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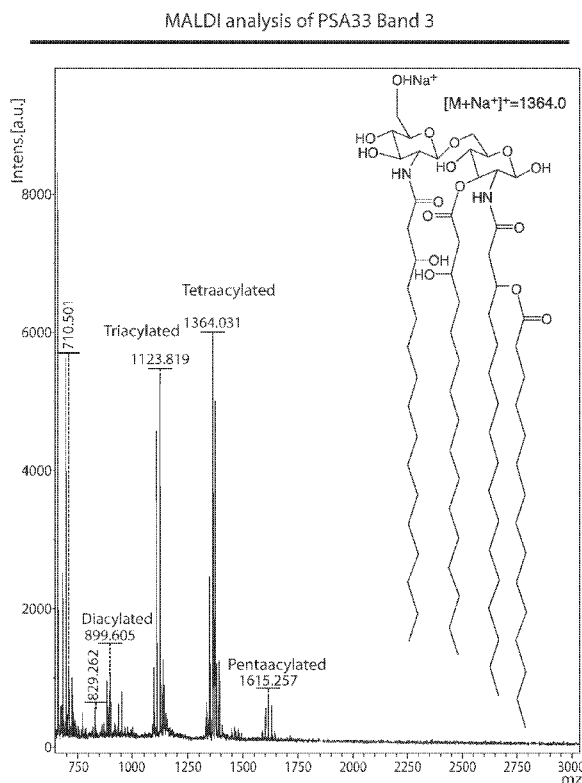
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[Continued on next page]

(54) Title: LIPIDATED PSA COMPOSITIONS AND METHODS



(57) Abstract: The invention provides various isolated and synthetic forms of lipidated PSA as well as isolated or synthetic forms of the glycolipid component of lipidated PSA, and compositions thereof, methods of making including methods of isolating such forms, and methods of use thereof.



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- 1 -

LIPIDATED PSA COMPOSITIONS AND METHODS

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application Serial No. 62/207,360, filed August 19, 2015, entitled “LIPIDATED PSA COMPOSITIONS AND METHODS”, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to lipidated capsular polysaccharide A (PSA), glycolipids, compositions, methods of synthesis, isolation and/or purification, and methods of use thereof.

BACKGROUND OF THE INVENTION

Polysaccharide A (PSA) of *Bacteroides fragilis* (*B. fragilis*) has been reported to be an immunomodulator with therapeutic and prophylactic activities. U.S. Pat. Nos. 5,679,654 and 5,700,787; Tzianabos AO et al. (2000) J Biol Chem 275:6733-40. PSA was recently discovered to possess a lipid moiety. The lipid moiety was hypothesized to anchor the polysaccharide in the *B. fragilis* outer membrane. It was also recently discovered that this “lipidated PSA” was significantly more potent than non-lipidated PSA (referred to herein as “PSA”) forms provided in the prior art. The nature of the lipid moiety, however, has not been heretofore determined, and nor has the nature of its association with PSA.

SUMMARY OF THE INVENTION

The invention is based, in part, on the identification and characterization of the lipid moiety that is found conjugated to PSA using certain isolation methods. This disclosure provides the full structural identification and characterization of PSA conjugated to such lipid moiety (referred to herein as “lipidated PSA”). The invention is further premised, in part, on novel isolation methods and the recognition that such methods achieve greater yields of lipidated PSA than was heretofore possible. Significantly, the majority of prior art PSA isolation methods did not yield the lipidated form of the PSA at all. This is likely due, in part, to the use of a relatively stringent acid hydrolysis step late in the isolation process which released the lipid moiety from the lipidated PSA, thereby resulting in non-lipidated PSA.

- 2 -

The disclosure therefore provides, in some aspects, isolated lipidated PSA of a defined chemical structure, as well as compositions comprising isolated lipidated PSA. Such compositions may be further defined by the purity and/or concentration of isolated lipidated PSA contained therein. It has also been discovered that isolated lipidated PSA self assemble
5 into a micelle form. Significantly, lipidated PSA does not adopt such a conformation in vivo, and accordingly such micelle form is non-naturally occurring. Even more significantly, it has been discovered that such micelles are very stable and thus difficult to disrupt. This has led to the finding that the combined use of certain disruptive agents (such as for example detergents and bile salts) and isolated lipidated PSA having a purity or concentration
10 conducive to forming such stable micelles (as provided herein), renders the micelles less stable and makes more lipidated PSA accessible in vivo.

Also provided are synthetic forms of lipidated PSA and compositions thereof comprising one or more PSA polymers (each polymer comprising one or more of the repeating tetrasaccharide units of PSA), and one or more of the lipid or glycolipid
15 components of lipidated PSA. The PSA and lipid or glycolipid components may be conjugated to each other directly or indirectly. Such conjugation may be covalent or non-covalent conjugation. As an example, the invention provides compositions comprising the PSA and lipid or glycolipid components in an unconjugated form together with a substrate such as a nanoparticle. These synthetic forms of lipidated polysaccharides may comprise
20 PSA components (including tetrasaccharide units) and lipid or glycolipid components in ratios that are not found in nature.

The invention further provides methods of isolating lipidated PSA, methods of preparing the aforementioned synthetic forms of lipidated PSA, as well as in vitro and in vivo uses of the isolated and synthetic forms of lipidated PSA provided herein.

25 Thus, in one aspect, the invention provides an isolated lipidated polysaccharide A (PSA) comprising polysaccharide A (PSA) covalently conjugated to a glycolipid, wherein the glycolipid is di-acylated, tri-acylated, tetra-acylated or penta-acylated. In some embodiments, the glycolipid is tetra-acylated or penta-acylated.

In another aspect, the invention provides an isolated lipidated polysaccharide A (PSA)
30 comprising polysaccharide A (PSA) covalently conjugated to a glycolipid comprising one or more acyl chains ranging in length from 14-17 carbons.

- 3 -

In various embodiments, the glycolipid comprises a disaccharide substituted with the one or more acyl chains. In various embodiments, the glycolipid comprises a diglucosamine.

In another aspect, the invention provides an isolated lipidated polysaccharide A (PSA) comprising polysaccharide A (PSA) covalently conjugated to one or more acyl chains

5 ranging in length from 14-17 carbons.

In various embodiments, the one or more acyl chains range in length from 15-17 carbons.

In various embodiments, the isolated lipidated polysaccharide A (PSA) is substantially free of other components found in a *B. fragilis* capsule. In various
10 embodiments, the isolated lipidated polysaccharide A (PSA) is substantially free of LPS. In various embodiments, the isolated lipidated polysaccharide A (PSA) is substantially free of unconjugated glycolipid. In various embodiments, the isolated lipidated polysaccharide A (PSA) is free of non-lipidated PSA. In various embodiments, the isolated lipidated polysaccharide A (PSA) is in purified form. In various embodiments, the lipidated
15 polysaccharide A (PSA) is isolated from *B. fragilis* cells that overexpress PSA relative to polysaccharide B (PSB).

In various embodiments, the isolated lipidated polysaccharide A (PSA) is provided in a form is free of *B. fragilis* membrane, and thus is not provided as a *B. fragilis* cell or as a *B. fragilis* OMV. In various embodiments, the isolated lipidated polysaccharide A (PSA),
20 including synthetic, non-naturally occurring versions of lipidated polysaccharide A (PSA), may be provided in a liposome or micelle form, and such liposome or micelle form may be non-naturally occurring (e.g., it may lack naturally occurring components and/or it may further comprise non-naturally occurring components such as non-naturally occurring lipids, surfactants, stabilizers, and the like).

25 In various embodiments, the isolated lipidated polysaccharide A (PSA) is in a micelle form.

In various embodiments, the isolated lipidated polysaccharide A (PSA) is in lyophilized form. Lyophilized forms of lipidated PSA are particularly suitable for long-term storage, ranging from days, weeks, months or even years.

30 In various embodiments, the isolated lipidated polysaccharide A (PSA) is suitable for administration to a human.

- 4 -

In another aspect, the invention provides any of the foregoing lipidated polysaccharide A (PSA), wherein the PSA component comprises less than 100, less than 90, less than 80, less than 70, less than 60, or less than 50 repeating tetrasaccharide units.

5 In another aspect, the invention provides any of the foregoing lipidated polysaccharide A (PSA), wherein the PSA component comprises 1-10 repeating tetrasaccharide units.

In another aspect, the invention provides a pharmaceutical composition comprising any of the foregoing lipidated polysaccharide A (PSA), and less than 0.5% (w/w) of free glycolipid.

10 In another aspect, the invention provides a pharmaceutical composition comprising any of the foregoing lipidated polysaccharide A (PSA), and a pharmaceutically acceptable carrier.

In various embodiments, the composition comprises less than 1% or less than 0.5% free glycolipid (w/w). In various embodiments, the composition further comprises a
15 detergent or a bile salt. In various embodiments, the detergent or bile salt is present at a pharmaceutically acceptable level. In various embodiments, the detergent or bile salt is present at or less than 1%, 0.5% or 0.1%.

In various embodiments, the composition is in lyophilized form.

20 In various embodiments, the isolated lipidated polysaccharide A (PSA) is provided as a micelle or a liposome.

In another aspect, the invention provides a pharmaceutical composition comprising any of the foregoing lipidated polysaccharide A (PSA), and a detergent or a bile salt.

In another aspect, the invention provides a composition comprising any of the foregoing lipidated polysaccharide (PSA) in a micelle or a liposome.

25 In another aspect, the invention provides a composition comprising polysaccharide A (PSA) comprising 1 to 50 tetrasaccharide units, and a glycolipid, wherein PSA is covalently conjugated to the glycolipid.

In another aspect, the invention provides a composition comprising a polysaccharide comprising 1 to 50 tetrasaccharide units, each tetrasaccharide unit having a structure of
30 Formula I, and a glycolipid comprising one or more acyl chains ranging in length from 14-17 carbons, wherein the polysaccharide is covalently conjugated to the glycolipid.

- 5 -

In various embodiments, the polysaccharide comprises 1-40 tetrasaccharide units or 1-20 tetrasaccharide units. In various embodiments, the polysaccharide comprises 1-10 tetrasaccharide units or 1-5 tetrasaccharide units.

5 In various embodiments, the composition is formulated for parenteral or enteral or oral administration to a subject. In various embodiments, the composition is formulated for lipophilic delivery, including for example in a liposome or in an oil-based delivery system. The various compositions provided herein may be formulated as a capsule or other discrete dosage form, including those intended for oral or enteral administration.

10 In another aspect, the invention provides an isolated glycolipid comprising a diglucosamine covalently conjugated to 2-5 acyl chains, each independently ranging in length from 14-17 carbons. The glycolipid may be any of the glycolipids provided herein, or a combination thereof.

15 In various embodiments, the diglucosamine is covalently conjugated to 2-5 or 2-4 acyl chains. In various embodiments, the diglucosamine is covalently conjugated to 4 or 5 acyl chains. In various embodiments, the acyl chains range in length from 15-17 carbons. Other embodiments relating to the glycolipids are recited below.

In another aspect, the invention provides a composition comprising polysaccharide A (PSA) and a glycolipid, in or on a substrate, wherein PSA is not covalently conjugated to the glycolipid.

20 In another aspect, the invention provides a composition comprising a polysaccharide comprising one or more tetrasaccharide units, each tetrasaccharide unit having a structure of Formula I, and a glycolipid comprising one or more acyl chains ranging in length from 14-17 carbons, wherein the polysaccharide and glycolipid are provided in unconjugated to each other, in or on a substrate.

25 In various embodiments, the substrate is a nanoparticle.

In various embodiments, PSA and the glycolipid are present in a molecular weight ratio of 10:1 to less than 1:1.

In another aspect, the invention provides a composition comprising polysaccharide A (PSA) and a glycolipid, covalently conjugated to each other via a non-ketosidic bond.

30 In another aspect, the invention provides a composition comprising a polysaccharide comprising one or more tetrasaccharide units, each tetrasaccharide unit having a structure of Formula I, and a glycolipid comprising one or more acyl chains ranging in length from 14-17

- 6 -

carbons, wherein the polysaccharide is covalently conjugated to the glycolipid via a non-ketosidic bond.

In various embodiments, the non-ketosidic bond is an ester, amide or ether bond.

In various embodiments, the glycolipid comprises a disaccharide. In various
5 embodiments, disaccharide is diglucosamine.

In various embodiments, the glycolipid comprises 2-5 acyl chains. In various
embodiments, the glycolipid comprises 4 or 5 acyl chains.

In various embodiments, at least one of the one or more acyl chains is unmodified. In
various embodiments, at least one of the one or more acyl chains is modified. In various
10 embodiments, at least one of the one or more acyl chains is unmodified and at least one of the
one or more acyl chains is modified. In various embodiments, at least one of the one or more
acyl chains is modified with a hydroxyl group.

In various embodiments, at least one of the one or more acyl chains is C16:0-OH. In
various embodiments, at least one of the one or more acyl chains is C17:0-OH. In various
15 embodiments, at least one of the one or more acyl chains is C14:0. In various embodiments,
at least one of the one or more acyl chains is C15:0.

In various embodiments, at least one of the one or more acyl chains is N-substituted
on a disaccharide. In various embodiments, at least one of the one or more acyl chains is O-
substituted on a disaccharide. In various embodiments, at least one of the one or more acyl
20 chains is N-substituted on a disaccharide and at least one of the one or more acyl chains is O-
substituted on a disaccharide.

In various embodiments, the polysaccharide has a molecular weight of about 150
kiloDaltons. In various embodiments, the polysaccharide comprises 1-10 tetrasaccharide
units.

25 In various embodiments, the glycolipid has a structure of Formula II. In various
embodiments, the glycolipid has a structure of Formula III.

In various embodiments, polysaccharide and the glycolipid are present in or on a
substrate. In various embodiments, the substrate is a film, a matrix or a particle. In various
embodiments, the substrate is biodegradable. In various embodiments, the substrate is a
30 nanoparticle.

In various embodiments, the composition further comprises a pharmaceutically
acceptable carrier. In various embodiments, the composition is a pharmaceutical composition.

- 7 -

In various embodiments, the composition is formulated for parenteral, enteral or oral administration. In various embodiments, the composition is effective in the treatment of an autoimmune disorder. In various embodiments, the composition is substantially free of other components found in a *B. fragilis* capsule and is suitable for administration to a human.

5 In another aspect, the invention provides a micelle consisting essentially of lipidated PSA. In various embodiments, the lipidated PSA is isolated lipidated PSA.

In another aspect, the invention provides a composition comprising a micelle consisting essentially of lipidated PSA and a detergent or bile salt.

10 In various embodiments, the detergent or bile salt is present in a pharmaceutically acceptable amount.

In various embodiments, the composition is a pharmaceutical composition.

15 In another aspect, the invention provides a non-hydrolytic method for isolating lipidated polysaccharide A (PSA) from *B. fragilis*, comprising extracting, into an aqueous phase, capsular complex from *B. fragilis* using a mixture of phenol and water, precipitating a polysaccharide fraction from the aqueous phase using ethanol, and isolating lipidated PSA from the polysaccharide fraction by size exclusion.

20 In various embodiments, isolating by size exclusion comprises using a chromatographic column comprising a detergent or a bile salt. In various embodiments, the chromatographic column comprises deoxycholate. In various embodiments, the method is performed in the presence of sodium deoxycholate.

In various embodiments, the method is performed at a pH less than about 9.

In various embodiments, the method further comprises dialyzing the isolated lipidated PSA.

25 In various embodiments, extraction occurs at 60-75°C. In various embodiments, extraction occurs at about 68°C.

In various embodiments, *B. fragilis* is a mutant form of *B. fragilis* that over-expresses PSA relative to PSB.

In various embodiments, the isolated lipidated PSA is substantially free of unconjugated glycolipid.

30 In another aspect, the invention provides a composition comprising isolated lipidated polysaccharide A produced by any of the foregoing methods.

- 8 -

In various embodiments, the composition is formulated for parenteral, enteral or oral administration to a subject.

In another aspect, the invention provides a method comprising administering, to a subject having or at risk of developing a condition associated with inflammation, an effective amount of any of the foregoing lipidated PSA or any of the foregoing compositions.

In various embodiments, the condition is an autoimmune disease. In various embodiments, the autoimmune disease is multiple sclerosis, Crohn's disease, ulcerative colitis, rheumatoid arthritis, or type I diabetes. In various embodiments, the condition is asthma.

In various embodiments, the condition is a post-surgical adhesion. In various embodiments, the composition is administered prior to, during, and/or after surgery. In various embodiments, the condition is an abscess. In various embodiments, an antibiotic is administered to the subject. In various embodiments, the condition is obesity.

In various embodiments, the composition is parenterally or enterally administered to the subject.

It is to be understood that various foregoing aspects and embodiments overlap. It is intended that the embodiments recited above apply equally to the various aspects recited above.

It should be appreciated that all combinations of the foregoing concepts and additional concepts discussed in greater detail below (provided such concepts are not mutually inconsistent) are contemplated as being part of the inventive subject matter disclosed herein. In particular, all combinations of claimed subject matter appearing at the end of this disclosure are contemplated as being part of the inventive subject matter disclosed herein. It should also be appreciated that terminology explicitly employed herein that also may appear in any disclosure incorporated by reference should be accorded a meaning most consistent with the particular concepts disclosed herein.

BRIEF DESCRIPTION OF THE FIGURES

It is to be understood that the Figures are not necessarily to scale, emphasis instead being placed upon generally illustrating the various concepts discussed herein.

- 9 -

FIG. 1A provides representative mass spectrometry (MS) spectra for tetra-acylated (bottom) and penta-acylated (top) glycolipids released from lipidated PSA.

FIG. 1B provides LC-MS/MS profiles for *B. fragilis* PSA glycolipid anchors showing monophosphorylated and unphosphorylated glycolipid species having different numbers of acyl chains. Species with the same number of acyl chains may still differ from each other with respect to total chain length and/or nature (composition) of such acyl chains. In some instances, ~30 total glycolipid species have been identified within a group of species having the same number of acyl chains.

FIG. 2A provides a comparison of the MS spectra for penta-acylated glycolipids (from the top, panels 1 and 2) and tetra-acylated glycolipids (from the top, panels 3 and 4) released from lipidated PSA. The material in panels 1 and 3 was obtained using a mild hydrolysis step earlier in the isolation process. The material in panels 2 and 4 was obtained using a harsher hydrolysis step later in the isolation process. The lipid moieties are detectable in panels 1 and 3 but not detectable in panels 2 and 4, evidencing the detrimental effect of the harsher and later in time acid hydrolysis step on the lipid moieties.

FIG. 2B provides MS/MS assignment of the structure of a species isolated from *B. fragilis*. The species at $m/z=1674.2$ has been determined to be a pentaacylated and monophosphorylated with saturated or monohydroxylated C15-C17 fatty acids.

FIG. 3 is a MALDI-MS spectrum of lipid moieties released from lipidated PSA showing peaks for the di-acylated, tri-acylated, tetra-acylated and penta-acylated lipid moieties. The structure on the right is an example of a tetra-acylated glycolipid from lipidated PSA. The structure comprises hydroxyl groups at carbons C1 and C4 (carbons on the right-most substituted glucosamine (or reducing sugar)) and on carbons C3' and C4' (carbons on the left-most substituted glucosamine (or non-reducing sugar)). This disclosure embraces variants thereof that comprise a phosphate group (e.g., $-OPO_3H$) in place of the hydroxyl ($-OH$) at the C1 or C4' position.

FIG. 4 is a bar graph showing quantitative analysis of lipid moieties conjugated to PSA. Six different lipid moieties are shown, each having a different MS position. The tetra-acylated lipid moieties, having an m/z in the range of about 1350-1378, and the penta-acylated lipid moieties, having an m/z in the range of about 1604-1634, are shown. For each lipid moiety, there are two bars shown: the first corresponds to a material generated using the harsher and later acid hydrolysis step (PSA 23), and the second corresponds to a material

- 10 -

generated using the milder and earlier acid hydrolysis step (PSA 24). The Figure shows that the lipid moieties are preserved when the milder and earlier acid hydrolysis step is used. It also shows the relative proportion of the different moieties, with the m/z 1378 moiety being the most prevalent tetra-acylated version and the m/z 1618 moiety being the most prevalent penta-acylated version.

FIG. 5 is a table listing molecular species of glycolipids derived from lipidated PSA and their proposed acyl chain composition. The table lists 5 different penta-acylated species, 5 different tetra-acylated species, 4 different tri-acylated species, and 2 different di-acylated species. The highlighted species within each grouping (for example, 1632, 1618 and 1604) represent the most abundant species. Of the penta-acylated species, the 1632 and 1618 species are the most prevalent, followed by the 1604 species, followed by the 1646 and 1590 species. Of the tetra-acylated species, the 1378 and 1364 species are the most prevalent, followed by the 1350 species, followed by the 1392 and 1336 species. Of the tri-acylated species, the 1123 and 1109 species are the most prevalent, followed by the 1095 species, followed by the 1081 species. Of the di-acylated species, the 899 and 885 species are about equally prevalent. Of all the species, the abundance of the various groups is as follows (from most to least abundant): tetra-acylated, penta-acylated, tri-acylated and di-acylated. A typical ratio of these glycolipids hydrolyzed from lipidated PSA is approximately di: tri: tetra: penta = trace: 0.5: 3: 2. The species listed in the Figure are typically observed in a preparation of lipidated PSA isolated using the methods provided herein. Such methods preferably do not include an acid hydrolysis step, and thereby result in a greater proportion of fully lipidated PSA being isolated relative to prior art methods. Such methods also include in some instances sodium deoxycholate or other bile salt.

FIG. 6A provides representative structures of glycolipids from lipidated PSA. A representative tetra-acylated diglucosamine having a molecular weight of about 1341.1 is shown on the left (referred to herein as Formula II), and a representative penta-acylated diglucosamine having a molecular weight of about 1595.3 is shown on the right (referred to herein as Formula III). The tetra-acylated structure at the left comprises hydroxyl groups at the C1, C4, C3' and C4' positions. The penta-acylated structure at the right comprises hydroxyl groups at the C1, C4 and C4' positions. Monophosphorylated variants of these compounds are also provided herein comprising a phosphate group in place of the hydroxyl at the C1 or the C4' position.

FIG. 6B provides a pentaacylated, monophosphorylated species of glycolipid. The phosphorylation exists at the C1 position.

FIG. 7 is a bar graph showing the results of an IL-10 induction assay. Material prepared using the milder and early acid hydrolysis step is identified as PSA24. Material prepared using the harsher and later acid hydrolysis step is identified as PSA 23. CPC represents capsular polysaccharide complex from wild-type *B. fragilis* NCTC 9373.

FIG. 8 is a bar graph showing the results of a IL-10 induction assay in splenic DC+Tcell coculture. The Figure uses an isolated form of lipidated PSA that is considered to be more fully lipidated than prior art preparations, intending a higher purity of lipidated PSA as compared to prior art methods. This preparation is referred to herein as “fully lipidated PSA” because it apparently contains no free (or released) glycolipid component. The Figure shows that the isolated fully lipidated PSA preparation (denoted PSA Lot 34) aggregates in the absence of deoxycholate, and such aggregation results in less IL-10 inducing activity. Addition of small amounts of deoxycholate, which disperses the aggregates or renders them less stable, results in a significant increase in the ability of Lot 34 to stimulate IL-10 production by T cells. This observation was not made using PSA Lot 28 which was prepared by mild acid treatment. This suggests that the fully lipidated PSA isolated using the methods provided herein, adopts a different conformation than lipidated PSA isolated using prior art methods.

FIG. 9 provides chromatographic elution profiles of fully lipidated PSA Lot 40 using a PBS column (top) and a deoxycholate column (bottom). Monitoring molecular size of fractions by refractive index demonstrates a major reduction in size of Lot 40 when a column equilibrated with deoxycholate is used. This is due to the disruption of micelles formed by isolated, fully lipidated PSA.

FIG. 10 is a photograph of a zinc sulphate/imidazole stained SDS PAGE gel. Isolated fully lipidated PSA preparations (PSA Lot 34) display much less free lipids as compared to PSA prepared by mild acid treatment (PSA Lot 28).

FIG. 11 is a bar graph showing EAE cumulative score of PBS and PSA Lot 40 treated mice. PBS mice, n= 8; PSA mice, n=7. PSA treatment: oral gavages every other day (75 microgram dose), starting on day 2.

- 12 -

FIG. 12 provides MS spectra for a number of penta-, tetra- and tri-acylated glycolipids obtained from a lipidated PSA preparation prepared using a non-hydrolytic method (PSA Lot 40).

FIG. 13 provides MS spectra for a number of penta-acylated glycolipids obtained from a lipidated PSA preparation prepared using a non-hydrolytic method (PSA Lot 40). These various glycolipid species differ from each other in acyl chain lengths.

FIG. 14 provides a table listing various glycolipid species obtained from a lipidated PSA preparation prepared using a non-hydrolytic method (PSA Lot 40). The table demonstrates the complexity of glycolipid component of lipidated PSA. Such complexity is imparted by differences in chain length and hydroxylation.

FIG. 15 provides representative structures for glycolipids species obtained from a lipidated PSA preparation prepared using a non-hydrolytic method (PSA Lot 40). Monophosphorylated variants of the illustrated compounds are also provided herein comprising a phosphate group in place of the hydroxyl at the C1 or C4' positions.

FIG. 16 provides an elution profile and a structure for glycolipid species obtained from a lipidated PSA preparation prepared using a non-hydrolytic method (PSA Lot 40). Monophosphorylated variants of the illustrated compound are also provided herein comprising a phosphate group in place of the hydroxyl at the C1 or C4' positions.

DETAILED DESCRIPTION OF THE INVENTION

Provided herein is the structural identification and characterization of the lipid moieties of lipidated PSA. It has been found in accordance with the invention that these lipid moieties are glycolipids comprised of a diglucosamine substituted with one or more acyl chains. In the naturally occurring form, the glycolipid is conjugated to its neighbouring tetrasaccharide unit through a ketosidic bond, an acid labile bond that is susceptible to acid hydrolysis. Lipidated PSA has been shown to be more immunologically potent than its non-lipidated counterpart, PSA. For example, as demonstrated in the Examples, lipidated PSA is better able to induce IL-10 production (and therefore better able to interact with Treg cells) than non-lipidated PSA.

Lipidated PSA

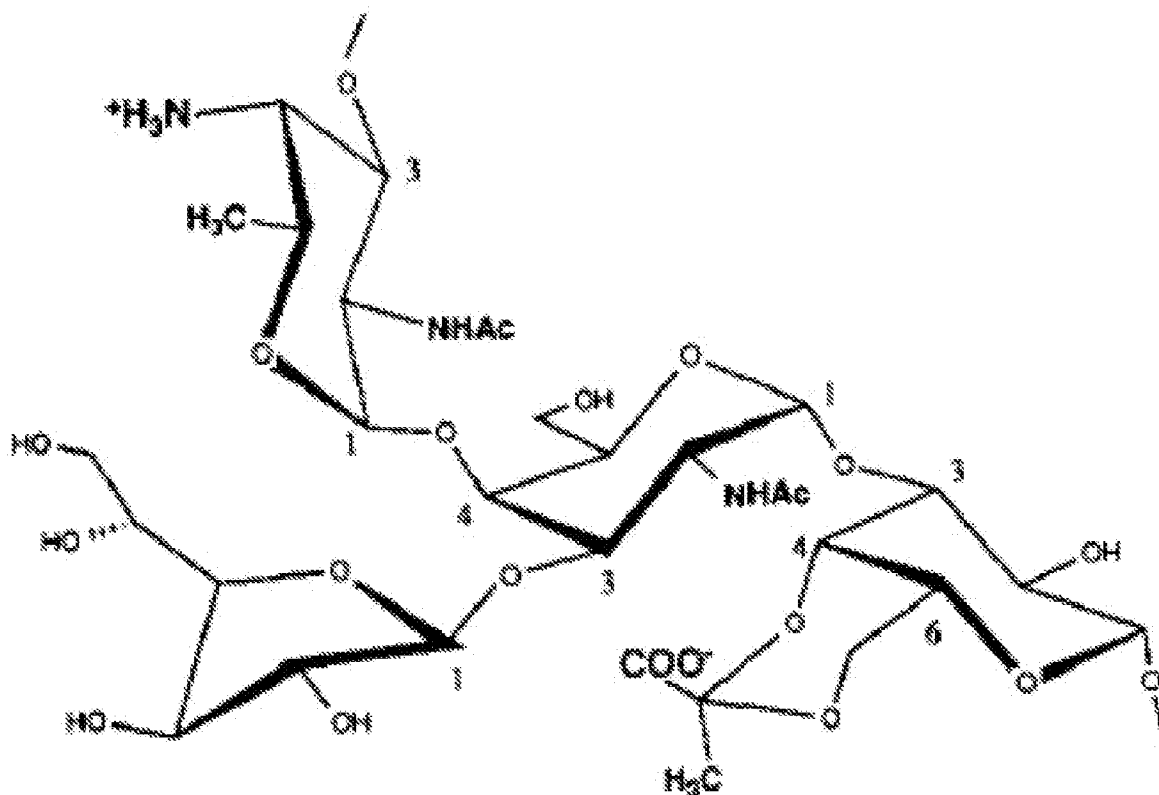
- 13 -

The invention relates in part to the characterization of the lipid moiety of lipidated PSA and the newly recognized glycolipid structure and lipid complexity of lipidated PSA, and the nature of the conjugation of this glycolipid structure to PSA. It has now been discovered that lipidated PSA comprises a glycolipid moiety at the reducing end of its polysaccharide component. This glycolipid comprises a disaccharide substituted with one and typically more than one acyl chains.

Polysaccharide component

The polysaccharide component of lipidated PSA, referred to herein as PSA, comprises a tetrasaccharide repeating unit shown below. It possesses zwitterionic behavior as conferred by a positive charge on its free amine group and a negative charge on its free carboxyl group (per repeating tetrasaccharide unit). Its naturally occurring state has been reported to comprise over 60 tetrasaccharide repeating units (e.g., up to and including in some instances about 100, or about 200, or about 300 repeated units on average), and it has an average molecular size of about 150 kD (with a range of about 75 kD to 240 kD).

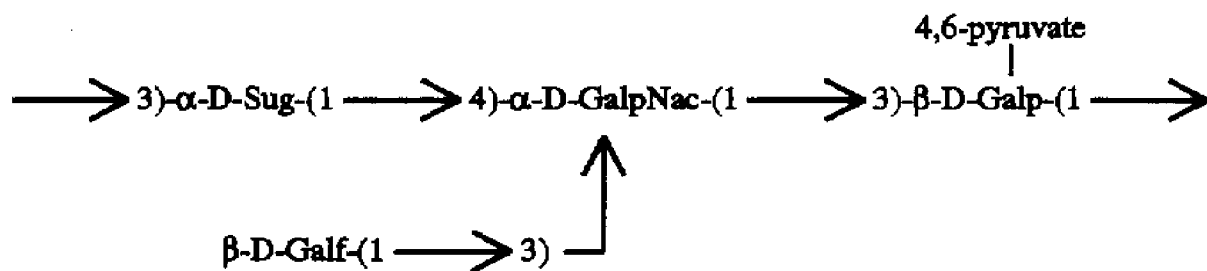
The repeating tetrasaccharide unit of PSA has a structure as follows:



- 14 -

(Formula I).

The tetrasaccharide repeating unit may also be expressed as follows:



The invention contemplates synthetic forms of lipidated PSA comprising fewer tetrasaccharide units (e.g., 1-60, 1-50, 1-40, 1-30, 1-20, 1-10, 1-9, 1-8, 1-7, 1-6, or 1-5 tetrasaccharide units, or any number of units therebetween as is explicitly recite herein including but not limited to 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 units, for example). Such shorter variants can be obtained by depolymerizing naturally occurring lipidated PSA or by depolymerizing PSA obtained from lipidated PSA. PSA can be depolymerized using for example chemical means (e.g., using reactive oxygen species or reactive nitrogen species such as but not limited to nitrogen monoxide, as described in Duan and Kasper, *Glycobiology*, 2011, 21(4):401-409), mechanical means, and/or enzymatic means that are known in the art.

The invention further contemplates synthetic forms of lipidated PSA comprising more than 300 repeating tetrasaccharide units, including without limitation 350, 400, 500, 600, 700, 800, 900 or 1000 units or more.

As described herein, the polysaccharide component may be covalently conjugated to the glycolipid, or in certain synthetic forms it may be unconjugated to the glycolipid. If covalently conjugated, it may be conjugated via a ketosidic bond or other acid labile bond or via a bond such as an ester, an amide, or an ether bond to form a non-naturally occurring lipidated PSA.

Glycolipid component

The glycolipid component comprises a diglucosamine substituted with one or more acyl chains. An exemplary diglucosamine in the context of a glycolipid is provided in FIGs. 3 and 6. It is now recognized in accordance with the invention that the diglucosamine is

conjugated to the polysaccharide component via a ketosidic bond that is acid-labile and thus susceptible to the stringent hydrolysis steps of the prior art methods.

The diglucosamine, in some instances, may or may not be phosphorylated. In some instances, the diglucosamine is monophosphorylated. Phosphorylation may occur at the C1 position (the reducing end) or at the C4' position of the diglucosamine.

The disaccharide may be conjugated to one or more acyl chains, including two, three, four, five or more acyl chains in some instances via for example ester or amide linkages, and thus may be referred to as "O" substituted (e.g., acylated) or "N" substituted (e.g., acylated) respectively. Each lipidated PSA molecule therefore comprises one, two, three, four, five or more acyl chains. Accordingly, the disaccharides, glycolipid components and ultimately lipidated PSA molecules may be referred to herein as di-acylated, tri-acylated, tetra-acylated or penta-acylated forms, respectively.

The acyl chains of isolated lipidated PSA may range in length from 14 to 17 carbons, in some instances. Such species are thought to represent greater than 95% of naturally occurring total lipidated PSA. The acyl chains may be unmodified or they may be modified. If modified, the acyl chains may be hydroxy-modified. Thus, in some instances, the lipidated PSA may comprise one or more acyl chains characterized as C14:0, C14:0-OH, C15:0, C15:0-OH, C16:0, C16:0-OH, C17:0, and C17:0-OH.

FIGs. 1, 4 and 12-16 illustrate that a single preparation of lipidated PSA may yield a number of differently acylated glycolipids. For example, in FIG. 1A, each of the peaks on the mass spectrometry (MS) spectra represents a different species of glycolipid, wherein the species differ in their acyl chain composition. The Figure illustrates this to be the case for both the tetra-acylated and the penta-acylated glycolipids. FIG. 4 illustrates the absolute amounts of a different tetra-acylated (shown as m/z 1350, 1364 and 1378) and penta-acylated (shown as m/z 1604, 1618 and 1634) glycolipid species. Thus, a bulk preparation of lipidated PSA isolated from *B. fragilis* will yield a heterogeneous mixture of lipidated PSA molecules, potentially comprising without limitation a plurality of di-acylated species and/or a plurality of tri-acylated species and/or a plurality of tetra-acylated species and/or a plurality of penta-acylated species.

FIG. 5 provides a list of glycolipid species and their acyl chain composition. For example, the Table provides penta-acylated species comprising the following combinations of acyl chains:

- 16 -

- (1) one chain of C16:0—OH, three chains of C17:0—OH, and one chain of C15:0,
- (2) two chains of C16:0—OH, two chains of C17:0—OH, and one chain of C15:0,
- (3) three chains of C16:0—OH, one chain of C17:0—OH, and one chain of C15:0,
- (4) four chains of C16:0—OH, and one chain of C15:0, and
- (5) four chains of C16:0—OH, and one chain of C14:0.

The table similarly provides various species of tetra-acylated, tri-acylated and di-acylated acyl chains.

It will therefore be appreciated the lipidated PSA forms of the invention, whether isolated from *B. fragilis* or synthetic, and whether of conjugated or unconjugated form, may comprise any of the foregoing combinations of acyl chains, without limitation:

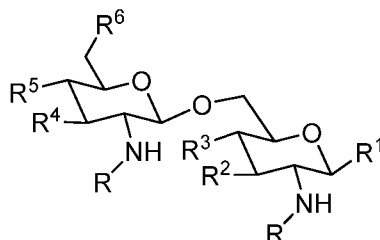
- (1) C16:0—OH acyl chain(s) only,
- (2) C17:0—OH acyl chain(s) only,
- (3) C16:0—OH and C17:0—OH chain(s) only,
- (4) C16:0—OH and C17:0—OH and C15:0 chain(s) only,
- (5) C16:0—OH and C17:0—OH and C14:0 chain(s).

The number of each type of chain may vary, and may include without limitation the following options

- (1) 0-4 C16:0—OH chains,
- (2) 0-4 C17:0—OH chains,
- (3) 0 or 1 C14:0 chains, and
- (4) 0 or 1 C15:0 chains.

Similar diversity is apparent in another lipidated PSA preparation obtained using a non-hydrolytic method (PSA Lot 40), as illustrated in FIGs. 12-16.

The disclosure therefore provides compounds each having the following structure:



Formula I

wherein:

R¹ and R⁵ each independently comprises or is —OH or a phosphate such as —OPO₃H⁻;

- 17 -

R^2 , R^3 , and R^4 each independently comprises or is $-OH$ or $-OR$;

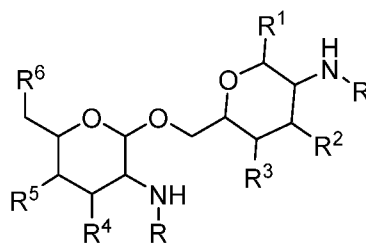
R^6 is $-OH$ or $-OR^7$;

each instance of R is independently hydrogen or an optionally substituted acyl chain;

and

5 R^7 is or comprises a polysaccharide.

The disclosure therefore provides compounds having the following structure:



Formula II

wherein:

10 R^1 and R^5 each independently comprises or is $-OH$ or a phosphate such as $-OPO_3H^-$;

R^2 , R^3 , and R^4 each independently comprises or is $-OH$ or $-OR$;

R^6 is $-OH$ or $-OR^7$;

each instance of R is independently hydrogen or an optionally substituted acyl chain;

and

15 R^7 is or comprises a polysaccharide.

In some embodiments, the phosphate is $-OPO_3H^-$. In some embodiments, the phosphate is $-OPO_3H_2^-$.

In some embodiments, the acyl chains are selected from any of the acyl chains provided herein, including straight and branched acyl chains.

20 In some embodiments, the polysaccharide is PSA or is a polysaccharide that comprises 1 or more tetrasaccharide repeating units of PSA, as described herein.

In some embodiments, R_3 is OH .

In some embodiments, either R_1 or R_5 is or comprises a phosphate (i.e., only one is or comprises a phosphate).

25

The foregoing examples are not to be considered limiting, and rather the invention contemplates various combinations, and combinations of the foregoing, to be used in lipidated PSA compositions.

- 18 -

The invention provides defined lipidated PSA mixtures, having known, and thus optionally pre-defined, glycolipid content and composition, as well as known, and thus optionally pre-defined, polysaccharide to glycolipid ratios. Thus, the lipidated PSA of the invention and compositions thereof may be characterized in terms of any of these structural features, thereby further distinguishing these compositions from those of the prior art. For example, based on the teachings provided herein, the invention provides compositions comprising lipidated PSA species that are only or predominantly (e.g., greater than 50%, or at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%) di-acylated, or tri-acylated, or tetra-acylated, or penta-acylated, or some combination thereof including but not limited to tetra- and penta-acylated. Such chemically defined compositions were not heretofore contemplated or possible.

The invention further provides isolated glycolipids obtained from lipidated PSA and compositions thereof for use in vivo and in vitro. Any of the foregoing glycolipids and any combination of the foregoing lipids are contemplated for such use.

Isolated forms

As described herein, it has been found, in accordance with the invention, that the method of isolation can significantly impact the abundance, impacting yield and purity, of isolated lipidated PSA. For example, it has been found that isolation methods that exclude an acid hydrolysis step yield more intact, fully lipidated PSA species than do methods that include an acid hydrolysis step, even if that acid hydrolysis step occurs earlier in the isolation process. In other words, when the lipidated PSA is harvested from *B. fragilis* using an acid hydrolysis step, some fraction of the originally lipidated PSA will become delipidated in the process. This can be seen for example by running the preparation on a 16.5% Tris-Tricine SDS-PAGE gel reverse stained with zinc sulphate/imidazole staining, as shown for example in FIG. 10 which compares a lipidated PSA isolated using a mild acid hydrolysis (Lot 28) and lipidated PSA isolated without an acid hydrolysis step (Lot 34). This staining protocol allows one to observe both the polysaccharide and lipid moieties of lipidated PSA in the same gel system. It has been found that the lipidated PSA preparations of the prior art contained a higher degree of released glycolipid than do the lipidated PSA preparations of the instant invention.

- 19 -

The preparations provided herein therefore can be characterized by their content of released or free glycolipids. Such content can be less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, less 0.1%, less than 0.05%, less than 0.001%, less than 0.0005%, less than 0.0001% (w/w of released glycolipid to lipidated PSA). In some instances, the compositions or preparations have undetectable levels of released or free glycolipids, as determined for example using the gel electrophoresis methods described herein. In these instances, the lipidated PSA may be considered free or substantially free of released glycolipid. The lipidated PSA may also be considered to be pure (i.e., it is free or substantially free of released glycolipid and any other naturally occurring contaminant). The degree of purity may be at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9%, or higher.

Accordingly, the invention provides compositions comprising isolated lipidated PSA, including compositions comprising isolated lipidated PSA at a purity and/or a concentration that has not been heretofore achieved. Also provided are compositions comprising or consisting essentially of particular species of lipidated PSA or particular subsets of species of lipidated PSA. These species may be characterized and thus distinguished from other species and from bulk isolated lipidated PSA in terms of their glycolipid components. The glycolipid components may be characterized by the number, position and type of acyl chains they possess. For example, they may comprise an increased amount, relative to naturally occurring proportion, of di-acylated, tri-acylated, tetra-acylated, or predominantly penta-acylated forms of lipidated PSA. These increased amounts may exceed the naturally occurring representation of the particular species, and thus such amounts will vary depending on the particular species. For example, a composition may comprise at least 5%, 10%, 15%, 20%, or more of a di-acylated lipidated PSA, or it may comprise at least 70%, 75%, 80%, 85%, 90%, 95%, or more of a tetra-acylated and/or penta-acylated lipidated PSA.

The compositions may be defined by their degree of purity, for example with respect to their glycolipid components, or with respect to their content of contaminants such as non-lipidated PSA. The compositions may be defined by their concentration of lipidated PSA, or by their concentration of PSA components and/or glycolipid components, or by their ratio of PSA to glycolipid components.

Synthetic forms

- 20 -

The invention further provides additional synthetic, non-naturally occurring species of lipidated PSA. In some instances, these non-naturally occurring species are characterized as having a lower tetrasaccharide/glycolipid ratio (or a lower PSA/glycolipid ratio, wherein the PSA is the polymer comprised of one or more repeating tetrasaccharide units) than is
5 observed in isolated forms of lipidated PSA. Such ratio may be a molar ratio or a molecular weight ratio. The invention further provides other compositions comprising polysaccharide (PSA) or tetrasaccharide and glycolipid and/or lipid components obtained or derived from lipidated PSA, in a non-naturally occurring conjugated form. For example, the polysaccharide and glycolipid components may be conjugated to each other via a non-
10 naturally occurring linkage. The linkage may be a non-ketosidic linkage, and may be an ester or an amide or an ether, without limitation. In other compositions, the components, such as the polysaccharide and glycolipid components, may be unconjugated. In still other compositions, a substrate in or on which the polysaccharide (or tetrasaccharide) and glycolipid (or lipid) components, whether conjugated or unconjugated, are present in or on a
15 substrate or delivery vehicle.

All of these various forms of lipidated PSA, including for example those isolated from *B. fragilis* cells, those made synthetically and having different polysaccharide/glycolipid ratios from isolated forms, those provided as unconjugated polysaccharide and glycolipid
20 components, and the like, are considered to be active agents. Various aspects and embodiments relating and referring to “lipidated PSA” apply equally to these various forms and are not meant to apply solely to an isolated form or to a covalently conjugated form unless otherwise indicated or apparent.

25 ***Lipidated PSA compositions***

The invention further provides compositions comprising isolated lipidated PSA. As used herein, with respect to lipidated PSA, the term “isolated” intends that the lipidated PSA is prepared or obtained from *B. fragilis*, and is physically separated from its natural environment (e.g., a *B. fragilis* cell, components of the *B. fragilis* cell, and/or components of
30 the *B. fragilis* cell capsular complex such as but not limited to PSB).

In some embodiments, the compositions are substantially free of naturally occurring contaminants such as nucleic acids (e.g., DNA and RNA), proteins, and other components of

B. fragilis and/or the *B. fragilis* capsule. Substantially free, as used herein, intends that these contaminants represent about or less than 5%, less than 1%, less than 0.5%, or less than 0.1% (or less) by weight (weight of the contaminant to weight of the lipidated PSA form). In some instances, such contaminants may be undetectable.

5 Various compositions may or may not contain LPS. LPS may be present in an amount of about 0.5% (w/w of LPS to lipidated PSA components).

 Some compositions may comprise at least about 95%, 96%, 97%, 98%, 99%, or more (w/w) of lipidated PSA and less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, or less of free, released glycolipid. In some embodiments, the free,
10 released glycolipid is undetectable.

 Thus, certain compositions comprising lipidated PSA, whether of isolated or synthetic form, may or may not comprise other components including LPS and/or free, released glycolipid. In some embodiments, the amount of LPS present in such compositions is about 0.5% (w/w) or less. In some embodiments, the amount of released or free glycolipid to
15 lipidated PSA is about 0.5% (w/w) or less. In various other embodiments, the amount of non-lipidated PSA present in such compositions is about 10% (w/w) or less, including 5% or less, or 1% or less. In some embodiments, the compositions are substantially free of non-lipidated PSA.

 The presence and amount of these various components and contaminants including
20 lipidated PSA, released (unconjugated) glycolipid, and/or LPS can be determined using a gel system such as that described herein.

 Some compositions of lipidated PSA may comprise at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or more of the polysaccharide component (non-lipidated PSA) (weight of polysaccharide to combined weight of polysaccharide and
25 glycolipid). Some compositions of lipidated PSA may comprise about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 1%, or less of the glycolipid component (weight of glycolipid to combined weight of polysaccharide and glycolipid). Some compositions of lipidated PSA may comprise about 99% polysaccharide component (non-lipidated PSA) and about 0.5% glycolipid component. Some compositions of lipidated PSA may comprise about
30 80% polysaccharide component (non-lipidated PSA) and about 20% glycolipid component. These may be isolated or synthetic forms of lipidated PSA. Accordingly, they may be forms in which the polysaccharide and glycolipid components are conjugated to each other or they

- 22 -

may be forms in which these components are not conjugated to each other. Conjugation may be direct or indirect conjugation, and additionally it may be covalent or non-covalent conjugation. It is to be understood that the polysaccharide and glycolipid components are lipidated PSA components (i.e., the polymer formed of one or more tetrasaccharide units of Formula I and the glycolipid described herein and comprising a disaccharide conjugated to one or more acyl chains).

As an example, in one instance, the composition may comprise a synthetic form of lipidated PSA having 6 tetrasaccharide units and one tetra-acylated glycolipid unit (see for example Formula II in FIG. 6A). The compositions may comprise about 20% glycolipid and 80% polysaccharide (w/w as defined above). Synthetic compositions may be defined by their glycolipid and polysaccharide components, amounts and ratios, whether such components are conjugated or unconjugated to each other. Other compositions and combinations are contemplated and will be readily appreciated by those of ordinary skill in the art.

It is to be understood that the compositions of the invention typically comprise a plurality of lipidated PSA molecules, and that in some instances the plurality may exhibit variation in the degree or nature of lipidation. The invention contemplates compositions have particular proportions of particular species of lipidated PSA and/or particular subsets of lipidated PSA. The proportions may be w/w proportions (e.g., weight of the particular species to weight of all lipidated PSA in the composition). Such proportions may be about or more than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more of a defined species or subset of species. For example, a particular species may be a lipidated PSA comprising the glycolipid of Formula II or a lipidated PSA comprising the glycolipid of Formula III, or a lipidated PSA comprising any one of the acyl chain combinations listed in FIG. 5. A particular subset of lipidated PSA species may be lipidated PSA comprising tetra-acylated glycolipids, or lipidated PSA comprising penta-acylated glycolipids, or lipidated PSA comprising tetra- or penta-acylated glycolipids. Various subsets are contemplated and will be apparent based on this disclosure.

The invention provides compositions for use in vitro and in vivo. In vitro, the compositions may be used as analytical tools or assay standards. In vivo, the compositions may be used or in experimental models, such as animal models, of human disease or in humans or other subjects in need of immune regulation. When used in vivo, the compositions are pharmaceutically acceptable, intending that they are suitable for

- 23 -

administration into a subject. They may or may not be used prophylactically or therapeutically in such subjects.

The lipidated PSA forms may be used as stand-alone active agents or they may be used in combination with other active agent(s). The combined use of agents may be additive or may be supra-additive (e.g., synergistic). The lipidated PSA forms may be formulated together with or separately from the other active agent(s). The lipidated PSA forms may be administered via the same or a different route from the other active agent(s). If not formulated together, the lipidated PSA forms and the other active agent(s) may be administered on the same or substantially the same administration regimen (including being administered substantially simultaneously although not formulated together) or they may be administered according to different regimen. The lipidated PSA forms may be administered acutely and/or chronically.

Isolation and synthesis methods

The invention further provides methods of isolating and purifying lipidated PSA from *B. fragilis* as well as methods of making the various synthetic forms of lipidated PSA described herein.

It was recently recognized that prior art methods used to isolate and purify PSA removed the lipid moiety from the polysaccharide, thereby previously yielding only a polysaccharide structure for PSA. See published PCT application WO 2013/009945. However, the nature of the lipid moiety and its particular attachment to PSA was not known until the present invention.

Isolation methods

In accordance with the invention, it was found that lipidated PSA could be isolated in the absence of a hydrolysis step. Thus provided herein are non-hydrolytic methods for isolating lipidated-PSA from *B. fragilis* strains (i.e., methods that lack a hydrolysis step such as an acid hydrolysis step). It was not previously recognized that lipidated PSA could be isolated from *B. fragilis* strains without a hydrolysis step.

It was also found that the lipidated PSA could be isolated in the presence of a bile salt such as deoxycholate. Previously it was thought that the presence of detergents or bile salts such as sodium deoxycholate were detrimental to the isolation process, resulting in lower

- 24 -

yields of the lipidated PSA. Unexpectedly, various methods provided herein which lack a hydrolysis step and optionally which utilize a detergent (such as deoxycholate) provide suitable yields of lipidated PSA and in some instances higher yields of lipidated PSA.

Excluding the acid hydrolysis step entirely from the isolation process prevents hydrolysis of the glycolipid from PSA, thereby resulting in a greater proportion of lipidated PSA versus non-lipidated PSA. This is evidenced by the reduced and in most instances undetectable amount of released (unconjugated) glycolipid in such preparations. The released glycolipid can be observed using any of the analysis techniques described herein including gel electrophoresis or mass spectrometry.

In some instances, such methods involve isolating lipidated PSA using the isolation methods described herein, and purifying the isolated lipidated PSA to arrive at purity and/or concentration levels not heretofore achieved. Such purity and/or concentration levels may be apparent by the degree of aggregation of the lipidated PSA (e.g., as micelles), and optionally the effect of disaggregating agents such as deoxycholate to increase the biological activity of such compositions, as demonstrated herein.

Since the lipidated PSA is considered more biologically active than non-lipidated PSA, the isolation methods provided herein yield lipidated PSA having a higher activity per weight than previously described preparations. Such activity may be IL-10 inducing activity, for example.

The method provides general and specific methods for isolating and purifying lipidated PSA from *B. fragilis*. It is to be understood that these methods may be performed on any strain of *B. fragilis* provided it produces lipidated PSA. Such strains include naturally occurring strains or non-naturally occurring strains. One example of a non-naturally occurring strains is the delta44 mutant of *B. fragilis* strain 9343. This mutant strain expresses only PSA, rather than PSA and PSB as found in wild type strains such as 9343.

The methods of isolation generally involve growth of *B. fragilis* (wild type or mutant strains) under anaerobic conditions, extraction of the polysaccharide capsular complex from *B. fragilis*, isolation of a polysaccharide fraction, and purification of lipidated PSA from that fraction. The extraction step may be accomplished using a phenol/water extraction, which optionally can be carried out at an elevated temperature (e.g., about 60-80°C). The aqueous phase, which contains the capsular polysaccharides, is then dialyzed versus water, following which it may be partially lyophilized in order to reduce total volume. The resulting solution

- 25 -

is then typically treated with nucleases such as DNase and RNase and proteinases such as pronase in order to further purify the polysaccharide fraction. The polysaccharide fraction is then ethanol precipitated, and the precipitate is collected, washed, and subjected to size exclusion techniques to further isolate the lipidated PSA from other polysaccharides

5 including for example LPS. A typical size exclusion technique is column chromatography. A suitable column is a S-400 size exclusion column. A chromatographic column containing a biological detergent in the form of sodium deoxycholate was used to isolate lipidated PSA, as described in the Examples. Column fractions containing the lipidated PSA are identified and pooled, and the pooled mixture can then be further dialyzed and lyophilized if desired.

10 Additionally, after reconstitution, the mixture may be further dialyzed, ethanol precipitated and/or lyophilized for storage or delayed use. The pH throughout the isolation preferably is 9 or less (e.g., about 4 to about 9 or less), and in most steps is maintained in a neutral range.

The foregoing method may be performed without an acid hydrolysis step. However, it is to be understood that lipidated PSA, and species and subsets of species thereof, may be
15 harvested using methods that include an acid hydrolysis step and/or a size exclusion step without the use of a detergent such as sodium deoxycholate.

If an acid hydrolysis step is used, it is preferably a mild hydrolysis (e.g., at a pH of about 4, or in the range of 4-5) and it is preferably incorporated at an early step in the purification process (e.g., following the first ethanol precipitation). The acid hydrolysis, if
20 used, may be performed using dilute acid (e.g., 1-2% acetic acid) at elevated temperature. The elevated temperature may range from 80-100°C, 85-95°C, and in some instances may be about 90°C. The treatment may last for 1 hour, 2 hours, 3 hours or longer. In some instances, the acid treatment is performed using 2% acetic acid at 90°C for 3 hours.

The foregoing method may be performed using a detergent or a bile salt such as
25 deoxycholate (e.g., sodium deoxycholate) in the chromatographic column and/or the eluent. Sodium deoxycholate may be present at a strength of less than 5%, less than 4%, less than 3%, less than 2%, or about or less 1%. However, it is to be understood that lipidated PSA may be harvested using methods that include a size exclusion step that does not use a detergent such as deoxycholate.

30 Thus, an exemplary isolation method comprises extracting, into an aqueous phase, a capsular complex from *B. fragilis* using a mixture of phenol and water optionally at high temperature, precipitating a polysaccharide fraction from the aqueous phase using ethanol

- 26 -

optionally following DNA and/or RNA and/or protein digestion, and isolating lipidated PSA from other polysaccharides by size exclusion, such as for example a chromatographic column containing sodium deoxycholate.

5 The lipidated PSA forms prepared from *B. fragilis* once isolated may be further modified. For example, the polysaccharide component may be depolymerized to produce another non-naturally occurring form having fewer tetrasaccharide units than are found in nature. This can be accomplished through mechanical, chemical or enzymatic means. An example of chemical depolymerization involves reactive oxygen species or reactive nitrogen species such as but not limited to nitrogen monoxide, as described in Duan and Kasper,
10 Glycobiology, 2011, 21(4):401-409). Glycosidases can be used for enzymatic depolymerization. Mechanical depolymerization may involve shearing. As an example, the PSA polymer may be shortened by 25%, 50%, 75%, or more, thereby rendering a lipidated PSA that is structurally different from naturally occurring lipidated PSA. Such modification may generate PSA or lipidated PSA having a length of about 50, 40, 30, 20, or to about 1-10
15 tetrasaccharide units.

Lipidated and non-lipidated versions of PSA can be depolymerized and fractionated according to polysaccharide length using for example liquid chromatography, ion chromatography, or other size-based or ion-based separation techniques.

Isolated forms of lipidated PSA may be formulated alone, in which case they form
20 tight micelle-like structures. Significantly, lipidated PSA does not form such micelle-like structures when present in vivo. Such altered structure is only observed (and thus possible) upon isolation of the lipidated PSA in a relatively pure form. Furthermore, these micelle-like structures are so stable that detergent or other disaggregating agent may be necessary to disrupt them or to render them less stable, thereby making the lipidated PSA contained
25 therein accessible.

In these various non-naturally occurring forms, the acyl chains are situated externally in the micelle-like structure and thus are accessible to target cells and their receptors. Such cells include antigen presenting cells and such receptors include TLRs such as TLR2. See Wang et al. J. Exp. Med 203(13): 2853-63 and Round et al. Science 2011, 332(6032):974-7.

30 The isolated lipidated PSA may also be fractionated based on the nature of the glycolipid such that resulting compositions comprise non-naturally occurring ratios of di-, tri-, tetra-, and penta-acylated PSA.

- 27 -

Thus, this disclosure contemplates additional non-naturally occurring species of lipidated PSA having fewer tetrasaccharide units compared to naturally occurring lipidated PSA.

5 ***Other methods***

The synthetic lipidated PSA forms of the invention may be prepared using naturally occurring or synthetically produced forms of the polysaccharide and glycolipid components. Naturally occurring forms may be prepared by deliberating hydrolyzing (e.g., acid-treating) lipidated PSA thereby cleaving the ketosidic linkage between the polysaccharide and glycolipid. The polysaccharide and glycolipid components may be separated from each other and thereby isolated using liquid chromatography, ion chromatography, gel electrophoresis or other size-based or charge-based separation technique.

The polysaccharide may be further modified by mechanical, chemical and/or enzymatic means. This may serve to reduce the length of the polysaccharide component, if desired. The polysaccharide (PSA) can be depolymerized using for example mechanical and/or enzymatic means known in the art and described herein.

Similarly, the invention contemplates fractionating the naturally occurring glycolipids obtained from lipidated PSA according to their degree of acylation and then recombining particular subsets with the polysaccharide component. For example, the penta-acylated or the tetra-acylated glycolipid subsets may be isolated and recombined with the polysaccharide components. The end-products may comprise the naturally occurring linkage between glycolipid and polysaccharide or they may comprise non-naturally occurring linkages such as esters, amides, ethers or a combination thereof. Polysaccharides and glycolipids can also be conjugated via a bifunctional linker molecule, such as, but not limited to, 2-(Boc-amino)ethyl bromide. Other linker molecules may be used, and are known in the art.

Alternatively, components of lipidated PSA may be synthesized and then combined. For example, the glycolipid may be synthesized and then used with the polysaccharide component isolated from *B. fragilis*. For example, glycolipid may be synthesized using techniques described in Imoto et al (Tet. Lett. 1984, 25:25, 2667-2670). These techniques may be used to produce both conjugated and unconjugated forms of synthetic lipidated PSA. It is to be understood that for brevity, as used herein, the term "lipidated PSA" embraces

synthetic forms that comprise polysaccharide and glycolipid components provided together but in an unconjugated form (e.g., in or on a substrate, as described below).

Synthetic lipidated PSA forms may be provided with, including in and/or on a substrate. The substrate may be a solid or semi-solid and it may take any one of a variety of shapes or forms. The substrate may be biodegradable and itself may be composed of naturally occurring and/or non-naturally occurring components such as but not limited to naturally occurring and/or non-naturally occurring polymers. One example of a suitable substrate is a particle. The particle may be a microparticle (average diameter in the range of 1-999 microns) or a nanoparticle (average diameter in the range of 1-900 nanometers). The particle may be a porous particle or it may be a non-porous particle. Methods for producing such particles having active agents therein or thereon are known in the art. Thus, the invention contemplates the use of any of such methods and any of such particles to prepare certain compositions of lipidated PSA as contemplated herein.

In some instances, the glycolipid or PSA components are conjugated to the surface of the particle.

In some instances, glycolipid and PSA components are provided in a liposome or liposome-like structure. The glycolipid component may be external and the polysaccharide component may be internal to the liposome. The liposome may comprise solely the glycolipid and PSA components from lipidated PSA or it may comprise other components such as but not limited to other lipids. The externally facing lipid component is believed to interact with TLR2 on antigen-presenting cells, thereby facilitating entry into such cells of the liposome and intracellular release of the PSA component.

The liposome or micelle forms of the naturally or non-naturally occurring lipidated PSA will typically comprise the glycolipid component at the surface and available for interaction with cells and particular receptor such as TLR2.

The lipidated PSA may be formulated to target specific cell types, for greater therapeutic efficacy. For example, if the lipidated PSA, whether isolated or synthetic, is provided in the context of a substrate such as a nanoparticle, then the substrate may further comprise moieties that increasing homing or binding of the substrate to immune cells such as antigen-presenting cells (APC) including dendritic cells and B cells.

Examples of non-naturally occurring forms of lipidated PSA include those comprising a non-naturally occurring linkage between the glycolipid and polysaccharide components,

those having PSA polymers that are shorter or longer than naturally occurring forms of lipidated PSA, those that are present in a proportion lower or higher than their naturally occurring proportion, or those that have some combination or all of these features.

5 ***Methods for analysis and characterization of lipidated PSA***

The invention provides methods for detecting the presence of lipidated PSA and in some instances quantitating the amount of lipidated PSA in a sample or a composition.

Structural characterization

10 Purity of the isolated fractions may be assessed by proton NMR and/or SDS PAGE gel. Proton NMR profiles may be generated using 600 MHz NMR. Other compositions may be tested for the presence of lipidated PSA in a similar manner.

These approaches can also be used to characterize the glycolipid component of lipidated PSA. For example, as shown in FIGs. 1 and 2, the tetra-acylated and penta-acylated
15 glycolipids of lipidated PSA can be distinguished from each other using mass spectrometry. Similarly, MADLI-TOF-TOF can be used to detect and distinguish between di-, tri-, tetra- and penta-acylated glycolipids from lipidated PSA. Thus, these approaches can be used to test for the presence of specific glycolipids.

It is also possible to identify the presence of lipidated PSA using an acid treatment
20 time course, whereby the released (unconjugated) lipid moiety can be visualized using a 16.5% Tris-Tricine SDS-PAGE gel reverse stained with zinc sulphate/imidazole staining. This staining protocol allows one to observe both the polysaccharide and lipid moieties of lipidated PSA in the same gel system. As an example, a sample being tested for lipidated PSA content may be treated with 2% acetic acid at 90°C for various periods of time, followed
25 by neutralization with NaOH and dialysis. One hundred micrograms of the resultant product is then run on a 16.5% Tris-Tricine SDS-PAGE gel and reverse-stained as described above. The reduction in intensity of the lipidated PSA band (the major band above 60 kD which represents lipidated and non-lipidated versions of PSA) with the concomitant emergence of one or more lipid bands at about 5 kD with increasing hydrolysis time evidences the presence
30 of lipidated PSA. LPS bands of about 6 and 8 kD also reduce in intensity with increasing time.

- 30 -

This approach can also be used to test a lipidated PSA preparation for the presence of free, released glycolipid. FIG. 10 illustrates results of an SDS PAGE gel analysis of a preparation prepared with mild acid hydrolysis (PSA Lot 28) and a preparation prepared with no acid hydrolysis step at all (PSA Lot 34). Lot 34, isolated using a non-hydrolytic method, contains no free, released glycolipid, while Lot 28, isolated using a mild hydrolysis, does. It is to be understood that the free, released glycolipid refers to the glycolipid unconjugated to the polysaccharide PSA and such glycolipid may be referred to as being free, released or unconjugated interchangeably.

Functional characterization

Lipidated PSA has been shown to be more potent than its non-lipidated counterpart (i.e., non-lipidated PSA). The immunological activity of lipidated PSA can be assayed in vitro and in vivo. An example of an in vitro test is the induction of IL-10 production in a splenic dendritic cell (DC) and T cell co-culture. This assay can be performed as follows:

- (1) splenic DCs are isolated using mouse anti-CD11c microbeads (Miltenyi Biotec cat#130-052-001);
- (2) CD4⁺ T cells are isolated using Mouse T cell CD4 Subset Column Kit (R&D systems cat#MCD4C-1000);
- (3) 2x10⁴ CD11c⁺ DCs and 10⁵ CD4⁺ T cells are mixed and 1 µg/ml anti-CD3 (BD Pharmingen cat#553057) is added;
- (4) the culture is then stimulated with 100 µg/ml lipidated PSA and the cells are incubated for 5 days; and
- (5) supernatants are harvested and analyzed by ELISA for the presence of IL-10.

FIG. 7 demonstrates, using this co-culture system, that lipidated PSA prepared using the milder and early acid hydrolysis step (shown as PSA 24) is approximately 3 fold more potent than material made using a harsher and later acid hydrolysis step (shown as PSA 23).

FIG. 8 shows the activity in this same assay of a lipidated PSA prepared without an acid hydrolysis step (shown as PSA 34, second bar of each bar pair). The IL-10 inducing activity of this preparation is increased about 2-fold following the addition of deoxycholate (DOC). A similar increase is not observed with a lipidated PSA prepared using a milder acid hydrolysis step (shown as PSA 28, first bar of each bar pair). The increase in activity upon addition of deoxycholate suggests that the lipidated PSA is tightly aggregated and that such aggregated form is less stable, and thus the lipidated PSA is more accessible, in the presence of deoxycholate.

- 31 -

An animal model of multiple sclerosis (EAE) may be used to study the immunological activity of lipidated PSA in vivo. In this model, mice are treated with lipidated PSA (on the order of about 75 – 100 µg per mouse) or control (saline, PBS) every three days starting 6 days before EAE induction. Mice are challenged subcutaneously with 250 µg of MOG₃₃₋₅₅ (Peptides International) in 200 µl of complete Freund's adjuvant (Sigma). On days 0 and 2 after challenge, mice receive intraperitoneal injections of 250 ng of *Bordetella pertussis* toxin (List Biological Laboratories). Disease is scored on an established 0 to 5 scale, with 5 being advanced neurological disease. Mice are monitored and scored daily for disease progression.

Methods of use

Also provided are methods of using the various forms of lipidated PSA in vitro and in vivo. The various forms of lipidated PSA are more potent than the previously described non-lipidated form of PSA. The various forms provided herein can be used as immunomodulators, particularly in view of their enhanced IL-10 inducing activity and Treg maturation activity. These forms are contemplated for use in vitro and in vivo. In vitro uses include use as an analytical tool (e.g., as a marker of the presence of *B. fragilis*) and as an assay standard or control (e.g., as a positive marker of lipidated PSA or a comparator in an in vitro assay such as a IL-10 induction assay). In vivo uses include uses in animal models and also clinically to treat or prevent inflammatory conditions such as but not limited to autoimmune disorders (e.g., multiple sclerosis and inflammatory bowel disease).

The invention further contemplates use of the individual polysaccharide and glycolipid components of lipidated PSA. For example, the glycolipid component may be used as a single agent. As another example, the polysaccharide and glycolipid components may be used together in an unconjugated form.

In vivo uses include but are not limited to those involving human subjects. For example, in vivo uses include administration of the lipidated PSA molecule and compositions thereof to a non-human subject in order to modulate an immune response, for example as a positive control or a comparator.

Also contemplated are methods of modulating immune responses in a subject by administering to such subject the lipidated PSA described herein. The subject may be one having or likely to develop an aberrant immune response. Typically, the aberrant immune

- 32 -

response is an enhanced immune response and the lipidated PSA acts to down-regulate the immune response. Enhanced immune responses are typically associated with inflammatory conditions, such as but not limited to autoimmune diseases.

Accordingly, the compositions of the invention, comprising for example isolated or synthetic forms of lipidated PSA, conjugated or unconjugated forms of lipidated PSA, or the glycolipid component of lipidated PSA as a single agent or in combination with a polysaccharide other than PSA, may be used to modulate (and typically down-regulate) immune responses in subjects having or at risk of developing autoimmune diseases. As will be understood by those of ordinary skill in the art, subjects having autoimmune diseases typically experience one or more “events” or recurrences associated with the autoimmune disease. For example, a subject having inflammatory bowel disease may experience temporally isolated attacks of the disease, characterized by the presence of symptoms or increased severity of symptoms. The invention contemplates that the compositions may be used in such subjects to reduce the likelihood of such future recurrences of the disease or to reduce the severity of symptoms associated with the disease (e.g., pain, fever, discomfort, fatigue, etc.). Thus, the compositions may be administered prior to such recurrence, and in this manner may be chronically administered, optionally at a regular frequency. Examples include once a day, once every 2, 3, 4, 5 or 6 days, or once a week, etc. The invention also contemplates that the compositions may be administered to the subject during a recurrence in order to reduce the severity of symptoms or shorten the time of the recurrence.

Thus, as an example, the invention provides a method comprising administering to a subject at risk of a recurrence of a condition associated with inflammation an effective amount of a lipidated PSA in any of the forms provided herein such as but not limited to isolated or synthetic forms of lipidated PSA and/or conjugated or unconjugated forms of lipidated PSA, or an effective amount of the glycolipid component of lipidated PSA as a single agent or in combination with an agent other than PSA. The method may reduce the likelihood of a recurrence of the condition or may reduce the frequency of future recurrences. The method may reduce the severity of symptoms associated with the condition, whether such symptoms are present in the first manifestation, in a recurrence, or chronically.

Autoimmune diseases are known in the art. Examples of autoimmune diseases include but are not limited to multiple sclerosis, inflammatory bowel disease including Crohn’s Disease and ulcerative colitis, rheumatoid arthritis, psoriasis, type I diabetes, uveitis,

Celiac disease, pernicious anemia, Sjogren's syndrome, Hashimoto's thyroiditis, Graves' disease, systemic lupus erythematosus, acute disseminated encephalomyelitis, Addison's disease, Ankylosing spondylitis, Antiphospholipid antibody syndrome, Guillain-Barre syndrome, idiopathic thrombocytopenic purpura, Goodpasture's syndrome, Myasthenia
5 gravis, Pemphigus, giant cell arteritis, aplastic anemia, autoimmune hepatitis, Kawasaki's disease, mixed connective tissue disease, Ord throiditis, polyarthritis, primary biliary sclerosis, Reiter's syndrome, Takaysu's arteritis, vitiligo, warm autoimmune hemolytic anemia, Wegener's granulomatosis, Chagas' disease, chronic obstructive pulmonary disease, and sarcoidosis.

10 In important embodiments, the autoimmune disease is multiple sclerosis. In other important embodiments, the autoimmune disease is an inflammatory bowel disease including but not limited to ulcerative colitis and Crohn's disease. In other embodiments, the autoimmune disease may be rheumatoid arthritis or type I diabetes.

In some instances, the compositions of the invention may be administered to a subject
15 who has yet to manifest an autoimmune disease (including symptoms thereof) yet is at risk of developing such as disease based on a known genetic or familial predisposition. Such a subject may have one or more family members that are afflicted with the disease.

In some instances, the compositions of the invention are administered to subject having or at risk of developing graft-versus-host disease. Administration may occur prior to,
20 during and/or after transplantation of an organ or tissue (including blood or a blood product) into the subject.

In still other instances, the compositions may be administered to subjects having or at risk of developing a conditions associated with inflammation.

As an example, the composition may be administered to a subject having asthma. As
25 will be understood in the art, subjects having asthma typically experience asthmatic attacks or events characterized by impaired breathing. The invention contemplates that the compositions described herein may be administered acutely (e.g., a single large dose) or chronically (e.g., repeated, smaller doses) to asthmatic subjects. Accordingly, in some instances, the compositions may be administered prior to an asthmatic attack in order to
30 prevent the occurrence of the attack, reduce the frequency of attacks, and/or to lessen the severity of the attack. In some instances, the compositions may be administered during an attack in order to reduce its severity and/or reduce its duration.

- 34 -

Another condition associated with inflammation is a post-surgical adhesion. The invention contemplates administration of the compositions described herein to subjects having or at risk of developing a post-surgical adhesion. The compositions may be administered prior to, during, and/or immediately following surgery, or any combination thereof including but not limited to prior to and during surgery, in order to prevent the occurrence of such adhesions and/or reduce their severity. The compositions may be administered repeatedly following surgery, including for example every day, every two days, every three days, etc. for a week, two weeks, three weeks, a month, or several months post-surgery.

Another condition associated with inflammation is an abscess, including but not limited to an abdominal abscess as may occur upon leakage of intestinal contents into the peritoneum. In these instances, the subjects being treated may also be administered anti-bacterial agents such as antibiotics.

Thus, as another example, a method is provided that comprises administering to a subject having or at risk of developing an abscess an effective amount of any of the lipidated PSA forms described herein or the glycolipid component of lipidated PSA (separate from the polysaccharide component) or compositions thereof. In some embodiments, the subject is also administered an anti-bacterial agent such as an antibiotic. In some embodiments, the lipidated PSA is administered prior to development of an abscess and/or prior to the manifestation of symptoms associated with an abscess. In some embodiments, the lipidated PSA or glycolipid is administered after an abscess has been detected or diagnosed and/or after symptoms associated with an abscess are manifested.

Another condition associated with inflammation is obesity, and accordingly the invention also contemplates administration of the compositions described herein in subjects that are obese. Such subjects are typically defined as having a body mass index (BMI) of 30 or more. In some instances, the compositions may be administered to a subject having a BMI greater than 20 or greater than 25. The compositions are intended to prevent further weight gain and/or induce weight loss in such subjects.

A subject intends any subject that would benefit from administration of a composition of the invention or that could be administered the composition of the invention. In important embodiments, the subject is a human subject. The subject may also be a companion animal such as a dog or cat, agricultural livestock such as horses, cattle, pigs, sheep, etc., laboratory

- 35 -

animals such as mice, rats, rabbits, monkeys, etc., or animals such as those maintained in zoos or otherwise in captivity.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular condition being treated, the severity of the condition being treated, and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, inhalation (e.g., inhaler or nebulization), or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, intraperitoneal, or infusion.

Formulations

When administered, the active agents of the invention are formulated as pharmaceutically acceptable compositions or preparations. Such compositions or preparations may routinely contain pharmaceutically acceptable carriers, concentrations of salt, buffering agents, preservatives, other immune modulators, and optionally other therapeutic agents. The term "pharmaceutically-acceptable carrier" as used herein, and described more fully below, means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active agent(s) is combined to facilitate administration, long-term storage, stability and the like. The active agents of the present invention may be comingled with the other components of the pharmaceutical compositions, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may be presented in unit dosage form and may be prepared by any of the methods known in the art of pharmacy. All methods include the step of bringing the active agents into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active agent(s) into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each

- 36 -

containing a predetermined amount of the active agent. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

The active agent(s) may be administered per se (neat) or in the form of a pharmaceutically acceptable salt. Pharmaceutically acceptable salts may be used for in vivo applications as well as in vitro applications. Non-pharmaceutically acceptable salts may be used to prepare pharmaceutically acceptable salts thereof and are not excluded from the scope of the invention. Pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonc, tartaric, citric, methane sulphonc, formic, malonic, succinic, naphthalene-2-sulphonc, and benzene sulphonc. Also, pharmaceutically acceptable salts can be prepared as alkyl metal or alkyl earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active agent(s), which can be isotonic with the blood of the recipient. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for subcutaneous, intramuscular, intraperitoneal intravenous, etc. administrations may be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa.

In some embodiments, the lipidated PSA or the glycolipid component is formulated with a detergent such as but not limited to Tween or a bile salt such as but not limited to deoxycholate (e.g., sodium deoxycholate) in order to limit or prevent lipidated PSA aggregation. Such detergent or bile salt may be used at a low concentration such that it is still pharmaceutically acceptable. For example, it may be present at about or less than 0.0001%, 0.0005%, 0.001%, 0.002%, 0.005%, 0.01%, 0.02%, 0.05%, 0.07%, 0.1%, 0.2%,

- 37 -

0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, or more. FIG. 8 provides a composition comprising about 0.0025% deoxycholate and isolated lipidated PSA generated without acid hydrolysis.

Degradation of the lipidated PSA into glycolipid and polysaccharide components can be determined using mass spec or zinc gels as described herein (e.g., in the latter case, the released lipid is clearly identified as a faster migrating band). This is illustrated in FIG. 10.

The pharmaceutical preparations, as described above, are administered in effective amounts. For therapeutic applications, it is generally that amount sufficient to achieve a medically desirable result. In general, a therapeutically effective amount is that amount necessary to delay the onset of, inhibit the progression of, or halt altogether the particular condition being treated, including reducing the likelihood, frequency and/or severity of a recurrence of the condition. As an example, the effective amount may be that amount which serves to reduce, alleviate, or delay the onset of the symptoms (e.g., pain, fever, etc.) of the disorder being treated or prevented. The effective amount will depend upon the mode of administration, the particular condition being treated and the desired outcome. It will also depend upon the stage of the condition, the severity of the condition, the age and physical condition of the subject being treated, the nature of concurrent therapy (if any), the duration of the treatment, the specific route of administration and like factors within the knowledge and expertise of the medical practitioner. For prophylactic applications, it is that amount sufficient to delay the onset of, inhibit the progression of, or halt altogether the particular condition being prevented, and may be measured by the amount required to prevent the onset of symptoms.

Generally, doses of active agent(s) of the present invention may be from about 0.01 mg/kg per day to 1000 mg/kg per day, preferably from about 0.1 mg/kg to 200 mg/kg and most preferably from about 0.2 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or more days. It is expected that doses ranging from 1-500 mg/kg, and preferably doses ranging from 1-100 mg/kg, and even more preferably doses ranging from 1-50 mg/kg, will be suitable. The preferred amount can be determined by one of ordinary skill in the art in accordance with standard practice for determining optimum dosage levels of the agent. It is generally preferred that a maximum dose is the highest safe dose according to sound medical judgment be used.

- 38 -

In some instances, the total daily dose for a human subject may range from about 50-100 micrograms of lipidated PSA or the glycolipid component isolated from the polysaccharide component.

The pharmaceutical preparation may be administered alone or in conjunction with one or more other active agents.

The pharmaceutical preparation may be used or administered in conjunction with active agents that are suitable for autoimmune disorders such as multiple sclerosis, Crohn's disease, ulcerative colitis, asthma, rheumatoid arthritis, and the like.

An example of such agents include anti-inflammatory agents. Examples include steroids and corticosteroids such as cortisone; non-steroidal anti-inflammatory drugs such as aspirin, salsalate, celecoxib, diclofenac, etodolac, ibuprofen, indomethacin, ketoprofen, ketorolac, nabumetone, naproxen, oxaprozin, piroxicam, sulindac, and tolmetin; aminosalicylates such as sulfasalazine and 5-aminosalicylates including mesalamine, balsalazide, and olsalazine; azathioprine; mercaptopurine; cyclosporine; beta interferons; glatiramer acetate; dimethyl fumarate; fingolimod; mitoxantrone; disease-modifying antirheumatic drugs (DMARDs) such as methotrexate, leflunomide, hydroxychloroquine and sulfasalazine.

Another example of such agents include antibodies or antibody fragments. Examples include TNF alpha inhibitors such as infliximab (Remicade), adalimumab (Humira), and golimumab (Simponi); natalizumab (Tysabri), vedolizumab (Entyvio); ustekinumab (Stelara); abatacept (Orencia); anakinra (Kineret); certolizumab (Cimzia), etanercept (Enbrel), rituximab (Rituxan), tocilizumab (Actemra), and tofacitinib (Xeljanz).

The invention contemplates that the combined use of lipidated PSA in the various forms described herein or isolated glycolipid component of lipidated PSA together with standard treatments such as those recited above will allow a lower dose of the standard treatment to be used for the same or better therapeutic effect, and/or will result in reduced incidence and/or severity of side effects associated with such standard treatments.

In one embodiment the pharmaceutical preparation is given in conjunction with one or more anti-bacterial agents including antibiotics selected from the group consisting of penicillin G, penicillin V, ampicillin, amoxicillin, bacampicillin, cyclacillin, epicillin, hetacillin, pivampicillin, methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin, carbenicillin, ticarcillin, avlocillin, mezlocillin, piperacillin, amdinocillin,

- 39 -

cephalexin, cephadrine, cefadroxil, cefaclor, cefazolin, cefuroxime axetil, cefamandole, cefonicid, cefoxitin, cefotaxime, ceftizoxime, cefmnenoxine, ceftriaxone, moxalactam, cefotetan, cefoperazone, ceftazidme, imipenem, clavulanate, timentin, sulbactam, neomycin, erythromycin, metronidazole, chloramphenicol, clindamycin, lincomycin, vancomycin, 5 trimethoprim-sulfamethoxazole, aminoglycosides, quinolones, tetracyclines and rifampin.

The following Examples are included for purposes of illustration and are not intended to limit the scope of the invention.

EXAMPLES

Example 1. Isolation of lipidated PSA

Briefly, *B. fragilis* was grown in anaerobic conditions. The capsular complex from *B. fragilis* was isolated with hot phenol/water extraction. The polysaccharide fraction was precipitated with ethanol after DNase, RNase and pronase treatments. The precipitate was subjected to size exclusion chromatography in order to separate the lipidated PSA from other polysaccharide constituents. The fractions of interest were analyzed and pooled, then dialyzed and lyophilized. The purity of lipidated PSA was assessed by nuclear magnetic resonance spectroscopy and mass spectroscopy.

The isolation and purification process of lipidated PSA is provided below in greater detail.

The *B. fragilis* delta44 mutant strain was derived experimentally from strain 9343 and upon further characterization it was found to over-express PSA relative to PSB. Delta44 was plated onto a blood agar plate and grown overnight at 37°C. A swab from a heavily colonized plate was sub-cultured into a 500 ml starter culture of peptone yeast broth. The starter culture was inoculated into 16 liter culture of the same media and pH was titrated to neutrality with 5M NaOH. An anaerobic gas mix was bubbled into the sealed culture.

After an overnight culture maintained at pH 7, bacteria were checked by Gram stain and subculture. Organisms were collected by centrifugation at 8,000x g for 20 minutes.

Bacterial pellets were washed two times with saline yielding approximately one liter of bacterial pellet.

The bacterial pellet was suspended in 68°C melted crystalline phenol to a final concentration of phenol of about 37% v/v (yielding a phenol/water preparation) and mixed for 30 minutes at 68°C followed by stirring at 4°C for 48 hours. The phenol/water preparation was aliquoted into glass bottles which were then centrifuged at 1500 rpm. The upper water layer was harvested. Any residual phenol contained in the harvested aqueous phase was extracted with an equal volume of ethyl ether. The ether phase was then removed using a separatory funnel and any residual ether in the aqueous phase was evaporated, yielding the final aqueous phase from the phenol/water preparation.

The aqueous phase was dialyzed versus water with multiple changes over 5 days at 4°C and subsequently lyophilized until it was nearly dry (approximately 5 ml water

- 41 -

remaining). A solution of 0.05M Tris with magnesium, calcium and sodium azide (total volume 61 ml) was added to the lyophilized product to bring the total volume to about 66 ml.

To the dissolved product was added 10 ml of Tris buffer with DNase (0.07 mg/ml) and RNase (0.33 mg/ml). The entire suspension was filtered through a 0.45 micron filter and the filtrate was stirred at 37°C. The DNase/RNase treatment was repeated by adding fresh enzymes to the mixture, at similar concentrations, and stirred for two hours.

The mixture was then combined with 25 mg pronase in 10 ml Tris/magnesium/calcium solution, and the mixture stirred for 24 hours at 37°C. This step was repeated.

The polysaccharide fraction was precipitated by adding 5 volumes of ethanol at 4°C to the mixture. The solution was then centrifuged at 12,000x g for 30 minutes to pellet the polysaccharide fraction. The supernatant was removed and the pellet was resuspended in 392 ml type 1 H₂O.

The dissolved fraction was then dialyzed against two changes of 16 liters type 1 H₂O at 4°C. The volume was reduced by lyophilization to approximately 50 mls.

Twenty ml aliquots were chromatographed on a 5x200 cm column of S400 suspended in PBS and 1% sodium deoxycholate, and fractions were collected. Fractions were tested by double diffusion in agar with an antibody that reacts with both lipidated and non-lipidated PSA to determine where lipidated PSA eluted. Aliquots were tested for UV absorption at 280 nm and it was determined that lipidated PSA-containing fractions had no UV absorbable material.

Fractions containing lipidated PSA were then pooled, concentrated and dialyzed against type 1 H₂O on a Minitan concentrator (Millipore) with 10,000 mw cutoff membranes until conductivity of 100 ml was less than 50 µS. Lipidated PSA was then lyophilized.

Polysaccharide and glycolipid purity and structure was determined by proton nuclear magnetic resonance spectroscopy on a 600 MHz spectrometer and mass spectroscopy. For MALDI-TOF-TOF and LC-MS analysis, the lipidated PSA sample was resuspended to 10 µg/µL in 2% acetic acid and heated at 90 degrees for 90 minutes. For MALDI-TOF-TOF analysis, samples were mixed 1:1 to 1% matrix (CHCA or DHB) solution and directly loaded to stainless steel MALDI plate. For LC-MS analysis, samples did or did not undergo liquid-liquid extraction (chloroform-water) step, and then were dried and resuspended in 50:50 isopropanol:acetonitrile and injected.

EQUIVALENTS

5 While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those
10 skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many
15 equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit,
20 and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

25 All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

 All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

30 The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, i.e., “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e. “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one,

- 44 -

A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

- 45 -

What is claimed is:

CLAIMS

1. An isolated lipidated polysaccharide A (PSA) comprising polysaccharide A (PSA) covalently conjugated to a glycolipid, wherein the glycolipid is di-acylated, tri-acylated, tetra-acylated or penta-acylated.
2. The isolated lipidated polysaccharide A (PSA) of claim 1, wherein the glycolipid is tetra-acylated or penta-acylated.
3. An isolated lipidated polysaccharide A (PSA) comprising polysaccharide A (PSA) covalently conjugated to a glycolipid comprising one or more acyl chains ranging in length from 14-17 carbons.
4. The isolated lipidated polysaccharide A (PSA) of claim 3, wherein the one or more acyl chains range in length from 15-17 carbons.
5. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-4, wherein the glycolipid comprises a disaccharide substituted with the one or more acyl chains.
6. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-5, wherein the glycolipid comprises a diglucosamine.
7. An isolated lipidated polysaccharide A (PSA) comprising polysaccharide A (PSA) covalently conjugated to one or more acyl chains ranging in length from 14-17 carbons.
8. The isolated lipidated polysaccharide A (PSA) of claim 7, wherein the one or more acyl chains range in length from 15-17 carbons.
9. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-8, substantially free of other components found in a *B. fragilis* capsule.

- 46 -

10. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-9, substantially free of LPS.

11. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-10,
5 substantially free of unconjugated glycolipid.

12. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-11, free of non-lipidated PSA.

10 13. The lipidated polysaccharide A (PSA) of any one of claims 1-12, in purified form.

14. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-13, in a micelle form.

15

15. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-14, in lyophilized form.

16. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-15,
20 wherein the isolated lipidated polysaccharide A (PSA) is suitable for administration to a human.

17. The isolated lipidated polysaccharide A (PSA) of any one of claims 1-16,
isolated from *B. fragilis* cells that overexpress PSA relative to polysaccharide B (PSB).

25

18. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-17, wherein at least one of the one or more acyl chains is unmodified.

19. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-17,
30 wherein at least one of the one or more acyl chains is modified.

- 47 -

20. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-17, wherein at least one of the one or more acyl chains is unmodified and at least one of the one or more acyl chains is modified.

5 21. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-20, wherein at least one of the one or more acyl chains is modified with a hydroxyl group.

22. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-21, wherein at least one of the one or more acyl chains is C16:0-OH.

10 23. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-22, wherein at least one of the one or more acyl chains is C17:0-OH.

15 24. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-7 or 9-23, wherein at least one of the one or more acyl chains is C14:0.

25. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-24, wherein at least one of the one or more acyl chains is C15:0.

20 26. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-25, wherein at least one of the one or more acyl chains is N-substituted on a disaccharide.

27. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-26, wherein at least one of the one or more acyl chains is O-substituted on a disaccharide.

25 28. The isolated lipidated polysaccharide A (PSA) of any one of claims 3-27, wherein at least one of the one or more acyl chains is N-substituted on a disaccharide and at least one of the one or more acyl chains is O-substituted on the disaccharide.

30 29. A lipidated polysaccharide A (PSA) of any one of claims 1-28, wherein PSA comprises less than 60 repeating tetrasaccharide units.

- 48 -

30. A lipidated polysaccharide A (PSA) of any one of claims 1-28, wherein PSA comprises 1-10 repeating tetrasaccharide units.

31. A pharmaceutical composition comprising
the lipidated polysaccharide A (PSA) of any one of claims 1-30, and
less than 0.5% (w/w) of free glycolipid.

32. A pharmaceutical composition comprising
the lipidated polysaccharide A (PSA) of any one of claims 1-30, and
a pharmaceutically acceptable carrier.

33. The pharmaceutical composition of claim 32, wherein the composition comprises less than 1% or less than 0.5% free glycolipid (w/w).

34. The pharmaceutical composition of any one of claims 31-33, further comprising a detergent or a bile salt.

35. The pharmaceutical composition of claim 34, wherein the detergent or bile salt is present at present at or less than 1%, 0.5% or 0.1%.

36. The pharmaceutical composition of any one of claims 31-35, in lyophilized form.

37. The pharmaceutical composition of any one of claims 31-36, wherein the isolated lipidated polysaccharide A (PSA) is provided as a micelle or a liposome.

38. A pharmaceutical composition comprising
the lipidated polysaccharide A (PSA) of any one of claims 1-30, and
a detergent or a bile salt.

39. A composition comprising

- 49 -

the lipidated polysaccharide (PSA) of any one of claims 1-30 in a micelle or a liposome.

40. The composition of any one of claims 31-39, formulated for parenteral
5 administration to a subject.

41. An isolated glycolipid comprising a diglucosamine covalently conjugated to 2-
5 acyl chains, each independently ranging in length from 14-17 carbons.

10 42. The isolated glycolipid of claim 41, wherein the diglucosamine is covalently
conjugated to 2-4 acyl chains.

43. The isolated glycolipid of claim 41, wherein the diglucosamine is covalently
conjugated to 4 acyl chains.

15

44. The isolated glycolipid of any one of claims 41-43, wherein the acyl chains
range in length from 15-17 carbons.

45. A non-hydrolytic method for isolating lipidated polysaccharide A (PSA) from
20 *B. fragilis*, comprising

extracting, into an aqueous phase, capsular complex from *B. fragilis* using a
mixture of phenol and water,

precipitating a polysaccharide fraction from the aqueous phase using ethanol,
and

25

isolating lipidated PSA from the polysaccharide fraction by size exclusion.

46. The method of claim 45, wherein isolating by size exclusion comprises using a
chromatographic column comprising a detergent or a bile salt.

30

47. The method of claim 46, wherein the chromatographic column comprises
deoxycholate.

- 50 -

48. The method of any one of claims 45-47, wherein the method is performed at a pH less than about 9.

49. The method of any one of claims 45-48, further comprising dialyzing the
5 isolated lipidated PSA.

50. The method of any one of claims 45-49, wherein extraction occurs at 60-75°C.

51. The method of any one of claims 45-50, wherein extraction occurs at about
10 68°C.

52. The method of any one of claims 45-51, wherein the method is performed in the presence of sodium deoxycholate.

53. The method of any one of claims 45-52, wherein *B. fragilis* is a mutant form of *B. fragilis* that over-expresses PSA relative to PSB.
15

54. The method of any one of claims 45-53, wherein the isolated lipidated PSA is substantially free of unconjugated glycolipid.
20

55. A composition comprising isolated lipidated polysaccharide A produced by the method of any one of claims 45-54.

56. The composition of claim 55, wherein the composition is formulated for
25 parenteral administration to a subject.

57. A composition comprising
polysaccharide A (PSA) and a glycolipid, in or on a substrate,
wherein PSA is not covalently conjugated to the glycolipid.
30

58. A composition comprising

- 51 -

a polysaccharide comprising one or more tetrasaccharide units, each tetrasaccharide unit having a structure of Formula I, and

a glycolipid comprising one or more acyl chains ranging in length from 14-17 carbons,

5 wherein the polysaccharide and glycolipid are provided in unconjugated to each other, in or on a substrate.

59. The composition of claim 58 or 59, wherein the substrate is a nanoparticle.

10 60. The composition of claim 58, 59 or 60, wherein PSA and the glycolipid are present in a molecular weight ratio of 10:1 to less than 1:1.

61. A composition comprising:
polysaccharide A (PSA) and a glycolipid, covalently conjugated to each other
15 via a non-ketosidic bond.

62. A composition comprising:
a polysaccharide comprising one or more tetrasaccharide units, each
tetrasaccharide unit having a structure of Formula I, and
20 a glycolipid comprising one or more acyl chains ranging in length from 14-17 carbons,
wherein the polysaccharide is covalently conjugated to the glycolipid via a non-ketosidic bond.

25 63. The composition of claim 61 or 62, wherein the non-ketosidic bond is an ester, amide or ether bond.

64. The composition of any one of claims 61-63, wherein the glycolipid comprises a disaccharide.

30 65. The composition of claim 64, wherein the disaccharide is diglucosamine.

- 52 -

66. The composition of any one of claims 61-65, wherein the glycolipid comprises 2-5 acyl chains.

67. The composition of any one of claims 61-66, wherein the glycolipid comprises 4 or 5 acyl chains.

68. The composition of any one of claims 61-67, wherein at least one of the one or more acyl chains is unmodified.

69. The composition of any one of claims 61-68, wherein at least one of the one or more acyl chains is modified.

70. The composition of any one of claims 61-69, wherein at least one of the one or more acyl chains is unmodified and at least one of the one or more acyl chains is modified.

71. The composition of any one of claims 61-70, wherein at least one of the one or more acyl chains is modified with a hydroxyl group.

72. The composition of any one of claims 61-71, wherein at least one of the one or more acyl chains is C16:0-OH.

73. The composition of any one of claims 61-72, wherein at least one of the one or more acyl chains is C17:0-OH.

74. The composition of any one of claims 61-73, wherein at least one of the one or more acyl chains is C14:0.

75. The composition of any one of claims 61-74, wherein at least one of the one or more acyl chains is C15:0.

76. The composition of any one of claims 61-75, wherein at least one of the one or more acyl chains is N-substituted on a disaccharide.

- 53 -

77. The composition of any one of claims 61-76, wherein at least one of the one or more acyl chains is O-substituted on a disaccharide.

5 78. The composition of any one of claims 61-77, wherein at least one of the one or more acyl chains is N-substituted on a disaccharide and at least one of the one or more acyl chains is O-substituted on a disaccharide.

10 79. The composition of any one of claims 61-78, wherein the polysaccharide has a molecular weight of about 150 kiloDaltons.

80. The composition of any one of claims 61-79, wherein the polysaccharide comprises 1-10 tetrasaccharide units.

15 81. The composition of any one of claims 61-80, wherein the glycolipid has a structure of Formula II.

20 82. The composition of any one of claims 61-81, wherein the glycolipid has a structure of Formula III.

83. The composition of any one of claims 61-82, wherein the polysaccharide and the glycolipid are present in or on a substrate.

25 84. The composition of claim 57, 58, 60 or 83, wherein the substrate is a film, a matrix or a particle.

85. The composition of claim 57, 58, 59, 60, 83 or 84, wherein the substrate is biodegradable.

30 86. The composition of claim 57-60, 83, 84 or 85, wherein the substrate is a nanoparticle.

- 54 -

87. The composition of any one of claims 61-86, wherein the composition further comprises a pharmaceutically acceptable carrier.

5 88. The composition of any one of claims 61-87, wherein the composition is a pharmaceutical composition.

89. The composition of any one of claims 61-88, wherein the composition is formulated for parenteral administration.

10 90. The composition of any one of claims 61-89, wherein the composition is effective in the treatment of an autoimmune disorder.

15 91. The composition of any one of claims 61-90, wherein the composition is substantially free of other components found in a *B. fragilis* capsule and is suitable for administration to a human.

92. A composition comprising:
polysaccharide A (PSA) comprising 1 to 50 tetrasaccharide units, and a glycolipid, wherein PSA is covalently conjugated to the glycolipid.

20

93. A composition comprising:
a polysaccharide comprising 1 to 50 tetrasaccharide units, each tetrasaccharide unit having a structure of Formula I, and
a glycolipid comprising one or more acyl chains ranging in length from 14-17
25 carbons,
wherein the polysaccharide is covalently conjugated to the glycolipid.

25

94. The composition of claim 93 or 94, wherein the polysaccharide comprises 1-40 tetrasaccharide units or 1-20 tetrasaccharide units.

30

95. The composition of claim 93 or 94, wherein the polysaccharide comprises 1-10 tetrasaccharide units or 1-5 tetrasaccharide units.

- 55 -

96. A micelle consisting essentially of lipidated PSA.

97. The micelle of claim 96, wherein the lipidated PSA is isolated lipidated PSA.

5

98. A composition comprising
a micelle consisting essentially of lipidated PSA and
a detergent or bile salt.

10 99. The composition of claim 98, wherein the detergent or bile salt is present in a
pharmaceutically acceptable amount.

100. The composition of claim 98 or 99, wherein the composition is a
pharmaceutical composition.

15

101. A method comprising
administering, to a subject having or at risk of developing a condition associated with
inflammation, an effective amount of the lipidated PSA or the composition of any one of
claims 1-44, 55-95 and 98-100.

20

102. The method of claim 101, wherein the condition is an autoimmune disease.

103. The method of claim 102, wherein the autoimmune disease is multiple
sclerosis, Crohn's disease, ulcerative colitis, rheumatoid arthritis, or type I diabetes.

25

104. The method of claim 101, wherein the condition is asthma.

105. The method of claim 101, wherein the condition is a post-surgical adhesion.

30 106. The method of claim 105, wherein the composition is administered prior to,
during, and/or after surgery.

- 56 -

107. The method of claim 101, wherein the condition is an abscess.

108. The method of claim 107, wherein an antibiotic is administered to the subject.

5 109. The method of claim 101, wherein the condition is obesity.

110. The method of any one of claims 101-109, wherein the composition is parenterally administered to the subject.

1/19

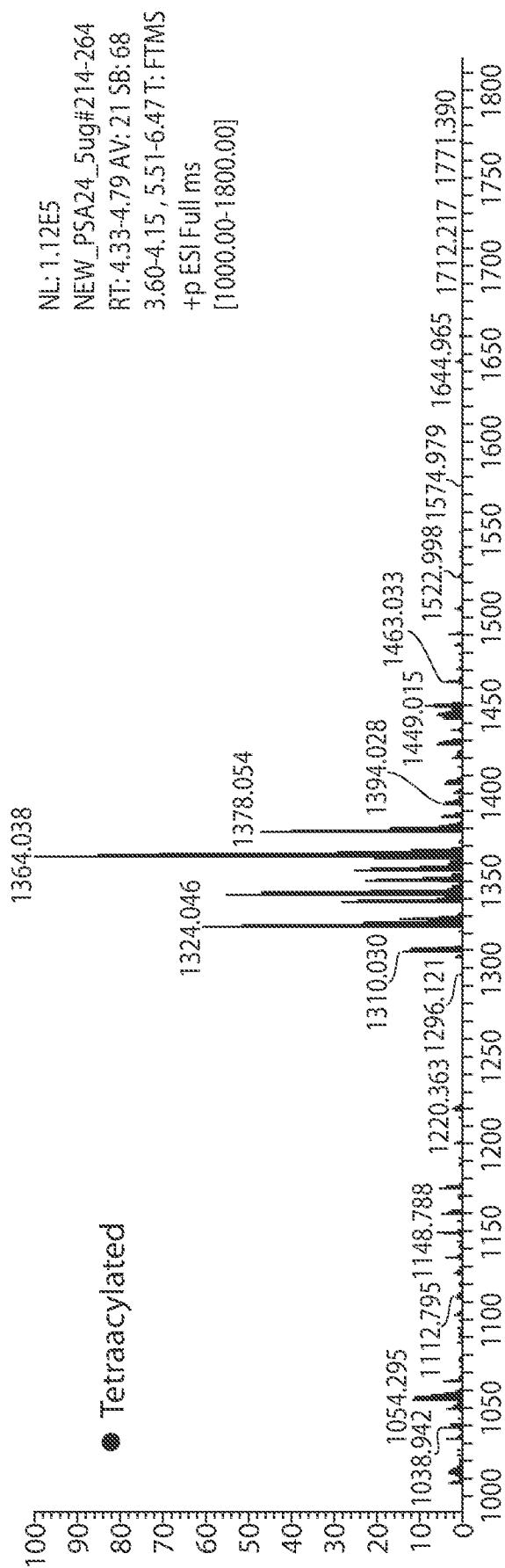
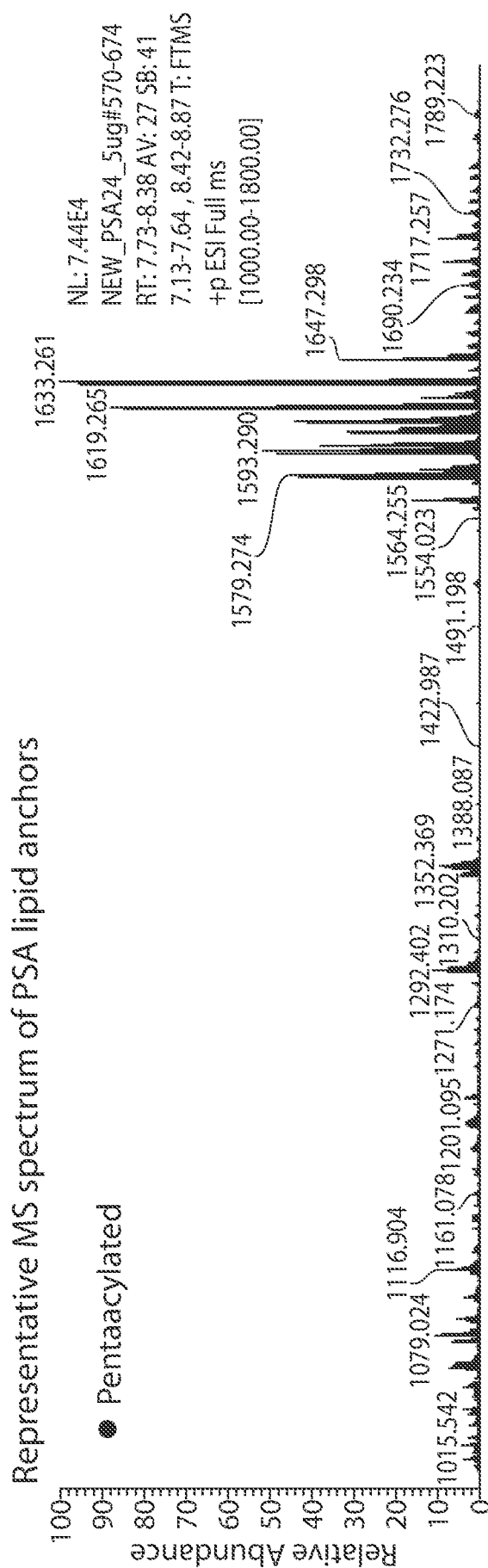


FIG.1A

2/19

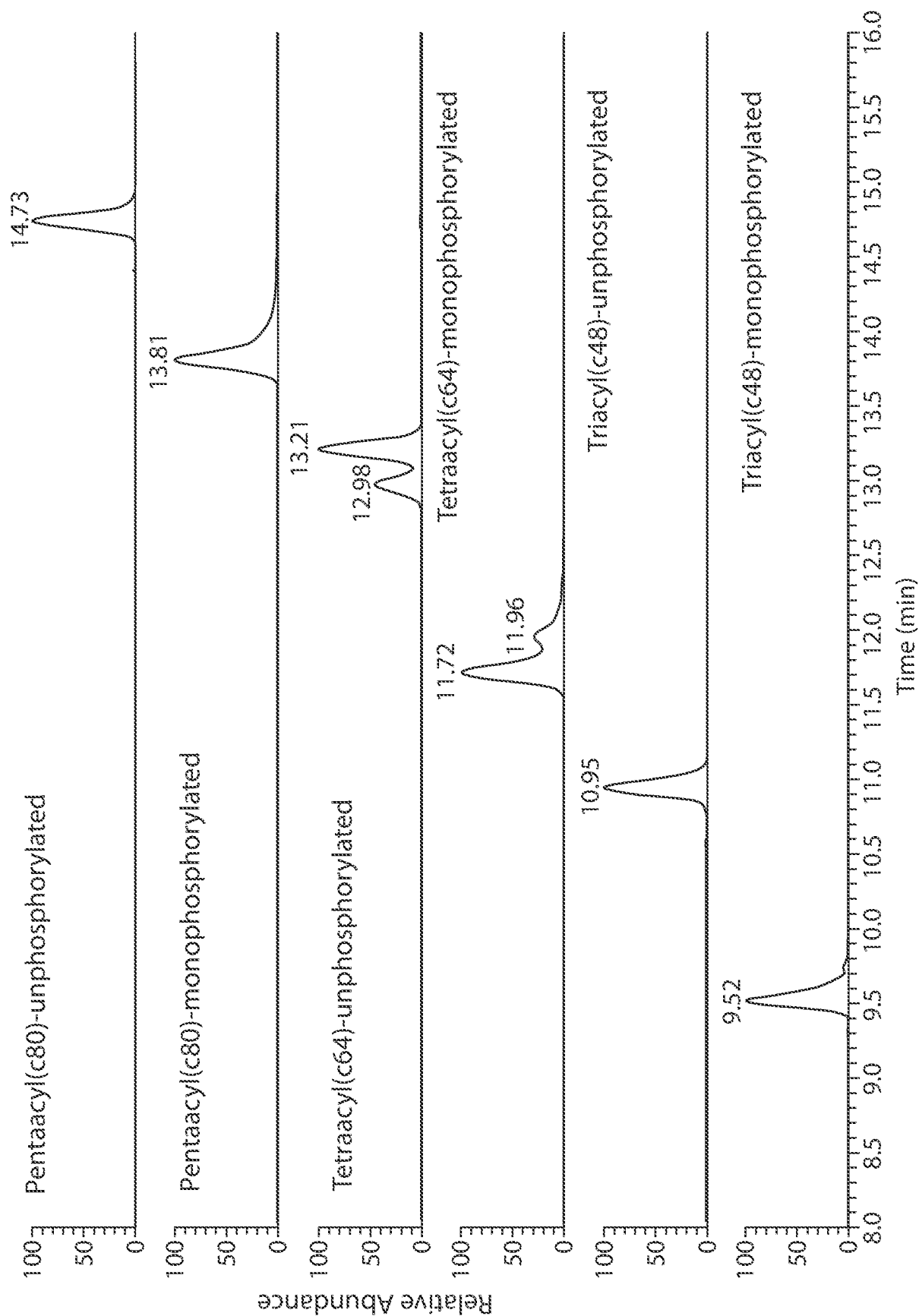


FIG. 1B

3/19

Comparison between PSA24(lipidated) and PSA23(delipidated)

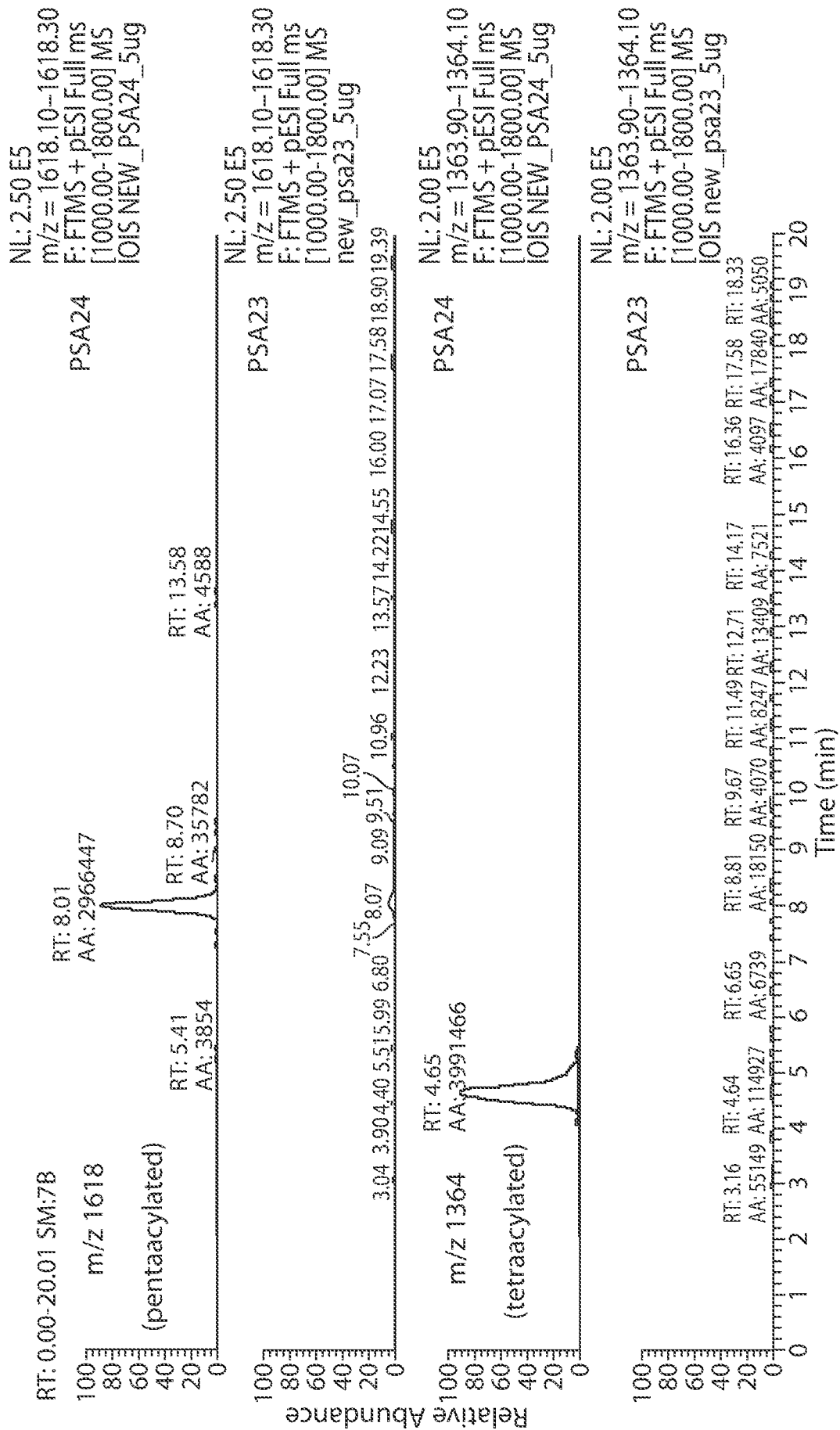


FIG. 2A

4/19

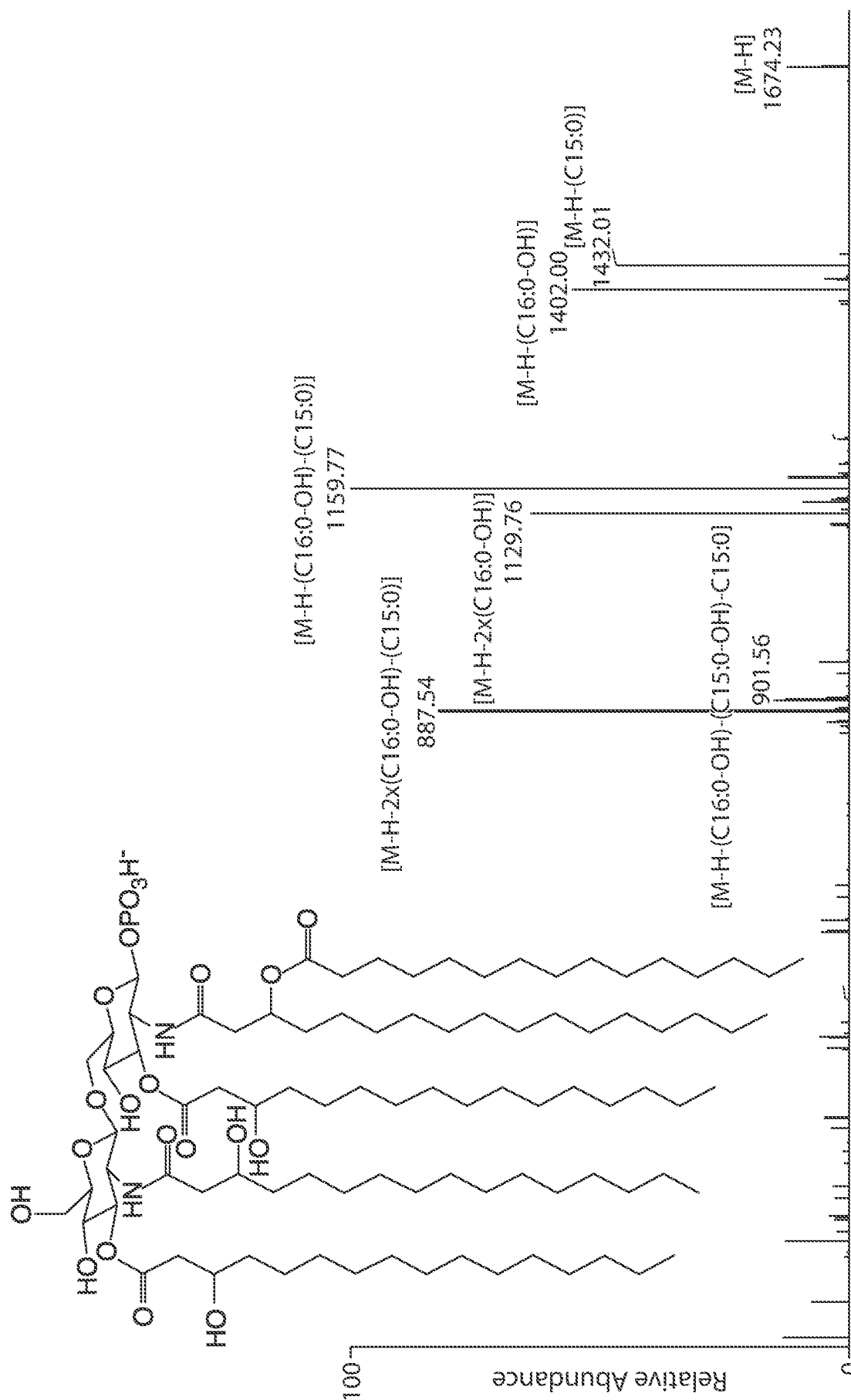


FIG. 2B

5/19

MALDI analysis of PSA33 Band 3

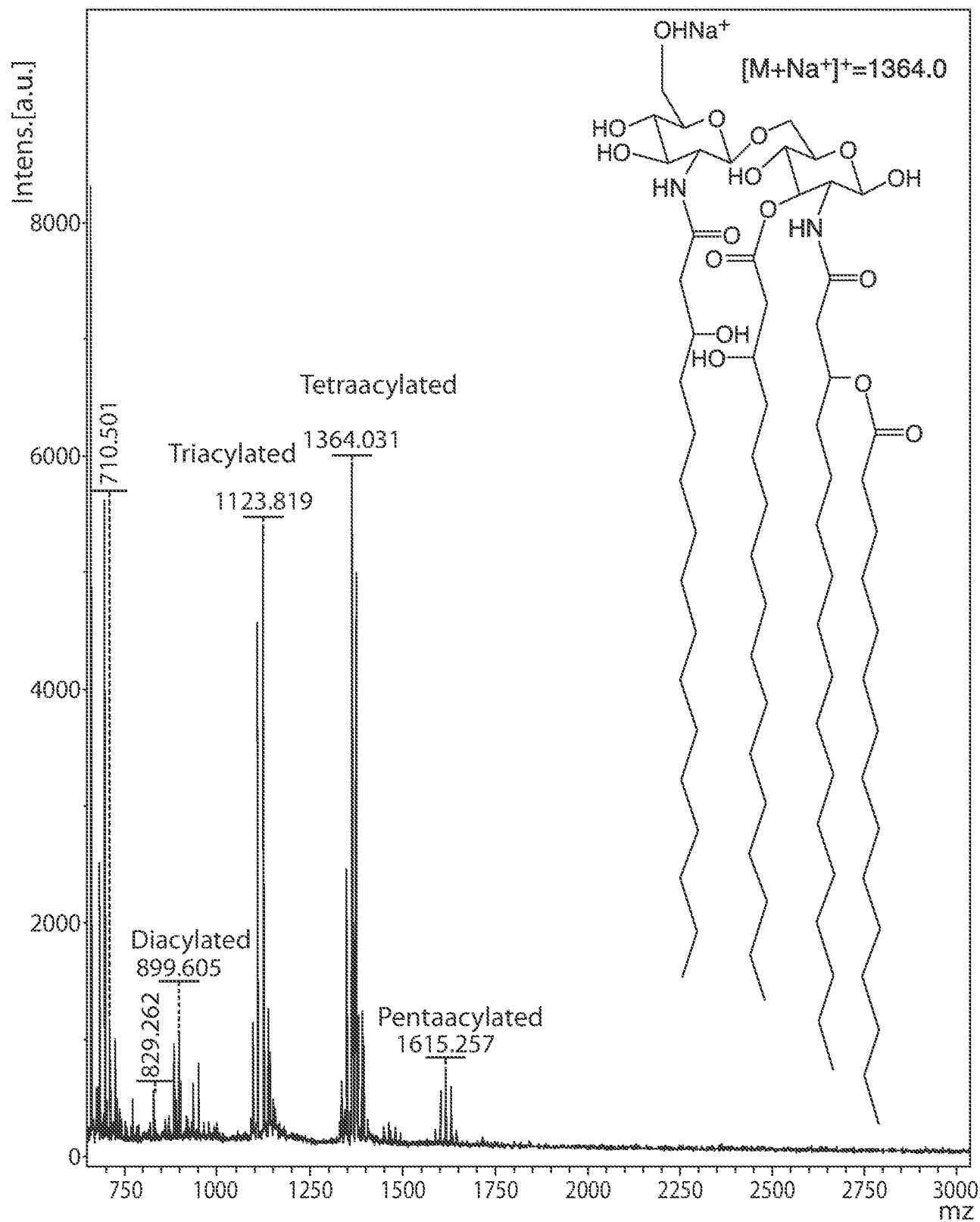


FIG. 3

Quantitative analysis of lipid anchor species

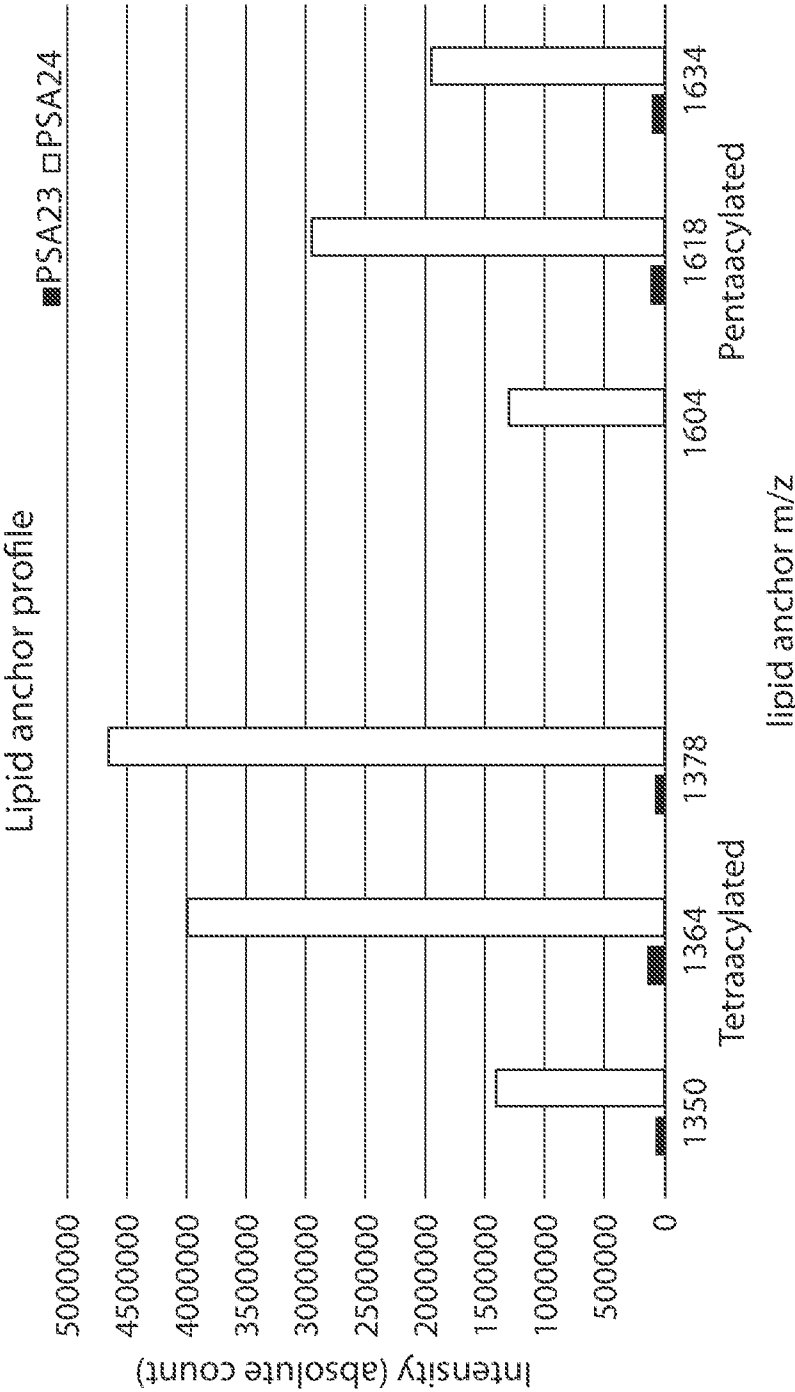


FIG. 4

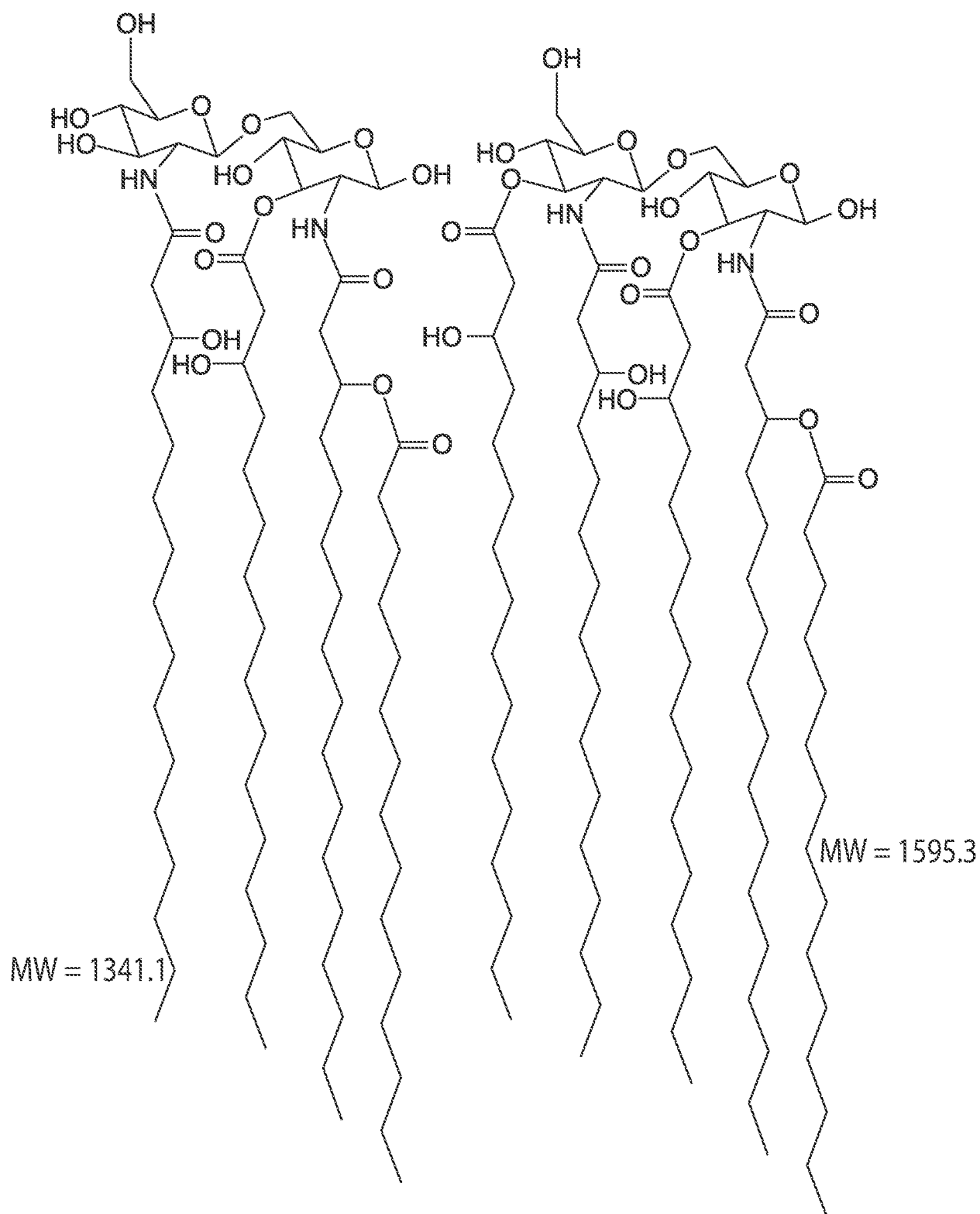
7/19

Molecular species of PSA glycolipids and proposed acyl
chain composition

# of acyl group	M/Z of [M+Na ⁺] ⁺	Proposed acyl chain composition				
Penta	1646	c16:0-OH	c17:0-OH	c17:0-OH	c17:0-OH	c15:0
	1632	c16:0-OH	c16:0-OH	c17:0-OH	c17:0-OH	c15:0
	1618	c16:0-OH	c16:0-OH	c16:0-OH	c17:0-OH	c15:0
	1604	c16:0-OH	c16:0-OH	c16:0-OH	c16:0-OH	c15:0
	1590	c16:0-OH	c16:0-OH	c16:0-OH	c16:0-OH	c14:0
Tetra	1392		c17:0-OH	c17:0-OH	c17:0-OH	c15:0
	1378		c16:0-OH	c17:0-OH	c17:0-OH	c15:0
	1364		c16:0-OH	c16:0-OH	c17:0-OH	c15:0
	1350		c16:0-OH	c16:0-OH	c16:0-OH	c15:0
	1336		c16:0-OH	c16:0-OH	c16:0-OH	c14:0
Tri	1123			c17:0-OH	c17:0-OH	c15:0
	1109			c16:0-OH	c17:0-OH	c15:0
	1095			c16:0-OH	c16:0-OH	c15:0
	1081			c16:0-OH	c16:0-OH	c14:0
Di	899			c17:0-OH	c17:0-OH	
	885			c16:0-OH	c17:0-OH	

FIG. 5

8/19



Representative structures of PSA lipid anchors, a tetraacylated (left) and pentaacylated (right) diGluNs.

FIG. 6A

9/19

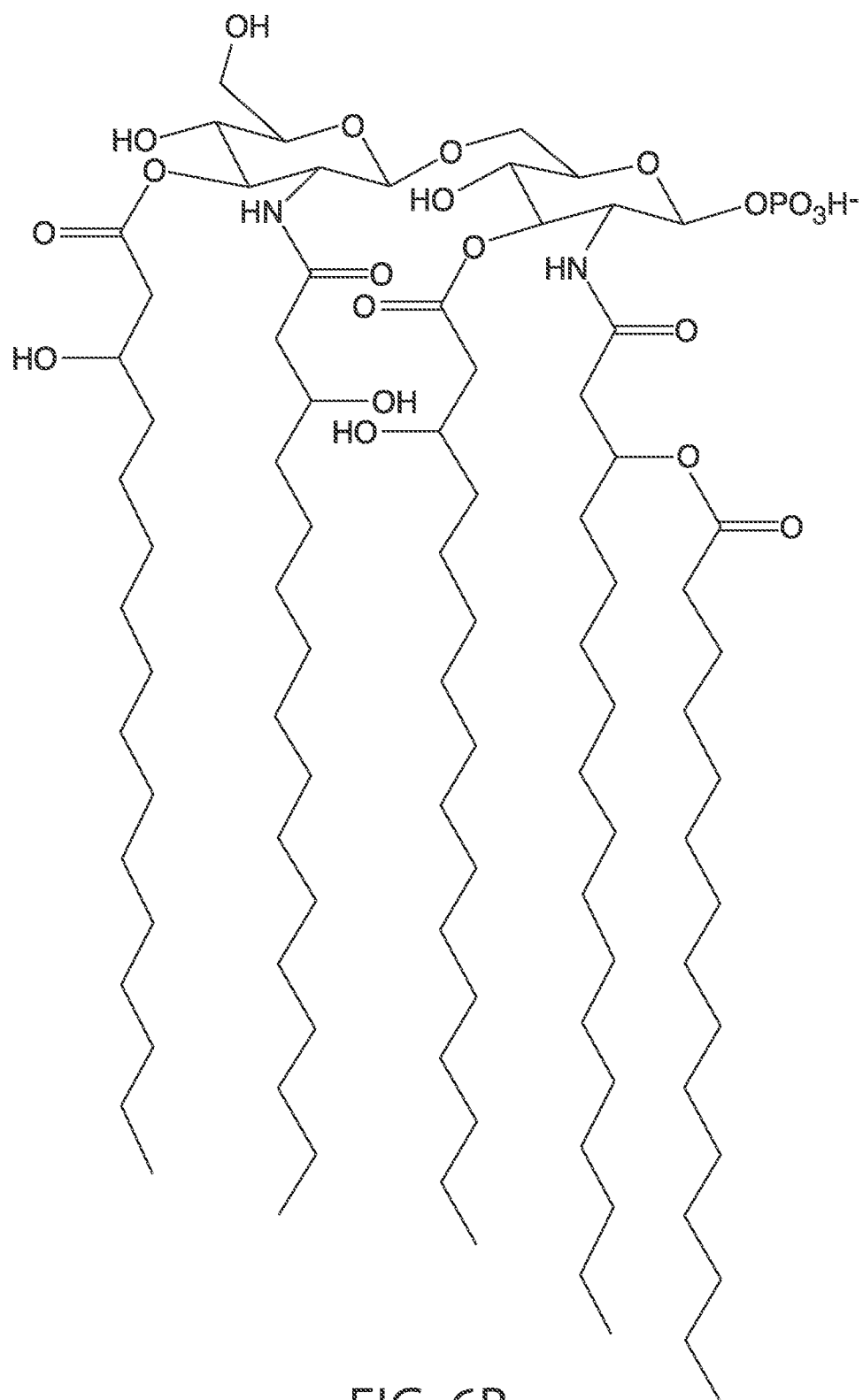


FIG. 6B

10/19

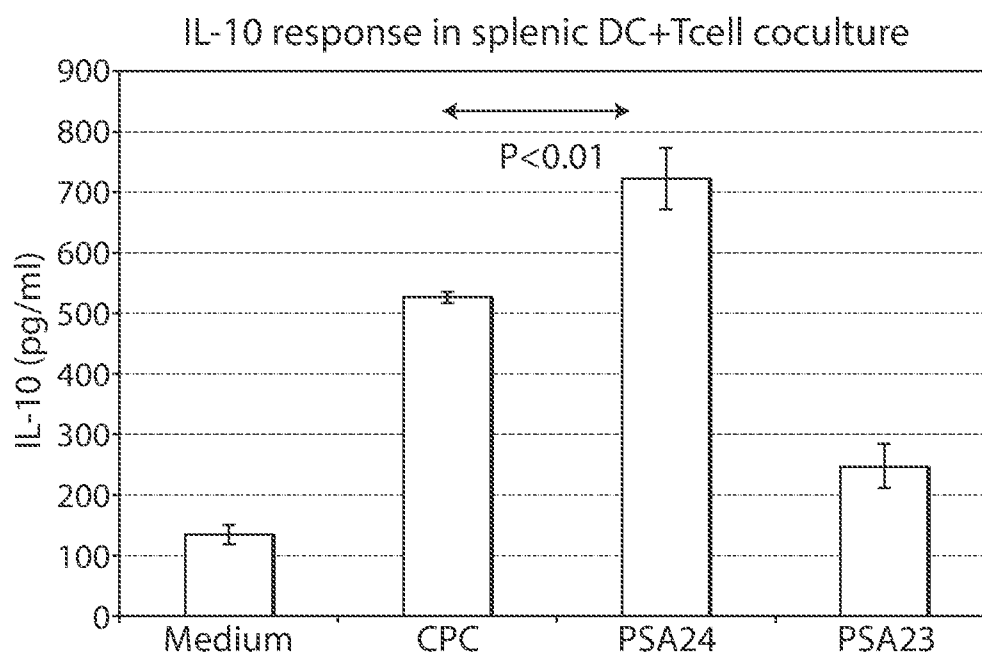
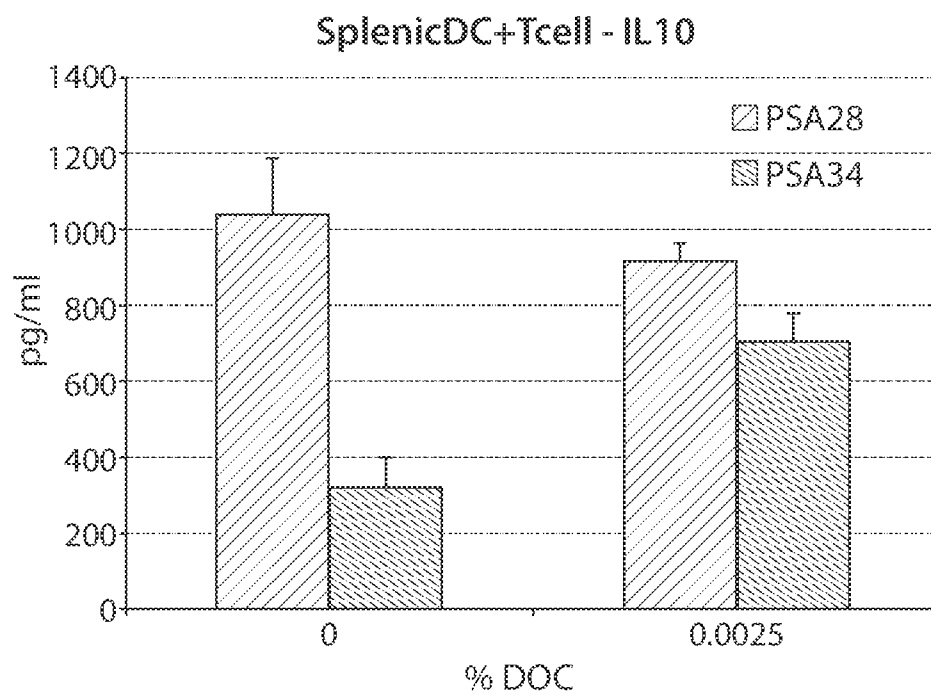


FIG. 7

11/19

**FIG. 8**

12/19

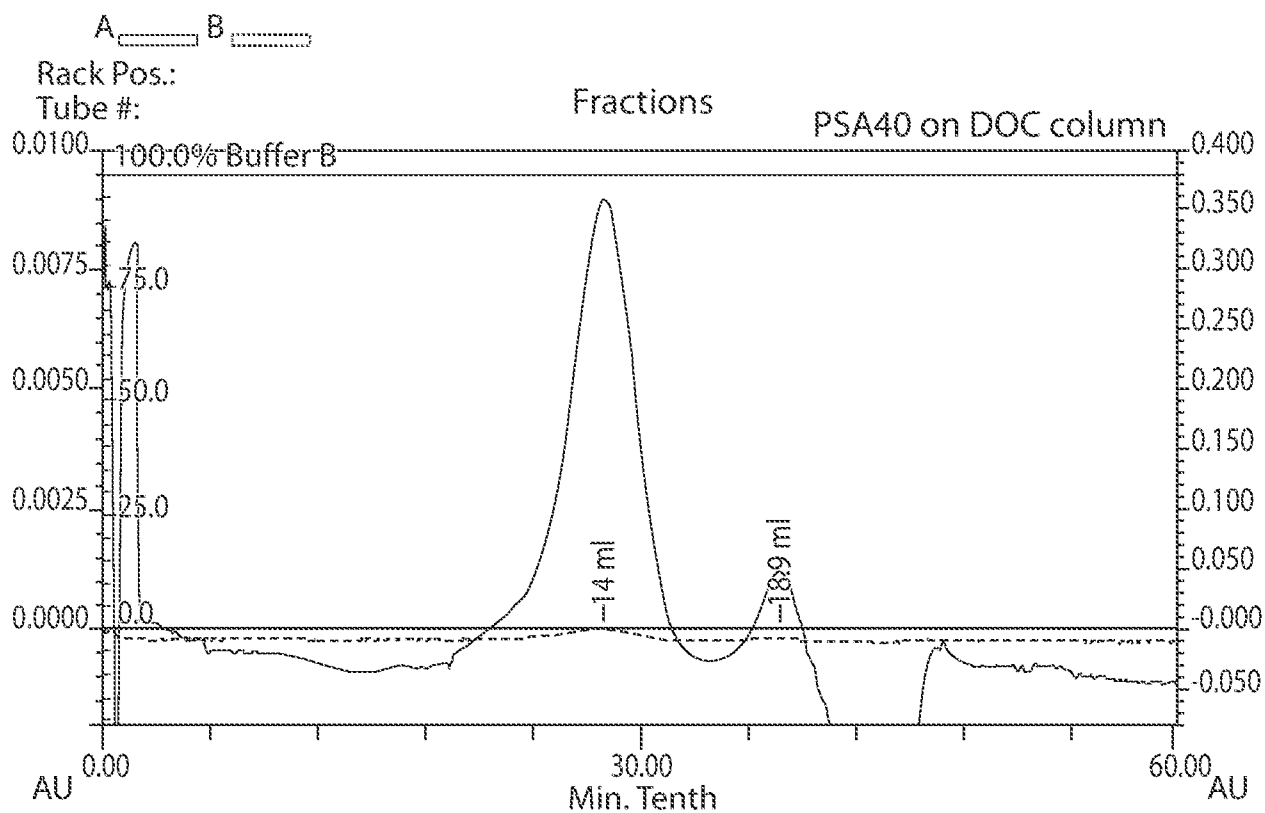
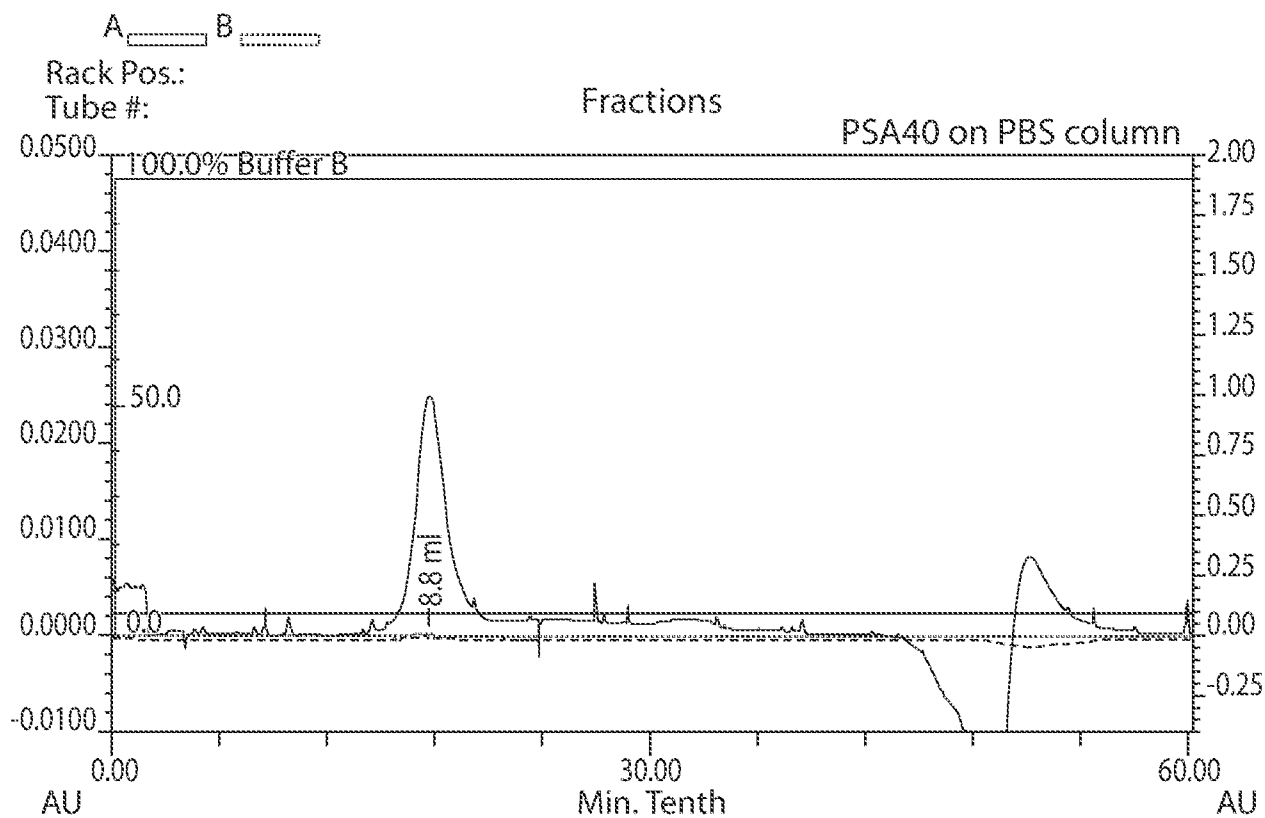


FIG. 9

13/19

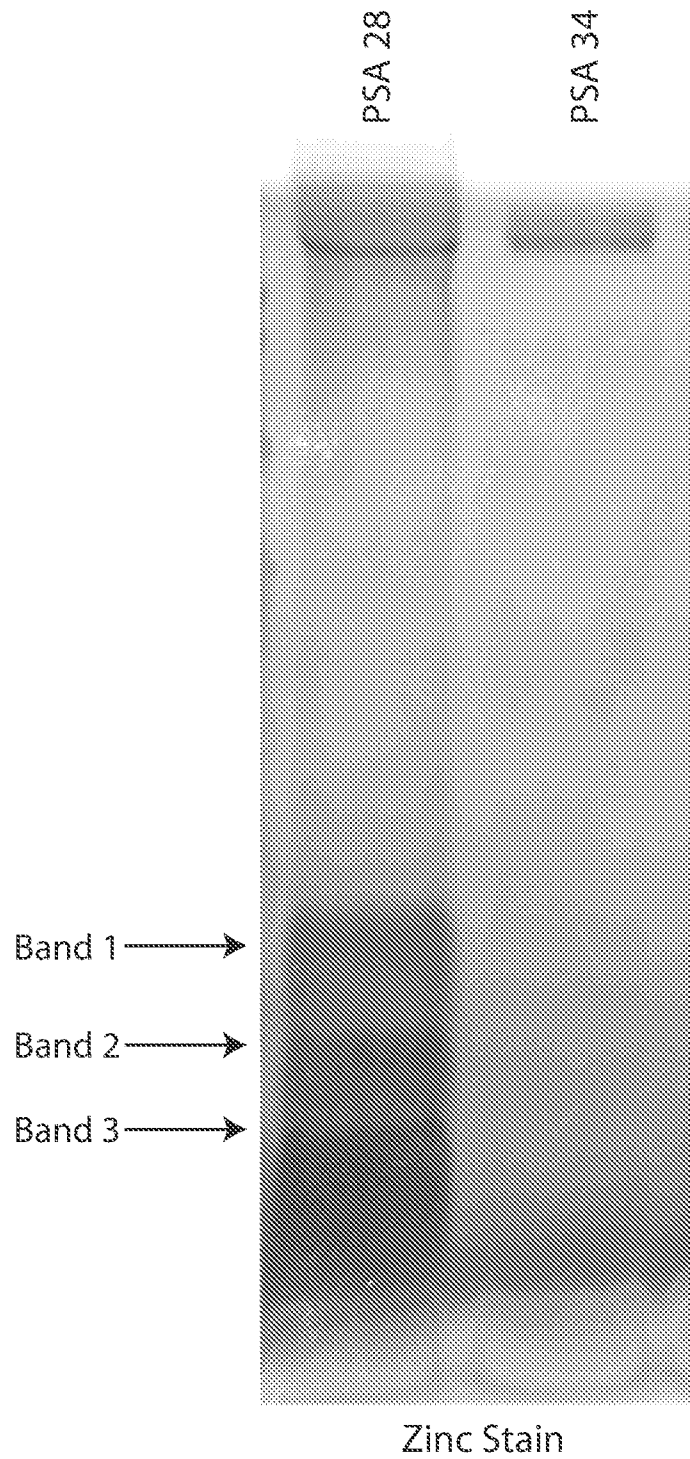


FIG. 10

14/19

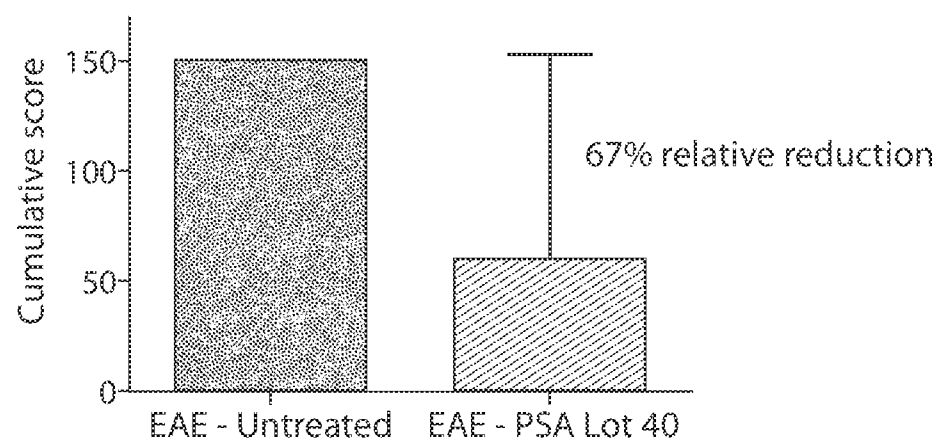


FIG. 11

15/19

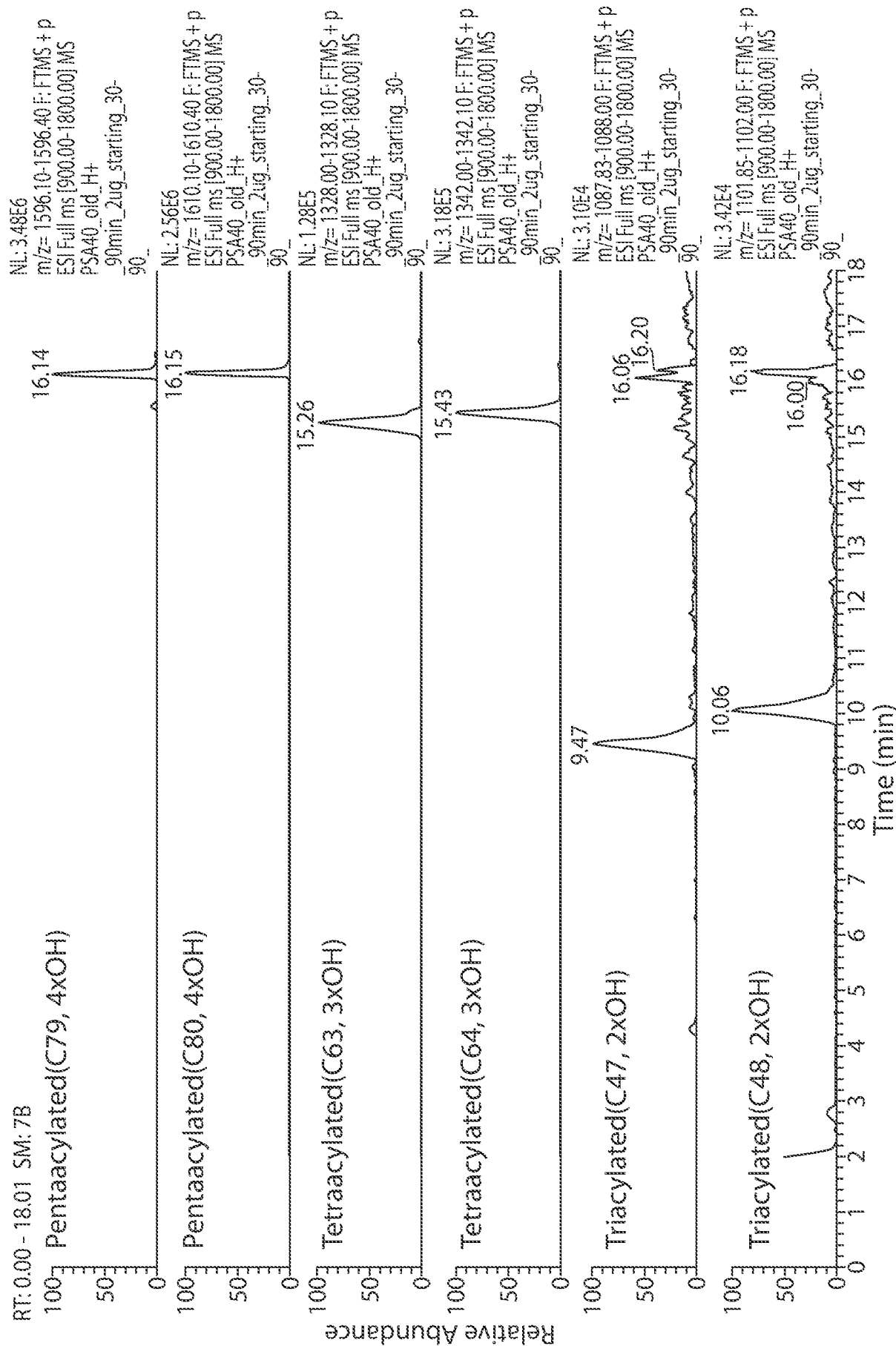


FIG. 12

16/19

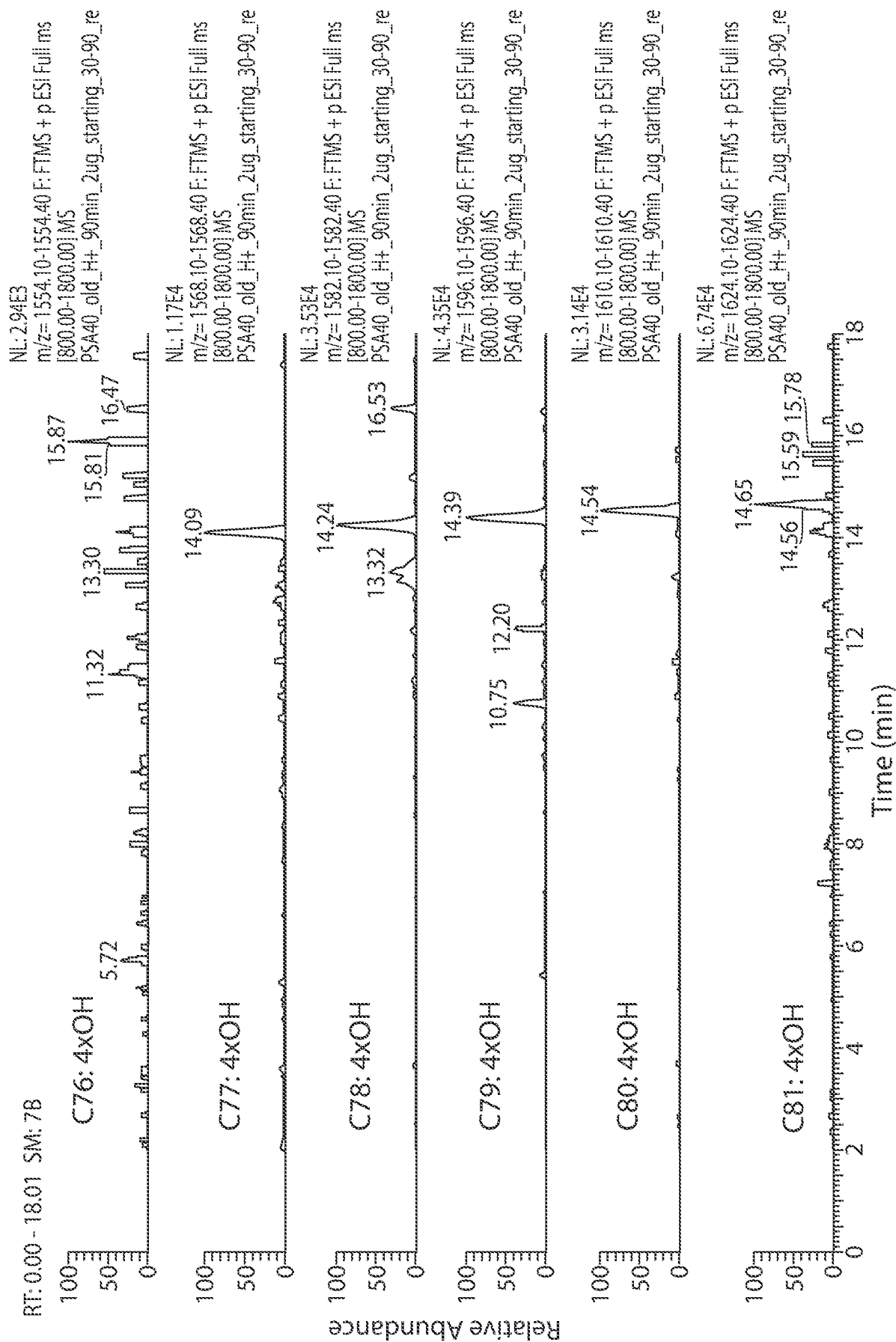


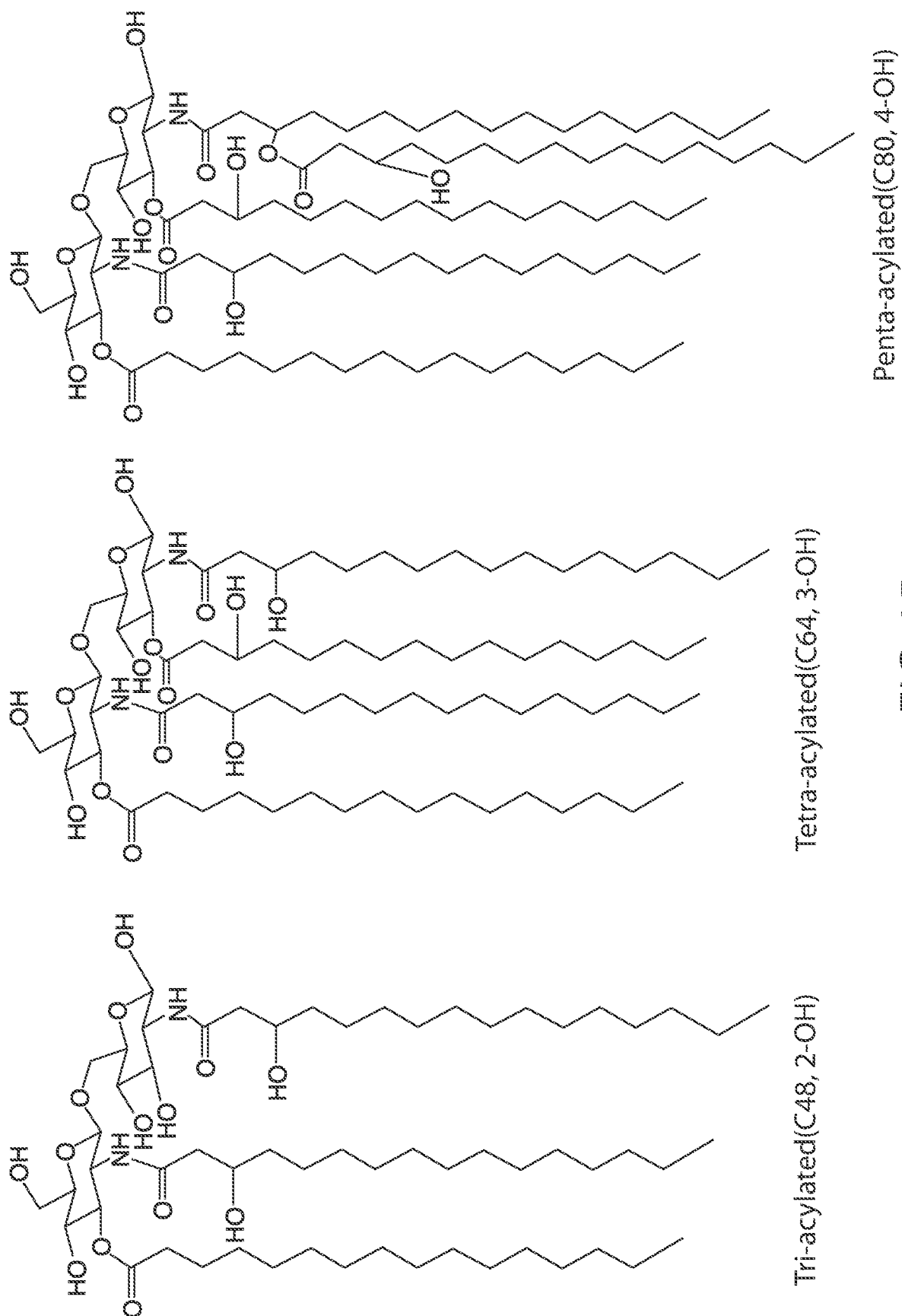
FIG. 13

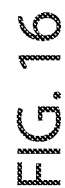
17/19

pentaacylated		5xOH				4xOH				3xOH		
		exact mass	CPS			exact mass	CPS			exact mass	CPS	
total Acyl Carbon		1542.3	ND			1526.3	999290			1510.3	2187535	
74		1556.3	786504			1540.3	1261489			1524.3	3431369	
75		1570.3	2673445			1554.3	1220670			1538.3	1691385	
76		1584.3	8338616			1568.3	5992380			1552.3	4055057	
77		1598.3	13803256			1582.3	16539240			1566.3	10375662	
78		1612.3	11357658			1596.3	25412060			1580.3	19631384	
79		1626.3	2033195			1610.3	18401147			1594.3	12449433	
80		1640.3	ND			1624.3	3358855			1608.3	2835742	
81						1638.3	nd			1622.3	1517435	
82										1636.3	1162429	
83												
84												
85												
tetraacylated												
		4xOH				3xOH				2xOH		
		exact mass	CPS			exact mass	CPS			exact mass	CPS	
total Acyl Carbon		1300.0	ND			1284.0	ND			1268.0	ND	
60		1314.0	561145			1298.0	ND			1282.0	ND	
61		1328.0	3001367			1312.0	979210			1296.0	177876	
62		1342.0	7747490			1326.0	2814859			1310.0	2931125	
63		1356.0	6193798			1340.0	2509823			1324.0	7130550	
64		1370.0	462989			1354.0	353662			1338.0	6665907	
65		1384.0	ND			1368.0	ND			1352.0	1447857	
66		1398.0				1382.0				1366.0	2428419	
67		1412.0				1396.0				1380.0	2469079	
68										1394	335522	
69												

FIG. 14

18/19





INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/47787

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A01N 43/04; A61K 31/715 (2016.01) CPC - A61K 31/715; A61K 31/728; A61K 47/36 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC(8): A01N 43/04; A61K 31/715 (2016.01) CPC: A61K 31/715; A61K 31/728; A61K 47/36				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC: 514/54				
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatBase, Google Scholar, Google Patents: isolated lipidated polysaccharide, covalently conjugated, glycolipid, di-acylated, tri-acylated, tetra-acylated, penta-acylated, lipid moiety, acyl chain				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X --- Y	US 2004/0185057 A1 (Kirkby et al.) 23 September 2004 (23.09.2004); para [00226]-[0227]	1 ----- 2 and 5/(1-2)		
X --- Y	US 2010/0221269 A1 (Boons et al.) 02 September 2010 (02.09.2010); para [0078], [0080], [0083], [0197], [0210]	3-4, 5/(3-4) and 7-8 ----- 2 and 5/(1-2)		
A	US 2006/0147624 A1 (Jennings et al.) 07 July 2005 (07.07.2005); para [002]; Abstract	1		
☐ Further documents are listed in the continuation of Box C. ☐				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;"> * 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 application or patent 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 </td> <td style="width: 50%; vertical-align: top;"> "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 </td> </tr> </table>			* 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 application or patent 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
* 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 application or patent 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 12 December 2016		Date of mailing of the international search report 06 JAN 2017		
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774		

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/47787

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.: 6,9-44, 49-56, 60, 64-91, and 101-110
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:
---please see supplemental box ---

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 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.:
1-5, 7-8

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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/47787

Box III: lack of unity

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-5 and 7-8, directed to an isolated lipidated polysaccharide A (PSA) comprising polysaccharide A (PSA) covalently conjugated to a glycolipid.

Group II: Claims 45-48 directed to a non-hydrolytic method for isolating lipidated polysaccharide A (PSA) from *B. fragilis*, comprising extracting, into an aqueous phase, capsular complex from *B. fragilis* using a mixture of phenol and water, precipitating a polysaccharide fraction from the aqueous phase using ethanol, and isolating lipidated PSA from the polysaccharide fraction by size exclusion

Group III: Claims 57-59, directed to a composition comprising polysaccharide A (PSA) and a glycolipid, in or on a substrate, wherein PSA is not covalently conjugated to the glycolipid.

Group IV: Claims 61-63, 92-95, directed to a composition comprising: polysaccharide A (PSA) and a glycolipid, covalently conjugated to each other via a non-ketosidic bond

Group V: claims 96-100, directed to a micelle consisting essentially of lipidated PSA

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group II requires a non-hydrolytic method for isolating lipidated polysaccharide A (PSA) from *B. fragilis*, comprising extracting, into an aqueous phase, capsular complex from *B. fragilis* using a mixture of phenol and water, precipitating a polysaccharide fraction from the aqueous phase using ethanol, and isolating lipidated PSA from the polysaccharide fraction by size exclusion, not required by group I and III-V

Group III requires a composition comprising polysaccharide A (PSA) and a glycolipid, in or on a substrate, wherein PSA is not covalently conjugated to the glycolipid, not required by groups I-II and IV-V.

Group IV requires a composition comprising: polysaccharide A (PSA) and a glycolipid, covalently conjugated to each other via a non-ketosidic bond, not required by group I-III and V.

Group V requires a micelle consisting essentially of lipidated PSA not required by group I-IV.

Common Technical Features:

Groups I-V share the technical feature of lipidated polysaccharide A (PSA).

Groups III-IV share the technical feature of a composition comprising polysaccharide A (PSA) and a glycolipid. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is being anticipated by US 2004/0185057 A1 to Kirkby et al. (hereinafter "Kirkby"). Kirkby teaches an isolated lipidated polysaccharide A (PSA) comprising: polysaccharide A (PSA) covalently conjugated to a glycolipid ([0226]-[0227]; Attachment can be via a functional group on a lipophilic moiety that covalently reacts ... glycolipids), wherein the glycolipid is di-acylated, tri-acylated, tetra-acylated or penta-acylated ([0226]; By, way of example... di-acylglycerols can be covalently attached).

As the shared technical features were known in the art at the time of the invention, they cannot be considered common technical features that would otherwise unify the groups. Therefore, Groups I-V lack unity under PCT Rule 13.

Note:

Claim 94 is improperly depending from claim 93 or 94. For the purpose of completing this ISR, claim 94 is interpreted as it is depending from claim 93.