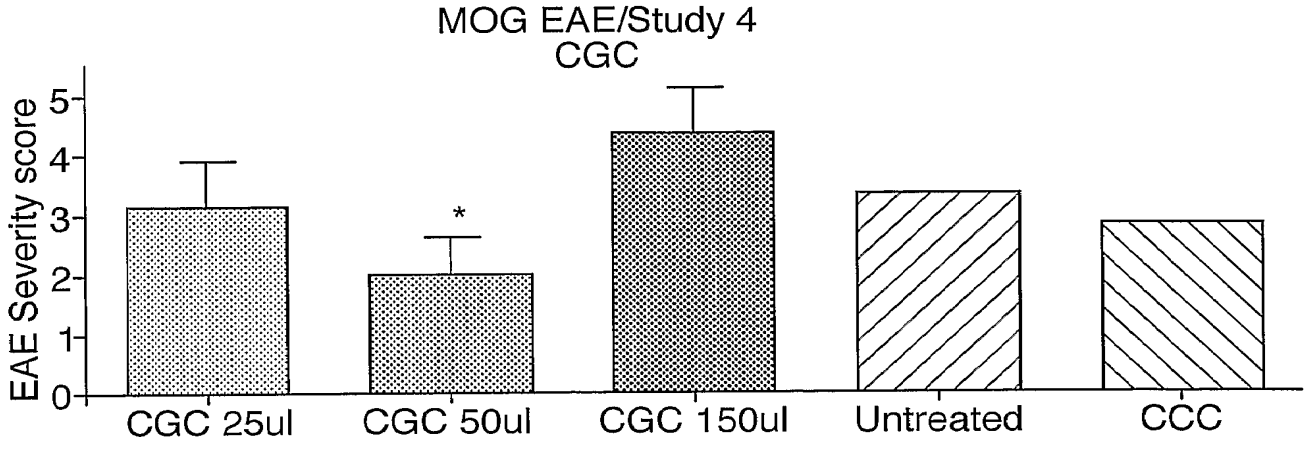
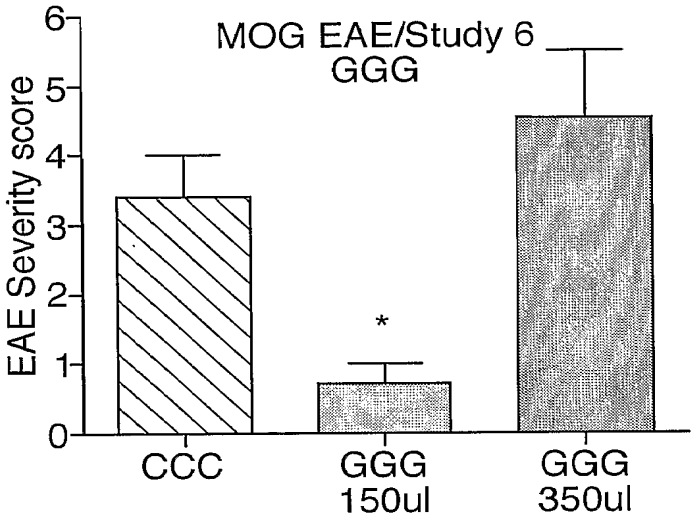


Fig.14.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GGG
EAE Severity score
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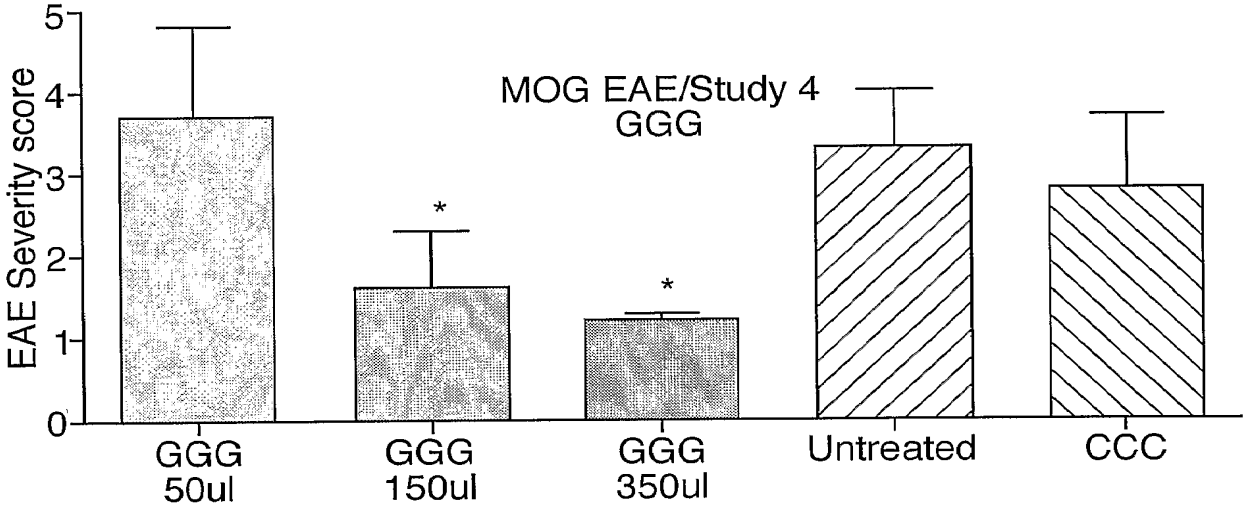
1
2
3
4
5
6
*
CCC
GGG 150ul
GGG 350ul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GGG
EAE Severity score
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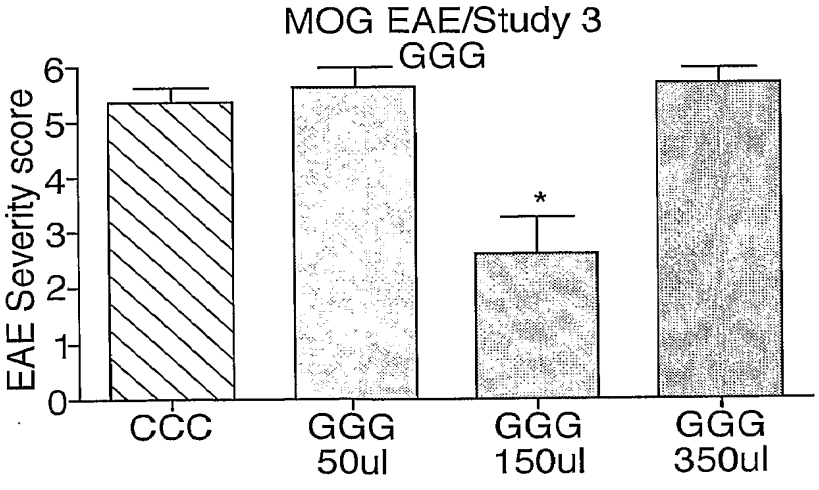
1
2
3
4
5
*
*
GGG 50ul
GGG 150ul
GGG 350ul
Untreated
CCC


MOG EAE/Study 3
GGG
EAE Severity score
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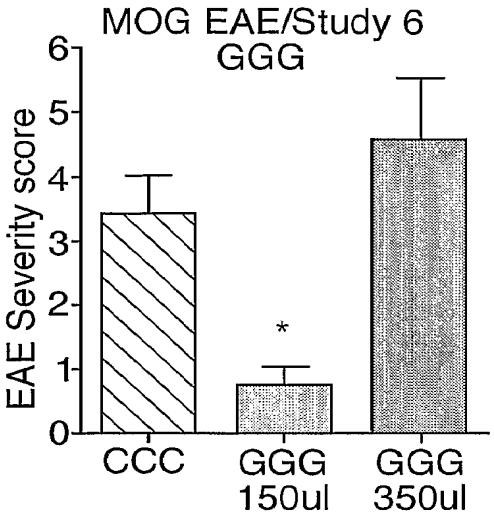
2
3
4
5
6
*
CCC
GGG 50ul
GGG 150ul
GGG 350ul

Fig.15.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GGG
EAE Severity score
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CCC
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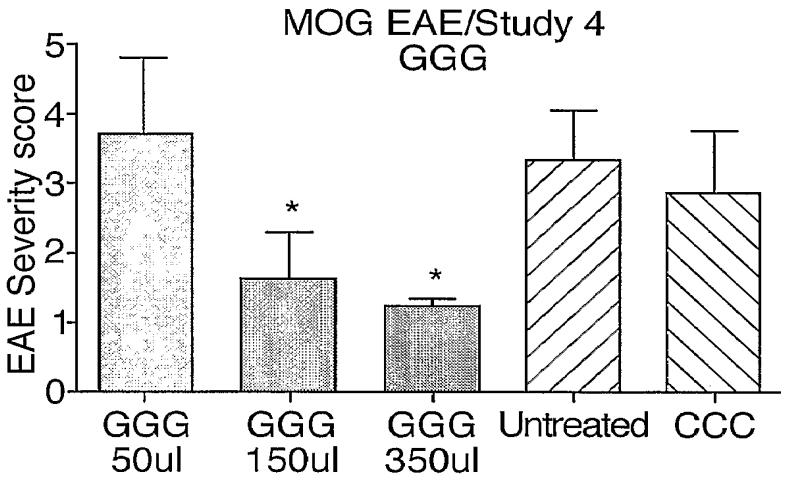
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EAE Severity score
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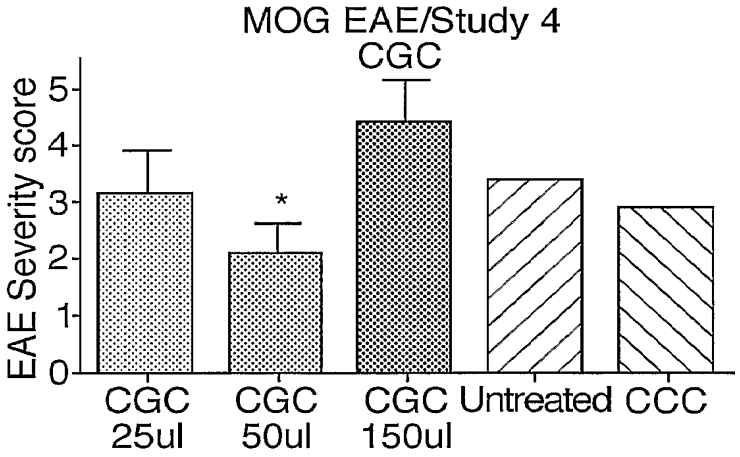
3
4
5
*
*
GGG 50ul
GGG 150ul
GGG 350ul
Untreated
CCC


MOG EAE/Study 4
CGC
EAE Severity score
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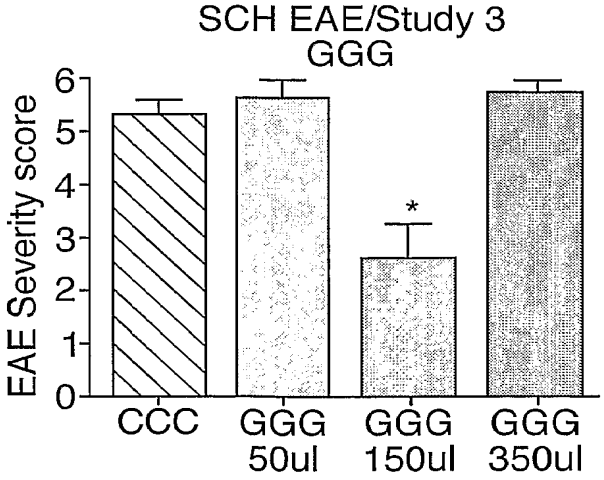
2
3
4
5
*
CGC 25ul
CGC 50ul
CGC 150ul
Untreated
CCC


SCH EAE/Study 3
GGG
EAE Severity score
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2
3
4
5
6
*
CCC
GGG 50ul
GGG 150ul
GGG 350ul

Fig.16.

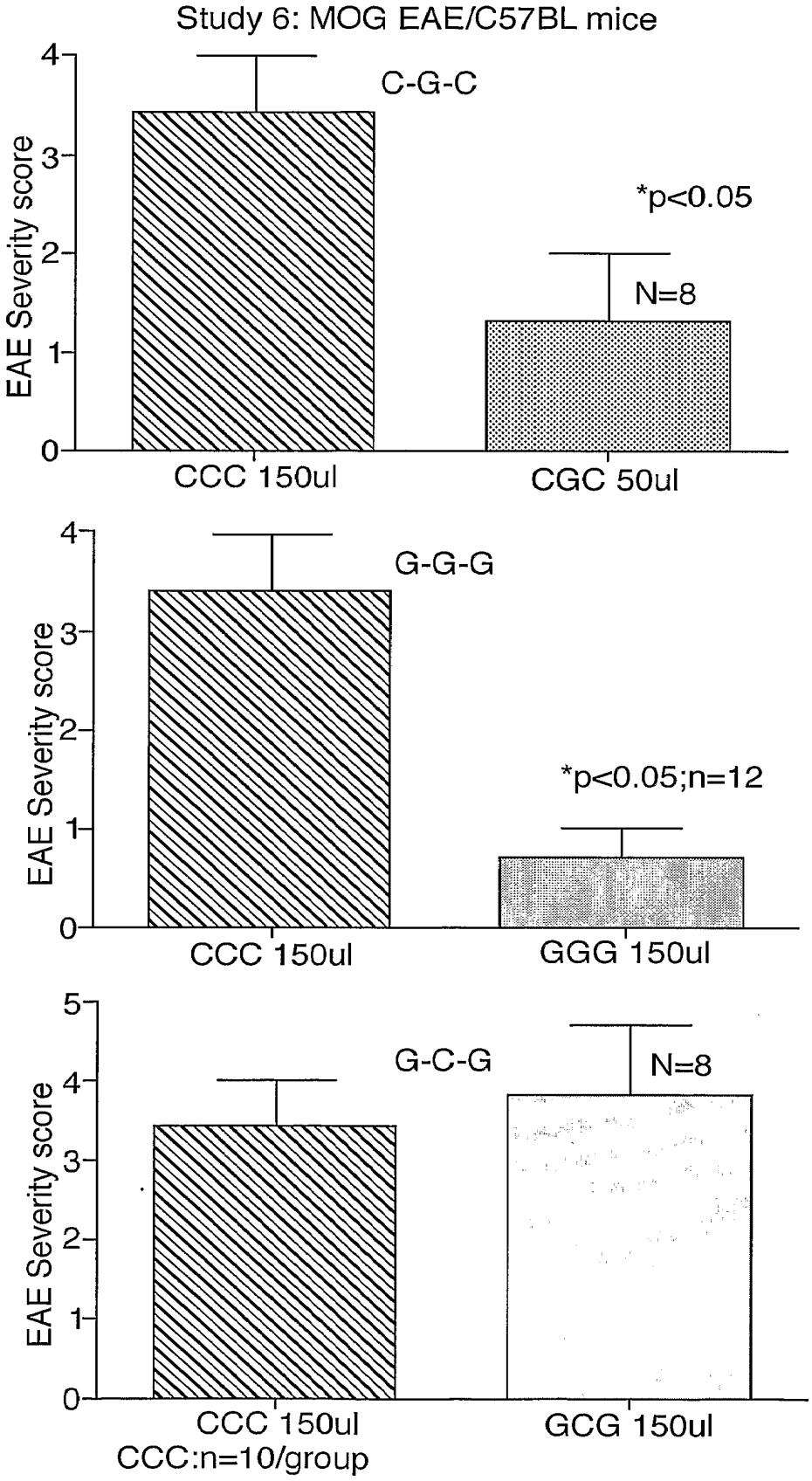


Fig.17.

Study 6:MOG EAE/C57BL mice

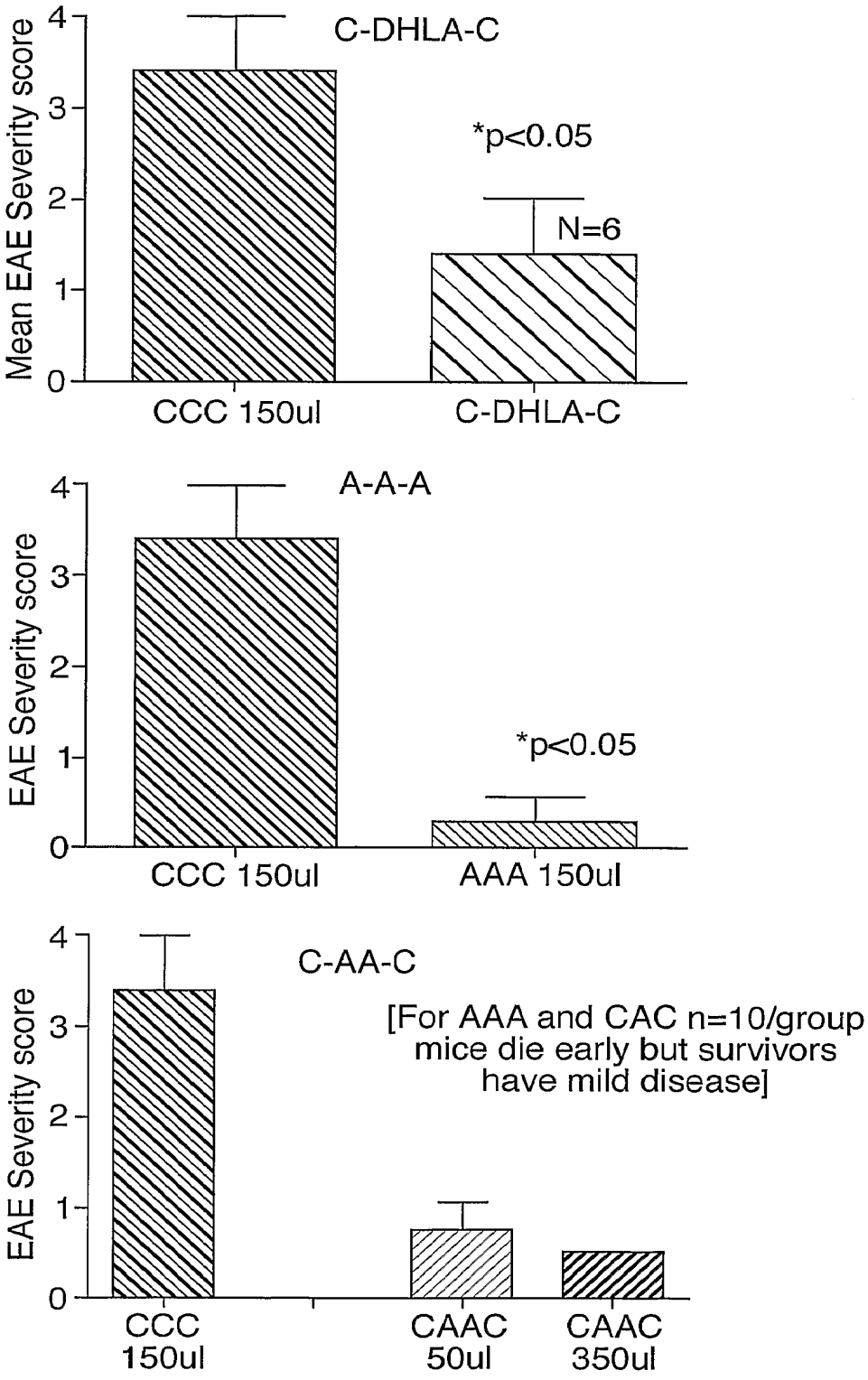
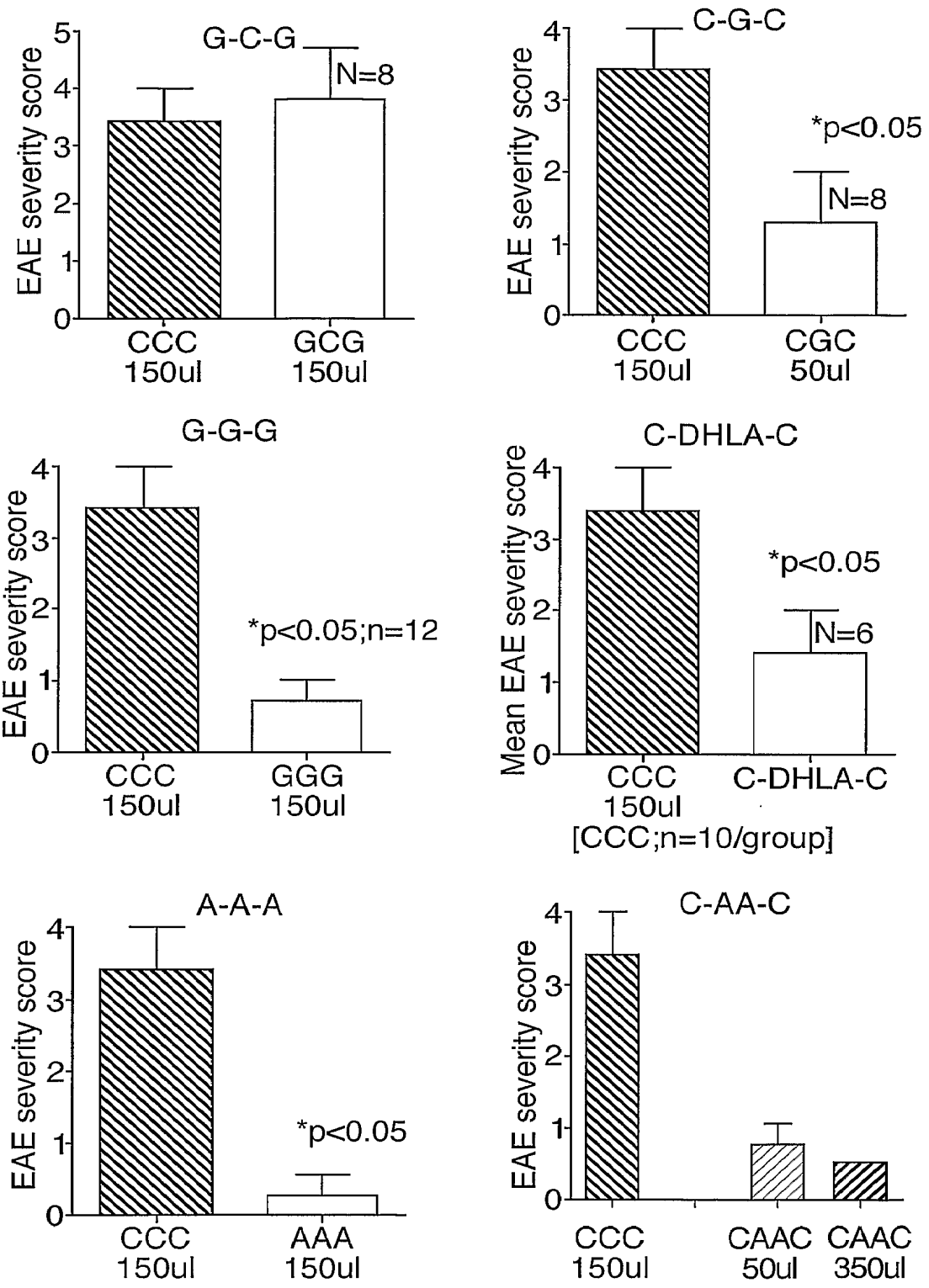


Fig.18.

Study 6. MOG EAE in C57BL Mice

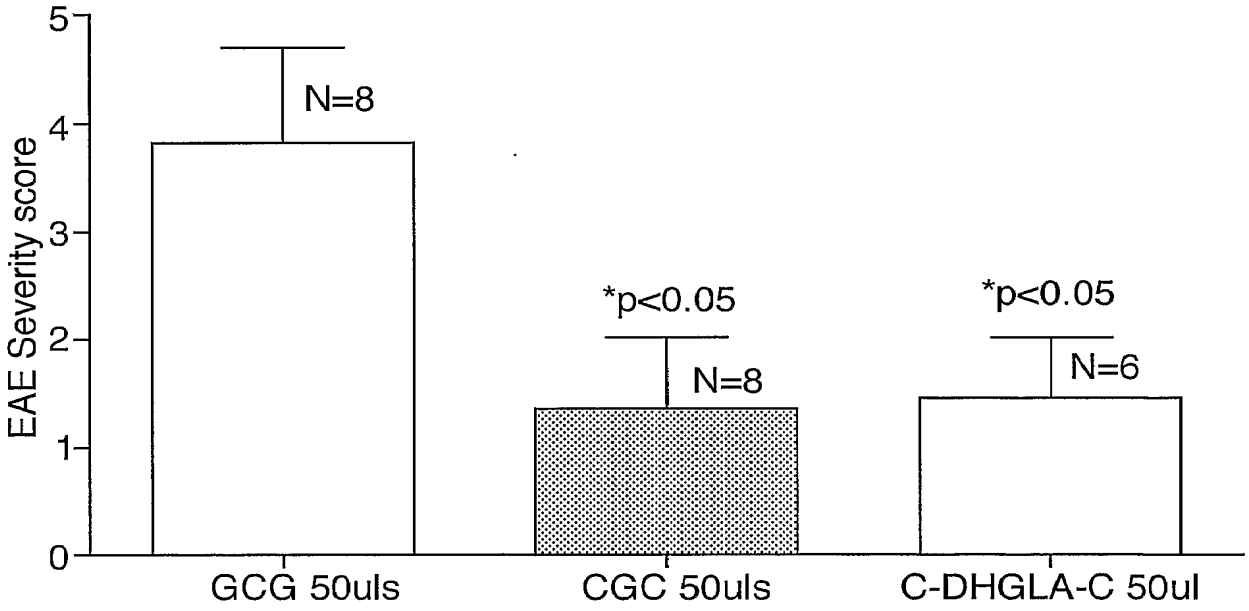


[For AAA and CAC n=10/group,mice die early but surviving mice have mild disease]

Fig.19.

MOG EAE C57BL MICE

Test of SN-2 Principle



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/003524

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/232 C07C69/587 A61P25/28

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)
IPC 7 A61K A61P C07C

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/013497 A (KAWASHIMA HIROSHI ; OKAICHI HIROSHIGE (JP); AKIMOTO KENGO (JP); OKAICH) 20 February 2003 (2003-02-20) the whole document	1-38
X	WO 01/97793 A (MECHOULAM RAPHAEL ; YISSUM RES DEV CO (IL); BREUER AVIVA (IL); GALLILY) 27 December 2001 (2001-12-27) page 8, paragraph 2-5; claim 1	1-21, 23-34
X	US 4 701 469 A (BARTHELEMY PIERRE ET AL) 20 October 1987 (1987-10-20) cited in the application column 5, line 27; claims 1-3	1,3-5, 9-23, 26-28, 35,36

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in 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 but 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	Date of mailing of the international search report
9 December 2004	21/12/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Friederich, M

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2004/003524

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

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

Although claims 1-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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 III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

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.:

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

Information on patent family members

International Application No
PCT/GB2004/003524

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03013497 A	20-02-2003	JP 2003048831 A	21-02-2003
		CA 2456049 A1	20-02-2003
		EP 1419768 A1	19-05-2004
		WO 03013497 A1	20-02-2003
WO 0197793 A	27-12-2001	AT 277608 T	15-10-2004
		AU 6628901 A	02-01-2002
		DE 60106026 D1	04-11-2004
		EP 1292288 A2	19-03-2003
		WO 0197793 A2	27-12-2001
		JP 2003535892 T	02-12-2003
		US 2002072539 A1	13-06-2002
US 4701469 A	20-10-1987	US 4607052 A	19-08-1986
		US 4701468 A	20-10-1987

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(19) World Intellectual Property Organization
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(43) International Publication Date
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PCT

(10) International Publication Number
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(21) International Application Number: PCT/GB2004/003524

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(71) Applicant *(for all designated States except US)*: **BTG INTERNATIONAL LIMITED** [GB/GB]; 10 Fleet Place, Limeburner Lane, London EC4M 7SB (GB).

(72) Inventors; and

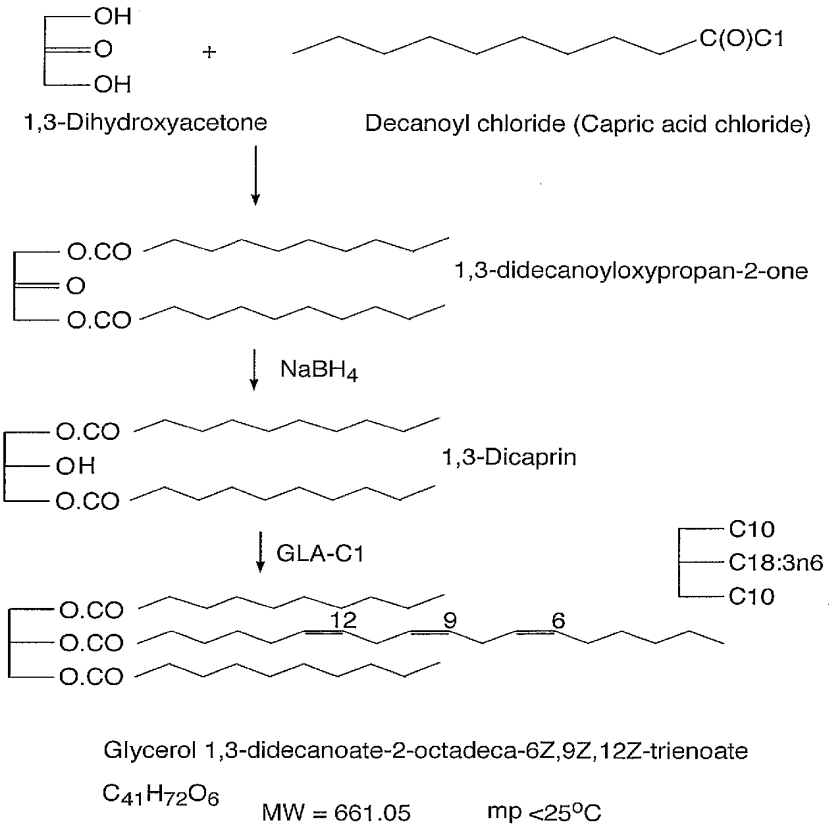
(75) Inventors/Applicants *(for US only)*: **HARBIGE, Laurence, S.** [GB/GB]; The University of Greenwich, Old Royal Naval College, Park Row, Greenwich, London SE10 9LS (GB). **LEACH, Michael, J.** [GB/GB]; The University of Greenwich, Old Royal Naval College, Park Row, Greenwich, London SE10 9LS (GB). **SHARIEF, Mohammed** [GB/GB]; Dept. of Clinical Neurosciences, Guy's, King's & St Thoma's School of Medicine, Hodgkin Building, Guy's Hospital, London SE1 9RT (GB). **BARRACLOUGH, Paul** [GB/GB]; Synnovation Ltd, Unit 8, Nonsuch Industrial Estate, Kiln Lane, Epsom, Surrey KT17 1DH (GB).

(74) Agent: **DOLAN, Anthony, Patrick**; BTG International Limited, 10 Fleet Place, Limeburner Lane, London EC4M 7SB (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,

[Continued on next page]

(54) Title: TREATMENT OF NEURODEGENERATIVE CONDITIONS



Glycerol 1,3-didecanoate-2-octadeca-6Z,9Z,12Z-trienoate

$C_{41}H_{72}O_6$ MW = 661.05 mp <25°C

(57) Abstract: A method is provided for treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a lipid glyceride comprising a glycerol moiety and a fatty acid moiety, the fatty acid moiety being selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid characterised in that the selected fatty acid moiety is attached to the glycerol moiety at its sn-2 position. Preferably the method is that wherein the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF-β1 levels in the patient to therapeutic levels.

WO 2005/018632 A1

KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TREATMENT OF NEURODEGENERATIVE CONDITIONS.

The present invention relates to a method for treating neurodegenerative conditions, particularly those in which increase in transforming growth factor β (TGF-β) is beneficial, particularly TGF-β1. More particularly the present invention provides treatment for neurodegenerative conditions, particularly those such as demyelinating diseases, such as multiple sclerosis, Alzheimer's and Parkinsons diseases and the degenerative sequelae associated with head trauma, stroke and intracranial bleeds, whereby neuronal function may be improved or restored from an impaired condition, eg. by remyeleination.

Further provided are novel use of known and novel compounds comprising unsaturated fatty acid moieties for the manufacture of medicaments capable of effectively treating such conditions, more particularly being capable of achieving previously unattained levels of success with regard to recovery of neurological function.

The inventor's copending unpublished patent application PCT/GB04/002089, incorporated herein by reference, relates to the use of plant and fungal oils for the treatment of neurodegenerative diseases. These oils have high percentages of the essential fatty acid γ-linolenic acid (GLA) at the sn-2 position of their lipids, typically being over 40% of the sn-2 fatty acid total of the oil.

It is well reported in the literature that essential fatty acids (EFAs) of the n-3 and n-6 unsaturation pattern have beneficial effect in a wide variety of human physiological disorders, including autoimmune diasese (WO 02/02105). Harbige (1998) Proc. Nut. Soc. 57, 555-562 reviewed the supplementation of diet with n-3 and n-6 acids in autoimmune disease states, and particularly noted evidence of benefit of γ-linolenic (GLA) and/or linoleic acid (LA) rich oils.

Bates et al noted that lipid oils comprising a mixture of linoleic acid and γ-linolenic acid residues had been suggested back in 1957 to be possibly more efficacious in treating inflammation and autoimmune diseases, but found that at 3g oil per day (Naudicelle Evening Primrose oil 7:1 LA:GLA) patients who had relapses became more ill on the trial oil than on the control.

Although the aetiology of MS remains unknown studies have shown that MS patients have higher than normal neuro-antigen autoreactive T-cells levels. These T-cells react *inter alia* to myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) and are in an increased state of activation compared with healthy controls. The actual processes of axonal damage e.g. chronic inflammation, demyelination and astrogliosis in MS is complex but white matter inflammation and demyelination are considered to determine disease severity, whilst recent studies suggested that axonal damage in MS begins in the early stages of the disease and contributes to disability (De Stefano et al, 2001).

Experimental autoimmune encephalomyelitis (EAE) is the most frequently used animal model for immune mediated effects of MS. Studies in the guinea-pig have shown that linoleic acid partially suppresses the incidence and severity of EAE (Meade et al (1978)). (Harbige et al (1995), 1997b) demonstrated disease modifying effects of linoleic acid and γ-linolenic acid on clinical and histopathological manifestations of EAE. Depending on dose, γ-linolenic acid was fully protective in acute rat EAE whereas linoleic acid had dose-dependent action on the clinical severity but did not abolish it.

Despite these experimental findings, it is recognised that the human disease, multiple sclerosis, is highly complex and can be conversely exacerbated and ameliorated by the activity of T-cells and other immune response factors. It is thought that the n-6 fatty acids promote autoimmune and inflammatory disease based upon results obtained with linoleic acid only. TGF-β1 and PGE_2 production has been shown to be increased non-specifically in γ-linolenic acid fed mice *ex vivo*. TGF-β1 has been reported to protect in acute and relapsing EAE ((Racke et al (1993); Santambrogio et al (1993)), and PG inhibitors such as indomethacin augment, and thus worsen, the disease (Ovadia & Paterson (1982)).

Cytokines are implicated in the pathogenesis of MS, with many studies showing an increase in myelinotoxic inflammatory cytokines (TNF-α, IL-1β and IFN-γ) coinciding with the relapse phase of the disease. Conversely, levels of the anti-inflammatory and immunosuppressive cytokine transforming growth factor-beta1

(TGF-β1) appear to be reduced during a phase of relapse and increase as the patient enters remission. Thus the balance between biologically active TGF-β1 and the pro-inflammatory TNF-α, IL-1β and IFN-γ appears to be dysregulated during MS relapse-remission.

During natural recovery phase from EAE, TGF-β1-secreting T-cells inhibit EAE effector cells, TGF-β1 is expressed in the CNS and, in oral-tolerance-induced protection in EAE, TGF-β and PGE_2 are expressed in the brain (Karpus & Swanborg (1991); Khoury et al (1992)). Harbige ((1998) concluded that dietary γ-linolenic acid effects on EAE are mediated through Th_3-like mechanisms involving TGF-β1 and possibly through superoxide dismutase antioxidant activity.

Borage oil (typically 20% to 23% γ-linolenic acid and 34 to 40% linoleic acid per 100% fatty acid content) and Mucor javanicus fungal oil (see Figure 1) have been shown to be effective in the EAE animal model used to identify MS candidates, whilst never having been shown to be significantly effective in the human disease. High levels of linoleic rich oil containing low levels of γ-linolenic acid (EPO: linoleic acid:γ-linolenic acid 7:1) partially suppressed the incidence and severity of EAE in rat (Mertin & Stackpoole, 1978) whereas the Bates' Naudicelle study referred to above led to worsening of patients. In spite of the use of Borage oil and other GLA/LA containing oils such as Evening Primrose oil by multiple sclerosis sufferers over the past 30 years or so, the vast majority of patients fail to recover from the disease, showing no significant improvement, with the underlying disease continuing to progress to death.

It has been suggested to use, *inter alia*, γ-linolenic acid and linoleic acid rich Borage oil as a means to provide immuno-suppression in multiple sclerosis (US 4,058,594). Critially, the dose suggested is 2.4 grams of oil per day and no actual evidence of efficacy is provided. This is much lower than the low 5g/day dose found to be ineffective *in vivo* in man in the PCT/GB04/002089 study.

Other more dramatic immunosuppressant treatments, including T cell depleters and modulators such as cyclophosphamide, are also shown to be effective in

the EAE model, but where these are employed in the human multiple sclerosis disease symptoms improve, but the underlying disease continues to progress. T-cells indeed produce beneficial cytokines, such as TGF-β1, as well as deleterious ones in man. David Baker of Institute of Neurology, UK summed up the disparity between what is effective in the EAE and in MS with a paper entitled '*Everything stops EAE, nothing stops MS*' at the 10th May 2004 UK MS Frontiers meeting of the UK MS Society.

It is clear that immunosuppression alone cannot cure MS. This is almost certainly due to a fundamental underlying metabolic disorder in MS patients, in addition to the autoimmune disease, that leads to membrane abnormality, cytokine dysregulation and subsequent immune attack and lesioning. Although patients go into remission in relapse-remitting disease, the underlying demyelination proceeds.

The 'gold standard' treatment for MS remains interferon, such as with β-Avonex ®, Rebif ® and other interferon preparations. This gold standard treatment only addresses needs of some, eg 30%, of the patients and even in these symptom improvement is restricted to reduced severity of relapses. Whilst symptoms may be reduced in a proportion of patients, the disease tends to progress to further disability and death due to underlying degeneration.

In their as yet unpublished PCT/GB04/002089 study the present inventors have surprisingly determined that with compliance to a 'high dose' treatment with triglyceride oil containing high levels of sn-2 γ-linolenic acid (>40% of residues at the sn-2 being of γ-linolenic acid) with suitable accompanying fatty acid content, remarkable levels of improvement in almost all symptoms of MS can be achieved, way surpassing that provided by the current gold standard treatment. Such success is particularly surprising in the light of the prior use of other γ-linolenic acid containing preparations without success, such as the Naudicelle study.

The PCT/GB04/002089 study shows that over an 18-month period, patients taking high dose (15g/day) selected high sn-2 γ-linolenic acid borage oil showed significant ($p<0.001$) and marked improvements in EDSS score, a reduced rate of relapse, symptomatic relief of muscle spasticity and painful sensory symptoms, and improved objective measures of cognitive functions. Low doses of 5g/day of this

borage oil were without effect.

Patients taking the highest dose of this borage oil maintained their level of peripheral blood mononuclear cell production (PBMC) of TGF-β1 during the trial period, their pro-inflammatory cytokines TNF-α and IL-1β were significantly and markedly (<70%) reduced and they either maintained or increased the PBMC membrane long chain omega-6 fatty acids dihomo-γ-linolenic acid (DHLA) and arachidonic acid (AA) in contrast to patients taking placebo who demonstrated loss of these fatty acids over the course of the trial period.

This whilst immuno-suppression would be expected to reduce increase of active lesioning and neurodegeneration, the high sn-2 GLA oil treatment apparently targeted maintenance and/or increase of key membrane lipid components that are otherwise specifically lost in MS, being consistent with a correction of a metabolic defect not otherwise effectively treated by current therapies. The fact that the low dose (5 grams/day) had no effect on this supports such determination.

γ-Linolenic acid (18:3n-6, or GLA) is known to be rapidly converted to longer-chain omega-6 polyunsaturated fatty acids dihomo-γ-linolenic acid and arachidonic acid *in vivo* (Phylactos et al 1994, Harbige et al 1995, 2000). Therefore to determine how to increase the level of membrane long chain omega-6 fatty acids in MS the inventors have reviewed their results obtained with several GLA-containing oils:- both fungal (from Mucor javanicus) and plant (Borago officianalis), Evening primrose Oenothera spp. or Blackcurrant Ribes spp) as well as a synthetic tri-GLA oil as GLA delivery systems in an *in vivo* experimental animal model of MS known as chronic relapsing experimental autoimmune encephalomyelitis (CREAE).

Induction of EAE in rats does ***not*** produce histological features of demyelination (Brosnan et al 1988) but induces an acute mono-phasic disease pattern, unlike MS which is characterised by CNS demyelination and is in the majority of cases clinically relapsing-remitting. Chronic relapsing and demyelinating EAE models (CREAE) however are characterised by demyelination and relapse phases. With the demonstration that myelin oligodendrocyte glycoprotein (MOG) is an important neuroantigenic target in MS (Genain et al 1999) and the demonstration of

far greater responses of peripheral blood auto-reactive lymphocytes to this neuroantigen, compared with MBP, in MS (Kerlero de Rosbo et al 1993, 1997) MOG induced CREAE has become the animal model of choice with features closely resembling those observed in MS (Fazakerely et al 1997, Genain et al 1999, Amor et al 1994).

Evidence from the inventor's CREAE and rat EAE feeding studies indicates that an enriched blackcurrant seed oil (72 % w/w 18:3n-6, GLA) did not protect against EAE (see Table 3). Importantly blackcurrant seed oil has a low sn-2 GLA with most of the GLA in the sn-1 and sn-3 positions (Lawson and Hughes 1988). Furthermore a structured triacylgcerol containing three GLA moieties (TG-GLA) provided protective effects similar to that of the borage oil used in CREAE (Table 2). This would also be consistent with the sn-2 GLA being important i.e. the outer pair sn-1 and sn-3 GLA being enzymatically removed *in vivo* and probably undergoing oxidation leaving the sn-2 GLA only. This selective hydrolysis arises from the known ability of specific lipases to remove the sn-1 and sn-3 fatty acids from triacylgycerol molecules but an apparent protection of the sn-2 position *in vivo* (Lawson and Hughes 1988, Kyle 1990).

This review has led the inventors to postulate that glycerides having sn-2-γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid residues will be superior in correcting MS metabolism even to the high sn-2-γ-linolenic acid Borage oil of their earlier trial. This would allow lower doses of lipid to be taken and/or possibly decrease the time of treatment which would result in beneficial effect.

Table 3 of EP 0520624 (Efamol Holdings) compares the triglyceride content of Evening Primrose and Borage Oils, the former being taught to be more therapeutically effective than the latter for a variety of GLA responsive disorders. This document indicates Borage oil to have twenty seven different trigyceride components, only 20% of which have sn-2 GLA. Page 3, lines 40-42 notes that biological testing has shown that equal amounts of GLA may indeed have very different effects when that GLA is supplied as different oil sources. Crucially, it then directs the reader to one particular fraction present in Evening Primrose Oil (EPO),

but not Borage Oil, as being responsible for the former's superior effect in raising PGE1 (see EP 0520624 Chart page 4 and Table 2) and thus anti-inflammatory effect: that fraction being identified as di-linoeoyl-mono-gamma-linolenyl-glycerol (DLMG) which it states to be 18 to 19% of the total triglyceride in EPO. Crictically, page 6 clearly teaches that the position of the GLA, in sn-1, 2 or 3, is not important to this effect.

Dines et al (1994) Proceedings of the Physiological Society, Aberdeen Meeting 14-16 September 1994 report on studies of treatment of diabetic neuropathy neuronal damage with γ-linolenic acid containing oils of the type advocated by EP 0520624 and again note that Borage Oil was not very effective in treating this neurodegeneration whereas Evening primrose oil was. The paper concludes that Borage Oil contains other constituents that interfere with GLA activity.

The present inventors now set out, in view of their results for high sn-2-γ-linolenic acid Borage Oil, to demonstrate that it is indeed the presence of an sn-2-γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid residue in a glyceride, particularly a triglyceride, that gives it efficacy in treating EAE, CREAE and the human disease MS.

In a first aspect the present invention provides a method of treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group of residues consisting of residues of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid.

Particularly advantageously treated neurodegenerative diseases are those involving demyelination. The present method specifically arrests underlying neurodegeneration and restores neuronal function. Particularly the method normalises neuronal membrane composition, and restores healthy PBMC spontaneuosly released TGF-β1/TNFα ratios and the ratios of TGF-β1 with other PBMC released cytokines. Most advantageously the method arrests neurodegeneration in multiple sclerosis of

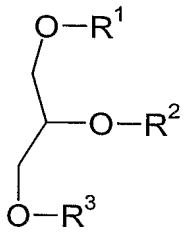
all types but particularly relapsing remitting, primary progressive and chronic progressive MS and the restoration, in part or completely, of neuronal function such as measured, eg. By MRI or CAT scan or by EDSS score. Such method may also be used in treatment of cerebral impairment after stroke, head trauma and intracranial bleeding where there is demyelination or neuronal damage. Further application is provided in treating other chronic demyelination such as in Alzheimer's and Parkinson's disease.

Preferably the the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF-β levels in the patient to therapeutic levels. By therapeutic levels is meant levels at least consistent with healthy subjects. Preferably the dose is such as to produce a TGF-β1/TNF-α ratio spontaneously released from peripheral blood mononuclear cells (PBMCs) isolated from blood of a patient, after 18 months of daily dosing, of 0.4 to 3.0, at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably the dose is such as to produce a TGF-β1/IL-1β ratio in blood of a patient, after 18 months of daily dosing, of at least 0.5, more preferably at least 0.75 and most preferably at least 1. Preferably said levels are produced after 12 months and more preferably after 6 months.

Typically the amount of lipid administered daily will be between 0.5 and 30 grams, orally dosed, still more preferably between 1 and 20 grams and most preferably between 1 and 18 grams, typically 3 to 5 grams.

Where the sn-2 moiety is that of a γ-linolenic acid residue, the dose may be toward the higher end of these ranges, particuarly where the sn-1 and sn-3 moieties are relatively inert, eg. being metabolically utilised acids such as saturated fatty acids. Where the sn-2 moiety is that of a dihomo-γ-linolenic acid residue, the dose may be less, whilst where the sn-2 moiety is that of an aracidonic acid residue, efficacy is higher, but dosing should be more cautious, due to possibilities of unwanted side effects at higher levels.

More preferably the method is characterised in that the lipid is a monoglyceride, diglyceride or triglyceride containing the at least one sn-2 γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety of general Formula I below:



Formula I

wherein R^1 and R^3 are independently selected from hydrogen and acyl groups, and R^2 is selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid residues.

For the purpose of the present invention acyl groups are defined as comprising at least one carbonyl group on the end of an optionally substituted hydrocarbyl chain selected from alkyl and alkenyl chains, the carbonyl group being directly attached by its carbon to the oxygen of the glycerol residue shown in Formula 1.

Preferred acyl groups R^1 and R^3 are saturated fatty acid moities of formula -$CO-(CH_2)_n-CH_3$, wherein n is an integer selected from 1 to 22, more preferably being 4 to 16, still more preferably being from 5 to 12, most preferably being from 6 to 10. Particularly preferred acyl groups are those of caprylic and capric acids, particularly being 1,3-dicaprylic or 1,3-dicapric glycerols having the γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety at the sn-2 position..

Preferred glycerides for use in the invention are triglycerides.

US 4701469 describes some potential triglycerides for nutraceutical use that the present inventors have determined may be used in the method of the invention, although it only specifically describes 1,3-dioctanyl triglycerides wherein the sn-2 acid is of an EFA, only 1,3-dioctanoyl eicosapenta glycerol is described as having been prepared. These are said to useful in *inter alia* immunomodulation, but although a number of diseases are specified, use in immunosuppresion in neurodegeneration and MS are not listed.

Whilst most preferred groups R^1 to R^3 for inclusion in the compound of formula I are simple saturated fatty acids or naturally occurring fatty acids with structural or metabolic function, such as medium chain or long chain fatty acids, there are other possibilities. Particularly preferred fatty acids are those that are utilised primarily by the metabolism for producing energy. Where fatty acids are structural, that is utilised in membranes, they are conveniently such as γ-linolenic acid, linoleic acid, dihomo-γ-linolenic acid and arachidonic acid residues. By residue is meant the moiety that remains after the fatty acid carboxyl group esterifies to one of the hydroxy groups of the glycerol molecule.

Other preferred acids for sn-1 and sn-3 are selected from fatty acids that are metabolised in the human to yield energy as opposed to a fatty acid that is primarily directed to the structural membrane pool: such preferred acids include oleic acid and palmitic acid.

Where the sn-1 and sn-3 fatty acid chain (R^1 and R^3) is unsaturated it may also be that of other essential fatty acids, such as the n-3 acids such as stearidonic acid, eicosapentanoic acid and docosahyexanoic acid. Where the fatty acid is optionally substituted these will preferably be by hydroxy, oxo, carboxyl, alkyl, alkenyl and alkoxy groups. The hydrocarbyl chain is preferably one of from 1 to 30 carbon atoms in length, more preferably from 4 to 28 carbon atoms in length, still more preferably 4 to 24 carbon atoms in length. Most preferably the hydrocarbyl chain is that of a fatty acid, more particularly a mono or polyunsaturated fatty acid.

Many of the preferred lipids for use in the method of the invention are known and may be prepared by chemical process known in the art. For example, many are commercially available, such as trigamma-linolenin, known as TLG, but herein referred to as GGG, reflecting the identity of groups $R^1R^2R^3$ where G represents γ-linolenic acid residues.

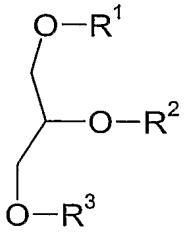
GGG is commercially available from Nu-Check-Prep Inc. EP 0300844 describes its synthesis using a base-catalysed trans-esterification of triacetin with methyl gamma linolenate to give a mixture containing 80% GGG, unreacted methyl γ-linolenate and 10% mono- and di-glycerides.

Triarachidin is known and small quantities can be obtained commercially eg. from Sigma AAA has been synthesised from arachidonic acid by using immobilised lipase patented for angiogenisis-enhancing activity US 4888324.

However, whilst the tri and di-γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid di or triglycerides may be used, the present inventors prefer the use of the mono-γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid sn-2 ester triglycerides because they administer less of the immunomodulatory and proinflammatory fatty acids γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid whilst retaining the enhanced activity that the sn-2 γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety provides with regard to the desired membrane normalising and disease modifying effect.

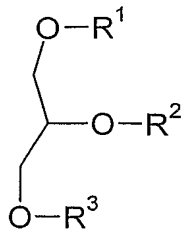
Novel lipids which are preferred are accessible by processes and methods set out in the Examples herein. Most preferred lipids are those where there is just a single γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety esterified to the glycerol at sn-2, with the flanking sn-1 and sn-3 acids being unsaturated medium chain or long chain acids.

Thus a further aspect of the present invention provides novel lipids disclosed herein including compounds of formula II



wherein R^1 and R^3 are the same and are $-C(O)(CH_2)_nCH_3$ wherein n is selected from 4 to 14, more preferably 6 to 10 and most preferably 7, 8 or 9 and R^2 is selected from γ-linolenyl, dihomo-γ-linolenyl and arachidonyl.

A further aspect of the present invention provides a method for synthesis of a compound of general formula III



wherein R^1 and R^3 are the same and are $-C(O)(CH_2)_nCH_3$ wherein n is selected from 4 to 14, more preferably 6 to 10 and most preferably 7, 8 or 9 and R^2 is γ-linolenyl residue, dihomo-γ-linolenyl residue or arachidonyl residue

comprising

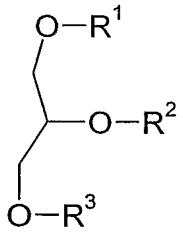
reacting 1,3-dihydroxyacetone with a compound of formula $X\text{-}C(O)(CH_2)_nCH_3$ wherein X is selected from Cl, Br and I,

to give the corresponding 1,3-di-($C(O)(CH_2)_nCH_3$) 2-keto compound

reducing the keto group to the corresponding 1,3-di-($C(O)(CH_2)_nCH_3$) 2-ol

and reacting that with γ-linolenyl chloride or dihomo-γ-linolenyl chloride or arachidonyl chloride.

A still further aspect of the present invention provides a method for synthesis of a compound of general formula IV



wherein R^1 to R^3 are the same and selected from γ-linolenyl residue, dihomo-γ-linolenyl residue or arachidonyl residue

comprising reacting the corresponding γ-linolenyl chloride, dihomo-γ-linolenyl chloride or arachidonyl chloride with glycerol.

Synthesis of some of these compounds is described below and schemes shown in the figures below.

For example, a single-step esterification of glycerol using GLA and a coupling agent, such as DCCI/DMAP (1.1-Dicylcohexylcarbodiimide/ 4-dimethylaminopyridine) coupling reagents may be carried out. This method gives a good yield but generates impurities that, unless removed, make the final oil cloudy. This may be circumvented by using a coupling agent such as EDCI (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) which gives rise to water-soluble by-products that are easier to remove. Jpn. Kokai Tokkyo Koho JP 05310638 A2 22Nov 1993 Heisei, 6pp. describes the preparation of tri-α-linolenin (LnLnLn where Ln is linoleic acid) using DCCI, and analogous but different reaction.

A alternative approach provides a two-step sequence that utilises reaction of GLA-Cl (prepared from γ-linolenic acid and oxalyl chloride) and glycerol in dichloromethane/pyridine with good yields at scale-up to 250 g purified by column chromatography. Jpn. Kokai Tokkyo Koho JP 04328199 A2 17 Nov 1992 Heisei, 5pp. (Japan) Concentration of a-linolenic acid triglyceride by flash chromatography. Ando, Yukiki, Watanebe, Yoichi, Takagi, Yoshiaki (Nisshin Oil Mills Ltd, Japan) describes a related but different technique for purification of tri-α-linolenin (LnLnLn).

Comparative example tricaprin (glycerol tridecanate) is a known compound commercially available from Sigma. It has been prepared by reaction of methyl decanoate and sodium glyceroxide with subsequent purification of the crude product by column chromatography (see E. S. Lutton and A. J. Fehl, *Lipids*, **5**, 90-99 (1970))

An alternative method involves the acid-catalysed reaction of glycerol with decanoic acid followed by four crystallisations (see L. H. Jenson and A. J. Mabis, *Acta Cryst.*, **21**, 770 (1966)).

The applicant further provides an improved process which allows glycerol to react with more than 3 equivalents of decanoyl chloride and purified the tricaprin product by recrystallisation.

Further aspects of the present invention provide use of triglyceride oils as described above for the manufacture of a medicament for the treatment of neurodegenerative diseases as set out for the method of the invention. Particularly preferred medicaments are for the arresting and reversing of neurodegeneration in multiple sclerosis of all types but particularly relapsing remitting, primary progressive and chronic progressive and the restoration, in part or completely, of neuronal integrity function such as measured, eg. By MRI or CAT scan or by EDSS score. Other TGF-β1 responsive diseases may be treated as set out previously.

The lipids for use in the present invention may be administered by any of the conventional vehicles known in pharmacy. Most conveniently they are administered as neat oils or in admixture with foodstuffs, in the form of capsules containing such oils, or in enterically coated forms. Other forms will occur to those skilled in the art but Remington Pharmaceutical Sciences 19th Edition.

It will be realised by those skilled in the art that other beneficial agents may be combined with the lipids for use in the present invention or otherwise form part of a treatment regime with the lipids. These might be ion channel blockers, eg. sodium channel blockers, interferons (α, β, or γ), T-cell depleters, steroids or other palliative agents. It will further be realsied that where the immune and inflammatory responses are being modulated, such combinations will need to be made carefully, given the complex nature of these systems. However, given the delayed response to the present oils, shorter acting agents might be beneficial in the first months of treatment before the TGF-β1 levels are normalised, as long as the additional treatment does not impede this normalization process.

The synthesis of structured lipids for use in the present invention is described below together with synthesis of comparative examples. Some of these lipids are novel while others are known but have not been used for the treatment of the invention.

The present invention will now be described by way of Example only by reference to the following non-limiting Tables, Examples and Figures. Further

embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

TABLES

Table 1: Shows the compositional % Total fatty acid content of various triglyceride oils and protective effect in EAE.

Table 2: Shows the parameters of the three treatment groups in high sn-2 GLA Borage Oil trial described in PCT/GB04/002089.

Table 3: Shows the effect of various forms of GLA on EAE incidence and clinical score in SJL mice: lower score indicating improved therapeutic effect.

Table 4: Shows the failure of enriched Blackcurrent oil, a high GLA, but low sn-2-GLA, plant oil, to match fungal and Borage oils in EAE.

FIGURES

Figure 1: Shows spontaneous peripheral blood mononuclear cell cytokine production in placebo and high sn-2 γ-linolenic acid, PCT/GB04/002089 trial oil treated human MS patients at 18 months.

Figure 2: Shows the effect of placebo and low dose (5g/day) high sn-2 GLA Borage oil on human MS patient EDSS score as compared to high dose (15g/day) displayed as a histogram with months treatment on the x axis.

Figure 3: Shows the effect of placebo, low dose and high dose high sn-2 GLA Borage oil on human MS patient Mean Relapse rate (%) as histogram with months on x axis.

Figure 4: Shows the reaction scheme for synthesis of a single fatty acid triacylglyceride for use in the method and use of this invention.

Figure 5: Shows the reaction scheme for synthesis of control compound tricaprin.

Figure 6: Shows the reaction scheme for synthesis of CGC, a mixed fatty acid triacylglyceride of the invention.

Figure 7: Shows the reaction scheme for synthesis of C-DHGLA-C, a mixed fatty acid triacylglyceride of the invention.

Figure 8: Shows the reaction scheme for synthesis of control compound GCG, 1,3-dicapryl, 2-γ-linolenic acid.

Figure 9: Shows the reaction scheme for synthesis of C-AA-C, a mixed fatty acid traiacylglyceride of the invention.

Figure 10 to 19 show the results of EAE studies in SJL and C57BL mice as set out in the examples below. (DHLA=DHGLA: A=AA)

EXAMPLES

High sn-2 Borage oil (PCT/GB04/002089) trial.

Twenty-eight active relapsing-remitting (two relapses in the preceding 18 months) multiple sclerosis patients (ages ranging from 18 to 65 yrs) were entered into a double-blind placebo controlled trial to investigate the effects of encapsulated borage oil on clinical activity and laboratory parameters over 18 months. This oil was of high sn-2 γ-linolenic (GLA) content (>40% of sn-2 residues being γ-linolenic acid) with low monene (eg. erusic acid) content and had no added Vitamin E, a known immunomodulator.

Patients were recruited from neurology out-patient clinics at two inner city hospitals; hospital informed consent was obtained on first (baseline) visit. Exclusion criteria include any form of steroid or immunosuppressive drug treatment, pregnancy, hyperlipidemia, regular use of aspirin or related drugs and vitamin or fatty acid supplementation within the previous three months.

Only patients meeting all the following criteria were included in the trial: (a) able to provide informed consent prior to treatment, with the full understanding that consent may be withdrawn at any time without prejudice; (b) male or female out-patients aged 18 to 60 years inclusive; (c) have confirmed diagnosis of clinically definite relapsing MS; (d) have had at least three documented clinical relapses in the past two years; (e) have a baseline Expanded Disability Scoring Scale (EDSS) score of 0.0-5.5 inclusive, provided they have well documented exacerbations; and (f) healthy, apart from the MS-related symptoms, as confirmed by the medical history, physical examination and clinical chemistry, urine and haematological tests.

Patients were randomly allocated by the Pharmacy Department to one of three groups each containing 12 patients:

- One clinical group (n=12) to receive placebo (5 g of Polyethylene Glycol 400)
- Second clinical group (n=12) to receive low-dose (5 g) refined *Borage officinalis*
- Third clinical group (n=12) to receive high-dose (15 g) refined *Borage officinalis*

Supplementation was in the form of one gram oil capsules daily (5/day for low dose, 15/day high dose) for 18 months duration. *Borage officinalis* oil and omega-6 polyunsaturated fatty acids are food ingredients that are generally recognised as safe for human consumption (GRAS). There are no classification or labelling requirements under EC regulations. Clinical assessment included: Extended Disability Scale Scores (EDSS) and clinical relapse record. Venous blood (50 mls) was obtained for laboratory studies on the 1^{st}, 3^{rd}, 6^{th}, 12^{th}, 15^{th}, and 18^{th} month of supplementation.

The following biochemical and immunological parameters were investigated on each visit for comparison with pre-treatment data and between group data:

- Stimulated and unstimulated *ex vivo* peripheral blood mononuclear cell cytokine production: changes in TGF-β1, IFN-γ, TNF-α, IL-1β, IL-6 and IFN-β, which are implicated in the pathogenesis of MS. Cytokine and related gene expression.
- Soluble adhesion molecules in serum particularly ICAM-1 and VCAM-1
- Peripheral blood mononuclear cell membrane fatty acids and plasma phospholipid fatty acid composition.

Results are shown in Tables 1 and 2 and Figures 1 to 5.

The primary outcome parameter was the number of clinical relapses between baseline (Month 0) and the end of treatment (Month 18). Secondary outcome parameters included: the time to first clinical relapse; severity of relapses, as assessed by EDSS score and the use of steroid treatment; and changes in EDSS at Month 3, 6, 9, 12, and 18 compared to baseline and defined as at least 1.0 point increase in the EDSS that is sustained for 3 months or at least 1.5 point increase on the EDSS from the baseline EDSS that is sustained for 3 months.

Eleven patients were in the placebo group, seven patients had been taking low-dose Borage oil, and ten patients had been taking high-dose Borage oil. The study drug was well-tolerated, and there were no serious adverse events during the 18-month trial.

Isolation and Culture of PBMC

Heparinised whole blood was diluted with an equal volume of Hanks' balanced salt solution (Sigma, UK) and the resulting diluted blood layered onto Lymphoprep (Nycomed, Oslo, Norway). Following density centrifugation at 800g for 30 minutes the PBMC were removed from the interface and diluted in Hanks' solution. The cells were then washed twice by centrifugation for 10 minutes at 250g. The resulting final pellet was then resuspended in culture medium consisting of RPMI-1640 medium (Sigma, UK) supplemented with 2mM L-glutamine, 100U

penicillin and 100μg streptomycin (Sigma, UK) and 10% autologous plasma. $2x10^6$ per ml PBMC, >95% viable as judged by trypan blue exclusion, were added to tissue culture tubes (Bibby Sterilin Ltd, Stone, UK) and incubated for 24h at 37°C with 5% CO_2. The concentration of antigen, cell density and time of culture were all determined in previous kinetic experiments to determine maximum cytokine production (data not shown). Routine cytospin preparations were also prepared for subsequent differential counts. Following incubation the cells were removed from culture by centrifugation at 250g for 10 minutes, the resulting supernatants were then removed, aliquoted and stored at -70°C.

Preparation of Plasma Samples

10ml of heparinised blood was spun at 250g for 10 minutes. The resulting plasma layer was then removed, aliquoted and stored at -70°C.

Detection of Pro-inflammatory Cytokines

TNF-α, IL-1β and IFN-γ in cell culture supernatants and plasma were detected using commercially available paired antibodies enabling cytokine detection in an ELISA format (R&D systems Ltd, Abingdon, UK). The sensitivities for the TNF-α and IFN-γ ELISAs were 15.6-1000pg/ml and 3.9-250pg/ml for IL-1β.

Detection of Biologically Active TGF-β1

Biologically active TGF-β1 in cell culture supernatants and plasma were detected using the commercially available E_{max} ELISA system with a sensitivity of 15.6-1000pg/ml (Promega, Southampton, UK).

Statistical Analysis

Differences in cytokine production were compared using Student's *t*-test and Mann-Whitney *U*-test and were considered significant when p values were less than 0.05.

RESULTS

Two patients had developed diarrhoea, both of whom were later confirmed to have been taking high-dose Borage oil. The diarrhoea was mild in one patient, but was moderately severe in the second patient, who later discontinued the study drug. The code was not broken and the diarrhoea had stopped after the discontinuation of the drug, but reappeared upon re-challenge. Therefore, this patient was withdrawn from the trial. The remaining patients who were treated with high-dose Borage oil showed excellent clinical improvement on all primary and secondary outcome criteria. For example, their mean EDSS score after 6 months of treatment had improved from baseline EDSS (Figure 1). More importantly, the mean number of clinical relapses had significantly reduced after 6 months of treatment when compared to the number of relapses in the placebo group (Figure 2). In contrast, patients who had been receiving low-dose Borage oil did not show any clinical improvement when compared to the placebo group. In addition to its beneficial effect on MS disease activity, high dose Borage oil provided some symptomatic relief of muscle spasticity (stiffness) and painful sensory symptoms, and also improved cognitive functions.

As can be seen for the figures below, relapse rate after 9, 12 and 18 months was down to zero in the high dose group. The increase seen at 15 months was due to the patient dropping out of this group.

The following are three brief case histories to illustrate the therapeutic benefits of high dose high sn-2 GLA Borage oil. The first two are from the trial while the third is a post trial patient for whom MRI studies were obtained.

Patient 1 (Treatment):

The first patient was a 48 year old woman who had had a clinically active, relapsing remitting MS for 9 years. She had originally worked as a full-time administrator at the local Health Authority, but she was unable to perform her duties

because of her severe MS. Therefore, she later worked as a part-time secretary, but still had difficulties in mobilization because of muscles stiffness and sensory disturbances. She was also experiencing severe clinical relapses at an average of one relapse every nine months. Most of these relapses had resulted in hospital admissions for steroid therapy. In view of her active MS, she was recruited into the Borage oil trial. There were no adverse events relating to the study, and after taking the medication for four months, she experienced good improvement in her walking and sensory symptoms.

About nine months after therapy, she was well enough to start full-time employment. In addition, she remained relapse-free for the 18-month duration of the clinical trial. Following the conclusion of the trial, the treatment code revealed that she was taking high-dose Borage oil.

Patient 2 (Control):

The second case was a 46-year old woman who also had a clinically active relapsing remitting MS for 8 years. She had originally worked as a shop assistant, but became unemployed after MS was diagnosed.

Her symptoms included difficulty with mobilisation and painful sensory symptoms in both legs. She had experienced three clinical relapses in the two years preceding the clinical trial, and had been admitted to hospital twice for steroid therapy. Consequently, she was recruited into the Borage oil trial, but her walking continued to deteriorate. Six months into the trial, she need to use a walking stick and also received treatment with Baclofen to reduce low limb spasticity. Approximately ten months after starting the Borage oil trial, she was admitted to hospital because of severe clinical relapse, which was treated with steroids. She later developed bladder disturbances and began to use a wheelchair for long journeys. The treatment code was broken after the conclusion of the 18-month trial, and she was found to have been taking placebo. Since then, she started using a walking frame for journeys exceeding 50 yards.

Patient 3: Treatment (additional to trial)

The third case was a 26 year-old man who was diagnosed with definite MS in April 2001. His symptoms had started in 1999 when he complained of diffuse, intractable pain affecting various parts of his body, particularly the left side of the chest and abdomen. This was followed by intermittent numbness in the hands and feet, associated with fluctuating weakness. There were also distressing bladder symptoms in the form of urinary frequency and urgency. The diagnosis of MS in 2001 was based on his relapsing remitting symptoms, and was confirmed by positive cerebrospinal fluid analysis and magnetic resonance imaging (MRI) of the brain, which showed multiple white matter abnormalities in both cerebral hemispheres. Symptoms did not respond to various pharmaceutical therapies.

In April 2003, oral supplementation with the present high dose Borage oil was commenced. The patient reported dramatic improvement in his symptoms within three months of starting this oral supplementation. His painful sensory symptoms disappeared completely. He reported no numbness or weakness since May 2003, and noticed significant improvement in his bladder control. The oral supplementation caused no adverse events. A repeat brain MRI was undertaken to verify the reported improvement in Mr N's symptoms. The repeat MRI showed a reduction in the size and distribution of the white matter abnormalities.

EXAMPLES; Structured sn-2 lipids

In all the examples below higher purity is obtained by use of higher purity starting material γ-linolenic, dihomo-γ-linolenic or arachidonic acid, such as is available eg from Sigma Aldrich. GLA 95 indicates 95% pure γ-linolenic acid.

Synthesis Example 1: synthesis of Trigammalinolenin

1) Acid chloride method

2.0 g (7.2 mmol, 3.1 equiv) GLA95 (95% pure γ-linolenic acid) was dissolved in 10 ml DCM. 1.01g (0.71 ml, 8.0 mmol, 3.4 equiv) oxalyl chloride in 5

ml DCM added dropwise over 2-3 min under nitrogen. Stirred at RT overnight. Reaction mixture concentrated in vacuo to remove DCM and excess oxalyl chloride. This acid chloride was then added dropwise over 2-3 min to a stirred mixture of 215 mg (2.3 mmol, 1 equiv) of glycerol, 0.58 ml (3.1 equiv) pyridine and 10 ml DCM under nitrogen. The mixture was stirred at RT overnight. The pyridine hydrochloride formed was then filtered off and washed with DCM. The solution was washed 1 x 4 ml water, 0.1N HCl, 5% sodium bicarbonate and 5% NaCl. Dried over magnesium sulphate, filtered and concentrated in vacuo to a yellow oil. This oil was purified on a silica column using 10% ether in hexane as eluting solvent. A clear colourless oil was obtained, a sample of which was trans-esterified and subsequently analysed by GC. The product contained 96.3% GLA

2) DCCI method

2.19 g GLA95 (3.15 equiv), 230 mg (1 equiv) glycerol, 153 mg DMAP (0.5 equiv) were stirred in 10 ml DCM under nitrogen. 1.85 g DCCI (3.6 equiv) in 5 ml DCM was added. The reaction mixture was stirred at RT under nitrogen overnight. The DCU formed was filtered and washed with DCM. DCM washed 1 x 5mls N HCl, water, 5% sodium bicarbonate and water. Dried over magnesium sulphate, filtered and concentrated in vacuo to an oil. This oil was then purified on a silica column using 10% ether in hexane as eluting solvent. 1.47 g (67%) of a slightly cloudy oil was obtained. A sample of this product was trans-esterified and subjected to GC analysis. The product contained 95.8% GLA.

Scale-up

20 g (0.072 mol, 3.1 equiv) of GLA95 (gamma linolenic acid, 95%) was dissolved in 100 ml DCM. 13.7g (9.3 ml, 0.11 mol, 4.78 equiv) oxalyl chloride was added over 3-4 min under nitrogen. The reaction mixture was stirred under nitrogen overnight. It was then concentrated in vacuo to remove DCM and excess oxalyl chloride. This oil was then added dropwise over ca 5 min to a stirred mixture of

2.14g (0.023 mol, 1 equiv) of glycerol, 100 ml DCM and 5.8 ml (5.68 g, 0.072 mol, 3.1 equiv) of pyridine under nitrogen. 85 mg (0.7 mmol, 0.03 equiv) of DMAP (4-dimethylaminopyridine) catalyst was added.. The mixture was stirred at RT overnight. Pyridine hydrochloride was filtered off and washed with DCM. The DCM solution was washed 1x 25 ml: water, 10% sodium bicarbonate, 0.1N HCl, 5% NaCl. (Emulsions formed during this process, especially at first). The DCM was dried over magnesium sulphate, filtered and concentrated in vacuo to a brown oil (~21 g).

The oil was purified on a silica column using 5% ether in hexane at first and then 10%. 15.6g (77% yield) of a clear oil was obtained. By tlc this material contained a small amount of free GLA. (This material was repurified at a later date)

Large Scale-up

The above reaction was repeated on 10 times scale. Thus, 200 g of GLA95, 1L DCM, 137 g of oxalyl chloride, and 21.4 g of glycerol were used. On the addition of the acid chloride the reaction mixture was cooled in a cold water bath and the temperature kept below 35°C. 250g of a brown oil were produced. This was initially purified on a 500 gram silica column. The oil was dissolved in 200 ml hexane and applied to the column. The column was eluted at first with hexane, then 5% ether in hexane and then 10%. Fractions were collected and analysed by tlc eventually yielding two batches of oils. The first A (66 g) contained a small amount of front running impurity and a little GLA (slower running than TGL), the second fraction B (99g) was clear of front running impurity and contained a little GLA.

The large scale reaction was repeated using 169 g of GLA and gave two fractions as above. This time there was 85g of 'A' fraction and 54g of 'B' fraction. Both batches of 'A' were combined and re-purified on a 500g silica column. The 'B' fractions were treated in a similar manner (15g of material from the small-scale reaction were also added to this batch).

Some fractions from the above were again re-purified to eventually give 259 grams of oil. The oil was pumped down on a rotary evaporator under high vacuum to constant weight – 256g. This represents an overall yield of 65%.

Analysis of product.

GC

A small sample was trans-esterified and subjected to GC analysis:

The GLA content was 97.1%. The main impurity was linoleic acid – 1.91%.

Note: The original GLA95 that was used for the synthesis contained 96.2% GLA and 2.42% linoleic acid.

HPLC

An HPLC method was developed using a reversed phase column (Hypersil C18 4.6 x 100 mm), eluting with 80/20 acetonitrile/THF. Detection was by UV at 210 nm. This showed the product to be a mixture of three components. The main peak (93.6%) was the required product. A slower running impurity (representing 5.0% of the product) was probably a **GGLl** triglyceride (Ll = linoleic acid). A second impurity was slightly faster running and represented 1.4% of the product.

Note: Absorption at 210 nm varies considerably between triglycerides of differing fatty acid content. For example trigammalinolenin has a UV absorbtion 5-6 times greater than that of trilinolenin

Summary

254 g of glycerol tri-6,9,12-linolenate (gamma linolenic acid triglyceride, trigammalinolenin, **GGG**) was prepared from 96.2 % GLA by a two-step acid chloride route. It is a clear, pale yellow oil and was stored under nitrogen in the freezer. The GLA content was 97.1 % and no C20:1, C22:1, or C24:1 acids were detected). The HPLC purity was 93.6 %.

Synthesis of higher purity **GGG** would is readily achievable using GLA 98 (98% γ-linolenic acid: Scotia) or higher starting material.

Comparative lipid 1: synthesis Tricaprin (Glycerol tridecanoate)

Small Scale

Glycerol (3.0 g, 0.0325 mol, 1 eq) pyridine (8.1 ml, 0.10 mol, 3.1 eq) and dichloromethane (100 ml) were stirred at room temperature under nitrogen. Decanoyl chloride (21 ml, 19.25 g, 0.10 mol, 3.1 equiv) was then added dropwise over 5 min, with external cooling in a water bath to keep the temperature at 30-35 °C. When the addition was complete 4-dimethylaminopyridine (DMAP (0.12 g, 1 mmol, 0.03 eq) was added and the mixture stirred under nitrogen at room temperature overnight. The precipitated pyridine hydrochloride was removed by filtration and washed with dichloromethane. The combined washing and filtrate was then washed with aqueous solutions (20 ml) of 5% sodium chloride, 5% sodium bicarbonate, 0.1N hydrochloric acid, and 5% sodium chloride. The dichloromethane layer was then dried over $MgSO_4$ and the solvent removed *in vacuo*. The residual oil crystallised on standing. This material was recrystallised from isopropanol (40 ml) to give 15.6 g (86% yield) of a waxy white solid.

Analysis

GC – 99.8% pure

HPLC

(C18 4.6 x 100 mm, ACN/THF 85/15 1 ml/min, λ 210 nm) – 94.9% pure

Large Scale

The above was repeated on 15 times the scale. Glycerol (45.0 g, 0.49 mol, 1 eq), pyridine (121.5 ml, 1.50 mol, 3.1 eq) and dichloromethane (1.5 L) were stirred at room temperature under nitrogen. Decanoyl chloride (315 ml, 288.8 g, 1.50 mol, 3.1 equiv) was then added dropwise over 15 min, with external cooling in a water bath to keep the temperature at 30-35 °C. When the addition was complete 4-dimethylaminopyridine (DMAP (1.8 g, 15 mmol, 0.03 eq)

was added and the mixture stirred under nitrogen at room temperature overnight. The precipitated pyridine hydrochloride was removed by filtration and washed with dichloromethane. The combined washing and filtrate was then washed with aqueous solutions (300 ml) of 5% sodium chloride, 5% sodium bicarbonate, 0.1N hydrochloric acid, and 5% sodium chloride. The dichloromethane layer was then dried over $MgSO_4$ and the solvent removed *in vacuo*. The residual oil crystallised on standing. This material was recrystallised from isopropanol (400 ml) to give 228 g (86% yield) of a waxy white solid.

Analysis

GC – 99.8% pure

HPLC

(C18 4.6 x 100 mm, ACN/THF 85/15 1 ml/min, λ 210 nm) – 94.9% pure

A further batch was made and combined with the small-scale batch above and recrystallised from isopropanol to give 44 g of product. The above batches were combined (268 g) and reanalysed:

GC

99.9% pure

HPLC

97.9%

Summary

263 g of glycerol tridecanoate (tricaprin, CCC) was been prepared from decanoyl chloride (98 %) by a one-step process (scheme given below). It is a white, low-melting solid and was stored under nitrogen in the freezer. The **C** content was 99.9 % of fatty acid content and the HPLC purity was 97.9 %.

Synthesis Example 2: 1,3-Dicaprin 2-gammalinolenoate (Glycerol 1,3-didecanoate 2-octadecatri(6-Z,9-Z,12-Z)enoate or CGC)

This triglyceride is novel. Unlike CGC, its isomer CLnC (Ln = α-linolenic acid), has been identified (see K. Long et al *Biotechnol. Lett.*,**20**, 369-372 (1998). and H. Mu, P. Kalo et al, *Eur. J. Lipid Sci. Technol.*, **102**, 202-211(2000). as a component of coconut oil. In addition, CLxC (Lx = a linolenic acid of unspecified double bond position) has been described (see J. Gresti et al. *J. Dairy Sci.*, **76**, 1850-1869 (1993)),

The two intermediates used in the synthesis of **CGC** are known (see L. El Kihel et al *Arzneim -Forsch./Drug Res.*, **46**, 1040-1044 (1996) and US 4178299. The last step described below is novel and the first two stages are also inventive since they are more suitable for large scale production than those previously reported.

CGC was prepared by reaction of 1,3-Dicaprin with GLA-chloride in dichloromethane-pyridine. 1,3-Dicaprin was prepared by sodium borohydride reduction of 1,3-didecanoyloxypropan-2-one, which was in turn prepared by reaction of decanoyl chloride with 1,3-dihydroxyacetone. The intermediate 1,3-dicaprin must be handled with care since it can undergo acyl migration on exposure to acids , bases and heat. An older method of making 1,3-dicaprin has been described (see A. P. J. Mank et al *Chem. Physics Lipids*, **16**, 107-114 (1976).

A versatile, flexible synthesis of 1,3-diglycerides and triglycerides. by catalysed addition of decanoic acid to a glycidol ester (from epichlorohydrin) is less attractive because of more severe reaction conditions and acyl migration problems. The final product, **CGC**, was purified by careful column chromatography on silica which removed by-products.

Small Scale

1,3-didecanoyloxypropan-2-one

Decanoyl chloride (40.0 ml, 36.8 g, 0.19 mol, 1.98 equiv) was added dropwise over 10-15 min to a stirred suspension of 1,3-dihydroxyacetone dimer (8.68 g, 0.048 mol, 1.0 equiv), pyridine (15.6 ml, 0.19 mol), 4-dimethylaminopyridine (0.18 g, 0.0014 mol, 0.03 equiv) and dichloromethane (DCM, 150 ml) at room temperature

under nitrogen. The temperature of the reaction mixture was kept below 30°C by cooling in a cold water bath. The reaction mixture was stirred at RT under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 25ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and concentrated in vacuo to a yellowish semi-solid. This was then crystallised from methanol (150 ml) to give a white solid. The yield was 28.2 g (73%).

1,3-Dicaprin

The above ketone (28.2 g, 0.071 mol) was dissolved in tetrahydrofuran (THF, 200 ml). Water (10 ml) was then added, the solution cooled to 5°C, and sodium borohydride (5.38 g, 0.14 mol) added portionwise below 10°C. The reaction mixture was stirred at RT for 1h and then concentrated in vacuo to remove THF. The residue was partitioned between ethyl acetate and 5% sodium chloride solution. The aqueous phase was re-extracted with ethyl acetate and the combined extracts dried over $MgSO_4$ and concentrated in vacuo to a waxy solid. This was crystallised twice from hexane to give 11.2g (40%) of a white solid. (99%+ pure by HPLC)

*1,3-Dicaprin 2-gammalinolenoate (**CGC**)*

Gamma-linolenic acid (GLA95, 8.34 g, 0.03 mol) was dissolved in dichloromethane (DCM, 60 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (3.9 ml, 5.67 g, 0.044 mol) added dropwise over 5 mins. The mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 15 min (ice /water cooling) to a stirred solution of 1,3-dicaprin (11.2g, 0.028 mol), DCM (50 ml), pyridine (2.42 ml, 2.37 g, 0.03 mol) and 4-dimethylaminopyridine (0.10 g, 0.0008 mol, 0.03 equiv) at 10-15°C . The temperature was maintained by ice-water cooling. The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed

with DCM. The combined washing and filtrate was washed with 1 x 20ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and the solvent removed in vacuo.The residual brown oil was purified by column chromatography on silica. Elution with hexane and then with 5% ether/hexane gave 10.3g (56%) of a colourless oil. The structure was confirmed by ^{13}C NMR and GLC. Purity determined by HPLC.

Large Scale

1,3-didecanoyloxypropan-2-one

Decanoyl chloride (272 ml, 250 g, 1.3 mol, 2 equiv) was added dropwise over 10-15 min to a stirred suspension of 1,3-dihydroxyacetone dimer (59.1 g, 0.65 mol, 1.0 equiv), pyridine (106 ml, 103.7g 1.3 mol), 4-dimethylaminopyridine (2.38 g, 0.02 mol, 0.03 equiv) and dichloromethane (DCM, 750ml) at room temperature under nitrogen. The temperature of the reaction mixture was kept below 30°C by cooling in a cold water bath. The reaction mixture was stirred at RT under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 150ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and concentrated in vacuo to a yellowish semi-solid. This was then crystallised from methanol (500ml) to give a white solid. The yield was 158 g (60%).

1,3-Dicaprin

The above ketone (158 g, 0.40 mol) was dissolved in tetrahydrofuran (THF, 2.25 L). Water (50 ml) was then added, the solution cooled to 5°C, and sodium borohydride (5.66 g, 1.5eq) added portionwise below 10°C. The reaction mixture was monitored by HPLC (C18, eluted with ACN at 1ml/min λ210nm) (Note: only about 4.5g of the borohydride was in fact added, as all SM had reacted).The reaction mixture was stirred at RT for 1h and then concentrated in vacuo to remove THF. The residue was partitioned between ethyl acetate and 5% sodium chloride solution. The aqueous phase was re-extracted with ethyl acetate and the combined extracts dried

over $MgSO_4$ and concentrated in vacuo to a waxy solid. This was crystallised twice from hexane to give 96g (60%) of a white solid. (98% pure by HPLC)

*1,3-Dicaprin 2-gammalinolenoate (**CGC**)*

Gamma-linolenic acid (GLA95, 120.2g, 0.43mol) was dissolved in dichloromethane (DCM, 750 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (55.7 ml, 82.3 g, 0.65 mol, 1.5eq) added dropwise at 15-20°C over 15 mins. The mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 30-40 min at 10-15°C (ice /water cooling) to a stirred solution of 1,3-dicaprin (164.7g, 0.41 mol), DCM (650 ml), pyridine (33.3 ml, 32.5 g, 0.41 mol) and 4-dimethylaminopyridine (1.50 g, 0.012 mol, 0.03 equiv) at 10-15°C .The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed with DCM. The combined washing and filtrate was washed with 1 x 150 ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and the solvent removed in vacuo to a brown oil (275g).

The scale of the above three reactions was the largest on which each was carried out. The borohydride reduction produced, in addition to 1,3-dicaprin, a by-product in variable yield. The presence of this by-product greatly affected the yield of the isolated pure 1,3-dicaprin; the by-product could only be removed by two crystallisations of the crude product. Since the final product, **CGC**, is purified by column chromatography, it is imperative that the 1,3-dicaprin used for the final step is as pure as possible!

From the above reactions about 440g of crude CGC was produced as a brown oil. This was purified on a series of silica columns using hexane followed by 2-3% ether/hexane. The purification required 7 or 8 columns, using 3-4 kilos of silica, 25-30 litres of solvent (recycling solvent kept this figure low – in practice over 100 litres were used)

The resulting product, a clear almost colourless oil, (264grams) was 96.4% pure by HPLC (C18 4.6 x 100mm, eluted with 85/15 ACN/THF at 1ml/min. UV detection λ210nm). GC indicated a ratio of 66.1/33.9 C/G. NMR analysis indicated the product to have the correct CGC structure and be of at least 95% purity: δ_c (500 MHz, $CDCl_3$) 172.65 (2-GLA carbonyl), 173.25 (1,3- capric carbonyl). Ratio of signals 2.04:1. No signal at 173.0 indicating absence of 1.3-GLA. Trace signal at 172.79 could be oleic acid impurity in GLA or 2-capric acid.

Summary

264 g of glycerol 1,3-didecanoate-2-gammalinolenoate (1,3-dicaprin-2-GLA, CGC) has been prepared from decanoyl chloride (98 %) by a three-step process (scheme given below). It is an almost colourless oil (slight yellow tinge) and was stored under nitrogen in the freezer. The HPLC purity was 96.4 %.

Synthesis Example 3

1,3-Didecanoate-2-dihomo-γ-linolenoate (Glycerol 1,3-didecanoate2- eicosa-(8Z,11Z,14Z)-trienoate or C(DHLA)C

This triglyceride appears to be novel - no reference to it has been found.

DHLA (3.93g, 12.8 mmol, 1 eq) was dissolved in dichloromethane (DCM, 20 ml) and stirred at room temperature under a nitrogen atmosphere. Oxalyl chloride (1.69 ml, 2.46 g, 19.4 mmol, 1.5 eq) was added dropwise over 1-2 min, and left stirring at room temperature overnight. The resulting solution was concentrated *in vacuo* to remove DCM and excess oxalyl chloride. The residual oily acid chloride (DHLA-Cl) was then added dropwise over 5 min at 25°C to a stirred mixture of 1,3-dicaprin (4.91 g, 12.2 mmol, 0.95 eq), pyridine (0.98 ml, 0.96 g 12.1 mmol, 0.95 eq) and 4-dimethylam inopyridine (DMAP, 8 mg, 0.07 mmol, 0.03 eq). The reaction temperature rose to 32 °C during the addition. The reaction was stirred at 30-35°C and monitored by HPLC. The reaction was stopped after 1.5h. The precipitated pyridine hydrochloride was filtered off and washed with DCM. The combined filtrate and washings were then

washed with 1 x 10 ml portions of 5% NaCl, 5% $NaHCO_3$, 0.1N HCl, 5% NaCl. The solution was then dried over $MgSO_4$ and concentrated *in vacuo* to give the crude product as a yellow-orange oil (8.9 g, 86% purity by HPLC). This oil was chromatographed on silica gel (250 g). Elution with hexane and diethyl ether-hexane (2-6%) gave a purified product as a pale yellow oil. Treatment of a hexane solution with decolourising charcoal and removal of the solvent *in vacuo* gave **C(DHLA)C** as a clear colourless oil (6.48g, 98.9% purity by HPLC).

Synthesis Example 4

Triarachidin (Glycerol trieicosotetra5-Z,8-Z,11-Z,14Z-eneoate) or AAA

Arachidonic acid (50.9 g, 0.17 mol, 3 eq) was dissolved in dichloromethane (DCM, 175 ml) and stirred at room temperature under a nitrogen atmosphere. Oxalyl Chloride (21.9 ml, 31.9 g, 0.25 mol, 4.4 eq) was then added to the stirred solution over 5 min and the temperature increased by 4 °C. The resulting yellow-green mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (A-Cl) was then added dropwise over 15 min to a pre-warmed (25 °C) stirred mixture of glycerol (5.11 g, 0.055 mol, 1 eq), pyridine (13.5 ml, 13.2 g, 0.17 mol, 3 eq) and 4-dimethylamino pyridine (DMAP, 0.20 g, 0.002 mol, 0.03 eq). The temperature of the reaction mixture rose to 42 °C during the addition and a gentle reflux was observed. The mixture was stirred at 30-40 °C and monitored by HPLC. After 2 h, no further product formation was observed. The precipitated pyridine hydrochloride was filtered off and washed with DCM. The combined filtrate and washings were then washed with 1 x 50 ml portions of 5% NaCl, 5% $NaHCO_3$, 0.1N HCl, 5% NaCl. The solution was then dried over $MgSO_4$ and concentrated *in vacuo* to give the crude product as a yellow-orange oil (57 g). This oil was purified by column chromatography on silica gel (*ca.* 600 g). Elution with hexane and diethyl ether(2-4%)-hexane gave 22.8 g of the product as an oil. A second batch (17.8 g) was produced from 39.8 g of arachidonic acid, The two batches were combined and residual solvents removed

under vacuo to give 40.5 g (43%) of a mobile pale yellow oil. HPLC purity 84.8% GLC analysis 94.3% AA (arachidonic acid).

Comparative Lipid 2

1,3-Di(octadeca-6Z,9Z,12Z-enoyloxy)propan-2-one

(1,3-Di(γ-linolenoyloxy)propan-2one ,**GonG**) Stage 1 intermediate for **GCG**

Gamma-linolenic acid (GLA95, 197g, 0.71 mol, 2.2 equiv) was dissolved in dichloromethane (DCM, 600 ml) contained in a 2L 3 necked flask. The resulting solution was stirred at RT under nitrogen. Oxalyl chloride (93 ml, 136 g, 1.07 mol, 3.3eq) was added dropwise at 15-20°C over 15 min. The brown mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 20 min at 25°C to a stirred mixture of 1,3-dihydroxyacetone dimer (28.99 g, 0.32 mol, 1.0 equiv), pyridine (52 ml, 50.9 g 0.64 mol, 2.0 equiv), 4-dimethylaminopyridine (2.36 g, 0.02 mol, 0.06 equiv) and dichloromethane (DCM, 600 ml) at room temperature under nitrogen. The temperature of the reaction mixture was allowed to rise to 40°C and the mixture was stirred for a further 2 h under nitrogen (monitored by HPLC). The pyridine hydrochloride that formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 150 ml portions of 5% NaCl, 5% $NaHCO_3$, 0.1N HCl, 5% NaCl. The solution was then dried over $MgSO_4$ and concentrated *in vacuo* to give *ca.* 200 g of a yellow oil. This material was partially purified by column chromatography on silica (600 g). Elution with hexane and then ether-hexane mixtures (2–15%) gave 42 g of a pale yellow oil. This oil was chromatographed again on silica (600 g) and eluted with hexane and then 1-10% ether-hexane to give the product (95.9% purity) as a pale yellow oil. The yield was 42 g (17%).

1,3-Di(octadeca-6Z,9Z,12Z-enoyloxy)propan-2-ol

(1,3-Di(γ-linolenoyloxy)propan-2-ol or 1,3-Di-gamma-linolenin **GolG**) Stage 2 intermediate for **GCG**

13-Di(γ-linolenoyloxy)propan-2-one (**GonG**, 25.5 g, 0.04 mol, 1 eq) was dissolved in tetrahydrofuran (THF, 375 ml) and water (12.7 ml). The solution was vigorously stirred at –20°C, care was taken to keep the reaction temperature below –15°C. Sodium borohydride (790 mg, 0.02 mol, 1.25 eq) was added portionwise to the stirred solution over 3 mins. The reaction mixture was stirred for a further 10 mins at –20°C and hexane (380 ml) then added. The still cold mixture was then washed with water (2 x 200 ml), dried over $MgSO_4$ and concentrated *in vacuo* to give the title compound as a brown oil (27.8g) (82.6 % purity by HPLC, less than 1% migrated material). Another batch was prepared and combined with the first to give 50 g of crude product. This material was purified by column chromatography on silica gel (400 g). Elution with hexane and diethyl ether-hexane mixture (5-20%) gave 36.1 g of the product as a pale oil (91.5 % purity).

(N.B. Care should be taken not to leave the compound on the silica overnight as it appears to undergo a migration reaction, giving **GGol**)

<u>1,3-Di-γ-linolenin 2-decanoate (Glycerol 1,3-dioctadeca-(6Z,9Z,12Z)-trienoate 2-decanoate or **GCG**)</u>

Decanoyl chloride (13.5 ml, 12.4 g, 0.065 mol, 1.1 eq) was added to a stirred solution of 1,3-di-γ-linolenin (36.1 g, 0.059 mol, 1eq), dry pyridine (5.7 ml, 5.6 g, 0.07 mol, 1.1eq), 4-dimethylaminopyridine (0.2 g, 0.002 mol, 0.03 eq) and dichloromethane (DCM, 150 ml) over *ca.* 10 mins. The temperature was maintained at 17 °C –23 °C during addition. The reaction was then stirred at 30-35 °C and monitored by HPLC. A further 1-2 ml of decanoyl chloride was added after 1 h, 1.5 h and 2 h. Further addition appeared to increase the conversion to product as determined by HPLC. After 3 h the reaction mixture was filtered and the filtrate washed with DCM. The combined filtrate and washings were then washed with 1 x 50 ml portions of 5% NaCl, 5% $NaHCO_3$, 0.1N HCl, 5% NaCl. The DCM extract was then dried over $MgSO_4$ and concentrated *in vacuo* to give the crude product as a pale yellow oil; (purity 90% by HPLC). The oil was purified by column chromatography on silica gel (600 g). Elution with hexane and diethyl ether-hexane

(1.5-2.5 then 3.5%) gave the product (**GCG.**) as a clear oil; (35.5 g 96.1% purity by HPLC). Another 7.5 g of pure lipid was obtained by further chromatography on some of the fractions containing only a small amount of impurity.

Synthesis Example 5

1,3-Dicaprin 2-arachidonate (Glycerol 1,3-didecanoate 2-eicosatetra-(5-Z,8-Z,11-Z,14-Z)enoate or **CAC**)

This triglyceride is known. **CAC** has been identified as a constituent of lymph lipids following administration of safflower oil to rats. WO 03 013,497 describing an arachidonic acid containing triglyceride (produced by culturing Mortierella alpina) useful for diseases caused by brain hypofunction, but specifically for cognition enhancement. The two intermediates used in the synthesis of **CAC** are known.

The synthesis of CAC from 1,3-dicaprin, and the purification of this are all novel.

Here **CAC** was prepared by reaction of 1,3-Dicaprin with arachidonyl chloride in dichloromethane-pyridine. 1,3-Dicaprin was prepared by sodium borohydride reduction of 1,3-didecanoyloxypropan-2-one, which was in turn prepared by reaction of decanoyl chloride with 1,3-dihydroxyacetone. The intermediate 1,3-dicaprin must be handled with care since it can undergo acyl migration on exposure to acids , bases and heat. An older method [6] of making 1,3-dicaprin, by catalysed addition of decanoic acid to a glycidol ester (from epichlorohydrin) was deemed less attractive because of more severe reaction conditions and acyl migration problems. The final product, **CAC**, was purified by careful column chromatography on silica which removed by-products.

1,3-Dicaprin 2-arachidonate (CAC)

Arachidonic acid (AA96, 8.34 g, 0.03 mol) was dissolved in dichloromethane (DCM, 60 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (3.9 ml, 5.67 g, 0.044 mol) added dropwise over 5 mins. The mixture was

stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 15 min (ice /water cooling) to a stirred solution of 1,3-dicaprin (11.2g, 0.028 mol), DCM (50 ml), pyridine (2.42 ml, 2.37 g, 0.03 mol) and 4-dimethylaminopyridine (0.10 g, 0.0008 mol, 0.03 equiv) at 10-15°C . The temperature was maintained by ice-water cooling. The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed with DCM. The combined washing and filtrate was washed with 1 x 20ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and the solvent removed in vacuo.The residual brown oil was purified by column chromatography on silica. Elution with hexane and then with 5% ether/hexane gave 10.3g (56%) of a colourless oil. The structure was confirmed by ^{13}C NMR and GLC. Purity determined by HPLC.

Large Scale

1,3-didecanoyloxypropan-2-one

Decanoyl chloride (272 ml, 250 g, 1.3 mol, 2 equiv) was added dropwise over 10-15 min to a stirred suspension of 1,3-dihydroxyacetone dimer (59.1 g, 0.65 mol, 1.0 equiv), pyridine (106 ml, 103.7g 1.3 mol), 4-dimethylaminopyridine (2.38 g, 0.02 mol, 0.03 equiv) and dichloromethane (DCM, 750ml) at room temperature under nitrogen. The temperature of the reaction mixture was kept below 30°C by cooling in a cold water bath. The reaction mixture was stirred at RT under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 150ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and concentrated in vacuo to a yellowish semi-solid. This was then crystallised from methanol (500ml) to give a white solid. The yield was 158 g (60%).

1,3-Dicaprin

The above ketone (158 g, 0.40 mol) was dissolved in tetrahydrofuran (THF, 2.25 L). Water (50 ml) was then added, the solution cooled to 5°C, and sodium borohydride (5.66 g, 1.5eq) added portionwise below 10°C. The reaction mixture was monitored by HPLC (C18, eluted with ACN at 1ml/min λ210nm) (Note: only about 4.5g of the borohydride was in fact added, as all SM had reacted).The reaction mixture was stirred at RT for 1h and then concentrated in vacuo to remove THF. The residue was partitioned between ethyl acetate and 5% sodium chloride solution. The aqueous phase was re-extracted with ethyl acetate and the combined extracts dried over $MgSO_4$ and concentrated in vacuo to a waxy solid. This was crystallised twice from hexane to give 96g (60%) of a white solid. (98% pure by HPLC)

1,3-Dicaprin 2-arachidonate (**CAC**)

Arachidonic acid (AA96, 78.8 g, 0.26 mol) was dissolved in dichloromethane (DCM, 425 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (33.9 ml, 49.4 g, 0.39 mol, 1.5eq) added dropwise at 15-20°C over 15 mins. The mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 30-40 min at 10-15°C (ice /water cooling) to a stirred solution of 1,3-dicaprin (94.2 g, 0.24 mol), DCM (450 ml), pyridine (19.1 ml, 18.6 g, 0.24 mol) and 4-dimethylaminopyridine (1.72 1.50 g, 0.014 mol, 0.06 equiv) at 10-15°C .The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed with DCM. The combined washing and filtrate was washed with 1 x 150 ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and the solvent removed in vacuo to a brown oil (171 g).

The scale of the above three reactions was the largest on which each was carried out. The borohydride reduction produced, in addition to 1,3-dicaprin, a by-product in variable yield. The presence of this by-product greatly affected the yield of the isolated pure 1,3-dicaprin; the by-product could only be removed by two

crystallisations of the crude product. Since the final product, **CAC**, is purified by column chromatography, it is imperative that the 1,3-dicaprin used for the final step is as pure as possible!

412 g of crude **CAC** was produced as a brown oil from the above reactions. This material was purified on a series of silica columns using hexane followed by 1-3% ether/hexane. The purification required 7 or 8 columns, using 3-4 kilos of silica, and 100 litres of solvent.

The resulting product, a clear very pale yellow oil, (295grams) was 95.8% pure by HPLC (C18 4.6 x 100mm, eluted with 85/15 ACN/THF at 1ml/min. UV detection λ210nm). GC indicated a ratio of 66.3/32.1 C/A (1.6% impurity carried through from the 5% impurity in A).

Summary

295 g of glycerol 1,3-didecanoate-2-arachidonate (1,3-dicaprin-2-AA, **CAC**) has been prepared from decanoyl chloride (98 %) and Arachidonic acid (95%) by a three-step process (scheme given below). It is a very pale yellow oil and was stored under nitrogen in the freezer. The HPLC purity is 95.8 %.

Synthesis Example 7

1,3-Dioleoin 2-gammalinolenoate (Glycerol 1,3-dioctadeca-9Z-enoate 2-octadecatri(6-Z,9-Z,12-Z)enoate or **OGO**)

This triglyceride is known: a carbon-14 labelled version has been prepared by normal chemical synthesis and the normal unlabelled form by biochemical synthesis using lipases. **OGO** is not a major component of borage oil but its isomer **OOG** is (9%). The two intermediates used in the synthesis of **CGC** are known. The last step is novel.

The use of, the synthesis of from 1,3-dioleoin, and the purification of CGC are all believed novel. In general triglycerides CXC are preferred over OXO on patent and cost of goods grounds.

OGO was here prepared by reaction of 1,3-Doleoin with GLA-chloride in dichloromethane-pyridine. 1,3-Diolein was prepared by sodium borohydride reduction of 1,3-dioleoylpropan-2-one, which was in turn prepared by reaction of oleoyl chloride with 1,3-dihydroxyacetone. The intermediate 1,3-dioleolin must be handled with care since it can undergo acyl migration on exposure to acids , bases and heat. Older methods [7,8] of making 1,3-dioleoin, via mono-tritylglycerols or glycidyl esters was deemed less attractive because of more steps and acyl migration problems. The final product, **OGO**, was purified by careful column chromatography on silica which removed by-products.

Small Scale

1,3-dioleoylpropan-2-one

155.1g Oleic acid (155.1 g, 0.55 mol, 1.0 equiv, Croda 094 RV05192) was dissolved in dichloromethane (DCM, 500 ml). The solution was stirred at room temperature (RT) under nitrogen and 104.4g (1.5eq 71mls) oxalyl chloride (104.4 g, 71.8 ml, 0.82 mol, 1.5 equiv) was added dropwise at 15-20°C over about 20 mins. The reaction mixture was stirred overnight at RT. The excess oxalyl chloride and DCM were removed in vacuo and the residual oily acid chloride was added dropwise over 15-20 min to a stirred suspension of 1,3-dihydroxyacetone dimer (22.5g, 0.24 mol of monomer), pyridine (40.4 ml), 4-dimethylaminopyridine (1.83g) and dichloromethane (DCM, 500ml) at room temperature under nitrogen. The temperature of the reaction mixture was kept below 20°C by cooling in an ice/water bath. The reaction mixture was stirred at RT under nitrogen overnight. The pyridine hydrochloride formed was removed by filtration and washed with DCM. The combined filtrate and washings were then washed with 1 x 150ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$ and concentrated in vacuo to an orange/brown semi-solid. This was triturated in methanol and stored in the 'fridge overnight. The solid deposited (90% pure by HPLC) was then crystallised from diisopropyl ether (DIPE) and methanol to give

51.3g of an off white solid which was 95% pure by HPLC. Further crystallisation from DIPE/methanol yielded 41g (27%) of a 98% pure product.

1,3-Diolein

The above ketone (32.8 g, 0.053 mol) was dissolved in tetrahydrofuran (THF, 250 ml). Water (10 ml) was then added, the solution cooled to 5°C, and sodium borohydride added portionwise below 10°C. The reaction was followed by HPLC (C18, ACN/THF 90/10 at 2mls/min ,λ210nm) and after all the starting ketone had reacted the addition of the borohydride was stopped (830mg ,0.022 mol added). The mixture was then concentrated in vacuo to remove THF. The residue was partitioned between ethyl acetate and water. The aqueous phase was re-extracted with ethyl acetate and the combined extracts dried over $MgSO_4$ and concentrated in vacuo to an oil (~33g) which solidified on cooling.The product (68% pure by HPLC) was crystallised from 100ml hexane at –20°C (in the freezer) overnight This product (92% pure 21.1g) was recrystallised from hexane (50ml) to give 18.28g (56%yield) of a product 97.5% pure by HPLC.

1,3-Diolein 2-gammalinolenoate (**O-G-O**)

γ-Linolenic acid (GLA95, 41.2 g, 0.15 mol, 1.1 equiv) was dissolved in dichloromethane (DCM, 250 ml). The resulting solution was stirred at RT under nitrogen and oxalyl chloride (19.1 ml, 28.2 g, 0.22 mol, 1.65 equiv) added dropwise over 5 mins. The mixture was stirred at RT overnight and then concentrated in vacuo to remove DCM and excess oxalyl chloride. The residual oily acid chloride (GLA-Cl) was then added dropwise over 15 min (ice /water cooling) to a stirred solution of 1,3-diolein (83.5g, 0.13 mol), DCM (250 ml), pyridine (10.9 ml, 10.6 g, 0.14 mol) and 4-dimethylaminopyridine (0.49 g, 0.004 mol, 0.15 equiv) at 10-15°C . The temperature was maintained by ice-water cooling. The reaction mixture was stirred at RT under nitrogen overnight. Pyridine hydrochloride was removed by filtration and washed with DCM. The combined washing and filtrate was washed with 1 x 80ml portions of 5%NaCl, 5%$NaHCO_3$, 0.1N HCl, 5%NaCl. The solution was then dried over $MgSO_4$

and the solvent removed in vacuo.The residual brown oil was purified by column chromatography on silica. Elution with hexane and then with 5% ether/hexane gave 63.6g (54%) of a colourless oil. Purity determined by HPLC.

Summary

64 g of glycerol 1,3-oleoate-2-gammalinolenoate (1,3-dioleate-2-GLA, OGO) was prepared from oleoyl chloride (98 %) by a three-step process (scheme given below). It was an almost colourless oil (slight yellow tinge) and is being stored under nitrogen in the freezer. The HPLC purity was 89.4 %.

^{13}C NMR Data for Structured lipids

GGG δ_C (125.7 MHz, $CDCl_3$) 172.69 (1C, C-2 carbonyl), 173.09 (2C, C-1, C-3 carbonyls)

CGC δ_C (125.7 MHz, $CDCl_3$) 172.76 (1C, C-2 carbonyl), 173.17 (2C, C-1, C-3 carbonyls)

CAC δ_C (125.7 MHz, $CDCl_3$) 172.65 (1C, C-2 carbonyl), 173.28 (2C, C-1, C-3 carbonyls)

C(DHLA)C δ_C (125.7 MHz, $CDCl_3$) 172.83 (1C, C-2 carbonyl), 173.30 (2C, C-1, C-3 carbonyls)

GCG δ_C (125.7 MHz, $CDCl_3$) 172.91 (1C, C-2 carbonyl), 173.11 (2C, C-1, C-3 carbonyls)

OGO δ_C (125.7 MHz, $CDCl_3$) 172.69 (1C, C-2 carbonyl), 173.25 (2C, C-1, C-3 carbonyls)

AAA δ_C (125.7 MHz, $CDCl_3$) 172.66 (1C, C-2 carbonyl), 173.04 (2C, C-1, C-3

carbonyls)

CCC δ_C (125.7 MHz, $CDCl_3$) 172.81 (1C, C-2 carbonyl), 173.21 (2C, C-1, C-3 carbonyls)

Experimental Procedure

The proton-decoupled ^{13}C NMR spectra with suppressed NOE were collected at 21 °C in a 5-mm broadband probe on a Joel 500 MHz spectrometer operating at 125.728 MHz. Waltz decoupling was the chosen mode of decoupling and was gated on only during the 14.89s acquisition time. The relaxation delay was set at 30 secs and the pulse angle was 90^O. The spectral window used was ca.35 ppm (from 173.5 to 172.6 ppm) with a 170 ppm offset. The spectra were internally referenced to $CDCl_3$ at 77.0 ppm. Typically, the approximate number of scans collected for adequate signal-to-noise ranged from 300 to 1200 scans depending on the concentration and purity of the sample. The total acquisition time for the experiments ranged between 2-8h e.g 1272 scans; data points 65,536. Concentrated solutions up to 20% w/v were employed when possible to reduce the acquisition time The chemical shifts quoted vary with the concentration of the solution.

BIOLOGICAL STUDIES.

Chronic Relapsing Experimental Autoimmune Encephalomyelitis (CREAE) Studies .

Induction and Clinical Assessment of EAE

CREAE was induced in C57B1/6 and SJL mice. Animals were injected subcutaneously with 100 μg of the neuroantigen peptide MOG 35-55 (amino acid sequence MEVGWYRSPFSRVVHLYRNGK Genemed Synthesis, Inc) or 1 mg of mouse spinal cord homogenate (SCH), in phosphate buffered saline (PBS), emulsified by sonication for 10 min at room temperature, in incomplete Freund's adjuvant (DIFCO, Detroit, USA) supplemented with 480 μg of *mycobacteria tuberculosis* and 60μg of *Mycobacteria butyricium* (DIFCO, Detroit, USA) on days 0 and 7 as

described previously (Morris-Downes, MM., et al 2002). In addition to optimise the disease mice also received 200 ng (intraperitoneally) of *Bordetella pertussis* toxin dissolved in PBS administered 1hr and 24 hrs after immunization with the MOG neuroantigen and for SCH days 0, 1, 7 and 8.

Animals were weighed from day 5 onwards and examined daily for clinical neurological signs by two experienced investigators and graded according to a previously validated grading scheme (Morris-Downes, MM. et al 2002 and others): 0 = normal; 1 = limp tail and feet; 2 = impaired righting reflex; 3 = partial hind limb paralysis; 4 = complete hindlimb paralysis; 5 = moribund; 6 = death. Animals exhibiting clinical signs of a lesser severity grade than typically observed were scored as 0.5 less than the indicated grade.

Reference

Morris-Downes, MM., et al (2002). Pathological and regulatory effects of anti-myelin antibodies in experimental allergic encephalomyelitis in mice. ***J. Neuroimmunol.*** 125. 114-124.

The mean group EAE score was compared for each test group compared to a respective control group by non-parametric statistical analysis (Mann Whitney U Test).

All MOG-CREAE studies comprised a treatment control group (**C-C-C** or **saline** as selected from the above study). Each structured lipid was tested at 3 dose levels, all treaments being orally administered for 2 weeks from day 7 after inoculation. All treatment groups will contained 10 animals. On completion of studies (day 21), brain and spinal cord were be removed and half of the samples were processed for signs of CNS perivascular mononuclear leucocyte-infiltrated sites and demyelination.

Studies were as follows:

Study 2: Spinal cord homogenate(SCH) EAE in SJL mice.

EAE Induction: 1mg SCH day 0 +day 7 sc. 200ng Pertussis toxin day 0,1, 7 & 8 ip.10mice/group. Mice were treated from day 7 to 21 with CCC or CGC.

Study 3: SCH EAE in SJL mice: Treatment was from PSD 7 to 21, both days inclusive.

Study 4: MOG EAE in C57BL mice: Treatment was from PSD 7 to 21, both days inclusive.

Study 5: SCH EAE in SJL mice: Treatment was from PSD 5 to 18, both days inclusive.

Study 6: MOG EAE in C57BL mice: Treatment was from Days 5 to 21 inclusive except C-DHLA-C group where treatment was from days 5 to 15 inclusive. Animals were culled on PSD 25. [Five animals from an untreated group, 3 animals from control CCC treatment group, 5 animals from GGG 150ul treatment group and 2 animals from GGG 350ul treatment group were sampled for histological analysis on PSD 20].

Study 7: SCH EAE in SJL mice
Treatment was from Days 6 to 20 inclusive.

Study 2 – Spinal cord homogenate (SCH) in SJL mice :-tested

***CGC** (50/150/350ul); **CCC** (350ul).*

***GGG**.(50/350ul)*

[Severe disease observed]

Study 3 – SCH/SJL mice:- tested

***CCC** (50/150/350ul)*

***CGC** (25/50/150/350ul)*

***GGG** (50/150/350ul)*

***OGO**.* (25/50/150/350ul)

[Severe disease observed]

Study 4 – MOG/ C57BL mice:- tested

***CCC** (50/150/350ul)*

***CGC** (25/50/150/350ul)*

***GGG** (50/150/350ul)*

***OGO**.* (25/50/150/350ul)

Study 6 – MOG/C57BL mice:- tested

***CCC** (150ul)*

***C-DHLA-C** (50ul)*

***CAC** (50/350ul)*

***AAA** (50/150ul)*

***GCG** (50ul)*

***CGC** (50ul)*

***GGG**.(150/350ul)*

[Pathology: CCC; GGG]

Histological examination of the submitted samples of brain and spinal cord showed lesions typical of experimental allergic encephalomyelitis.

Localised and diffuse lesions were characterised by gliosis, myelin vacuolation, axonal degeneration and perivascular cuffing with lymphocytes, macrophages and neutrophils.

Spinal cord lesions were mostly located in subpial white matter and brain lesions mostly occurred in the cerebellar white matter. Lesions were more severe in the spinal cords than in the brains and whereas all animals with brain lesions had lesions in the spinal cord, not all animals with cord lesions had lesions in the brain.

Variation in the severity of changes between individual mice is summarised using a semi-quantitative five point grading system.

Untreated mice had histological scores of 3-4 which correlated with EAE scores of 1.5-3. One mouse showed little pathological change with a zero score. In the GGG treated mice, the majority showed no abnormalities. Two mice from this group had histological scores of 2 and 3 respectively which correlated with EAE severity. scores of 1 and 1.5

The results of the four studies are shown in Figures 11 to 20 below

These show that the compounds G-G-G, A-A-A, C-G-C, C-DHGLA-C, and C-A-C are all capable of reducing severity of CREAE whereas compounds G-C-G and C-C-C failed to treat the condition. Compound O-G-O is believed to work if the dose is adjusted.

As cautioned in the description, the arachidnoic acid compounds are effective, but lead to death of some animals. Surviving animals had much reduced disease. It is believed that the dose of these compounds may be reduced still further to provide survival with satisfactory treatment.

Some of the studies show a bell shaped response curve for compounds C-G-C and G-G-G, suggesting that very high doses are not optimal, as set out above. Such dosing can be conveniently determined by those skilled art, eg. By dose escalatio and monitoring TGF-β1/TNF-α spontaneously release ratio changes from PBMCs.

Given the PCT/GB04/002089 high sn-2 γ-linolenic acid results, the lack of efficacy of low sn-2 black-current oil and G-C-G in CREAE and the low dose efficacy of C-G-C and C-DHGLA-C in Figure 20, it can be seen that sn-2 -γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid lipids provide a novel treatment for MS that far exceeds any current therapy outcome in that lesions are repaired and difficult symptoms are resolved: decreasing EDSS over a period of years being so far unachieved in other treatments.

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TABLE 1

Compositional (% Total FAs) Characteristics of Various Oils and their Protective Effects in EAE

Treatment	18:2n-6	18:3n-6	18:2n-6/18:3n-6	18:1n-9	INCIDENCE OF EAE
FGO	17	20	0.6	35	0/10
BOO	37	24	1.5	15	3/10
EPO	71	9.4	7.5	9	7/10
SAF	66	-	-	17	9/10
Controls	-	-	-	-	9/10

FGO, Fungal Oil; BOO, Borage Oil; EPO, Evening Primrose Oil, SAF, Safflower Oil.

TABLE 2

Treatment Groups- PCT/GB04/002089 Borage oil-MS trial

		Female	Male	Mean Relapse Rate (in past two years)	Mean Base EDSS	Number
Group	Placebo	7	4	2.6	3.9	11
	Low Dose	5	2	2.9	3.5	7
	High Dose	8	2	3.4	2.8	10
Total		20	8	2.9	3.4	28

Table 3. Molecular Species Comparison of Triacylglycerol-GLA (TG-GLA), Ethyl-Ester-GLA (EE-GLA) and PCT/GB04/002089 *Borago Officinalis* Oil-GLA (BOR-GLA) in MOG-induced CREAE in SJL Mice

Treatment	No. with EAE	Mean Clinical Score
Control	10/11	3.3±1.3
EE-GLA[a]	5/6	3.0±0.8
TG-GLA[a]	3/6	1.0±1.3[c]
BOR-GLA[b]	3/6	1.0±1.2[c]

[a] Animals given 100 µl of test lipid; [b]250µl BOR-GLA given. Significance of difference compared with controls, [c] $p < 0.05$

Table 4. Effect of enriched black-currant seed oil (73% GLA) on the incidence of EAE

	% Incidence of EAE (Days after immunisation)		
	13	17	21
Controls (n=10)	60	90	10
Blackcurrant (n=10)	10	80	70

Note: Blackcurrant oil delays the incidence but does not provide full protection. Animals were fed 7 days after sensitization (immunisation).

CLAIMS.

1. A method of treating a patient in need of therapy for a neurodegenerative disease comprising administering to that patient a therapeutically effective dose of a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid

2. A method as claimed in Claim 1 wherein the neurodegenerative disease involves demyelination.

3. A method as claimed in Claim 1 wherein the treatment specifically arrests underlying neurodegeneration and restores neuronal function.

4. A method as claimed in Claim 1 which normalises neuronal membrane composition with respect to γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid lipid content.

5. A method as claimed in Claim 1 which restores healthy TGF-β1/TNFα ratios as measured from spontaneous release from peripheral blood mononuclear cell release.

6. A method as claimed in Claim 1 wherein the disease is multiple sclerosis.

7. A method as claimed in Claim 1 wherein the disease is relapsing remitting multiple sclerosis, primary progressive multiple sclerosis or chronic progressive multiple sclerosis.

8. A method as claimed in Claim 1 wherein the disease is multiple sclerosis and the treatment restores, in part or completely, neuronal function or neuronal integrity as measured by one or more of MRI scan, CAT scan or by EDSS score.

9. A method as claimed in Claim 1 wherein the treatment is of cerebral impairment after stroke, head trauma and intracranial bleeding, Alzheimer's disease or Parkinson's disease where there is demyelination or neuronal damage.

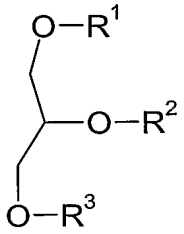
10. A method as claimed in Claim1 wherein the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF-β1 levels in the patient to therapeutic levels.

11. A method as claimed in Claim 1 wherein the lipid is administered for a duration and at a dose sufficient to maintain or elevate TGF-β1 levels in the patient to a TGF-β1/TNF-α ratio released spontaneously from peripheral blood mononuclear cells isolated from the blood of a patient, after 18 months of daily dosing, of 0.4 to 3.0, at least 0.5, more preferably at least 0.75 and most preferably at least 1.

12. A method as claimed in Claim11 wherein the dose is such as to produce a TGF-β1/IL-1β ratio in PBMCs isolated from blood of a patient, after 18 months of daily dosing, of at least of at least 0.75.

13. A method as claimed in Claim 1 wherein the amount of lipid administered is between 0.5 and 30 grams, typically 3 to 5 grams, per day.

14. A method as claimed in Claim 1 wherein the lipid is a monoglyceride, diglyceride or triglyceride containing the at least one sn-2 γ-linolenic acid, dihomo-γ-linolenic acid or arachidonic acid moiety, the lipid being of of general Formula I



Formula I

wherein R^1 and R^3 are independently selected from hydrogen and acyl groups, and R^2 is selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid residues having their carbonyl carbon attached to the oxygen of the glycerol moiety.

15. A method as claimed in Claim 1 wherein R^1 and R^3 are saturated fatty acid moieties of formula $-CO-(CH_2)_n-CH_3$, wherein n is an integer selected from 1 to 22,

16. A method as claimed in Claim 15 wherein R^1 and R^3 are the same and n is an integer of from 5 to 12.

17. A method as claimed in Claim 16 wherein n is an integer of from 6 to 10.

18. A method as claimed in Claim 14 wherein R^1 and R^3 are selected from the group consisting of essential fatty acids or physiologically acceptable fatty acids metabolisable by the human body.

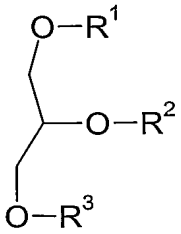
19. A method as claimed in Claim 14 wherein R^1, R^2 and R^3 are all the same and are selected from the group consisting of of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid residues.

20. A pharmaceutial composition characterised in that it comprises a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid.

20. A pharmaceutial composition for treating neurodegeneration characterised in that it comprises a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid.

21. A pharmaceutical composition for treating demyelinating disease comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid.

22. A lipid of formula II



wherein R^1 and R^3 are the same and are $-C(O)(CH_2)_nCH_3$ wherein n is selected from 4 to 14, more preferably 6 to 10 and most preferably 7, 8 or 9 and R^2 is selected from γ-linolenyl, dihomo-γ-linolenyl and arachidonyl residues.

23. Use of a defined structure lipid glyceride comprising a glycerol moiety esterifed with one or more fatty acid moieties, characterised in that the lipid has a fatty acid moiety at the sn-2 position selected from the group consisting of γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid, for the manufacture of a medicament for the treatment of neurodegenerative disease

24. Use as claimed in Claim 23 wherein the degenerative disease is a demyelinating disease.

25. Use as claimed in Claim 23 wherein the disease is multiple sclerosis.

26. Use as claimed in Claim 23 wherein the medicament normalises neuronal membrane composition with respect to lipid γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid levels.

27. Use as claimed in Claim 23 wherein the medicament restores TGF-β1/TNFα ratios spontaneously released from peripheral blood mononuclear cells of a patient to healthy levels.

28. Use as claimed in Claim 23 wherein treatment is for multiple sclerosis or the degenerative sequelae associated with head trauma, stroke and intracranial bleeds or neuronal damage caused by Alzheimer's or Parkinson's disease.

29. Use as claimed in Claim 23 wherein the medicament repairs CNS lesions.

30. Use as claimed in Claim 23 wherein the medicament relieves muscle spasticity and/or pain.

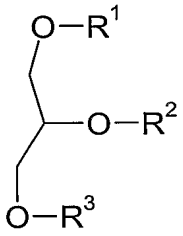
31. Use as claimed in Claim 23 wherein the medicament eliminates relapses.

32. Use as claimed in Claim 23 wherein the medicament improves EDSS score by at least 1 unit over a period of 1 years treatment.

33. Use as claimed in Claim 23 wherein the medicament is sufficient to restore EDSS of a patient with EDSS above 2.5 to below 2 over a period of 1 years treatment.

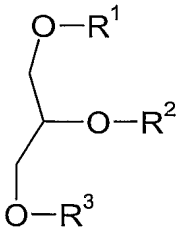
34. Use as claimed in Claim 23 wherein there is improvement in bladder control.

35. A method for synthesis of a compound of general formula III



wherein R^1 and R^3 are the same and are $-C(O)(CH_2)_nCH_3$ wherein n is selected from 4 to 14, more preferably 6 to 10 and most preferably 7, 8 or 9 and R^2 is γ-linolenyl residue, dihomo-γ-linolenyl residue or arachidonyl residue
comprising
reacting 1,3-dihydroxyacetone with a compound of formula $X\text{-}C(O)(CH_2)_nCH_3$
wherein X is selected from Cl, Br and I,
to give the corresponding 1,3-di-($C(O)(CH_2)_nCH_3$) 2-keto compound
reducing the keto group to the corresponding 1,3-di-($C(O)(CH_2)_nCH_3$) 2-ol
and reacting that with γ-linolenyl halide or dihomo-γ-linolenyl halide or arachidonyl halide, wherein halide is chloride, bromide or iodide.

36. A method for synthesis of a compound of general formula IV



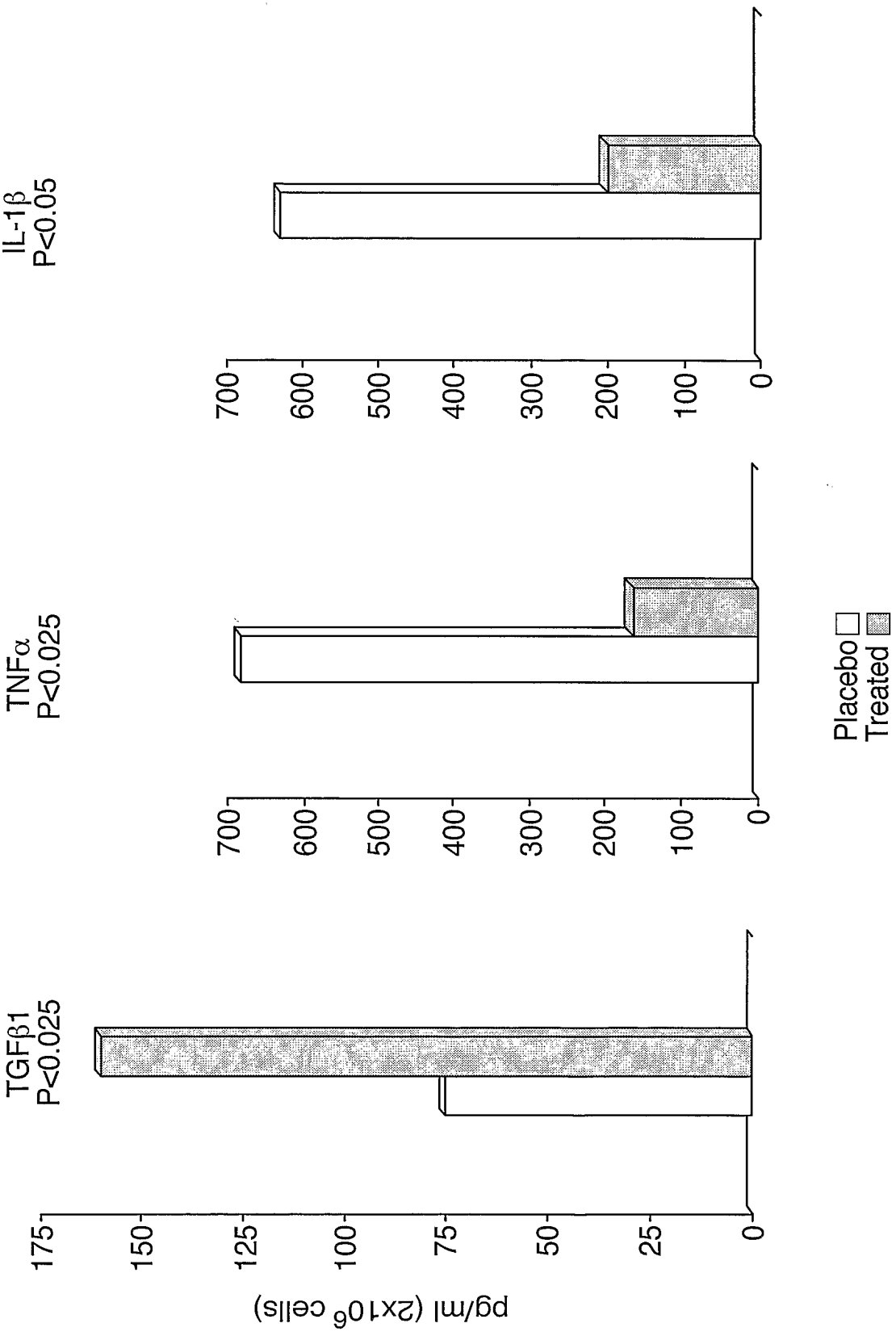
wherein R^1 to R^3 are the same and selected from γ-linolenyl residue, dihomo-γ-linolenyl residue or arachidonyl residue

comprising reacting the corresponding γ-linolenyl halide, dihomo-γ-linolenyl halide or arachidonyl halide, wherein halide is chloride, bromide or iodide, with glycerol

37. A lipid selected from the group consisting of
Glycerol 1,3-didecanoate- 2-octadecatri(6-Z,9-Z,12-Z)enoate
Glycerol 1,3-didecanoate-2- eicosa-(8Z,11Z,14Z)-trienoate
Glycerol trieicosotetra5-Z,8-Z,11-Z,14Z-eneoate

38. A lipid as claimed in Claim 37 for use in therapy.

Fig.1.



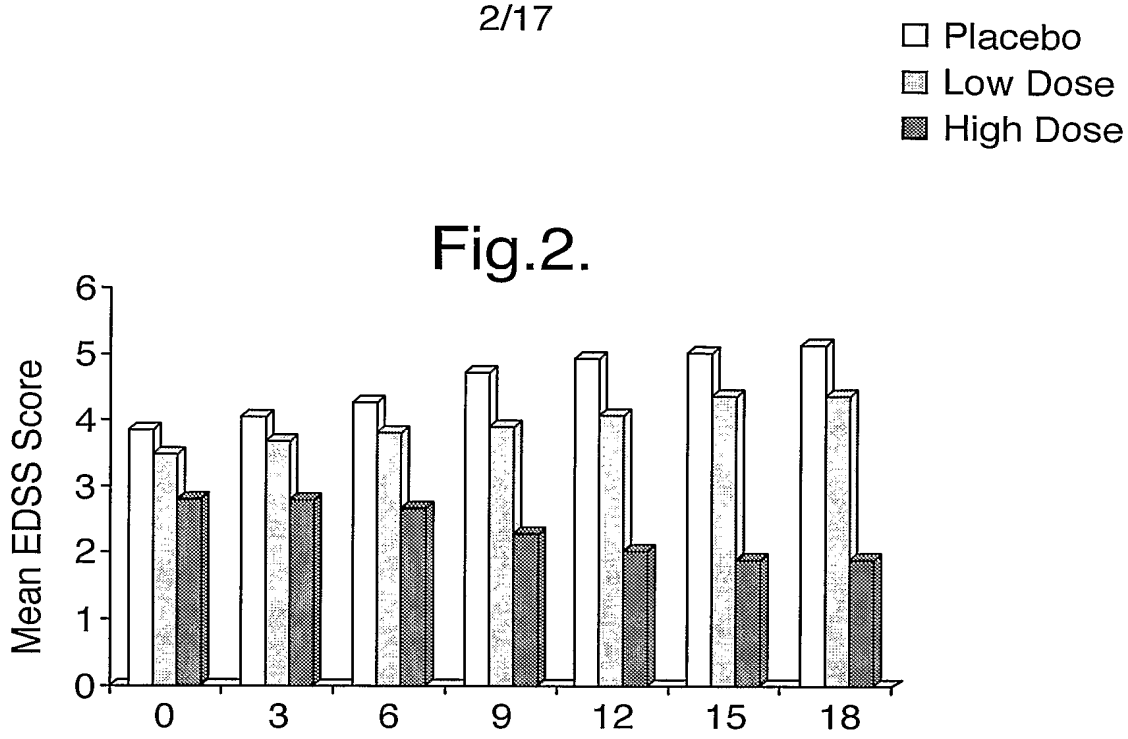

Placebo
Low Dose
High Dose
Mean EDSS Score
0
1
2
3
4
5
6
0
3
6
9
12
15
18

Fig.2.

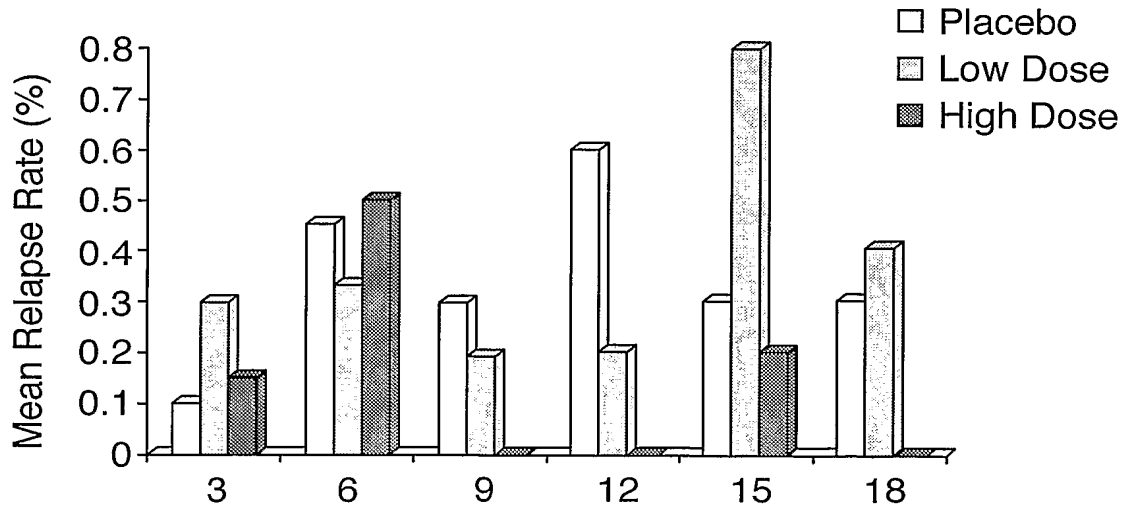
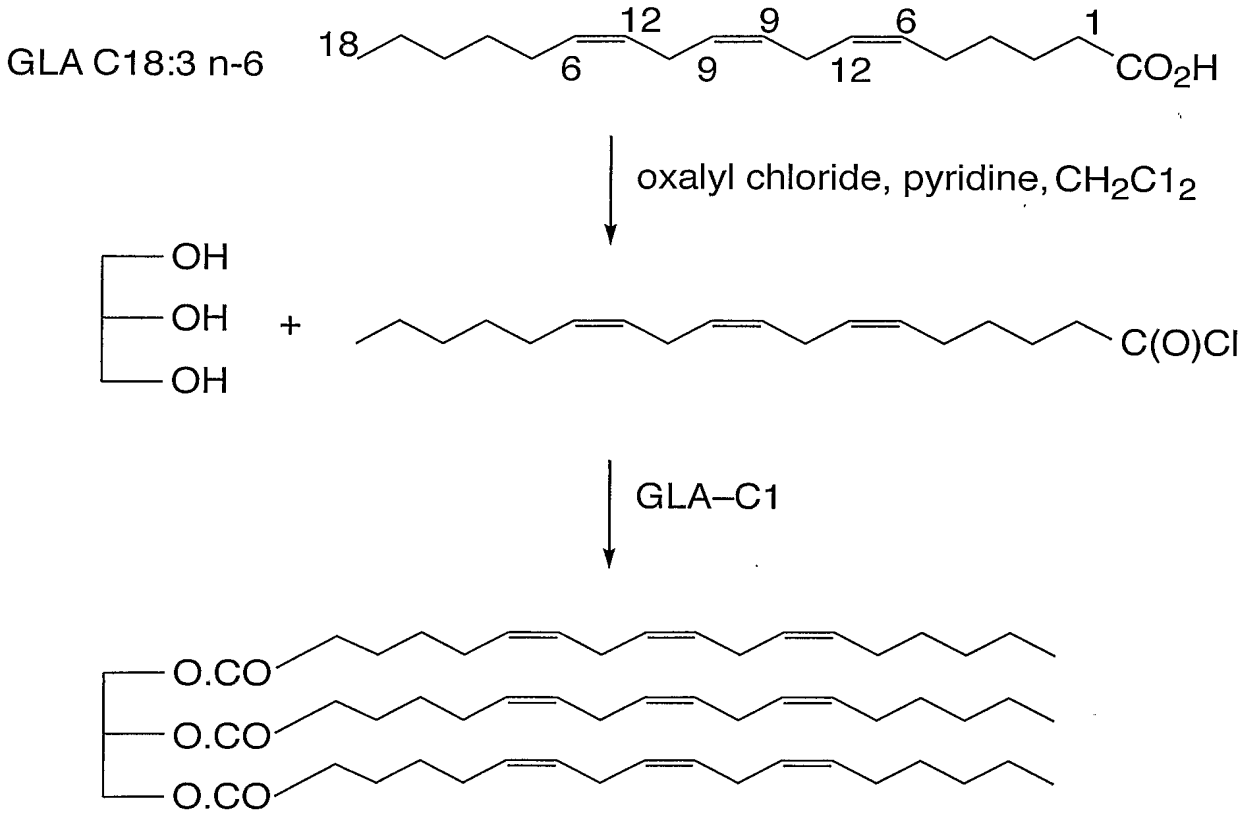

Placebo
Low Dose
High Dose
Mean Relapse Rate (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
3
6
9
12
15
18

Fig.3.

Fig.4.



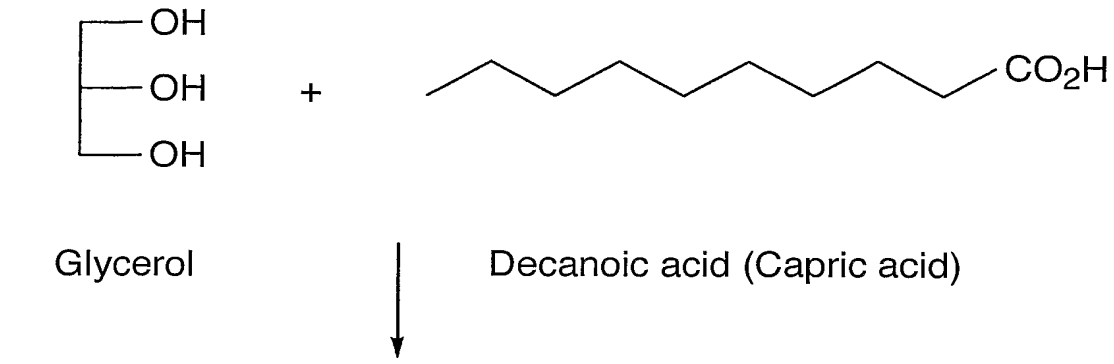
2

Glycerol tris-(6-Z,9-Z,12-Z-octadecatrienoate) $C_{57}H_{92}O_6$

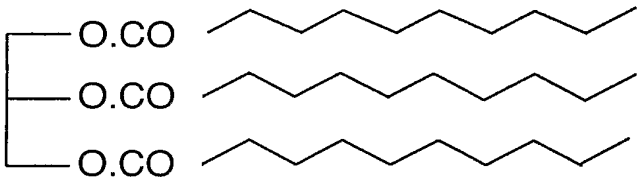
Tri-gamma-linolenin MW = 873.4

Numbering systems: black for biologists; blue for chemists

Fig.5.



Glycerol Decanoic acid (Capric acid)



Glycerol tridecanoate $C_{33}H_{62}O_6$

4

MW=554.85 mp 32°C Exhibits polymorphism

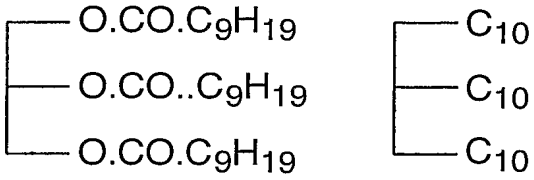
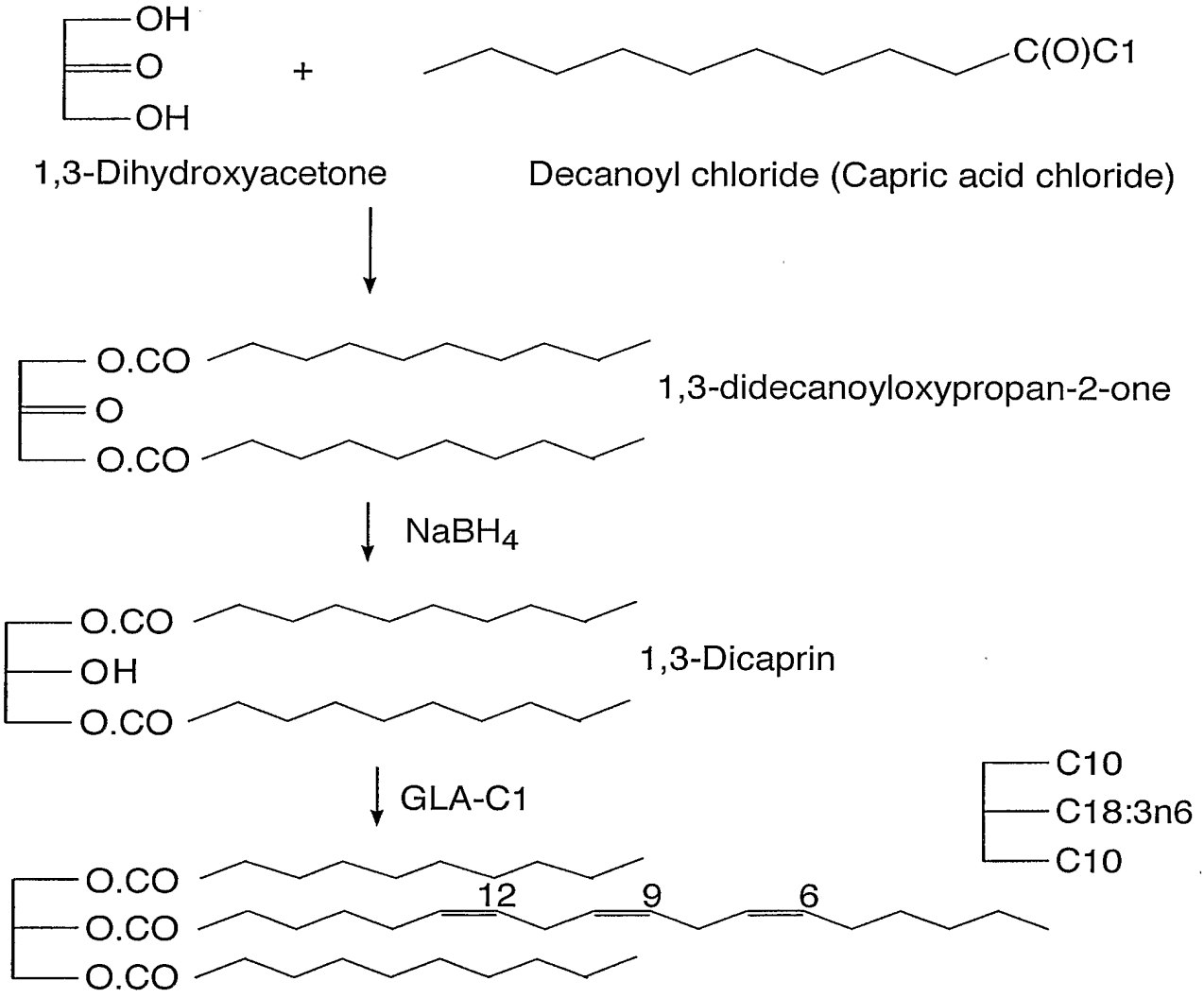


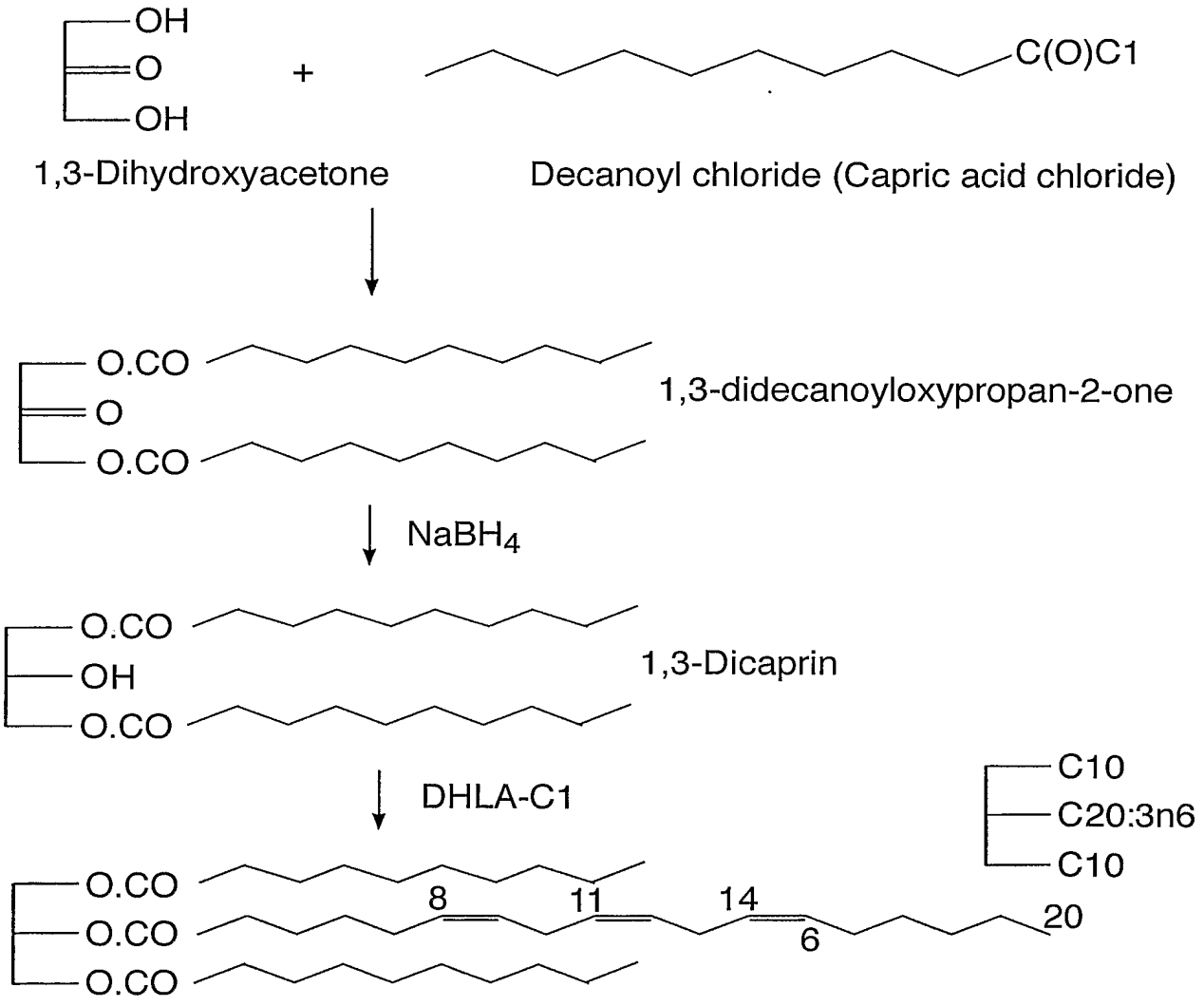
Fig.6.



Glycerol 1,3-didecanoate-2-octadeca-6Z,9Z,12Z-trienoate

$C_{41}H_{72}O_6$ MW = 661.05 mp <25°C

Fig.7.

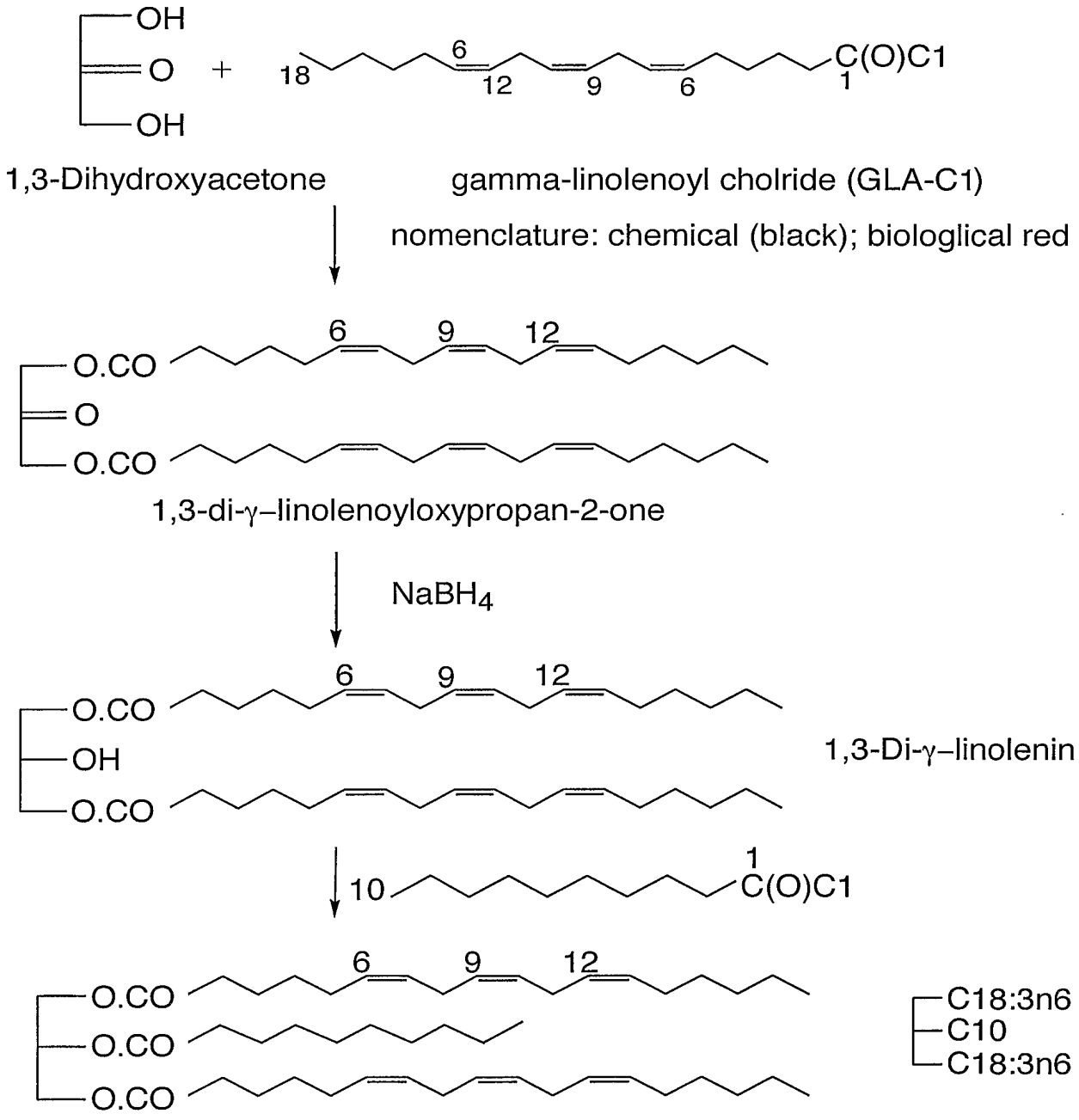


Glycerol 1,3-didecanoate-2-eicosa-8Z,11Z,14Z-trienoate

$C_{43}H_{76}O_6$

MW = 689.1 mp <25°C

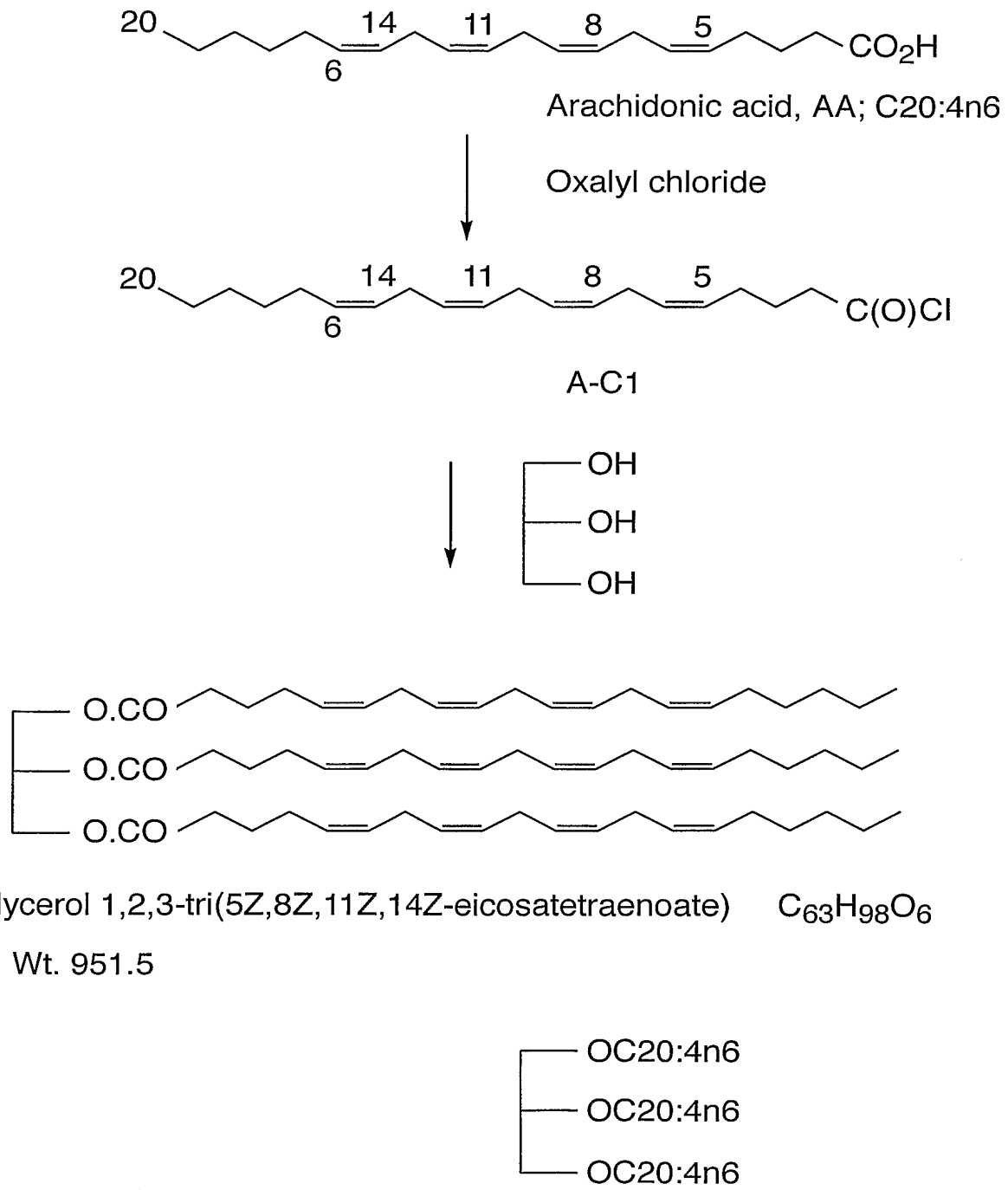
Fig.8.



Glycerol 1,3-dioctadeca-6Z,9Z,12Z-trienoate-2-decanoate

$C_{49}H_{82}O_6$ MW = 766.1 mp <25°C

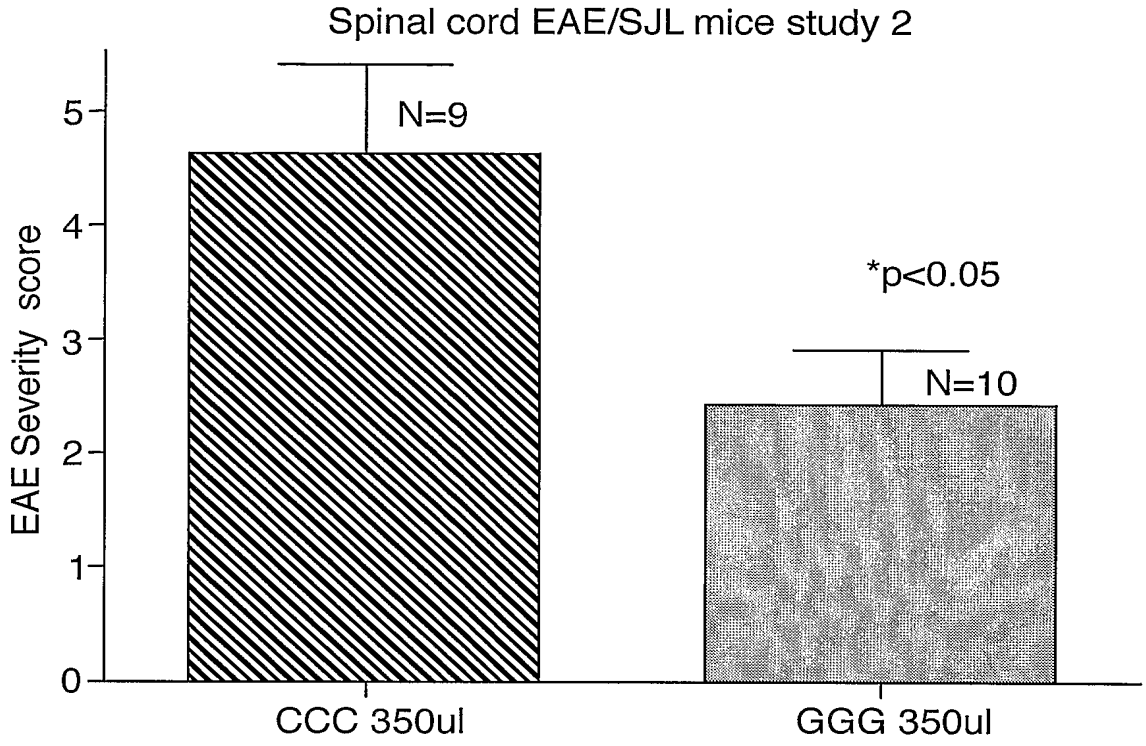
Fig.9.



Glycerol 1,2,3-tri(5Z,8Z,11Z,14Z-eicosatetraenoate) $C_{63}H_{98}O_6$

M. Wt. 951.5

Fig.10.



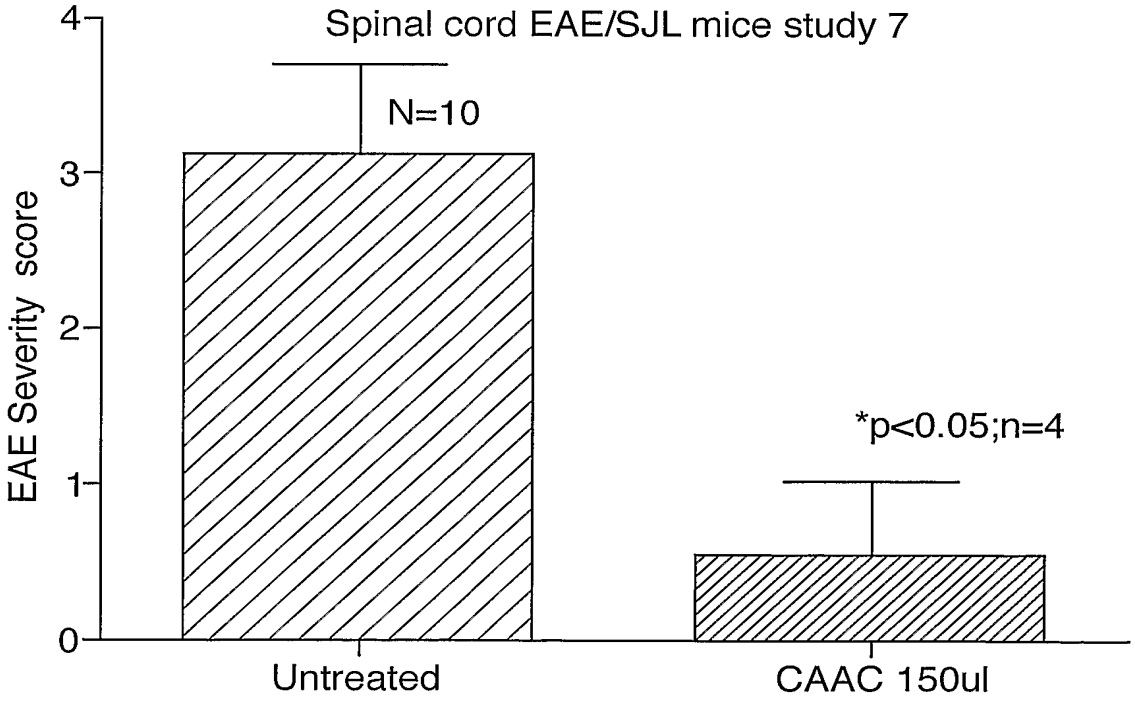
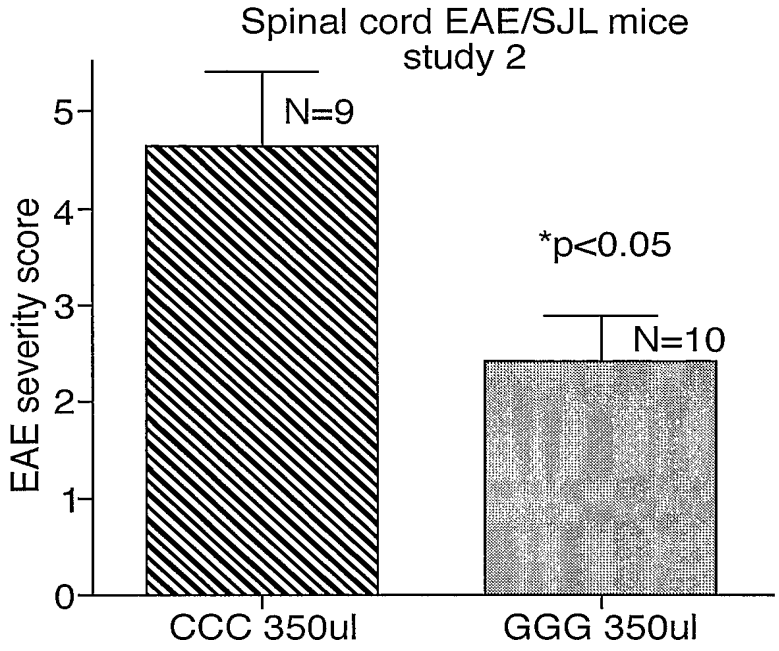
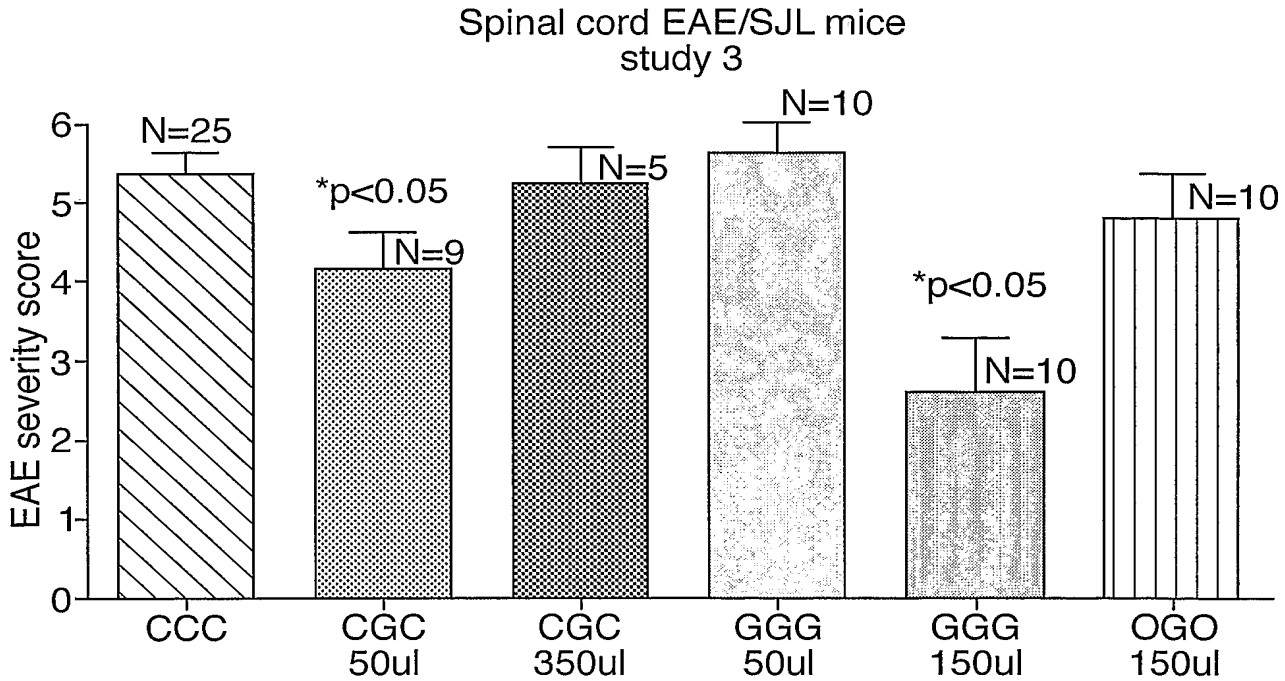


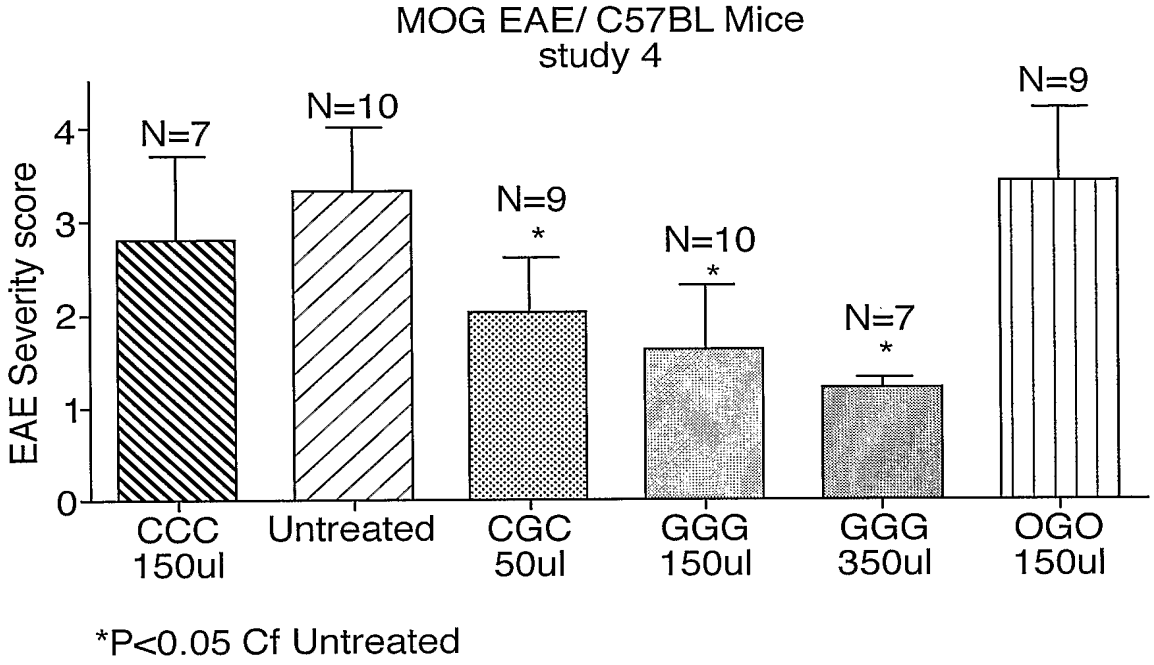
Fig.11.





CCC pooled from 50/150/350ul doses
CGC 25/50/350ul
GGG 50/150/350ul
OGO 25/50/150/350ul

Fig.12.



*P<0.05 Cf Untreated

Fig.13.

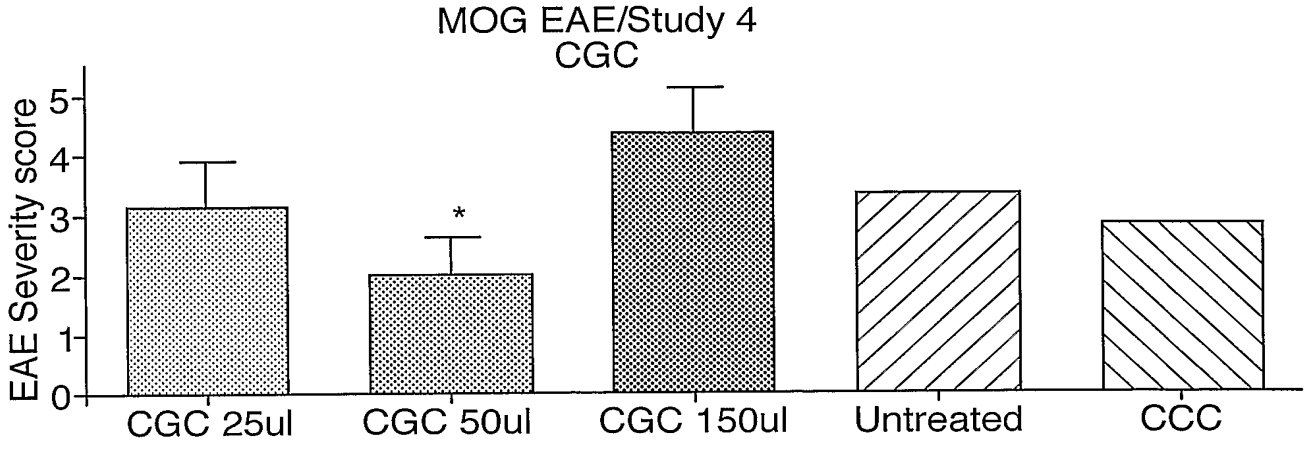


Fig.14.

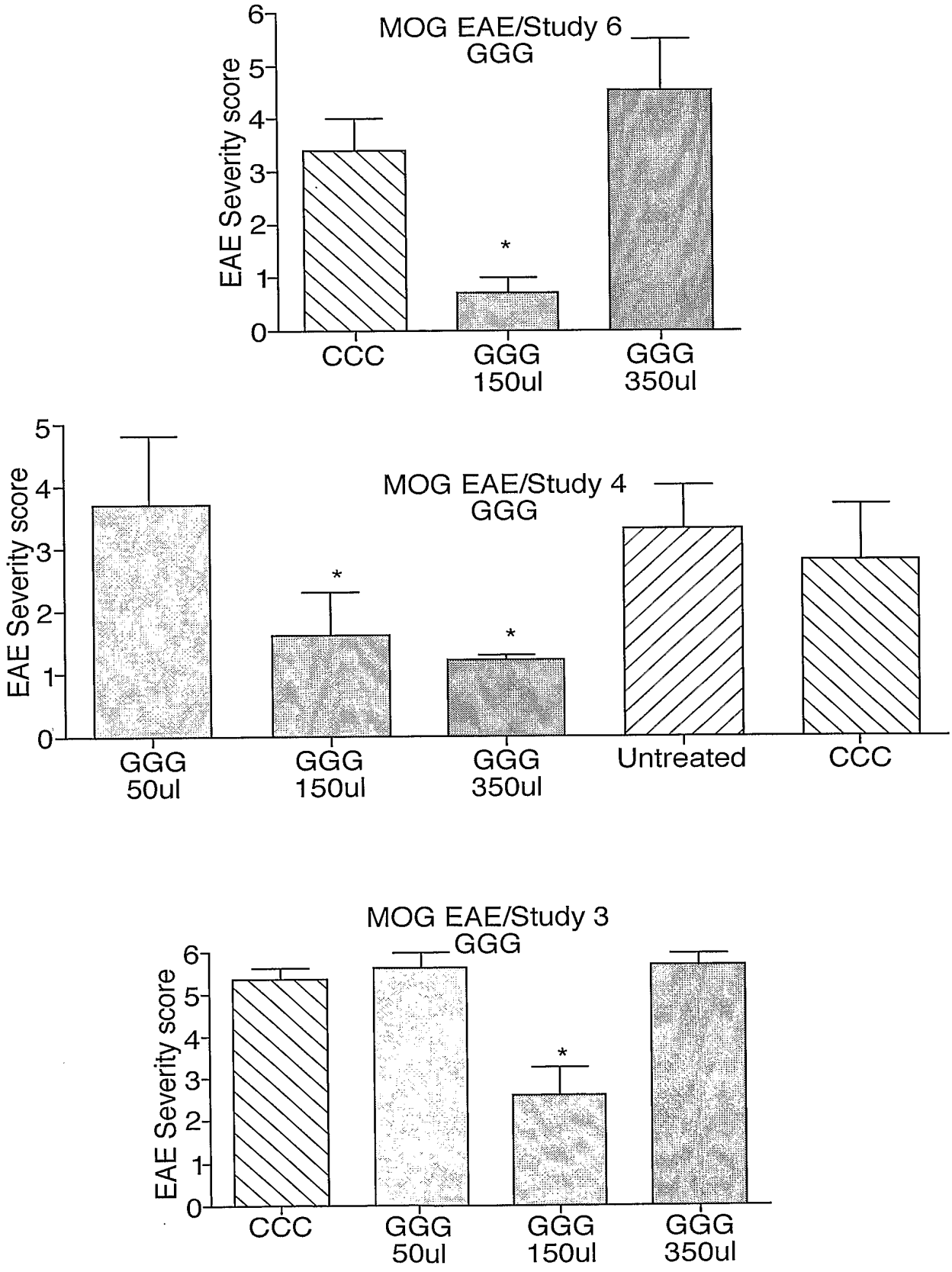
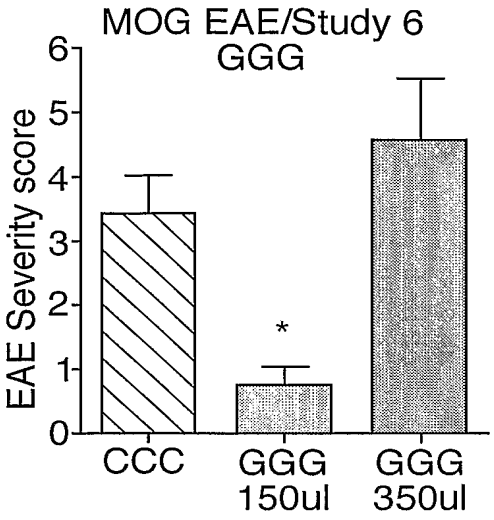
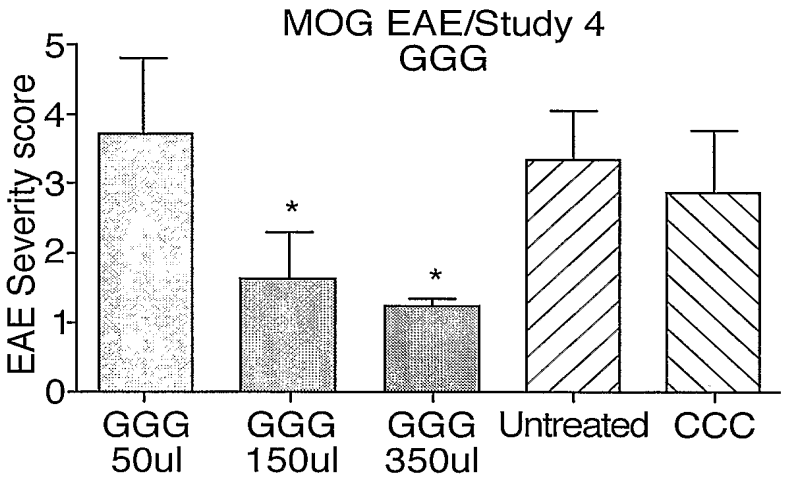
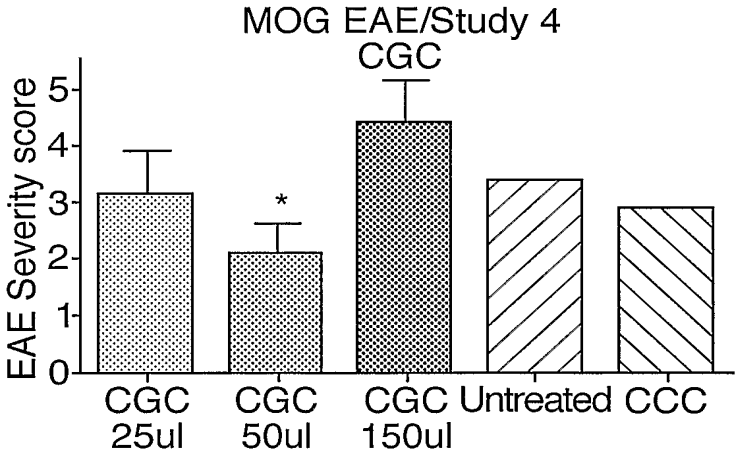


Fig.15.


MOG EAE/Study 6
GGG
EAE Severity score
0
1
2
3
4
5
6
*
CCC
GGG 150ul
GGG 350ul


MOG EAE/Study 4
GGG
EAE Severity score
0
1
2
3
4
5
*
*
GGG 50ul
GGG 150ul
GGG 350ul
Untreated
CCC


MOG EAE/Study 4
CGC
EAE Severity score
0
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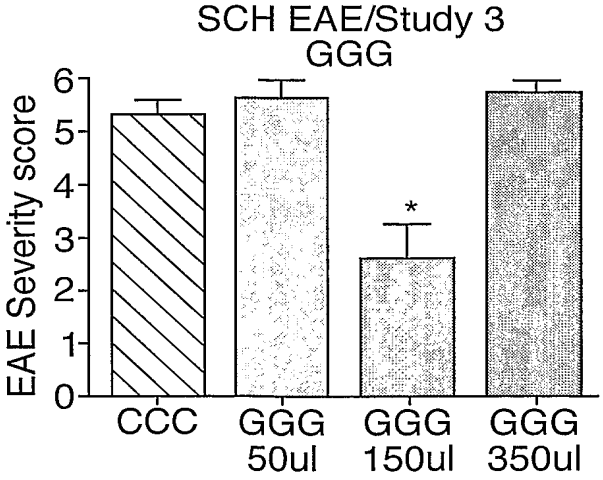
2
3
4
5
*
CGC 25ul
CGC 50ul
CGC 150ul
Untreated
CCC


SCH EAE/Study 3
GGG
EAE Severity score
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1
2
3
4
5
6
*
CCC
GGG 50ul
GGG 150ul
GGG 350ul

Fig.16.

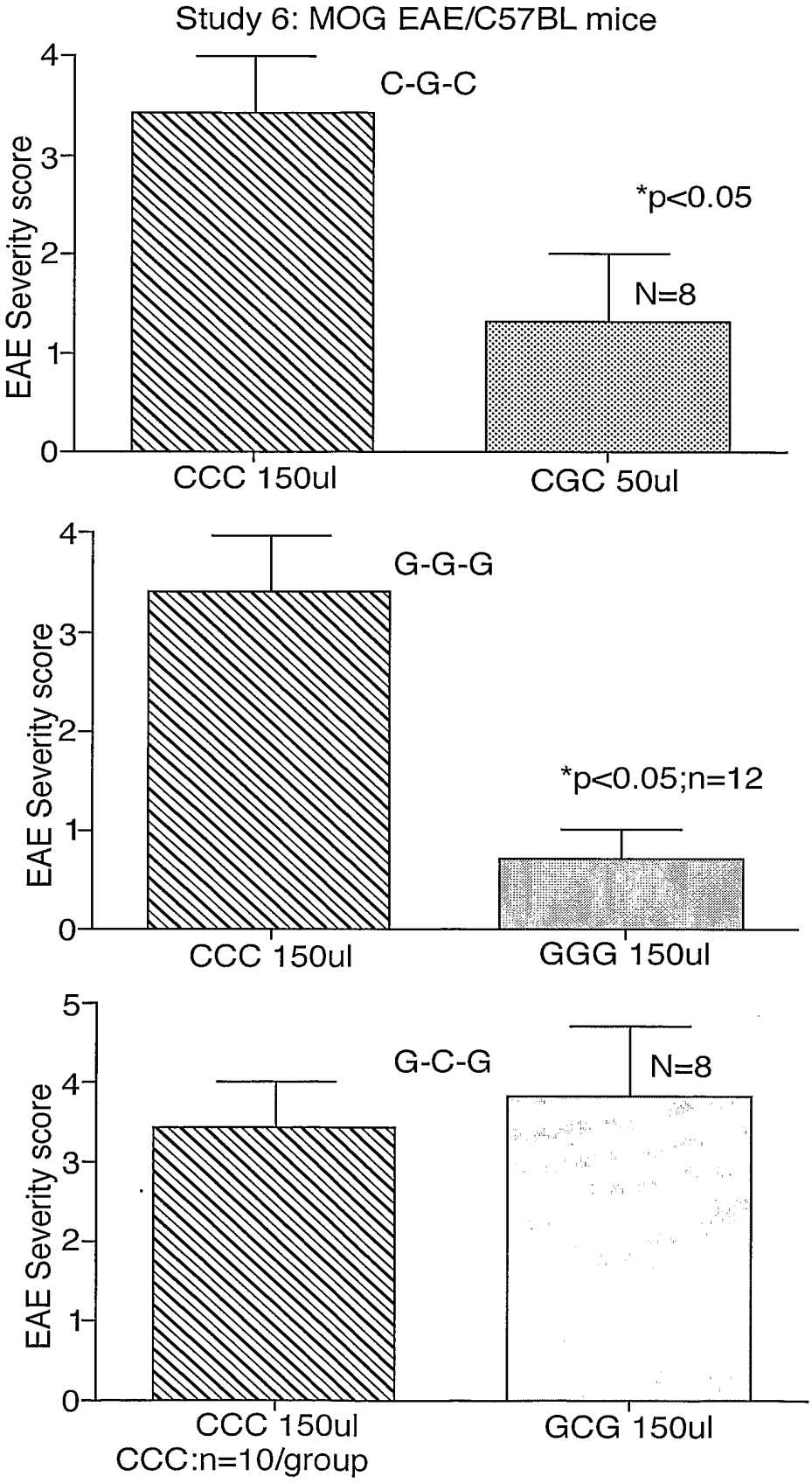


Fig.17.

Study 6:MOG EAE/C57BL mice

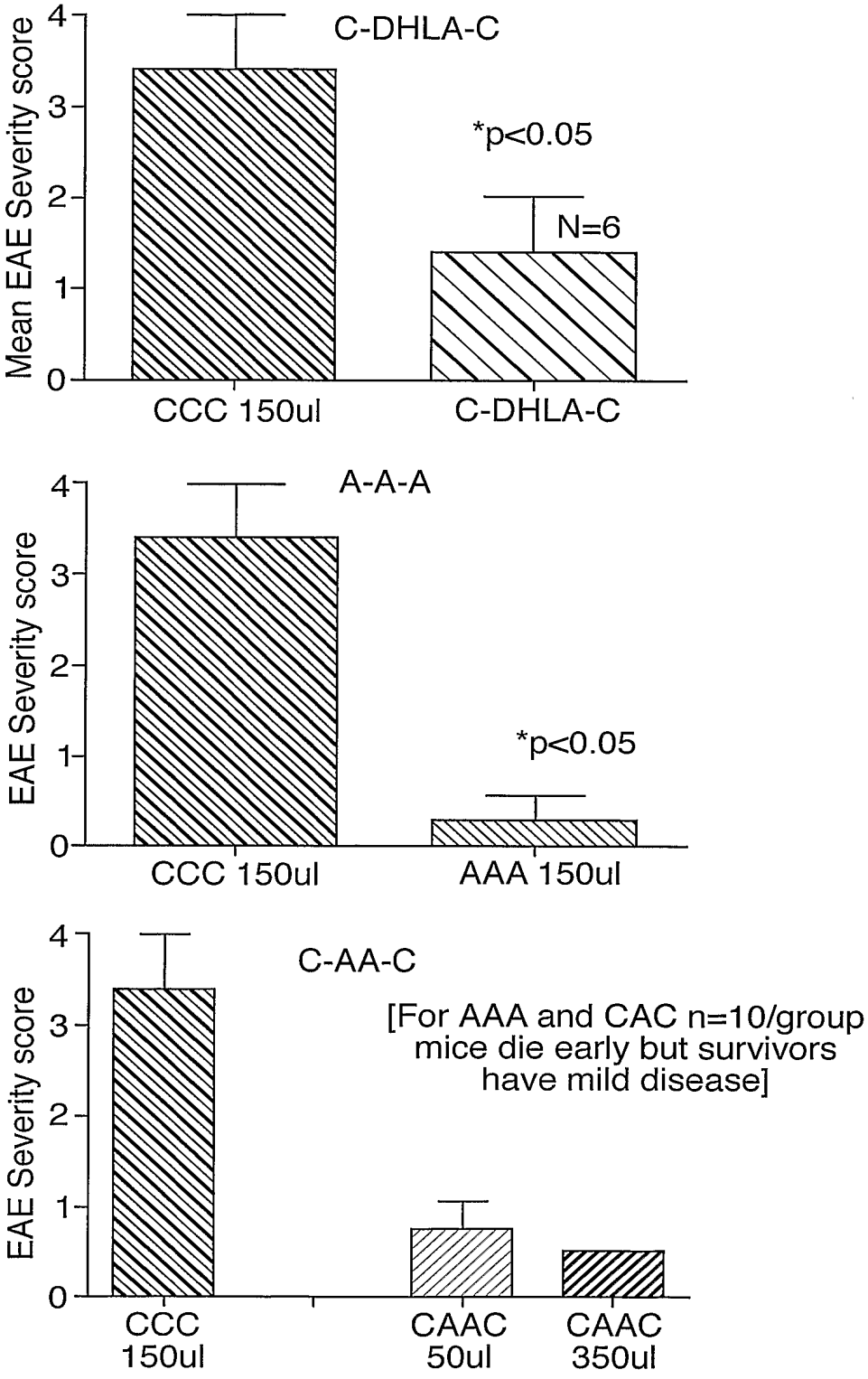
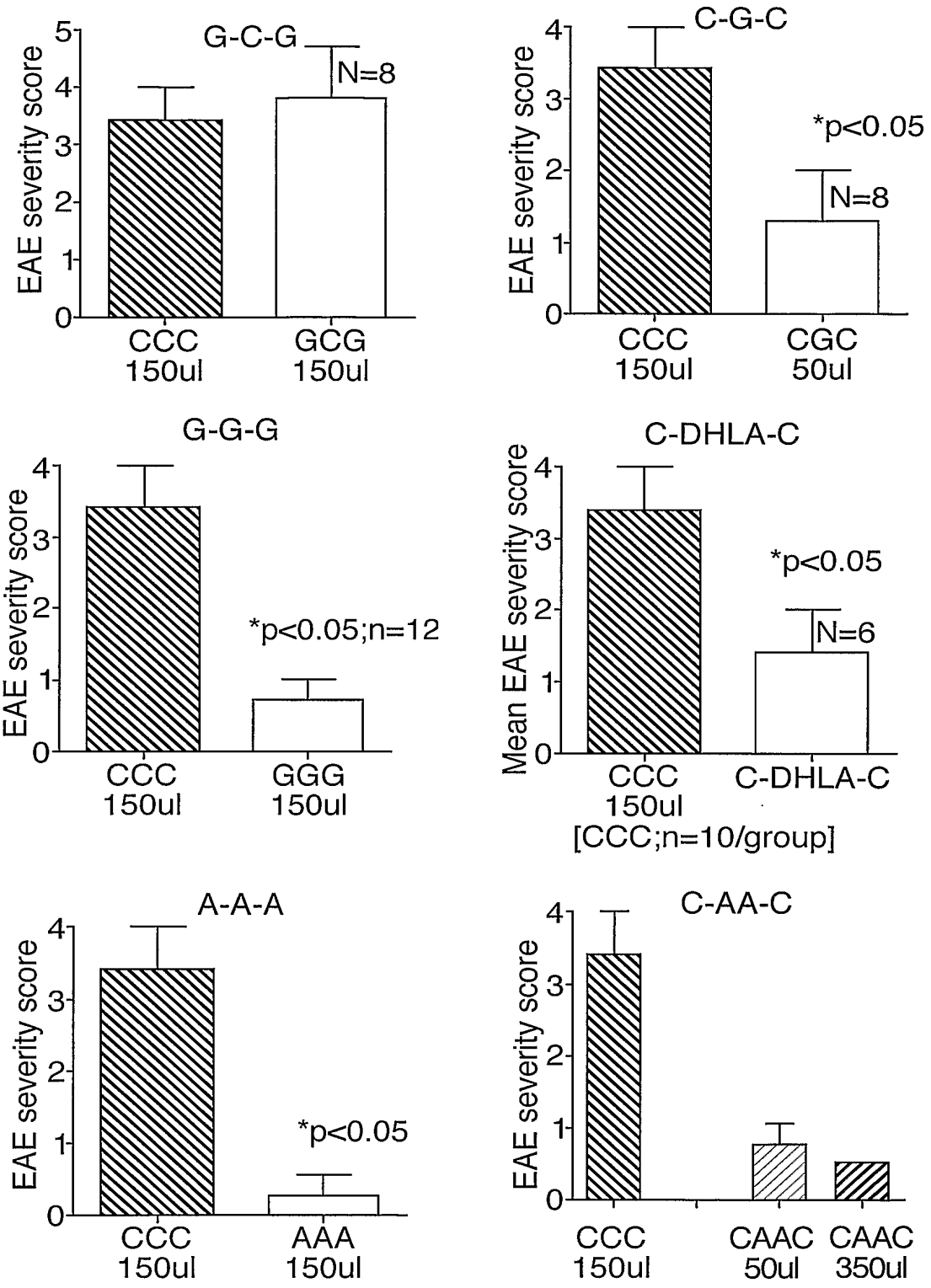


Fig.18.

Study 6. MOG EAE in C57BL Mice

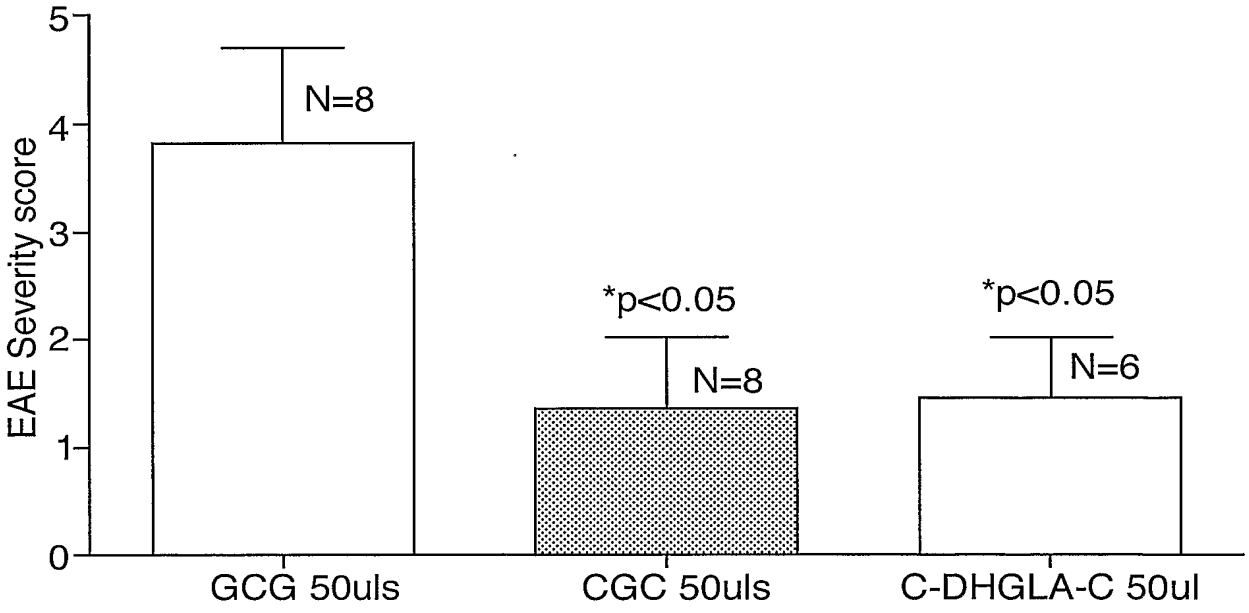


[For AAA and CAC n=10/group,mice die early but surviving mice have mild disease]

Fig.19.

MOG EAE C57BL MICE

Test of SN-2 Principle



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/003524

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/232 C07C69/587 A61P25/28

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)
IPC 7 A61K A61P C07C

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/013497 A (KAWASHIMA HIROSHI ; OKAICHI HIROSHIGE (JP); AKIMOTO KENGO (JP); OKAICH) 20 February 2003 (2003-02-20) the whole document	1-38
X	WO 01/97793 A (MECHOULAM RAPHAEL ; YISSUM RES DEV CO (IL); BREUER AVIVA (IL); GALLILY) 27 December 2001 (2001-12-27) page 8, paragraph 2-5; claim 1	1-21, 23-34
X	US 4 701 469 A (BARTHELEMY PIERRE ET AL) 20 October 1987 (1987-10-20) cited in the application column 5, line 27; claims 1-3	1,3-5, 9-23, 26-28, 35,36

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in 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 but 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	Date of mailing of the international search report
9 December 2004	21/12/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Friederich, M

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2004/003524

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

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

Although claims 1-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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 III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

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.:

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

Information on patent family members

International Application No
PCT/GB2004/003524

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03013497 A	20-02-2003	JP 2003048831 A	21-02-2003
		CA 2456049 A1	20-02-2003
		EP 1419768 A1	19-05-2004
		WO 03013497 A1	20-02-2003
WO 0197793 A	27-12-2001	AT 277608 T	15-10-2004
		AU 6628901 A	02-01-2002
		DE 60106026 D1	04-11-2004
		EP 1292288 A2	19-03-2003
		WO 0197793 A2	27-12-2001
		JP 2003535892 T	02-12-2003
		US 2002072539 A1	13-06-2002
US 4701469 A	20-10-1987	US 4607052 A	19-08-1986
		US 4701468 A	20-10-1987

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(19) World Intellectual Property Organization
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(21) International Application Number:
PCT/JP2005/005622

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(30) Priority Data:
2004-271958 17 September 2004 (17.09.2004) JP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITION WITH PREVENTIVE OR IMPROVEMENT EFFECT ON STRESS-INDUCED BRAIN FUNCTION IMPAIRMENT AND RELATED SYMPTOMS OR DISEASES

(57) Abstract: A composition with a preventive or improvement effect on stress-induced brain function impairment and related symptoms or diseases, comprising arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid.

WO 2006/030552 A1

DESCRIPTION

COMPOSITION WITH PREVENTIVE OR IMPROVEMENT EFFECT ON STRESS-INDUCED BRAIN FUNCTION IMPAIRMENT AND RELATED SYMPTOMS OR DISEASES

Technical Field of the Invention

The present invention relates to a preventive or improvement agent for stress-induced brain function impairment and related symptoms or diseases, comprising as an active ingredient arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid, as well as to a composition with a preventive or improvement effect on stress-induced brain function impairment and related symptoms or diseases, and a method for its production. More specifically, the invention relates to a preventive or improvement agent for stress-induced memory and learning ability impairment, emotional disorders (such as depression) and the like, comprising as an active ingredient at least one selected from the group consisting of arachidonic acid, arachidonic acid alcohol esters, and triglycerides, phospholipids or glycolipids wherein all or a portion of the constituent fatty acid is arachidonic acid, as well as to a composition with such a preventive or improvement effect and a method for its production.

Background Art

Stress is recognized as a response which can lead to brain disorders. After a recorded event in which the death of apes resulted from overcrowding stress during long-distance transport fifteen years ago, the dead apes were examined and found to have signs of serious stress, including gastric ulcers, immunodeficiency and hypertrophic adrenal glands, while exfoliation of pyramidal cells of the CA3 region of the hippocampus was also reported (J. Neurosci. 9, 1705, 1989). Since publishing of this report, researchers began to focus on

the psychological causes of brain disorders, and in particular, advances have been made in research on brain function impairment caused by stress.

Highly frequent stimulation of the brain hippocampus is known to lead to a phenomenon which includes synapse excitation and subsequent highly persistent synapse response. This is known as hippocampal LTP (long-term potentiation), a result of synaptic plasticity and one of the indicators for brain function evaluation. M.A. Lynch et al. reported that the hippocampal LTP of rats subjected to mild stress induced by separately breeding is demonstrably reduced compared to group-housed controls (J. Neurosci. 18, 2974, 1998). Thus, stress clearly contributes to brain function impairment.

Blood cortisol levels increase during periods of stress, and McEwen et al. have reported that Type 1 glucocorticoid receptors function in the hippocampus under physiological conditions, while Type 2 glucocorticoid receptors are active during times of corticosterone increase by stress; Type 1 receptors are protective in the hippocampal dentate gyrus, whereas Type 2 receptors tend to exacerbate neuropathy (Ann. NY Acad. Sci. 512, 394, 1987). Recently, increased blood IL-1β has been reported in post-traumatic stress disorder patients (Biol. Psychiatry 42, 345, 1997), and as the relationship between IL-1β and neuropathy has attracted researcher's attention, the possibility has been suggested that glucocorticoid receptor-mediated IL-1β increase in the hippocampus may contribute to neuropathy; however, much still remains unknown at the current time.

Research and development are also progressing in the area of discovering agents effective for the treatment of brain disorders (cerebral circulation/metabolism enhancers, anti-dementia drugs, etc.). Specifically, studies have focused on methods of improving brain energy metabolism through more efficient neuronal absorption of

nutrients for activation of cellular function (increasing intracerebral glucose, for example), methods of improving brain circulation with the aim of more adequately providing the necessary nutrients and oxygen to neurons (cerebral blood flow enhancement methods, for example), methods of activating neurotransmission at the synaptic cleft by neurotransmitters (providing neurotransmitter precursors (for example, choline or acetyl CoA supplementation), inhibiting conversion of released neurotransmitters (for example, acetylcholinesterase inhibition), increasing neurotransmitter release (for example, augmentation of acetylcholine or glutamate release), and activating neurotransmitter receptors), or methods of protecting neurocyte membranes (for example, antioxidant treatment, membrane component supplementation or anti-atherosclerotic treatment). To date, however, no satisfactorily effective therapeutic agent has been discovered.

It has also become apparent that the pharmacological mechanism by which conventional drugs are efficacious for treatment of brain function is distinct from the pharmacological mechanism of stress-related brain function impairment, for which reason, presumably, the conventional agents by themselves have not been effective for prevention or improvement of stress-induced brain function impairment.

The progression of stress-related brain function impairment can be slowed by removing the cause of stress, and this is one obvious course for prevention and improvement; however, such a method is difficult to realize given the stressful nature of modern society. Thus, absolutely no drug has existed which is safe enough to be readily administered even to infants or the elderly, and which has a preventive or improvement effect on stress-related brain function impairment and its associated symptoms or diseases.

The brain consists of a lipid mass-like tissue, with

phospholipids constituting about 1/3 of the white matter and about 1/4 of the gray matter. The polyunsaturated fatty acids in phospholipids of the various cell membranes in the brain consist primarily of arachidonic acid and docosahexaenoic acid. However, arachidonic acid and docosahexaenoic acid cannot be synthesized *de novo* in animal bodies and must be directly or indirectly obtained through diet (for example, as the arachidonic acid and docosahexaenoic acid precursors, linoleic acid and α-linolenic acid). Consequently, while it has been supposed that arachidonic acid plays an important role in maintaining cerebral function, this has not been concretely substantiated because of a lack of adequate sources of arachidonic acid.

Several inventions have been disclosed which utilize arachidonic acid for maintenance of brain function. In Japanese Unexamined Patent Publication HEI No. 10-101568, "Brain function improvement and nutritive composition", there is disclosed a ganglioside and arachidonic acid combination, as a means for providing a novel brain function improvement agent and a nutritive composition comprising it. Also, Japanese Unexamined Patent Publication No. 2003-048831, "Composition with preventive or improvement effect on symptoms and diseases associated with brain function impairment", describes as test examples experiments wherein brain function decline in aged rats is improved by arachidonic acid. Still, these inventions are based on the conventional mode of improving brain function, whereas nothing is indicated regarding an effect of arachidonic acid against stress-induced brain function impairment.

Patent document 1: Japanese Unexamined Patent Publication HEI No. 10-101568

Patent document 2: Japanese Unexamined Patent Publication No. 2003-048831

Non-patent document 1: J. Neurosci. 9, 1705, 1989

Non-patent document 2: J. Neurosci. 18, 2974, 1998

Non-patent document 3: Ann. NY Acad. Sci. 512, 394, 1987

Non-patent document 4: Biol. Psychiatry 42, 345, 1997

DISCLOSURE OF THE INVENTION

Thus, a strong demand exists for development of pharmaceuticals which prevent and improve stress-induced brain function impairment and its related symptoms or diseases, as well as such compounds which are highly suitable for consumption and lacking notable side effects.

As a result of much diligent research conducted with the purpose of elucidating the preventive or improvement effects on stress-induced brain function impairment and its associated symptoms and diseases by agents comprising as active ingredients arachidonic acid and/or compounds including arachidonic acid as a constituent fatty acid, the present inventors found, surprisingly, that the active ingredients of the invention exhibit apparent behavioral pharmacologic effects in mice subjected to restraint stress and evaluated by a Morris water maze learning test.

We also succeeded in realizing industrial production of a triglyceride containing at least 10% microorganism-generated arachidonic acid, and supplied the triglyceride for testing in order to elucidate the effect of the invention.

Specifically, the present invention provides a preventive or improvement agent for stress-induced brain function impairment and related symptoms or diseases and a composition with a preventive or improvement effect on stress-induced brain function impairment and related symptoms or diseases, comprising as an active ingredient arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid, as well as a method for their production.

More specifically, the invention provides a preventive or improvement agent for stress-induced memory and learning ability impairment or emotional disorders (such as depression or melancholia), comprising as an active ingredient at least one selected from the group consisting of arachidonic acid, arachidonic acid alcohol esters, and triglycerides, phospholipids or glycolipids wherein all or a portion of the constituent fatty acid is arachidonic acid, as well as to a composition with such a preventive or improvement effect and a method for its production.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the results for Example 3, indicating the effect of arachidonic acid on the spatial recognition of stressed mice.

Best Mode for Carrying Out the Invention

The present invention relates to a preventive or improvement agent for stress-induced brain function impairment and related symptoms or diseases and a composition with a preventive or improvement effect on stress-induced brain function impairment and related symptoms or diseases, comprising as an active ingredient arachidonic acid and/or a compound comprising arachidonic acid as a constituent fatty acid, as well as a method for their production.

As "stress-induced brain function impairment and related symptoms or diseases" there may be mentioned memory and learning ability impairment, emotional disorders (such as depression or melancholia), and the like, but the symptoms and diseases are not limited to these and include all symptoms and diseases associated with stress-induced brain function impairment.

The active ingredient of the invention is arachidonic acid, but any compound comprising arachidonic acid as a constituent fatty acid may be used. As compounds comprising arachidonic acid as a constituent fatty acid there may be mentioned arachidonic acid salts,

such as calcium or sodium salts. There may also be mentioned arachidonic acid lower alcohol esters such as arachidonic acid methyl ester and arachidonic acid ethyl ester. There may also be used triglycerides, phospholipids or glycolipids wherein all or a portion of the constituent fatty acid is arachidonic acid. However, the invention is not limited to the compounds mentioned above, and includes any compound comprising arachidonic acid as a constituent fatty acid.

For application to food products, the arachidonic acid is preferably in the form of a triglyceride or phospholipid, and most preferably in the form of a triglyceride. While virtually no natural sources of arachidonic acid-containing triglycerides (i.e., triglycerides including a triglyceride wherein all or a portion of the constituent fatty acid is arachidonic acid) exist, the present inventors have been the first to demonstrate that it is possible to industrially utilize triglycerides comprising arachidonic acid as a constituent fatty acid, that the active ingredients of the invention exhibit apparent behavioral pharmacologic effects in mice subjected to restraint stress and evaluated by a Morris water maze learning test and have preventive or improvement effects for stress-induced brain function impairment and related symptoms or diseases, and that the effects are attributable to arachidonic acid.

According to the invention, therefore, triglycerides including a triglyceride wherein all or a portion of the constituent fatty acid is arachidonic acid (arachidonic acid-containing triglycerides) may be used as the active ingredients of the invention. For application in foods, the arachidonic acid-containing triglycerides are preferably oils or fats (triglycerides) in a form wherein the arachidonic acid content of the total constituent fatty acid of the triglycerides is at least 10 wt% (w/w), more preferably at least 20 wt%, even more preferably at

least 30 wt%, and most preferably at least 40 wt%. Thus, the present invention may employ any such compounds which are obtained by culturing microorganisms capable of producing arachidonic acid-containing oils or fats (triglycerides).

As microorganisms capable of producing oils or fats (triglycerides) containing arachidonic acid, there may be mentioned microorganisms belonging to the genera *Mortierella*, *Conidiobolus*, *Pythium*, *Phytophthora*, *Penicillium*, *Cladosporium*, *Mucor*, *Fusarium*, *Aspergillus*, *Rhodotorula*, *Entomophthora*, *Echinosporangium* and *Saprolegnia*.

As examples of microorganisms belonging to the genus *Mortierella*, subgenus *Mortierella*, there may be mentioned *Mortierella elongata*, *Mortierella exigua*, *Mortierella hygrophila* and *Mortierella alpina*. More specifically, there may be mentioned the strains *Mortierella elongata* IFO8570, *Mortierella exigua* IFO8571, *Mortierella hygrophila* IFO5941, and *Mortierella alpina* IFO8568, ATCC16266, ATCC32221, ATCC42430, CBS219.35, CBS224.37, CBS250.53, CBS343.66, CBS527.72, CBS529.72, CBS608.70, CBS754.68, etc.

All of these strains may be acquired without any special restrictions from the Institute for Fermentation, Osaka (IFO), American Type Culture Collection (ATCC) or Centralbureau voor Schimmelcultures (CBS). There may also be used the strain *Mortierella elongata* SAM0219 (FERM-P 8703) (deposited under the provisions of the Budapest Treaty on March 19, 1986 with the Patent Microorganism Depository of National Institute of Industrial Science and Technology at Chuo 6, 1-1, Higashi 1-chome, Tsukuba city, Ibaraki pref., Japan, as FERM BP-1239), isolated from soil by the research group for the present invention.

For culturing of a strain to be used for the invention, spores, hypha or a pre-culture solution obtained by pre-culturing the strain may be seeded in a

liquid medium or solid medium for culturing. In the case of liquid culturing, the carbon source used may be a common one such as glucose, fructose, xylose, saccharose, maltose, soluble starch, molasses, glycerol or mannitol, although there is no limitation to these.

As nitrogen sources there may be used organic nitrogen sources including urea, and natural nitrogen sources such as peptone, yeast extract, malt extract, meat extract, casamino acid, corn steep liquor, soybean protein, defatted soybean and cotton seed meal, or inorganic nitrogen sources such as sodium nitrate, ammonium nitrate and ammonium sulfate. Trace nutrient sources including inorganic salts such as phosphoric acid salts, magnesium sulfate, iron sulfate and copper sulfate, or vitamins, may also be used if necessary. The medium components are not particularly restricted so long as they are in concentrations which do not prevent growth of the microorganisms. For most practical applications the carbon source may be used at a concentration of 0.1-40 wt% and preferably 1-25 wt%. The initial nitrogen source addition may be at 0.1-10 wt% and preferably 0.1-6 wt%, with optional further feeding of the nitrogen source during culturing.

By controlling the carbon source concentration of the medium it is possible to obtain oils or fats (triglyceride) containing at least 45 wt% arachidonic acid as the active ingredient of the invention. The cell growth phase is the culturing period up to the 2nd-4th day of culturing, while the fat/oil accumulation phase is from the 2nd-4th day of culturing. The initial carbon source concentration is 1-8 wt% and preferably 1-4 wt%, with successive supplemental addition of the carbon source only between the cell growth phase and the early fat/oil accumulation phase, for a total supplemental carbon source addition of 2-20 wt% and preferably 5-15 wt%. The amount of carbon source added between the cell growth phase and the early fat/oil accumulation phase

will depend on the initial nitrogen source concentration, and if the carbon source concentration in the medium is 0 from the 7th day of culturing, preferably from the 6th day of culturing and more preferably from the 4th day of culturing, it will be possible to obtain oils or fats (triglyceride) containing at least 45 wt% arachidonic acid, as the active ingredient of the invention.

The culturing temperature for the arachidonic acid-producing cells will differ depending on the microorganism used, but is 5-40°C, preferably 20-30°C, while culturing at 20-30°C for proliferation of the cells may also be followed by continued culturing at 5-20°C to produce unsaturated fatty acids. Such temperature control can also be utilized to increase the proportion of polyunsaturated fatty acids among the produced fatty acids. The pH of the medium may be 4-10 and preferably 5-9, for jar fermentor culturing, shake culturing or stationary culturing. The culturing is normally carried out for 2-30 days, preferably 5-20 days and more preferably 5-15 days.

In addition to controlling the carbon source concentration of the medium as a strategy for increasing the proportion of arachidonic acid in the arachidonic acid-containing oils or fats (triglyceride), arachidonic acid-rich oils or fats may also be obtained by selective hydrolysis of the arachidonic acid-containing oils or fats. Since lipases used for such selective hydrolysis do not have specificity for triglycerides and the hydrolytic activity decreases in proportion to the number of double bonds, the ester bonds of the fatty acids other than the polyunsaturated fatty acids are preferentially hydrolyzed. Furthermore, ester-exchange reaction between the produced PUFA glycerides may be used to produce triglycerides with an increased polyunsaturated fatty acid content ("Enhancement of Arachidonic Acid: Selective Hydrolysis of a Single-Cell Oil from *Mortierella* with

Candida cylindracea Lipase": J. Am. Oil Chem. Soc., 72, 1323, 1998).

Thus, oils or fats (triglyceride) with a high content of arachidonic acid obtained by selective hydrolysis of arachidonic acid-containing oils or fats can be prepared as the active ingredient of the invention. The proportion of arachidonic acid with respect to the total fatty acid content of the arachidonic acid-containing oils or fats (triglyceride) of the invention is preferably higher from the standpoint of eliminating the effect of other fatty acids, but it does not necessarily have to be a high proportion, and in fact the absolute amount of arachidonic acid can pose a problem for application to some foods. Oils or fats (triglycerides) containing arachidonic acid at 10 wt% or greater can be suitably used in most cases.

As triglycerides wherein all or a portion of the constituent fatty acid is arachidonic acid according to the invention, there may be used triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position. The oils or fats (triglycerides) used may also comprise at least 5 mole percent, preferably at least 10 mole percent, more preferably at least 20 mole percent and most preferably at least 30 mole percent, of triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position. The medium chain fatty acids bonded at the 1,3-positions of the triglyceride may be selected from among C6-12 fatty acids. As examples of C6-12 fatty acids there may be mentioned caprylic acid or capric acid, with 1,3-capryloyl-2-arachidonoyl-glycerol (hereinafter, "8A8") being particularly preferred.

Such triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position are optimum oils or fats (triglycerides) for elderly persons. Generally speaking,

ingested oils or fats (triglycerides) are hydrolyzed by pancreatic lipases upon entering the small intestine, but since pancreatic lipases are 1,3-specific, the 1,3-positions of the triglycerides are cleaved to form two free fatty acids while simultaneously producing a single 2-monoacylglycerol (MG). As 2-MG has extremely high bile solubility and is highly absorbable, the 2-position fatty acid is generally considered to be better absorbed. In addition, 2-MG dissolved in bile acid acts as a surfactant and thus increases the absorption of the free fatty acids.

The free fatty acids and 2-MG then form bile acid complex micelles together with cholesterol, phospholipids and the like and are incorporated into the intestinal epithelial cells where triacylglycerols are resynthesized, being finally released into the lymph as chylomicrons. However, the fatty acid specificity of pancreatic lipases is higher for saturated fatty acids, whereas arachidonic acid is not as easily cleaved. Another problem is that pancreatic lipase activity declines with age, and therefore triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position are more optimal oils or fats (triglycerides) for the elderly.

One specific production method for triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position is a method using a lipase which acts only on the 1,3-position ester bonds of triglycerides, in the presence of arachidonic acid-containing oils or fats (triglyceride) and a medium chain fatty acid.

The oils or fats (triglyceride) starting material are a triglyceride comprising arachidonic acid as a constituent fatty acid, but in the case of a high proportion of arachidonic acid with respect to the total constituent fatty acid of the triglycerides, reduced reaction yield due to excess unreacted oils or fats (the

triglyceride starting material and triglycerides wherein only one of the 1,3-position fatty acids has been converted to a medium chain fatty acid) can be prevented if the temperature is above the normal enzyme reaction temperature of 20-30°C, such as 30-50°C and preferably 40-50°C.

As examples of lipases which act specifically on the 1,3-position ester bonds of triglycerides there may be mentioned lipases produced by microorganisms such as *Rhizopus*, *Rhizomucor* and *Aspergillus*, as well as porcine pancreatic lipases. Any such commercially available lipases may be used. For example, there may be mentioned *Rhizopus delemar* lipase (Talipase, Tanabe Pharmaceutical Co., Ltd.), *Rhizomucor miehei* lipase (Ribozyme IM, Novo Nordisk Co., Ltd.) and *Aspergillus niger* lipase (Lipase A, Amano Pharmaceutical Co., Ltd.), although there is no limitation to these enzymes and any 1,3-specific lipases may be used.

The form of the lipase used is preferably an immobilized form on an immobilizing support in order to impart heat resistance to the enzyme, since the reaction temperature is 30°C or above and preferably 40°C or above for increased reaction efficiency. The immobilizing support may be a porous (highly porous) resin, for example, an ion-exchange resin with pores of approximately 100 Å or greater such as Dowex MARATHON WBA. However, this condition is not restrictive on the immobilizing support, and any immobilizing support capable of imparting heat resistance may be used.

The immobilizing support may be suspended in an aqueous solution of a 1,3-specific lipase at a weight proportion of 0.5-20 of the latter with respect to the former, and a 2- to 5-fold amount of cold acetone (for example, -80°C) may be slowly added to the suspension while stirring to form a precipitate. The precipitate may then be dried under reduced pressure to prepare the

immobilized enzyme. As a simpler method, a 1,3-specific lipase in a proportion of 0.05-0.4 with respect to the immobilizing support may be dissolved in a minimal amount of water and mixed with the immobilizing support while stirring and dried under reduced pressure to prepare the immobilized enzyme. This procedure can immobilize approximately 90% lipase on the support, but since absolutely no ester exchange activity will be exhibited in that state, pretreatment may be carried out in a substrate containing 1-10 wt% (w/v) water and preferably a substrate containing 1-3 wt% water, in order to activate the immobilized enzyme to maximum efficiency before it is provided for production.

The amount of water added to the reaction system is extremely important depending on the type of enzyme, because a lack of water will impede ester exchange while an excess of water will cause hydrolysis and a reduced glyceride yield (since hydrolysis will produce diglycerides and monoglycerides). However, if the immobilized enzyme used has been activated by pretreatment the amount of water added to the reaction system is no longer crucial, and an efficient ester exchange reaction can be carried out even in a completely water-free system. Also, selection of the type of enzyme agent may allow the pretreatment step to be omitted.

Thus, by using a heat-resistant immobilized enzyme and raising the enzyme reaction temperature, it is possible to efficiently produce triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position (8A8), without lowering the reaction efficiency even for arachidonic acid-containing oils or fats (triglycerides) with low reactivity for 1,3-specific lipases.

A method for production of a dietary product having a preventive or improvement effect on stress-induced brain function impairment and related symptoms or diseases, involves adding arachidonic acid and/or a

compound including arachidonic acid as a constituent fatty acid alone, or in combination with a dietary material containing substantially no arachidonic acid or only a slight amount thereof. Here, a "slight amount" means that even if arachidonic acid is present in the dietary product material and a food composition containing it is ingested by a human, the amount does not reach the daily amount of arachidonic acid consumption according to the invention, as described hereunder.

An unlimited number of uses exist for oils or fats (triglycerides) wherein all or a portion of the constituent fatty acid is arachidonic acid: for example, they may be used as starting materials and additives for foods, beverages, cosmetics and pharmaceuticals. The purposes of use and amounts of use are also completely unrestricted.

As examples of food compositions there may be mentioned ordinary foods, as well as functional foods, nutritional supplements, food for specified health uses, preterm infant formula, term infant formula, infant foods, maternal foods or geriatric foods. As examples of fat/oil-containing foods there may be mentioned natural fat/oil-containing foods such as meat, fish and nuts, foods to which oils or fats are added during preparation, such as soups, foods employing oils or fats as heating media, such as donuts, oils or fats foods such as butter, processed foods to which oils or fats are added during processing, such as cookies, or foods which are sprayed or coated with oils or fats upon finishing, such as hard biscuits. Such compositions may also be added to agricultural foods, fermented foods, livestock feeds, marine foods and beverages which contain no oils or fats. They may also be in the form of functional foods or pharmaceuticals, and for example, in processed form such as enteral nutrients, powders, granules, lozenges, oral solutions, suspensions, emulsions, syrups and the like.

A composition of the invention may also contain

various carriers or additives ordinarily used in foods and beverages, pharmaceuticals or quasi drugs, in addition to the active ingredient of the invention. Antioxidants are particularly preferred as additives to prevent oxidation of the active ingredient of the invention. As examples of antioxidants there may be mentioned natural antioxidants such as tocopherols, flavone derivatives, phyllodulcins, kojic acid, gallic acid derivatives, catechins, fukiic acid, gossypol, pyrazine derivatives, sesamol, guaiaol, guaiac acid, p-coumaric acid, nordihydroguaiaretic acid, sterols, terpenes, nucleotide bases, carotenoids, lignans and the like, and synthetic antioxidants including ascorbic palmitic acid esters, ascorbic stearic acid esters, butylhydroxyanisole (BHA), butylhydroxytoluene (BHT), mono-t-butylhydroquinone (TBHQ) and 4-hydroxymethyl-2,6-di-t-butylphenol (HMBP).

As tocopherols there may be mentioned α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, ε-tocopherol, ζ-tocopherol, η-tocopherol and tocopherol esters (tocopherol acetate and the like), as well as tocopherol analogs. As examples of carotenoids there may be mentioned β-carotene, cantaxanthine, astaxanthine and the like.

The composition of the invention may also contain, in addition to the active ingredient of the invention, supports such as carrier supports, extenders, diluents, bulking agents, dispersing agents, excipients, binder solvents (for example, water, ethanol and vegetable oils), dissolving aids, buffering agents, dissolving accelerators, gelling agents, suspending agents, wheat flour, rice flour, starch, corn starch, polysaccharides, milk protein, collagen, rice oil, lecithin and the like. As examples of additives there may be mentioned vitamins, sweeteners, organic acids, coloring agents, aromatic agents, moisture-preventing agents, fibers, electrolytes,

minerals, nutrients, antioxidants, preservatives, fragrances, humectants, natural food extracts, vegetable extracts and the like, although there is no limitation to these.

Arachidonic acid is the main active ingredient of the compound which is either arachidonic acid or comprises arachidonic acid as a constituent fatty acid. The daily intake of arachidonic acid from dietary sources has been reported to be 0.14 g in the Kanto region and 0.19-0.20 g in the Kansai region of Japan (Shishitsu Eiyougaku 4, 73, 1995), and in consideration of reduced oils or fats intake and reduced pancreatic lipase function in the elderly, a correspondingly greater amount of arachidonic acid must be ingested. Thus, the daily intake of the arachidonic acid or the compound comprising arachidonic acid as a constituent fatty acid according to the invention for an adult (for example, 60 kg body weight) is 0.001-20 g, preferably 0.01-10 g, more preferably 0.05-5 g and most preferably 0.1-2 g, based on the arachidonic acid content.

When the active ingredient of the invention is to be actually applied for a food or beverage product, the absolute amount of arachidonic acid in the product is an important factor. However, since the absolute amount added to foods and beverages will differ depending on the amount of consumption of those foods or beverages, triglycerides including a triglyceride wherein all or a portion of the constituent fatty acid is arachidonic acid may be added to food products in amounts of at least 0.001 wt%, preferably at least 0.01 wt% and more preferably at least 0.1 wt% in terms of arachidonic acid. For addition to food and beverage products of triglycerides having medium chain fatty acids bonded at the 1,3-positions and arachidonic acid bonded at the 2-position, the amount may be at least 0.0003 wt%, preferably at least 0.003 wt% and more preferably at least 0.03 wt%.

When the composition of the invention is to be used as a pharmaceutical, it may be produced according to a common method in the field of pharmaceutical preparation techniques, such as according to a method described in the Japanese Pharmacopeia or a similar method.

When the composition of the invention is to be used as a pharmaceutical, the content of the active ingredient in the composition is not particularly restricted so long as the object of the invention is achieved, and any appropriate content may be employed.

When the composition of the invention is to be used as a pharmaceutical, it is preferably administered in the form of an administrable unit, and especially in oral form. The dosage of the composition of the invention will differ depending on age, body weight, symptoms and frequency of administration, but for example, the arachidonic acid and/or compound including arachidonic acid as a constituent fatty acid according to the invention may be administered at about 0.001-20 g, preferably 0.01-10 g, more preferably 0.05-5 g and most preferably 0.1-2 g (as arachidonic acid) per day for adults (approximately 60 kg), either once a day or divided among multiple doses, such as three separate doses.

The major fatty acid components of phospholipid membranes in the brain are arachidonic acid and docosahexaenoic acid, and therefore from the standpoint of balance, a combination with docosahexaenoic acid is preferred. Also, since the proportion of eicosapentaenoic acid in brain phospholipid membranes is very small, a combination of arachidonic acid and docosahexaenoic acid containing virtually no eicosapentaenoic acid is especially preferred. Furthermore, the arachidonic acid/docosahexaenoic acid ratio in the combination of the arachidonic acid and docosahexaenoic acid is preferably in the range of 0.1-15, and more preferably in the range of 0.25-10. Also,

the amount of eicosapentaenoic acid in the food or beverage preferably does not exceed 1/5 of the arachidonic acid (weight ratio).

EXAMPLES

The present invention will now be explained in greater detail by the following examples, with the understanding that the invention is not limited to these examples.

Example 1 Method for production of arachidonic acid-containing triglycerides

Mortierella alpina CBS754.68 was used as the arachidonic acid-producing strain. After preparing 6 kL of medium containing 1.8% glucose, 3.1% defatted soybean powder, 0.1% soybean oil, 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2 \cdot 2H_2O$ and 0.05% $MgCl_2 \cdot 6H_2O$ in a 10 kL culturing tank, the initial pH was adjusted to 6.0. A 30 L portion of the preculturing solution was transferred for 8 days of jar fermentor culturing under conditions with a temperature of 26°C, an airflow of 360 m^3/h and an internal pressure of 200 kPa. The stirring rate was adjusted to maintain a dissolved oxygen concentration of 10-15 ppm. Also, the glucose concentration was adjusted by the feeding culture method for a glucose concentration in the range of 1-2.5% in the medium up to the 4th day, with 0.5-1% maintained thereafter (where the percentage values are weight (W/V)%).

After completion of the culturing, the cells containing triglycerides having arachidonic acid as a constituent fatty acid were collected by filtration and drying, and the oils or fats portion was extracted from the collected cells by hexane extraction and subjected to dietary oils or fats purification steps (degumming, deoxidation, deodorization, decolorizing) to obtain 150 kg of arachidonic acid-containing triglycerides (triglycerides including a triglyceride wherein all or a portion of the constituent fatty acid is arachidonic