(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
09 May 2019 (09.05.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/090203 A1

(51) International Patent Classification:
A61K 31/70 (2006.01)

(21) International Application Number:
PCT/US2018/059152

(22) International Filing Date:
05 November 2018 (05.11.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/581,443 03 November 2017 (03.11.2017) US

(71) Applicants: THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; Office of Technology Commercialization, 109 Church Street, Chapel Hill, NC 27516 (US). THE RESEARCH FOUNDATION FOR THE STATE UNIVERSITY OF NEW YORK; Technology Transfer, University at Buffalo, 520 Lee Entrance, Suite 109, UB Commons, Amherst, NY 14228 (US).

(72) Inventors: ARNOLD, Katelyn; 324 Cobblestone Ct., Chapel Hill, NC 27514 (US). XU, Ding; 10 Santa Clara Ct., East Amherst, NY 14051 (US). XU, Yongmei; 5501 Weston Downs Dr., Durham, NC 27707 (US). PAWLINSKI, Rafal; 102 Brunswick Ct, Chapel Hill, NC 27516 (US). LIU, Jian; 217 Huntington Drive, Chapel Hill, NC 27514 (US).

(74) Agent: TAYLOR, Jr., Arles, A.; Jenkins, Wilson, Taylor & Hunt, P.A., 3015 Carrington Mill Boulevard, Suite 550, Morrisville, NC 27560 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

(54) Title: SULFATED OLIGOSACCHARIDES HAVING ANTI-INFLAMMATORY ACTIVITY

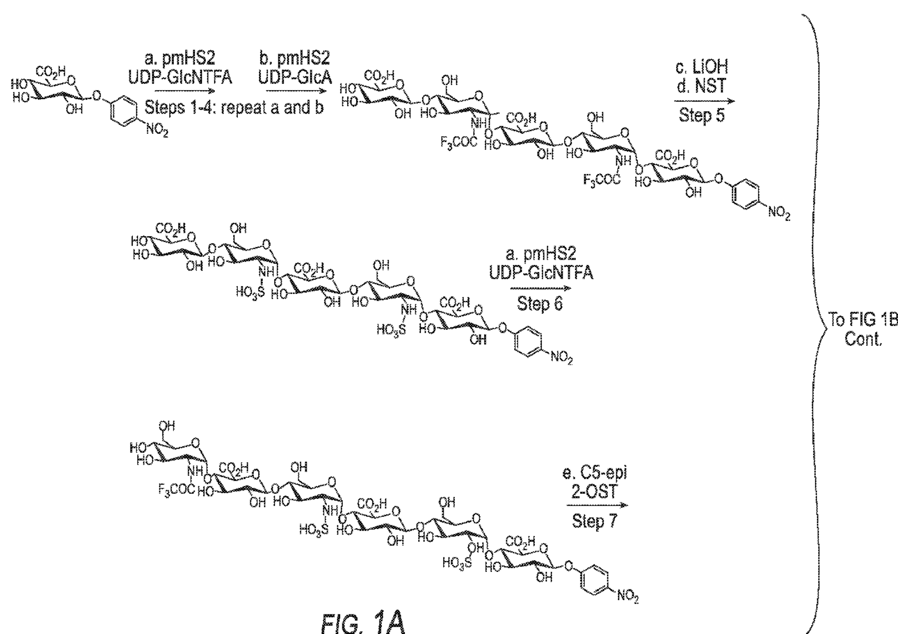


FIG. 1A

(57) Abstract: Provided herein are small molecule compounds, including non- anticoagulant heparan sulfate oligosaccharide molecules, having anti- inflammatory properties and capable of interacting with high mobility group box 1 (HMGB1) proteins in a manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation end products (RAGE). Also provided herein are methods of treating Paracetamol (APAP) overdose in subjects.

WO 2019/090203 A1

[Continued on next page]

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

DESCRIPTION

SULFATED OLIGOSACCHARIDES HAVING ANTI-INFLAMMATORY ACTIVITY

5 ## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Patent Application Serial No. 62/581,443, filed November 3, 2017, herein incorporated by reference in its entirety.

10 ## TECHNICAL FIELD

Disclosed herein in some embodiments are new sulfated heparan sulfate oligosaccharide compounds having anti-inflammatory activity. For example, in some embodiments it is observed that the compounds display a protective effect on drug-induced liver damage. While it is not desired to be 15 bound by any particular theory of operation, the compounds likely inhibit the interaction of HMGB1/RAGE, a key signaling interaction involved in sterile inflammatory injury. This discovery can be applied for a wide range of diseases that are caused by over reaction of inflammation responses.

20 ## BACKGROUND

Sterile inflammation is a natural process that initiates tissue repair in response to cellular damage. However, exaggerated inflammation after an initial insult often damages surrounding healthy tissues and is a key contributor to many disease processes. High mobility group box 1 (HMGB1) 25 is a DNA binding protein that regulates transcription and is released from the nucleus during necrotic cell death (Zitvogel, 2010; Chen, 2010). Extracellularly, HMGB1 is a damage associated molecular pattern (DAMP) protein that acts as a pro-inflammatory molecule, orchestrating migration and activation of inflammatory cells to the injury site (Bianchi, 2017). 30 HMGB1-dependent inflammation has been associated with ischemia-reperfusion and drug-induced liver injury (Huebener, 2015).

Acetaminophen (APAP), also known as paracetamol, is a widely used analgesic and is the active pharmaceutical ingredient of Tylenol®. Ingestion

of a supratherapeutic dose causes acute liver failure (ALF) (Heard, 2008). The misuse of Vicodin® or Percocet®, co-formulations of opioids and APAP, can also cause ALF. In the US, nearly 50% of drug-induced liver injury has been attributed to APAP toxicity (Lee, 2007), which accounts for ~80,000 emergency room visits annually (Blieden, 2014). The mechanism for APAP toxicity begins with its metabolic conversion to the reactive chemical species, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which causes hepatocyte necrosis. (Tacke, 2015). Necrotic hepatocytes release HMGB1 which drives chemotaxis of neutrophils through the receptor for advanced glycation end-products (RAGE), activating sterile inflammation and amplifying liver injury (Huebener 2015).

However, additional compositions and methods for treating inflammation in subject in need thereof remain a need in the art.

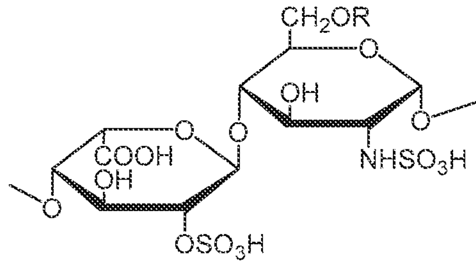
SUMMARY

This summary lists several embodiments of the presently disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently disclosed subject matter, whether listed in this summary or not. To avoid excessive repetition, this summary does not list or suggest all possible combinations of such features.

In some embodiments, provided herein are small molecule compounds having an anti-inflammatory property, the small molecule compound comprising a non-anticoagulant heparan sulfate oligosaccharide molecule, optionally wherein the non-anticoagulant heparan sulfate oligosaccharide molecule is configured to interact with a high mobility group box 1 (HMGB1) protein in a manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation end products (RAGE). The oligosaccharide molecule can comprise about 10 to

about 20 saccharide units, or about 12 to about 18 saccharide units. The oligosaccharide molecule can comprise about 18 saccharide units.

In some embodiments, provided herein is a small molecule compound comprising a disaccharide structure unit as shown:

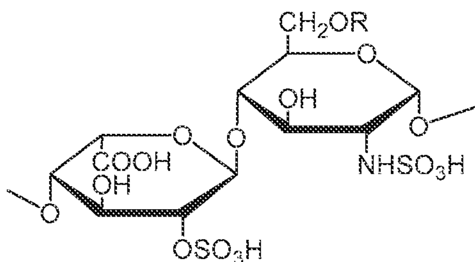


5 , wherein R = -H or $-SO_3H$. The disaccharide structure unit can be selected from the group consisting of non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin, and O-desulfated heparin (ODSH). In some embodiments, the oligosaccharide molecule protects against liver injury *in vivo*. In some embodiments, the
10 oligosaccharide molecule decreases neutrophil infiltration *in vivo*. In some embodiments, the oligosaccharide molecule decreases inflammation *in vivo*.

Provided herein are methods of treating a subject, comprising providing a subject to be treated, wherein the subject is suffering from inflammation, and administering to the subject a non-anticoagulant heparan
15 sulfate oligosaccharide molecule having an anti-inflammatory property, optionally wherein the non-anticoagulant heparan sulfate oligosaccharide molecule is configured to interact with a high mobility group box 1 (HMGB1) protein in a manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation endproducts (RAGE). In some
20 embodiments, the subject suffers from any injury resulting in increased inflammation. In some embodiments, the subject suffers from liver injury. In some embodiments, the subject in need of treatment is a subject suffering from an overdose of Paracetamol (APAP). In some embodiments, the subject in need of treatment is a human subject. In some embodiments, the
25 small molecule compound comprises a disaccharide structure unit selected from the group consisting of non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin, and O-desulfated heparin (ODSH). In some embodiments, the oligosaccharide molecule comprises about 10 to about 20 saccharide units, or about 12 to about 18 saccharide units. In some

embodiments, the oligosaccharide molecule comprises about 18 saccharide units. Administration of the oligosaccharide molecule can decrease neutrophil infiltration in the subject. Administration of the oligosaccharide molecule can decrease inflammation in the subject. Administration of the
5 oligosaccharide molecule can protect against liver damage and multi-organ system failure in the subject. In some aspects, the subject to be treated suffers from drug-induced inflammation.

In some aspects, provided herein are methods of treating Paracetamol (APAP) overdose in a subject, the method comprising providing
10 a subject in need of treatment for APAP overdose, administering to the subject a non-anticoagulant heparan sulfate oligosaccharide molecule having an anti-inflammatory property, optionally wherein the non-anticoagulant heparan sulfate oligosaccharide molecule is configured to interact with a high mobility group box 1 (HMGB1) protein in a manner
15 sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation endproducts (RAGE), wherein damage from the APAP overdose in the subject is mitigated. The treatment for the APAP overdose in the subject can in some embodiments be effective between 0 hours and 24 hours after the overdose, or at least within 12 hours after the
20 overdose. The treatment for the APAP overdose in the subject can comprise protection against liver injury and/or multi-organ system failure. The treatment for the APAP overdose in the subject can comprise a decrease in neutrophil infiltration in the subject. The treatment for the APAP overdose in the subject can comprise blocking the interaction between the
25 HMGB1 protein and RAGE. In some aspects, the small molecule compound comprises a disaccharide structure unit as shown:



, wherein R = -H or $-SO_3H$. The small molecule compound can comprise a disaccharide structure unit is selected from the group consisting of non-anticoagulant heparin, non-anticoagulant

low-molecular weight heparin, and O-desulfated heparin (ODSH). The oligosaccharide molecule comprises about 10 to about 20 saccharide units, or about 12 to about 18 saccharide units. In some aspects, the oligosaccharide molecule comprises about 18 saccharide units.

5 Accordingly, it is an object of the presently disclosed subject matter to provide sulfated heparan sulfate oligosaccharide compounds having anti-inflammatory activity and methods for making and using the same.

This and other objects are achieved in whole or in part by the presently disclosed subject matter. Further, an object of the presently 10 disclosed subject matter having been stated above, other objects and advantages of the presently disclosed subject matter will become apparent to those skilled in the art after a study of the following description, Drawings and Examples.

15 BRIEF DESCRIPTION OF THE DRAWINGS

The presently disclosed subject matter can be better understood by referring to the following figures. The components in the figures are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the presently disclosed subject matter (often schematically). In 20 the figures, like reference numerals designate corresponding parts throughout the different views. A further understanding of the presently disclosed subject matter can be obtained by reference to an embodiment set forth in the illustrations of the accompanying drawings. Although the illustrated embodiment is merely exemplary of systems for carrying out the 25 presently disclosed subject matter, both the organization and method of operation of the presently disclosed subject matter, in general, together with further objectives and advantages thereof, may be more easily understood by reference to the drawings and the following description. The drawings are not intended to limit the scope of this presently disclosed subject matter, 30 which is set forth with particularity in the claims as appended or as subsequently amended, but merely to clarify and exemplify the presently disclosed subject matter.

For a more complete understanding of the presently disclosed subject matter, reference is now made to the following drawings in which:

Figures 1A-1G are schematic illustrations of a chemoenzymatic synthetic pathway for the synthesis of 18-mer oligosaccharides;

5 Figure 2 is a schematic illustration of a chemical structure of an 18-mer oligosaccharide as disclosed herein;

Figures 3A through 3D are graphical depictions of data showing the effects of an 18-mer oligosaccharide on liver injury after APAP overdose;

Figures 4A through 4D are graphical depictions of data showing 18-
10 mer oligosaccharide targeting HMGB1 to decrease inflammation,

Figure 4E show symbolic structures of HS oligosaccharides;

Figure 4F is a legend for Figure 4E;

Figures 5A through 5D are graphical depictions of data showing the effects of delayed 18-mer oligosaccharide treatment on APAP overdose;

15 Figures 6A-6D are graphical depictions of data showing biological parameters of APAP overdosed mice;

Figure 7 is a schematic of the chemical structures of biotinylated and non-biotinylated oligosaccharides: 6-mer, 12-mer, 18-mer and 18-mer AXa;

Figures 8A-8D are graphical depictions of data showing the protective
20 effects of 6-mer, 12-mer, 18-mer and 18-mer AXa oligosaccharide;

Figures 9A and 9B are graphical depictions of data showing APAP overdose results in increased levels of ALT, migrating neutrophils and shed syndecan-1; and

Figures 10A through 10C are graphical depictions of data based on
25 the analysis of syndecan-1 from ALF patients and the plasma concentration of ALT and HMGB1 in ALF patients.

DETAILED DESCRIPTION

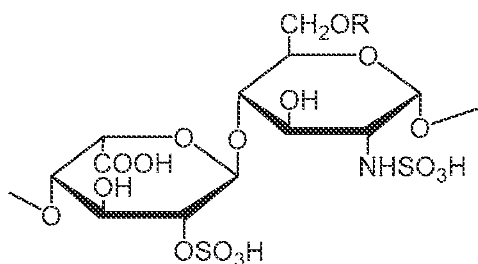
The presently disclosed subject matter now will be described more
30 fully hereinafter, in which some, but not all embodiments of the presently disclosed subject matter are described. Indeed, the presently disclosed subject matter can be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these

embodiments are provided so that this disclosure will satisfy applicable legal requirements.

Heparan sulfate (HS) is a sulfated polysaccharide abundantly present on cell surface and in extracellular matrix. Many DAMPs, including HMGB1, are HS-binding proteins (Xu, 2011). HS participates in various aspects of inflammation including chemokine presentation and neutrophil transendothelial migration (Proudfoot, 2003; Wang, 2005; Axelsson, 2013; and Sarris, 2012). HS is comprised of disaccharide repeating units of glucuronic acid (GlcA) or iduronic acid (IdoA) linked to glucosamine residues that carry sulfo groups. The chain length and sulfation pattern of HS determine its biological functions (Gama, 2006). In accordance with aspects of the presently disclosed subject matter, synthesis of a specially designed HS octadecasaccharide (18-mer) to exploit the anti-inflammatory effect is provided. The 18-mer protects APAP-induced acute liver failure through neutralizing the pro-inflammatory activity of HMGB1 in a murine model. The results presented offer a new chemical space to curb HMGB1-mediated inflammatory diseases.

In some embodiments, a small molecule compound with anti-inflammatory properties is disclosed. In some embodiments, the small molecule compound comprises a non-anticoagulant heparan sulfate oligosaccharide molecule with anti-inflammatory properties. In some embodiments, the non-anticoagulant heparan sulfate oligosaccharide molecule is configured to interact with a high mobility group box 1 (HMGB1) protein in a manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation endproducts (RAGE).

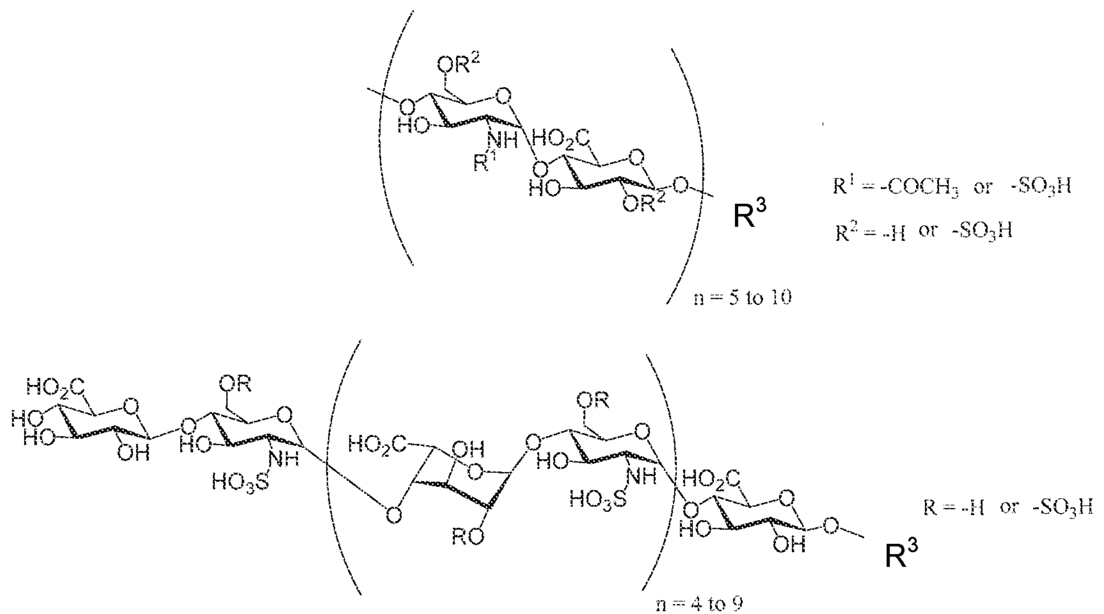
In some embodiments, a small molecule compound in accordance with the presently disclosed subject matter comprises, or can be part of a composition comprising, a disaccharide structure unit as shown:



, wherein R = -H or $-SO_3H$.

By way of example and not limitation, this disaccharide composition can be found in non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin or O-desulfated heparin (ODSH). A composition in accordance with the presently disclosed subject matter can comprise a non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin or O-desulfated heparin (ODSH).

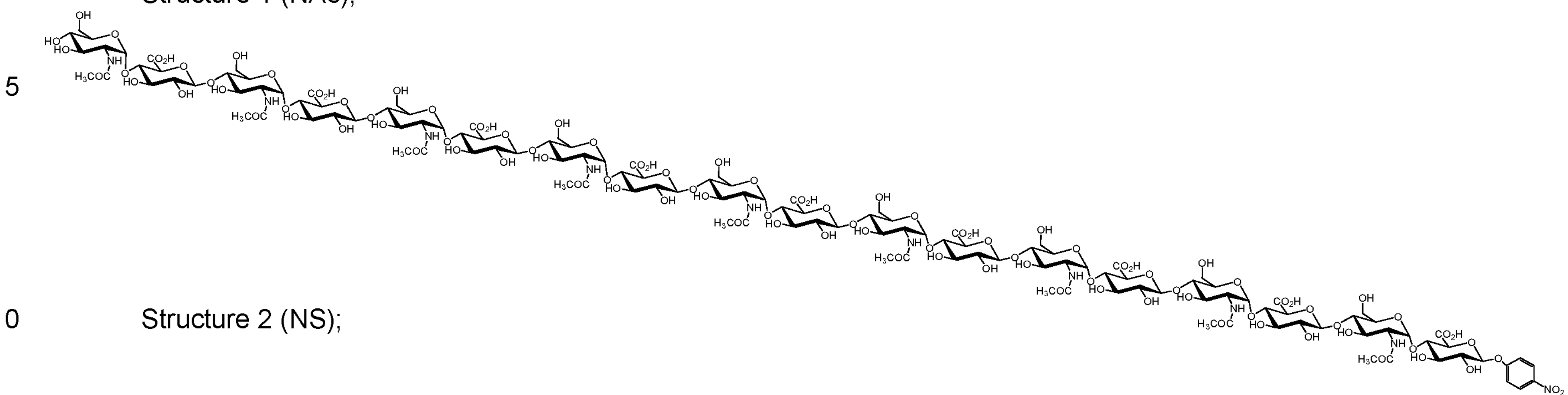
In some embodiments, the oligosaccharide molecule comprises about 10 to about 20 saccharide units, or about 12 to about 18 saccharide units. In some embodiments, the oligosaccharide molecule comprises about 18 saccharide units. In some embodiments, generic structures of heparan sulfate oligosaccharides displaying anti-inflammatory effects comprise:



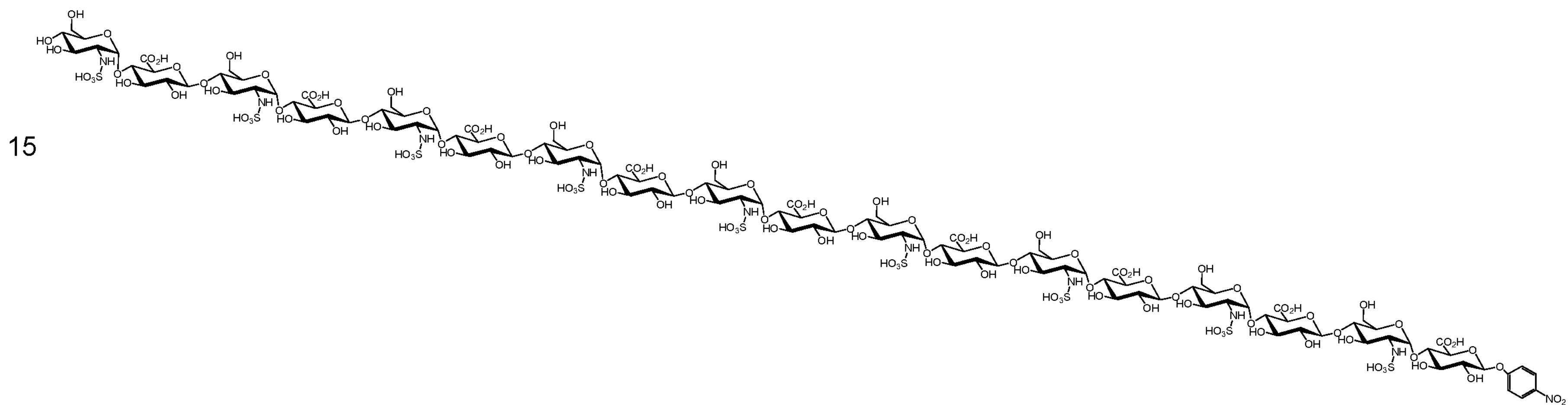
, wherein R^3 is H or a detectable tag. In some embodiments, the detectable tag comprises para-nitrophenyl.

In some embodiments, the oligosaccharide molecule comprises one of the following structures:

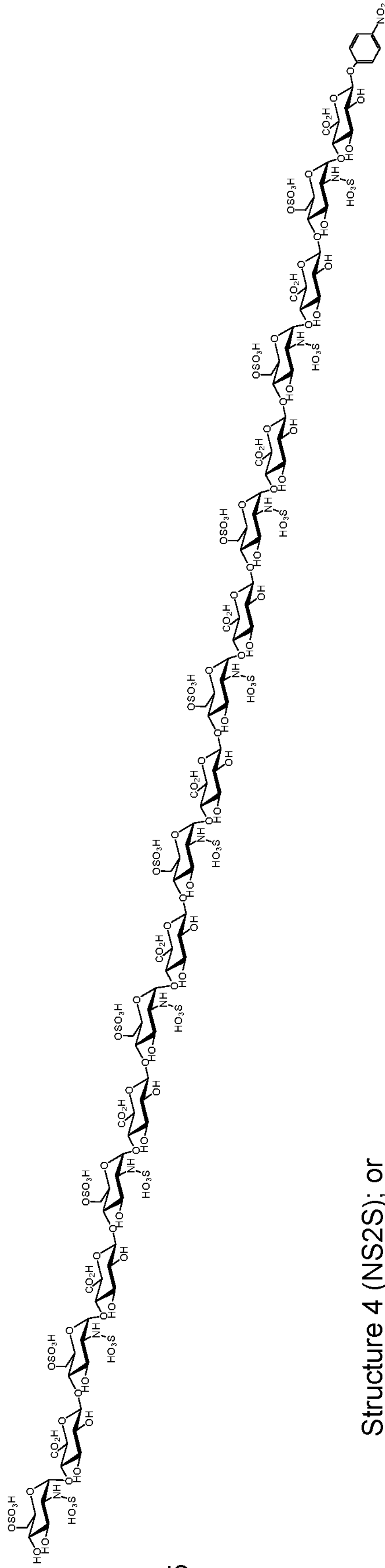
Structure 1 (NAc);



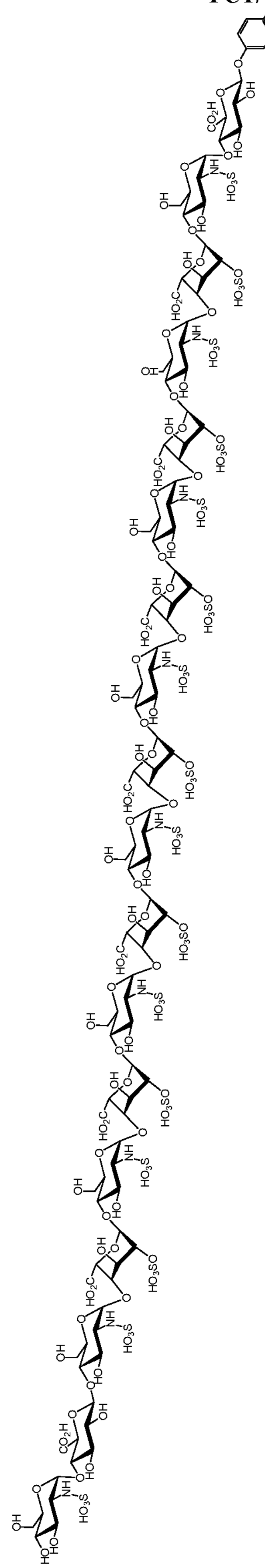
Structure 2 (NS);

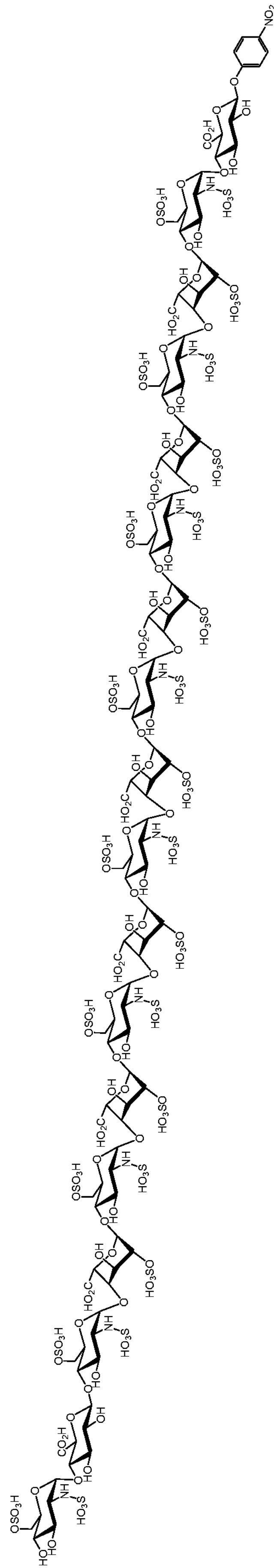


Structure 3 (NS6S);



Structure 4 (NS2S); or





Structure 5 (NS6S2S).

The compositions of the presently disclosed subject matter comprise in some embodiments a composition that includes a pharmaceutically acceptable carrier. Any suitable pharmaceutical formulation can be used to prepare the compositions for administration to a subject. In some embodiments, the composition and/or carriers can be pharmaceutically acceptable in humans.

For example, suitable formulations can include aqueous and non-aqueous sterile injection solutions that can contain anti-oxidants, buffers, bacteriostatics, bactericidal antibiotics, and solutes that render the formulation isotonic with the bodily fluids of the subject; and aqueous and non-aqueous sterile suspensions that can include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of sterile liquid carrier, for example water for injections, immediately prior to use. Some exemplary ingredients are sodium dodecyl sulfate (SDS), in one example in the range of 0.1 to 10 mg/ml, in another example about 2.0 mg/ml; and/or mannitol or another sugar, for example in the range of 10 to 100 mg/ml, in another example about 30 mg/ml; and/or phosphate-buffered saline (PBS).

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this presently disclosed subject matter can include other agents conventional in the art having regard to the type of formulation in question. For example, sterile pyrogen-free aqueous and non-aqueous solutions can be used.

Methods of treating a subject are also provided in accordance with the presently disclosed subject matter, including but not limited to methods that treat conditions comprising inflammation. The therapeutic methods of the presently disclosed subject matter can comprise administering to the subject a non-anticoagulant heparan sulfate oligosaccharide molecule. In some embodiments, the non-anticoagulant heparan sulfate oligosaccharide molecule is configured to interact with a high mobility group box 1 (HMGB1) protein in a

manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation endproducts (RAGE).

In some embodiments, the oligosaccharide molecule protects against liver injury *in vivo*. In some embodiments, the oligosaccharide molecule decreases neutrophil infiltration *in vivo*. In some embodiments, the oligosaccharide molecule decreases inflammation *in vivo*.

In some embodiments, the subject is suffering from inflammation. In some embodiments, the subject suffers from any injury resulting in increased inflammation. In some embodiments, the subject suffers from liver injury. In some embodiments, the subject in need of treatment is a subject suffering from an overdose of Paracetamol (acetaminophen or APAP).

In some embodiments, the subject treated in the presently disclosed subject matter is desirably a human subject, although it is to be understood the methods described herein are effective with respect to all vertebrate species (e.g., mammals, birds, etc.), which are intended to be included in the term "subject."

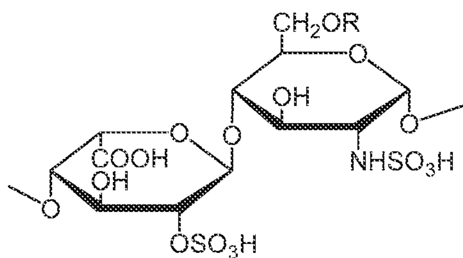
More particularly, provided herein is the treatment of mammals, such as humans, as well as those mammals of importance due to being endangered (such as Siberian tigers), of economical importance (animals raised on farms for consumption by humans) and/or social importance (animals kept as pets or in zoos) to humans, for instance, carnivores other than humans (such as cats and dogs), swine (pigs, hogs, and wild boars), ruminants (such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels), and horses. Thus, the methods described herein include the treatment of livestock, including, but not limited to, domesticated swine (pigs and hogs), ruminants, horses, and the like.

In some embodiments, the oligosaccharide molecule decreases neutrophil infiltration in the subject. In some embodiments, the administration of the oligosaccharide molecule decreases inflammation in the subject. In some embodiments, the administration of the oligosaccharide molecule protects against liver damage and multi-organ system failure in the subject. In some embodiments, the subject to be treated suffers from drug-induced inflammation.

In some embodiments, damage from the APAP overdose in the subject is mitigated.

In some embodiments, the treatment for the APAP overdose in the subject is effective between 0 hours and 24 hours after the overdose, or in some embodiments at least within 12 hours after the overdose, and in some embodiments within 6 hours after overdose. In some embodiments, the treatment for the APAP overdose in the subject comprises protection against liver injury and/or multi-organ system failure. In some embodiments, the treatment for the APAP overdose in the subject comprises a decrease in neutrophil infiltration in the subject. In some embodiments, the treatment for the APAP overdose in the subject comprises blocking the interaction between the HMGB1 protein and RAGE.

In some embodiments, a small molecule compound in accordance with the presently disclosed subject matter comprises a disaccharide structure unit as shown:

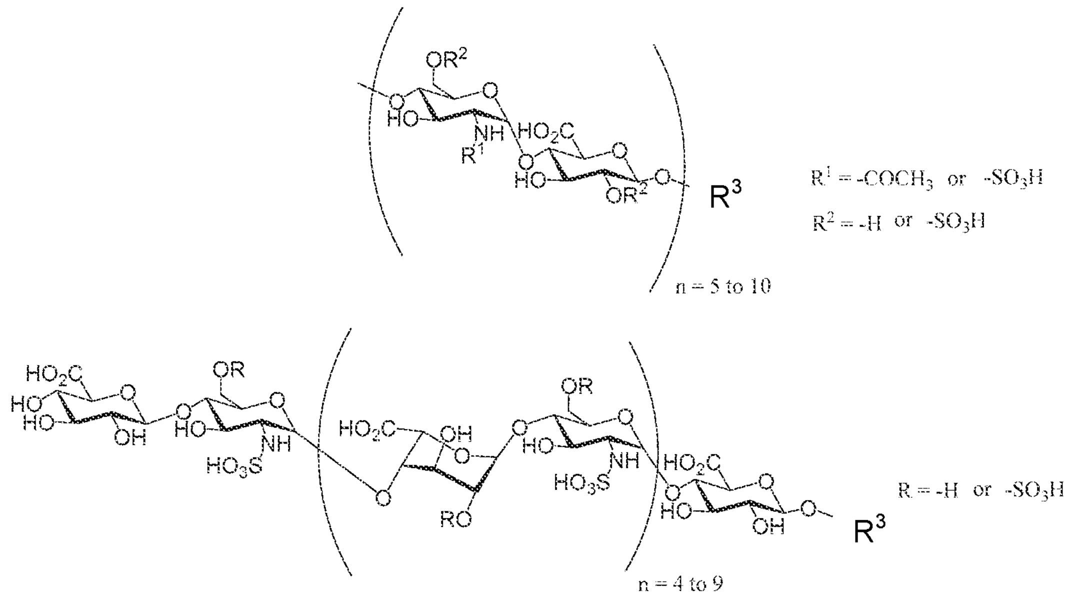


, wherein R = -H or $-SO_3H$.

By way of example and not limitation, this disaccharide structure unit can be found in non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin or O-desulfated heparin (ODSH). A composition employed in accordance with the presently disclosed methods can comprise a non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin or O-desulfated heparin (ODSH). In some embodiments, the composition protects against liver injury *in vivo, e.g.,* provides hepatoprotection *in vivo*. In some embodiments, the oligosaccharide molecule decreases neutrophil infiltration *in vivo*. In some embodiments, the subject suffers from liver injury. In some

embodiments, the subject in need of treatment is a subject suffering from an overdose of Paracetamol (acetaminophen or APAP).

In some embodiments, the oligosaccharide molecule comprises about 10 to about 20 saccharide units, or about 12 to about 18 saccharide units. In some
5 embodiments, the oligosaccharide molecule comprises about 18 saccharide units. In some embodiments, generic structures of heparan sulfate oligosaccharides displaying anti-inflammatory effects comprise:



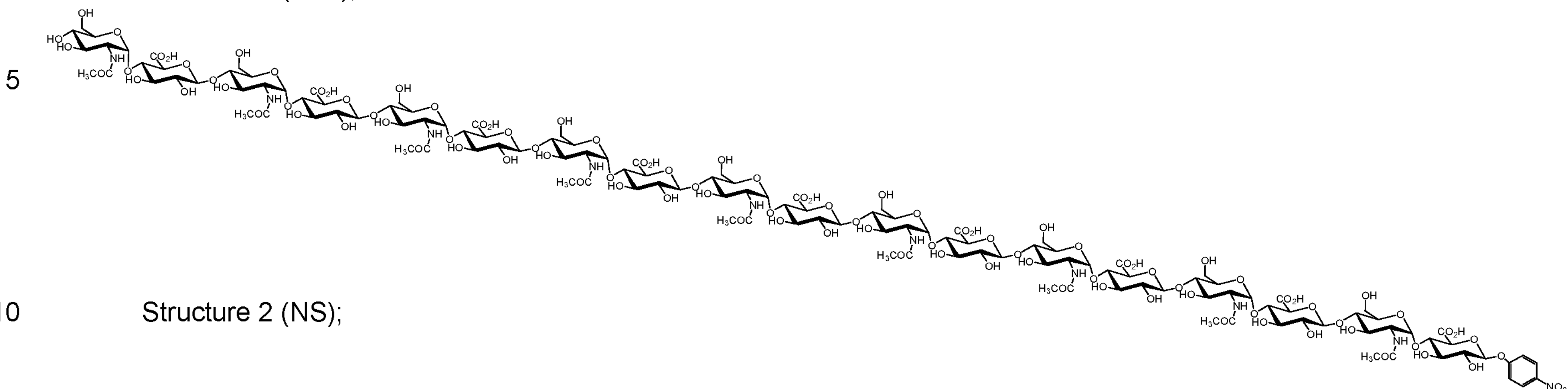
5

, wherein R^3 is H or a detectable tag. In some embodiments, the detectable tag comprises para-nitrophenyl.

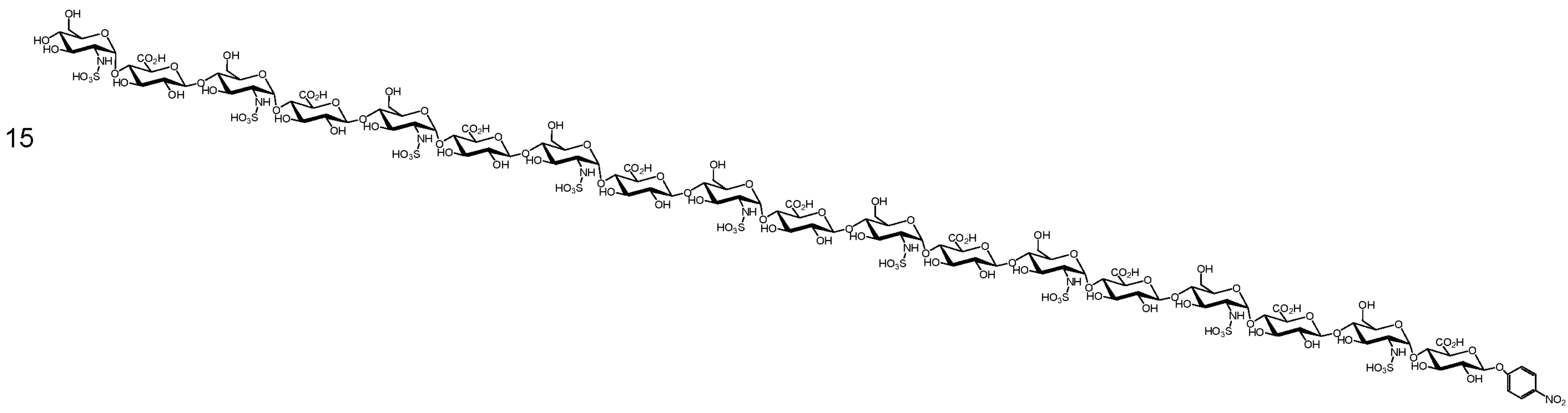
In some embodiments, the oligosaccharide molecule comprises one of the following structures:

Structure 1 (NAc);

5

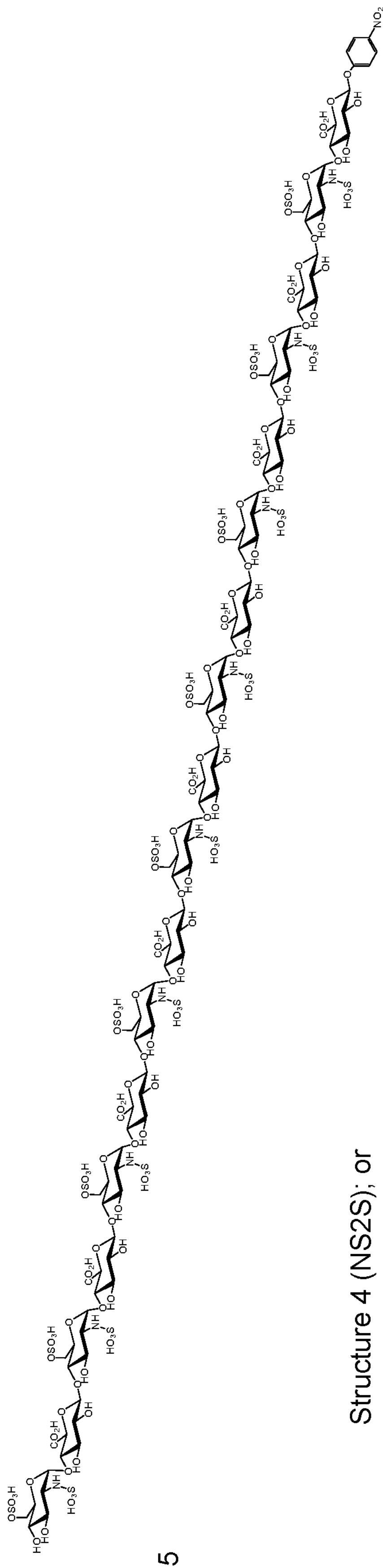


10 Structure 2 (NS);



15

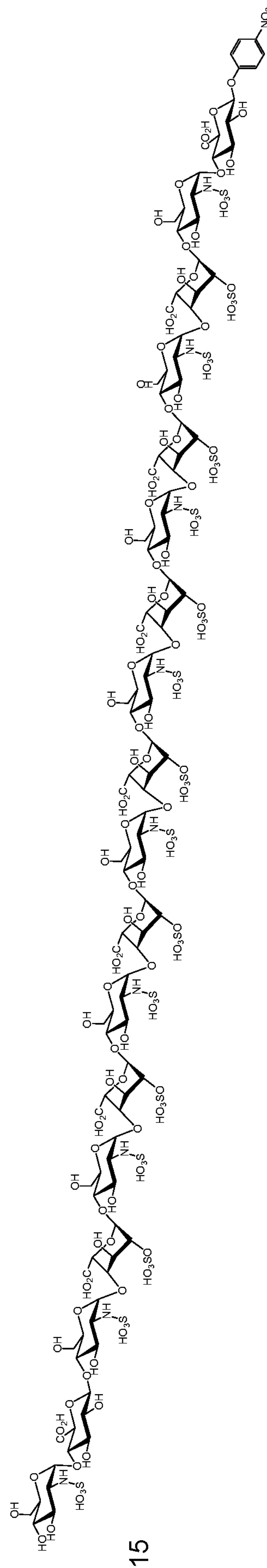
Structure 3 (NS6S);



5

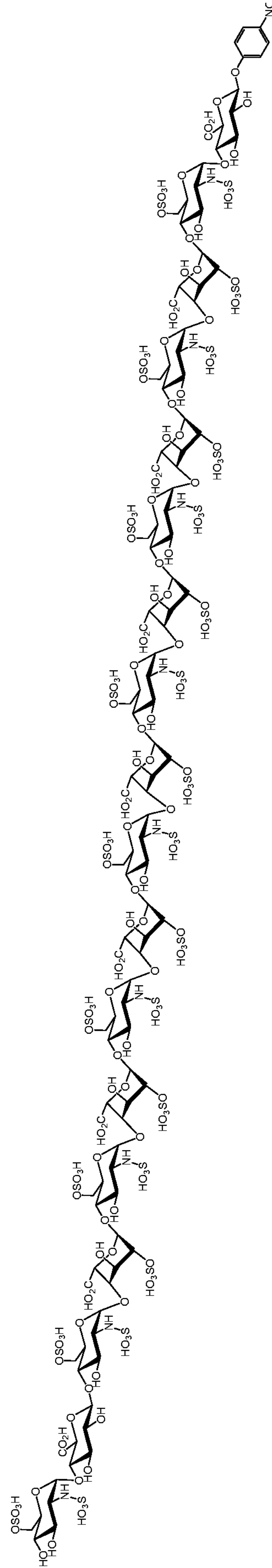
10

Structure 4 (NS2S); or



15

20



Structure 5 (NS6S2S).

DEFINITIONS

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the presently disclosed subject matter.

5 While the following terms are believed to be well understood by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the presently disclosed subject matter.

All technical and scientific terms used herein, unless otherwise defined below, are intended to have the same meaning as commonly understood by 10 one of ordinary skill in the art. References to techniques employed herein are intended to refer to the techniques as commonly understood in the art, including variations on those techniques or substitutions of equivalent techniques that would be apparent to one skilled in the art. While the following terms are believed to be well understood by one of ordinary skill in the art, the following 15 definitions are set forth to facilitate explanation of the presently disclosed subject matter.

The term “non-anticoagulant” and phrase “without anticoagulant” used in this application describes presently disclosed subject matter having less than or equal to about 50 units/mg anticoagulant activity where the anticoagulant 20 activity is attributed to inhibition of Factor Xa and/or Factor IIa.

In describing the presently disclosed subject matter, it will be understood that a number of techniques and steps are disclosed. Each of these has individual benefit and each can also be used in conjunction with one or more, or in some cases all, of the other disclosed techniques.

25 Accordingly, for the sake of clarity, this description will refrain from repeating every possible combination of the individual steps in an unnecessary fashion. Nevertheless, the specification and claims should be read with the understanding that such combinations are entirely within the scope of the invention and the claims.

30 Following long-standing patent law convention, the terms “a”, “an”, and “the” refer to “one or more” when used in this application, including the claims.

Thus, for example, reference to "a unit cell" includes a plurality of such unit cells, and so forth.

Unless otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about". Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently disclosed subject matter.

As used herein, the term "about," when referring to a value or to an amount of a composition, mass, weight, temperature, time, volume, concentration, percentage, *etc.*, is meant to encompass variations of in some embodiments ±20%, in some embodiments ±10%, in some embodiments ±5%, in some embodiments ±1%, in some embodiments ±0.5%, and in some embodiments ±0.1% from the specified amount, as such variations are appropriate to perform the disclosed methods or employ the disclosed compositions.

The term "comprising", which is synonymous with "including" "containing" or "characterized by" is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. "Comprising" is a term of art used in claim language which means that the named elements are essential, but other elements can be added and still form a construct within the scope of the claim.

As used herein, the phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. When the phrase "consists of" appears in a clause of the body of a claim, rather than immediately following the preamble, it limits only the element set forth in that clause; other elements are not excluded from the claim as a whole.

As used herein, the phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps, plus those that do not materially affect the basic and novel_characteristic(s) of the claimed subject matter.

With respect to the terms “comprising”, “consisting of”, and “consisting essentially of”, where one of these three terms is used herein, the presently disclosed and claimed subject matter can include the use of either of the other two terms.

5 As used herein, the term “and/or” when used in the context of a listing of entities, refers to the entities being present singly or in combination. Thus, for example, the phrase “A, B, C, and/or D” includes A, B, C, and D individually, but also includes any and all combinations and subcombinations of A, B, C, and D.

10 **EXAMPLES**

The following examples are included to further illustrate various embodiments of the presently disclosed subject matter. However, those of ordinary skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed 15 and still obtain a like or similar result without departing from the spirit and scope of the presently disclosed subject matter.

Example 1

Acetaminophen/paracetamol (APAP) overdose is the leading cause of 20 drug-induced acute liver failure (ALF) in the US and Europe. The progression of the disease is attributed to sterile inflammation induced by the release of high mobility group box 1 (HMGB1). A specific, effective and safe approach to neutralize the pro-inflammatory activity of HMGB1 is highly desirable. Disclosed herein in some embodiments is a HS octadecasaccharide (18-mer) 25 that displays potent hepatoprotection by targeting to HMGB1. Investigation of the role of endogenous syndecan-1 in response to APAP overdose substantiated the mechanism for 18-mer’s protection. The presently disclosed data suggest that 18-mer potentiates the host anti-inflammation effect mediated by syndecan-1. Finally, it is demonstrated that 18-mer administered six hours 30 after APAP overdose is still protective, and therefore offers a therapeutic advantage over *N*-acetyl cysteine for late-presenting patients. Synthetic HS

opens a new approach for the treatment of ALF and other HMGB1-involved inflammatory diseases

A series of novel sulfated heparan sulfate oligosaccharide compounds were synthesized. In particular, chemoenzymatic synthesis was employed to
5 obtain a pure HS/heparin-like oligosaccharide without anticoagulant activity One exemplary compound, termed 18-mer NS2S, was used for in depth *in vivo* studies in mice. 18-mer NS2S significantly decreased injury in the context of acetaminophen (APAP)-induced acute liver failure. Although it is not desired to be bound by any particular theory of operation, the mechanism of action of 18-
10 mer NS2S likely involves the HMGB1/RAGE. 18mer NS2S treatment is still effective 6 hours after APAP overdose in mice whereas the clinically used antidote, NAC, loses effectiveness at this time. Additional description of these observations can be found in the Examples that follow.

Chemoenzymatic synthesis was used to generate novel, pure
15 oligosaccharide compounds without anticoagulant activity. Representative synthetic routes are disclosed in the Examples that follow. A series of 18-mer compounds and one 12-mer, and one 6-mercompound were used *in vivo*. One exemplary 18-mer NS2S was shown to be protective against liver injury *in vivo*. In this example, 18-mer NS2S significantly increases the survival rate *in vivo*.
20 18-mer NS2S decreases neutrophil infiltration. RAGE knockout mice were observed to be insensitive to 18-mer NS2S's protective effects. This observation supports that delayed treatment after acetaminophen (APAP) overdose is possible.

25

Example 2

Materials and Methods

Study design

This study was designed to synthesize a HS octadecasaccharide (18-mer) and evaluate its anti-inflammatory effect in an APAP-induced liver failure
30 murine model. The chemoenzymatic synthesis of two HS 18-mers, including 18-mer NS2S (nonanticoagulant compound; referred to as 18-mer) and 18-mer

AXa (anticoagulant compound) is demonstrated herein. The structures of two 18-mers were confirmed using both nuclear magnetic resonance (NMR) and high resolution mass spectroscopy (MS). Demonstration of the binding between HMGB1 and biotinylated 18-mer and 18-mer AXa was achieved using avidin-agarose column followed by western analysis. HMGB1 binds to 18-mer and 18-mer AXa in nanomolar ranges as determined by surface plasmon resonance (SPR). The hepatoprotection effect of the synthesized 18-mers was evaluated in a well-established APAP-induced liver failure murine model. The liver damage was assessed by two methods, including the plasma ALT level and examination of hematoxylin and eosin (H&E) stained liver sections. The inflammation responses after APAP overdose were assessed by determining the neutrophil migration to the injury sites. Factor Xa (FXa) activity was used as a surrogate to assess the anticoagulant activity of 18-mers, 12-mer and 6-mer in both *in vitro* and *in vivo* experiments. The number of animals in the tested groups and controls groups as well as statistical analysis are presented in the figure legends. Anonymous patient ALF plasma samples were obtained from the Acute Liver Failure Study Group (ALFSG) biorepository (of the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland, United States of America) to determine the plasma level of shed syndecan-1. The analyses were stopped after analyzing 31 patient samples as a clear statistical difference between healthy control group (n = 11) and AFL patients was observed.

Expression of HS biosynthetic enzymes

A total of nine enzymes was used for the synthesis, including NST, C_5-epi, 2-OST, 6-OST-1, 6-OST-3, 3-OST-1, 3-OST-5, and pmHS2. All enzymes, with the exception of C_5-epi and 2-OST, were expressed in *E. coli* and purified by appropriate affinity chromatography as described previously (Renpeng et al., 2010; Xu et al., 2008). Recombinant C_5-epi and 2-OST were expressed in insect cells using the Bac-to-Bac baculovirus expression approach (from Invitrogen) to obtain high expression levels (*19*). Three enzyme co-factors, including 3'-phosphoadenosine 5'-phosphosulfate (PAPS), uridine 5'-

diphosphoglucuronic acid (UDP-GlcA), uridine 5'-diphospho N-trifluoroacetyl glucosamine (UDP-GlcNTFA), were all synthesized in-house using enzymatic approaches as described previously (Nam, 2017).

Synthesis of non-biotinylated 18-mer AXa

5 The synthesis of 18-mer AXa was initiated from 18-mer. Two enzymatic modifications were added, including 6-*O*-sulfotransferases (6-OST) modification and 3-*O*-sulfotransferase isoform 1 (3-OST-1) modification. In the 6-*O*-sulfation step, the 18-mer (0.5 mM) was incubated in a buffer containing MES (50 mM, pH 7.0), 6-OST-1 (50 μg ml^{-1}), 6-OST-3 (50 μg ml^{-1}) and PAPS (1.5 equiv. of 6-
10 hydroxyl group amount) at 37°C overnight in 60 ml. The product was then purified by Q-Sepharose column for the subsequent 3-*O*-sulfation step.

The 3-*O*-sulfation step was completed by 3-OST-1 enzyme. The 6-*O*-sulfated 18-mer (0.5 mM) was incubated in a solution containing MES (50 mM) buffer (pH 7.0), 3-OST-1 (20 μg ml^{-1}) and PAPS (0.675 mM) in 20 ml at 37°C for
15 2 hours. The product was purified by Q-Sepharose column. During the synthesis, the products were monitored by HPLC using a DEAE-NPR column (4.6 mm × 75 mm, from Tosoh).

The synthesis of non-biotinylated 6-mer and non-biotinylated 12-mer was completed using the enzymatic method, and was reported previously (Xu,
20 2017).

Conversion of nonbiotinylated oligosaccharides to biotinylated oligosaccharides

Four nonbiotinylated oligosaccharides, 6-mer, 12-mer, 18-mer and 18-mer AXa, were converted to biotinylated counterparts. 6-mer, 12-mer, 18-mer
25 and 18-mer AXa with a pNP tag (5-10 mg) and 0.5 mg Pd/C were dissolved in 20 mM NaOAc, pH 5.0 in a total volume of 4 ml. Reaction mixture was vacuumed and refilled with H_2 three times. The reaction was then incubated at room temperature for 4 h. After that, it was filtered to remove charcoal. The filtered solution was adjusted to pH 8.5 using 500 mM Na_2HPO_4. Succinimidyl
30 6-azidohexanoate (20 molar equivalent of starting oligosaccharides) was added and incubated at 37 °C overnight. Reaction was purified by DEAE-HPLC

column to generate azido tagged oligosaccharides. PBS (pH7.4) buffer was bubbled with N_2 for 5 min to prepare the sample solution of 0.1M $CuSO_4$, 0.1M Tris(3-hydroxypropyl-triazolylmethyl)amine (THPTA) (Sigma, Burlington, Massachusetts, United States of America), 0.15M sodium ascorbate, 0.01M
5 azido tagged oligosaccharides and 0.02 M biotin-PEG_4-alkyne (Sigma). The mixture of 400 µl THPTA and 80 µl $CuSO_4$ was vortexed, then 160 µl sodium ascorbate, 200 µl azido tagged oligomers and 200 µl biotin-PEG4-alkyne was added and bubbled with N_2 for 2 min, then incubated at 37 °C overnight. The reaction was purified by DEAE -HPLC column to generate biotinylated products.
10 The reactions were monitored using HPLC and MS.

HPLC analysis

Both DEAE-NPR HPLC and polyamine-based anion exchange (PAMN)-HPLC were used to analyze the purity of the products. The elution conditions for the HPLC analysis were described elsewhere (Renpeng et al., 2010).
15 Briefly, for DEAE-HPLC method, the column TSK gel DNA-NPR (4.6 × 75 mm, from Tosoh Bioscience) was eluted with a linear gradient of NaCl in 20 mM sodium acetate buffer (pH 5.0) from 0 to 1M for 60 min at a flow rate of 0.4 ml min^{-1}. As for PAMN-HPLC, the column (Polyamine II-HPLC, 4.6 × 250 mm, from YMC) was eluted with a linear gradient of KH_2PO_4 from 0 to 1M for 40 min
20 then remained at 1M for 30 min at a flow rate of 0.5 ml min^{-1}.

MS Analysis of oligosaccharides

The low-resolution analyses were performed at a Thermo LCQ-Deca. Oligosaccharides were directly diluted in 200:l of mixture of MeOH/H_2O (9:1, vol/vol). A syringe pump (Harvard Apparatus) was used to introduce the
25 sample by direct infusion (50 µl min^{-1}). Experiments were carried out in negative ionization mode. Synthetic nonsulfated oligosaccharides were diluted in 200 µl of H_2O with the electrospray source set to 5 kV and 275°C. Sulfated oligosaccharides were diluted in 200 µl of 10 mM ammonium bicarbonate with the electrospray source set to 3 kV and 150°C. The automatic gain control was
30 set to 1×10^7 for full scan MS. The MS data were acquired and processed using Xcalibur 1.3.

High resolution ESI-MS analysis was conducted on Thermo LTQ XL Orbitrap (Breman, Germany) under the following conditions. A Luna hydrophilic liquid interaction chromatography (HILIC) column (2.0 × 50 mm^2, 200 Å, Phenomenex, Torrance, California, United States of America) was used to separate the oligosaccharide mixture. Mobile phase A was 5 mM ammonium acetate prepared with high performance liquid chromatography (HPLC) grade water. Mobile phase B was 5 mM ammonium acetate prepared in 98% HPLC grade acetonitrile with 2% of HPLC grade water. After injection of 5.0 µl 12-mer mixture (1.0 µg µl^{-1}) through an Agilent 1200 autosampler, HPLC binary pump was used to deliver the gradient from 3% A to 80% A over 10 min at a flow rate of 250 µl min^{-1}. The LC column was directly connected online to the standard electrospray ionization source of LTQ-Orbitrap XL Fourier transform (FT) mass spectrometer (MS) (Thermo Fisher Scientific, San-Jose, California, United States of America). The optimized parameters, used to prevent in-source fragmentation, included a spray voltage of 4.2 kV, a capillary voltage of −40 V, a tube lens voltage of −50 V, a capillary temperature of 275° C, a sheath flow rate of 40, and an auxiliary gas flow rate of 20. External calibration of mass spectra routinely produced a mass accuracy of less than 3 ppm. All FT mass spectra were acquired at a resolution of 60,000 with 200−2000 Da mass range.

LC/MS disaccharide analysis of human plasma samples

Pooled individual plasma samples of healthy control subjects and APAP-ALF patients were digested in 300 µl digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride adjusted to pH 7.0). Recombinant heparin lyase I, II, III (pH optima 7.0−7.5) and recombinant chondroitin lyase ABC (10 mU each, pH optimum 7.4) were added to each sample and mixed well. The samples were all placed in a water bath at 37°C for 12 h, after which enzymatic digestion was terminated by removing the enzymes by centrifugation. The filter unit was washed twice with 250 µl distilled water and the filtrates, containing the disaccharide products, were dried by vacuum centrifuge. The dried samples were AMAC-labeled by adding 10 µl of 0.1 M 2-aminoacridone (AMAC) in DMSO/acetic acid (17/3, vol/vol) incubating at room

temperature for 10 min, followed by adding 10 μl of 1 M aqueous sodium cyanoborohydride and incubating for 1 h at 45°C. A mixture containing all 17-disaccharide standards purchased from Iduron (UK) prepared at 0.5 ng μl^{-1} was similarly AMAC-labeled and used for each run as an external standard. After
5 the AMAC-labeling reaction, the samples were centrifuged, and each supernatant was recovered. LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 ECC18 (2.7 μm, 3.0 × 50 mm) column. Mobile phase A was 50 mM ammonium acetate aqueous solution, and the mobile phase B was methanol. The mobile phase passed through the column at
10 a flow rate of 300 μl min^{-1}. The gradient was 0-10 min, 5-45 % B; 10-10.2 min, 45-100 % B; 10.2-14 min, 100 % B; 14-22 min, 100-5% B. Injection volume is 5 μl. A triple quadrupole mass spectrometry system equipped with an ESI source (Thermo Fisher Scientific) was used a detector. The online MS analysis was at the Multiple Reaction Monitoring (MRM) mode. MS parameters: negative
15 ionization mode with a spray voltage of 3000 V, a vaporizer temperature of 300°C, and a capillary temperature of 270°C.

NMR analysis

The NMR spectra of 18-mer and 18-mer AXa were obtained on Bruker 800 MHz standard-bore NMR spectrometer with TopSpin 2.1.6 software
20 (Bruker, Billerica, Massachusetts, United States of America). Samples (3.0 to 6.0 mg) were each dissolved in 0.4 ml of 99.9% D_2O centrifuged at 5000 x *g* for 1 min and lyophilized. The process was repeated twice and the final sample was dissolved in 0.45 mL of 99.99% D_2O. 1H spectroscopy, ^1H-^1H correlated spectroscopy (COSY), ^1H-^{13}C heteronuclear single quantum coherence
25 spectroscopy (HSQC), ^1H-^1H total correlation spectroscopy (TOCSY) and ^1H-^1H nuclear Overhauser effect spectroscopy (NOESY) experiments were all carried out at 298 K.

Expression of HMGB1

The complete open reading frame of human HMGB1 was cloned into
30 pcDNA3.1(+)-C-6His (GenScript, Piscataway, New Jersey, United States of America). Transfection was performed using FectoPRO transfection reagent

(Polyplus transfection). Recombinant HMGB1-his was produced in 293-freestyle cells (Thermo Fisher Scientific) at 31°C. Purification of HMGB1-his from cell lysate was carried out using Ni Sepharose™ 6 Fast Flow gel (GE Healthcare, Little Chalfont, England) followed by Superdex200 gel filtration
5 chromatography. After purification, HMGB1-his was >99% pure as determined by SDS-PAGE followed by silver staining. Endotoxin removal was performed using Detoxi-Gel (Thermo Fisher Scientific) and the final endotoxin level was < 0.1 EU/μg protein by a Chromogenic LAL Endotoxin Assay (GenScript, Piscataway, New Jersey, United States of America). The recombinant HMGB1-
10 his had both cytokine and chemokine activities, which were assessed by a cell signaling assay and an air pouch assay (described in detail below), respectively.

Removal of endotoxin from HS oligosaccharides

Endotoxin was removed from HS oligosaccharides by using a 50 ml
15 centrifugal filter unit (Amicon Ultra-15, Ultracel-100k; Merck Millipore, Darmstadt, Germany) at 4,000 rpm for 30 min. The process was repeated three times by refilling the filter insert with 1 ml of endotoxin-free water each time. The filtered solution was collected. The level of endotoxin was measured using the Limulus Amebocyte Lysate (LAL) kit (Associated of Cape Cod Inc.). The LAL
20 test was performed by adding 100 μl of reconstituted Pyrotell (sensitivity 0.03 endotoxin units ml^{-1}) to 100 μl of 0.1mg ml^{-1} HS oligosaccharides in sterile saline which is the concentration used for animal injections. The Pyrotell and HS oligosaccharides were added to a 10 mm x 75 mm depyrogenated glass reaction tube (Associates of Cape Cod Inc.) and incubated at 37ºC for 1 h. At
25 the end of the incubation time, the tubes were inverted. If a gel clot forms, the sample is positive for endotoxin. If the sample remains in solution, the negative result implies that any endotoxin in the sample is below the sensitively of Pyrotell. All HS oligosaccharides solutions tested negative.

Histology/Immunohistochemistry

30 Liver tissues were fixed in 10% neutral buffered formalin for 24 h at room temperature, paraffin-embedded, and sectioned. Liver sections (4 μm) were

stained with hematoxylin-eosin (H&E) or immunostained with monoclonal antibodies anti-neutrophil (Abcam, Ab 2557, NIMP-R14) (Abcam, Cambridge, United Kingdom) or anti-syndecan-1 (StemCell Technologies, 60035, clone 281.2) (StemCell Technologies, Vancouver, Canada) and goat anti-rat biotinylated secondary antibodies (Abcam). For fibrin(ogen) staining, polyclonal anti-fibrin(ogen) (Dako of Agilent, Santa Clara, California, United States of America) was used followed by goat anti-rabbit biotinylated secondary antibody (Sigma). Embedding, sectioning and H&E staining were performed at the Animal Histopathology and Laboratory Medicine Core Facility at UNC Chapel Hill. H&E analyses were performed by the Translational Pathology Laboratory Core Facility at UNC Chapel Hill using Aperio ImageScope Software (Leica Biosystems, Concord, Canada). IHC images were captured using an HD camera attached to a bright field microscope (Leica DM 1000 LED, Leica Microsystems Inc., IL, USA) and were processed using ImageJ. For neutrophil quantitation, five 100x images were randomly selected for each sample and the average neutrophils/field were reported.

Mouse models of inflammation: peritonitis and air pouch

Two models were used to study *in vivo* neutrophil migration: peritonitis and an air pouch inflammation model. For the peritonitis model, 30 mg of liver lysate was injected into the peritoneal cavity of the mouse in the absence or presence of 20 µg of 18-mer. After 20 h, the mice were euthanized via inhalation of isofluorane and the peritoneal cavity was washed with 10 ml of ice cold PBS. The peritoneal lavage was used to determine neutrophil migration using flow cytometry.

In the air pouch technique, 3 ml of sterile air is injected under the skin on the back of the mouse. Three days later, the pouch is refilled with sterile air. On the sixth day, 5 µg of recombinant HMGB1 in the absence or presence of 22.3 µg of 18-mer were injected into the air pouch. Control mice were injected with 2 mg ml^{-1} BSA in PBS. After 4 hours, mice were euthanized by isofluorane inhalation and the air pouch was washed with PBS only. The lavage was used to determine neutrophil migration using flow cytometry.

Flow cytometry

Peritoneal and air pouch lavages were stained with fluorescently labeled antibodies against Ly-6G/Ly-6C (monoclonal anti-mouse RB6-8C5, PE-Cy7, eBioscience) and Cd11b (monoclonal anti-mouse M1/70, FITC, eBioscience).
5 Samples were run on a Stratedigm S1000Exi flow cytometer.

Immunoblot

Liver lysate was prepared by snap freezing tissue in liquid nitrogen at the time of sacrifice. The tissue was mechanically homogenized in buffer containing 200 mM MES, 500 mM phosphate, and 1 mM EDTA at pH 6 followed by three
10 rounds of freeze thawing. The lysed sample were centrifuged at 10000 x g for 15 min at 4°C. Biotinylated HS oligosaccharides (final concentration 15 μM) were mixed with 100 μl of fresh liver lysate (~12.5 mg) and incubated overnight at 4 °C. Pierce High Capacity Streptavidin Agarose (Thermo Fisher Scientific) was used to isolated biotinylated HS oligosaccharide bound complexes. After
15 washing with 50 mM MES, 50 mM NaCl pH 6, samples were eluted with LDS buffer. Eluted samples were separated using NuPAGE 4-12% Bis-tris protein gels and assayed using anti-HMGB1 antibody (Abcam, rabbit monoclonal EPR3507) and goat anti-rabbit HRP (Abcam).

Preparation of ^{35}S-labeled HS and binding studies to recombinant
20 ***HMGB1***

[^{35}S]HS was prepared using [^{35}S]PAPS (4 × 10^7 cpm) and *N*-sulfotransferase (NST) (100 μg ml^{-1}), and HS from bovine kidney in 50 mM MES in a total volume of 2 ml. The [^{35}S]HS (1.35 x 10^5 cpm) was incubated with 1 μg of recombinant HMGB1 in 50 mM MES, 70 mM NaCl, 10 mM
25 imidazole at pH 6 and incubated at room temperature for 30 min. 5 μg chromatin immunoprecipitation grade anti-HMGB1 (Abcam) was added and incubated for 1 h at 4 °C. Reaction mixtures were purified using Dynabeads Protein A (Thermo) and eluted in a buffer containing 25 mM Tris, 150 mM NaCl at pH 7.5. The eluted samples were measured for [^{35}S] counts using a liquid
30 scintillation analyzer (Packard; GMI, Ramsey, Minnesota, United States of

America). [^{35}S]HS in the absence of recombinant HMGB1 serves as a negative control.

Surface Plasmon Resonance

The biotinylated HS 18-mers (18-mer and 18-mer AXa) were immobilized to streptavidin (SA) sensor chips (BIAcore, GE Healthcare, Uppsala, Sweden) based on the manufacturer's protocol. In brief, 20 µl of biotinylated HS oligosaccharides were injected over flow cells 2,3, and 4 (FC2, FC3, and FC4) of the SA chip at a flow rate of 10 µl min^{-1}. The successful immobilization of biotinylated oligosaccharides were confirmed by the observation of a 563, 823, 505, 553 and 176 resonance unit (RU) increase, respectively on the SA chip. The control flow cell (FC1) was prepared by 1 min injection with saturated biotin.

Recombinant HMGB1 was diluted in 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20 at pH 7.4. Recombinant HMGB1 at concentrations of 1000, 500, 250, 125 and 63 nM were injected at a flow rate of 30 µl min^{-1}. At the end of the recombinant HMGB1 injection, the same buffer was flowed over the SA surface to facilitate dissociation. After a 3 min dissociation time, the SA surface was regenerated by injecting 30 µl of 2 M NaCl. The response was monitored as a function of time (sensogram) at 25 ºC. The sensograms of various recombinant HMGB1 concentrations were globally fitted with 1:1 Langmuir model. SPR measurements were performed on a BIAcore 3000 operated using BIAcore 3000 control and BIAevaluation software (version 4.0.1)

APAP-ALF Patient Plasma

APAP-ALF patient plasmas, containing 1.8 mg ml^{-1} EDTA, were obtained from the Acute Liver Failure Study Group (ALFSG) biorepository (of the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland, United States of America). Details on the study design and collection methods are described previously (*35*). Briefly, starting in 1998, adult patients who met the inclusion and exclusion criteria were enrolled in the ALFSG Registry. Plasma samples were obtained on admission to the Registry. In this

study, only the plasma from APAP-overdosed patients was analyzed. Clinical data including the estimated quantity of APAP ingested, estimated time from ingestion to hospitalization, intentionality of overdose and patient demographics (age, gender, race, comorbidities, etc.), were not revealed for this study.
5 Plasma syndecan-1 levels were measured using an ELISA kit (Human syndecan-1 ELISA, CellSciences, Newburyport Massachusetts, United States of America) according to the manufacturer's protocol. Plasma HMGB1 levels were measured using an HMGB1 ELISA according the manufacturer's protocol. Plasma ALT levels were measured using ALT Infinity Reagent.

10 ***Determination of the in vitro and ex vivo anti-FXa activity***

Assays were based on a previously published method (*19*). Briefly, human FXa (Enzyme Research Laboratories, South Bend, Indiana, United States of America) was diluted to 50 U ml^{-1} with PBS. The chromogenic substrate S-2765 (Diapharma, West Chester, Ohio, United States of America)
15 was diluted to 1 mg ml^{-1} in water. For *in vitro* studies, Fondaparinux and HS oligosaccharides (18-, 12-, 6-, and 18-mer AXa) were dissolved in PBS at various concentrations (11 – 131 nM). 16 µl of sample was incubated with 60 µl of 35 µl ml^{-1} antithrombin (Cutter Biologics) for 2 min at room temperature. Next, 100 µl of FXa was added and incubated for 4 min at room temperature.
20 30 µl of S-2765 substrate was added and the absorbance of the reaction mixture was measured at 405 nm continuously for 5 min. PBS serves as a control sample. The maximum slope for each sample was convert to percent FXa activity by dividing by the maximum slope for the control sample.

For *ex vivo* studies, mouse plasma collected 24 h post-APAP overdose
25 in mice treated with APAP only, 18-mer, and 18-mer AXa were used and the same protocol was following.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical significance between experimental and control groups were analyzed by two-tailed unpaired Student *t*
30 test, between multiple groups by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison's test, and Kaplan-Meier survival curves by log-

rank test using GraphPad Prism software (version 7.03; GraphPad Software, Inc., LaJolla, California, United States of America).

Example 3

5

Synthesis of HS 18-mer

The synthesis of 18-mer was completed according to the chemoenzymatic method published previously (*17, 31*). Briefly, heparosan synthase-2 (PmHS2) from *Pasteurella multocida* was used to elongate the monosaccharide, GlcA-pNP, to appropriate sized backbones. The backbone
10 was then subjected to the modification of *N*-sulfotransferase (NST), C_5-epimerase (C_5-epi), and 2-*O*-sulfotransferase (2-OST). There were five major steps involved in the overall synthesis, including Step a (elongation step to add GlcNTFA), Step b (elongation step to add GlcA), Step c (detrifluoroacetylation using LiOH), step d (*N*-sulfation step), step e (2-*O*-sulfation/epimerization)
15 (Figs.1A-1G).

Step **a** was to elongate the oligosaccharide backbone to the desired size, involving the addition of GlcNTFA residue. Briefly, GlcA-pNP (3.2 mM) was dissolved in buffer containing Tris (25 mM, pH 7.2), $MnCl_2$ (5 mM), pmHS2 (60 μg ml^{-1}) and UDP-GlcNTFA (4.5 mM), then incubated at 30°C overnight. The
20 total reaction volume was 4 L. A C_{18}-column (3 × 15 cm, or 120 g, Biotage) was used for purification with gradient elution method (0–100% methanol in H_2O, 0.1% trifluoroacetic acid, 5 ml min^{-1}).

Step **b** was to elongate with a GlcA residue. A disaccharide (3.2 mM) was dissolved in a buffer containing Tris (25 mM, pH 7.2), $MnCl_2$ (5 mM),
25 pmHS2 (30 μg ml^{-1}) and UDP-GlcA (1.5 mM), then incubated at 30°C overnight. The total reaction volume was 3 l. A C_{18}-column (3 × 15 cm, or 120 g, Biotage) was used for purification as described above.

Step **c** was to convert a GlcNTFA residue to a $GlcNH_2$ residue under alkaline conditions. The detrifluoroacetylation of oligosaccharide (13 mM) was
30 conducted in 0.1 M LiOH under ice bath for 0.5 h. The products were monitored by electrospray ionization mass spectrometry (ESI-MS). After the reaction was

completed, the pH was immediately adjusted to 7.0 using hydrochloric acid (1 M).

Step **d** was to convert a $GlcNH_2$ residue to a GlcNS residue using NST enzyme. In one example, the detrifluoroacetylated tetrasaccharide (1.3 mM, $GlcNH_2$-GlcA -$GlcNH_2$-GlcA-pNP) incubated with *N*-sulfotransferase (32 μg ml^{-1}) and PAPS (1.5 equiv. of free amino group amount) in a solution containing 2-(*N*-morpholino) ethanesulfonic acid (MES, 50 mM) pH 7.0 at 37°C overnight in a reaction volume of 5.6 L.

Step **e** was to convert an internal GlcA residue to an IdoA2S residue and involves both C_5-epimerase and 2-*O*-sulfotransferase (2-OST). For example, the oligosaccharide GlcNTFA-GlcA-GlcNS-GlcA-GlcNS-GlcA-pNP (1.2 mM) was incubated in a solution containing Tris (25 mM) buffer (pH 7.5) and semi-purified C_5-epimerase (3 μg ml^{-1}), 2-OST (6.5 μg ml^{-1}) and PAPS (1.8 mM) at 37°C overnight in a reaction volume of 4.9 L.

The sulfated products were purified using Q-Sepharose fast flow column (from GE Healthcare Life Science) and eluted with a linear gradient 0-100% 2 M NaCl in 20 mM NaOAc-HOAc, pH 5.0 in 3 h. Different sizes of Q-Sepharose columns and salt gradients were chosen based on the binding affinity of the product and reaction scale.

At every elongation synthesis step, the products were monitored by Shimadzu HPLC equipped with a polyamine II column (4.6 mm × 250 mm, from YMC). The structures of the intermediates from each step were characterized by electrospray ionization mass spectrometry (ESI-MS).

Example 4

Mouse model of APAP liver injury

All animal experiments were approved by the Institutional Animal Care and Use Committee of University of North Carolina at Chapel Hill (Chapel Hill, North Carolina, United States of America) and the IACUC of the University at Buffalo (Buffalo, New York, United States of America). *Ager*$^{-/-}$ mice were originally gifted by Dr. Angelika Bierhaus (University of Heidelberg, Heidelberg,

Germany) (*32*). C57BL/6J or *Ager*$^{-/-}$ mice were fasted overnight (12-15 h) to deplete glutathione stores before APAP (Sigma) administration. Fresh APAP was dissolved in warm (~50 ºC) sterile 0.9% sodium chloride solution (sterile saline), cooled to 37 ºC, and injected intraperitoneal at 400 or 600 mg kg^{-1}. In some experiments, mice were injected subcutaneously at 30 min post-APAP with 9.5 µM HS oligosaccharide in ~200 µl sterile saline and again at 12 hours post-APAP with 4.75 µM HS oligosaccharide in ~100 µl or equivalent volumes of sterile saline.

To compare the 18-mer's effectiveness to *N*-acetyl cysteine (NAC), 300 mg kg^{-1} NAC at pH 7.5 in sterile saline was injected intraperitoneal 30 min post-APAP. In a separate experiment, 300 mg kg^{-1} NAC or 0.34 mg/kg 18-mer were administered 6 h post-APAP, then at 18 h post-APAP, 0.17 mg kg^{-1} 18-mer was administered to the 18-mer treated group. At 24 h post-APAP, mice were euthanized and blood and liver tissue were collected.

In the survival study (600 mg kg^{-1} APAP), mice were injected 30 min post-APAP with 0.4 mg kg^{-1} 18-mer followed by repeat injections every 12 h or equivalent volumes of sterile saline for 96 h.

Example 5

Evaluation of APAP-induced liver injury

Plasma ALT was measured using the ALT Infinity reagent (Thermo Fisher Scientific) following the manufacturer's instructions. Plasma TNF-α was measured using Mouse TNF-α DuoSet Kit (R&D Systems, Minneapolis, Minnesota, United States of America) according to the manufacturer's instructions. Plasma HMGB1 levels were determined using HMGB1 ELISA Kit (Tecan US of Tecan Trading AG, Mannedorf, Switzerland) according to the manufacturer's instructions. Plasma syndecan-1 levels were determined using Mouse Syndecan-1 ELISA (CellSciences) according to manufacturer's instructions. Hepatic GSH levels were determined using Glutathione Assay Kit (Cayman Chemicals, Ann Arbor, Michigan, United States of America) according to the manufacturer's instructions.

Example 6

18-mers significantly decrease liver injury 36 hours after APAP overdose

Similar to the above examples, C57Bl/6J male mice, at about 10 weeks of age and about 25 grams of body weight, were overdoses with acetaminophen (APAP; 400mg/kg) via intraperitoneal injection at time 0 hr.

Mice received repeat subcutaneous injections of 18-mers 30 minutes, 12 hours and 24 hours after APAP. The mice were sacrificed via cervical dislocation, blood was drawn from the vena cava, and liver tissue was harvested for histology. Alanine aminotransferase (ALT) was used as a biomarker for liver injury. Tumor necrosis factor-α (TNF-α) was used as a marker for inflammation. Hematoxylin and Eosin staining (H&E) was used as the histological staining technique to quantitate regions of tissue necrosis.

The resulting data showed that all 18-mer compounds (Table 1) decreased plasma ALT levels. Although all 18-mers tested were effective, the NS2S sulfation pattern provided the most consistent decrease in ALT (largest difference of means compared to APAP control), results of which are presented in Table 1.

Table 1.

Comparison	***N APAP; N=19***	***Difference of Means***	***P***
18mer NS2S	*17*	*2928*	*<0.001*
18mer NS6S	*17*	*2261*	*<0.001*
18mer NS6S2S	*14*	*1779*	*<0.001*
18mer NS	*12*	*1682*	*<0.001*
18mer NAc	*7*	*1356*	*0.014*

Example 7

Results and Discussion

HS isolated from natural sources is highly complex mixtures with different polysaccharide chain lengths and sulfation patterns. Lack of structurally homogeneous HS oligosaccharides, especially long HS oligosaccharides that display similar functions of full length HS, hampers the efforts to exploit the interests of HS as a therapeutic agent (Liu, 2014). We have recently developed a chemoenzymatic method to synthesize HS oligosaccharides with excellent efficiency (Xu, 2011, Xu 2014, Xu, 2017). Here, HS oligosaccharides, including 323 mg of an HS octadecasaccharide (18-mer), were synthesized (Figs. 1A-1G and Fig. 2). This represents one of the longest HS oligosaccharides synthesized to date. The structure of 18-mer was confirmed by high resolution mass spectrometry and NMR. The purity was determined to be > 98% by high resolution anion exchange high-performance liquid chromatography.

The hepatoprotective effects of 18-mer were examined in an APAP-induced ALF murine model. Mice treated with 18-mer after APAP overdose had significantly healthier livers than APAP control mice as indicated by a higher population of normal hepatocytes (Figs. 3A) and a lower plasma level of alanine aminotransferase (ALT), a biomarker of liver damage (Fig. 3C). 18-mer treatment showed reduced neutrophil infiltration into the liver and decreased plasma levels of tissue necrosis factor α (TNF-α) (Figs. 3B and 6A), suggesting attenuation of local and systemic inflammation. 18-mer also decreased mortality following a lethal APAP overdose (600 mg kg^{-1}), resulting in 90% survival at 96 hours compared to 42% survival in the control group (Fig 3D). Hepatic glutathione (GSH) levels in APAP control and 18-mer treated mice were essentially the same during the course of the studies (Fig. 6B). GSH levels decrease as NAPQI forms (Tacke, 2015; Nam, 2017), therefore the data suggest that 18-mer does not affect the metabolic conversion of APAP to the cytotoxic intermediate, NAPQI. These results demonstrate that 18-mer

ameliorates liver injury by reducing the inflammatory responses rather than by affecting APAP metabolism.

Two lines of evidence suggest that 18-mer targets HMGB1 to reduce inflammation after APAP overdose. First, we discovered that 18-mer diminishes the HMGB1-mediated neutrophil infiltration in two *in vivo* models. In an air pouch model, injection of recombinant HMGB1 induced extensive neutrophil infiltration (Fig. 4A), an effect that was significantly reduced by co-administration of 18-mer. 18-mer also reduced neutrophil infiltration in a peritonitis model induced by liver lysate (Fig. 6C), a process known to be mediated by HMGB1(*4*). Second, RAGE knockout mice, or *Ager*$^{-/-}$ mice, were used to demonstrate that 18-mer targets the HMGB1/RAGE axis. Because the interaction of HMGB1 and RAGE is essential for the pro-inflammatory response in APAP overdose (Huebener, 2015), the hepatoprotective effect of 18-mer is expected to be dependent on the presence of RAGE. Hepatic neutrophil infiltration was increased, and the increase was significantly greater in wild type (WT) than *Ager*$^{-/-}$ mice after APAP overdose (Fig. 4B). 18-mer treated *Ager*$^{-/-}$ mice failed to show a decrease in neutrophil infiltration, while WT mice responded to 18-mer treatment (Fig. 4B). Furthermore, 18-mer treatment in *Ager*$^{-/-}$ mice was incapable of lowering the ALT level (Fig. 6D). This data suggest that 18-mer lost its protective effect in the absence of RAGE.

Structure-activity relationship studies were conducted to examine binding to HMGB1 and protection in the APAP model using different forms of HS. Four biotinylated oligosaccharides were synthesized, including 6-, 12-, and two 18-mers (Fig. 4E) (for structures and characterization see Fig. 7). Among these, 6-, 12- and 18-mer varied only in chain length but not in sulfation pattern. These oligosaccharides are non-anticoagulant because they lack anti-factor Xa activity. 18-mer AXa is a highly sulfated anticoagulant octadecasaccharide that possesses potent anti-factor Xa activity (Figs. 8A and 8B). The 18-mer and 18-mer AXa, but not the 6-mer or 12-mer, were observed to bind HMGB1 from mouse liver lysate, suggesting that there is a minimum chain length requirement for binding (Fig. 4C). The HMGB1-binding constants (K_D) were determined to be

186 nM (for 18-mer) and 65 nM (18-mer AXa) by surface plasmon resonance, yet only the 18-mer displayed the hepatoprotective effects as measured by ALT (Fig. 4D) and by neutrophil infiltration (Fig. 8C). It is apparent that the lack of hepatoprotection from the 6- and 12-mer correlates with the inability to bind
5 HMGB1. However, the 18-mer AXa, while capable of binding HMGB1, also lacked hepatoprotection. This may be due to its anticoagulant activity since anticoagulant unfractionated heparin also lacked hepatoprotection after APAP-overdose (Fig. 8D). Kopec and colleagues have reported that fibrin is required to activate liver repair after APAP overdose (*20*). Administering 18-mer AXa
10 reduces fibrin formation, and thus, results in loss of hepatoprotection.

To further understand the protective role of 18-mer, the role of syndecan-1, a major HS proteoglycan present on hepatocytes, was investigated in response to APAP overdose. Syndecan-1 comprises a core protein attached with HS chains and is shed from the cell surface by matrix metalloproteases in
15 pathological conditions (Park, 2000). Higher but fluctuating levels of plasma syndecan-1 were observed over a period of 24 hours after APAP overdose in mice (Fig. 5A). Concurrently, plasma levels of HMGB1 (Fig. 5A), ALT and hepatic neutrophil infiltration increased over time (Figs. 9A and 9B). Loss of cell surface syndecan-1 was confirmed by immunostaining liver section. Using ^{35}S-
20 labeled HS from bovine kidney as a surrogate molecule for the HS chains on shed syndecan-1, we demonstrated that HMGB1 binds to HS (Fig. 5B), which is consistent with a previous report using HS from different sources (*9*). These results suggest that shed syndecan-1 binds to HMGB1 through its HS chains. A significant increase in plasma syndecan-1 levels was also observed from
25 patients with APAP-induced ALF (Fig. 5C). Notably, shed syndecan-1 levels in APAP-ALF patients were about 200-fold higher than in APAP overdosed mice as measured by core protein analysis. HS chain analysis confirmed the heightened levels of shed syndecan-1 in ALF patients (Fig. 10A). HMGB1 and ALT in APAP-ALF patients were also higher than the healthy control group
30 (Figs. 10B and 10C), consistent with a previous report (*22*).

The relationship between syndecan-1 shedding and HMGB1 release in humans and mice after APAP overdose underscores an endogenous protective pathway. Syndecan-1 sheds after the initial insult and neutralizes the pro-inflammatory activities of HMGB1, thereby limiting sterile inflammation. Indeed, *syndecan-1$^{-/-}$* mice are more susceptible to APAP-induced ALF than WT animals (Nam, 2017). During extensive APAP damage, it is likely that shed syndecan-1 is inadequate to neutralize all HMGB1, and the addition of 18-mer, a mimetic of HS on syndecan-1, provides further protection.

Since sterile inflammation occurs after NAPQI-induced cell damage, we examined the possibility of further delaying 18-mer treatment after APAP overdose. *N*-acetyl-cysteine (NAC) is a NAPQI-neutralizing antioxidant that is the standard of care for the treatment of APAP overdose. However, NAC treatment is only effective if given within 8 hours of APAP ingestion (Bailey, 2016). While NAC loses its protective effect when administered at six hours post-APAP in mice, the 18-mer is still able to decrease ALT (Fig. 5D). These data suggest that 18-mer treatment has a potential advantage for late-presenting APAP overdose patients by providing a wider therapeutic window.

Example 8
Conclusions

The use of synthetic HS to treat APAP-induced ALF by targeting HMGB1-mediated sterile inflammation is disclosed herein. The availability of homogeneous oligosaccharides has made it possible to identify candidate targets and compounds underlying this effect. In addition to neutralizing HMGB1, HS of syndecan-1 can also activate liver repair and modulate the activity of chemokines. 18-mer could also contribute to these functions. HS is well-tolerated by patients (Shriver, 2004) and unfractionated heparin, a highly sulfated form of HS, has been used as an anticoagulant for nearly a century (Szajek, 2016). Now, the synthesis of HS oligosaccharides can be achieved on a large scale and in a cost-effective manner. HMGB1-neutralizing synthetic oligosaccharides should offer a promising therapeutic approach for ALF.

Because HMGB1 has been implicated in diverse disease states including cancers, stroke and arthritis (Venereau, 2016), novel HS-based therapeutics provide promising opportunities as inhibitors of HMGB1.

REFERENCES

All references listed herein including but not limited to all patents, patent applications and publications thereof, scientific journal articles, and database entries (*e.g.*, GENBANK® database entries and all annotations available therein) are incorporated herein by reference in their entireties to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

1. L. Zitvogel, O. Kepp, G. Kroemer, Decoding cell death signals in inflammation and immunity. *Cell* **140**, 798-804 (2010).

2. G. Y. Chen, G. Nuñez, Sterile inflammation: sensing and reacting to damage. *Nat. Immunol.* **10**, 826-837 (2010).

3. M. E. Bianchi *et al.*, High-mobility group box 1 protein orchestrates responses to tissue damage via inflammation, innate and adaptive immunity, and tissue repair. *Immunol. Rev.* **280**, 74-82 (2017).

4. P. Huebener *et al.*, The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. *J. Clin. Invest.* **125**, 539-550 (2015).

5. K. J. Heard, Acetylcystein for acetaminophen poisoning. *N. Eng. J. Med.* **359**, 285-292 (2008).

6. W. M. Lee, Acetaminophen toxicity: changing perceptions on a social/medical issue. *Hepatology* **46**, 966-970 (2007).

7. M. Blieden, L. C. paramore, D. Shah, R. Ben-Joseph, A perspective on the epidemiology of acetaminophen exposre and toxicity in the United States. *Expert Rev. Clin. Pharmacol.* **7**, 341-348 (2014).

8. F. Tacke, J. C. Mossanen, Acetaminophen-induced acute liver injury in mice. *Lab. Anim.* **49**, 30-36 (2015).

9. D. Xu, J. Young, D. Song, J. D. Esko, Heparan sulfate is essential for high mobility group protein 1 (HMGB1) signaling by the receptor for advanced glycation end products (RAGE). *J. Biol. Chem.* **286**, 41736-41744 (2011).

10. A. E. Proudfoot *et al.*, Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc. Natl. Acad. Sci. USA* **100**, 1885-1890 (2003).

11. L. Wang, M. Fuster, P. Sriramarao, J. D. Esko, Edothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inlammatoray responses. *Nat. Immunol.* **6**, 902-910 (2005).

5 12. J. Axelsson *et al.*, Inactivation of heparan sulfate 2-O-sulfotransferase accentuates neutrolphil infiltration during acute inflammation in mice. *Blood* **120**, 1742-1751 (2013).

13. M. Sarris *et al.*, Inflammatory chemokines direct and restrict leukocyte migration within live tissues as glycan-bound gradients. *Curr. Biol.* **22**,
10 2375-2382 (2012).

14. C. Gama *et al.*, Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. *Nat. Chem. Biol.* **2**, 467-473 (2006).

15. J. Liu, R. J. Linhardt Chemoenzymatic synthesis of heparan sulfate and heparin. *Nat. Prod. Rep.* **31**, 1676-1685 (2014).

15 16. Y. Xu *et al.*, Chemoenzymatic synthesis of homogeneous ultra-low molecular weight heparin. *Science* **334**, 498-501 (2011).

17. Y. Xu *et al.*, Homogeneous and reversible low-molecular weight heparins with reversible anticoagulant activity. *Nat. Chem. Biol.* **10**, 248-250 (2014).

20 18. Y. Xu *et al.*, Synthetic oligosaccharides can replace animal-sourced low-molecular weight heparins. *Sci. Transl. Med.* **9**, eaan5954 (2017).

19. E. J. Nam *et al.*, Syndecan-1 Limits the Progression of Liver Injury and Promotes Liver Repair in Acetaminophen-Induced Liver Injury. *Hepatology*, doi: 10.1002/hep.29265 (2017).

25 20. A. Kopec *et al.*, Fibrin(ogen) drives repair after acetaminophen-induced liver injury via leukocyte aMb2 integrin-dependent upregulation of Mmp12. *J. Hepatol.* **66**, 787-797 (2017).

21. P. W. Park, O. Reizes, M. Bernfield, Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. *J. Biol.*
30 *Chem.* **275**, 29923-29926 (2000).

22. D. J. Antoine *et al.*, Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. *Hepatology* **58**, 777-787 (2013).

23. G. P. Bailey *et al.*, Delays during the administration of acetylcysteine for
35 the treatment of paraacetamol overdose. *Br. J. Clin. Pharmacol.* **62**, 1358-1363 (2016).

24. K. C. Wildhagen *et al.*, Nonanticoagulant heparin prevents histone-mediated cytotoxicity in vitro and improves survival in sepsis. *Blood* **123**, 1098-1101 (2014).

25. Y. Monneau, F. Arenzana-Seisdedos, H. Lortat-Jacob, The sweet spot: how GAGs help chemokines guide migrating cells. *J. Leukoc. Biol.* **99**, 935-953 (2016).

26. P. Lundback *et al.*, A novel high mobility group box 1 neutralizing chimeric antibody attenuates drug-induced liver injury and postinjury inflammation in mice. *Hepatology* **64**, 1699-1710 (2016).

27. T. Yamamoto, Y. Tajima, HMGB1 is a promising therapeutic target for acute liver failure. *Expert Rev. Gatroenterol. Hepatol.* **11**, 673-682 (2017).

28. Z. Shriver, S. Raguram, R. Sasisekharan, Glycomics: A pathway to a class of new and improved therapeutics. *Nat Rev Drug Discov* **3**, 863-873 (2004).

29. A. Szajek *et al.*, The US regulatory and pharmacopeia responses to the global heparin contamination crisis. *Nat. Biotechnol.* **34**, 625-630 (2016).

30. E. Venereau *et al.*, HMGB1 as biomarker and drug target. *Pharmacol. Res.* **111**, 534-544 (2016).

31. P. Hsieh, Y. Xu, D. A. Keire, J. Liu, Chemoenzymatic synthesis and structural characterization of 2-O-sulfated glucuronic acid containing heparan sulfate hexasaccharides. *Glycobiology* **24**, 681-692 (2014).

32. B. Liliensiek *et al.*, Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response. *J. Clin. Invest.* **113**, 1641-1650 (2004).

33. PCT Publication No. WO2012/088416, published June 28, 2012

34. Published U.S. Patent Application No. US2013/0296540, published November 7, 2013

35. PCT Publication No. WO2014204929, published December 24, 2014

36. Published U.S. Patent Application No. US2016/0122446, published May 5, 2016

37. Xu, Y., Chandarajoti, K., Zhang, X., Pagadala, V., Dou, W., Hoppensteadt, D.M., Sparkenbaugh, E., Colley, B., Daily, S., Key, N., Severynse-Stevens, D., Fareed, J., Linhardt, R.J., Pawlinksi, R., and Liu, J. (2017) Synthetic oligosaccharides can replace animal-sourced low-molecular weight heparins *Sci. Transl. Med.*, 9, eaan5954.

38. Xu, Y, Cai, C., Chandarajoti, K, Li, L., Hsieh, P., Pham, T., Sparkenbaugh, E.M., Sheng, J., Key, N., Pawlinski, R., Harris, E., Linhardt, R.J., and Liu, J. (2014) Homogeneous and reversible low-molecular weight heparins with reversible anticoagulant activity. *Nat. Chem Biol*. 10: 248-250.

39. Renpeng Liu, Yongmei Xu, Miao Chen, Michel Weïwer, Xianxuan Zhou, Arlene S. Bridges, Paul L. DeAngelis, Qisheng Zhang, Robert J. Linhardt and Jian Liu (2010) Chemoenzymatic design of heparan sulfate oligosaccharides *J. Biol. Chem.*285:34240-34249

40. Xu, D., Moon, A.F., Song, D., Pedersen, L.C. and **Liu, J.** (2008) Engineering the sulfotransferases to modify heparan sulfate *Nat. Chem. Biol*. 4: 200-202.

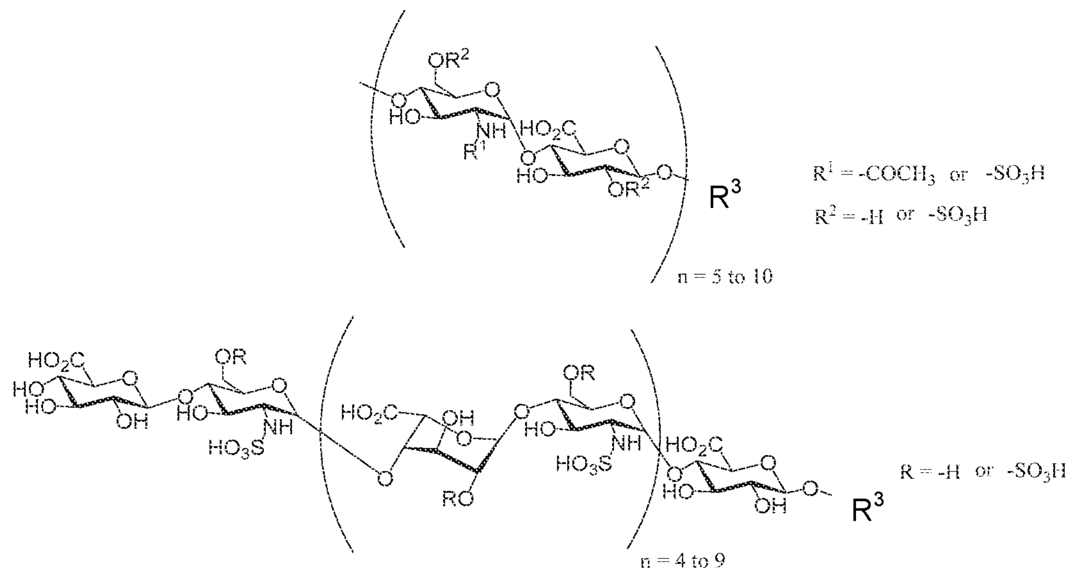
It will be understood that various details of the presently disclosed subject matter may be changed without departing from the scope of the presently disclosed subject matter. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

What is claimed is:

1. A small molecule compound having an anti-inflammatory property, the
5 small molecule compound comprising a non-anticoagulant heparan sulfate oligosaccharide molecule, optionally wherein the non-anticoagulant heparan sulfate oligosaccharide molecule is configured to interact with a high mobility group box 1 (HMGB1) protein in a manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation end
10 products (RAGE).

2. A small molecule compound having an anti-inflammatory property, the small molecule compound comprising a non-anticoagulant heparan sulfate oligosaccharide molecule comprising one of the following structural formulas:
15



.

, wherein R^3 is H or a detectable tag.

20

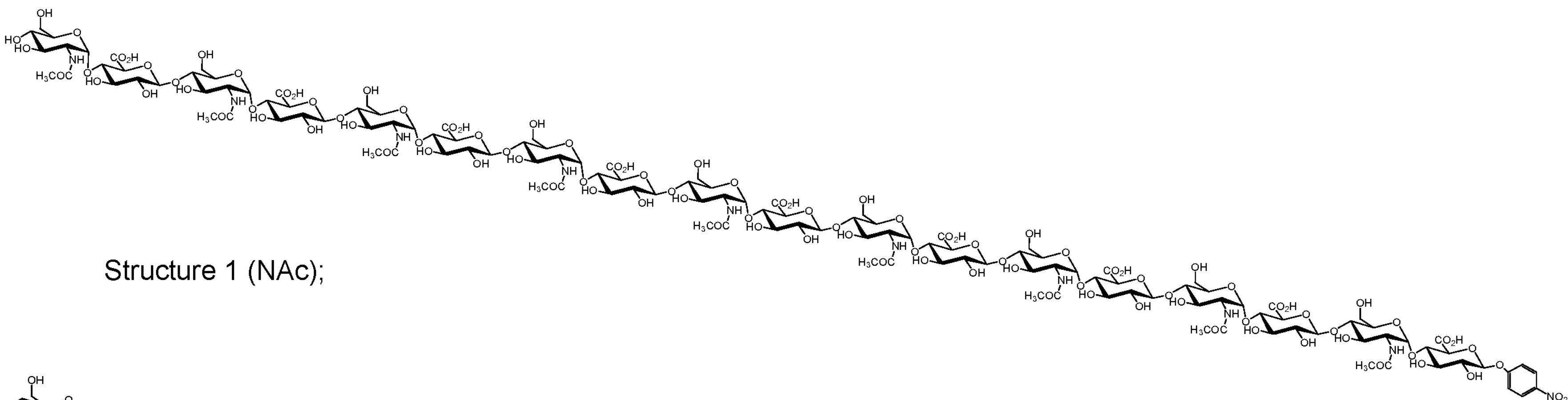
3. The small molecule compound of any of the above claims, wherein the oligosaccharide molecule comprises about 10 to about 20 saccharide units, or about 12 to about 18 saccharide units.

5

4. The small molecule compound of any of the above claims, wherein the oligosaccharide molecule comprises about 18 saccharide units.

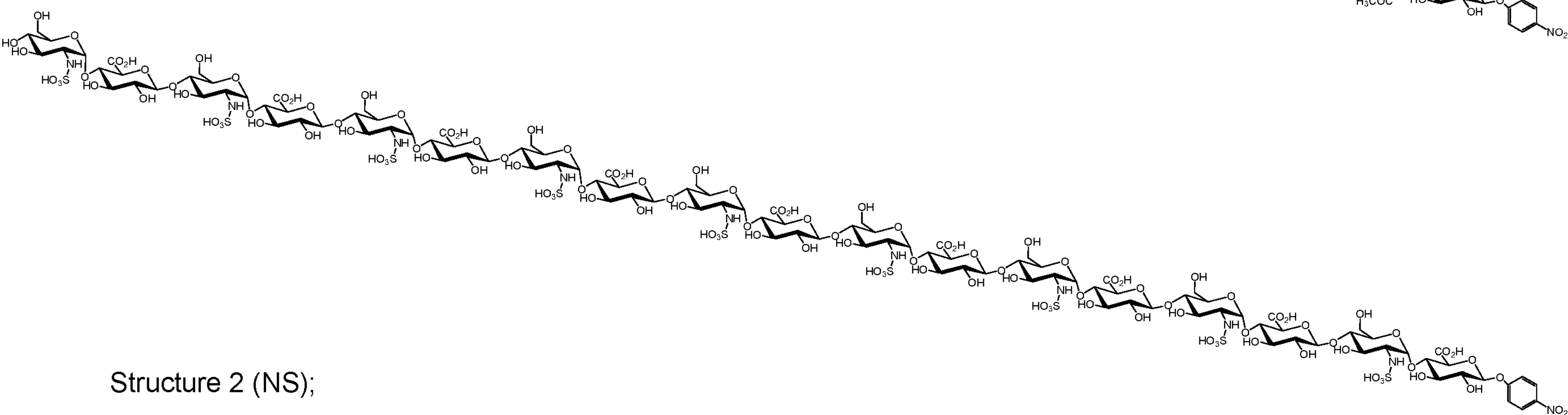
5. The small molecule compound of any of the above claims, wherein the oligosaccharide molecule comprises one of the following structures:

5



Structure 1 (NAc);

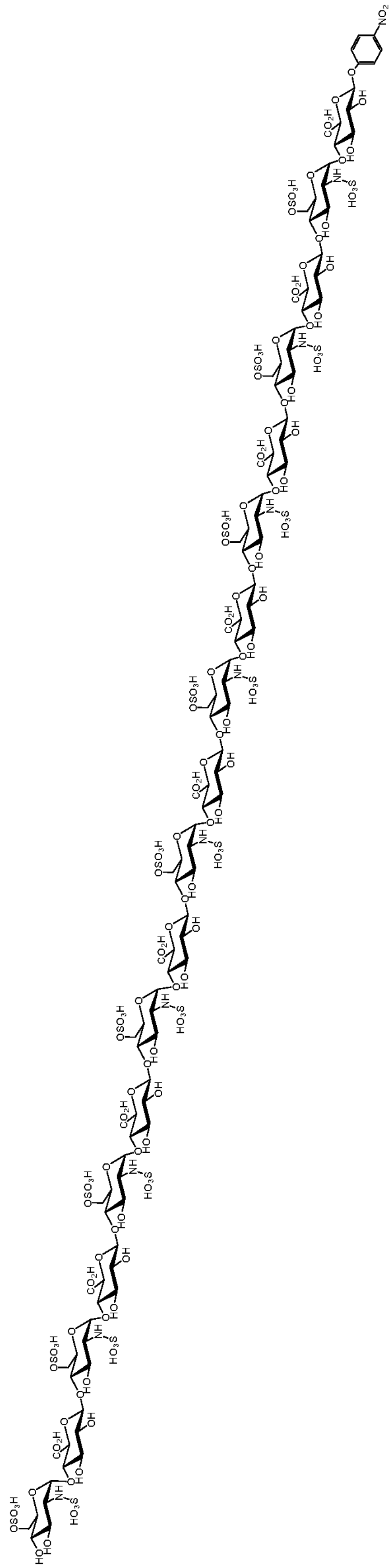
10



15

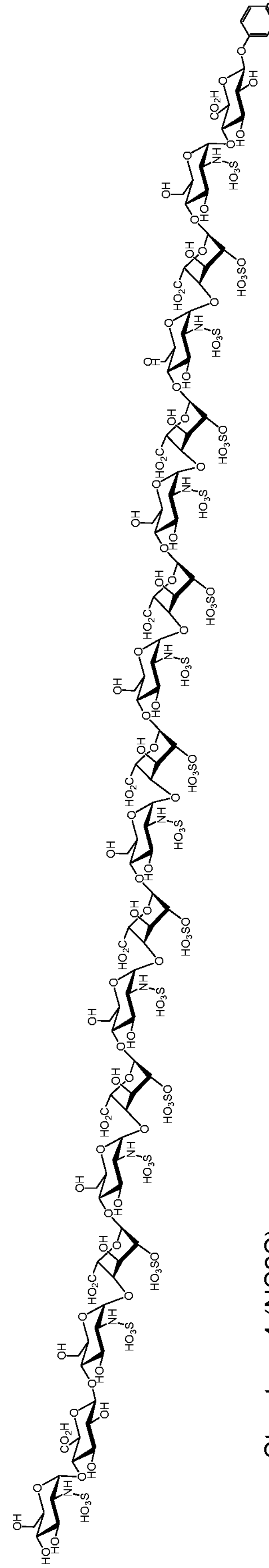
Structure 2 (NS);

5



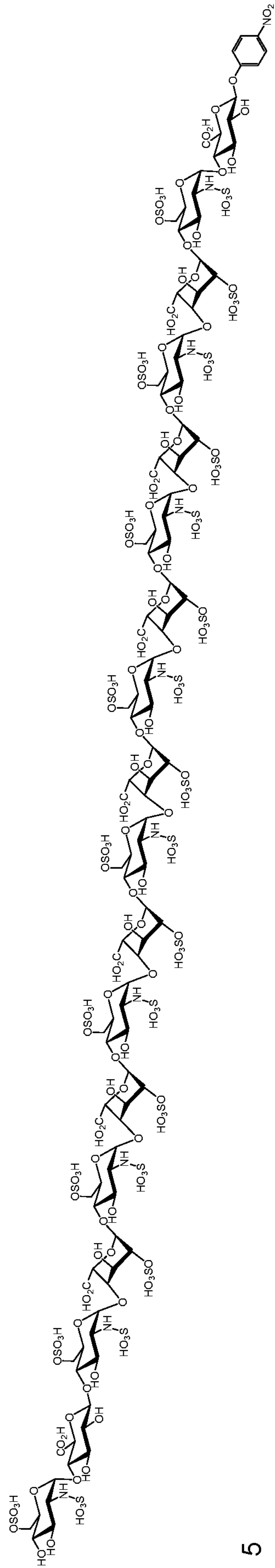
Structure 3 (NS6S);

10



Structure 4 (NS2S); or

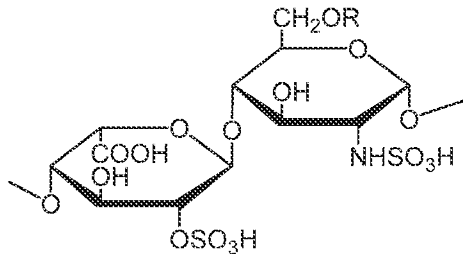
15



5

Structure 5 (NS6S2S).

6. The small molecule compound of any of the above claims, wherein the small molecule compound is provided in a composition further comprising a small molecule compound comprising a disaccharide structure unit as shown:



, wherein R = -H or $-SO_3H$.

5

7. The small molecule compound of claim 6, wherein the small molecule compound comprising a disaccharide structure unit is selected from the group consisting of non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin, and O-desulfated heparin (ODSH).

10

8. The small molecule compound of any of the above claims, wherein the oligosaccharide molecule protects against liver injury *in vivo*.

9. The small molecule compound of any of the above claims, wherein the
15 oligosaccharide molecule decreases neutrophil infiltration *in vivo*.

10. The small molecule compound of any of the above claims, wherein the oligosaccharide molecule decreases inflammation *in vivo*.

20 11. A method of treating a subject, the method comprising:

providing a subject to be treated, wherein the subject is suffering from inflammation; and

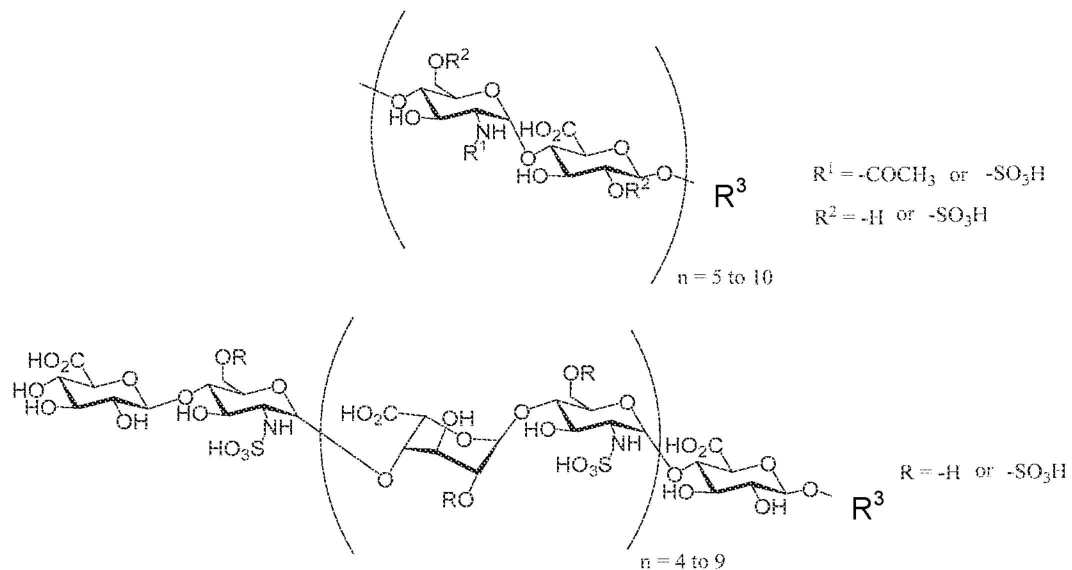
administering to the subject a non-anticoagulant heparan sulfate oligosaccharide molecule having an anti-inflammatory property, optionally
25 wherein the non-anticoagulant heparan sulfate oligosaccharide molecule is configured to interact with a high mobility group box 1 (HMGB1) protein in a

manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation endproducts (RAGE).

12. A method of treating a subject, the method comprising:

providing a subject to be treated, wherein the subject is suffering from inflammation; and

administering to the subject a small molecule compound having an anti-inflammatory property, the small molecule compound comprising a non-anticoagulant heparan sulfate oligosaccharide molecule comprising one of the following structural formulas:



, wherein R^3 is H or a detectable tag.

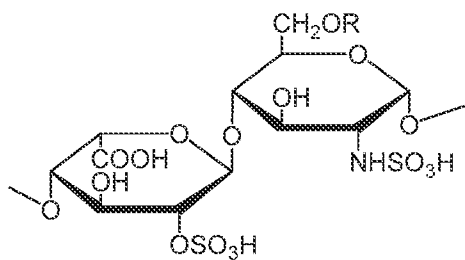
13. The method of any of the above claims, wherein the subject suffers from any injury resulting in increased inflammation.

14. The method of any of the above claims, wherein the subject suffers from liver injury.

15. The method of any of the above claims, wherein the subject in need of treatment is a subject suffering from an overdose of Paracetamol (APAP).

16. The method of any of the above claims, wherein the subject in need of
5 treatment is a human subject.

17. The method of any of the above claims, wherein the small molecule compound comprises a disaccharide structure unit as shown:



, wherein R = -H or $-SO_3H$.

10

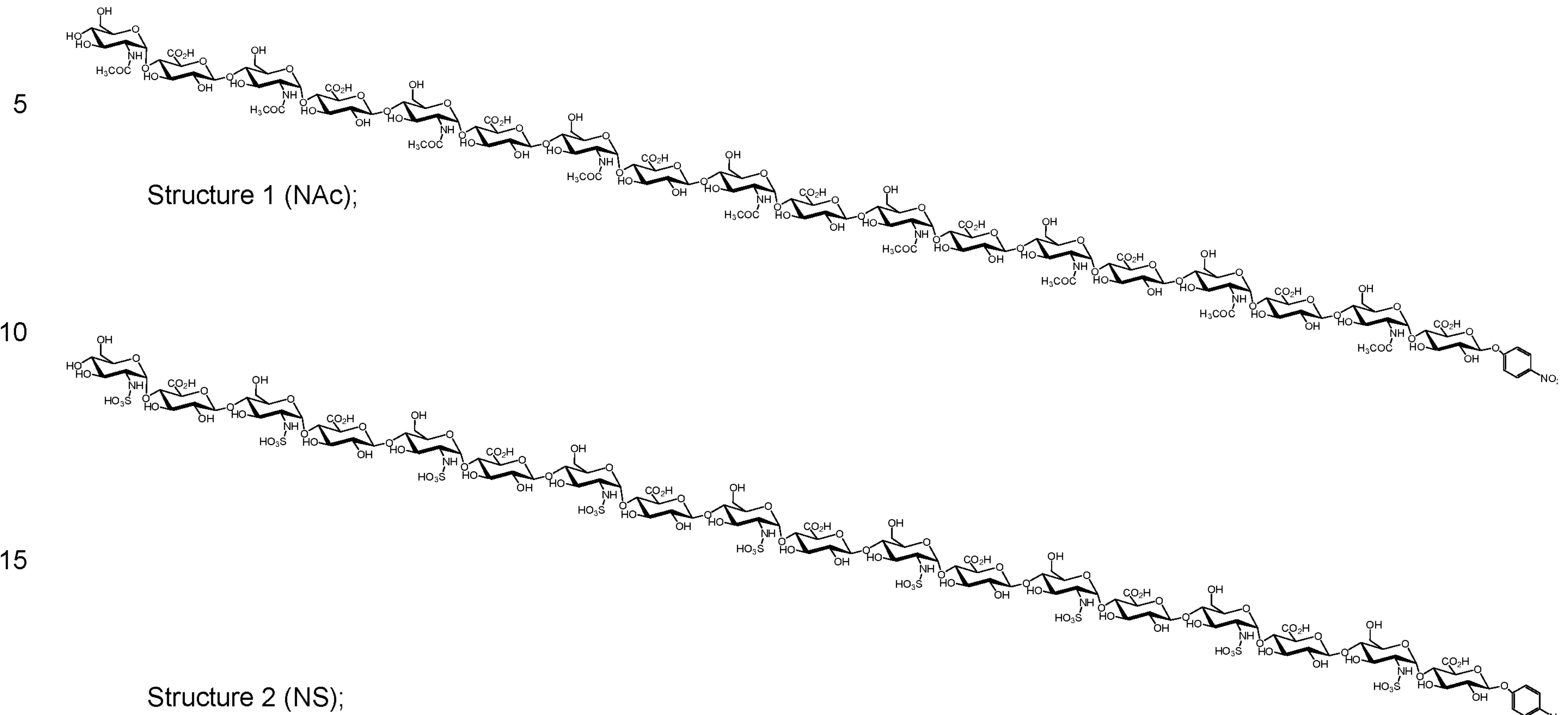
18. The method of claim 17, wherein the small molecule compound comprising a disaccharide structure unit is selected from the group consisting of non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin, and O-desulfated heparin (ODSH).

15

19. The method of any of the above claims, wherein the oligosaccharide molecule comprises about 10 to about 20 saccharide units, or about 12 to about 18 saccharide units.

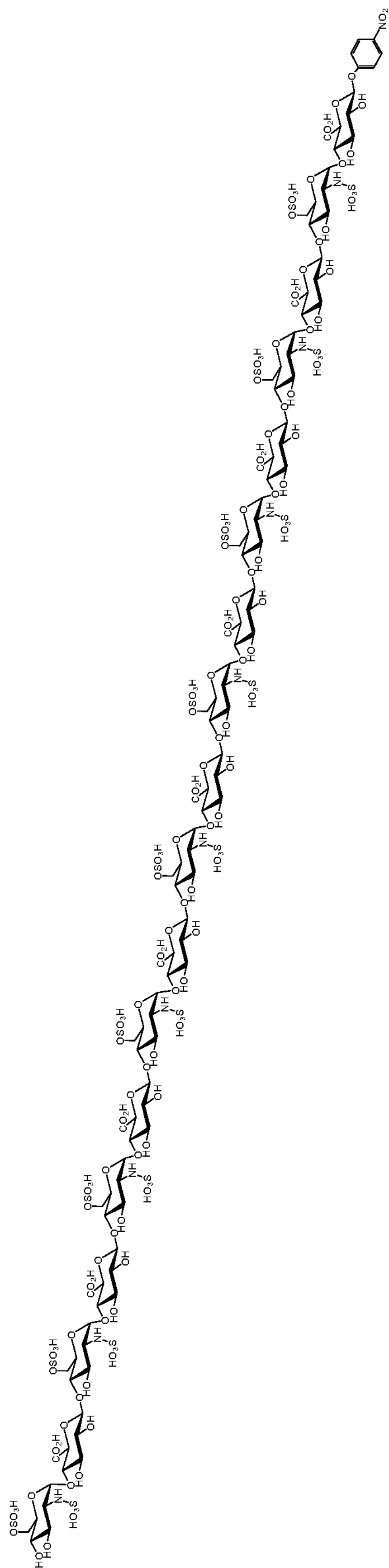
20 20. The method of any of the above claims, wherein the oligosaccharide molecule comprises about 18 saccharide units.

21. The method of any of the above claims, wherein the oligosaccharide molecule comprises one of the following structures:

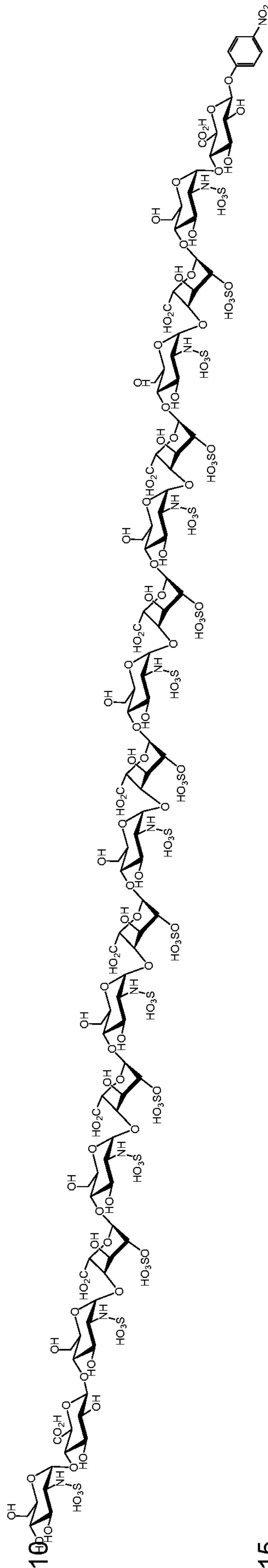


Structure 1 (NAc);

Structure 2 (NS);

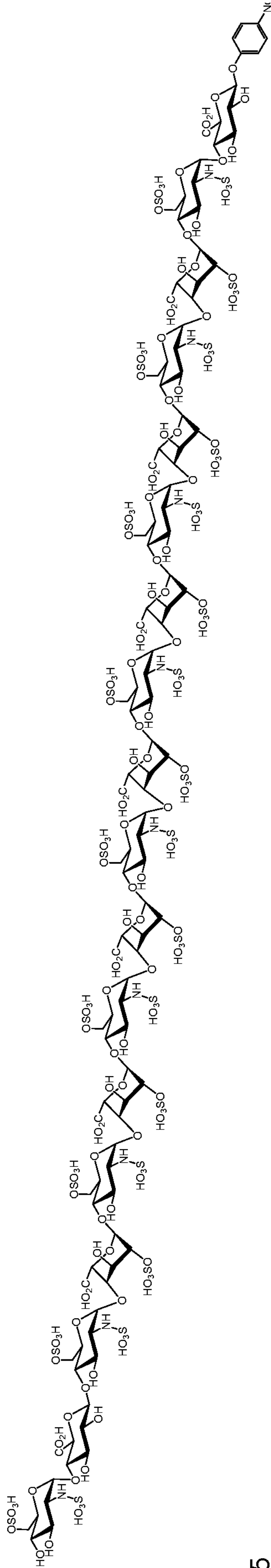


Structure 3 (NS6S);



Structure 4 (NS2S); or

5



Structure 5 (NS6S2S).

22. The method of any of the above claims, wherein administration of the oligosaccharide molecule decreases neutrophil infiltration in the subject.

23. The method of any of the above claims, wherein administration of the
5 oligosaccharide molecule decreases inflammation in the subject.

24. The method of any of the above claims, wherein administration of the oligosaccharide molecule protects against liver damage and multi-organ system failure in the subject.
10

25. The method of any of the above claims, wherein the subject to be treated suffers from drug-induced inflammation.

26. A method of treating Paracetamol (APAP) overdose in a subject, the
15 method comprising:

providing a subject in need of treatment for APAP overdose;

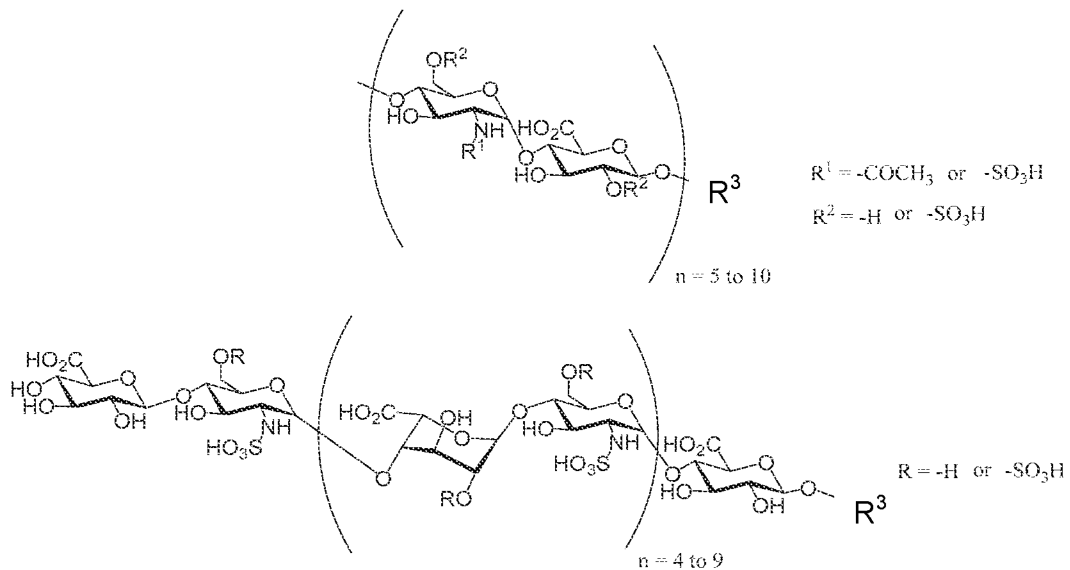
administering to the subject a non-anticoagulant heparan sulfate oligosaccharide molecule having an anti-inflammatory property, optionally wherein the non-anticoagulant heparan sulfate oligosaccharide molecule is
20 configured to interact with a high mobility group box 1 (HMGB1) protein in a manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation endproducts (RAGE),

wherein damage from the APAP overdose in the subject is mitigated.

25 27. A method of treating Paracetamol (APAP) overdose in a subject, the method comprising:

providing a subject in need of treatment for APAP overdose;

administering to the subject a small molecule compound having an anti-inflammatory property, the small molecule compound comprising a non-
30 anticoagulant heparan sulfate oligosaccharide molecule comprising one of the following structural formulas:



,

wherein R^3 is H or a detectable tag and wherein damage from the APAP
5 overdose in the subject is mitigated.

28. The method of any of the above claims, wherein the treatment for the APAP overdose in the subject is effective between 0 hours and 24 hours after the overdose, or at least within 12 hours after the overdose.

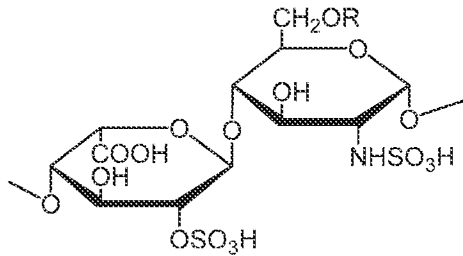
10

29. The method of any of the above claims, wherein the treatment for the APAP overdose in the subject comprises protection against liver injury and/or multi-organ system failure.

15 30. The method of any of the above claims, wherein the treatment for the APAP overdose in the subject comprises a decrease in neutrophil infiltration in the subject.

31. The method of any of the above claims, wherein the treatment for the
20 APAP overdose in the subject comprises blocking the interaction between the HMGB1 protein and RAGE.

32. The method of any of the above claims, wherein the small molecule compound comprises a disaccharide structure unit as shown:



, wherein R = -H or $-SO_3H$.

5

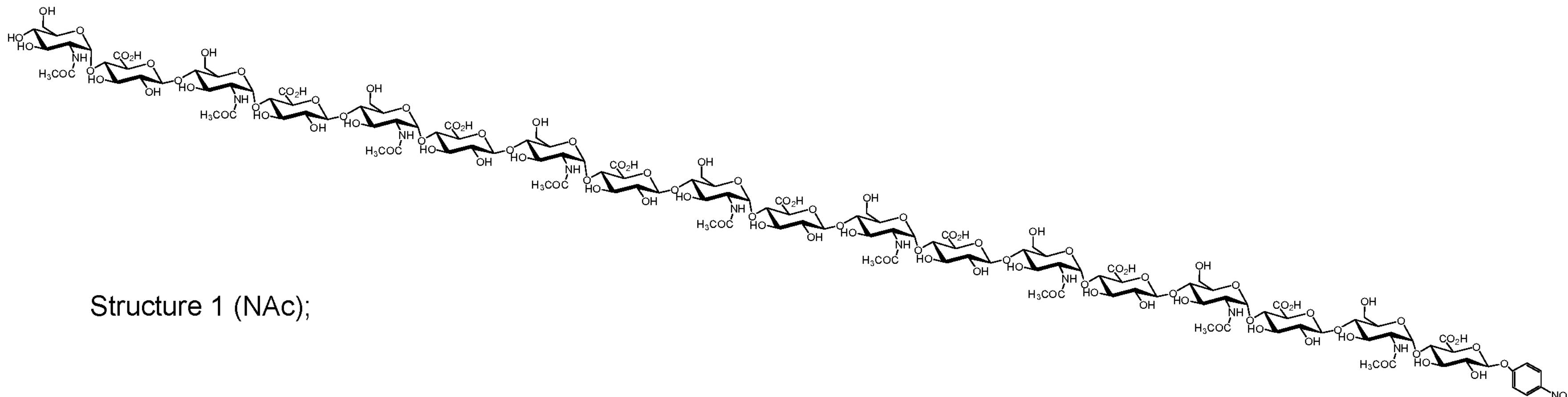
33. The method of claim 32, wherein the small molecule compound comprising a disaccharide structure unit is selected from the group consisting of non-anticoagulant heparin, non-anticoagulant low-molecular weight heparin, and O-desulfated heparin (ODSH).

10

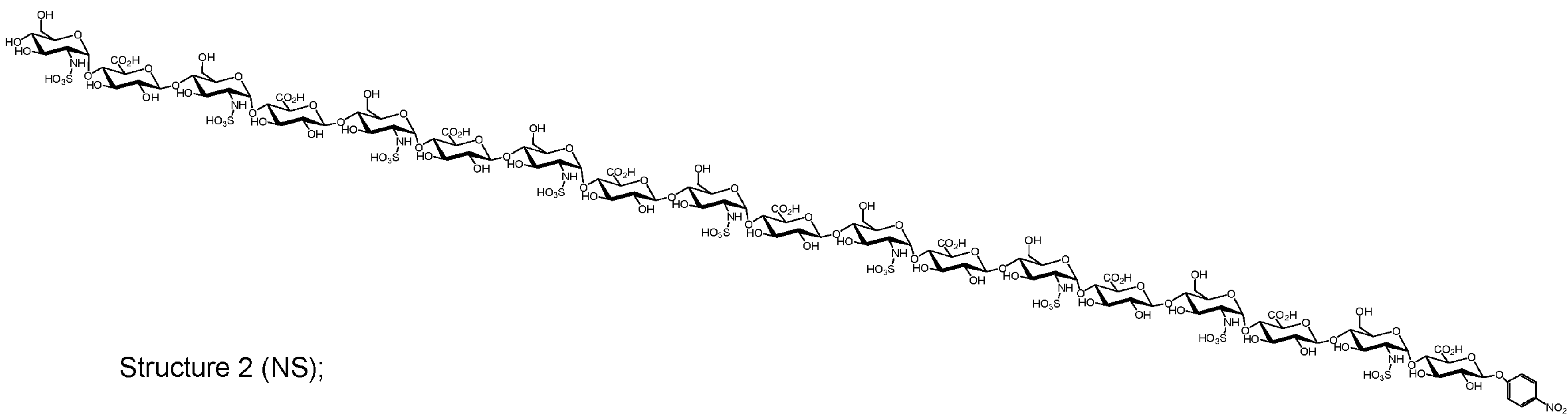
34. The method of any of the above claims, wherein the oligosaccharide molecule comprises about 10 to about 20 saccharide units, or about 12 to about 18 saccharide units.

15 35. The method of any of the above claims, wherein the oligosaccharide molecule comprises about 18 saccharide units.

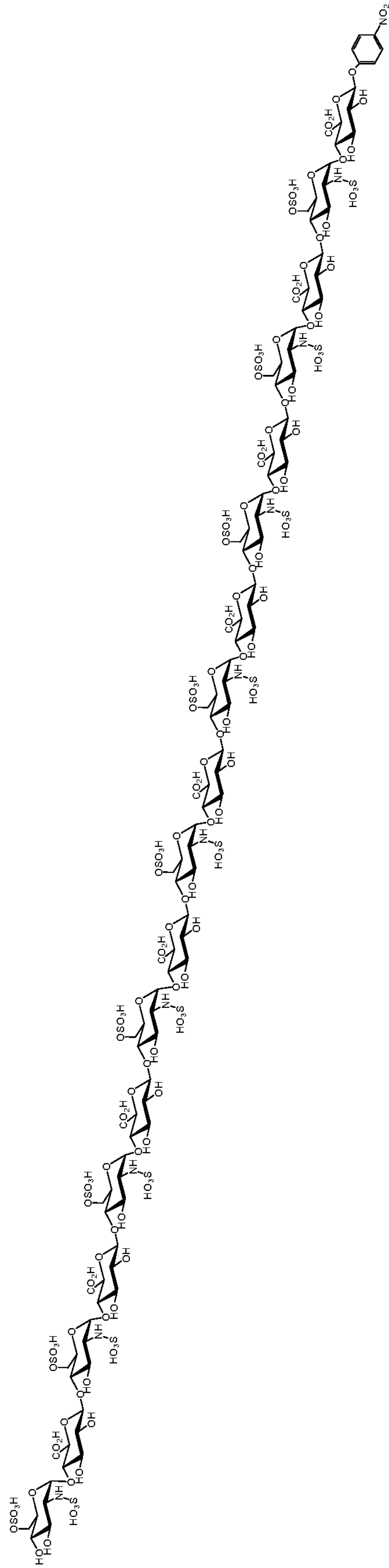
36. The method of any of the above claims, wherein the oligosaccharide molecule comprises one of the following structures:



Structure 1 (NAc);

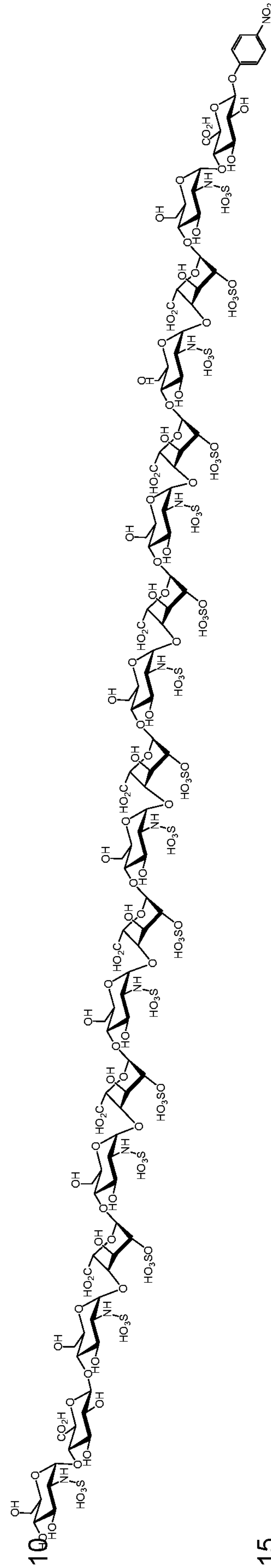


Structure 2 (NS);



Structure 3 (NS6S);

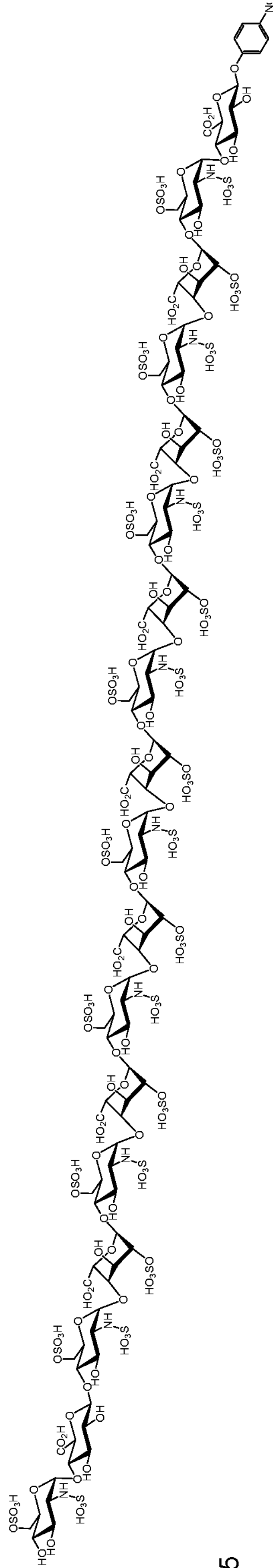
5



Structure 4 (NS2S); or

10

15



5

Structure 5 (NS6S2S).

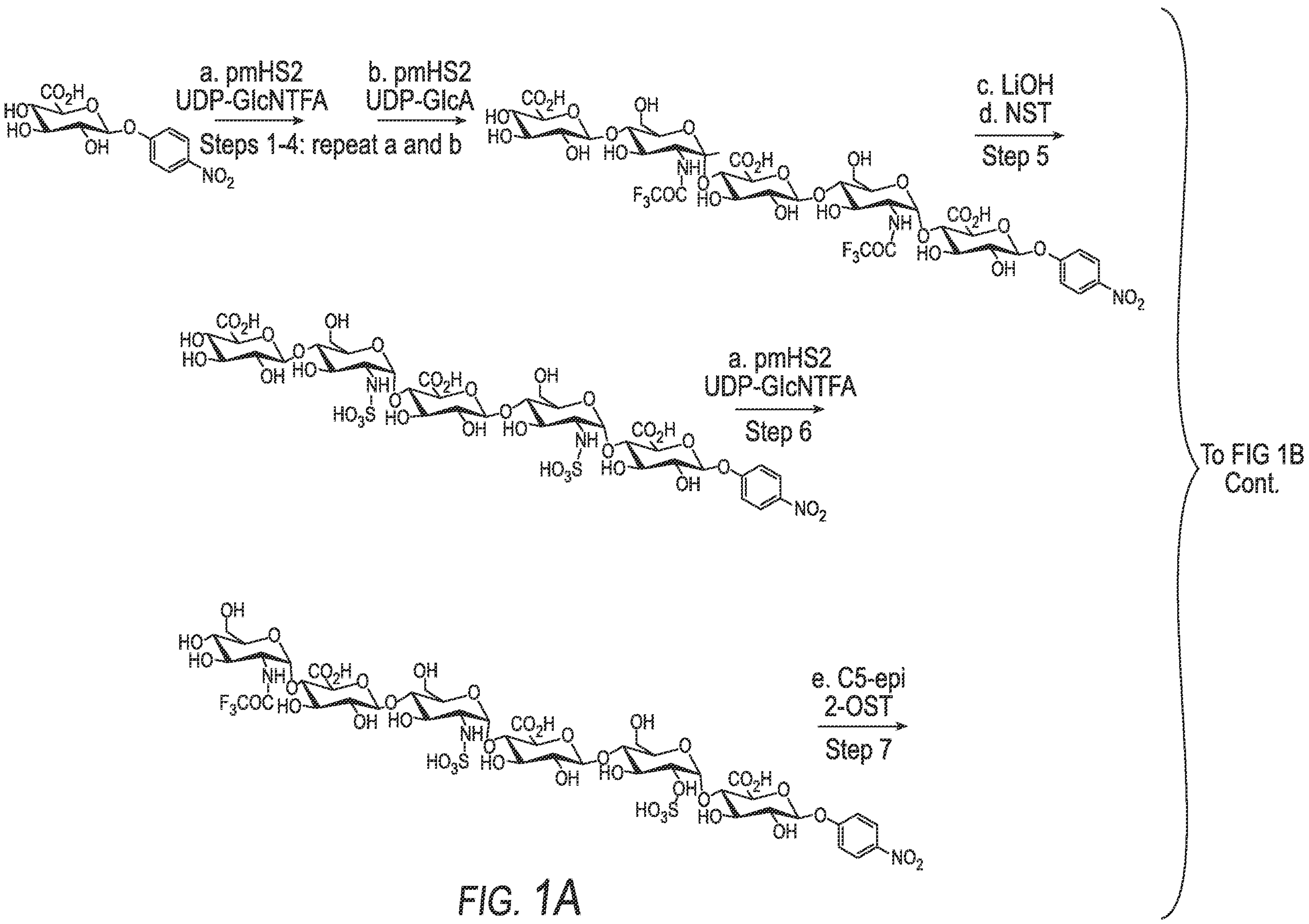


FIG. 1A

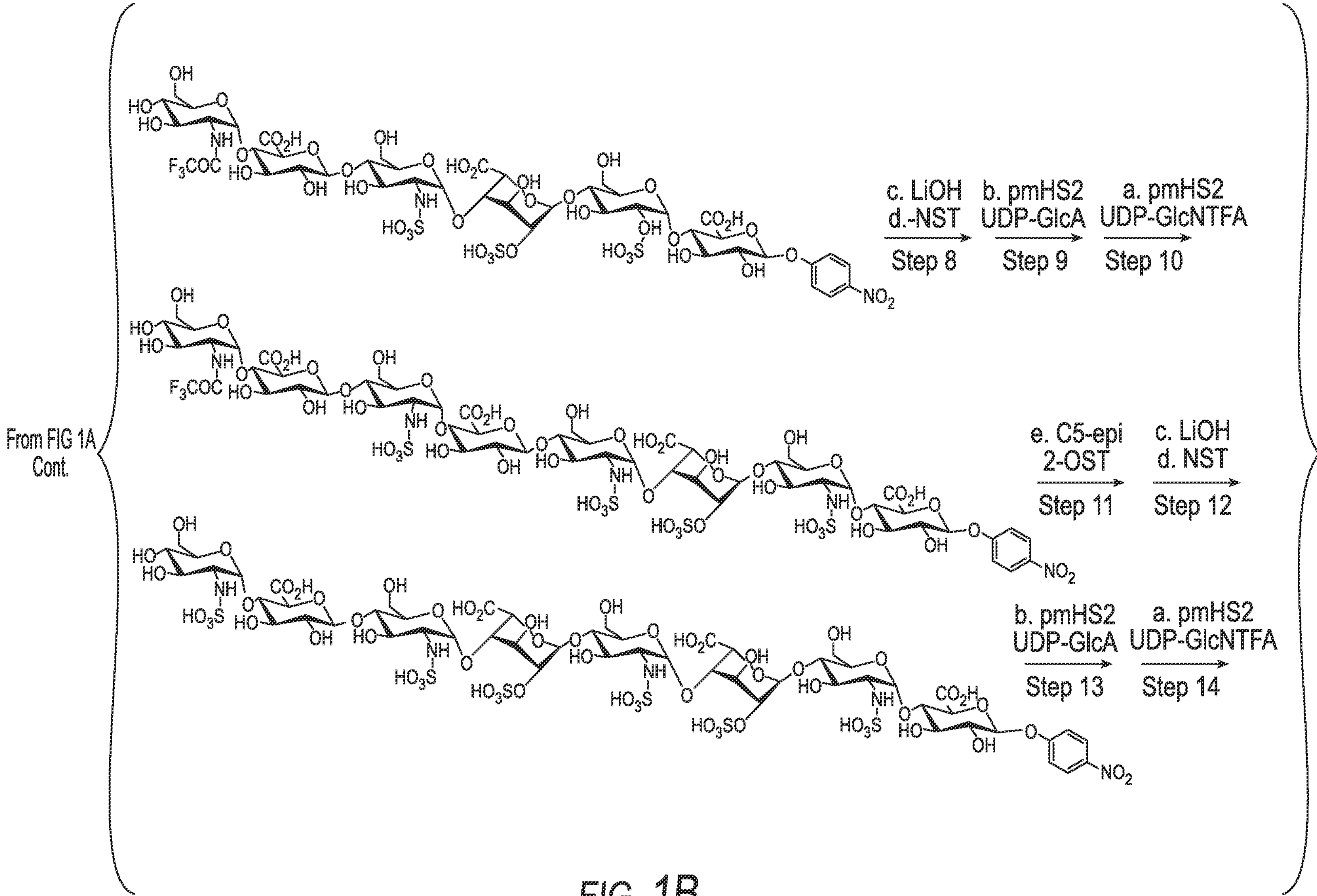


FIG. 1B

From FIG 1B Cont.

e. C5-epi
2-OST
Step 15

c. LiOH
d. NST
Step 16

To FIG 1D Cont.

FIG. 1C

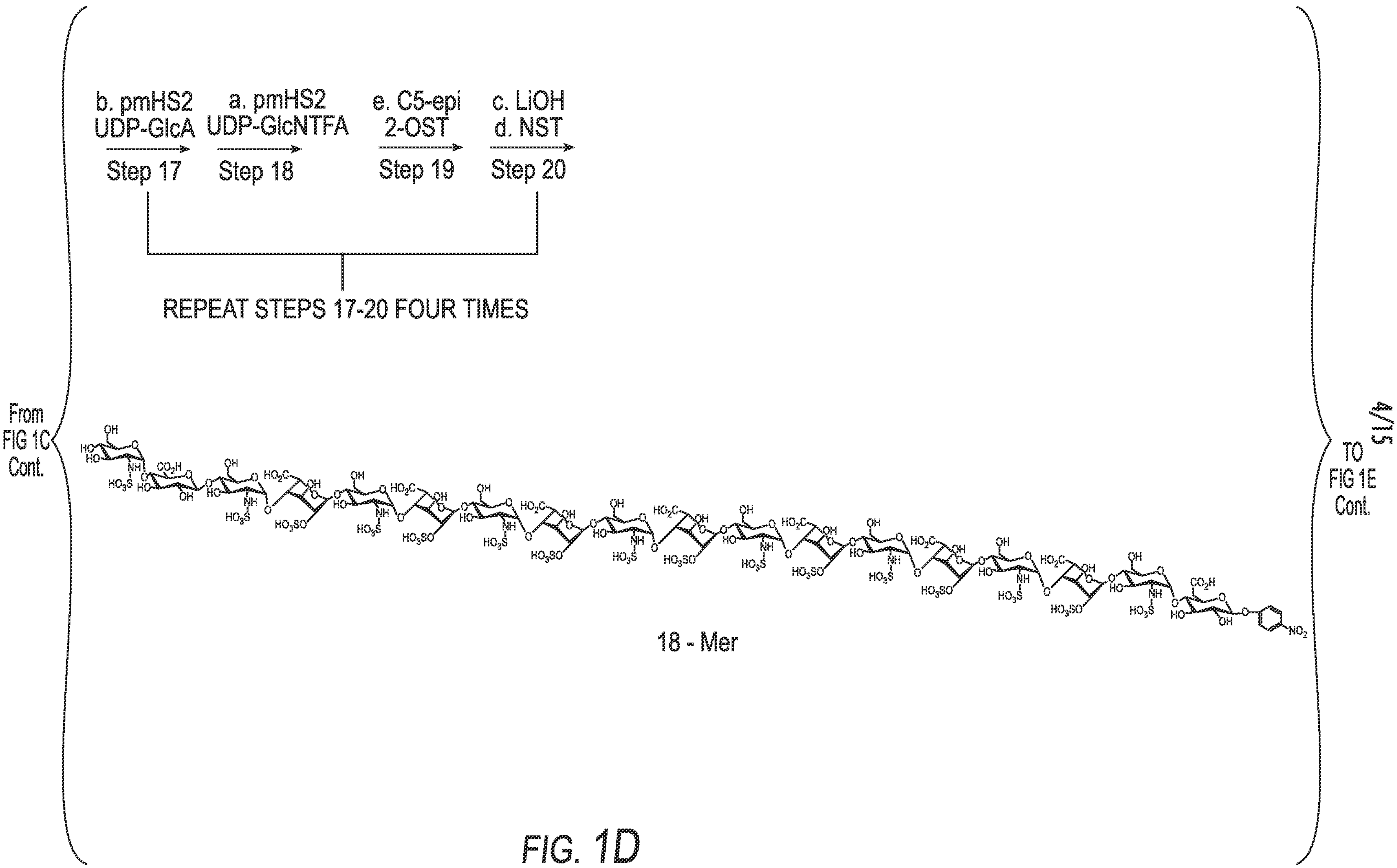


FIG. 1D

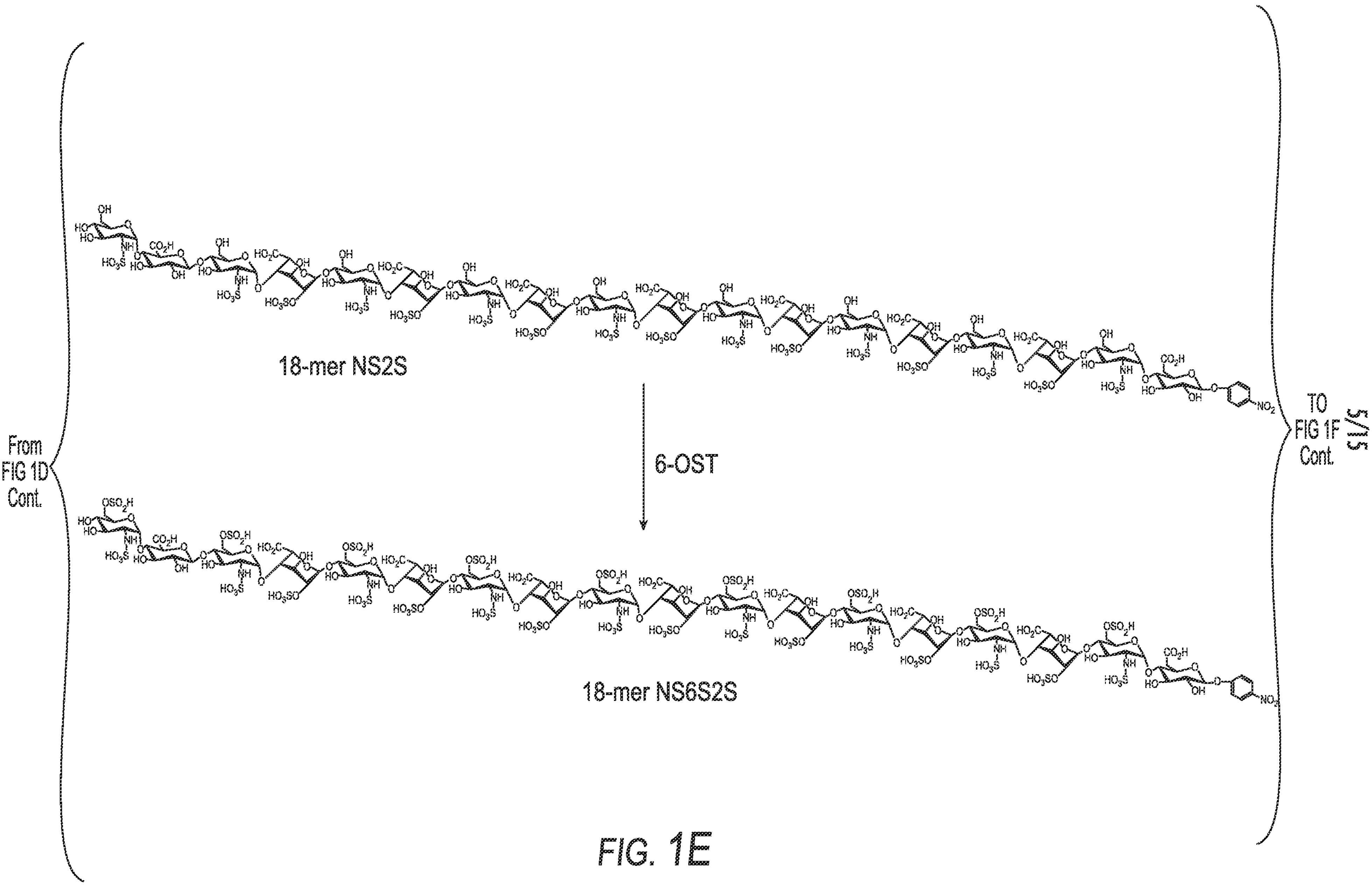


FIG. 1E

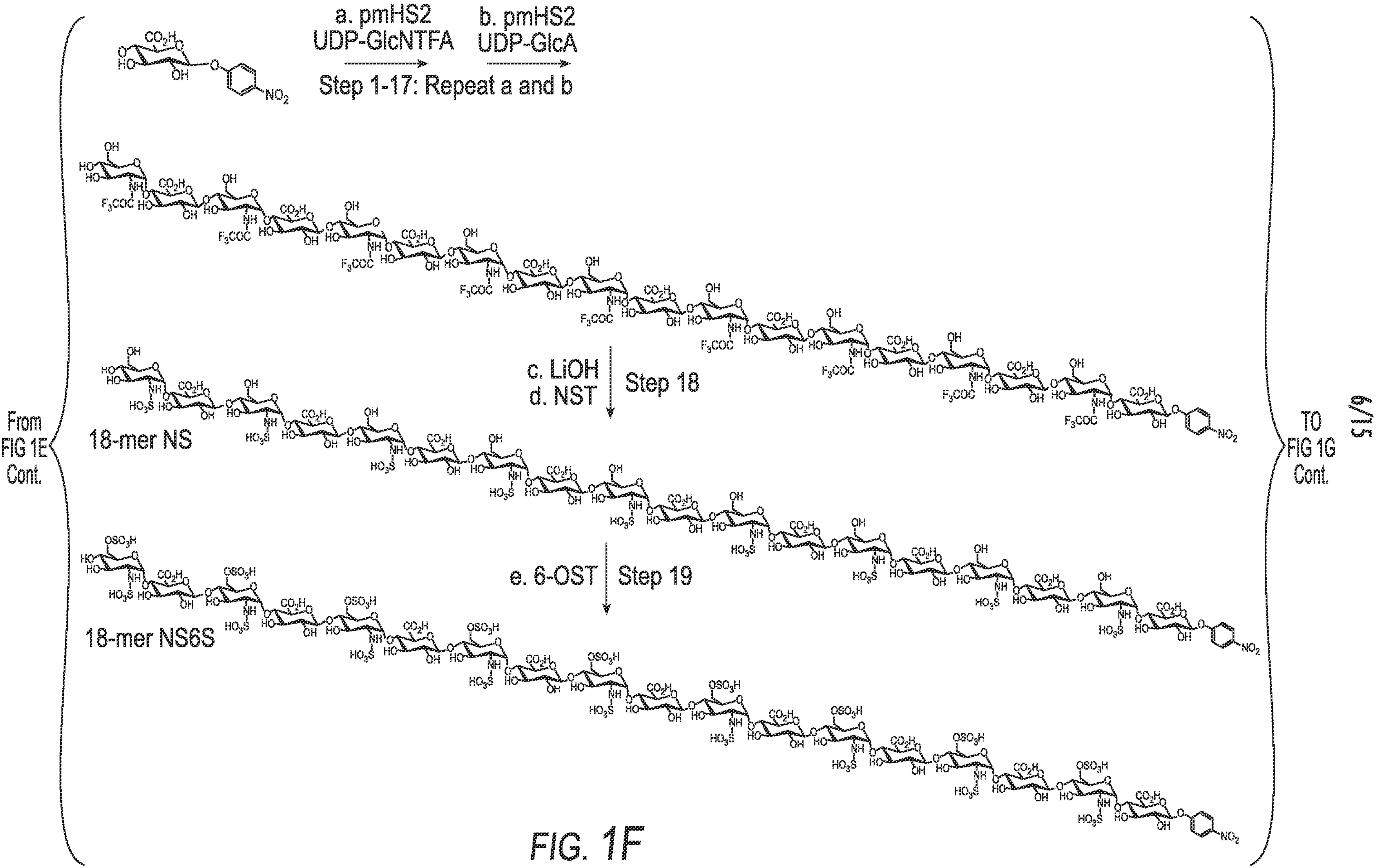


FIG. 1F

a. pmHS2
UDP-GlcNTFA

b. pmHS2
UDP-GlcA

Step 1-17: Repeat a and b

From FIG 1F Cont.

18-mer NAc

FIG. 1G

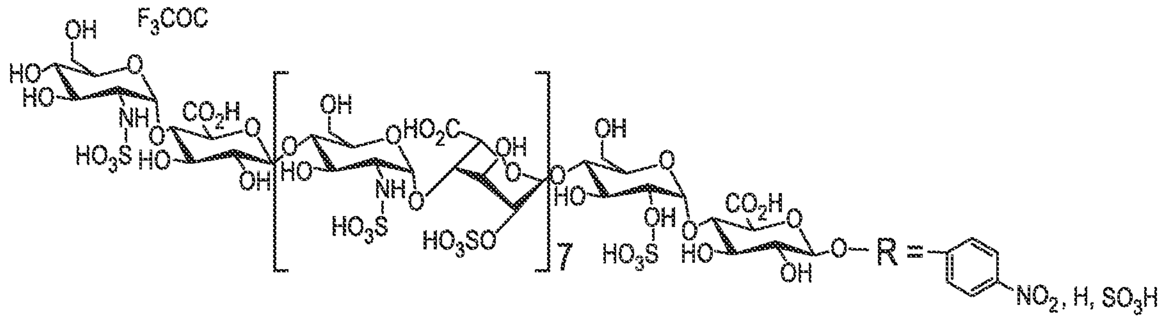


FIG. 2

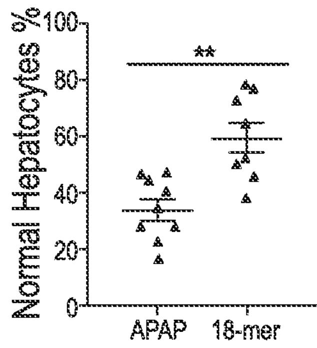


FIG. 3A

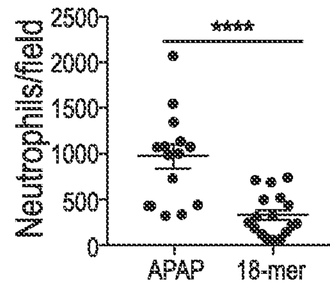


FIG. 3B

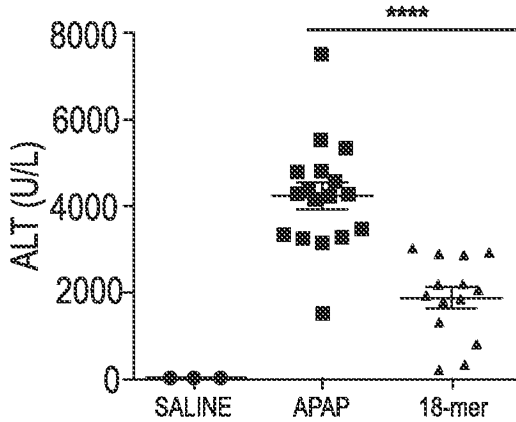


FIG. 3C

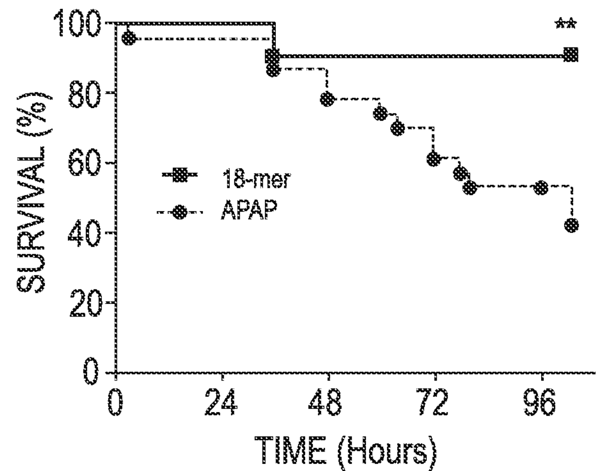


FIG. 3D

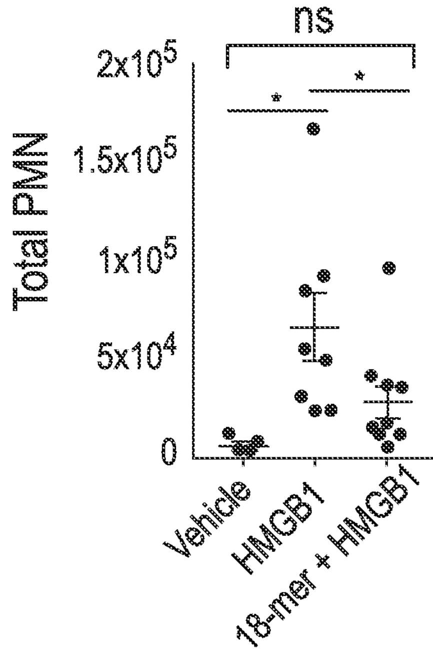


FIG. 4A

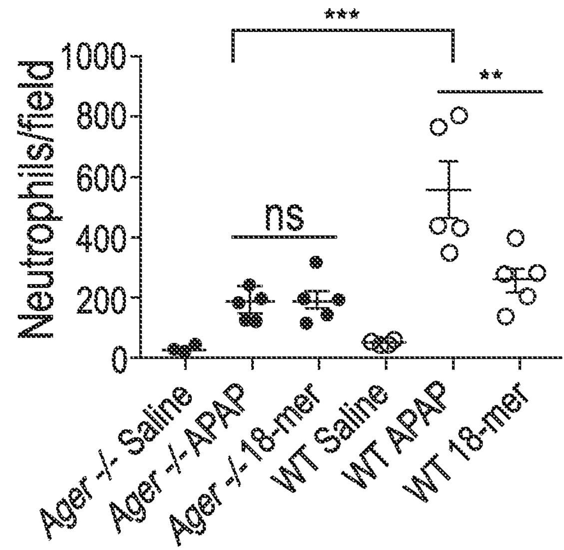


FIG. 4B

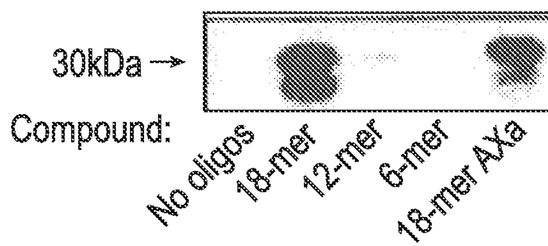


FIG. 4C

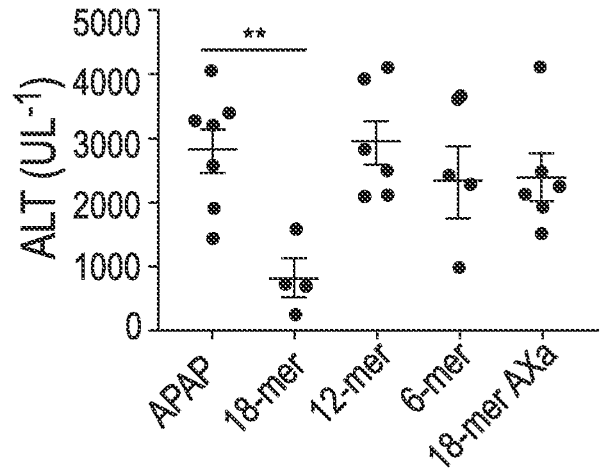


FIG. 4D

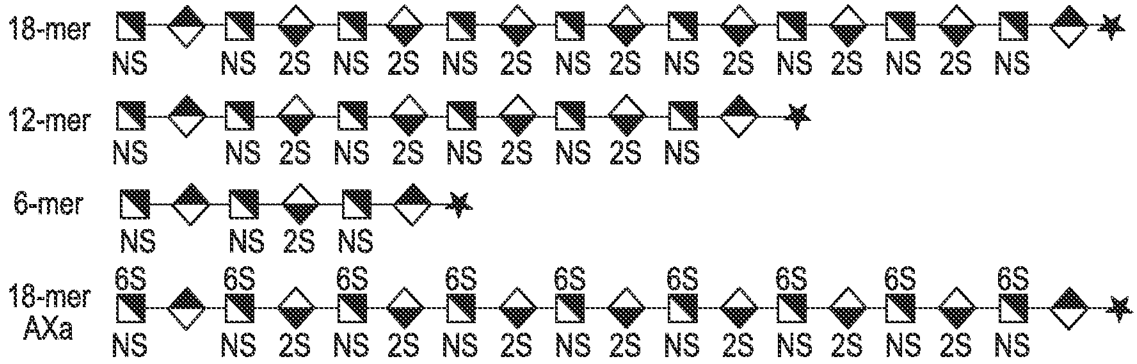


FIG. 4E

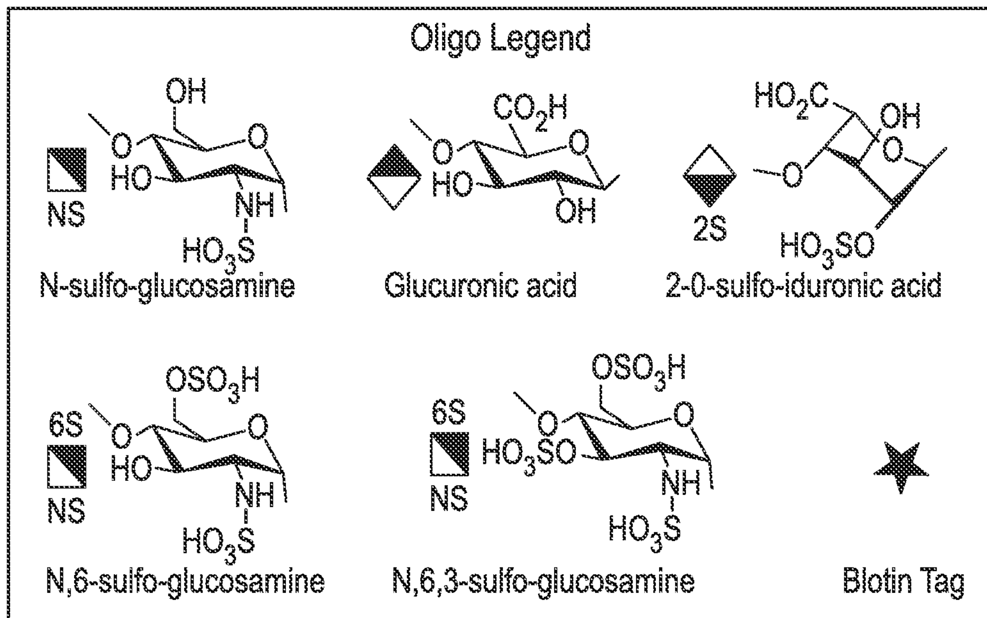


FIG. 4F

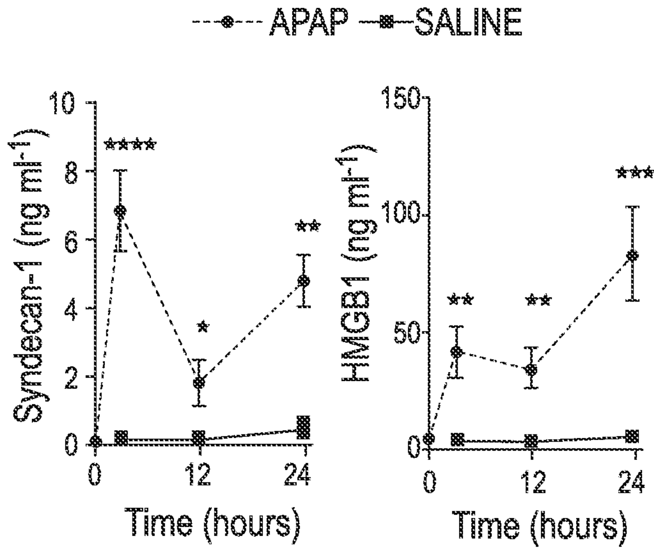


FIG. 5A

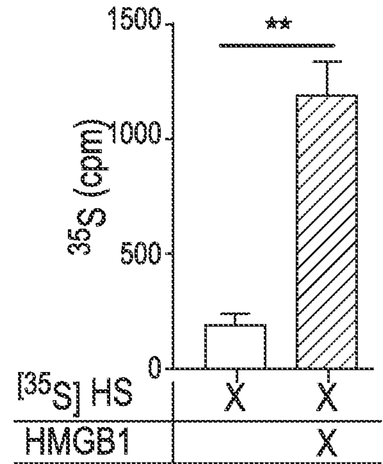


FIG. 5B

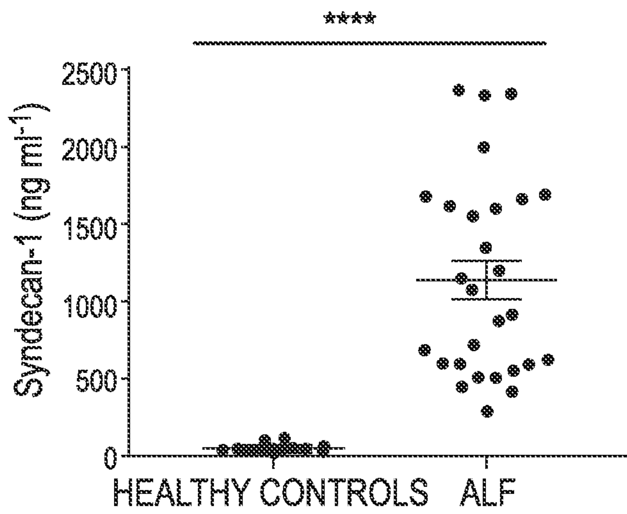


FIG. 5C

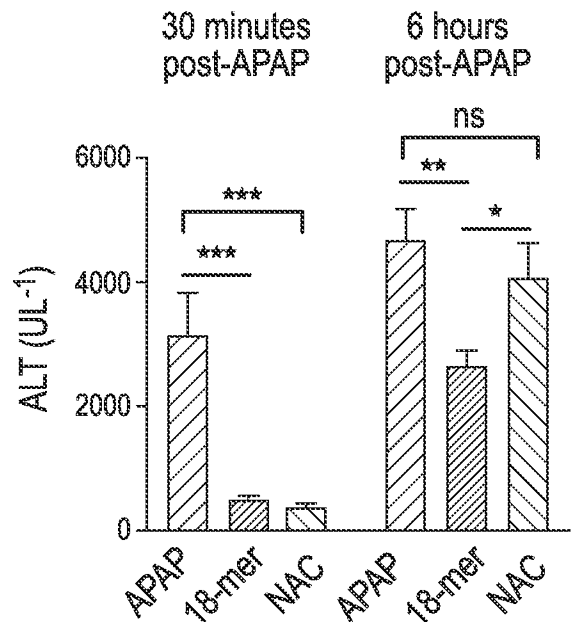


FIG. 5D

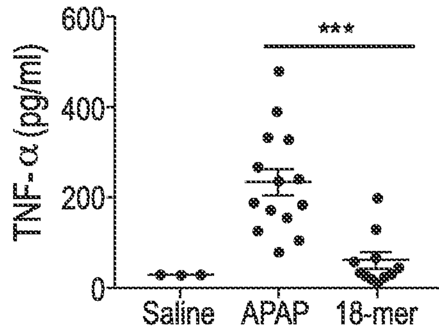


FIG. 6A

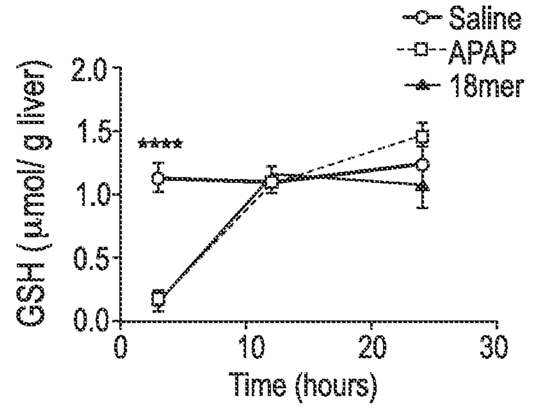


FIG. 6B

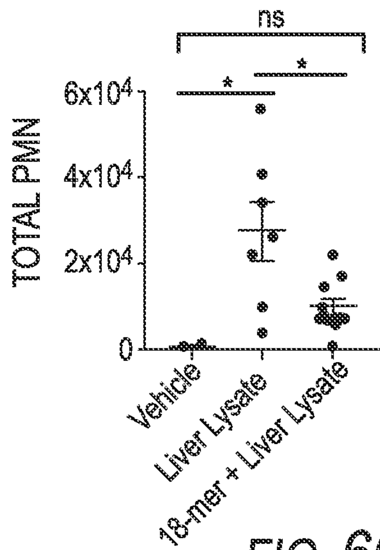


FIG. 6C

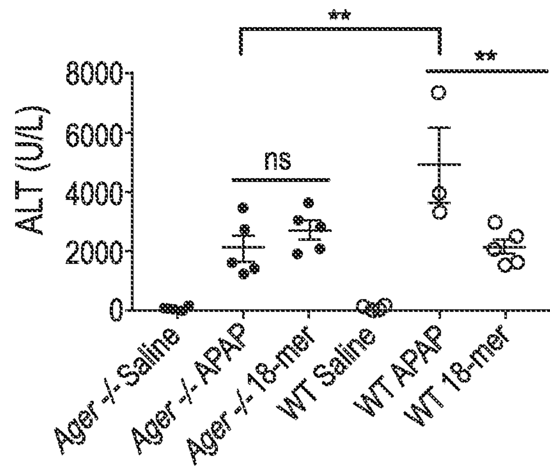


FIG. 6D

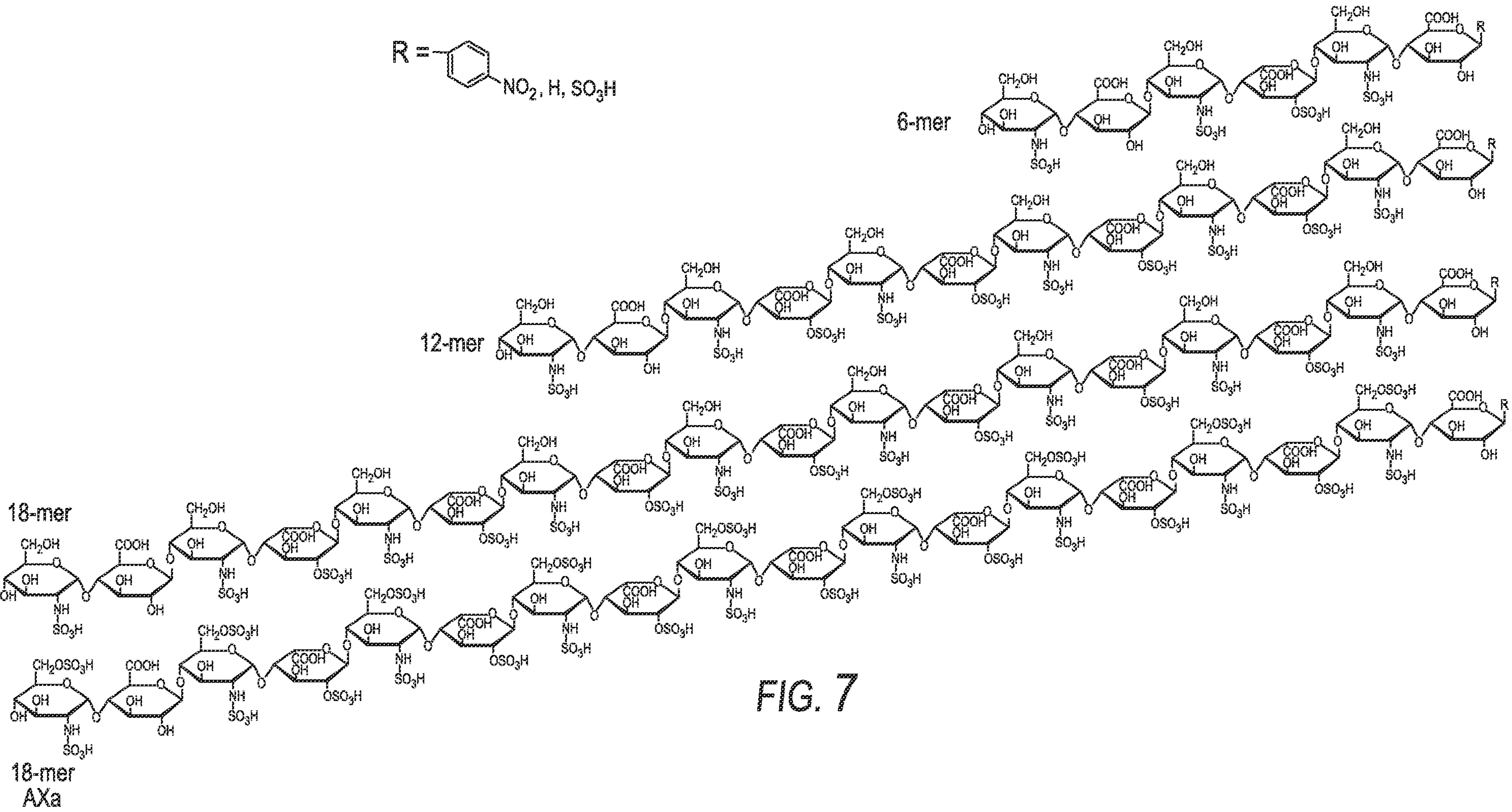


FIG. 7

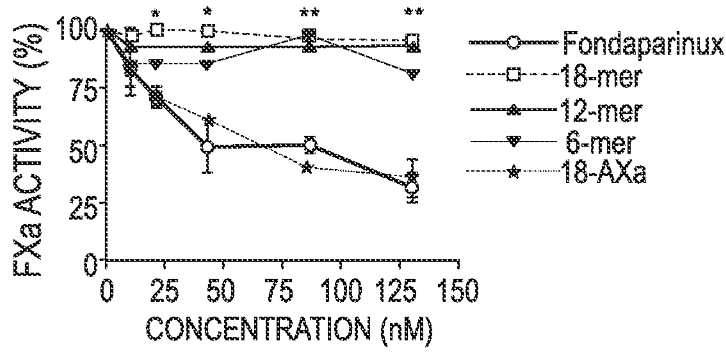


FIG. 8A

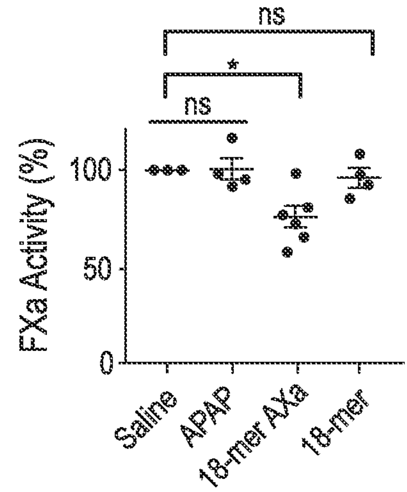


FIG. 8B

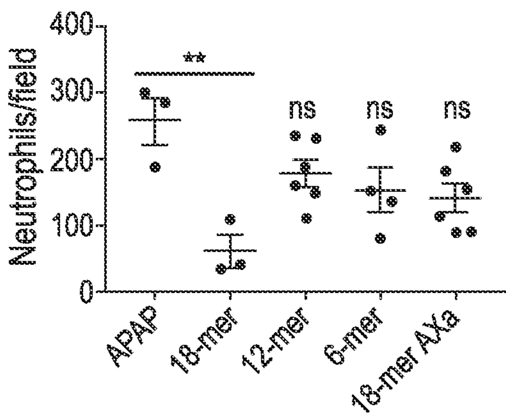


FIG. 8C

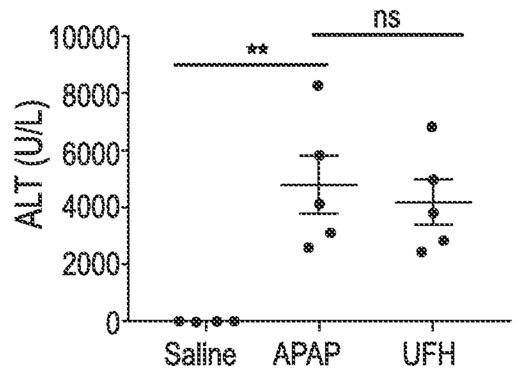


FIG. 8D

ALT (U/L)
5000
4000
3000
2000
1000
0
ns
*
****
0 10 20 30
Time (hours)

FIG. 9A

Neutrophils/field
2000
1500
1000
500
0
*
***
0 12 24
Time (hours)

FIG. 9B

HEPARAN SULFATE (ng/ml)
20000
16000
12000
8000
4000
0
n=2
166, 201
n=2
13650, 18670
HEALTHY CONTROLS
ALF

FIG. 10A

ALT (U/L)
5000
4000
3000
2000
1000
0
***
HEALTHY CONTROLS
ALF

FIG. 10B

HMGB1 (ng/ml)
20
15
10
5
0
**
HEALTHY CONTROLS
ALF

FIG. 10C

INTERNATIONAL SEARCH REPORT

International application No. PCT/US 18/59152

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/70 (2019.01)
CPC - A61K 31/70; A61K 31/715; A61K 31/726; A61K 31/727

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)
See Search History Document

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

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

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 2010/0298260 A1 (SUNDARAM et al.) 25 November 2010 (25.11.2010), entire document, especially: para [0001]; para [0003]; para [0004]; para [0005]; para [0019].	1, 3/1, 11 ----------- 26
Y	GANEY et al. "Role of the Coagulation System in Acetaminophen-Induced Hepatotoxicity in Mice", HEPATOLOGY. 2007. Vol. 46(4), pp1177-1186, entire document, especially: abstract; pg 1182, col 2, para 2.	26
A	CASSINELLI et al. "Old and new applications of non-anticoagulant heparin", International Journal of Cardiology. 2016. 212S1 pp S14-S21, entire document, especially: pg S17, Fig. 1.	1-3, 11-12, 26-27

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier 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	Date of mailing of the international search report
2 January 2019	06 MAR 2019

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/59152

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.: 4-10, 13-25, 28-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
--SEE ATTACHED EXTRA SHEET--

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

2. ☐ As all searchable claims could be searched without effort justifying 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, 3/1, 11, 26

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 18/59152

--BOX III--

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, 3/1, 11 and 26 drawn to a small molecule compound having an anti-inflammatory property, the small molecule compound comprising a non-anticoagulant heparan sulfate oligosaccharide molecule, high mobility group box 1 (HMGB1) protein in a manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation end products (RAGE).

Group II: Claims 2, 3/2, 12 and 27, drawn to a method of treating a subject with a compound comprising one of the structural formulas given in claim 2, the method comprising: providing a subject to be treated, wherein the subject is suffering from inflammation; and administering to the subject a non-anticoagulant heparan sulfate oligosaccharide molecule having an anti-inflammatory property.

The inventions listed as Groups I-II 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 includes the speciial technical feature of a method of treating a disease with a compound of structural formula given in claim 2, not required by group I

Group I includes a small molecule compound having an anti-inflammatory property, the small molecule compound comprising a non-anticoagulant heparan sulfate oligosaccharide molecule, high mobility group box 1 (HMGB1) protein in a manner sufficient to affect an interaction between the HMGB1 protein and a receptor for advanced glycation end products (RAGE), not required by group II

Shared Common Features

The only feature shared by Groups I and II that would otherwise unify the groups is a method of treating a disease with an anti-inflammatory non-anticoagulant heparan sulfate. However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is anticipated by the article entitled "Old and new applications of non-anticoagulant heparin" by Cassinelli et al. (hereinafter 'CASSINELLI'). Cassinelli teaches a non-anticoagulant heparan sulfate oligosaccharide molecule (pg S17, Fig. 1) with anti-inflammatory properties (abstract, The aim of this chapter is to provide an overview of non-anticoagulant effects of heparins and their potential use in new therapeutic applications. Heparin and heparin derivatives have been tested in inflammatory, pulmonary and reproductive diseases, in cardiovascular, nephro- and neuro-tissue protection and repair, but also as agents against angiogenesis, atheroschlerosis, metastasis, protozoa and viruses. Targeting and inhibition of specific mediators involved in the inflammatory process, promoting some of the above mentioned pathologies, are reported along with recent studies of heparin conjugates and oral delivery systems).

As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups. Groups I, and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note reg. item 4: Claims 4-10, 13-25 and 28-36 are unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a). These claims are therefore, not included in the above analysis.

Note: Group I was described incompletely/incorrectly. The correct definition for Group I is as follows:

Group I: Claims 1, 3/1, 11 and 26, directed to a method of treating Paracetamol (APAP) overdose in a subject, the method comprising: providing a subject in need of treatment for APAP overdose; administering to the subject a non-anticoagulant heparan sulfate oligosaccharide molecule having an anti-inflammatory property.