

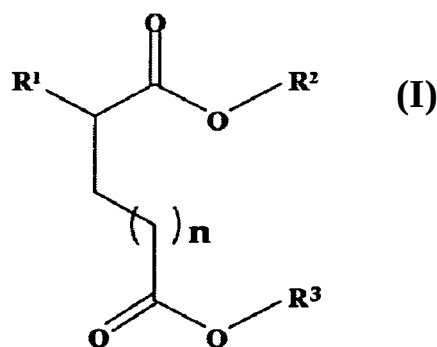


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- (71) Applicants (for all designated States except US):
FUKUOKA PREFECTURAL GOVERNMENT [JP/JP]; 7-7, Higashikoen, Hakata-ku, Fukuoka-shi, Fukuoka, 8128577 (JP). Kyoto University [JP/JP]; 36-1, Yoshidahonmachi, Sakyo-ku, Kyoto-shi, Kyoto, 6068501 (JP). DOJINDO LABORATORIES [JP/JP]; 2025-5, Tabaru, Mashikimachi, Kamimashiki-gun, Kumamoto, 8612202 (JP). ASTEC CO., LTD. [JP/JP]; 6-15, Minamizato 4-chome, Shimemachi, Kasuya-gun, Fukuoka, 8112207 (JP).

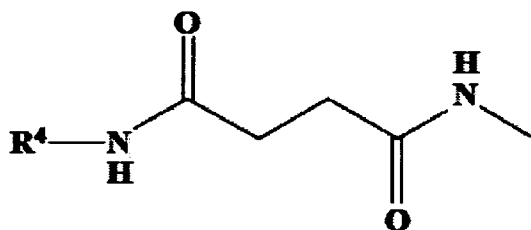
- (72) Inventors; and
(75) Inventors/Applicants (for US only): KUSUMOTO, Ken-ichi [JP/JP]; 147-1-602, Nonakamachi, Kurume-shi, Fukuoka, 8390862 (JP). HAMACHI, Itaru [JP/JP]; c/o Graduate School of Engineering, Kyoto University, Kyotodaigaku-katsura, Nishikyo-ku, Kyoto-shi, Kyoto, 6158510 (JP). SHIGA, Masanobu [JP/JP]; c/o DOJINDO LABORATORIES, 2025-5, Tabaru, Mashikimachi, Kamimashiki-gun, Kumamoto, 8612202 (JP). SAKAI, Takanori [JP/JP]; c/o ASTEC CO., LTD., 6-15, Minamizato 4-chome, Shimemachi, Kasuya-gun, Fukuoka, 8112207 (JP). IKEGAMI, Takashi [JP/JP]; c/o DOJINDO LABORATORIES, 2025-5, Tabaru, Mashikimachi, Kamimashiki-gun, Kumamoto, 8612202 (JP). TAKENAKA, Satoshi [JP/JP]; c/o DOJINDO LABORATORIES, 2025-5, Tabaru, Mashikimachi, Kamimashiki-gun, Kumamoto, 8612202 (JP). KANAZAWA, Eiichi [JP/JP]; 1-9-6-302, Daizenji Ohashi, Kurume-shi, Fukuoka, 8300079 (JP). ISHIKAWA, Tomoyuki [JP/JP]; 15-15-401, Goguchimachi, Higashi-ku, Fukuoka-shi, Fukuoka, 8120069 (JP). KOGA, Shintaro [JP/JP]; 1-6-24, Koyanose, Yahatanishiku, Kitakyushu-shi, Fukuoka, 8071261 (JP).

[Continued on nextpage]

- (54) Title: AGENT FOR PROMOTING GENE TRANSFER AND METHOD OF GENE TRANSFER USING THE SAME



(57) Abstract: Provided is a compound represented by the formula (I): wherein R^1 represents $R^5=N-$ (R^5 represents a saccharide residue) or (R^4 represents a mono- or polyethylene glycol residue); each of R^2 and R^3 , whether identical or not, represents a hydrocarbon group having 1-30 carbon atoms; and n represents 0 or 1.



(74) **Agent:** TAKASHIMA, Hajime; Meiji Yasuda Seimei
Osaka Midosuji Bldg., 1-1, Fushimimachi 4-chome,
Chuo-ku, Osaka-shi, Osaka, 5410044 (JP).

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DESCRIPTION

AGENT FOR PROMOTING GENE TRANSFER AND METHOD OF GENE TRANSFER
USING THE SAME

Technical Field

5 [0001]

The present invention relates to a glycolipid and an ethylene glycol lipid capable of promoting introduction of a compound of interest such as a nucleic acid into cells, a lipofection carrier comprising the glycolipid or the ethylene
10 glycol lipid, and a method of introducing a compound of interest into cells using the carrier and the like.

Background Art

[0002]

15 In recent years, against the background of the advances in life science research, proteins for biopharmaceuticals have been growing remarkably as a solution to a lack of new drugs developed by major pharmaceutical companies in Japan, the EU and the USA. The dramatic expansion of proteins for
20 biopharmaceuticals is thanks to the rapid progress of the establishment of groundbreaking therapies with pharmaceuticals comprising a monoclonal antibody, cytokine, functionally modified protein or the like. Many of these biopharmaceuticals have been obtained by artificially mass-producing recombinants
25 of bioactive proteins playing key roles in living organisms, by using mammalian cells. However, there are some technical problems to be solved in developing and producing biopharmaceuticals .

[0003]

30 While bioactive proteins that occur only in trace amounts in living organisms can be produced using a production system based on genes that serve as blue prints and mammalian cells, microorganisms, insect cells and the like, the importance of production systems based on mammalian cells is increasing due
35 to the growing needs for the production of proteins with

improved sugar chain structures or functional regions, with advances in the recent years in biopharmaceuticals. However, production systems based on mammalian cells, which are higher organisms, pose some problems to be solved, including
5 difficulty with establishing a long-spanning continuous manufacturing process and relatively high manufacturing cost, compared with those based on microorganisms or insect cells.
[0004]

Because mammalian cells are normally unable to stably
10 maintain the expression level of any gene exogenously introduced, a cell clone maintaining a preferred expression level cannot be acquired without performing selection culture for a long time. In many cases, selection culture for 3 to 6 months is needed until cells that stably produce a protein at
15 high levels are obtained. Therefore, to efficiently produce a protein in a short time using mammalian cells, it is important to establish a technology for efficiently introducing a particular gene into a large number of cells at one time.
[0005]

20 A wide variety of techniques are available for transferring a particular gene into mammalian cells (gene transfer technology). Known such methods include the lipofection method, wherein a gene is allowed to be incorporated in cells by phagocytosis or membrane fusion using
25 a lipid molecule, the virus method, wherein cells are infected using a retrovirus and the like, the electroporation method, wherein a small pore is electrically made in the cell surface, the microinjection method, wherein a gene is directly injected using a glass capillary, the particle gun method, wherein a
30 gene is fired into cells using microparticles such as of gold, and the ionic polymer association method, wherein a complex comprising a polyarginine, a polylysine, a virus-derived peptide, a nuclear localization peptide or the like is allowed to be incorporated by endocytosis. Generally, regarding gene
35 transfer technology for producing proteins for

biopharmaceuticals, the lipofection method and the electroporation method are chosen since the material used does not exhibit infectivity or pathogenicity, and also since a reasonable number of cells can be accommodated.

5 [0006]

The lipofection method poses the problems of low gene transfer efficiency and high product prices. Many of the existing reagents (gene transfer reagents) that have been developed and brought into practical applications to date are
10 intended for cultivation on small scales of up to 10 ml, with little reagents intended for gene transfer into a large number of cells on a liter scale. Furthermore, as the situation stands in the production of proteins for biopharmaceuticals, because of the use of a totally synthesized medium consisting
15 of a chemically defined ingredient of non-animal origin that does not exhibit infectivity or pathogenicity, the capacities of lipofection reagents that have been developed for use in basal media or serum media are insufficient to allow the use of a totally synthesized medium. Although some gene transfer
20 reagents intended for liter-scale production of biopharmaceutical proteins are available, their capacity of introduction is about 50% and they are expensive. In lipofection using a totally synthesized medium, alterations of cell membrane surface structure and medium components can
25 interfere with the lipofection.

[0007]

Although the electroporation method is unlikely to undergo the influences of medium components on the introduction efficiency, it poses the problems of difficulty
30 with introducing a gene into a large number of cells at one time, and of inducing cell death due to alterations of the structure of the cell membrane by electric pulses. Electroporation apparatuses that have been developed and brought into practical applications are designed to treat up
35 to 5×10^6 cells in one operation. Therefore, for treating 1×10^9

cells (1×10^6 cells/ml) to be used for gene transfer on the 1-liter cultivation scale, several hundreds of times of transfer operation are required, and this is unrealistic.

[0008]

5 The present inventors attempted to create lipid compounds that allow DNA or RNA to be introduced into a wide variety of mammalian cells, and have reported on a cationic lipid molecule and plasmid DNA transfection using the same [Non Patent Literature 1], gene transfer to normal neural stem
10 cells by a film-like self-organized lipid molecule [Non Patent Literature 2], a cationic lipid molecule and siRNA transfection using the same [Non Patent Literature 3], and a protein production system based on transfection [Non Patent Literature 4].

15 [0009]

Furthermore, the present inventors have searched their own cationic lipid repertoire (Patent Literature 1) and discovered a compound suitable for introduction of DNA (Patent Literature 2), a compound suitable for introduction of siRNA
20 (Patent Literature 3), a glycolipid molecule that improves lipofection efficiency (Patent Literature 4), and a peptide lipid that allows a compound to be introduced with high efficiency (Patent Literature 5).

25 Citation List

Patent Literature

[0010]

[PTL 1] JP1984767 B

[PTL 2] W02005/054486 A1

30 [PTL 3] JP2006-158314 A

[PTL 4] JP2006-254877 A

[PTL 5] W02007/099650 A1

Non Patent Literature

[0011]

35 [NPL 1] K. Kusumoto, T. Akao, E. Mizuki, and O. Nakamura.

Gene Transfer Effects on Various Cationic Amphiphiles in CHO Cells. Cytotechnology 51(2), 57-66 (2006)

[NPL 2] K. Kusumoto, S. Yamashita, T. Nagata, T. Ido, I. Hamachi, and T. Akao. Thin-film Assembly of Diethanolamine-based Lipidic Material as Potential Gene Carrier in Mouse Embryonic Neural Stem Cells. Journal of Biomedical Materials Research Part A 91(1), 1-10 (2009)

[NPL 3] K. Kusumoto. Structural Features of Glutamate-based Lipidic Materials for Small Interfering RNA Delivery System. Journal of Biomedical Materials Research Part A 89(3), 739-750 (2009)

[NPL 4] K. Kusumoto, T. Nagata, E. Kanazawa, T. Sakai, Y. Yoshikawa, T. Ogata, N. Nakatani, T. Ishikawa, S. Koga, A. Shirasu, S. Morishita, T. Emura, and I. Hamachi. Transfection system using a cocktail of totally designed lipidic materials in Chinese hamster ovary cells. Animal Cell Technology: Basic & Applied Aspects 16, 444-450 (2010)

Summary of Invention

20 Technical Problem

[0012]

It is an object of the present invention to provide a novel lipid compound carrier capable of promoting introduction of a compound such as a nucleic acid into cells conveniently and safely without using special equipment, and a method of introducing a compound into cells using the carrier.

Solution to Problem

[0013]

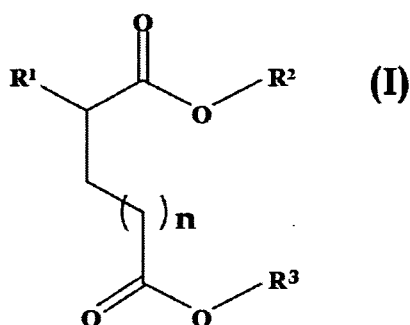
30 The present inventors conducted extensive investigations to accomplish the above-described object, and discovered, in a repertoire of independently designed and synthesized glycolipid compounds and ethylene glycol lipid compounds, a compound that increases the transfection efficiency when mixed in a complex of a nucleic acid (plasmid DNA/siRNA) and a

cationic lipid or peptide lipid. The present inventors conducted further investigations based on these findings, and have developed the present invention.

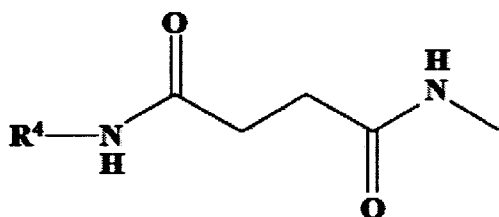
[0014]

5 Accordingly, the present invention relates to the following:

(1) A compound represented by the formula (I) :



wherein R^1 represents $R^5=N-$ (wherein R^5 represents a saccharide
10 residue) or

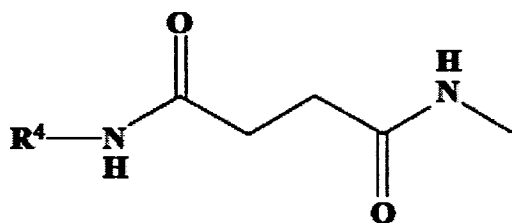


(wherein R^4 represents a mono- or polyethylene glycol residue);
each of R^2 and R^3 , whether identical or not, represents a
hydrocarbon group having 1 to 30 carbon atoms; and

15 n is 0 or 1.

(2) The compound according to (1), wherein R^1 is $R^5=N-$ and R^5 is
a monosaccharide or disaccharide residue.

(3) The compound according to (1), wherein R^1 is,



20 and R^4 is a group represented by $-CH_2CH_2(OCH_2CH_2)_m-OH$ (wherein m
represents an integer of 0 to 20) .

(4) The compound according to (1), wherein each of R² and R³ is a C₆₋₂₀ linear alkyl group or linear unsaturated hydrocarbon group.

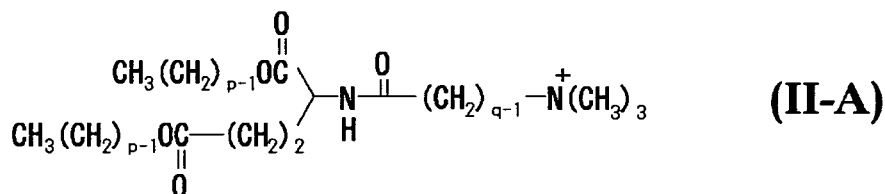
(5) The compound according to (1), wherein the compound is
 5 Glc-EC12, Glc-EC6, Glc-EC8, Glc-EC10, Glc-EC14, Gal-EC12, Man-EC[^], A11-EC12, Fuc-EC12, Xyl-EC12, GlcNAc-EC12, 2EG-EC10, 2EG-EC12, 4EG-EC10, 4EG-EC12, 2EG-EC14, or 4EG-EC14.

(6) An agent for promoting introduction of a compound of interest into cells, comprising the compound according to any
 10 one of (1) to (5).

(7) A composition comprising the compound according to any one of (1) to (5) and a lipid having an activity to introduce a compound of interest into cells.

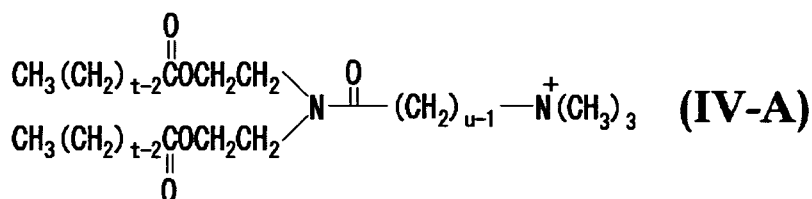
(8) The composition according to (7), wherein the lipid is a
 15 cationic lipid or a peptide lipid.

(9) The composition according to (7), wherein the lipid is a compound represented by the formula (II-A):



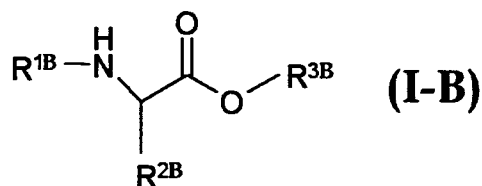
wherein p represents an integer of 12 to 16, and q represents
 20 an integer of 2 to 11;

a compound represented by the formula (IV-A):



wherein t represents an integer of 12 to 16, and u represents an integer of 2 to 11; or

25 a compound represented by the formula (I-B):



wherein R^{1B} represents an amino acid or peptide having 1 to 10 amino acid residues, R^{2B} represents an optionally chosen amino acid side chain, R^{3B} represents a hydrocarbon group having 1 to 30 carbon atoms, and with the provision that when R^{2B} has a carboxyl group, the carboxyl group may be esterified with a saturated or unsaturated alcohol having 1-30 carbon atoms.

(10) The composition according to (9), wherein the lipid is TMA-EC12, TMA-EC14, TMA-DEAC12, TMA-DEAC14, R-EC12, R-EC14, K-EC12, or K-EC14.

(11) The composition according to (7), wherein the composition is for introducing a compound of interest into cells.

(12) The composition according to (11), wherein the compound of interest is a nucleic acid.

(13) The composition according to (12), wherein the nucleic acid is a plasmid DNA, cDNA or antisense DNA, or an siRNA, miRNA, shRNA, mRNA, antisense RNA or RNA replicon.

(14) The composition according to (7), wherein the composition further comprises the compound of interest.

(15) A method of introducing a compound of interest into cells, comprising contacting the composition according to (14) and the cells.

(16) A method of introducing a compound of interest into cells in a human or non-human subject, comprising administering the composition according to (14) to the subject.

(17) A kit for introducing a compound of interest into cells, comprising the compound according to any one of (1) to (5), and a lipid having an activity to introduce the compound of interest into cells.

(18) A complex comprising the compound according to any one of (1) to (5), a lipid having an activity to introduce a compound of interest into cells, and the compound of interest.

Advantageous Effects of Invention

[0015]

It is possible to increase the efficiency of introduction

of a compound into cells by a lipid using the compound of the present invention. Using the compound of the present invention also makes it possible to introduce a compound of interest into cells with high efficiency even under conditions of basal
5 medium, serum medium, or totally synthesized medium. The compounds of the present invention are superior in safety because of the absence of infectious or pathogenic materials such as animal-derived ingredients and virus-derived ingredients .

10

Brief Description of Drawings

[0016]

Fig. 1 shows Mass Spectrum data of Glc-EC12.

[0017]

15

Fig. 2 shows Mass Spectrum data of Gal-EC12.

[0018]

Fig. 3 shows Mass Spectrum data of Man-EC12 .

[0019]

Fig. 4 shows Mass Spectrum data of A11-EC12.

20 [0020]

Fig. 5 shows Mass Spectrum data of Fuc-EC12.

[0021]

Fig. 6 shows Mass Spectrum data of Xyl-EC12.

[0022]

25

Fig. 7 shows Mass Spectrum data of GlcNAc-EC12.

[0023]

Fig. 8 shows Mass Spectrum data of Glc-EC6.

[0024]

Fig. 9 shows Mass Spectrum data of Glc-EC8 .

30 [0025]

Fig. 10 shows Mass Spectrum data of Glc-EC10.

[0026]

Fig. 11 shows Mass Spectrum data of Glc-EC14.

[0027]

35

Fig. 12 shows Mass Spectrum data of 2EG-EC10.

[0028]

Fig. 13 shows Mass Spectrum data of 4EG-EC10.

[0029]

Fig. 14 shows Mass Spectrum data of 2EG-EC12.

5 [0030]

Fig. 15 shows Mass Spectrum data of 4EG-EC12 .

[0031]

Fig. 16 shows Mass Spectrum data of 2EG-EC14 .

[0032]

10 Fig. 17 shows Mass Spectrum data of 4EG-EC14.

[0033]

Fig. 18 shows results of an experiment of introduction of plasmid DNA (pCMVIE-GFP vector) into CHO cells using various glycolipids and cationic lipids. The vertical axis indicates
15 the ratio (%) of the number of transfected and GFP-expressing cells to the total cell count.

[0034]

Fig. 19 shows results of an experiment of introduction of plasmid DNA (pCMVIE-GFP vector) into CHO cells using various
20 glycolipids and cationic lipids. The vertical axis indicates the ratio (%) of the number of transfected and GFP-expressing cells to the total cell count.

[0035]

Fig. 20 shows results of an experiment of introduction of
25 plasmid DNA (pCMVIE-GFP vector) into CHO cells using various ethylene glycol lipids and cationic lipids. The vertical axis indicates the ratio (%) of the number of transfected and GFP-expressing cells to the total cell count.

[0036]

30 Fig. 21 shows effects of various glycolipids and cationic lipids on the introduction of siRNA into CHO-EGFP cells, wherein the bar graph shows the relative suppression rate (%) of the expression of EGFP.

[0037]

35 Fig. 22 shows results of an experiment of introduction of

plasmid DNA (pCMVIE-GFP vector) into CHO cells using various glycolipids and cationic lipids. The vertical axis indicates plasmid DNA introduction efficiency as values relative to the introduction efficiency of 1 obtained by introducing each

5 cationic lipid alone.

[0038]

Fig. 23 shows results of an experiment of introduction of plasmid DNA (pCMVIE-GFP vector) into A549 cells using Glc-EC12 and TMA-EC12. The vertical axis indicates plasmid DNA

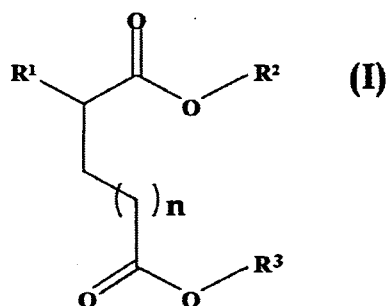
10 introduction efficiency as values relative to the introduction efficiency of 1 obtained by introducing TMA-EC12 alone.

Description of Embodiments

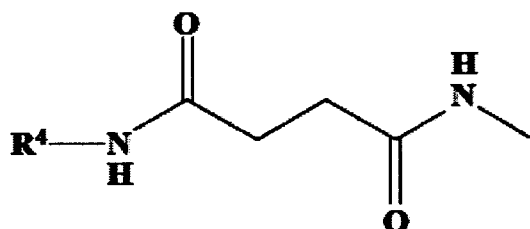
[0039]

15 1. Compound that promotes introduction of compound into cells by lipid

The present invention provides a compound represented by the formula (I) :



20 wherein R^1 represents $\text{R}^5\text{=N-}$ (wherein R^5 represents a saccharide residue) or



(wherein R^4 represents a mono- or polyethylene glycol residue) ; each of R^2 and R^3 , whether identical or not, represents a

hydrocarbon group having 1 to 30 carbon atoms; and
n represents 0 or 1.

[0040]

In the compounds of the present invention, a head moiety
5 containing a saccharide or ethylene glycol and a tail moiety
containing a hydrocarbon chain are joined via a connector
moiety containing glutamic acid or aspartic acid. Hence, R⁴
and R⁵ correspond to the head moiety, and R¹ corresponds to the
tail moiety.

10 [0041]

The term "hydrocarbon group" used herein includes
hydrocarbon groups having 1-30 carbon atoms, for example,
"alkyl group", "cycloalkyl group", "alkenyl group",
"cycloalkenyl group", "alkynyl group", "aryl group", "aralkyl
15 group", "cycloalkylalkyl group" and the like. These
hydrocarbon groups may be substituted with one or more
suitable substituents. Examples of such substituents include,
but are not limited to, Ci-C₆ alkyl, Ci-C₆ alkenyl, Ci-C₆ alkynyl,
C₆ aryl, C₂-C₅ heteroaryl, C₃-C₆ cycloalkyl, Ci-C₆ alkoxy, CN, OH,
20 oxo, halo, COOH, NH₂, NH (C₁-C₆ alkyl), N (C₁-C₆ alkyl)₂, NH(C₆
aryl), N (C₆ aryl)₂, CHO, CO(Ci-C₆ alkyl), CO(C₆ aryl), COO(Ci-C₆
alkyl), COO(C₆ aryl), and the like.

[0042]

Examples of "alkyl group" include, but are not limited
25 to, "unbranched or branched ci-₃₀ alkyl groups" such as methyl,
ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-
butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl,
dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl,
heptadecyl, octadecyl, nonadecyl, icosanyl, henicosanyl,
30 docosanyl, tricosanyl, tetracosanyl, pentacosanyl, hexacosanyl,
heptacosanyl, octacosanyl, nonacosanyl, triacontyl and the
like.

[0043]

Examples of "cycloalkyl group" include, but are not
35 limited to, "C₃₋₈ cycloalkyl groups" such as cyclopropyl,

cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl and the like.

[0044]

Examples of "alkenyl group" include, but are not
5 limited to, "unbranched or branched C_{2-30} alkenyl groups" such
as vinyl, allyl, isopropenyl, 1-butenyl, 2-butenyl, 3-butenyl,
pentenyl, hexenyl, heptenyl, octenyl, nonenyl, decenyl,
undecenyl, dodecenyl, tridecenyl, tetradecenyl, pentadecenyl,
hexadecenyl, heptadecenyl, octadecenyl, nonadecenyl, icosenyl,
10 heneicosenyl, docosenyl, tricosenyl, tetracosenyl, pentacosenyl,
hexacosenyl, heptacosenyl, octacosenyl, nonacosenyl,
triacontenyl and the like.

[0045]

Examples of "cycloalkenyl group" include, but are not
15 limited to, " C_{3-8} cycloalkenyl groups" such as cyclopropenyl,
cyclobutenyl, cyclopentenyl, cyclohexenyl, cycloheptenyl,
cyclooctenyl and the like.

[0046]

Examples of "alkynyl group" include, but are not
20 limited to, "unbranched or branched C_{2-30} alkynyl groups" such
as ethynyl, propynyl, 1-butyne, 2-butyne, 1-pentyne, 2-
pentyne, 3-pentyne, hexynyl, heptyne, octyne, nonyne,
decynyl, undecynyl, dodecynyl, tridecynyl, tetradecynyl,
pentadecynyl, hexadecynyl, heptadecynyl, octadecynyl,
25 nonadecynyl, icosynyl, heneicosynyl, docosynyl, tricosynyl,
tetracosynyl, pentacosynyl, hexacosynyl, heptacosynyl,
octacosynyl, nonacosynyl, triacontynyl and the like.

[0047]

Examples of "aryl group" include, but are not limited
30 to, " C_{6-14} aryl groups" such as phenyl, 1-naphthyl, 2-naphthyl,
phenanthryl, anthryl and the like.

[0048]

Examples of "aralkyl group" include, but are not
limited to, " C_{7-30} aralkyl group (i.e., C_{6-24} aryl- C_{1-6} alkyl
35 group)" such as benzyl, phenethyl, 3-phenylpropyl, 4-

phenylbutyl, (1-naphthyl) methyl, 2- (1-naphthyl) ethyl, 2- (2-naphthyl) ethyl and the like.

[0049]

Examples of "cycloalkylalkyl group" include, but are
5 not limited to, "C₃₋₈ cycloalkyl-C₁₋₆ alkyl groups" such as
cyclopropylmethyl, cyclobutylmethyl, cyclopentylmethyl,
cyclohexylmethyl, cycloheptylmethyl, cyclooctylmethyl, 2-
cyclopropylethyl, 2-cyclobutylethyl, 2-cyclopentylethyl, 2-
cyclohexylethyl, 2-cycloheptylethyl, 2-cyclooctylethyl and the
10 like.

[0050]

R² and R³ are preferably unbranched saturated
hydrocarbon groups having 6-20 carbon atoms (i.e., n-hexyl, n-
heptyl, n-octyl, n-nonyl, n-decyl, n-undecyl, n-dodecyl, n-
15 tridecyl, n-tetradecyl, n-pentadecyl, n-hexadecyl, n-
heptadecyl, n-octadecyl, n-nonadecyl, n-eicosanyl, n-
heneicosanyl, n-docosanyl, n-tricosanyl, n-tetracosanyl, n-
pentacosanyl, n-hexacosanyl, n-heptacosanyl, n-octacosanyl, n-
nonacosanyl and n-triacontyl) or unbranched unsaturated
20 hydrocarbon groups (e.g., mono-unsaturated hydrocarbon groups
such as trans-2-buten-1-yl, cis-9-tetradecen-1-yl, cis-9-
hexadecen-1-yl, cis-9-octadecen-1-yl, cis-11-octadecen-1-yl,
cis-9-eicosaen-1-yl, cis-13-docosaen-1-yl and cis-15-
tetracosaen-1-yl, di-unsaturated hydrocarbon groups such as
25 cis-9-cis-12-octadecdien-1-yl, tri-unsaturated hydrocarbon
groups such as cis-9-cis-12-cis-15-octadectrien-1-yl and cis-
9-cis-11-cis-13-octadectrien-1-yl, tetra-unsaturated
hydrocarbon groups such as cis-4-cis-8-cis-12-cis-15-
octadectetraen-1-yl and cis-5-cis-8-cis-11-cis-14-
30 eicosatetraen-1-yl, penta-unsaturated hydrocarbon groups such
as cis-7-cis-10-cis-13-cis-16-cis-19-docosapentaen-1-yl, hexa-
unsaturated hydrocarbon groups such as cis-4-cis-7-cis-10-cis-
13-cis-16-cis-19-docosahexaen-1-yl and the like). More
preferably, R² and R³ are unbranched alkyl having 6-14 carbon
35 atoms (i.e., n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decyl, n-

undecyl, n-dodecyl, n-tridecyl, n-tetradecyl) or unbranched unsaturated hydrocarbon (e.g., cis-9-tetradecen-1-yl, cis-9-hexadecen-1-yl, cis-9-octadecen-1-yl, cis-9-octadecen-1-yl, cis-11-octadecen-1-yl, cis-9-eicosaen-1-yl, cis-13-docosaen-1-yl, cis-9-cis-12-octadecdien-1-yl, cis-9-cis-12-cis-15-octadectrien-1-yl, cis-9-cis-11-cis-13-octadectrien-1-yl, cis-4-cis-8-cis-12-cis-15-octadectetraen-1-yl, cis-5-cis-8-cis-11-cis-14-eicosatetraen-1-yl, cis-7-cis-10-cis-13-cis-16-cis-19-docosapentaen-1-yl, cis-4-cis-7-cis-10-cis-13-cis-16-cis-19-docosa-hexaen-1-yl and the like) groups having 6-14 carbon atoms .

[0051]

Further preferably, R^2 and R^3 are unbranched alkyl having 6-14 carbon atoms (i.e., n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decyl, n-undecyl, n-dodecyl, n-tridecyl, n-tetradecyl) .

[0052]

R^2 is preferably a group identical to R^3 .

[0053]

The R^4 mono- or polyethylene glycol residue is exemplified by groups represented by $-\text{CH}_2\text{CH}_2(\text{OCH}_2\text{CH}_2)_m-\text{OH}$ (wherein m represents an integer of 0 to 20, preferably an integer of 0 to 15, more preferably an integer of 0 to 10, still more preferably an integer of 0 to 7, yet still more preferably an integer of 1 to 5). Examples of mono- or polyethylene glycols include ethylene glycol, diethylene glycol, triethylene glycol, tetraethylene glycol, pentaethylene glycol, hexaethylene glycol, heptaethylene glycol, octaethylene glycol, nonaethylene glycol, decaethylene glycol, undecaethylene glycol, dodecaethylene glycol, tridecaethylene glycol, tetradecaethylene glycol, pentadecaethylene glycol, hexadecaethylene glycol, heptadecaethylene glycol, octadecaethylene glycol, nonadecaethylene glycol, eicosaethylene glycol, polyethylene glycol and the like.

[0054]

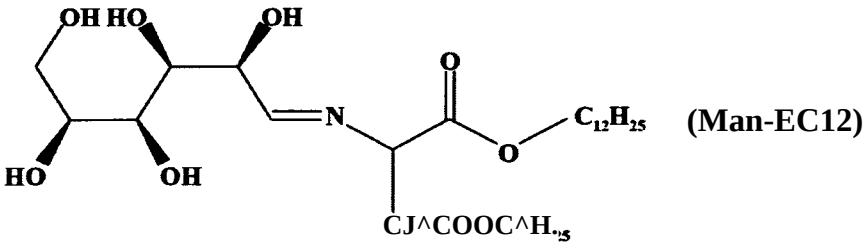
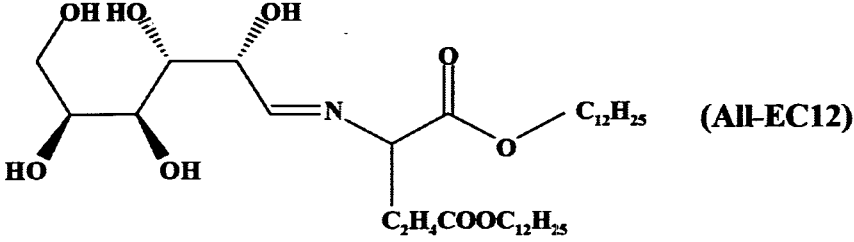
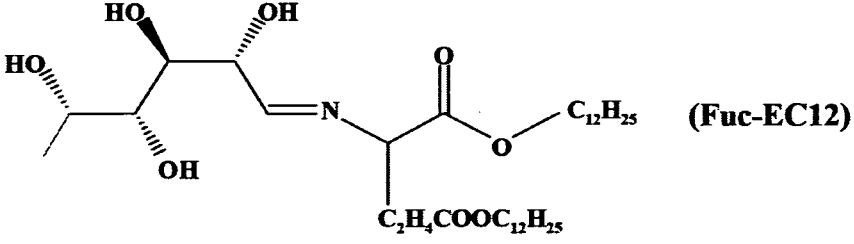
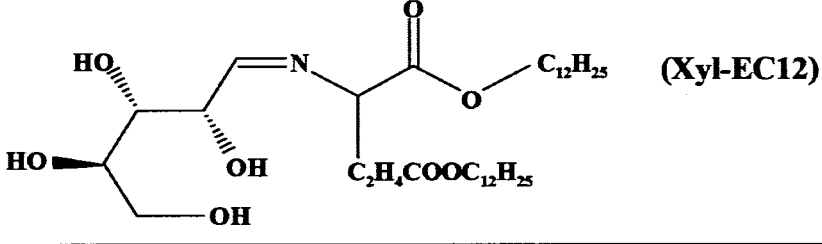
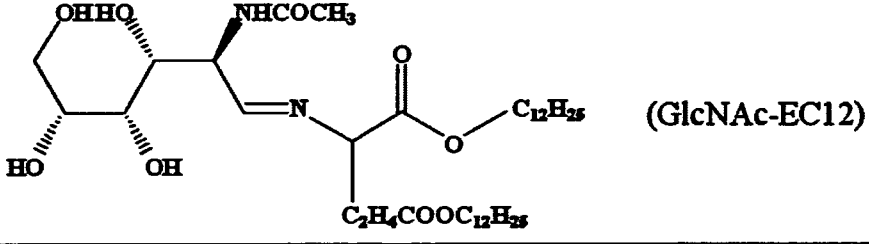
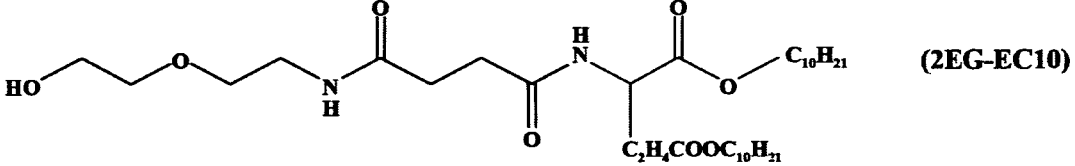
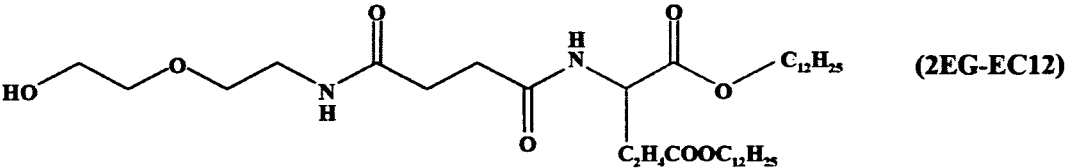
The R⁵ saccharide residue is a group resulting from elimination of oxygen atoms from the aldehyde group present in the saccharide of the open-ring structure. This saccharide may be any saccharide having an aldehyde group when assuming the open-ring structure, and is exemplified by monosaccharides (aldoses), saccharides comprising two or more monosaccharides bound via glycosidic bond, such as disaccharides, trisaccharides, tetrasaccharides, oligosaccharides, and polysaccharides. Monosaccharides (aldoses) include glucose, galactose, mannose, allose, talose, gulose, altose, idose, glyceraldehyde, erythrose, threose, ribose, lyxose, xylose, arabinose, deoxyribose, fucose, rhamnose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine, N-acetylneuraminic acid, N-glycolylneuraminic acid and the like. Disaccharides include sucrose, lactose, maltose, trehalose, turanose, cellobiose and the like. Trisaccharides include raffinose, melezitose, maltotriose and the like. Tetrasaccharides include acarbose, stachyose and the like. Oligosaccharides include fructooligosaccharides, galactooligosaccharides, mannanooligosaccharides and the like. The saccharide is preferably a monosaccharide (aldose) or a disaccharide. Preferred monosaccharides (aldoses) include glucose, galactose, mannose, allose, fucose, xylose, N-acetylglucosamine and the like. Preferred disaccharides include lactose, cellobiose and the like.

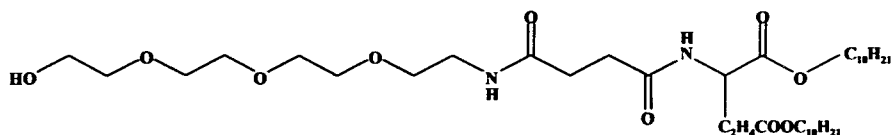
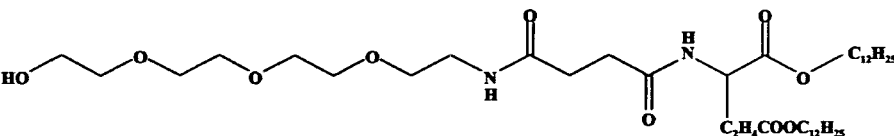
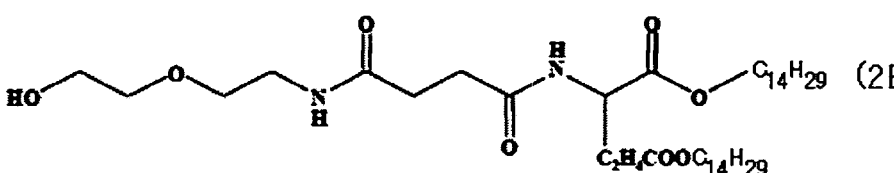
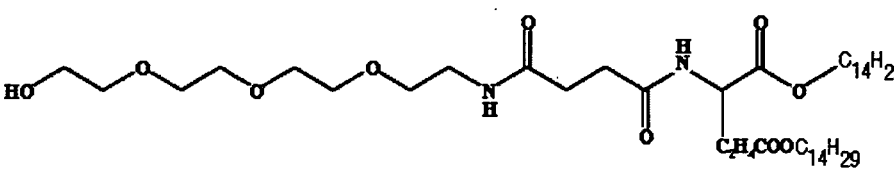
[0055]

Examples of suitable compounds of the present invention include the compounds shown below:

Table 1

	Structure
1	(Glc-EC12)
2	(Glc-EC6)
3	(Glc-EC8)
4	(Glc-EC10)
5	(Glc-EC14)
6	(Gal-EC12)

7	 (Man-EC12)
8	 (All-EC12)
9	 (Fuc-EC12)
10	 (Xyl-EC12)
11	 (GlcNAc-EC12)
12	 (2EG-EC10)
13	 (2EG-EC12)

14		(4EG-EC10)
15		(4EG-EC12)
16		(2EG-EC14)
17		(4EG-EC14)

[0056]

Of the compounds listed in the table above, $-C_6H_{13}$ contained in Glc-EC6; $-C_8H_{17}$ contained in Glc-EC8; $-C_{10}H_{21}$ contained in Glc-EC10, 2EG-EC10 and 4EG-EC10; $-C_{12}H_{25}$ contained in Glc-EC12, Gal-EC12, Man-EC12, A11-EC12, Fuc-EC12, Xyl-EC12, GlcNAc-EC12, 2EG-EC12 and 4EG-EC12; and $-C_{14}H_{29}$ contained in Glc-EC14, 2EG-EC14 and 4EG-EC14 are all linear alkyl groups.

[0057]

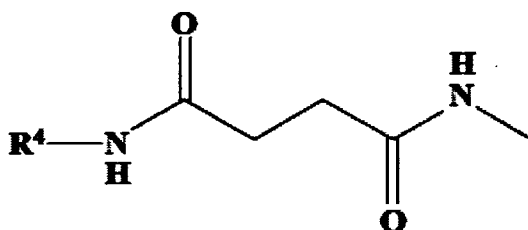
The compound of the present invention can be produced using a combination of a publicly known method of peptide synthesis and esterification. The compound of the present invention can be produced by condensing the acidic amino acid and the remaining moiety, and, if the product has a protecting group, removing the protecting group. For example, the α -carboxyl group and β -carboxyl group of the acidic amino acid (aspartic acid or glutamic acid) at the connector moiety is condensed with a desired alcohol (R^2 -OH and R^3 -OH) to yield an amino acid ester, after which a saccharide corresponding to the R^5 saccharide residue is bound to the amino group in the amino acid ester to give the compound of the present invention wherein

R^1 is $R^5=N-$ (wherein R^5 represents a saccharide residue) .

Alternatively a saccharide corresponding to the R^5 saccharide residue is bound to the amino group in the amino acid (aspartic acid or glutamic acid) , then the obtained product is further
5 condensed with an alcohol.

[0058]

Alternatively, the above-described amino acid ester is mixed with succinic anhydride and N,N-diisopropylethylamine in an appropriate inert organic solvent (e.g. chloroform) to give
10 a condensed product. The condensed product is reacted with N-hydroxysuccinimide and 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride in an appropriate inert organic solvent (e.g. dimethyl formamide) . Then, monoethylene glycol-monoamine or polyethylene glycol-
15 monoamine is added to the obtained reaction mixture to give the compound of the present invention, wherein R^1 is the group represented by the following formula:



[0059]

20 Condensation and removal of the protecting group can be achieved by methods known per se, for example, the methods described in (i) and (ii) below.

(i) M. Bodanszky and M.A. Oridetti: Peptide Synthesis, Interscience Publishers, New York (1966)

25 (ii) Schroeder and Luebke: The Peptide, Academic Press, New York (1965)

[0060]

Specifically, for example, the compound can be synthesized according to the method disclosed in Kanegae and
30 Akao ("Design and Synthesis of the Artificial Peptide-Lipids

Having Adamant ane Group", in *The Research Reports of Fukuoka Industrial Technology Center in 1998*, pp. 113-116) .

[0061]

2. Agent for promoting the introduction of a compound of
5 interest into cells by a lipid

The compound of the present invention has an effect to promote the introduction of a compound of interest into cells by a lipid. Accordingly, the present invention provides an agent for promoting the introduction of a compound of interest
10 into cells by a lipid, comprising the aforementioned compound of the present invention (the agent of the present invention) .

[0062]

The compound of interest can be any compound that can be introduced into cells by a lipid; examples include nucleic
15 acids, peptides, lipids, peptide lipids, sugars, bioactive substances, drugs [doxorubicin (antitumor drug), daunorubicin (antitumor drug), vincristine (antitumor drug), vinblastine (antitumor drug), idarubicin (antitumor drug), dibucaine (local anesthetic) , propranolol (β blocker) , quinidine
20 (antiarrhythmic therapeutic), dopamine (cardiotonic hypertensive), imipramine (antidepressant), diphenhydramine (antihistamine) , quinine (antimalarial) , chloroquine (antimalarial) , diclofenac (anti-inflammatory drug) and the like] , moisturizers for cosmetics and the like (mannitol and
25 the like), other synthetic or natural compounds and the like.

[0063]

Preferably, the compound of interest is a nucleic acid. Any nucleic acid can be used, whether it is a DNA, an RNA, a DNA-RNA chimeric nucleic acid, a DNA/RNA hybrid or the like.
30 While the nucleic acid may be single-stranded to triple-stranded, it is preferably single-stranded or double-stranded. The nucleic acid may be another type of nucleotide that is an N-glycoside of the purine or pyrimidine base, or another oligomer having a non-nucleotide skeleton (e.g., commercially
35 available peptide nucleic acid (PNA) and the like) or another

oligomer containing a special bond (however, the oligomer contains a nucleotide having an arrangement that allows base pairing or base attachment as found in DNA and RNA) and the like. Furthermore, the nucleic acid may also be one having a
5 known modification added thereto, for example, one with a marker known in the art, one with a cap, one methylated, one having one or more naturally occurring nucleotides substituted by analogues, one modified with an intramolecular nucleotide, for example, one having a non-charge bond (e.g.,
10 methylphosphonate, phosphotriester, phosphoramidate, carbamate and the like), one having a charged bond or a sulfur-containing bond (e.g., phosphorothioate, phosphorodithioate and the like), for example, one having a side chain group of a protein (nuclease, nuclease inhibitor, toxin, antibody, signal
15 peptide, poly-L-lysine and the like), a sugar (e.g., monosaccharide and the like) and the like, one having an intercalating compound (e.g., acridine, psoralen and the like), one containing a chelate compound (e.g., metals, radioactive metals, boron, oxidizing metals and the like), one containing
20 an alkylating agent, or one having a modified bond (e.g., a anomer type nucleic acid and the like) .

[0064]

Any kind of DNA can be chosen as appropriate according to the purpose of use; examples include plasmid DNA, cDNA,
25 antisense DNA, chromosome DNA, PAC, BAC and the like, with preference given to plasmid DNA, cDNA and antisense DNA, more preferably plasmid DNA. A circular DNA such as plasmid DNA can also be used as a linear DNA after being digested as appropriate with a restriction endonuclease and the like. Also,
30 any kind of RNA can be chosen as appropriate according to the purpose of use; examples include siRNA, miRNA, shRNA, antisense RNA, messenger RNA, single strand RNA genome, double strand RNA genome, RNA replicon, transfer RNA, ribosomal RNA and the like, with preference given to siRNA, miRNA, shRNA,
35 mRNA, antisense RNA and RNA replicon.

[0065]

The size of the nucleic acid is not subject to limitation; while a broad range of nucleic acids, from giant nucleic acid molecules (e.g., about 10^7 kbp in size) such as
5 chromosomes (artificial chromosome and the like) to low-molecular nucleic acids (e.g., about 5 bp in size), can be introduced, the size is preferably not more than 15 kbp in consideration of the efficiency of nucleic acid introduction into cells. For example, the size of a high-molecular nucleic
10 acid like plasmid DNA is, for example, 2 to 15 kbp, preferably 2 to 10 kbp. The size of a low-molecular nucleic acid like siRNA is, for example, 5 to 1000 bp, preferably 5 to 500 bp, and more preferably 5 to 200 bp.

[0066]

15 The nucleic acid may be a naturally-occurring one or a synthetic one; when the size is not more than about 100 bp, the nucleic acid can be synthesized by the phosphotriethyl method, the phosphodiester method and the like using a commonly used automated nucleic acid synthesizer.

20 [0067]

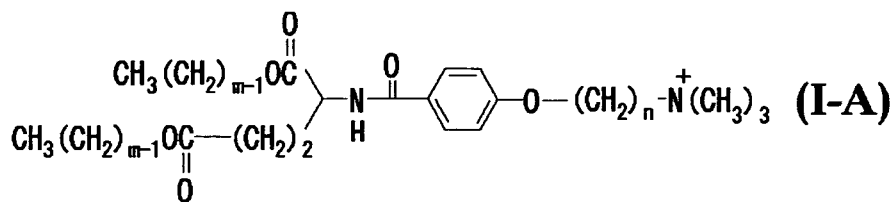
While the nucleic acid used in the present invention is not subject to limitation, it is preferably purified by a method commonly used by those skilled in the art.

[0068]

25 The lipid having an activity to introduce a compound of interest into cells is suitably a cationic lipid or a peptide lipid. A cationic lipid refers to a lipid containing in its molecular structure a moiety having a cationic charge in a polar solvent. The cationic lipid may be a molecule
30 constituted by one or more, typically two or more, fatty acid chains or hydrocarbon chains, and a group having a cationic charge. Groups having a cationic charge include quaternary ammonium groups, choline groups and the like. A peptide lipid refers to a lipid containing a peptide residue in its
35 molecular structure.

[0069]

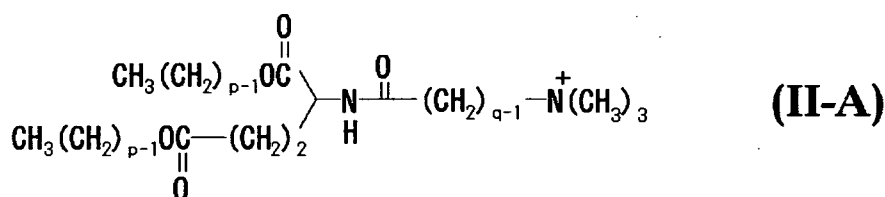
In an embodiment, preferred cationic lipids include, for example, a compound selected from the group consisting of a compound represented by the formula (I-A) :



5

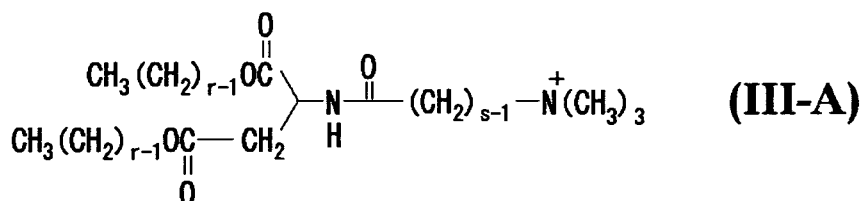
wherein m represents an integer of 12 to 16, and n represents an integer of 2 to 11;

a compound represented by the formula (II-A) :



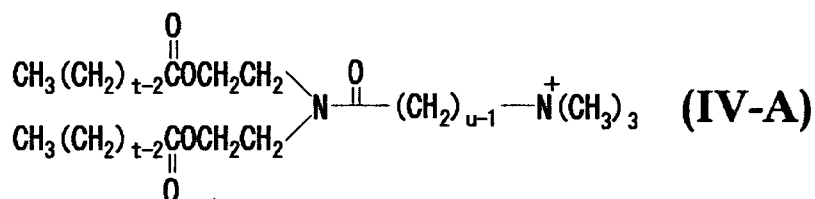
10 wherein p represents an integer of 12 to 16, and q represents an integer of 2 to 11;

a compound represented by the formula (III-A) :



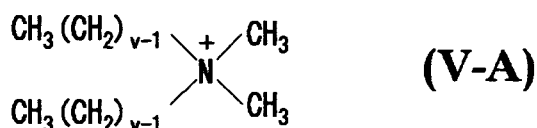
15 wherein r represents an integer of 12 to 16, and s represents an integer of 2 to 11;

a compound represented by the formula (IV-A) :



wherein t represents an integer of 12 to 16, and u represents an integer of 2 to 11; and

20 a compound represented by the formula (V-A) :



wherein v represents an integer of 12 to 16.

[0070]

In the formula (I-A), m is preferably 12 to 14, more preferably 12 or 14; and n is preferably 2 to 8, more preferably an integer selected from the group consisting of 2, 4, 6 and 8.

[0071]

In the formula (II-A), p is preferably 12 to 14, more preferably 12 or 14; and q is preferably 2 to 6, more preferably an integer selected from the group consisting of 2, 4 and 6.

[0072]

In the formula (III-A), r is preferably 12 to 14, more preferably 12 or 14; and s is preferably 2 to 6, more preferably an integer selected from the group consisting of 2, 4 and 6, still more preferably 2.

[0073]

In the formula (IV-A), t is preferably 12 to 14, more preferably 12 or 14, still more preferably 14; and u is preferably 2 to 6, more preferably an integer selected from the group consisting of 2, 4 and 6, still more preferably 2.

[0074]

In the formula (V-A), v is preferably an integer selected from the group consisting of 12, 14 and 16.

[0075]

The compounds represented by the aforementioned formulas (I-A) to (V-A) may have formed a salt with an appropriate monovalent anion. Anions include halogen ions (F^- , Cl^- , Br^- or I^-) and the like.

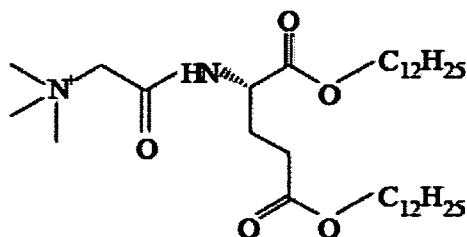
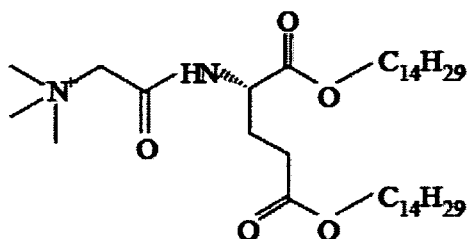
[0076]

For use in the present invention, out of the compounds represented by the formulas (I-A) to (V-A), those represented by the formula (II-A) or (IV-A) are more preferable.

[0077]

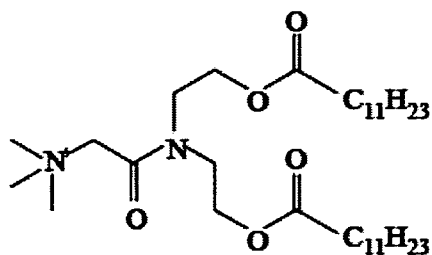
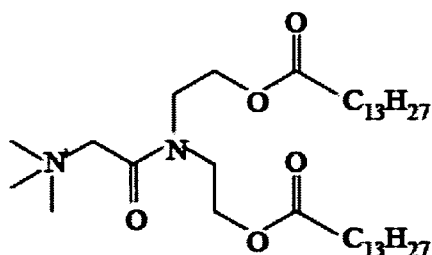
Examples of suitable compounds represented by the formula

(II-A) include TMA-EC12 and TMA-EC14 below.

**TMA-EC12****TMA-EC14**

[0078]

Examples of suitable compounds represented by the formula
5 (IV-A) include TMA-DEAC12 and TMA-DEAC14 below.

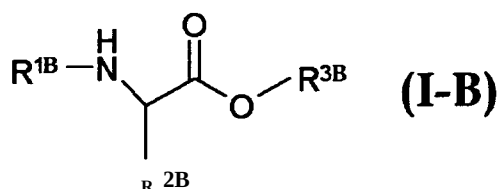
**TMA-DEAC12****TMA-DEAC14**

[0079]

The compounds represented by the formulas (I-A) to (V-A)
can be produced with reference to, for example, the
10 description in W02005/054486 and the like.

[0080]

In an embodiment, examples of preferable peptide lipids
include a compound represented by the following formula (I-B) :



[0081]

wherein $\text{R}^{1\text{B}}$ is an amino acid or peptide having 1-10 amino acid residues, $\text{R}^{2\text{B}}$ is a side chain of any amino acid, provided that
 5 $\text{R}^{2\text{B}}$ has a carboxyl group, the carboxyl group may be esterified with a saturated or unsaturated alcohol having 1-30 carbon atoms, and $\text{R}^{3\text{B}}$ is a hydrocarbon group having 1-30 carbon atoms.

[0082]

Preferably, $\text{R}^{1\text{B}}$ is an amino acid or peptide having 1-5
 10 amino acid residues. The amino acid constituting $\text{R}^{1\text{B}}$ may be 20 naturally-occurring amino acids (Gly, Ara, Leu, Ile, Val, Arg, Lys, Glu, Gln, Asp, Asn, Cys, Met, His, Pro, Phe, Tyr, Thr, Ser, Trp), or modified or nonnative amino acids (e.g., 2-aminoadipic acid, 3-aminoadipic acid, β -alanine, 2-aminobutyric
 15 acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, norvaline, norleucine, ornithine and the like). When the amino acid has a
 20 carboxyl group (or carboxylate) at a position other than the C-terminus, the carboxyl group may be amidated or esterified. As examples of the ester used in this case, C_{1-6} alkyl groups, for example, methyl, ethyl, n-propyl, isopropyl, n-butyl and the like; C_{3-6} cycloalkyl groups, for example, cyclopentyl, cyclohexyl and the like; C_{6-12} aryl groups, for example, phenyl,
 25 *a*-naphthyl and the like; phenyl- C_{1-2} alkyl groups, for example, benzyl, phenethyl and the like; C_{7-14} aralkyl groups, for example, *a*-naphthyl- C_{1-2} -alkyl groups such as *a*-naphthylmethyl; pivaloyloxymethyl groups; and the like can be mentioned.

[0083]

30 Furthermore, the amino group of the N-terminal amino acid or any constituting amino acid of $\text{R}^{1\text{B}}$ may be protected with a protecting group (for example, C_{1-6} acyl groups such as

ci-6 alkanoyls such as formyl group and acetyl group) ; and a substituent (for example, -OH, -SH, amino group, imidazole group, indole group, guanidino group and the like) on the side chain of the amino acid in a molecule may be protected with an appropriate protecting group (for example, ci-6 acyl groups such as ci-6 alkanoyl groups such as formyl group and acetyl group, and the like) .

[0084]

R^{1B} may be an unbranched or branched (dendrimer type) peptide. For example, when R^1 comprises an amino acid having an amino group on its side chain such as Arg and Lys, a branched chain can be formed by binding the amino group with a carboxyl group of other amino acid or peptide. Since a dendrimer type peptide can have two or more Arg/Lys at the N-terminus, it can be more positively charged and advantageous when, for example, the peptide directly binds to a nucleic acid or protein having a negative charge. Also, when R^{1B} comprises an amino acid having a carboxyl group on its side chain such as Glu and Asp, a branched chain can be formed by binding the carboxyl group with an amino group of other amino acid or peptide. Furthermore, when R^1 comprises Cys, a branched chain can be formed via a disulfide bond between the Cys and other Cys or a peptide comprising the same.

[0085]

In a preferable embodiment, R^{1B} can comprise a positively charged amino acid (e.g., Arg and Lys), in view that it can directly bind to a nucleic acid or protein having a negative charge.

[0086]

In another preferable embodiment, R^{1B} can comprise an amino acid having a thiol group in the side chain (e.g., Cys), in view that it can bind to a thiolated nucleic acid or protein via a disulfide bond. The disulfide bond is reduced within a cell, and a compound of interest, nucleic acid or protein can be easily released. In addition, the behavior of

the carrier itself in a tissue or cell and the structural change of the carrier when binding to a compound of interest such as nucleic acid can be observed by modifying the thiol group of Cys and the like with a fluorescent (e.g., FITC, 5 rhodamine, Cy3, etc.).

[0087]

In another preferable embodiment, R^{1B} can comprise an amino acid having an high affinity for a metal (e.g., Met and His), in view that it can bind to a nucleic acid and the like 10 modified with a metal (e.g., chelation).

[0088]

In another preferable embodiment, R^{1B} can comprise an amino acid having a hydroxyl group in the side chain (e.g., Thy, Thr and Ser), in view that it can bind to a functional 15 group in the side chain of a compound of interest, nucleic acid or protein via a hydrogen bond and the like.

[0089]

In another preferable embodiment, R^{1B} can comprise a negatively charged amino acid (e.g., Glu and Asp), in view 20 that it can bind to a nucleic acid modified with a nucleic acid-binding protein and the like such as histone, which has a net positive charge due to the binding protein.

[0090]

It is also preferable to use an amino acid other than 25 those mentioned above as appropriate. For example, a signal peptide for cell recognition, a neurotransmitter γ -aminobutyric acid (GABA) or the like can be utilized to improve the interaction between the carrier and a target cell.

[0091]

30 Preferably, R^{1B} comprises one or more residues of at least one amino acid selected from the group consisting of Arg, Lys, Cys, Met, His, Tyr, Glu and Asp. While these amino acids can be placed at any positions in R^{1B} as long as they can interact with a compound of interest, nucleic acid or protein, 35 or a target cell, it is desirable that at least one of them is

placed at N-terminus. Therefore, the N-terminal amino acid of R^{1B} is preferably either Arg, Lys, Cys, Met, His, Tyr, Glu or Asp, more preferably Arg or Lys.

[0092]

5 Examples of R^{2B} include the side chains of the naturally occurring amino acids or the modified or nonnative amino acids mentioned above for R^{1B} , preferably, amino acids having a carboxyl group on the side chain, for example, Glu and Asp. More preferably, the peptide lipid of the present invention is
10 a compound in which the carboxyl group on the side chain of the connector is esterified with a saturated or unsaturated alcohol having 1-30 carbon atoms. Namely, it is preferable that R^{2B} be $-\text{CH}_2\text{COOR}^{4B}$ or $-\text{C}_2\text{H}_4\text{COOR}^{4B}$, wherein R^{4B} is a hydrocarbon group having 1-30 carbon atoms.

15 [0093]

The term "hydrocarbon group" includes hydrocarbon groups having 1-30 carbon atoms, for example, "alkyl group", "cycloalkyl group", "alkenyl group", "cycloalkenyl group", "alkynyl group", "aryl group", "aralkyl group",
20 "cycloalkylalkyl group" and the like. These hydrocarbon groups may be substituted with one or more suitable substituents. Examples of such substituents include, but are not limited to, Ci-C_6 alkyl, Ci-C_6 alkenyl, Ci-C_6 alkynyl, C_6 aryl, $\text{C}_2\text{-C}_5$ heteroaryl, $\text{C}_3\text{-C}_6$ cycloalkyl, Ci-C_6 alkoxy, CN, OH, oxo, halo,
25 COOH , NH_2 , $\text{NH}(\text{Ci-C}_6 \text{ alkyl})$, $\text{N}(\text{Ci-C}_6 \text{ alkyl})_2$, $\text{NH}(\text{C}_6 \text{ aryl})$, $\text{N}(\text{C}_6 \text{ aryl})_2$, CHO , $\text{CO}(\text{d-C}_6 \text{ alkyl})$, $\text{CO}(\text{C}_6 \text{ aryl})$, $\text{COO}(\text{d-C}_6 \text{ alkyl})$, $\text{COO}(\text{C}_6 \text{ aryl})$, and the like.

[0094]

Examples of "alkyl group" include, but are not limited
30 to, "unbranched or branched Ci_{30} alkyl groups" such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, icosanyl, henicosanyl,
35 docosanyl, tricosanyl, tetracosanyl, pentacosanyl, hexacosanyl,

heptacosanyl, octacosanyl, nonacosanyl, triacontyl and the like.

[0095]

Examples of "cycloalkyl group" include, but are not
5 limited to, "C₃₋₈ cycloalkyl groups" such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl and the like.

[0096]

Examples of "alkenyl group" include, but are not
10 limited to, "unbranched or branched C₂₋₃₀ alkenyl groups" such as vinyl, allyl, isopropenyl, 1-butenyl, 2-butenyl, 3-butenyl, pentenyl, hexenyl, heptenyl, octenyl, nonenyl, decenyl, undecenyl, dodecenyl, tridecenyl, tetradecenyl, pentadecenyl, hexadecenyl, heptadecenyl, octadecenyl, nonadecenyl, icosenyl,
15 heneicosenyl, docosenyl, tricosenyl, tetracosenyl, pentacosenyl, hexacosenyl, heptacosenyl, octacosenyl, nonacosenyl, triacontenyl and the like.

[0097]

Examples of "cycloalkenyl group" include, but are not
20 limited to, "C₃₋₈ cycloalkenyl groups" such as cyclopropenyl, cyclobutenyl, cyclopentenyl, cyclohexenyl, cycloheptenyl, cyclooctenyl and the like.

[0098]

Examples of "alkynyl group" include, but are not
25 limited to, "unbranched or branched C₂₋₃₀ alkynyl groups" such as ethynyl, propynyl, 1-butyne, 2-butyne, 1-pentyne, 2-pentyne, 3-pentyne, hexynyl, heptyne, octyne, nonynyl, decynyl, undecynyl, dodecynyl, tridecynyl, tetradecynyl, pentadecynyl, hexadecynyl, heptadecynyl, octadecynyl,
30 nonadecynyl, icosynyl, heneicosynyl, docosynyl, tricosynyl, tetracosynyl, pentacosynyl, hexacosynyl, heptacosynyl, octacosynyl, nonacosynyl, triacontynyl and the like.

[0099]

Examples of "aryl group" include, but are not limited
35 to, "C₆₋₁₄ aryl groups" such as phenyl, 1-naphthyl, 2-naphthyl,

phenanthryl, anthryl and the like.

[0100]

Examples of "aralkyl group" include, but are not limited to, "C₇₋₃₀ aralkyl group (i.e., C₆₋₂₄ aryl-C₁₋₆ alkyl group)" such as benzyl, phenethyl, 3-phenylpropyl, 4-phenylbutyl, (1-naphthyl)methyl, 2-(1-naphthyl)ethyl, 2-(2-naphthyl)ethyl and the like.

[0101]

Examples of "cycloalkylalkyl group" include, but are not limited to, "C₃₋₈ cycloalkyl-C₁₋₆ alkyl groups" such as cyclopropylmethyl, cyclobutylmethyl, cyclopentylmethyl, cyclohexylmethyl, cycloheptylmethyl, cyclooctylmethyl, 2-cyclopropylethyl, 2-cyclobutylethyl, 2-cyclopentylethyl, 2-cyclohexylethyl, 2-cycloheptylethyl, 2-cyclooctylethyl and the like.

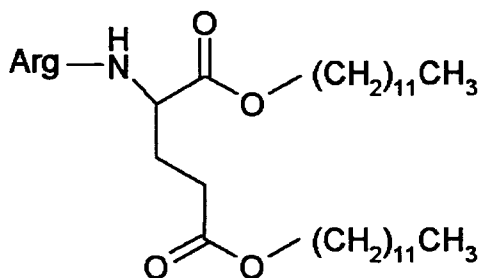
[0102]

R^{3B} and R^{4B} are preferably unbranched saturated hydrocarbon groups having 10-20 carbon atoms (i.e., n-butyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decyl, n-undecyl, n-dodecyl, n-tridecyl, n-tetradecyl, n-pentadecyl, n-hexadecyl, n-heptadecyl, n-octadecyl, n-nonadecyl, n-eicosanyl, n-henicosanyl, n-docosanyl, n-tricosanyl, n-tetracosanyl, n-pentacosanyl, n-hexacosanyl, n-heptacosanyl, n-octacosanyl, n-nonacosanyl and n-triacontyl) or unbranched unsaturated hydrocarbon groups (e.g., mono-unsaturated hydrocarbon groups such as trans-2-buten-1-yl, cis-9-tetradecen-1-yl, cis-9-hexadecen-1-yl, cis-9-octadecen-1-yl, cis-11-octadecen-1-yl, cis-9-eicosaen-1-yl, cis-13-docosaen-1-yl and cis-15-tetracosaen-1-yl, di-unsaturated hydrocarbon groups such as cis-9-cis-12-octadecdien-1-yl, tri-unsaturated hydrocarbon groups such as cis-9-cis-12-cis-15-octadectrien-1-yl and cis-9-cis-11-cis-13-octadectrien-1-yl, tetra-unsaturated hydrocarbon groups such as cis-4-cis-8-cis-12-cis-15-octadectetraen-1-yl and cis-5-cis-8-cis-11-cis-14-eicosatetraen-1-yl, penta-unsaturated hydrocarbon groups such

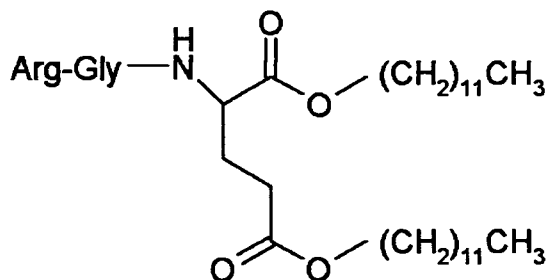
as cis-7-cis-10-cis-13-cis-16-cis-19-docosapentaen-1-yl, hexa-
unsaturated hydrocarbon groups such as cis-4-cis-7-cis-10-cis-
13-cis-16-cis-19-docosaheptaen-1-yl and the like). More
preferably, R^{3B} and R^{4B} are unbranched alkyl having 12-16 carbon
atoms (i.e., n-dodecyl, n-tridecyl, n-tetradecyl, n-pentadecyl
and n-hexadecyl) or unbranched unsaturated hydrocarbon (e.g.,
cis-9-tetradecen-1-yl, cis-9-hexadecen-1-yl, cis-9-octadecen-
1-yl, cis-9-octadecen-1-yl, cis-11-octadecen-1-yl, cis-9-
eicosaen-1-yl, cis-13-docosaen-1-yl, cis-9-cis-12-octadecdien-
1-yl, cis-9-cis-12-cis-15-octadectrien-1-yl, cis-9-cis-11-cis-
13-octadectrien-1-yl, cis-4-cis-8-cis-12-cis-15-
octadectetraen-1-yl, cis-5-cis-8-cis-11-cis-14-eicosatetraen-
1-yl, cis-7-cis-10-cis-13-cis-16-cis-19-docosapentaen-1-yl,
cis-4-cis-7-cis-10-cis-13-cis-16-cis-19-docosaheptaen-1-yl and
the like) groups having 10-20 carbon atoms. R^{3B} and R^{4B} can be
the same group.

[0103]

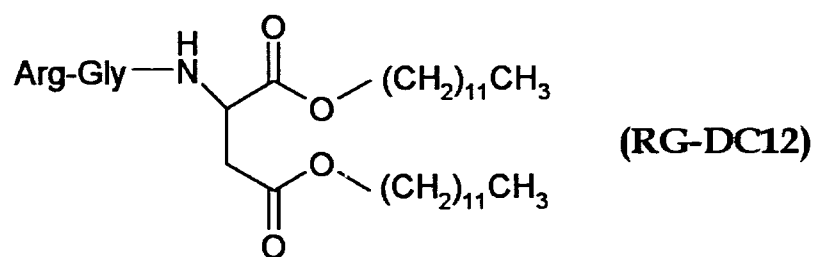
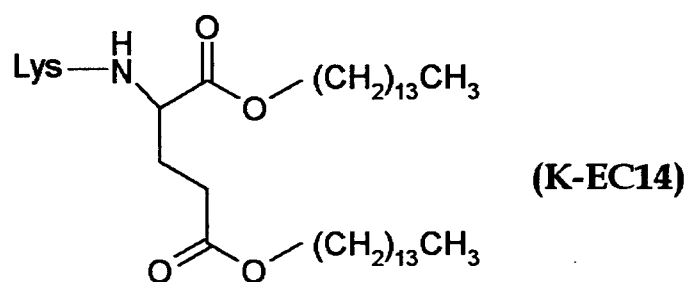
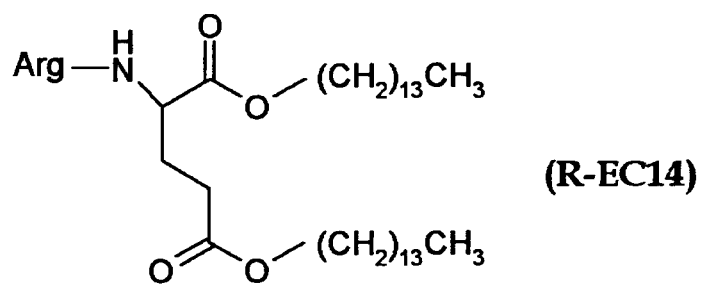
Examples of suitable compounds represented by the formula
(I-B) include the compounds shown below:



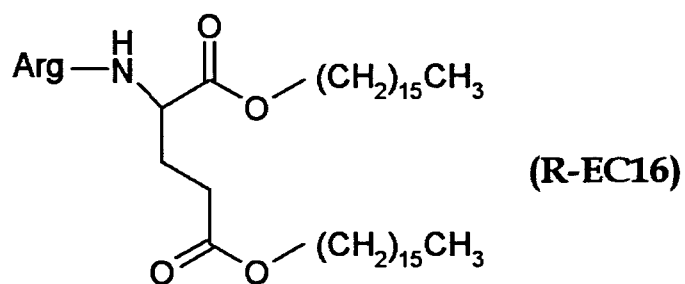
(R-EC12)

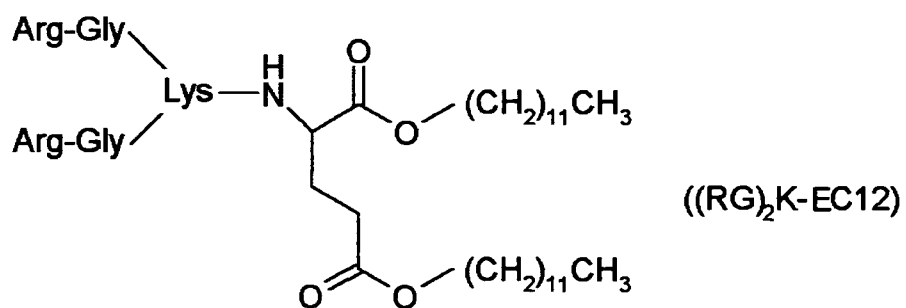
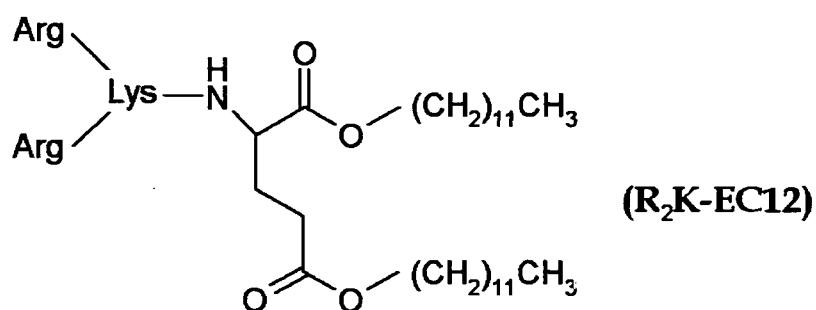
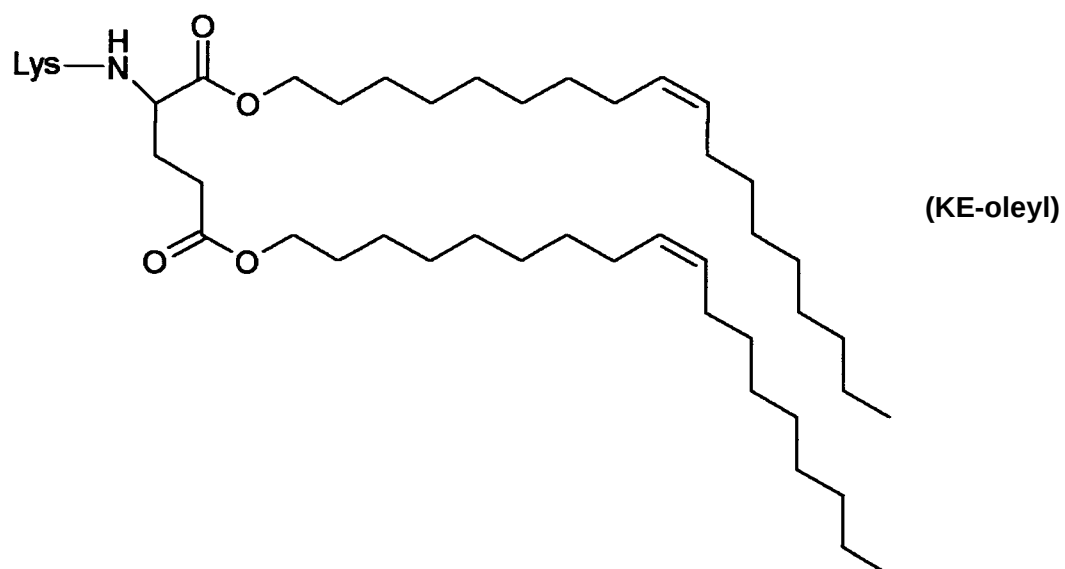


(RG-EC12)

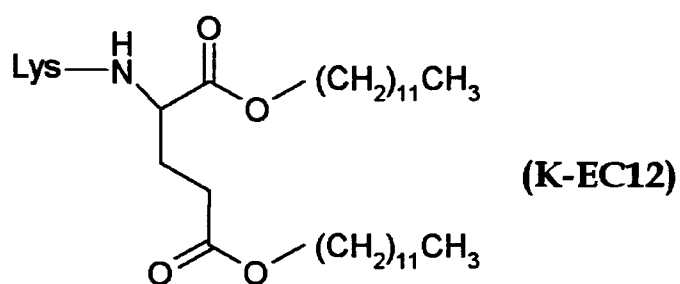


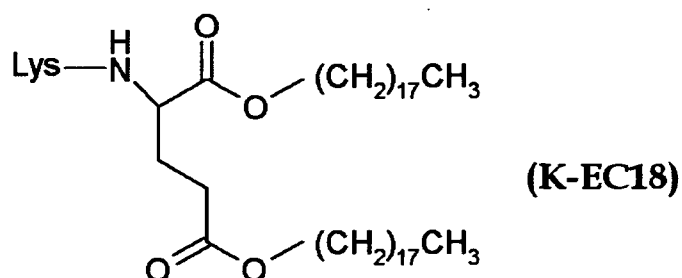
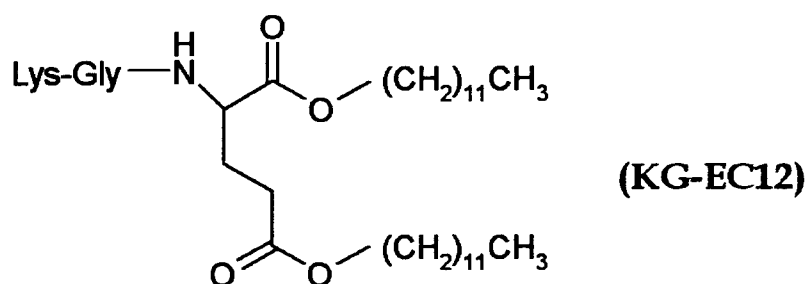
5





5





[0104]

5 The compound represented by the formula (I-B) is more preferably R-EC12, R-EC14, K-EC12 or K-EC14.

[0105]

The compound represented by the formula (I-B) can be produced by a method described in W02007/099650 .

10 [0106]

Other lipids having an activity to introduce a compound of interest into cells include, but are not limited to, N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-bis(oleoyloxy)-3-(trimethylammonium) propane (DOTAP), 1,2-dimyristyloxypropyl-3-dimethyl-
 15 hydroxyethyl ammonium bromide (DMRIE), dimethyldioctadecylammonium bromide (DDAB), dioctadecyldiammonium bromide (DODAB), dioctadecyldiammonium chloride (DODAC), 1-[2-(oleoyloxy)-ethyl]-2-oleoyl-3-(2-
 20 hydroxyethyl) imidazolinium chloride (DOIC), dioleoylphosphatidylcholine (DOPC), N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanammonium bromide (DLRIE), lipospermine, 2,3-dioleoyloxy-N-[sperminecarboxyamidoethyl]-N,N-dimethyl-1-propanalminum trifluoroacetate (DOSPA), 1,3-
 25 dioleoyloxy-2-(6-carboxyspermyl)-propylamide (DOSPER), di- and

tetra-alkyl-tetra-methylspermines [e.g. ,
tetramethyltetrapalmitoylspermine (TMTPS) ,
tetramethyltetraoleoylspermine (TMTOS) ,
tetramethyltetralaurylspermine (TMTLS) ,
5 tetramethyltetramyristylspermine (TMTMS) ,
tetramethyldioleoylspermine (TMDOS)] and the like.
[0107]

While the kind of the above-described "cells" is not
subject to limitation, whether they are derived from a
10 prokaryote or a eukaryote, a eukaryote is preferred. The kind
of eukaryote is also not subject to limitation, and is
exemplified by vertebrates such as mammals, including humans
(humans, monkeys, mice, rats, hamsters, cattle and the like),
birds (chickens, ostriches and the like) , amphibians (frogs
15 and the like), and fishes (zebrafish, killifish and the like),
invertebrates such as insects (silkworms, moths, drosophilas
and the like) , plants, microorganisms such as yeast, and the
like. More preferably, the subject cells in the present
invention are animal or plant cells, more preferably mammalian
20 cells.

[0108]

The cells may be cells of a cultured cell line,
including cancer cells, cells isolated from an individual or a
tissue, or cells of a tissue or a tissue fraction. The cells
25 may also be adherent cells or non-adherent cells.

[0109]

The agent of the present invention comprises the
aforementioned compound of the present invention as an active
ingredient, and may be the compound of the present invention
30 as it is alone or a composition thereof as blended with
another additive as appropriate, as far as the effect to
promote the introduction of the compound of interest into
cells by the lipid is not interfered with. Examples of the
composition include a dispersion (solution or suspension) of
35 the aforementioned compound of the present invention in an

appropriate dispersant. The dispersant is preferably an amphoteric solvent, an aqueous solvent or a mixture thereof. Amphoteric solvents include lower alcohols such as methanol, ethanol, propanol, isopropanol, and butanol; ketones such as
5 acetone, methyl ethyl ketone, and methyl isobutyl ketone; ethers such as dimethyl ether, ethylmethyl ether, and diethyl ether; polyhydric alcohols such as propylene glycol, ethylene glycol, 1,3-butylene glycol, dipropylene glycol, and glycerol ; dimethylsulfoxide (DMSO) ; N-methyl-2-pyrrolidone (NMP) ; N-N-
10 dimethyl formamide (DMF) and the like. In particular, from the viewpoint of low cytotoxicity, ethanol, isopropanol, DMSO and NMP are preferred. Aqueous solvents include purified water, distilled water, physiological saline, glucose solution, buffer solution (phosphate buffer solution, carbonate buffer
15 solution, Tris-buffer solution, acetate buffer solution) , cell culture media and the like.

[0110]

Other additives include biological molecules (e.g., phospholipids such as phosphatidylinositol, phosphatidylserine,
20 phosphatidylethanolamine, phosphatidylcholine and the like) , surfactants (e.g., CHAPS, sodium cholate, octylglucoside, N-D-glucosyl-N-methylalkanamide and the like) , polyethylene glycol, glycolipids, peptides, proteins or the like.

[0111]

25 The content of the compound of the present invention contained in the agent of the present invention is normally in the range of 0.001 to 100% by weight, preferably 0.01 to 100% by weight.

[0112]

30 The composition (A) or (B) of the present invention described in detail below is prepared using the agent of the present invention, and a compound of interest is introduced into cells using these compositions, whereby it is possible to promote the introduction of the compound of interest into
35 cells, and introduce the compound of interest into cells with

high efficiency.

[0113]

3. Composition comprising the compound of the present
invention and a lipid having an activity to introduce a
5 compound of interest into cells

The present invention provides a composition comprising the aforementioned compound of the present invention and a lipid having an activity to introduce a compound of interest into cells (the composition (A) of the present invention). The
10 composition (A) of the present invention preferably possesses an activity to introduce the compound of interest into cells. The composition (A) of the present invention can be one for introducing the compound of interest into cells (i.e., a carrier for introducing the compound of interest into cells).

15 [0114]

The composition (A) of the present invention comprises any one of the aforementioned compounds of the present invention. Alternatively, two or more of the aforementioned compounds of the present invention may be contained in
20 combination.

[0115]

The compound of the present invention contained in the composition (A) of the present invention is preferably Glc-EC12, G1C-EC6, Glc-EC8, Glc-EC10, Glc-EC14, Gal-EC12, Man-EC12,
25 A11-EC12, FUC-EC12, Xyl-EC12, GlcNAc-EC12, 2EG-EC10, 2EG-EC12, 2EG-EC14, 4EG-EC10, 4EG-EC12, or 4EG-EC14.

[0116]

Out of the compounds of the present invention, those offering high transfer efficiency can be chosen as appropriate
30 according to the choice of the compound of interest to be introduced. For example, when a nucleic acid such as plasmid DNA or siRNA is to be introduced, suitable compounds include, but are not limited to, Glc-EC12, Glc-EC6, Glc-EC8, Glc-EC10, G1C-EC14, Gal-EC12, Man-EC12, A11-EC12, Fuc-EC12, Xyl-EC12,
35 GlcNAc-EC12, 2EG-EC10, 2EG-EC12, 2EG-EC14, 4EG-EC10, 4EG-EC12,

4EG-EC14 and the like.

[0117]

The definition and modes of the lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, are the same as those described in the term "2. Agent for promoting the introduction of a compound of interest into cells by lipid" above .

[0118]

The lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, is preferably a compound represented by the aforementioned formula (I-A) to (V-A) or (I-B) .

[0119]

The lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, is more preferably a compound represented by the aforementioned formula (II-A) , (IV-A) , or (I-B).

[0120]

The lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, is more preferably TMA-EC12, TMA-EC14, TMA-DEAC12, TMA-DEAC14, R-EC12, RG-EC12, R-EC14, K-EC14, RG-DC12, R-EC16, KE-oleyl, R_2K -EC12, $(RG)_2K$ -EC12, K-EC12, KG-EC12, or K-EC18.

[0121]

The lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, is still more preferably TMA-EC12, TMA-EC14, TMA-DEAC12, TMA-DEAC14, R-EC12, R-EC14, K-EC12 or K-EC14.

[0122]

The lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, is most preferably TMA-EC12 .

[0123]

Examples of suitable combinations of compounds of the present invention and the lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, include the following:

Table 2

Compound of the present invention	Lipid having an activity to introduce a compound of interest into cells
Glc-EC12	TMA-EC12
Gal-EC12	TMA-EC12
Man-EC12	TMA-EC12
All-EC12	TMA-EC12
Fuc-EC12	TMA-EC12
Xyl-EC12	TMA-EC12
GlcNAc-EC12	TMA-EC12
Glc-EC6	TMA-EC12
Glc-EC8	TMA-EC12
Glc-EC10	TMA-EC12
Glc-EC14	TMA-EC12
2EG-EC10	TMA-EC12
4EG-EC10	TMA-EC12
2EG-EC12	TMA-EC12
4EG-EC12	TMA-EC12
2EG-EC14	TMA-EC12
4EG-EC14	TMA-EC12
Glc-EC12	TMA-EC14
Glc-EC12	TMA-DEAC12
Glc-EC12	TMA-DEAC14
Glc-EC12	R-EC12
Glc-EC12	R-EC14
Glc-EC12	K-EC12
Glc-EC12	K-EC14

[0124]

The composition (A) of the present invention may consist of the foregoing compound of the present invention and the lipid having an activity to introduce a compound of interest into cells, and may comprise, in addition to the compound of the present invention and the lipid having an activity to introduce the compound of interest into cells, another additive, as far as the activity of introducing the compound of interest into cells is not interfered with. The additive is preferably a pharmaceutically acceptable one.

[0125]

For example, the composition (A) of the present invention may comprise a dispersant for dispersing (dissolving or suspending) the aforementioned compound of the present invention and the lipid having an activity to introduce a compound of interest into cells. The dispersant is preferably an amphoteric solvent, an aqueous solvent or a mixture thereof. Amphoteric solvents include lower alcohols such as methanol, ethanol, propanol, isopropanol, and butanol; ketones such as acetone, methyl ethyl ketone, and methyl isobutyl ketone; ethers such as dimethyl ether, ethylmethyl ether, and diethyl ether; polyhydric alcohols such as propylene glycol, ethylene glycol, 1,3-butylene glycol, dipropylene glycol, and glycerol; dimethyl sulfoxide (DMSO); N-methyl-2-pyrrolidone (NMP); N-N-dimethylformamide (DMF) and the like. In particular, from the viewpoint of low cytotoxicity, ethanol, isopropanol, DMSO and NMP are preferred. Aqueous solvents include purified water, distilled water, physiological saline, glucose solution, buffer solution (phosphate buffer solution, carbonate buffer solution, Tris-buffer solution, acetate buffer solution), cell culture media and the like.

[0126]

The composition (A) of the present invention may further comprise a molecule other than the above-mentioned peptide lipid molecule, for example, biological molecule (e.g.,

phospholipids such as phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, etc.), surfactant (e.g., CHAPS, sodium cholate, octylglucoside, N-D-gluco-N-methylalkanamide, etc.), polyethylene glycol, glycolipid, peptide, protein or the like.

[0127]

The blending ratio of the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, is preferably a blending ratio that allows the compound of the present invention to promote the introduction of the compound of interest into cells by the lipid. For example, the compound of the present invention is blended in a ratio of 0.01 to 10 mole, preferably 0.1 to 5.0 mole, per one mole of the lipid having an activity to introduce a compound of interest into cells.

[0128]

The total content of the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells, contained in the composition (A) of the present invention, is not particularly limited, as far as the composition (A) of the present invention possesses an activity to introduce a compound of interest into cells, and the total content is normally in the range of 0.001 to 100% by weight, preferably 0.01 to 100% by weight.

[0129]

The composition (A) of the present invention is provided as an assembly in which the above-mentioned compound of the present invention and the lipid having an activity to introduce a compound of interest into cells are organized, while the assembly is in a state dispersed in the above-described dispersant (solution or suspension), or in a state in which the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells are completely dispersed in the above-described

dispersant (i.e., solution or suspension). "Organization" means that the component molecules of the composition (A) of the present invention, which comprises the compound of the present invention and the lipid having an activity to
5 introduce a compound of interest into cells, assemble via a non-covalent bond such as a hydrophobic bond. Examples of organized assemblies include bilayer membranes, liposomes, multilayer vesicles, ribbon-shaped assemblies, disc-shaped assemblies, lamellar assemblies, rod-shaped assemblies and the
10 like formed by hydrophobic bonding between the hydrophobic parts of the component molecules of the composition (A) of the present invention, and mixtures thereof.

[0130]

The composition (A) of the present invention can be
15 prepared in a state of an assembly of molecules by dispersing the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells (and another component molecule as required) in one of the above-described dispersants, for example, an aqueous solvent, and,
20 if required, performing an operation to induce organization. Examples of the "operation to induce organization" include, but are not limited to, various methods known per se, such as sonication, heating, vortexing, the ether injection method, the French press method, the cholic acid method, the Ca^{2+}
25 fusion method, the freeze-thawing method, and the reversed phase evaporation method [details of these methods are given in, for example, Chapter 2: Preparation of Liposomes (written by Sunamoto and Iwamoto) in "Liposomes", Nojima, Sunamoto, and Inoue, eds. (Nankodo, published in 1988), and elsewhere].
30 Under particular conditions, it is also possible to allow the component molecules of the composition (A) of the present invention, which comprises the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells, to autonomously assemble in
35 an aqueous solvent to form an assembly (self-organization)

without performing the operation to artificially induce organization described above. Although the assembly obtained by self-organization is normally a mixture of the various forms described above, it is also possible to form an assembly
5 in the form of a single substance by performing the operation to induce organization described above under particular conditions .

[0131]

Alternatively, the composition (A) of the present
10 invention can be prepared in a completely dispersed molecular state by dissolving the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells in one of the above-mentioned dispersants, for example, an amphiphilic solvent.

15 [0132]

In a preferred mode of embodiment, the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells, used to prepare the composition (A) of the present invention, are solid, gel,
20 liquid and the like, which, however, are not to be construed as limiting the invention. Examples of the dispersant for dispersing these compounds include, but are not limited to, aqueous solvents such as water (deionized water and the like), physiological saline, phosphate-buffered saline (PBS), and
25 media used by those skilled in the art for ordinary cell culture (e.g., RPMI1640, DMEM, HAM F-12, Eagle's medium and the like), amphiphilic solvents such as ethanol, methanol, and DMSO, mixed solvents of an aqueous solvent and an amphiphilic solvent, and the like. Although the aqueous solvent is
30 preferably free from protein components such as serum, it is also possible to prevent the inhibition of the organization of the component molecules of the composition (A) of the present invention, which comprises the compound of the present invention and a lipid having an activity to introduce a
35 compound of interest into cells, or the subsequent complex

formation between the compound of interest introduced into cells and the organized assembly, by previously removing protein components by polylysine treatment and the like. When the compound of interest to be introduced into cells is a nucleic acid such as an RNA or a DNA, or a peptidyl compound such as an oligopeptide or a protein, the stability of the compound of interest decreases due to the minglement of a nuclease such as RNase or DNase or a protein- (peptide) - decomposing enzyme such as a peptidase or a protease; therefore, the aqueous solvent preferably undergoes heat treatment to inactivate these enzymes before the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells are dispersed. Examples of the heat treatment include, but are not limited to, a treatment at about 50 to about 100°C for about 5 minutes to about 3 hours. Accordingly, the aqueous solvent is preferably one that permits the heat treatment.

[0133]

Although the pH of the aqueous solvent is not subject to limitation, it is preferably in the range of pH 4 to 10, more preferably in the range of pH 6 to 8.

[0134]

In preferred modes of embodiment, a lipid complexed liposome is prepared by (1) sonication or (2) heat treatment as described in detail below.

[0135]

(1) Sonication method

First, the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells are dissolved in an organic solvent (e.g., chloroform and the like), and the resulting solution is placed in a container such as an eggplant-shaped flask; the solvent is evaporated off under reduced pressure using a rotary evaporator and the like to form a thin membrane of lipid on the container wall surface. An aqueous solvent [e.g.,

phosphate buffer solution (pH 7.0) and the like] is added to the membrane, followed by shaking to swell the membrane, which is then detached using, for example, a vortex mixer and the like, to yield a suspension of multilayer liposome. To remove
5 the decomposed lipids and the like, gel filtration may be performed using a Sephadex 2B, 4B or G-50 column and the like.
[0136]

By sonicating the resulting suspension of multilayer liposome at a high output (e.g., about 100 to about 200 W) on
10 an ice bath or water bath using a sonicator (probe type, bathtub type and the like) for about 1 to about 2 minutes (e.g., a cycle of 1-minute sonication and 30-second interval repeated about two to four times and the like), a nearly uniform monolayer liposome can be prepared.

15 [0137]

A complexed liposome containing the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells can also be prepared solely by transferring an appropriate amount of a
20 powder of the compound of the present invention and the lipid having an activity to introduce the compound of interest into cells to a tube, adding MilliQ water and the like (to obtain a final concentration of about 20 mM), and performing the same sonication as described above.

25 [0138]

(2) Heat treatment method

A complexed liposome containing the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells can be prepared by
30 transferring an appropriate amount of a powder of the compound of the present invention and the lipid having an activity to introduce the compound of interest into cells to a tube, adding MilliQ water and the like (to obtain a final concentration of about 20 mM), and heating the mixture at
35 about 90°C for about 15 minutes.

[0139]

The combined concentration of the compound of the present invention and the lipid having an activity to introduce the compound of interest into cells in the complexed liposome
5 suspension can be set as appropriate in consideration of the choice of compounds used and the like, and is normally in the range of 0.01 to 200 mM, preferably 0.05 to 100 mM, and more preferably 0.1 to 50 mM.

[0140]

10 If the concentration is too low, no sufficient amount of lipid-complexed liposome is formed; if the concentration is too high, lipid molecules can precipitate.

[0141]

The composition (A) of the present invention, which can
15 be obtained as described above, is useful as an agent for efficiently introducing a compound into cells with low toxicity. Any compound of interest can be introduced into cells using the composition (A) of the present invention; for example, nucleic acids, peptides, lipids, peptide lipids,
20 sugars, bioactive substances, drugs (doxorubicin (antitumor drug), daunorubicin (antitumor drug), vincristine (antitumor drug), vinblastine (antitumor drug), idarubicin (antitumor drug), dibucaine (local anesthetic), propranolol (β blocker), quinidine (antiarrhythmic therapeutic), dopamine (cardiotonic
25 hypertensive), imipramine (antidepressant), diphenhydramine (antihistamine), quinine (antimalarial), chloroquine (antimalarial), diclofenac (anti-inflammatory drug) and the like), moisturizers for cosmetics and the like (mannitol and the like), other synthetic or natural compounds and the like
30 can be mentioned.

[0142]

A particularly preferable compound that can be introduced into cells using the composition (A) of the present invention is a nucleic acid. Any nucleic acid can be used,
35 whether it is a DNA, an RNA, a DNA-RNA chimeric nucleic acid,

a DNA/RNA hybrid or the like. While the nucleic acid may be single-stranded to triple-stranded, it is preferably single-stranded or double-stranded. The nucleic acid may be another type of nucleotide that is an N-glycoside of the purine or pyrimidine base, or another oligomer having a non-nucleotide skeleton (e.g., commercially available peptide nucleic acid (PNA) and the like) or another oligomer containing a special bond (however, the oligomer contains a nucleotide having an arrangement that allows base pairing or base attachment as found in DNA and RNA) and the like. Furthermore, the nucleic acid may also be one having a known modification added thereto, for example, one with a marker known in the art, one with a cap, one methylated, one having one or more naturally occurring nucleotides substituted by analogues, one modified with an intramolecular nucleotide, for example, one having a non-charge bond (e.g., methylphosphonate, phosphotriester, phosphor amidate, carbamate and the like), one having a charged bond or a sulfur-containing bond (e.g., phosphorothioate, phosphorodithioate and the like), for example, one having a side chain group of a protein (e.g., nuclease, nuclease inhibitor, toxin, antibody, signal peptide, poly-L-lysine and the like), a sugar (e.g., monosaccharide and the like) and the like, one having an intercalating compound (e.g., acridine, psoralen and the like), one containing a chelate compound (e.g., metals, radioactive metals, boron, oxidizing metals and the like), one containing an alkylating agent, or one having a modified bond (e.g., a anomer type nucleic acid and the like). [0143]

For example, any kind of DNA can be chosen as appropriate according to the purpose of use; examples include plasmid DNA, cDNA, antisense DNA, chromosome DNA, PAC, BAC and the like, with preference given to plasmid DNA, cDNA and antisense DNA, more preferably plasmid DNA. A circular DNA such as plasmid DNA can also be used as a linear DNA after being digested as appropriate with a restriction endonuclease

and the like. Also, any kind of RNA can be chosen as appropriate according to the purpose of use; examples include siRNA, miRNA, shRNA, antisense RNA, messenger RNA, single strand RNA genome, double strand RNA genome, RNA replicon, transfer RNA, ribosomal RNA and the like, with preference given to siRNA, miRNA, shRNA, mRNA, antisense RNA and RNA replicon.

[0144]

The size of the nucleic acid is not subject to limitation; while a broad range of nucleic acids, from giant nucleic acid molecules (e.g., about 10^7 kbp in size) such as chromosomes (artificial chromosome and the like) to low-molecular nucleic acids (e.g., about 5 bp in size), can be introduced, the size is preferably not more than 15 kbp in consideration of the efficiency of nucleic acid introduction into cells. For example, the size of a high-molecular nucleic acid like plasmid DNA is 2 to 15 kbp, preferably 2 to 10 kbp. For example, the size of a low-molecular nucleic acid like siRNA is 5 to 1000 bp, preferably 5 to 500 bp, and more preferably 5 to 200 bp.

[0145]

The nucleic acid may be a naturally-occurring one or a synthetic one; when the size is not more than about 100 bp, the nucleic acid can be synthesized by the phosphotriethyl method, the phosphodiester method and the like using a commonly used automated nucleic acid synthesizer.

[0146]

Although the nucleic acid used in the present invention is not subject to limitation, it is preferably purified by a method commonly used by those skilled in the art.

[0147]

Examples of modes of embodiment wherein the composition (A) of the present invention is used to introduce a prophylactic and/or therapeutic (hereinafter abbreviated as "prophylactic/therapeutic") compound into cells of a living

body include the use of a compound for the prevention and/or treatment, including what is called gene therapy, in administration in vivo, intended to prevent and/or treat a disease. Accordingly, in a preferred embodiment of the present invention, the compound introduced into cells using the composition (A) of the invention possesses prophylactic/therapeutic activity for a particular disease. Examples of such compound include nucleic acids, peptides, lipids, sugars, bioactive substances, drugs, and other natural or synthetic compounds.

[0148]

Gene therapies can be roughly divided into those intended to supplement lacking genetic information and those intended to control the expression of the causal gene (target gene) for a disease.

[0149]

For example, when the compound capable of controlling the expression of the target gene is a low-molecular nucleic acid, the low-molecular nucleic acid is exemplified by siRNA, miRNA, shRNA, an antisense oligonucleotide, ribozyme, a decoy oligonucleotide (e.g., an oligonucleotide comprising a base sequence that can be recognized and bound by a transcription factor or a transcription suppression factor) and the like.

[0150]

When the compound capable of controlling the expression of the target gene is a peptide or a protein, the peptide/protein is exemplified by a peptide/protein that binds to the target gene to control the transcription of the gene, or that binds to the mRNA or initial transcription product of the target gene to control the translation thereof into a protein, or a peptidyl ligand capable of enhancing a signal from a receptor that controls the expression of the target gene, or an antagonist-like peptide/protein capable of blocking the signal, and the like.

[0151]

Alternatively, the compound having prophylactic/therapeutic activity for a disease may be one capable of controlling the activity of the causal protein for the disease. Examples of such compound includes, but are not
5 limited to, peptides/proteins that are ligands for the target receptor protein, non-peptidyl compounds (e.g., fatty acids, steroid hormones and the like), various natural or synthetic compounds having agonist or antagonist activity, peptides that mimic a partial amino acid sequence of the phosphorylation
10 site of kinase and the like.

[0152]

To introduce a compound of interest into cells using the composition (A) of the present invention, a composition comprising the compound of the present invention, a lipid
15 having an activity to introduce a compound of interest into the cells, and a compound of interest (the composition (B) of the present invention) is prepared by adding the compound of interest to the composition (A). By contacting the composition (B) of the present invention with cells, the compound of
20 interest contained in the composition (B) of the present invention is introduced into the cells.

[0153]

In an embodiment, the composition (B) of the present invention comprises a complex comprising the compound of the
25 present invention, a lipid having an activity to introduce a compound of interest into cells, and the compound of interest (the complex of the present invention). The complex can be a complex formed as a result of complex formation of the compound of interest with an organized assembly comprising the
30 above-described compound of the present invention and the lipid having an activity to introduce a compound of interest into cells. For example, the assembly is capable of forming a complex with a compound of interest by adsorption or encapsulation. The complex may be formed by any interaction,
35 as far as the complex can be stably present, and as far as the

decomposition of the compound of interest (nucleic acid, peptide and the like) by, for example, nuclease, peptidase and the like, can be suppressed. For example, when the compound of interest is a negatively charged compound such as a nucleic acid or a peptide, the compound of interest can form a complex with an assembly containing a positively charged lipid via a non-covalent bond based on an electrostatic interaction. When the compound of interest is positively charged or non-charged, the compound of interest can form a complex with an assembly containing a negatively charged lipid via a non-covalent bond based on an electrostatic interaction. Alternatively, the compound of interest can be allowed to form a complex with an assembly by interlaying another interaction (e.g., hydrophobic bond, hydrogen bond and the like), or by being bound to a negatively charged compound in advance.

[0154]

When the assembly is a liposome, the compound of interest may be in a form adsorbed to the liposome via an interaction such as an electrostatic interaction, hydrophobic bond, or hydrogen bond, or in a form incorporated in the liposome. Preferably, the compound of interest is in a form incorporated in the liposome.

[0155]

A complex of the above-described assembly and a compound of interest can be obtained by mixing the composition (A) of the present invention, which comprises the assembly, and the compound of interest, and incubating the mixture (i.e., the composition (B) of the present invention) for a time sufficient to form the complex. Preferably, the composition (A) of the present invention, which comprises the assembly, and an aqueous solution of the compound of interest in an aqueous solvent are mixed. Examples of the aqueous solvent include those mentioned above as dispersants that can be contained in the composition (A) of the present invention.

[0156]

Incubation time after mixing the composition (A) of the present invention and the compound of interest can be set as appropriate in consideration of the kind of reagent used and other conditions, and is normally in the range of 0.5 to 500
5 minutes, preferably 0.5 to 180 minutes, more preferably 0.5 to 120 minutes, still more preferably 1 to 60 minutes, and most preferably 5 to 30 minutes.

[0157]

When the incubation time is too short, complex
10 formation between the compound of interest and the carrier is insufficient; when the incubation time is too long, the complex formed becomes unstable in some cases; in both cases, introduction efficiency of the compound of interest may decrease.

15 [0158]

The content of the compound of the present invention contained in the mixture (i.e., the composition (B) of the present invention) is not particularly limited, as far as the composition (B) of the present invention possesses an activity
20 to introduce the compound of interest contained in the composition into cells, and as far as the compound of the present invention exhibits an activity to promote the introduction of the compound of interest into cells by a lipid having an activity to introduce the compound of interest into
25 cells; the content is normally in the range of 0.001 to 1000 mM, preferably 0.01 to 200 mM, more preferably 0.05 to 100 mM.

[0159]

The content of the lipid having an activity to introduce a compound of interest into cells, contained in the
30 composition (B) of the present invention is not particularly limited, as far as the composition (B) of the present invention possesses an activity to introduce the compound of interest contained in the composition into cells, and as far as the compound of the present invention exhibits an activity
35 to promote the introduction of the compound of interest into

cells by the lipid having an activity to introduce the compound of interest into cells; the content is normally in the range of 0.001 to 1000 mM, preferably 0.01 to 200 mM, more preferably 0.05 to 100 mM.

5 [0160]

The blending ratio of the compound of the present invention and the lipid having an activity to introduce a compound of interest into cells, contained in the composition (B) of the present invention, is preferably a blending ratio
10 that allows the compound of the present invention to promote the introduction of the compound of interest into cells by the lipid. For example, the compound of the present invention is blended in a ratio of 0.01 to 10 mole, preferably 0.1 to 5.0 mole, per one mole of the lipid having an activity to
15 introduce a compound of interest into cells.

[0161]

The concentration of the compound of interest contained in the mixture (i.e., the composition (B) of the present invention) can be set as appropriate, taking into account the
20 choice, size (molecular weight) and the like of the compound of interest; when the compound is a nucleic acid, the concentration is normally in the range of about 0.001 to 2000 ng/ μ L, preferably about 0.002 to 500 ng/ μ L. Too low a concentration prevents the nucleic acid (DNA, RNA and the
25 like) introduced to the cells from exhibiting its function as expected; too high a concentration reduces the nucleic acid introduction efficiency.

[0162]

In a preferred embodiment, the blending ratio of the
30 compound of interest, contained in the composition (B) of the present invention, and the lipid having an activity to introduce a compound of interest into cells can be set as appropriate, taking into account the choice, size (molecular weight) and the like of the compound of interest; when the
35 compound is a nucleic acid, for example, the compound of

interest is blended in a ratio of 0.002 to 20 μg , preferably 0.02 to 2.0 μg , per one nanomole of the lipid having an activity to introduce the compound of interest into cells.

[0163]

5 Furthermore, by contacting the composition (B) of the present invention thus obtained with cells, the compound of interest contained in the composition (B) of the present invention can be introduced into the cells. When the composition (B) of the present invention is contacted with
10 cells, the above-described complex contained in the composition (B) of the present invention comes into contact with the cells, resulting in the introduction of the compound of interest contained in the complex into the cells.

[0164]

15 While the kind of the above-described "cells" is not subject to limitation, whether they are derived from a prokaryote or a eukaryote, a eukaryote is preferred. The kind of eukaryote is also not subject to limitation, and is exemplified by vertebrates such as mammals, including humans
20 (humans, monkeys, mice, rats, hamsters, cattle and the like), birds (chickens, ostriches and the like), amphibians (frogs and the like), and fishes (zebrafish, killifish and the like), invertebrates such as insects (silkworms, moths, drosophilas and the like), plants, microorganisms such as yeast, and the
25 like. More preferably, the subject cells in the present invention are animal or plant cells, more preferably mammalian cells.

[0165]

The cells may be cells of a cultured cell line,
30 including cancer cells, cells isolated from an individual or a tissue, or cells of a tissue or a tissue fraction. The cells may also be adherent cells or non-adherent cells.

[0166]

The step for bringing the composition (B) of the
35 present invention and the cells into contact with each other

can be described in more detail below.

[0167]

The cells are suspended in an appropriate medium several days before contact with the composition (B) of the present invention, and cultured under appropriate conditions. At the time of contact with the composition (B) of the present invention, the cells may or may not be in the logarithmic phase.

[0168]

While the culture broth used at the time of contact may be a serum-containing medium or a serum-free medium, it is preferable that the serum concentration in the medium be not more than 30%, preferably not more than 20%. This is because the presence of a protein such as serum in excess in the medium may inhibit the contact of the complex in the composition (B) of the present invention described above and the cells.

[0169]

Cell density at the time of the contact is not subject to limitation, and can be set as appropriate in consideration of the kind of cells and the like, and is normally in the range of 0.01×10^5 to 100×10^5 cells/mL, preferably 0.05×10^5 to 50×10^5 cells/mL, more preferably 0.1×10^5 to 10×10^5 cells/mL, still more preferably 0.5×10^5 to 7.5×10^5 cells/mL, and most preferably 1×10^5 to 5×10^5 cells/mL.

[0170]

The composition (B) of the present invention described above is added to the cell-containing medium thus prepared. The amount of the composition (B) of the present invention added is not subject to limitations, as far as the compound of interest contained in the composition (B) of the present invention can be introduced into cells; the amount can be set as appropriate in consideration of cell count and the like, and is normally in the range of 0.1 to 200 μ L, preferably 0.2 to 150 μ L, more preferably 0.5 to 100 μ L, still more preferably

0.75 to 50 μL , and most preferably 1 to 25 μL , per milliliter of the medium.

[0171]

After the composition (B) of the present invention is added to the medium, the cells are cultured. Temperature, humidity, CO_2 concentration and the like during the cultivation are set as appropriate in consideration of the kind of cells. In the case of mammalian cells, normal conditions are about 37°C temperature, about 95% humidity, and about 5% CO_2 concentration.

[0172]

Cultivation time can also be set as appropriate in consideration of the kind of cells used and other conditions, and is normally in the range of 0.1 to 72 hours, preferably 0.2 to 54 hours, more preferably 0.5 to 48 hours, still more preferably 1 to 24 hours, and most preferably 2 to 12 hours.

[0173]

The compound of interest is introduced into the cells by the above-described cultivation; the cultivation can be continued with the medium replaced with a fresh medium, or without replacing the medium. When the cells are of mammalian origin, the fresh medium preferably contains serum or a nutritive factor.

[0174]

Time for the further cultivation can be set as appropriate in consideration of the expected function of the compound of interest introduced and the like; when the compound is a plasmid DNA such as an expression vector, the time is normally in the range of 1 to 720 hours, preferably 2 to 600 hours, more preferably 4 to 480 hours, still more preferably 8 to 360 hours, and most preferably 12 to 240 hours. When the compound is a low-molecular nucleic acid capable of controlling the expression of a target gene such as siRNA, the time is normally in the range of 1 to 168 hours, preferably 2 to 144 hours, more preferably 4 to 120 hours, still more

preferably 6 to 72 hours, and most preferably 8 to 48 hours.

[0175]

As described above, by using the composition (B) of the present invention, the compound of interest can be introduced
5 into cells not only in vitro, but also in vivo. Hence, by administering the composition (B) of the present invention to a recipient, the above-mentioned complex reaches and comes in contact with target cells, thus resulting in the in vivo introduction of the compound of interest contained in the
10 complex into the cells.

[0176]

The recipient of the composition (B) of the present invention is not subject to limitation, and is exemplified by vertebrates such as mammals, including humans (humans, monkeys,
15 mice, rats, hamsters, cattle and the like), birds (chickens, ostriches and the like), amphibians (frogs and the like), and fishes (zebrafish, killifish and the like), invertebrates such as insects (silkworms, moths, drosophilas and the like), plants and the like. Preferably, the recipient of the
20 composition (B) of the present invention is a human or another mammal .

[0177]

The method of administering the composition (B) of the present invention is not subject to limitation, as long as the
25 above-mentioned complex reaches and comes in contact with target cells to allow the compound of interest contained in the complex to be introduced into the cells; a method of administration known per se (oral administration, parenteral administration (intravenous administration, intramuscular
30 administration, topical administration, percutaneous administration, subcutaneous administration, intraperitoneal administration, spraying and the like) and the like) can be chosen as appropriate in consideration of the kind of the compound of interest, the kind and site of target cells, and
35 the like.

[0178]

The dosage of the composition (B) of the present invention is not subject to limitation, as long as introduction of the compound of interest into the cells is
5 accomplishable, and can be chosen as appropriate in consideration of the kind of recipient, the method of administration, the kind of compound of interest, the kind and site of target cells, and the like. In the case of oral administration, the usual dosage per administration for a
10 human (weighing 60 kg), for example, is about 0.001 mg to 10000 mg, based on the above-described complex. In the case of parenteral administration (e.g., intravenous administration and the like), the usual dosage per administration for a human (weighing 60 kg), for example, is about 0.0001 mg to 3000 mg,
15 based on the complex. In the case of another animal, a dosage converted per 60-kg body weight can be administered.

[0179]

4. Kit

The present invention also provides a kit for introducing
20 a compound of interest into cells, comprising the above-described compound of the present invention and a lipid having an activity to introduce the above-described compound of interest into cells.

[0180]

25 The kit of the present invention can further comprise the above-described compound of interest. The compound of interest is preferably a nucleic acid.

[0181]

The definitions and modes of "the compound of the present
30 invention", "a lipid having an activity to introduce a compound of interest into cells", "a compound of interest", "cells", and "a nucleic acid" are as described in the terms 1 to 3 above .

[0182]

35 Examples of suitable combinations of the compound of the

present invention and the lipid having an activity to introduce a compound of interest into cells, contained in the kit of the present invention, are the same as the combinations contained in the above-described composition (A) of the
5 present invention.

[0183]

The kit of the present invention can further comprise a reagent for use in introducing a compound of interest into cells using the above-described composition (A) or composition
10 (B) of the present invention (e.g., cell culture medium, cell culture vessel), an instruction manual bearing a description of the protocol, and the like. The individual components contained in the kit of the present invention are separately (or, if possible, in the form of a mixture of some or all of
15 the components) placed in an appropriate container; all are packaged in a single or a plurality of packages.

[0184]

The compound of the present invention and the lipid having an activity to introduce a compound of interest into
20 cells may be contained as the above-described composition (A) of the present invention in the kit of the present invention.

[0185]

The compound of the present invention, the lipid having an activity to introduce a compound of interest into cells, and
25 the compound of interest may be contained as the above-described composition (B) of the present invention in the kit of the present invention.

[0186]

By using the kit of the present invention, it is possible
30 to easily introduce a compound of interest into cells according to the above-described method.

[0187]

All reference documents mentioned herein, including publications, patent documents and the like, are cited herein
35 by reference to the extent that they are individually cited as

specific references, and that all of them have been given expressly.

Examples

5 [0188]

The present invention is hereinafter described in more detail by means of the following Examples, to which, however, the present invention is not limited in any way.

[0189]

10 [Example 1]

Synthesis of lipids

(1) Synthesis of Glc-EC12

An amino acid (L-glutamic acid; ϵ), a fatty alcohol (dodecylalcohol; C12-OH), and p-toluenesulfonic acid were
15 mixed in a toluene solvent. This mixture was thermally refluxed in a reactor equipped with Dean-Stark trap, and allowed to form an ester bond by dehydration-condensation. The para-toluenesulfonate salt of EC12 was crystallized by cooling (4°C); the resulting crystal was separated by filtration and
20 washed with cold toluene to yield a white crystal. Next, EC12 and glucose (D-glucose; Glc) were condensed in a methanol solvent in the presence of TEA. Subsequently, the synthesized product (Glc-EC12) was purified by silica gel chromatography. The identification was achieved by NMR or TOF-MASS (Fig. 1).
25 Other glycolipids (Glc-EC6, Glc-EC8, Glc-EC10, Glc-EC14, Gal-EC12, Man-EC12, A11-EC12, Fuc-EC12, Xyl-EC12 and GlcNAc-EC12) were synthesized from corresponding raw materials (amino acid, lipid alcohol and saccharide) according to the same method as for Glc-EC12. The identification was achieved by NMR or TOF-
30 MASS (Figs. 2-11).

[0190]

(2) Synthesis of 4EG-EC10

An amino acid (L-glutamic acid; ϵ), a fatty alcohol (decanol; C10-OH), and p-toluenesulfonic acid were mixed in a
35 toluene solvent. This mixture was thermally refluxed in a

reactor equipped with Dean-Stark trap, and allowed to form an ester bond by dehydration-condensation. The para-toluenesulfonate salt of EC10 was crystallized by cooling (4°C); the resulting crystal was separated by filtration and washed with cold toluene to yield a white crystal. EC10, succinic anhydride and N,N-diisopropylethylamine were mixed and stirred in a chloroform solvent to obtain a reaction product. The reaction product was reacted with N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in a dimethylformamide solvent. Tetraethylene glycol-monoamine was added to the reaction mixture and stirred to obtain crude product of 4EG-EC10. Then, the product was applied on a silica gel chromatography to give purified 4EG-EC10. The identification was achieved by TOF-MASS (Fig. 13). Other ethylene glycol lipids (2EG-EC10, 2EG-EC12, 4EG-EC12, 2EG-EC14 and 4EG-EC14) were synthesized from corresponding raw materials (amino acid, lipid alcohol and polyethylene glycol-monoamine) according to the same method as for 4EG-EC10. The identification was achieved by NMR or TOF-MASS (Figs. 12 and 14-17).

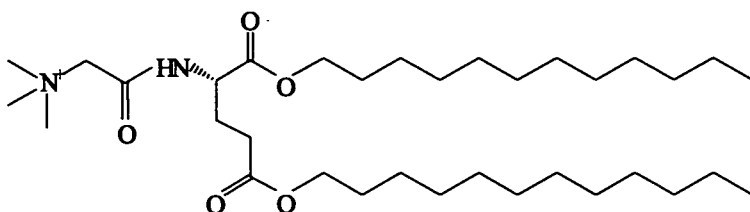
[0191]

[Experimental Example 1]

Introduction of plasmid DNA using various glycolipids and cationic lipids

25 (Preparation of lipid mixture solutions)

The cationic lipid TMA-EC12 (final concentration 1 mM) and each glycolipid (Glc-EC12, Gal-EC12, Man-EC12, A11-EC12, Fuc-EC12, Xyl-EC12 or GlcNAc-EC12) (final concentration 1 mM) were dissolved in methanol to yield a lipid mixture solution.



TMA-EC1 2

[0192]

(Introduction of plasmid DNA)

CHO cells (2×10^5 cells/well) were pre-cultured in Freestyle CHO medium (obtained from Invitrogen) on a 24-well plate for 2 hours, after which plasmid DNA was introduced. 1 μ g per well of plasmid DNA (pCMVIE-GFP vector; obtained from Nippon Gene Co., Ltd.) was dissolved in 50 μ l of Opti-MEM medium (obtained from Invitrogen); 5 μ l of each of the above-described lipid mixture solutions was added to the resulting plasmid DNA solution, and this was followed by incubation for 20 minutes, whereby a DNA-lipid complex was obtained. This complex was added to the above-described CHO cells, and the cells were cultured in a 5% CO₂ incubator at 37°C for 24 hours. Also, the commercially available gene transfer reagent MaxReagent (obtained from Invitrogen) was tested as directed in the instruction manual. The following day, the cells were examined under a fluorescence microscope, and the cells exhibiting fluorescence were counted using a flow cytometer.

[0193]

As shown in Fig. 18, the transfection efficiency rose when each glycolipid (Glc-EC12, Gal-EC12, Man-EC12, A11-EC12, Fuc-EC12, Xyl-EC12 or GlcNAc-EC12) was added to the cationic lipid TMA-EC12. These glycolipids were more effective than commercially available gene transfer reagents.

[0194]

[Experimental Example 2]

Introduction of plasmid DNA using various glycolipids and cationic lipids

(Preparation of lipid mixture solutions)

The cationic lipid TMA-EC12 (final concentration 1 mM) and each glycolipid (Glc-EC6, Glc-EC8, Glc-EC10 or Glc-EC14) (final concentration 1 mM) were dissolved in methanol to yield a lipid mixture solution.

[0195]

(Introduction of plasmid DNA)

Plasmid DNA (1 pg/well, pCMVIE-GFP vector) was introduced into CHO cells (2×10^5 cells/well) in a 24-well plate using each of the above-described lipid mixture solutions (5 μ l/well), by the same procedures as Experimental Example 1. After being
5 cultured in a 5% CO₂ incubator at 37°C for 24 hours, the cells were examined under a fluorescence microscope, and the cells exhibiting fluorescence were counted using a flow cytometer.
[0196]

As shown in Fig. 19, irrespective of the length of the
10 side chain of the glycolipid (C6 to C14), the transfection efficiency rose when each glycolipid (Glc-EC6, Glc-EC8, Glc-EC10, Glc-EC14) was added to the cationic lipid TMA-EC12, and GFP encoded in the plasmid DNA was expressed at high efficiency. This result suggests that the compounds of the
15 present invention, irrespective of the length of the side chain hydrocarbon group, possess an activity to increase the transfection efficiency.

[0197]

[Experimental Example 3]

20 Introduction of plasmid DNA using various ethylene glycol lipids and cationic lipids
(Preparation of lipid mixture solutions)

[0198]

The cationic lipid TMA-EC12 (final concentration 1 mM)
25 and each ethylene glycol lipid (2EG-EC10, 4EG-EC10, 2EG-EC12, 4EG-EC12, 2EG-EC14, 4EG-EC14) (final concentration 1 mM) were dissolved in methanol to yield a lipid mixture solution.

[0199]

(Introduction of plasmid DNA)

30 Plasmid DNA (1 μ g/well, pCMVIE-GFP vector) was introduced into CHO cells (2×10^5 cells/well) in a 24-well plate using the above-described lipid mixture solution (5 μ l/well) by the same procedures as Experimental Example 1. After being cultured for 24 hours, the cells were examined under a fluorescence
35 microscope, and the cells exhibiting fluorescence were counted

using a flow cytometer.

[0200]

As shown in Fig. 20, irrespective of the length of the side chain of the ethylene glycol lipid (C10 to C14) or the length of the ethylene glycol group (2EG to 4EG), the transfection efficiency rose when each ethylene glycol lipid (2EG-EC10, 4EG-EC10, 2EG-EC12, 4EG-EC12, 2EG-EC14, 4EG-EC14) was added to the cationic lipid TMA-EC12, and GFP encoded in the plasmid DNA was expressed at high efficiency. This result suggests that the compounds of the present invention, irrespective of the length of the ethylene glycol group or side chain hydrocarbon group, possess an activity to increase the transfection efficiency.

[0201]

[Experimental Example 4]

Introduction of siRNA using various glycolipids and cationic lipids

(Preparation of lipid mixture solutions)

The cationic lipid TMA-EC12 (final concentration 1 mM) and each glycolipid (Glc-EC12, Gal-EC12, Man-EC12, A11-EC12, Fuc-EC12, Xyl-EC12 or Glc-EC14) (final concentration 1 mM) were dissolved in methanol to yield a lipid mixture solution.

[0202]

(Study of introduction of siRNA)

CHO-EGFP cells (CHO cells that permanently express EGFP, prepared by a conventional method) at 1×10^5 cells/well were pre-cultured in a 24-well plate for 24 hours (DMEM medium supplemented with 10% FBS), after which, and just before introduction, the medium was replaced with 0.5 ml of a fresh supply of the same 10% FBS medium. 10 pmol per well of anti-EGFP-siRNA (obtained from Nippon Gene Co., Ltd.) was dissolved in 50 μ l of Opti-MEM medium; 5 μ l of the above-described lipid mixture solution was added to the resulting siRNA solution, followed by incubation for 20 minutes, whereby an RNA-lipid complex was obtained. This complex was added to the above-

described CHO-EGFP cells, and the cells were cultured in a 5% CO₂ incubator at 37°C for 24 hours. Also, the commercially available gene transfer reagent MaxReagent (obtained from Invitrogen) was tested as directed in the instruction manual.

5 The following day, the cells were examined under a fluorescence microscope, and the cells exhibiting fluorescence were counted using a flow cytometer.

[0203]

As shown in Fig. 21, the siRNA transfection efficiency rose when each glycolipid (Glc-EC12, Gal-EC12, Man-EC12, All-EC12, Fuc-EC12, Xyl-EC12 or Glc-EC14) was added to the cationic lipid TMA-EC12, and highly efficient gene suppression was observed.

[0204]

15 [Experimental Example 5]

Introduction of plasmid DNA using various glycolipids and cationic lipids or peptide lipids

(Preparation of lipid mixture solutions)

Each cationic lipid or peptide lipid (TMA-EC12, TMA-EC14, TMA-DEAC12, R-EC12, R-EC14, K-EC12 or K-EC14) (final concentration 1 mM) and the Glc-EC12 glycolipid (final concentration 1 mM) were dissolved in methanol to yield a lipid mixture solution.

[0205]

25 (Introduction of plasmid DNA)

Plasmid DNA (1 µg/well, pCMVIE-GFP vector) was introduced into CHO cells (2*10⁵ cells/well) in a 24-well plate using the above-described lipid mixture solution (5 µl/well) by the same procedures as in Experimental Example 1. The following day, the cells were examined under a fluorescence microscope, and the cells exhibiting fluorescence were counted using a flow cytometer.

[0206]

As shown in Fig. 22, the transfection efficiency rose when the glycolipid Glc-EC12 was added to each cationic lipid

or peptide lipid (TMA-EC12, TMA-EC14, TMA-DEAC12, R-EC12, R-EC14, K-EC12 or K-EC14) . This result suggests that the compounds of the present invention, irrespective of the choice of lipid, possess an activity to increase the efficiency of introduction of a compound of interest into cells by the lipid.

[0207]

[Experimental Example 6]

Increase in transfection efficiency by glycolipids added at different mixing ratios

10 (Preparation of lipid mixture solutions)

The cationic lipid TMA-EC12 (1 mM) and the Glc-EC12 glycolipid (final concentration 0.1 mM, 1.0 mM, 5.0 mM) were dissolved in methanol to yield a lipid mixture solution.

[0208]

15 (Introduction of plasmid DNA)

Human lung cancer cells, specifically A549 cells (1×10^5 cells/well) were pre-cultured in a 24-well plate for 24 hours (DMEM medium supplemented with 10% FBS), after which, and just before introduction, the medium was replaced with 0.5 ml of a fresh supply of the same 10% FBS medium. 1 μ g per well of plasmid DNA (pCMVIE-GFP vector) was dissolved in 50 μ l of Opti-MEM medium; 5 μ l of the lipid-mixed solution was added to the resulting DNA solution, and this was followed by incubation for 20 minutes, whereby a DNA-lipid complex was prepared. This complex was added to the A549 cells, and the cells were cultured in a 5% CO₂ incubator at 37°C. The following day, the cells were examined under a fluorescence microscope, and the cells exhibiting fluorescence were counted using a flow cytometer .

30 [0209]

As shown in Fig. 23, the transfection efficiency rose when the glycolipid Glc-EC12 was added to a cationic lipid (TMA-EC12) . In the range of 0.1 to 5.0 mM, based on the amount of Glc-EC12 added per 1 mM of TMA-EC12, a rise of the transfection efficiency with Glc-EC12 was observed. This

result suggests that the compounds of the present invention possess an activity to increase the transfection efficiency with a cationic lipid when added in a broad range of amounts added.

5

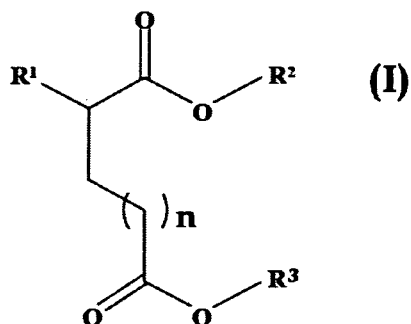
Industrial Applicability

[0210]

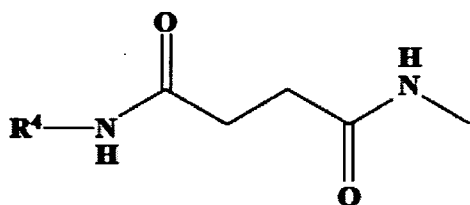
It is possible to increase the efficiency of introduction of a compound into cells by a lipid using the compound of the
10 present invention. Using the compound of the present invention also makes it possible to introduce a compound of interest into cells with high efficiency even under conditions of basal medium, serum medium, or totally synthesized medium. The compounds of the present invention are highly safe because of
15 the absence of infectious or pathogenic materials such as animal-derived ingredients and virus-derived ingredients.

CLAIMS

1. A compound represented by the formula (I) :



- 5 wherein R¹ represents R⁵=N- (wherein R⁵ represents a saccharide residue) or

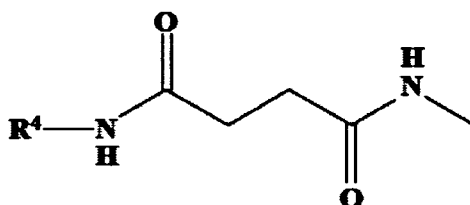


- (wherein R⁴ represents a mono- or polyethylene glycol residue) ;
each of R² and R³, whether identical or not, represents a
10 hydrocarbon group having 1 to 30 carbon atoms; and
n is 0 or 1.

2. The compound according to claim 1, wherein R¹ is R⁵=N-
and R⁵ is a monosaccharide or disaccharide residue.

15

3. The compound according to claim 1, wherein R¹ is,



- and R⁴ is a group represented by -CH₂CH₂ (OCH₂CH₂)_m OH (wherein m
represents an integer of 0 to 20) .

20

4. The compound according to claim 1, wherein each of R² and

R³ is a C₆₋₂₀ linear alkyl group or linear unsaturated hydrocarbon group.

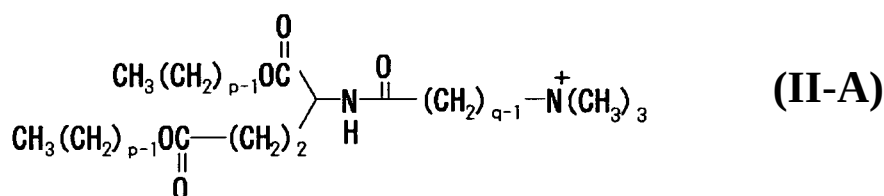
5. The compound according to claim 1, wherein the compound
 5 is G1C-EC12, G1C-EC6, Glc-EC8, Glc-EC10, Glc-EC14, Gal-EC12, Man-EC12, A11-EC12, Fuc-EC12, Xyl-EC12, GlcNAc-EC12, 2EG-EC10, 2EG-EC12, 4EG-EC10, 4EG-EC12, 2EG-EC14, or 4EG-EC14. .

6. An agent for promoting introduction of a compound of
 10 interest into cells, comprising the compound according to any one of claims 1 to 5.

7. A composition comprising the compound according to any one of claims 1 to 5 and a lipid having an activity to
 15 introduce a compound of interest into cells.

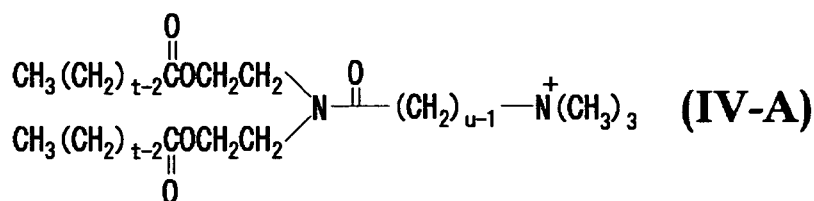
8. The composition according to claim 7, wherein the lipid is a cationic lipid or a peptide lipid.

20 9. The composition according to claim 7, wherein the lipid is a compound represented by the formula (II-A) :



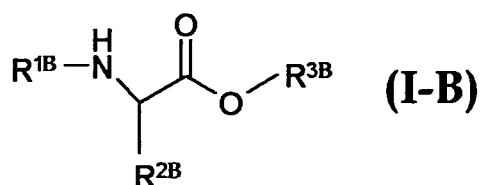
wherein p represents an integer of 12 to 16, and q represents an integer of 2 to 11;

25 a compound represented by the formula (IV-A) :



wherein t represents an integer of 12 to 16, and u represents an integer of 2 to 11; or

a compound represented by the formula (I-B) :



wherein $\text{R}^{1\text{B}}$ represents an amino acid or peptide having 1 to 10 amino acid residues, $\text{R}^{2\text{B}}$ represents an optionally chosen amino acid side chain, $\text{R}^{3\text{B}}$ represents a hydrocarbon group having 1 to 30 carbon atoms, and with the provision that $\text{R}^{2\text{B}}$ has a carboxyl group, the carboxyl group may be esterified with a saturated or unsaturated alcohol having 1-30 carbon atoms.

10. The composition according to claim 9, wherein the lipid is TMA-EC12, TMA-EC14, TMA-DEAC12, TMA-DEAC14, R-EC12, R-EC14, K-EC12, or K-EC14.

11. The composition according to claim 7, wherein the composition is for introducing a compound of interest into cells.

12. The composition according to claim 11, wherein the compound of interest is a nucleic acid.

13. The composition according to claim 12, wherein the nucleic acid is a plasmid DNA, cDNA or antisense DNA, or an siRNA, miRNA, shRNA, mRNA, antisense RNA or RNA replicon.

14. The composition according to claim 7, wherein the composition further comprises the compound of interest.

15. A method of introducing a compound of interest into cells, comprising contacting the composition according to claim 14 and the cells.

30

16. A method of introducing a compound of interest into cells in a human or non-human subject, comprising administering the

composition according to claim 14 to the subject.

17. A kit for introducing a compound of interest into cells,
comprising the compound according to any one of claims 1 to 5,
5 and a lipid having an activity to introduce the compound of
interest into cells.

18. A complex comprising the compound according to any one of
claims 1 to 5, a lipid having an activity to introduce a
10 compound of interest into cells, and the compound of interest.

INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2011/058663

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C07H15/12 A61K31/7008 C07C251/00
 ADD.

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)

C07H C07C 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 , WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Week 200674 Thomson Scientific, London, GB; AN 2006-711913 XP002668201, & JP 2006 254877 A (DOJIN KAGAKU KENKYUSHO KK) 28 September 2006 (2006-09-28) cited in the application abstract -----	1, 2, 4-18
A	wo 2007/099650 A1 (FUKUOKA PREFECTURE [JP]; UNIV KYOTO [JP]; DOJINDO LAB [JP]; KUSUMOTO K) 7 September 2007 (2007-09-07) cited in the application -----	1, 2, 4-18



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

5 April 2012

Date of mailing of the international search report

19/04/2012

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040,
 Fax: (+31-70) 340-3016

Authorized officer

Bardi I i , Burkhart

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/058663

Box No. 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. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

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

Box No. 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:

see additional sheet

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

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

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. :

2(compl etely) ; 1, 4-18(parti al ly)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims : 2 (completely) ; 1, 4-18 (partially)

a compound according to formula (I) wherein R represents R⁵=N, R⁵ being a saccharide residue, compositions comprising this compound and the use thereof

2. claims : 3 (completely) ; 1, 4-18 (partially)

a compound according to formula (I) wherein R represents R⁴NHC(O)-CH₂-CH₂-C(O)NH-, R⁴ being a mono- or polyethylene glycol residue, compositions comprising this compound and the use thereof

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/JP2011/058663

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2006254877	A	28-09-2006	NONE

W0 2007099650	AI	07-09-2007	CA 2644552 AI 07-09-2007
			CN 101421293 A 29-04-2009
			JP 2009528258 A 06- 08-2009
			US 2009170960 AI 02-07-2009
			W0 2007099650 AI 07- 09-2007
