

SMALL MOLECULE ICE RECRYSTALLIZATION INHIBITORS AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

[0001] The present application pertains to the field of cryopreservation and sub-zero storage of biological material. More particularly, the present application relates to small molecule ice recrystallization inhibitors, and methods of manufacture and uses thereof in cryopreservation and sub-zero storage.

BACKGROUND

[0002] Cryopreservation remains the most common method for the long-term storage of cells and can also be used for long-term storage of tissue. However, commonly during cryopreservation a significant number of cells experience irreparable damage due to the growth of ice, ultimately resulting in decreased post-thaw recoveries or impaired function. As cell-based therapeutics continue to define new models of care, for example, in stem cell therapy, regenerative medicine and transfusion, it is becoming increasingly important to ensure the highest level of post-thaw cell viability and functionality.

[0003] Currently, cryopreservation solutions include cryoprotective agents (CPAs), such as glycerol, dimethyl sulfoxide (DMSO) and hydroxyethyl starch (HES), which are used to mitigate cell damage during cyropreservation. However, cryopreservation solutions can include these CPAs at concentrations that can be toxic and damage the cells that they are meant to preserve.

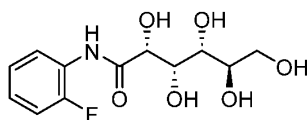
[0004] Apart from the cytotoxicity of currently used CPAs, a significant short fall of most current cryopreservation solutions and protocols is their failure to control ice recrystallization. These CPAs do not protect against cell damage that ice crystals can cause when samples are inappropriately stored or undergo unwanted transient warming during handling, storage, and shipment. Much of the cellular damage that occurs during cryopreservation results from uncontrolled growth of ice crystals during cooling and/or during warming and thawing following storage. This process is known as ice recrystallization

and ultimately results in decreased post-thaw viability and functional capacity of the cells and tissues that have been cryopreserved. Ice recrystallization is a form of ice crystal re-modeling that occurs during freezing, maintenance at sub-zero temperatures and during warming from sub-zero temperatures and which results in the growth of large ice crystals at the expense of small ice crystals. Ice recrystallization is a significant factor contributing to cell death from or during sub-zero storage of cells, tissues and organs. This is evidenced by the membrane damage observed following cryopreservation of cells.

[0005] Stem cell and regenerative therapy using cryopreserved cells has been hampered by decreased cell function and viability after thawing. Consequently, improved cryopreservation protocols that increase the yield of viable and functional cells are urgently required. Improved cryopreservation compositions and methods have the potential to revolutionize cell and gene therapies.

[0006] Freeze-tolerant organisms have developed mechanisms to avoid the problems associated with ice recrystallization by producing large quantities of biological antifreeze proteins and glycoproteins that function, at least in part, as ice recrystallization inhibitors (IRIs) *in vivo*. Unfortunately, biological antifreeze proteins and glycoproteins are typically not suitable for use as cryoprotectants in cryopreservation solutions because of their ice binding activity and/or dynamic ice shaping, which can exacerbate cellular damage. In addition, use of biological antifreeze proteins and glycoproteins is limited due to their poor solubility and the cost associated with isolation or manufacture.

[0007] Various polymers have been explored as an alternative to DMSO and glycerol, but thus far have failed to provide the high cell viabilities observed with DMSO or glycerol. Similarly, various sugars (mono- di- and oligosaccharides) have also been investigated as cryoprotectants. However, the structure of the carbohydrate, the freezing protocol, cell type and reported cell viabilities vary dramatically between studies making it difficult to ascertain the true ability of these compounds to protect cells against cryo-injury. More recently studies have pursued other small molecules that can function as IRIs. Following such studies, a particularly efficient ice recrystallization inhibitor, referred to as 2FA (depicted below), was identified to be useful in formulations for cryopreservation in cell and gene therapy applications.



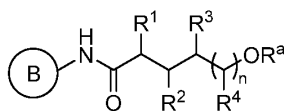
2FA

[0008] However, a need remains for alternative small molecule IRIs, and for small molecule IRIs that exhibit improved characteristics, for example improved solubility, while retaining the ability to control and/or reduce ice recrystallization.

[0009] The above information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

[0010] An object of the present application is to provide ice recrystallization inhibitors, and methods of synthesis and use thereof. In accordance with an aspect of the present application, there is provided an ice recrystallization inhibitor (IRI) compound of Formula A



A

where:

B is phenyl; phenyl mono- or di-substituted with C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F); or an alkyl;

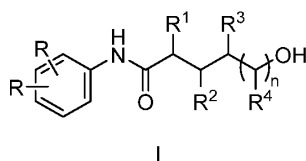
R^a is H when B is phenyl or substituted phenyl, and H or alkyl (e.g., a C₁-C₄ alkyl) when B is alkyl;

R^1 , R^2 , R^3 and each R^4 are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein at least one of the R^1 , R^2 , R^3 and R^4 substituents is not OH;

n is an integer from 1 to 7, wherein when n is greater than 1 each R^4 is the same or different; and

each $R^5 - R^{12}$ is independently H, alkyl or aryl.

[0011] In some embodiments, the IRI compound is a compound of Formula I



wherein:

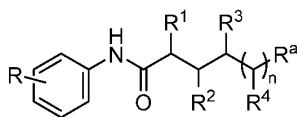
each R is independently hydrogen, C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F);

R^1 , R^2 , R^3 and each R^4 are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein at least one of the R^1 , R^2 , R^3 and R^4 substituents is not OH;

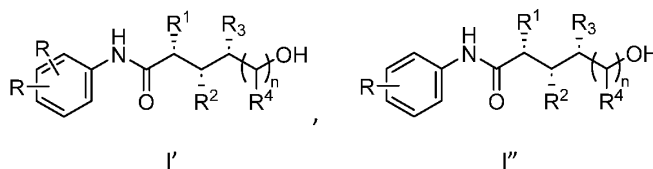
n is an integer from 1 to 7; and

each $R^5 - R^{12}$ is independently H, alkyl or aryl.

[0012] In some embodiments, the compound of Formula I has the structure of:

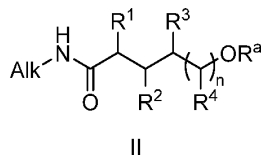


[0013] Optionally, the compound of Formula I has the structure of Formula I' or I''



In some examples of this embodiment, R³ is H.

[0014] In accordance with other embodiments, the IRI compound is a compound of Formula II



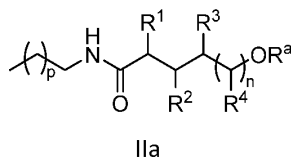
wherein:

Alk is a C₃ – C₉ linear or branched alkyl or a C₃ – C₁₀ mono- or poly-cyclic alkyl.

[0015] In some examples of this embodiment, Alk is a straight chain C₁ to C₉ alkyl.

Alternatively, Alk is a C₃ to C₉ branched alkyl (e.g., *t*-butyl) or a C₃-C₁₀ cycloalkyl, which is optionally a C₃ to C₈ monocyclic alkyl or adamantyl.

[0016] In accordance with some embodiments, the IRI compound is a compound of Formula IIa



where:

R¹, R², R³ and each R⁴ are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein at least one of the R¹, R², R³ and R⁴ substituents is not OH;

R^a is H or alkyl (e.g., a C₁-C₄ alkyl);

n is an integer from 1 to 7;

p is an integer from 1 to 7, or from 5 to 7, or p is 6; and

each R⁹ – R¹² is independently H, alkyl or aryl.

[0017] In accordance with another aspect of the present application, there is provided a composition, for example, a cryopreservation composition, wherein the composition

comprises an IRI compound as described herein and a solvent (e.g., water) or a buffer. Optionally, the composition further comprises a cryopreservation agent, such as, but not limited to DMSO, lactobionate, glycerol, polyvinylalcohol, hydroxyethyl starch (HES), ethylene glycol (EG), propylene glycol (PG), trehalose, mannitol, or a combination of two or more thereof. In addition, or in the alternative, the composition further comprises, a biological material. The biological material can be from, for example, a human, an animal, a plant, a fungus or a microorganism. In some non-limiting embodiments, the biological material is an organ, a tissue, cells or platelets, or a combination thereof. The cells can comprise, for example, stem cells, T cells (including, for example, CAR-T cells), peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells (including, for example, natural killer (NK) cells), endothelial cells, pancreatic islet cells, dendritic cells, fibroblasts or cells from a cell line, preferably the cells comprise stem cells, T cells (including, for example, CAR-T cells), red blood cells, progenitor cells, or liver cells. In some examples in which the composition is for use with cells or comprises cells, the composition additionally comprises a cell culture medium (e.g., phosphate buffered saline optionally comprising albumin, Eagle's minimum essential medium, Dulbecco's modified Eagle's medium, RPMI, fetal bovine serum, fetal calf serum, Ham's F-10, Ham's F-12, Medium 199, Hank's Buffered Salt Solution, Hank's Buffered Salt Solution and dextrose, or a combination thereof).

[0018] In accordance with another aspect of the present application, there is provided a method for cryopreserving a biological material (such as defined above) comprising:

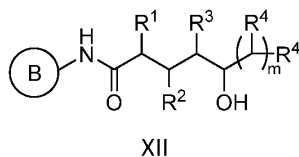
- a) combining (e.g., suspending, permeating, perfusing, immersing or infusing) the biological material with a solution comprising at least one IRI compound as defined herein;
- b) cooling the combination formed in step a) to a storage temperature at or below the freezing point of the solution; and
- c) storing the combination at the storage temperature.

[0019] In some embodiments of the above method, cooling comprises performing a rate-controlled cooling of about 1°C per minute over about 16 hours.

[0020] In accordance with another aspect of the present application, there is provided a use of an IRI compound as defined herein to:

- a. reduce toxicity during cryopreservation in comparison to cryopreservation in the absence of the IRI compound as defined herein;
- b. improve viability and/or functionality of biological material following cryopreservation in comparison to cryopreservation in the absence of the IRI compound as defined herein;
- c. improve stability of biological material during temperature cycling in cryopreservation in comparison to temperature cycling in cryopreservation in the absence of the IRI compound as defined herein; and/or
- d. facilitate cryopreservation at a warmer temperature in comparison to cryopreservation in the absence of the IRI compound as defined herein.

[0021] In accordance with another aspect of the present application, there is provided a method for synthesizing a compound of



wherein:

B is phenyl; phenyl mono- or di-substituted with C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F); or an alkyl;

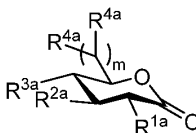
R¹, R², R³ and each R⁴ are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺ or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein the terminal R⁴ is OH;

m is an integer from 1 to 6; and

each R⁵ – R¹² is independently H, alkyl or aryl,

said method comprising:

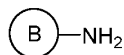
(a) providing a lactone of Formula VIII



VIII

wherein R^{1a} , R^{2a} , R^{3a} and each R^{4a} are independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, -[NH(R¹²)₂]⁺ where each R¹² is the same or different, a protected alcohol, or a protected amine, wherein the terminal R^{4a} is OH;

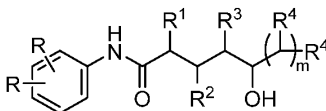
(b) ring opening the compound of Formula VIII with an aryl or alkyl amine of Formula VII



VII; and

(c) optionally, deprotecting the product of step (b).

[0022] In accordance with some embodiments, there is provided a method for synthesizing a compound of Formula IIIa



XIIa

wherein:

each R is independently hydrogen, C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO, haloalkyl or halogen (e.g., Cl, Br, I or F);

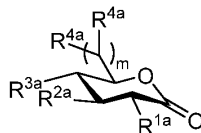
R¹, R², R³ and each R⁴ are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺ or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein the terminal R⁴ is OH;

m is an integer from 1 to 6; and

each R⁵ - R¹² is independently H, alkyl or aryl,

said method comprising:

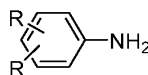
(a) providing a lactone of Formula VIII



VIII

wherein R^{1a} , R^{2a} , R^{3a} and each R^{4a} are independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, -[NH(R¹²)₂]⁺ where each R^{12} is the same or different, a protected alcohol, or a protected amine, wherein the terminal R^{4a} is OH;

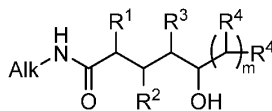
(b) ring opening the compound of Formula VIII with an aryl amine of Formula VIIa



VIIa ; and

(c) optionally, deprotecting the product of step (b).

[0023] In other embodiments, the synthetic method is for synthesizing a compound of formula IIIb:

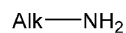


XIIb

wherein:

Alk is a C₃ – C₉ linear or branched alkyl or a C₃ – C₁₀ mono- or poly-cyclic alkyl; and

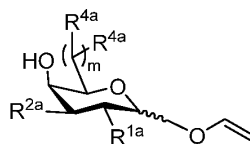
wherein the compound Formula VII is an alkyl amine having the structure of Formula VIIb:



VIIb.

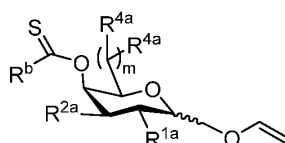
[0024] In some embodiments of the synthetic method, in which the compound of Formula III includes R^3 is H, prior to step (a), a compound of Formula VIII, in which R^{3a} is H, is first produced by steps comprising:

- i. providing a protected O-allyl pyranose of Formula IX



IX ;

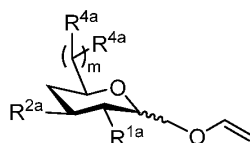
- ii. converting the protected O-allyl pyranose of Formula IX to a thiocarbonyl derivative of Formula X



X

wherein R^b is H, CH₃, SCH₃, OCH₃, phenyl (Ph), OPh or imidazolyl, preferably imidazolyl;

- iii. treating the thiocarbonyl derivative of Formula X with a radical reducing agent to produce a C4-deoxy pyranose derivative of Formula XI

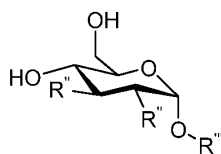


XI ;

- iv. converting the C1 allyl group in the compound of Formula XI to a C1 hydroxyl group; and
v. oxidizing the C1 hydroxyl group to form the lactone of Formula VIII.

[0025] In accordance with another aspect of the present application, there is provided a method for manufacture of a compound of Formula I, I as defined above, wherein n is 2, R³ is H and R⁴ is OH, comprising the steps of:

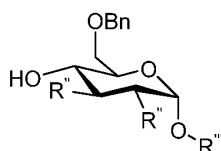
- (i) converting a protected D-pyranose of Formula III to a benzylidene acetal



III

where each R'' is independently H or OH and $O-R'''$ is a hydroxyl protecting group, such as methoxy or silyl ether (e.g., a bulky silyl ether);

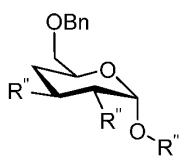
- (ii) when one or both of the R'' groups are OH, protecting the OH group or groups, and subsequently performing a selective ring opening of the benzylidene acetal to form a C4 acceptor compound of Formula IV



IV

wherein each R'' is independently H or OPr^1 , where Pr^1 is a hydroxyl protecting group (such as, but not limited to, a benzyl group);

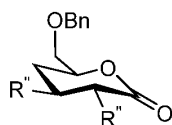
- (iii) converting the C4 hydroxyl group of the compound of Formula IV into a triflate and displacing the triflate to produce a C4 deoxy pyranose derivative of Formula V



V

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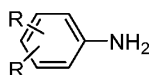
- (iv) deprotecting the anomeric protected group and oxidizing the resultant hydroxyl to produce the lactone of Formula VI



VI

; and

- (v) ring opening the lactone of Formula VI with an aryl amine of Formula VIIa



VIIa

wherein each R is independently hydrogen, C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F), and removing the hydroxyl protecting group(s) to form the compound of Formula I as defined above, wherein n is 2, R³ is H and R⁴ is OH.

BRIEF DESCRIPTION OF TABLES AND FIGURES

[0026] For a better understanding of the application as described herein, as well as other aspects and further features thereof, reference is made to the following description which is to be used in conjunction with the accompanying drawings, where:

[0027] Figure 1 depicts dose response curves generated for the known IRI, 2FA, and for C4 Deoxy 2FA, an example of an IRI compound of the present application, after analysis of crystal size using a splat cooling assay.

DETAILED DESCRIPTION

[0028] Definitions

[0029] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0030] As used in the specification and claims, the singular forms “a”, “an” and “the” include plural references unless the context clearly dictates otherwise.

[0031] The term “comprising”, as used herein, will be understood to mean that the list following is non-exhaustive and may or may not include any other additional suitable items, for example one or more further feature(s), component(s) and/or ingredient(s) as appropriate.

[0032] Reference throughout this specification to “one embodiment,” “an embodiment,” “another embodiment,” “a particular embodiment,” “a related embodiment,” “a certain embodiment,” “an additional embodiment,” or “a further embodiment” or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[0033] The term “and/or” as used in a phrase such as “A and/or B” herein is intended to include “A and B”, “A or B”, “A”, and “B”.

[0034] The term “alkoxy”, as used herein, refers to straight-chain or branched alkyl group bonded to an oxygen. In some embodiments, the alkoxy group includes an alkyl having 1 to about 10 carbons, or 1 to about 8 carbons, or 1 to 4 carbons. Examples include, but are not limited to, methoxy, ethoxy, propoxy, butoxy, pentoxy, hexoxy, heptoxy and octoxy. By way of example, the term “C1-C4-alkoxy” refers to an alkoxy having 1 to 4 carbon atoms, such as, but not limited to, methoxy, ethoxy, *n*-propoxy, 1-methylethoxy, *n*-butoxy, 1-methylpropoxy, 2-methylpropoxy and 1,1-dimethylethoxy. “Alkoxy” is intended to embrace all structural isomeric forms of an alkoxy group. For example, as used herein, propoxy encompasses both *n*-propoxy and isopropoxy, etc.

[0035] The term “alkyl”, as used herein, refers to a saturated hydrocarbon chain of 1 to about 12, or 1 to about 8, carbon atoms in length, such as, but not limited to, methyl, ethyl, propyl and butyl. The alkyl group may be a straight-chain, a branched-chain or cyclic. By way of example, the term “C1-C4-alkyl” as used herein refers to a saturated straight-chain or

branched hydrocarbon having 1 to 4 carbon atoms. "Alkyl" is intended to embrace all structural isomeric forms of an alkyl group. For example, as used herein, propyl encompasses both *n*-propyl and isopropyl; butyl encompasses *n*-butyl, *sec*-butyl, isobutyl and *tert*-butyl. Encompassed within cyclic alkyl (or "cycloalkyl") groups are mono- and poly-cyclic alkyl groups (e.g., spiro, fused or bridged), including, for example, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, and adamantyl.

[0036] The term "alkylamine", as used herein, refers to a group comprising one or two straight-chain or branched alkyl groups bonded to a nitrogen (-NHR or -NR₂, where R is an alkyl and each R is the same or different). In some embodiments, the alkyl(s) in the alkylamine includes 1 to about 10 carbons, or 1 to about 8 carbons, or 1 to 4 carbons. Examples include, but are not limited to, amino groups comprising a single methyl, ethyl, propyl or butyl and amino groups comprising two alkyl groups each independently selected from methyl, ethyl, propyl or butyl. By way of example, the term "C1-C4-alkylamine" refers to an alkylamine having 1 to 4 carbon atoms, such as, but not limited to, methylamine, ethylamine, *n*-propylamine, 1-methylethylamine, *n*-butylamine, methylpropylamine. "Alkylamine" is intended to embrace all structural isomeric forms of an alkylamine group. For example, as used herein, when alkyl(s) in the alkylamine is propyl, this encompasses both *n*-propoxy and isopropoxy, etc.

[0037] The term "aryl", as used herein, refers to a monocyclic or polycyclic aromatic group, such as, but not limited to, phenyl, naphthyl, thienyl and indolyl.

[0038] The term "arylamine", as used herein, refers to a group comprising one or two aryl groups bonded to a nitrogen (-NHR or -NR₂, where R is an aryl and each R is the same or different). In some embodiments, the aryl is phenyl or a substituted phenyl.

[0039] The term "biological material", as used herein, refers to any substance that can be or has been removed from a plant, a fungus, a human or a non-human animal that is suitable for cryopreservation, such as, but not limited to, organs, tissues (including, for example, organoids), cells, platelets, eggs, and, embryos. Alternatively, the biological material can be cultured cells, tissues or organs. Examples of "cells" include, but are not limited to, stem cells, T cells (including CAR-T cells), oocytes, sperm, peripheral blood mononuclear cells

(PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells (e.g., NK cells), endothelial cells, fungal cells, pancreatic cells (e.g., pancreatic islet cells) dendritic cells, fibroblasts or cells from a cell line. In another alternative, the “cells” can be unicellular microorganisms, including, for example, bacterial or unicellular fungal (e.g., yeast) cells. The cells can be in an isolated or a purified form, or they can be in a mixture with one or more other cell types.

[0040] In some embodiments, the biological material is from a non-human animal or human source. In alternative embodiments, the biological material is from a plant source or a fungal source. For example, the plant-sourced biological material can be a plant cell, tissue or organ or can be plant seed or fruit, or a part thereof.

[0041] In some embodiments, the biological material is food.

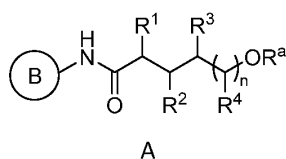
[0042] The terms “cell medium”, “cell culture medium”, and “growth medium”, are used interchangeably herein to refer to a liquid or gel composition used to support cellular growth of cells, tissues or organs in an artificial environment. Examples of cell culture media include, but are not limited to, Eagle's Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), Fetal Bovine Serum (FBS), Fetal Calf Serum (FCS), Ham's F-10, Ham's F-12, Hank's buffered salt solution (HBSS), HBSS and dextrose, and Medium 199.

[0043] The terms “cryopreservation agent”, “cyroprotecting agent” and “cyroprotectant” are used interchangeably herein to refer to a compound that assists in the cryopreservation of a biological material. Examples of suitable cryopreservation agents include, but are not limited to, DMSO, lactobionate, glycerol, trehalose, mannitol, hydroxyethyl starch (HES), ethylene glycol (EG), propylene glycol (PG), and other biopolymers useful in cryopreservation. Examples of suitable biopolymers include, but are not limited to, polyvinyl alcohols. As used herein, the term “cryopreservation agent” is not intended to include water, RPMI, DMEM, or MEM, HBSS or dextrose.

[0044] The present inventors have previously found that the aryl-glycosides, such as the ice recrystallization inhibitor (IRI) 2FA, shown above, can be successfully used in cryopreservation formulations, for example for use in cell and gene therapy applications. However, the present inventors have now identified a subset of aldonamides with improved solubility over the previously studied aryl-glycosides, while retaining good ice recrystallization inhibition activity. As used herein, “good” ice recrystallization inhibition is intended to refer to an IC_{50} < about 45 mM, or preferably an IC_{50} < about 35 mM.

[0045] The present inventors have now identified a specific class of deoxy aldonamide derivatives of 2FA, such as C4-deoxy N-aryl-aldonamides and C4-deoxy N-alkyl-aldonamides, having similar ice recrystallization inhibition activity to 2FA, but with higher solubility in aqueous buffers, in comparison to 2FA. The increased solubility of these compounds permits the use of higher concentrations of the compounds in cryopreservation formulations. This is of particular value since concentration is a function of temperature and cryopreservation formulations are used at subzero temperatures, which means increased solubility is useful for maximizing the amount of compound present in the formulations for cryopreservation. In addition to the ice recrystallization inhibitory activity of the present IRIs, the inventors have surprisingly found these compounds to have generally low cytotoxicity (e.g., in comparison to CPAs currently in use). Thus, in some embodiments, the combination of improved solubility and relatively low toxicity, further facilitates the use of these IRI compounds at higher concentrations in cryopreservation formulations.

[0046] Accordingly, the present application provides IRI compounds of Formula A:



where:

B is phenyl; phenyl mono- or di-substituted with C_1 - C_4 -alkyl, C_1 - C_4 -alkoxy, $-\text{NH}_2$, $-\text{NR}^5\text{H}$, $-\text{N}(\text{R}^6)_2$ where each R^6 is the same or different, $-\text{C}(\text{O})\text{R}^7$, $-\text{COOH}$, $-\text{COOR}^8$, $-\text{CHO}$ or halogen (e.g., Cl, Br, I or F); or an alkyl;

R^a is H when B is phenyl or substituted phenyl, and H or alkyl (e.g., a C_1 - C_4 alkyl) when B is alkyl;

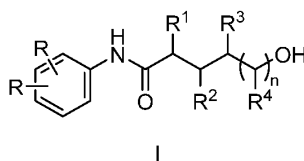
R^1 , R^2 and R^3 are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein at least one of the R^1 , R^2 , R^3 and R^4 substituents is not H;

n is an integer from 1 to 7; and

each $R^5 - R^{12}$ is independently H, alkyl or aryl.

[0047] For greater certainty, as used throughout the present application to reference selection of alternative substituents, use of the term “independently” indicates that each substituent is selected individually from the recited alternatives, without connection to or influence from the selection of other substituents.

[0048] In some embodiments of the present application the IRI compound of Formula A is a compound of Formula I:



where:

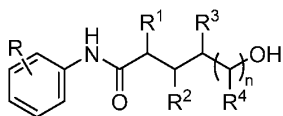
each R is independently hydrogen, C_1 - C_4 -alkyl, C_1 - C_4 -alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F);

R^1 , R^2 , R^3 and each R^4 are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein at least one of the R^1 , R^2 , R^3 and R^4 substituents is not OH;

n is an integer from 1 to 7, wherein when n is greater than 1 each R^4 is the same or different; and

each $R^5 - R^{12}$ is independently H, alkyl or aryl.

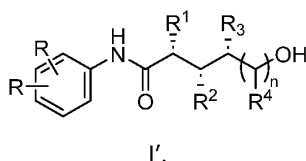
[0049] In some embodiments, the compound of Formula I includes a mono-substituted phenyl and has the following structure:



[0050] In some embodiments, R^1 , R^2 and R^3 are each independently H, -OH, or -OR⁹ wherein at least one of the R^1 , R^2 and R^3 substituents is not -OH or -OR⁹.

[0051] In some embodiments, R^3 is H. The C4-deoxy moiety has been found to contribute to improved solubility in buffers, for example PBS, in comparison to the corresponding C4-OH compounds.

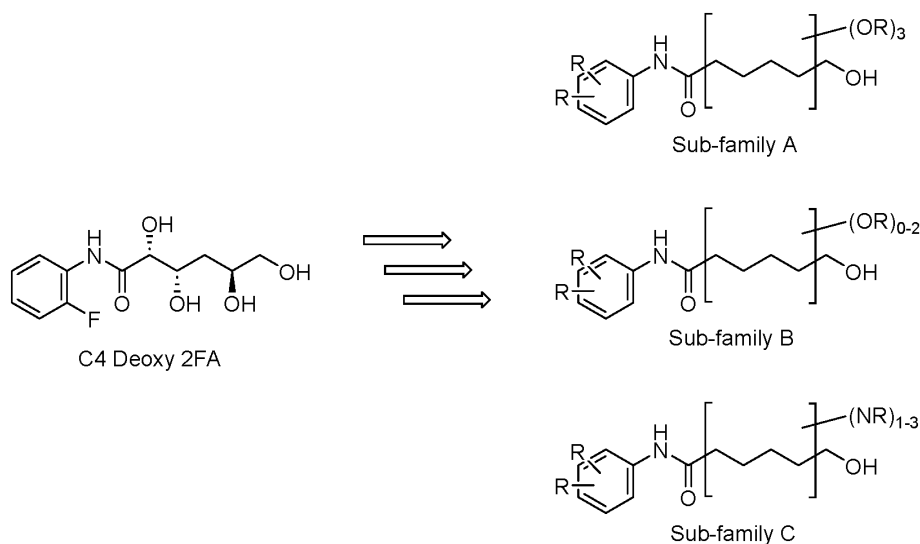
[0052] In certain embodiments, the IRI compound of Formula I has the stereochemistry as shown in Formula I'



[0053] However, it should be recognized that the compound of Formula I can also occur as a stereoisomer of the compound of Formula I', or as a mixture of stereoisomers, which optionally includes the stereoisomer of Formula I'. The compound of Formula I can be used in cryopreservation formulations as described herein, in a single stereoisomeric form or as a mixture of two or more stereoisomers.

[0054] In some embodiments, when $n = 2$, the IRI compound of Formula I of the present application can be grouped into three sub-families, as summarized in Scheme 1 below, which illustrates the three sub-families in relation to a specific C4-deoxy-derivative of 2FA, referred to herein as "C4 Deoxy 2FA". Sub-family A includes three oxygen-containing substituents (e.g., three hydroxy or alkoxy substituents, or a combination thereof), in the aldonamide chain. Sub-family B includes one or two oxygen-containing substituents (e.g., hydroxy and/or alkoxy substituents) in the aldonamide chain. In sub-families A and B, when

the compound includes two or more alkoxy substituents, these substituents may be the same or different from one another. Sub-family C includes one, two or three alkylamine substituents, where when there are two or three alkylamine substituents, they may be the same or different from one another. In specific embodiments of these sub-families, the compounds are C-4 deoxy aryl-aldonamide derivatives.

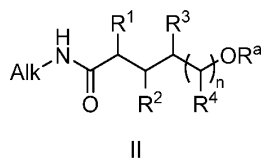


Scheme 1: Aryl aldonamide derivative ice recrystallization inhibitor Sub-families

[0055] Although Scheme 1 depicts compounds including a mono-substituted phenyl bound to the nitrogen of the amide group, it should be understood that the analogous compounds comprising a di-substituted phenyl or an alkyl group at this position are also encompassed by the present application, as detailed above.

[0056] In some embodiments, the compound of Formula I is not C4 Deoxy 2FA, as depicted in Scheme 1.

[0057] In some embodiments of the present application the IRI compound of Formula A is a compound of Formula II:



where:

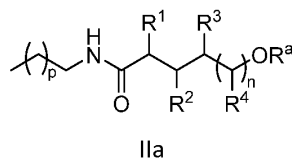
R^1 , R^2 , R^3 and R^4 are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein at least one of the R^1 , R^2 , R^3 and R^4 substituents is not OH;

R^a is H or alkyl (e.g., a C1-C4 alkyl);

n is an integer from 1 to 7, wherein when n is greater than 1 each R^4 is the same or different;

Alk is a C₃ – C₉ linear or branched alkyl or a C₃ – C₁₀ mono- or poly-cyclic alkyl.; and
each R⁹ – R¹² is independently H, alkyl or aryl.

[0058] In some embodiments, the compound of Formula II is a compound of Formula IIa:



where:

p is an integer from 1 to 7, or from 5 to 7, or p is 6.

[0059] In some embodiments, R^1 , R^2 and R^3 are each independently H, -OH, or -OR⁹ wherein at least one of the R^1 , R^2 and R^3 substituents is not -OH or -OR⁹.

[0060] In some embodiments, R^3 is H. The C4-deoxy moiety has been found to contribute to improved solubility in buffers, for example PBS, in comparison to the corresponding C4-OH compounds.

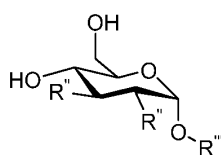
[0061] Synthesis of C4 Deoxy aldonamide IRI Compounds

[0062] The IRI compounds of the present application can be manufactured using a range of different synthetic routes using commercially available starting materials.

[0063] Provided below are non-limiting, examples of synthetic methods for manufacturing the compound of Formula I. However, the IRI compounds of the present application can be synthesized using alternative methods, as would be well appreciated by the skilled person.

[0064] In accordance with one embodiment, there is provided a method for manufacture of a compound of Formula I, I as defined above, wherein n is 2, R^3 is H and R^4 is OH, comprising the steps of:

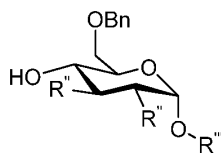
- (i) converting a protected D-pyranose of Formula III to a benzylidene acetal



III

where each R'' is independently H or OH and $O-R'''$ is a hydroxyl protecting group, such as methoxy or silyl ether (e.g., a bulky silyl ether);

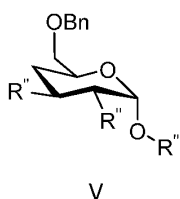
- (ii) when one or both of the R'' groups are OH, protecting the OH group or groups, and subsequently performing a selective ring opening of the benzylidene acetal, for example using sodium cyanoborohydride, or a related bulky borohydride reducing agent or TES/TFA, to form a C4 acceptor compound of Formula IV



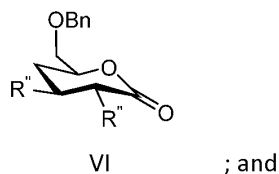
IV

wherein each R'' is independently H or OPr^1 , where Pr^1 is a hydroxyl protecting group (such as, but not limited to, a benzyl group);

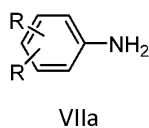
- (iii) converting the C4 hydroxyl group of the compound of Formula IV into a triflate and displacing the triflate, for example using sodium borohydride, to produce a C4 deoxy pyranose derivative of Formula V



- (iv) deprotecting the anomeric protected group and oxidizing the resultant hydroxyl (e.g., via an Albright-Goldman oxidation, Swern oxidation, a Dess-Martin or a PCC (pyridinium chlorochromate) oxidation) to produce the lactone of Formula VI



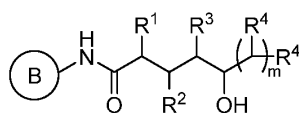
- (v) ring opening the lactone of Formula VI with an aryl amine of Formula VIIa



wherein R is hydrogen, C_1 - C_4 -alkyl, C_1 - C_4 -alkoxy, $-NH_2$, $-NR^5H$, $-N(R^6)_2$ where each R^6 is the same or different, $-C(O)R^7$, $-COOH$, $-COOR^8$, $-CHO$ or halogen (e.g., Cl, Br, I or F), and removing the hydroxyl protecting group(s) to form the compound of Formula I as defined above, wherein n is 2, R^3 is H and R^4 is OH.

[0065] In some embodiments of the synthetic method for manufacture of a compound of Formula I described above, the compound of Formula III employed in step (i) is a methoxy-D-pyranoside, for example, methoxy-D-glucose.

[0066] In another embodiment, there is provided an alternative method for manufacture of a compound of Formula I having the structure of Formula XII



XII

wherein:

B is phenyl; phenyl mono- or di-substituted with C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F); or an alkyl;

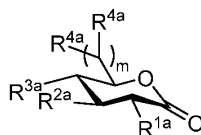
R¹, R², R³ and each R⁴ are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺ or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein the terminal R⁴ is OH;

m is an integer from 1 to 6; and

each R⁵ – R¹² is independently H, alkyl or aryl,

said method comprising:

(a) providing a lactone of Formula VIII

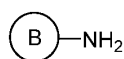


VIII

wherein R^{1a}, R^{2a}, R^{3a} and each R^{4a} are independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, -[NH(R¹²)₂]⁺ where each R¹² is the same or different, a protected alcohol, or a protected amine, wherein the terminal R^{4a} is OH;

(b) ring opening the compound of Formula VIII with an aryl amine of Formula

VII

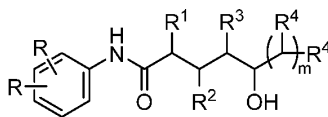


VII

; and

(c) optionally, deprotecting the product of step (b).

b) In one example of the above embodiment, the method is for manufacture of a compound of Formula XII has the structure of Formula XIIa:



XIIa

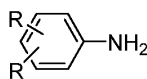
wherein:

each R is independently hydrogen, C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂

where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO, haloalkyl or halogen (e.g., Cl, Br, I or F), and

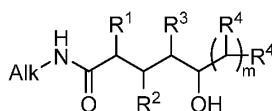
each R⁸ – R¹² is independently H, alkyl or aryl; and

wherein the compound of Formula VII is an aryl amine having the structure of Formula VIIa:



VIIa.

[0067] In another example of this embodiment, the method is for manufacture of a compound of Formula XII has the structure of Formula XIIb:

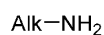


XIIb

wherein:

Alk is a C₃ – C₉ linear or branched alkyl or a C₃ – C₁₀ mono- or poly-cyclic alkyl; and

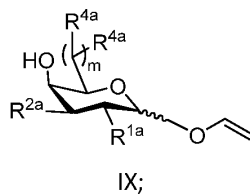
wherein the compound Formula VII is an alkyl amine having the structure of Formula VIIb:



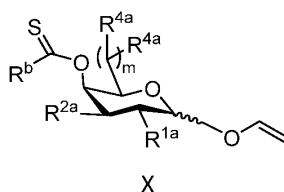
VIIb.

[0068] In one example of the above embodiments, the method for manufacture of a compound of Formula I, wherein R^3 is H and wherein prior to step (a), a compound of Formula VIII, in which R^{3a} is H, is first produced by the steps:

- (i) providing a protected O-allyl pyranose of Formula IX

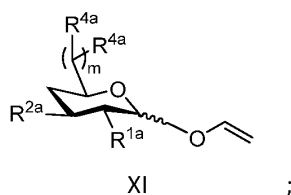


- (ii) converting the protected O-allyl pyranose of Formula IX to a thiocarbonyl derivative of Formula X



wherein R^b is H, CH_3 , SCH_3 , OCH_3 , phenyl (Ph), OPh or imidazolyl (preferably imidazolyl);

- (iii) treating the thiocarbonyl derivative of Formula X with a radical reducing agent to produce a C4 deoxy pyranose derivative of Formula XI



- (iv) converting the C1 allyl group of the derivative of Formula XI to a C1 hydroxyl group;
- (v) oxidizing the C1 hydroxyl group to form a lactone of Formula VII.

[0069] In some embodiments of the synthetic method for manufacture of a compound of Formula I described above, the protected O-allyl pyranose of Formula IX is a protected O-allyl galactose.

[0070] In other embodiments, the N-alkyl or N-aryl aldonamide IRI compounds of Formula I can be synthesized by an amidation of an alkyl or aryl amine of Formula VII (as defined above) with a protected aldonic acid or deoxyaldonic acid. For example, the aldonic acid or deoxyaldonic acid can include a terminal 1,3-dioxolane as a protecting group, alone or in combination with other hydroxyl or amine protecting groups, depending on the substituents on the aldonic acid. The resultant protected amide is then deprotected to produce an IRI compound of Formula I. In other examples, the 1,3-dioxolane protecting group is used to protect a hydroxyl group adjacent to the carboxylic acid moiety of the aldonic acid or deoxyaldonic acid.

[0071] As would be readily appreciated by the skilled person, the reagents and conditions used in the steps of the synthetic processes summarized above can be varied by incorporating standard alternatives.

[0072] Compositions for Preserving Biological Material

[0073] Also provided herein are compositions for preserving biological material comprising one or more IRI compounds of Formula I, optionally in combination with one or more cryoprotecting agents, as defined herein. The preservation compositions of the present application are suitable for use in cryopreservation, in which the compositions comprising the biological material are frozen, or for subzero preservation without freezing. In both instances, the presence of one or more IRI compounds of the present application aids in inhibiting ice recrystallization formation during cooling and during warming following cryopreservation. The preservation compositions of the present application can also be used in non-cryopreservation storage methods, such as, for example, hypothermic/vitrification storage methods and normalthermic storage methods.

[0074] In a particular embodiment, the at least one IRI compound is present in the composition at a concentration of less than about 400 mM, preferably less than about 200 mM, more preferably less than about 100 mM, even more preferably less than about 10

mM, still more preferably less than about 1 mM, and most preferably less than about 0.5 mM. In another embodiment, the IRI compound can be present in the composition at a concentration of about 0.5 mM to (and including) about 400 mM, or about 55 mM to (and including) about 220 mM.

[0075] As would be readily understood by the skilled person, the amount of IRI compound employed in a cryopreservation composition of the present application, will be determined, in part, based on the ratio between the toxicity of the IRI compound its IRI activity. This is analogous to a therapeutic index as employed in determining appropriate dosing of a drug. In the present application, the concentration of the IRI compound used in the cryopreservation composition will be determined, in part, based on the ratio of the LD₅₀ and the IC₅₀ of the IRI compound, with a higher ratio indicative of the ability to use a higher concentration of the IRI compound while minimizing or avoiding cytotoxicity during cryopreservation.

[0076] In a particular embodiment, compositions for cryopreserving a biological material are provided herein, wherein the composition additionally comprises at least one, at least two, or at least three, cryopreservation agents selected from the group consisting of DMSO, lactobionate, glycerol, trehalose, mannitol, hydroxyethyl starch (HES), ethylene glycol (EG), propylene glycol (PG), polyvinyl alcohol and other biopolymers useful in cryopreservation. The at least one cryopreservation agent is present in the composition in a concentration of about 0.1wt% to (and including) about 30wt%, preferably about 0.1wt% to (and including) about 20wt%, preferably about 5wt% to (and including) about 30wt%, and preferably about 5wt% to (and including) about 20wt% .

[0077] In a particular embodiment, compositions for cryopreserving a biological material are provided herein which further comprise a biological material. The biological material can be, for example, organs, tissues (e.g., organoids), food, cells, platelets, eggs, and embryos. Alternatively, the biological material can be cultured cells, tissues or organs. Examples of "cells" include, but are not limited to, stem cells, T cells (e.g., CAR-T cells), oocytes, sperm, peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells (e.g., NK cells), endothelial cells, pancreatic cells (e.g., pancreatic islet

cells), dendritic cells, fibroblasts or cells from a cell line. The cells can be in an isolated or a purified form, or they can be in a mixture with one or more other cell types.

[0078] In some embodiments, the biological material is from a non-human animal or human source. For example, the biological material from a non-human animal or human source can be a cell, tissue or organ. In alternative embodiments, the biological material is from a plant source. For example, the plant-sourced biological material can be a plant cell, tissue or organ or can be plant seed or fruit, or a part thereof. In other embodiments, the biological material is from a fungal source. In yet other embodiments, the biological material comprises microorganisms of one or more types or species.

[0079] In a particular embodiment, compositions for cryopreserving a biological material are provided herein further comprising a cell culture or growth medium. Examples of suitable cell culture media include, but are not limited to, Eagle's Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), Fetal Bovine Serum (FBS), Fetal Calf Serum (FCS), Ham's F-10, Ham's F-12, Hank's buffered salt solution (HBSS), HBSS and dextrose, Medium 199 and combinations thereof.

[0080] In another embodiment, the compositions for cryopreservation can contain both biological material and cell medium as provided above.

[0081] Kits for Preserving Biological Material

[0082] Kits for cryopreserving a biological material are provided herein comprising an IRI compound of Formula I, or composition comprising one or more IRI compound of Formula I, for cryopreserving a biological material as reported herein. The IRI compound and a further cryopreservation agent, if present, can be in the same composition or in separation compositions. Additionally, they can be co-packaged for common presentation or packaged individually. Instructions can also be provided in the kit for cryopreservation of various types of biological material. The kits provided herein can further comprise a cell culture or growth medium.

[0083] Methods for Cryopreserving Biological Material

[0084] Methods for cryopreserving a biological material are provided herein in which the biological material is combined with in a solution of at least one IRI compound of Formula I and the resulting combination is frozen or cooled to a sub-zero storage temperature. As would be readily understood by the skilled person, the technique by which the biological material is combined with a solution of the at least one IRI compound of Formula I will be dependent on the nature of the biological material. For example, cells may be suspended in the solution prior to storage, or an organ may be perfused with the solution using the existing vasculature of the organ, or a tissue may be perfused with or immersed in the solution. Selection of the appropriate technique for combining the biomaterial with the solution is a matter of routine for the skilled person having knowledge of cryopreservation.

[0085] Accordingly, in some embodiments, methods for cryopreserving a biological material, in which the biological material is cells, are provided herein which comprise suspending the biological material in a solution of at least one IRI compound of Formula I to form a suspension and freezing the suspension or cooling the suspension to a sub-zero storage temperature.

[0086] In some embodiments, the method comprises adding a biological material to a solution comprising at least one IRI compound of Formula I, and then cryopreserving the biological material in cryogenic vials or other suitable container. The vials can be frozen under rate controlled freezing conditions, such as freezing at 1°C per minute over 16 hours. The vials can be stored using standard cryopreservation techniques, and then they can be thawed when required by removing the vials from the cold storage, and thawing using standard protocols. Examples of standard cryopreservation techniques include freezing in liquid nitrogen to about -196 °C and freezing in dry ice to about -80 °C. Examples of standard thawing protocols include, but are not limited to, ambient thaw or rapid thaw in a water bath at a temperature that is at or between room temperature and 37°C. Alternatively, a controlled, slow thaw protocol can be used.

[0087] The present inventors have surprisingly found that use of the IRI compounds of Formula I in cryopreservation allows the use of storage temperatures that are warmer than

typically employed during cryopreservation in the absence of such IRI compounds. For example, cryopreservation can be performed using one or more IRI compounds of Formula I at sub-zero temperatures that are higher than -196°C . In some embodiments, the cryopreservation temperature is -100°C or higher, or -80°C or higher, or -40°C or higher, or -15°C or higher or -10°C or higher.

[0088] In some embodiments, temperatures of about -80°C have been found to be useful for cryopreservation of organoids or spinal cord tissue using a solution comprising one or more IRI compounds of Formula I.

[0089] In some embodiments, temperatures of about -40°C have been found to be useful for cryopreservation of platelets using a solution comprising one or more IRI compounds of Formula I.

[0090] In some embodiments, temperatures of from about -15°C to about -10°C have been found to be useful for high sub-zero cryopreservation of biological material using a solution comprising one or more IRI compounds of Formula I.

[0091] In a particular embodiment, there is further provided methods for cryopreserving a biological material by suspending biological material in a solution of at least one IRI compound of Formula I to form a suspension, which is contained in a vial or other suitable container, wherein the vial is frozen directly in a storage unit without a rate controlled freezing protocol.

[0092] Also provided herein is a method for inhibiting ice recrystallization during cryopreservation or sub-zero storage of a biological material, which method comprises combining, such as, by suspending, a biological material in a solution of at least one IRI compound of Formula I reported herein to form a combination (e.g., a suspension) and cryopreserving the resultant suspension or cooling the resultant suspension to a sub-zero storage temperature.

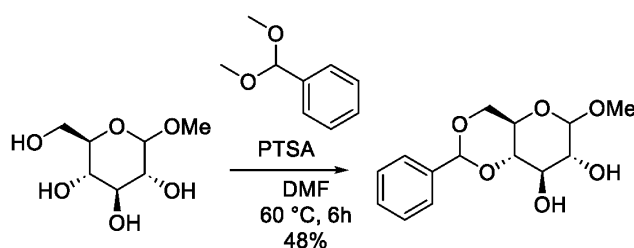
[0093] To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

[0094] EXAMPLE 1: Synthesis of IRI Compounds from Methyl- α -D-glucopyranoside

[0095] The IRI compounds used in the following Examples were synthesized according to the synthetic route summarized in this Example. However, it should be understood that this synthetic method is not limited to the specific starting material used in this Example; other pyranose compounds can be substituted for the methyl- α -D-glucopyranoside starting material in order to obtain other compounds according to Formula I, as defined herein.

[0096] Benzilidene acetal **1**



1

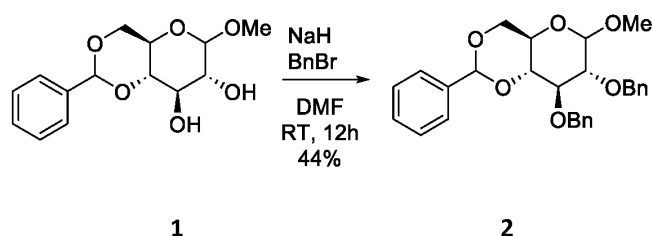
[0097] To a flame dried flask placed under an atmosphere of argon, methyl α -d-glucopyranoside (7.5120 g) was suspended in anhydrous N,N-dimethylformamide (DMF) (77 mL). Benzaldehyde dimethyl acetal (6.6 mL) and p-TSA (0.6700 g) were added. The resulting solution was stirred at room temperature under an atmosphere of argon for 6 hours after which the reaction mixture was diluted with ethyl acetate (EtOAc), and extracted with EtOAc / Brine. The organic phase was dried with Na₂SO₄ and concentrated under reduced pressure. The crude product was recrystallized with dichloromethane (DCM) / Hexanes to afford the benzylidene acetal **1** as a white solid.

[0098] $^1\text{H NMR}$ (400 MHz, DMSO-d_6): δ 7.45-7.39 (m, 2H), 7.36-7.31 (m, 3H), 5.53 (s, 1H), 5.16 (d, $J = 5.1$ Hz, 1H), 4.99 (d, $J = 6.7$ Hz, 1H), 4.59 (d, $J = 3.7$ Hz, 1H), 4.13 (dd, $J = 9.8, 4.7$ Hz, 1H), 3.66 (t, $J = 10.1$ Hz, 1H), 3.58-3.50 (m, 2H), 3.36-3.31 (m, 1H), 3.28 (s, 3H).

[0099] $^{13}\text{C NMR}$ (100 MHz, DMSO-d_6): δ 138.29, 129.31, 128.49 (2x CH), 126.86 (2x CH), 101.32, 101.01, 81.81, 72.88, 70.35, 68.64, 62.85, 55.22.

[00100] **LRMS (ESI)**: m/z calcd. for $\text{C}_9\text{H}_{14}\text{O}_5\text{Na}$ $[\text{M} + \text{Na}]^+$ 305.2, found 305.2.

[00101] Protected Benzilidene acetal **2**

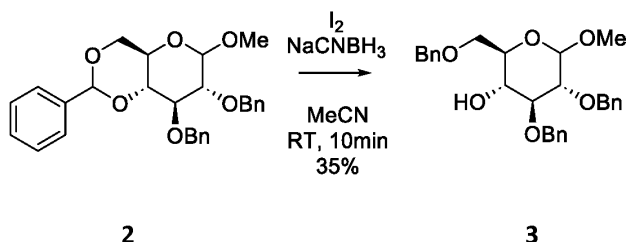


[00102] To a flame dried flask placed under an atmosphere of argon in a water / ice bath, benzilidene acetal **1** (1.1317 g) and anhydrous DMF (40 mL) were added. 60 % NaH suspension in mineral oil (0.4918 g), was added to the reaction portion-wise over 5 minutes, and the reaction was allowed to stir until homogeneous. Benzyl bromide (BnBr; 1.5 mL) was added dropwise to the solution and the water / ice bath was removed. The reaction was allowed to proceed at room temperature under argon atmosphere overnight. The resulting mixture was diluted with DCM and extracted with DCM / H_2O , the resulting organic phase was dried with Na_2SO_4 and concentrated under reduced pressure. The crude product was subjected to flash column chromatography with a gradient of 5 – 30 % EtOAc / Hexanes used as eluent to afford the benzyl-protected benzilidene acetal **2** as a clear oil.

[00103] $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 7.51-7.46 (m, 2H), 7.41-7.26 (m, 3H), 5.54 (s, 1H), 4.93-4.81 (m, 3H), 4.69 (d, $J = 12.2$ Hz, 1H), 4.59 (d, $J = 3.7$ Hz, 1H), 4.26 (dd, $J = 10.1, 4.7$ Hz, 1H), 4.04 (t, $J = 9.3$ Hz, 1H), 3.86-3.79 (m, 1H), 3.70 (t, $J = 10.2$ Hz, 1H), 3.63-3.53 (m, 2H), 3.39 (s, 3H).

[00104] ^{13}C NMR (100 MHz, CDCl_3): δ 138.74, 138.17, 137.42, 128.93, 128.48 (2x CH), 128.34 (2x CH), 128.25 (2x CH), 128.16 (2x CH), 128.06 (2x CH), 127.95, 127.62, 126.05 (2x CH), 101.28, 99.26, 82.15, 79.18, 18.63, 75.38, 73.83, 69.08, 62.34, 55.38.

[00105] C4-Hydroxy Glycosyl Acceptor **3**



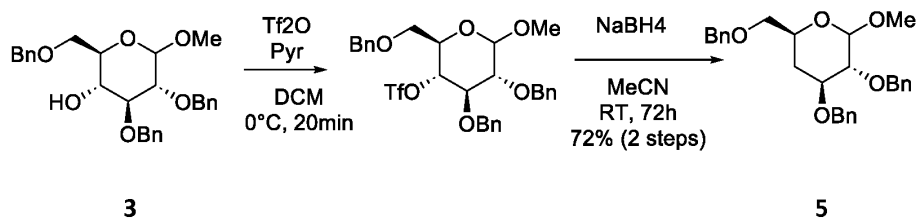
[00106] A flame dried flask placed under an atmosphere of argon was charged with benzyl-protected benzylidene acetal **2** (1.4633 g), 4Å molecular sieves, and anhydrous MeCN (7.5 mL). Sodium cyanoborohydride (1.0106 g) was added and allowed to stir 10 minutes. The reaction flask was placed into a water / ice bath and allowed to cool for 15 minutes. Iodide chips (3.0316 g) were added to the reaction flask portion-wise over 5 minutes, the resulting mixture was allowed to stir for 10 minutes after which the reaction mixture was filtered through a Celite™ bed, diluted with DCM and extracted with DCM / brine. The resulting organic phase was dried with Na_2SO_4 and concentrated under reduced pressure. The resulting crude oil was subjected to flash column chromatography with 30 % EtOAc / Hexanes used as the eluent to afford the C4-hydroxy glycosyl acceptor compound **3** as a clear oil.

[00107] ^1H NMR (400 MHz, CDCl_3): δ 7.37-7.24 (m, 15H), 4.99 (d, J = 11.4 Hz, 1H), 4.74 (dd, J = 15.9, 3.8 Hz, 2H), 4.68-4.60 (m, 2H), 4.55 (q, J = 11.2 Hz, 2H), 3.77 (t, J = 9.2 Hz, 1H), 3.71-3.64 (m, 3H), 3.59 (t, J = 9.1 Hz, 1H), 3.52 (dd, J = 9.5, 3.6 Hz, 1H), 3.37 (s, 3H).

[00108] ^{13}C NMR (100 MHz, CDCl_3): δ 138.81, 138.06, 138.01, 128.61 (2x CH), 128.49 (2x CH), 128.38 (2x CH), 128.15, 128.02, 127.98, 127.87, 127.66, 127.64, 98.21, 81.47, 79.59, 75.45, 73.59, 73.18, 70.72, 69.88, 69.47, 55.27.

[00109] LRMS (ESI): m/z calcd. for $\text{C}_9\text{H}_{14}\text{O}_5\text{Na}$ $[\text{M} + \text{Na}]^+$ 487.2, found 487.2.

[00110] Protected C4-Deoxy Pyranose Derivative 5



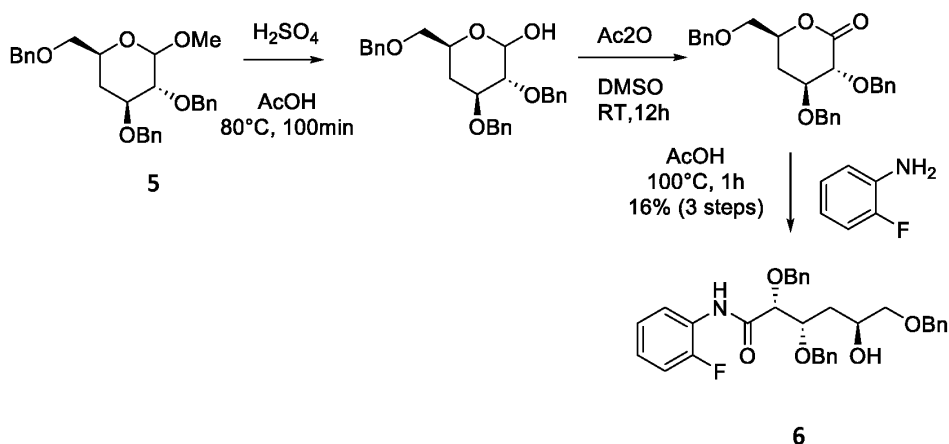
[00111] To a flame dried flask placed under an atmosphere of argon, C4-hydroxy glycosyl acceptor compound **3** (1.39 g) was dissolved in anhydrous DCM (10 mL). Pyridine (0.7 mL) was added and the reaction flask was placed into a water / ice bath. TiF_2O (0.5 mL) was added slowly and the resulting mixture was allowed to stir for 20 minutes. The reaction mixture was diluted with DCM and extracted with DCM / brine. The resulting organic solution was dried with Na_2SO_4 and concentrated under reduced pressure to afford a crude triflate intermediate (1.9 g) as a thick oil which was dried under high vacuum and placed under an atmosphere of argon. Anhydrous MeCN (160 mL), and NaBH_4 (1.0021 g) were added and allowed to stir overnight. The resulting mixture was diluted with EtOAc and extracted with EtOAc / H_2O . The organic phase was dried with Na_2SO_4 and concentrated under reduced pressure. The crude C4-deoxy pyranose derivative **5** was subjected to flash column chromatography with 10 % EtOAc / Hexanes used as the eluent, to afford the pure C4-deoxy pyranose derivative **5** as a clear oil.

[00112] $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 7.39-7.24 (m, 15H), 4.83 (d, $J = 12.2$ Hz, 1H), 4.74 (d, $J = 11.7$ Hz, 1H), 4.70-4.64 (m, 3H), 4.54 (s, 2H), 3.96-3.87 (m, 2H), 3.49-3.44 (m, 3H), 3.37 (s, 3H), 2.05 (ddd, $J = 12.8, 7.4, 2.9$ Hz, 1H), 1.50 (q, $J = 12.1$ Hz, 1H).

[00113] $^{13}\text{C NMR}$ (100 MHz, CDCl_3): δ 138.91, 138.56, 138.12, 128.38 (6x CH), 128.06 (2x CH), 127.74, 127.65 (3x CH), 127.62 (2x CH), 127.51, 99.07, 80.47, 75.32, 73.42, 73.33, 72.52, 72.42, 66.74, 55.19, 33.96.

[00114] **LRMS (ESI)**: m/z calcd. for $\text{C}_9\text{H}_{14}\text{O}_5\text{Na}$ $[\text{M} + \text{Na}]^+$ 471.3, found 471.3.

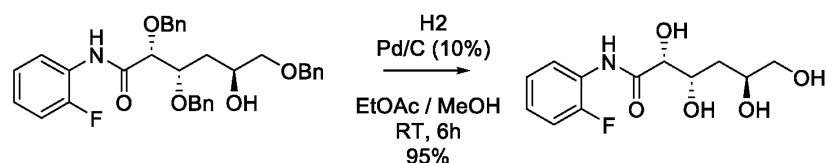
[00115] Protected C4 Deoxy-N-(2-Fluorophenyl)-D-gluconamide **6**



[00116] The C₄-deoxy pyranose derivative **5** (1.08 g) was dissolved in AcOH (17 mL) and 3 M H₂SO₄ (2 mL). The reaction flask was equipped with a condenser and brought to 80 °C. The reaction was allowed to stir at heat for 100 minutes after which the flask was removed from heat and allowed to cool to room temperature. The reaction mixture was diluted with DCM and extracted with DCM / H₂O. The organic phase was dried with Na₂SO₄ and concentrated under reduced pressure to afford the crude C₁ deprotected C₄-deoxy pyranose intermediate (0.9572 g) as a light brown oil which was dried under high vacuum and placed under an argon atmosphere. Anhydrous DMSO (6 mL) and Ac₂O (3.6 mL) were added, and the reaction mixture was allowed to stir at room temperature overnight. The reaction was quenched with H₂O (10 mL) and allowed to stir for 10 minutes. The solution was diluted with DCM and extracted with DCM / brine. The resulting organic phase was dried with Na₂SO₄, and concentrated under reduced pressure to afford crude C₄-deoxy lactone (0.8685 g) as a clear oil. A portion of crude C₄-deoxy lactone (0.6721 g) was dissolved in AcOH (5 mL) and 2-fluoroaniline (0.2 mL) was added. The reaction flask was equipped with a condenser and the reaction was brought to 100 °C. The reaction was allowed to stir at heat for 1 hour, after which time the flask was removed from heat and allowed to cool to room temperature. The crude mixture was concentrated under reduced pressure, and subjected to flask column chromatography using a gradient of 10 – 50 % EtOAc / Hexanes as the eluent. The protected C4 Deoxy-N-(2-Fluorophenyl)-D-gluconamide **6** was isolated as a white solid.

[00117] $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 8.77 (Br, 1NH), 8.34 (dt, $J = 8.0, 1.5$ Hz, 1H), 7.34-7.26 (m, 10H), 7.18 (s, 5H), 7.15-7.02 (m, 3H), 4.73 (d, $J = 11.4$ Hz, 1H), 4.59 (d, $J = 11.4$ Hz, 1H), 4.56 (s, 2H), 4.52 (s, 2H), 4.23-4.17 (m, 1H), 4.03 (d, $J = 3.1$ Hz, 1H), 4.00-3.93 (m, 1H), 3.46 (dd, $J = 9.4, 3.2$ Hz, 1H), 3.29 (dd, $J = 9.4, 7.6$ Hz, 1H), 2.29 (Br, 1OH), 1.82-1.74 (m, 1H), 1.65-1.55 (m, 1H).

[00118] C-4 Deoxy N-(2-Fluorophenyl)-D-Gluconamide (**C4 Deoxy 2FA**)



C4 Deoxy 2FA

[00119] Protected C4 Deoxy-N-(2-Fluorophenyl)-D-gluconamide **6** (0.1083 g) was dissolved in EtOAc (2 mL) and MeOH (2 mL). 10 % Pd/C (0.022 g) is added, and the hydrogenation flask was placed under an atmosphere of hydrogen. The reaction was allowed to stir until deemed complete by TLC (80 % EtOAc / Hexanes) and the hydrogen atmosphere was purged / cycled every 30 minutes. Once complete the reaction mixture was diluted with MeOH (10 mL) and filtered through a bed of Celite™. The resulting solution was concentrated under reduced pressure to afford the title compound **C4 Deoxy 2FA** as a white solid.

[00120] $^1\text{H NMR}$ (400 MHz, D_2O): δ 7.45 (dt, $J = 7.9, 1.5$ Hz, 1H), 7.23-7.17 (m, 1H), 7.15-7.08 (m, 2H), 4.17-4.10 (m, 2H), 3.82-3.74 (m, 1H), 3.50 (dd, $J = 11.7, 3.9$ Hz, 1H), 3.38 (dd, $J = 11.5, 6.8$ Hz, 1H), 1.72-1.63 (m, 1H), 1.53-1.44 (m, 1H).

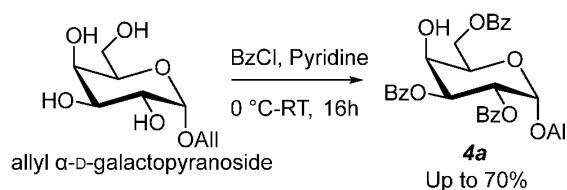
[00121] $^{13}\text{C NMR}$ (100 MHz, D_2O): δ 174.17, 128.21 (d, $J = 7.9$ Hz), 126.29, 124.61 (d, $J = 3.8$ Hz), 115.96 (d, $J = 19.5$ Hz), 74.85, 68.59, 68.38, 65.95, 35.95.

[00122] EXAMPLE 2: Synthesis of IRI Compounds from O-Allyl Pyranose

[00123] An alternative synthetic route was used to produce the IRI compounds described herein, which is more amenable to scale-up synthesis. In this example, the synthesis, was performed using commercially available O-allyl galactose as the starting material. It should be understood that this synthetic method is not limited to the specific starting material used in this Example; other O-allyl pyranose compounds can be substituted for the O-allyl galactose starting material in order to obtain other compounds according to Formula I, as defined above.

[00124] Details of the synthetic steps are provided below.

[00125] **Step 1: Benzoyl protection of the C2, C3 and C6 hydroxyl groups of allyl α -D-galactopyranoside**



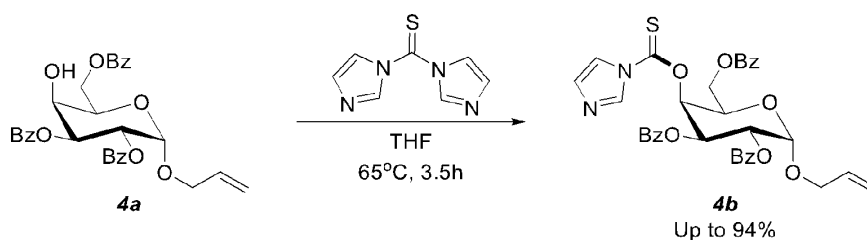
[00126] Allyl- α -D-galactopyranoside (10.0 g) was dissolved in dry pyridine (120 mL, 0.28 M) in a flame dried flask under argon and the mixture is cooled to 0 °C using an ice-water bath. After 15 minutes of cooling, benzoyl chloride (15.9 mL, 3.05 equiv) was added dropwise, and the mixture was stirred for 20 min. After the addition of benzoyl chloride, the ice bath was removed, and the mixture is left to gradually warm to room temperature for an additional 16 hours of stirring. The reaction was quenched by the slow addition an ice-cold saturated aq. NaHCO₃ solution while stirring. The solution is extracted three times with EtOAc, and the combined organic phase was washed 3 times with 1M HCl, ensuring that enough HCl was added to acidify the entire quantity of pyridine. The organic phase was then washed with saturated aq. NaCl solution and dried with Na₂SO₄. The organic phase was evaporated under reduced pressure to obtain a light-yellow solid. The crude product was dissolved in DCM (2 mL/g) at room temperature while mixing and hexanes (5 mL/g) was added after solubilization. The solution was placed in a freezer to recrystallize overnight, followed by filtration to give the purified product as an off-white solid. The recrystallization

mother liquor was collected, and the solvent was removed under reduced pressure. The recrystallization procedure was repeated with DCM (2 mL/g) hexanes (4 mL or 5 mL/g) to give additional pure product.

[00127] $^1\text{H NMR}$ (500 MHz, CDCl_3): δ 8.08 – 8.04 (m, 2H), 8.02–7.97 (m, 4H), 7.61 – 7.56 (m, 1H), 7.55 – 7.49 (m, 2H), 7.46 (t, J = 7.8 Hz, 2H), 7.41 – 7.35 (m, 4H), 5.85 (ddd, J = 22.6, 10.8, 5.7 Hz, 1H), 5.78 (dd, J = 10.6, 3.1 Hz, 1H), 5.71 (dd, J = 10.7, 3.7 Hz, 1H), 5.35 (d, J = 3.7 Hz, 1H), 5.27 (dq, J = 17.3, 1.7 Hz, 1H), 5.12 (dq, J = 10.4, 1.4 Hz, 1H), 4.68 (dd, J = 11.4, 6.1 Hz, 1H), 4.55 (dd, J = 11.4, 6.8 Hz, 1H), 4.44 – 4.39 (m, 2H), 4.25 (ddt, J = 13.1, 5.2, 1.5 Hz, 1H), 4.07 (ddt, J = 13.1, 6.0, 1.4 Hz, 1H)

[00128] $^{13}\text{C NMR}$ (126 MHz, CDCl_3): δ 166.6, 166.2, 165.9, 133.6, 133.5, 133.4, 133.4, 130.0, 129.9, 129.9, 129.8, 129.5, 129.4, 128.6, 128.6, 128.5, 117.8, 95.9, 71.1, 68.9, 68.9, 68.3, 68.1, 63.5

[00129] **Step 2: Thioacylation of C4-hydroxyl group for the subsequent Barton-McCombie deoxygenation**



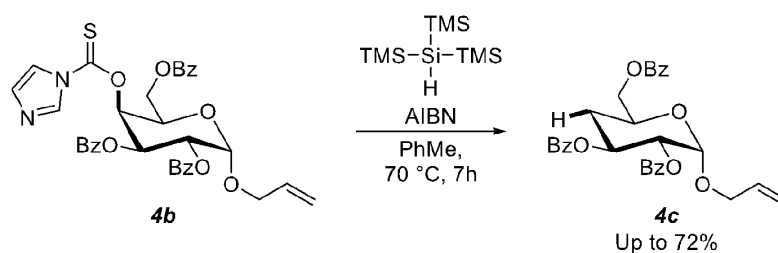
[00130] **4a** (23.91 g) was dissolved in dry THF (224 mL, 0.2 M) in a flame dried flask under argon. 1,1'-thiocarbonyldiimidazole (16.00 g) was added to the solution and the mixture was stirred at 65 °C with a reflux condenser for 3.5h. The solvent was evaporated under reduced pressure and the resulting crude was dissolved in DCM and washed in triplicate with 5% HCl. The organic phase was washed again with saturated aq. NaCl solution, dried with Na_2SO_4 and evaporated under reduced pressure to yield the pure product, a light green-white solid.

[00131] $^1\text{H NMR}$ (500 MHz, CDCl_3): δ 8.50 (s, 1H), 7.99 (ddd, J = 9.4, 8.3, 1.3 Hz, 4H), 7.77 (dd, J = 8.3, 1.2 Hz, 2H), 7.72 (s, 1H), 7.57 (tt, J = 7.4, 1.3 Hz, 1H), 7.53 (tt, J = 7.4, 1.2 Hz,

1H), 7.48 (tt, J = 7.4, 1.3 Hz, 1H), 7.43 (t, J = 8.0 Hz, 1H), 7.39 (t, J = 8.2 Hz, 1H), 7.33 – 7.28 (m, 2H), 7.17 (s, 1H), 6.63 (dd, J = 3.4, 1.2 Hz, 1H), 6.06 (dd, J = 10.8, 3.4 Hz, 1H), 5.87 (dddd, J = 17.2, 10.4, 6.0, 5.3 Hz, 1H), 5.62 (dd, J = 10.8, 3.7 Hz, 1H), 5.47 (d, J = 3.6 Hz, 1H), 5.31 (dq, J = 17.2, 1.6 Hz, 1H), 5.18 (dq, J = 10.4, 1.3 Hz, 1H), 4.77 – 4.71 (m, 1H), 4.57 (dd, J = 11.5, 6.4 Hz, 1H), 4.39 (dd, J = 11.5, 6.7 Hz, 1H), 4.30 (ddt, J = 13.0, 5.3, 1.4 Hz, 1H), 4.13 (ddt, J = 13.0, 6.1, 1.4 Hz, 1H)

[00132] ¹³C NMR (101 MHz, CDCl₃): δ 183.7, 166.0, 166.0, 165.7, 137.6, 133.7, 133.7, 133.5, 133.1, 131.6, 130.0, 129.9, 129.8, 129.4, 129.1, 128.8, 128.6, 128.6, 118.5, 117.9, 95.9, 77.7, 69.4, 69.1, 68.3, 66.9, 62.0

[00133] **Step 3: Barton-McCombie deoxygenation reaction with tris(trimethylsilyl)silane**

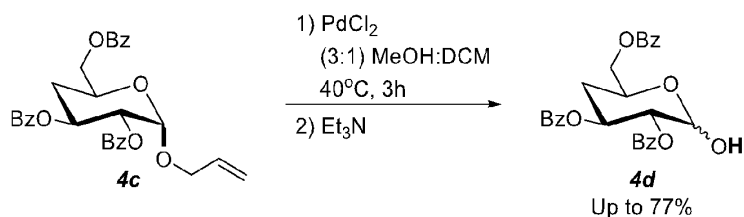


[00134] **4b** (12.30 g) was dissolved in dry toluene (260 mL, 74.6 mM) in a flame dried flask under argon. Tris(trimethylsilyl)silane (8.81 mL) was added to the solution followed by AIBN (0.629 g). The reaction was stirred at 70 °C with a reflux condenser for 6h. The solvent was evaporated under reduced pressure to give the crude product. The crude product was purified by flash column chromatography using a gradient of 10- 25% EtOAc/Hexanes as eluent to isolate the product as an off-white solid.

[00135] ¹H NMR (300 MHz, CDCl₃): δ 8.11 – 8.05 (m, 2H), 8.05 – 7.99 (m, 2H), 7.99 – 7.93 (m, 2H), 7.62 – 7.55 (m, 1H), 7.55 – 7.43 (m, 4H), 7.43 – 7.33 (m, 4H), 5.95 – 5.74 (m, 2H), 5.38 – 5.24 (m, 3H), 5.13 (dq, J = 10.4, 1.4 Hz, 1H), 4.50 – 4.37 (m, 3H), 4.26 (ddt, J = 13.2, 5.1, 1.5 Hz, 1H), 4.06 (ddt, J = 13.2, 5.9, 1.4 Hz, 1H), 2.54 – 2.43 (m, 1H), 1.99 – 1.81 (m, 1H)

[00136] ^{13}C NMR (101 MHz, CDCl_3): δ 166.4, 166.2, 166.0, 133.7, 133.4, 133.3, 133.3, 130.0, 129.9, 129.8, 129.8, 129.6, 128.6, 128.5, 128.5, 117.7, 96.2, 72.5, 68.7, 68.7, 66.2, 65.7, 33.3

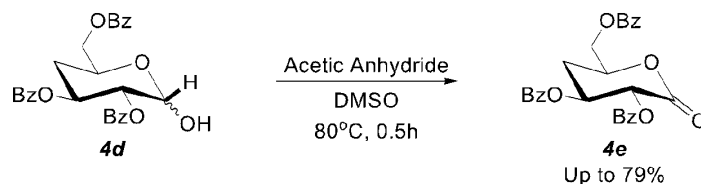
[00137] **Step 4: Allyl group removal from anomeric hydroxyl group**



[00138] **4c** (8.38 g) was dissolved in a mixture of MeOH:DCM (162 mL, 3:1) at a concentration of 0.1 M. A 0.15 M solution of PdCl_2 (0.432 g) in MeOH (16.2 mL) was added dropwise to the reaction vessel. The mixture was stirred at 40 °C with a reflux condenser for 3h. The reaction was quenched by the dropwise addition of triethylamine (3.3 mL). The solvent was evaporated under reduced pressure and the crude was purified by flash column chromatography using a gradient of 20% EtOAc/Hexanes as the eluent to isolate the product as a white solid.

[00139] ^1H NMR (300 MHz, CDCl_3): δ 8.15 – 8.05 (m, 2 aryl-H α + 2 aryl-H β), 8.05 – 7.93 (m, 4 aryl-H α + 4 aryl-H β), 7.64 – 7.55 (m, aryl-H α + aryl-H β), 7.55 – 7.42 (m, 4 aryl-H α + 4 aryl-H β), 7.42 – 7.33 (m, 4 aryl-H α + 4 aryl-H β), 5.91 – 5.80 (m, H-3 α), 5.69 (t, J = 3.6 Hz, H-1 α), 5.58 – 5.47 (m, H-3 β), 5.32 (ddd, J = 10.2, 3.6, 1.3 Hz, H-2 α), 5.24 (dd, J = 9.8, 7.9 Hz, H-2 β), 4.91 (t, J = 8.2 Hz, H-1 β), 4.69 – 4.57 (m, H-5 α), 4.51 – 4.47 (m, 2H-6 β), 4.44 (d, J = 4.7 Hz, 2H-6 α), 4.16 – 4.06 (m, H-5 β), 3.76 (d, J = 8.5 Hz, OH β), 3.04 (dd, J = 3.6, 1.3 Hz, OH α), 2.49 (ddd, J = 12.4, 5.2, 2.2 Hz, H-4eq α), 2.54 – 2.41 (m, H-4eq β), 1.93 (q, J = 12.0 Hz, H-4ax α), 1.90 (q, J = 11.9 Hz, H-4ax β)

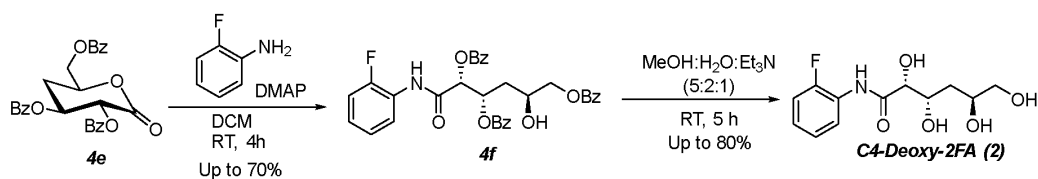
[00140] ^{13}C NMR (76 MHz, CDCl_3): δ 166.5, 166.2, 166.1, 133.5, 133.4, 133.3, 130.0, 129.9, 129.8, 129.5, 128.6, 128.5, 91.5, 72.9, 68.2, 66.2, 65.8, 33.4

[00141] **Step 5: Conversion to a lactone through Albright-Goldman oxidation**

[00142] **4d** (5.66 g) was dissolved in dry DMSO (30 mL, 0.4 M) in a flame dried flask under argon. Acetic anhydride (19.1 mL) was added to the solution, and the mixture was stirred at 80 °C with a reflux condenser for 30 min. Water was added to the reaction mixture (0.75x the solvent volume) and the solution obtained was extracted five times with DCM. The organic layer was dried over Na_2SO_4 , and the solvent was evaporated under reduced pressure. The crude was purified by flash column chromatography using a gradient of 20% EtOAc/Hexanes as the eluent to isolate the product as a white solid.

[00143] $^1\text{H NMR}$ (500 MHz, CDCl_3): δ 8.10 – 8.03 (m, 4H), 8.00 – 7.96 (m, 2H), 7.62 – 7.54 (m, 3H), 7.49 – 7.45 (m, 2H), 7.44 – 7.39 (m, 4H), 5.81 (ddd, $J = 11.8, 9.6, 4.7$ Hz, 1H), 5.65 (d, $J = 9.7$ Hz, 1H), 5.01 – 4.93 (m, 1H), 4.63 – 4.56 (m, 2H), 2.74 (ddd, $J = 13.6, 4.7, 2.7$ Hz, 1H), 2.28 (dt, $J = 13.6, 11.9$ Hz, 1H)

[00144] $^{13}\text{C NMR}$ (126 MHz, CDCl_3): δ 166.2, 166.1, 165.8, 165.6, 133.9, 133.8, 133.6, 130.2, 130.0, 129.9, 129.3, 129.0, 128.7, 128.7, 128.6, 128.6, 74.5, 71.8, 69.4, 65.1, 31.2

[00145] **Steps. 6 and 7: Final deprotection and addition of 2-fluoroaniline**

[00146] **4e** (5.68 g) was dissolved in DCM (30 mL, 0.4 M) in a flame dried flask under argon. DMAP (0.147 g) was added to the solution, followed by the dropwise addition of 2-fluoroaniline (3.5 mL) and the mixture was stirred at room temperature for 5 h. The solvent was evaporated under reduced pressure. The crude was purified by flash column

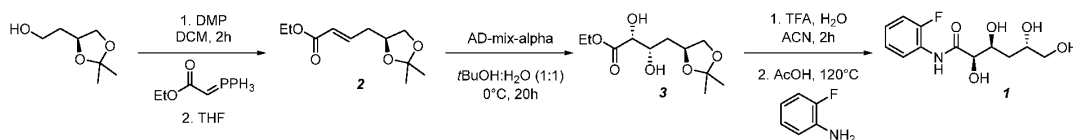
chromatography using a gradient of 10/25% EtOAc in Hexanes as the eluent to isolate the product as a white solid.

[00147] **4f** (4.78 g) is dissolved in MeOH:H₂O:Et₃N (410 mL, 5:2:1, 0.01 M) in a flask and the mixture was stirred at room temperature for 3h. The volatiles were evaporated under reduced pressure. The crude was purified by flash column chromatography using a gradient of 5% MeOH in DCM as the eluent to isolate the product as a white solid. Further purification by overnight EtOH recrystallization, followed by filtration, Hexanes wash and room temperature vacuum dried on the Büchner flask for 4h afforded over 99% purity of **2**.

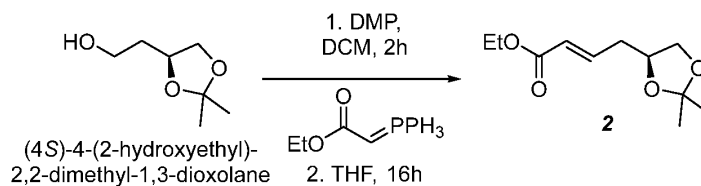
[00148] **¹H NMR (600 MHz, D₂O):** δ 7.56 (t, J = 7.3 Hz, 1H), 7.29 (t, J = 7.1 Hz, 1H), 7.22 (q, J = 8.3 Hz, 2H), 4.24 (q, J = 4.2 Hz, 2H), 3.88 (q, J = 6.6 Hz, 1H), 3.60 (ddd, J = 11.7, 4.0, 1.7 Hz, 1H), 3.55 – 3.43 (m, 1H), 1.85 – 1.73 (m, 1H), 1.60 (ddd, J = 14.4, 9.8, 2.4 Hz, 1H)

[00149] **¹³C NMR (151 MHz, D₂O):** δ 174.2, 155.60 (d, J = 246.5 Hz), 128.20 (d, J = 7.9 Hz), 126.2, 124.65 (d, J = 3.8 Hz), 123.30 (d, J = 12.2 Hz), 116.00 (d, J = 19.5 Hz), 74.9, 68.6, 68.4, 65.9, 36.0.

[00150] EXAMPLE 3: Alternative C4 Deoxy 2FA Synthesis



[00151] Step 1 and 2: Synthesis of unsaturated ester **2**



[00152] Dess-Martin periodinane (2.18 g, 5.13 mmol, 1.5 equiv) was dissolved in DCM (25 mL, 0.14 M) and (4S)-4-(2-hydroxyethyl)-2,2-dimethyl-1,3-dioxolane (0.487 mL, 3.42 mmol, 1 equiv) dissolved in DCM (2.5 mL) was added dropwise to the stirring solution. After 2h at room temperature, the reaction was quenched by a 0.5M Na₂S₂O₃ solution (25 mL)

then sat. NaHCO₃ solution (50 mL) and stirred until clear. The mixture was diluted with DCM (50 mL) and water (25 mL). The aqueous layer was further extracted with DCM, dried over MgSO₄ and the volatiles were carefully removed under reduced pressure (Caution, volatile product: 40°C bath, max 300 mbar pressure). Redissolve aldehyde oil in Hexanes, filter out excess DMP byproduct, remove solvent under reduced pressure. The aldehyde was used directly without further purification.

[00153] **¹H NMR (600 MHz, CDCl₃):** δ 9.81 (t, J = 1.5 Hz, 1H), 4.53 (p, J = 6.3 Hz, 1H), 4.19 (dd, J = 8.3, 6.1 Hz, 1H), 3.59 (dd, J = 8.3, 6.7 Hz, 1H), 2.84 (ddd, J = 17.2, 6.6, 1.8 Hz, 1H), 2.65 (ddd, J = 17.2, 6.1, 1.3 Hz, 1H), 1.42 (s, 3H), 1.37 (s, 3H).

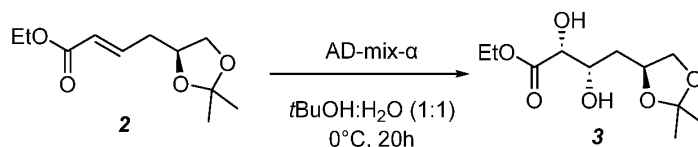
[00154] **¹³C NMR (151 MHz, CDCl₃):** δ 200.02, 109.10, 70.56, 68.99, 47.71, 26.69, 25.36.

[00155] Crude aldehyde (3.42 mmol, 1 equiv) is dissolved in THF (4.9 mL, 0.7 M) and ethyl (triphenylphosphoranylidene)acetate (1.19g, 3.42 mmol, 1 equiv.) was added to the stirring solution portion wise. The reaction was purged under inert atmosphere and the solution was stirred at room temperature for 16 h. The volatiles were removed under reduced pressure and the oily residue was purified by flash column chromatography (5% EtOAc in Pet. Ether) *via*. Celite dry loading, affording unsaturated ester **2** (280 mg, 38% over 2 steps)

[00156] **¹H NMR (600 MHz, CDCl₃):** δ 6.84 (dt, J = 15.7, 7.1 Hz, 1H), 5.82 (d, J = 15.7 Hz, 1H), 4.15 – 4.12 (m, 1H), 4.09 (q, J = 7.1 Hz, 2H), 3.97 (dd, J = 8.2, 6.0 Hz, 1H), 3.49 (dd, J = 8.2, 6.7 Hz, 1H), 2.42 (dtd, J = 15.1, 6.8, 1.6 Hz, 1H), 2.35 (dddd, J = 14.8, 7.3, 5.8, 1.6 Hz, 1H), 1.33 (s, 3H), 1.26 (s, 3H), 1.19 (t, J = 7.2 Hz, 3H).

[00157] **¹³C NMR (151 MHz, CDCl₃):** δ 166.0, 143.7, 123.8, 109.2, 74.2, 68.8, 60.2, 36.4, 26.8, 25.5, 14.2.

[00158] Step 3: Synthesis of dihydroxylated product **3**

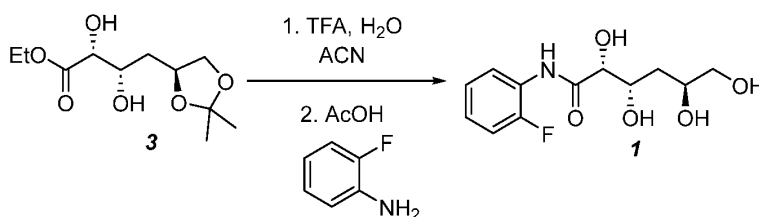


[00159] AD-mix- α (1.82 g, 1.4g/mmol) and MeSO_2NH_2 (124 mg, 1.3 mmol, 1 equiv) is dissolve in $t\text{BuOH}:\text{H}_2\text{O}$ (15.5 mL:15.5 mL, 0.082 M, 1:1) and stirred at 0°C for 5 mins. The α,β -unsaturated ester **2** (280 mg, 1.3 mmol, 1 equiv) was dissolved in minimal $t\text{BuOH}$ and added to the solution and stirred at 4°C for 20h. The reaction was quenched by the addition of sodium sulfite (1.17 g) and stirred at room temperature for 30 mins. The mixture was extracted with EtOAc (30 mL x 2), the combined organic fractions washed with 0.5M NaOH (20 mL), brine (20 mL), dried over MgSO_4 and the volatiles were removed under reduced pressure. The residue was purified by flash column chromatography to afford dihydroxylated product **3** (110 mg, 35%)

[00160] $^1\text{H NMR}$ (600 MHz, CDCl_3): δ 4.31 (dddd, $J = 8.2, 7.3, 6.0, 4.1$ Hz, 1H), 4.28 – 4.21 (m, 2H), 4.15 – 4.09 (m, 1H), 4.09 – 4.03 (m, 2H), 3.55 (dd, $J = 8.1, 7.3$ Hz, 1H), 3.39 (d, $J = 6.0$ Hz, 1H), 2.74 (d, $J = 7.5$ Hz, 1H), 1.88 (ddd, $J = 14.0, 9.7, 4.1$ Hz, 1H), 1.76 (ddd, $J = 14.3, 8.3, 3.4$ Hz, 1H), 1.38 (s, 3H), 1.32 (s, 3H), 1.28 (t, $J = 7.2$ Hz, 3H).

[00161] $^{13}\text{C NMR}$ (151 MHz, CDCl_3): δ 173.2, 109.0, 74.0, 73.3, 69.9, 69.6, 62.1, 37.5, 27.0, 25.7, 14.2.

[00162] Step 4 and 5: Synthesis of C4 Deoxy 2FA

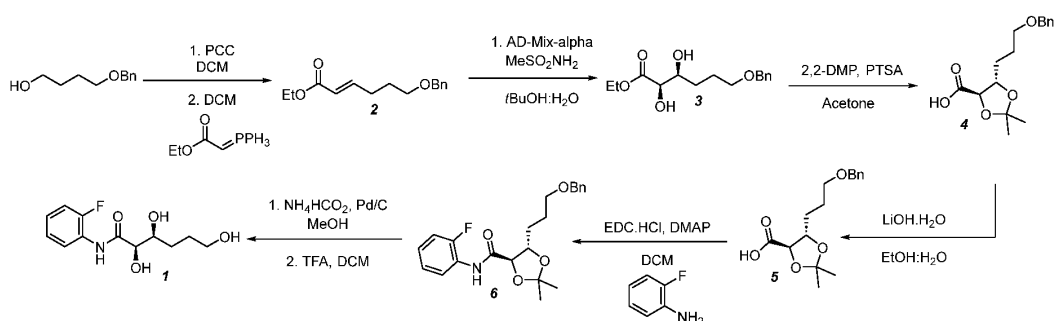


[00163] Diol **3** (100 mg, 0.403 mmol, 1 equiv) was dissolved in acetonitrile (5 mL, 0.08M). H_2O (0.20 mL) and TFA (0.70 mL) were added dropwise and the mixture was stirred at room temperature for 2h. The volatiles were removed under air to afford a crude residue which was used without purification.

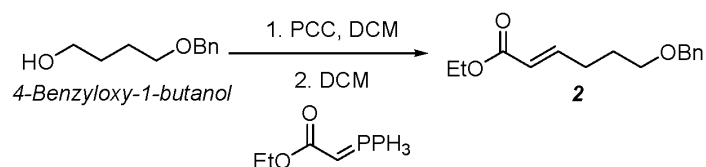
[00164] The residue was dissolved in AcOH (0.768 mL, 0.56 M) and 2-Fluoroaniline (0.125 mL, 1.3 mmol, 3 equiv.) was added dropwise. The solution was heated to 120 °C for 2h. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography (5-10% MeOH in DCM) to afford C4 Deoxy 2FA **1**.

[00165] ¹H NMR (600 MHz, D₂O): δ 7.56 (t, J = 7.3 Hz, 1H), 7.29 (t, J = 7.1 Hz, 1H), 7.22 (q, J = 8.3 Hz, 2H), 4.24 (q, J = 4.2 Hz, 2H), 3.88 (q, J = 6.6 Hz, 1H), 3.60 (ddd, J = 11.7, 4.0, 1.7 Hz, 1H), 3.55 – 3.43 (m, 1H), 1.85 – 1.73 (m, 1H), 1.60 (ddd, J = 14.4, 9.8, 2.4 Hz, 1H).

[00166] EXAMPLE 4: Synthesis of C4,C5 Deoxy IRI Compound



[00167] Step 1 and 2: Synthesis of α,β-unsaturated ester **2**

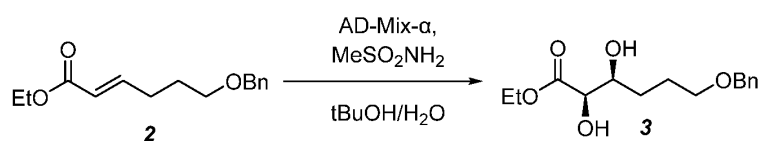


4-Benzyloxy-1-butanol (1g, 5.55 mmol, 1 eq.) was added to a stirring solution of DCM (14 mL, 0.4 M), Celite™ (4.1 g) and pyridinium chlorochromate (1.20 g, 5.55 mmol, 1 eq.). The solution was stirred at room temperature under inert atmosphere for 4h and the mixture was passed through a short silica plug and the solution was directly added to a stirring solution of ethyl (triphenylphosphoranylidene)acetate (1.9 g, 5.55 mmol, 1 eq.) in DCM (14 mL, 0.4 M). The mixture was stirred at room temperature overnight and the volatiles were removed under reduced pressure. The residue was purified by flash column chromatography (5% EtOAc in Hexanes), to of α,β-unsaturated ester **2** (2.54 g, 46% over two steps).

¹H NMR (600 MHz, CDCl₃): δ 7.36 – 7.30 (m, 4H), 7.26 (ddd, J = 5.6, 4.3, 2.8 Hz, 1H), 6.97 (dt, J = 15.7, 6.9 Hz, 1H), 5.87 – 5.78 (m, 1H), 4.48 (d, J = 1.5 Hz, 2H), 4.17 (qd, J = 7.0, 0.8 Hz, 2H), 3.47 (t, J = 6.3 Hz, 2H), 2.29 (qd, J = 7.1, 1.6 Hz, 2H), 1.76 (dq, J = 7.7, 6.3 Hz, 2H), 1.27 (td, J = 7.1, 0.8 Hz, 3H).

[00168] **¹³C NMR (101 MHz, CDCl₃):** δ 166.4, 148.4, 138.3, 128.2, 127.5, 127.4, 121.6, 72.8, 69.1, 60.0, 28.8, 28.1, 14.2.

[00169] Step 3: Synthesis of diol **3**



[00170] AD-mix-α (3.6 g, 1.4g/mmol starting material) and MeSO₂NH₂ (244 mg, 1 eq.) was dissolved in tBuOH:H₂O (15 mL:15 mL, 1:1, 0.082 M) and stirred at 0°C for 5 mins. The α,β-unsaturated ester **2** (630 mg, 2.57 mmol, 1 eq.) was dissolved in minimal tBuOH and added to the solution and stirred for 20h at 4°C. The reaction was quenched by the addition of sodium sulfite (1.17 g) and stirred at room temperature for 30 mins. The mixture was extracted with EtOAc (30 mL x 2), the combined organic fractions washed with 0.5 M NaOH (20 mL), brine (20 mL), dried over MgSO₄ and the volatiles were removed under reduced pressure. The residue was purified by flash column chromatography (20-40% EtOAc in Hexanes) to afford diol **3** (420 mg, 58%).

[00171] **¹H NMR (400 MHz, CDCl₃):** δ 7.36 – 7.23 (m, 5H), 4.50 (s, 2H), 4.24 (qd, J = 7.2, 1.3 Hz, 2H), 4.05 (d, J = 2.3 Hz, 1H), 3.90 (td, J = 6.4, 2.2 Hz, 1H), 3.54 – 3.47 (m, 2H), 1.85 – 1.66 (m, 4H), 1.27 (t, J = 7.1 Hz, 3H).

[00172] **¹³C NMR (101 MHz, CDCl₃):** δ 173.5, 138.2, 128.4, 127.7, 127.6, 73.6, 73.0, 72.4, 70.2, 61.8, 30.8, 26.2, 14.2.

[00173] Step 4: Synthesis of acetonide **4**

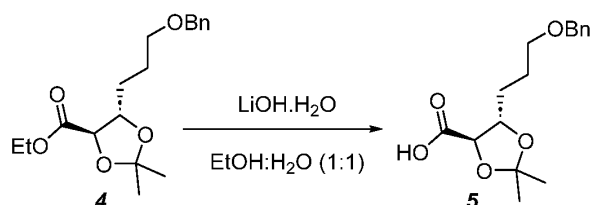


[00174] Diol **3** (510 mg, 1.83 mmol, 1 equiv) was dissolved in acetone (9.1 mL, 0.2 M). 2,2-Dimethoxypropane (0.448 mL, 3.66 mmol, 2 equiv) was added dropwise followed by the addition of p-Toluenesulfonic acid (32 mg, 0.183 mmol, 0.1 equiv). The solution was stirred at room temperature for 3h and the volatiles were removed under reduced pressure. The residue was purified by flash column chromatography (5% EtOAc in Hexanes) to afford acetonide **4** as a white powder (430 mg, 73%).

[00175] ¹H NMR (400 MHz, CDCl₃): δ 7.37 – 7.21 (m, 5H), 4.48 (s, 2H), 4.20 (qd, J = 7.1, 4.7 Hz, 2H), 4.15 – 4.07 (m, 2H), 3.50 (tt, J = 5.8, 3.1 Hz, 2H), 2.00 – 1.64 (m, 3H), 1.44 (s, 3H), 1.41 (s, 3H), 1.25 (dd, J = 7.5, 6.6 Hz, 3H).

[00176] ¹³C NMR (101 MHz, CDCl₃): δ 170.9, 138.6, 128.4, 127.6, 127.6, 110.8, 79.1, 79.0, 72.9, 69.9, 61.3, 30.3, 27.2, 25.9, 25.7, 14.2.

[00177] Step 5: Synthesis of carboxylic acid **5**

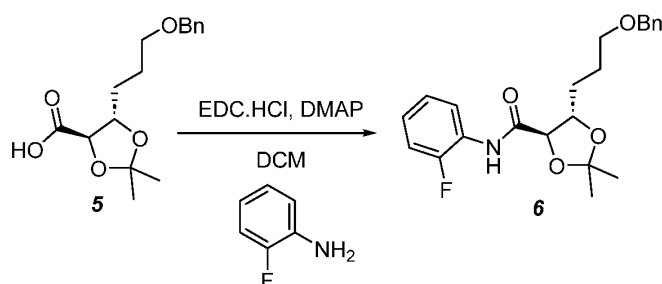


[00178] Acetonide **4** (430 mg, 1.34 mmol, 1 equiv) was dissolved in ethanol:H₂O (3.4 mL:3.4 mL, 0.2 M, 1:1) followed by the addition of LiOH·H₂O (112 mg, 2.68 mmol, 2 equiv). The solution was stirred at room temperature for 1.5 h. The solution was quenched with 1M HCl until the solution turned cloudy and the mixture extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with brine, dried over MgSO₄ and the volatiles were removed under reduced pressure to afford carboxylic acid **5**, used without further purification.

[00179] ^1H NMR (400 MHz, CDCl_3): δ 10.99 (s, 1H), 7.37 – 7.24 (m, 5H), 4.52 (s, 2H), 4.20 – 4.12 (m, 2H), 3.52 (dp, J = 6.3, 3.0 Hz, 2H), 2.01 – 1.70 (m, 4H), 1.47 (s, 3H), 1.43 (s, 3H).

[00180] ^{13}C NMR (101 MHz, CDCl_3): δ 175.4, 138.3, 128.5, 127.8, 127.7, 111.3, 79.0, 78.6, 72.9, 69.8, 30.3, 27.2, 25.8, 25.7.

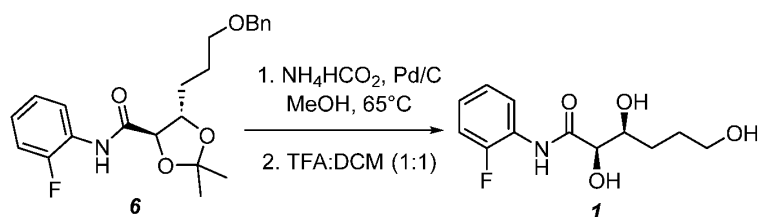
[00181] **Step 6:** Synthesis of amide **6**



[00182] Carboxylic acid **5** (284 mg, 0.966 mmol, 1 equiv) was dissolved in DCM (2.4 mL, 0.4 M) where EDC.HCl (277 mg, 1.44 mmol, 1.5 equiv) and DMAP (12 mg, 0.0966 mmol, 0.1 equiv) were added to the stirring solution. The solution was stirred at room temperature for 5 mins, followed by the dropwise addition of 2-Fluoroaniline (0.094 mL, 0.966 mmol, 1 equiv). The solution was stirred at room temperature overnight and quenched with 1M HCl solution (10 mL). The mixture was extracted with DCM (3 x 20 mL) and the combined organic fractions were washed with brine solution (25 mL). The organic fraction was dried over MgSO_4 and removed under reduced pressure. The residue was purified by dry loaded flash column chromatography (5-25% EtOAc in Hexanes), affording protected amide **6** (480 mg, 78%).

[00183] ^1H NMR (400 MHz, CDCl_3): δ 8.70 (s, 1H), 8.34 (td, J = 8.0, 1.7 Hz, 1H), 7.39 – 7.24 (m, 5H), 7.19 – 7.03 (m, 3H), 4.51 (s, 2H), 4.15 (d, J = 2.1 Hz, 2H), 3.54 (t, J = 6.0 Hz, 1H), 2.12 – 1.98 (m, 1H), 1.94 – 1.76 (m, 3H), 1.51 (s, 3H), 1.48 (s, 3H).

[00184] **Step 7 and 8:** Synthesis of C4,C5 Deoxy **1**



[00185] Protected amide **6** (82 mg, 0.21 mmol, 1 equiv) was dissolved in MeOH (1.7 mL, 0.127 M) and heated to 65°C while stirring. NH_4HCO_2 (66 mg, 1.05 mmol, 5 equiv) and 10% Pd/C (105 mg, 0.5 mg/mmol starting material) were added and the suspension was stirred at 65°C for 4h, with the addition of 5 more equivalents of NH_4HCO_2 at the halfway point. The mixture was cooled to room temperature, filtered over celite with MeOH washing and the solvent was removed under reduced pressure. The residue was used without further purification.

[00186] To the residue was added DCM (0.525 mL, 0.4 M) followed by the dropwise addition of TFA (0.525 mL, 0.4 M). The mixture was equipped with a balloon and stirred at room temperature for 1h. The volatiles were removed with air and the residue was purified by dry loaded flash column chromatography (50-100% EtOAc in DCM) to afford C4,C5 Deoxy compound **1** (25 mg, 46% over 2 steps).

[00187] **^1H NMR (400 MHz, D_2O):** δ 7.43 (td, $J = 7.8, 1.6$ Hz, 1H), 7.23 – 7.13 (m, 1H), 7.13 – 7.05 (m, 2H), 4.13 (d, $J = 2.5$ Hz, 1H), 3.89 (td, $J = 6.4, 3.0$ Hz, 1H), 3.54 – 3.44 (m, 2H), 1.63 – 1.42 (m, 4H).

[00188] **^{13}C NMR (151 MHz, D_2O):** δ 174.44, 155.61 (d, $J = 246.5$ Hz), 128.20 (d, $J = 7.9$ Hz), 126.24, 124.65 (d, $J = 3.7$ Hz), 123.31 (d, $J = 12.2$ Hz), 116.00 (d, $J = 19.6$ Hz), 74.08, 71.91, 61.52, 28.91, 27.84.

[00189] EXAMPLE 5: Ice Recrystallization Studies

[00190] A splat cooling assay was performed to demonstrate the inhibitory activity of one of the IRI compounds of the present application, C4-Deoxy-2FA as defined above, against ice recrystallization. To perform the assay it was necessary to first confirm solubility of C4-Deoxy-2FA in a buffer, which, in this study was phosphate buffered saline (PBS).

[00191] Solubility

[00192] C4 Deoxy 2FA was solubilized at its maximum concentration in a PBS solution during a splat cooling assay to determine its inhibitory activity against ice recrystallization. For comparison, the previously known IRI, 2FA, was also solubilized at its maximum concentration when performing the same assay.

[00193] Methodology:

[00194] In order to dissolve C4 Deoxy 2FA in PBS solution, a stock solution was prepared by adding a sufficient amount of the compound in PBS buffer to obtain a mixture having a concentration of 500 mM, and the solution was vortexed to achieve supersaturation. This stock mixture was used to prepare mixtures having lower concentrations through serial dilution, with each solution being further vortexed until a visual inspection indicated that the compound was fully solubilized at its maximal concentration. This procedure was repeated for 2FA.

[00195] Results:

[00196] C4-Deoxy-2FA was solubilized in PBS buffer solution at a concentration of 50 mM for the splat cooling assay, which corresponds with the maximum solubility of C4 Deoxy 2FA in PBS. The maximum solubility obtainable for 2FA in PBS was about 5 times lower than that obtainable for C4-Deoxy-2FA.

[00197] Conclusion:

[00198] C4-Deoxy-2FA was fully solubilized in PBS at a concentration of 50 mM, which is significantly higher than the solubility of 2FA in the same PBS solution, which had a maximum concentration of 12 mM. This means that a higher concentration of C4-Deoxy-2FA will remain in solution, as the temperature of the solution is lowered, than in solutions of 2FA.

[00199] Ice Recrystallization Inhibitory Activity

[00200] This study was performed to demonstrate the ice recrystallization inhibitory activity of C4-Deoxy-2FA, as an example of the IRI compounds of the present application.

The studies provide an evaluation of the concentration-dependence of the IC_{50} in the presence of the IRI using dose-response curves. The corresponding IC_{50} value of the IRI was then extrapolated from the dose-response curves using a four-parameter logistic regression.

[00201] Methodology:

[00202] A splat cooling assay was the initial step in the study to provide the ice recrystallization activity of C4-Deoxy-2FA. The details of the assay used in this study have been previously published (Abraham S.; Keillor, K.; Capicciotti, C. J.; Perley-Robertson, G. E.; Keillor, J. W.; Ben, R. N. Quantitative Analysis of the Efficacy and Potency of Novel Small Molecule Ice Recrystallization Inhibitors. *Cryst. Growth Des.* 2015, 15, 5034-5039) and shown to be appropriate for providing accurate ice recrystallization activity data.

[00203] Six solutions were initially prepared with a range of concentrations of C4-Deoxy-2FA (1, 5, 7.5, 10, 25, 50 mM) solubilized in PBS. The upper plateau of the curve was defined at 100 by the control sample, and the lower plateau at 0 by the maximum inhibitor concentration. The IC_{50} value and Hill slope for C4-Deoxy-2FA were then determined by nonlinear regression using a 4-parameter sigmoidal dose-response fitting equation. The lower bound was defined with the assay development in terms of what is considered rate = 0; the bottom plateau was not redefined to maximal inhibitor potency. The IC_{50} value in this case represents the concentration when 50% of the maximal ice recrystallization inhibition was achieved.

[00204] Results:

[00205] The IC_{50} value obtained using this method for C4-Deoxy-2FA was about 9.4 mM, which is very similar to the value obtained for 2FA (IC_{50} = 3.0 mM). Thus, the present study confirms that C4-Deoxy-2FA acts as a potent inhibitor of ice recrystallization (where a “potent” or “good” inhibitor of ice recrystallization is one that has an IC_{50} < about 45 mM, or an IC_{50} < about 35 mM). The 95% confidence interval for this obtained IC_{50} value of C4 Deoxy 2FA was 8.8 mM to 10.1 mM.

[00206] The dose response curves for both 2FA and C4 Deoxy 2FA are shown in Figure 1. C4 Deoxy 2FA has an ice recrystallization inhibitory activity that is approximately

equivalent to that of 2FA. This, in combination with the high solubility in aqueous solutions, is indicative of the value of the C4 deoxy derivatives of 2FA as improved IRIs.

[00207] EXAMPLE 6: Adsorption, Distribution, Metabolism and Excretion (ADME) Testing and Pharmacokinetic Studies

[00208] Adsorption, Distribution, Metabolism and Excretion (ADME) testing and pharmacokinetic studies were performed to assess the characteristics of the IRI compounds of the present application, as exemplified by C4 Deoxy 2FA. The studies illustrated that C4 Deoxy 2FA is a hydrophilic, low permeability compound, that is not a substrate for efflux transporters. Metabolite identification studies in mouse and human liver microsomes revealed that there were 2 metabolites in mouse liver samples and 1 metabolite in human liver samples. Moreover, upon IV and PO administration of C4 Deoxy 2FA to mice to identify the half-life, no changes in clinical signs or behaviour was observed. These results illustrate that the IRI compounds of the present application, as exemplified by C4 Deoxy 2FA, are suitable to use for clinical purposes.

[00209] Log D Determination

[00210] This study was performed to confirm the hydrophilic nature of the IRI compounds of the present application. LogD is a distribution coefficient used to measure the lipophilicity of ionizable compounds, where the partition is a function of the pH.

[00211] Experimental Procedure

[00212] Aliquots (10 μ L) of 1 mM working solutions, in DMSO, of each of C4 Deoxy 2FA and S-V1 (comparison compound) and a control compound, nifedipine, were placed into a 96-well deep well plate (Log D plate). Aliquots (500 μ L) of phosphate buffer, pH 7.4, saturated with octanol, and 500 μ L octanol, saturated with phosphate buffer, pH 7.4, were then added to each of the wells containing the working solutions and shaken at 2,000 rpm, 25°C, for 2 hours using the Eppendorf Thermomixer Comfort™ plate shaker. The samples were centrifuged to separate the layers of phosphate buffer and octanol. The layers were then analyzed by UPLC-MS/MS. All incubations were performed in duplicate.

[00213] Bioanalytical Method

[00214] UPLC-MS/MS was employed for analysis of the separated layers using the following conditions.

[00215] LC conditions:

Instrument: Shimadzu LC-30 AD

Column: Waters XSelect™ HSS T3, 2.5 μ m (2.1 $\times$ 50 mm)

Column temperature: 40°C

Injection volume: 3 μ L for C4 Deoxy 2FA and 0.5 μ L for S-V1

Mobile phase: A: 0.1% formic acid in water

B: 0.1% formic acid in acetonitrile

Elution gradient:

Time (min)	A (%)	B (%)	Elution rate (mL/min)
0	95	5	0.6
0.6	0	100	0.6
0.9	0	100	0.6
1.0	95	5	0.6
1.4	95	5	0.6

[00216] Mass conditions:

Instrument: Triple Quad 5500 (AB Sciex)

Ion source: Turbo spray

Ionization model: ESI

Scan type: MRM

Ionisation mode: Positive

[00217] Mass Conditions:

Test article	Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	DP (<i>v</i>)	EP (<i>v</i>)	CE (<i>v</i>)	CXP (<i>v</i>)
C4 Deoxy 2FA	274.169	112.061	60	10	25	19
S-V1	300.217	105.094	70	10	25	19

Tolbutamide (IS)	271.2	155.1	80	10	15	19
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[00218] Data Analysis

[00219] All calculations were carried out using Microsoft Excel. Peak areas were determined from extracted ion chromatograms.

[00220] Log D values of the control compound and the test compounds were calculated using the following equation:

$$LogD = Log \left(\frac{AREA\ Ratio_{Oct} \times DF}{AREA\ Ratio_{Buf} \times DF} \right)$$

where DF is the dilution factor, and AREA Ratio is peak area of sample / peak area of internal standard.

[00221] Acceptance Criteria for Control Compounds

[00222] The acceptance criteria of Log D values for control compound nicardipine was within 4.18 – 4.88.

[00223] Results

[00224] The Log D value for control compound nicardipine in 1-octanol/PBS at pH 7.4 was 4.59. The result was within the historical range. Therefore, the acceptance criteria were met, and the assay results for C4 Deoxy 2FA and S-V1 were acceptable.

[00225] The Log D values of C4 Deoxy 2FA and S-V1 in 1-octanol/PBS at pH 7.4 are summarised in Table 1.

Table 1: LogD Values for Control and Test Compounds in 1-octanol/PBS at pH 7.4

Compounds	Replicate	Log D
Nicardipine (control)	Replicate 1	4.59
	Replicate 2	4.59
	Mean	4.59
C4 Deoxy 2FA	Replicate 1	-0.451
	Replicate 2	-0.449
	Mean	-0.450

S-V1	Replicate 1	-0.460
	Replicate 2	-0.509
	Mean	-0.485

[00226] Conclusions

[00227] The Log D value for C4 Deoxy 2FA in 1-octanol/PBS at pH 7.4 was -0.450 and the Log D value for S-VI in 1-octanol/PBS at pH 7.4 was -0.485. The results indicated that C4 Deoxy 2FA and S-V1 were hydrophilic compounds.

[00228] Intestinal Mucosal Permeability

[00229] The objective of this study was to evaluate the bidirectional permeability and absorption mechanism of C4 Deoxy 2FA and S-V1 using Caco-2 cell monolayers.

[00230] Materials

[00231] The Caco-2 cells (passage 49) used in this study were obtained from American Type Culture Collection (ATCC® Number HTB-37).

[00232] All the reagents in this study were of at least the standard of analytical laboratory reagent grade and are listed below:

Item	Source	Lot No.
Acetonitrile	Fisher Chemical	197285
Alprazolam	Cerilliant	FE06102008
Caffeine	ChromaDex.Inc	00003027-300
Cimetidine	Tianjin Yifang Technology Co., Ltd.	100158
DMEM	Corning	20319014
DMSO	Solarbio	1209M031
Erythromycin	Tianjin Yifang Technology Co., Ltd.	R2004
FBS	Corning	35081003
Formic acid	DIKMA	4633515
H2O	Prepared by Water purification system	-
HBSS	Gibco	2085528
HEPES	Solarbio	625V023

Item	Source	Lot No.
Lucifer yellow	Sigma	MKCD2184
Metoprolol	Tianjin Yifang Technology Co., Ltd.	-
NaHCO ₃	Shanghai Lianshi Chemical Reagent Co., Ltd.	180302
Non-essential amino acids	Gibco	2163626
Penicillin/ Streptomycin	Solarbio	20190617
Tolbutamide	Sigma	SLBV1577
Trypsin/EDTA	Gibco	20191226

[00233] Experimental Procedure

[00234] Incubations with Caco-2 cell monolayers were performed in duplicate. Transport buffer solution (HBSS, 25 mM HEPES, pH 7.4) containing either a test compound, C4 Deoxy 2FA (10 μ M) and S-V1 (10 μ M), or a control compound, metoprolol (10 μ M), erythromycin (10 μ M) or cimetidine (10 μ M), was added to appropriate donor wells of the apical or basolateral plate. The transport buffer with DMSO was added to appropriate wells of receiver wells. Following incubation at 37°C for 2 hours, the cell plates were removed and 8 μ L samples were transferred from both apical and basolateral sides into new 96-well plates with 72 μ L transport buffer. Subsequently, 240 μ L of acetonitrile containing internal standard (100 nM alprazolam, 200 nM caffeine and 100 nM tolbutamide) was added to all samples to precipitate protein prior to analysis by UPLC-MS/MS to determine the peak area of C4 Deoxy 2FA, S-V1 and control compounds. The peak area ratio was used to calculate the apparent permeability (P_{app}) from apical to basolateral direction and basolateral to apical direction of the cell monolayers and the efflux ratio. Lucifer yellow was used as a marker to confirm the integrity of the cell monolayers after 2 hours incubation.

[00235] Bioanalytical Method

[00236] UPLC-MS/MS was used for sample analysis.

[00237] LC conditions:

Instrument: Shimadzu Nexera Series Pump LC-40D XS

Column: Waters XSelect™ HSS T3, 2.5 µm (2.1 × 50 mm)

Column temperature: 40°C

Injection volume: 10 µL

Mobile phase: A: 0.1% formic acid in water

B: 0.1% formic acid in acetonitrile

Elution gradient:

Time (min)	A (%)	B (%)	Elution rate (mL/min)
0	95	5	0.65
0.6	0	100	0.65
0.9	0	100	0.65
1.0	95	5	0.65
1.4	95	5	0.65

[00238] Mass conditions:

Instrument: Triple Quad 4500

Ion source: Turbo spray

Ionization model: ESI

Scan type: MRM

Ionisation mode: Positive

[00239] Mass conditions:

Test article	Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	DP (<i>v</i>)	EP (<i>v</i>)	CE (<i>v</i>)	CXP (<i>v</i>)
C4 Deoxy 2FA	274.17	112.06	60	10	25	6
S-V1	300.22	105.094	70	10	25	6
Caffeine (Internal Standard)	195.2	138.1	80	12	27	12

[00240] Data Analysis

[00241] All calculations were carried out using Microsoft Excel™. Peak areas were determined from extracted ion chromatograms.

[00242] *Apparent permeability* (P_{app} , $\text{cm/s} \times 10^{-6}$) was calculated for drug transport assays using the following equation:

$$P_{app} = \frac{V_A}{\text{Area} \times \text{time}} \times \frac{[\text{drug}]_{\text{receiver}}}{[\text{drug}]_{\text{initial, donor}}} \times 10^6$$

where:

- V_A is the volume (in mL) in the receiver well (0.3 mL for A→B flux and 0.1 mL for B→A flux)
- Area is the surface area of the membrane (0.143 cm^2 for HTS Transwell-96 Well Permeable Supports)
- Time is the total transport time (in seconds)

[00243] *Efflux ratio* was determined using the following equation:

$$\text{Efflux Ratio} = \frac{P_{app (B \rightarrow A)}}{P_{app (A \rightarrow B)}}$$

where:

- $P_{app (B \rightarrow A)}$ indicates the apparent permeability coefficient in basolateral to apical direction
- $P_{app (A \rightarrow B)}$ indicates the apparent permeability coefficient in apical to basolateral direction

[00244] *Mass balance (percentage recovery)* was determined using the following equation:

$$\text{Percentage recovery (\%)} = \frac{[\text{drug}]_{\text{receiver}} \times V_A + [\text{drug}]_{\text{donor}} \times V_D}{[\text{drug}]_{\text{initial, donor}} \times V_D} \times 100$$

where:

- V_A is the volume (in mL) in the receiver well
- V_D is the volume (in mL) in the donor well

[00245] *Percentage Lucifer yellow (LY) leakage* of monolayer was calculated using the following equation:

$$\text{Percentage LY leakage (\%)} = \left(\frac{I_{\text{receiver}} \times 0.3}{I_{\text{receiver}} \times 0.3 + I_{\text{donor}} \times 0.1} \right) \times 100$$

where:

- I_{receiver} is the fluorescence intensity in the receiver well (0.3 mL)
- I_{donor} is the fluorescence intensity in the donor well (0.1 mL)

[00246] Percentage Lucifer yellow leakage should be less than 1.5%.

[00247] Criteria for permeability assignment:

- if the $P_{\text{app(A} \rightarrow \text{B)}}$ is less than $1 \text{ cm/s} \times 10^{-6}$, the compound will be considered as low permeability;
- if the $P_{\text{app(A} \rightarrow \text{B)}}$ is greater than $10 \text{ cm/s} \times 10^{-6}$, the compound will be considered as high permeability;
- if the $P_{\text{app(A} \rightarrow \text{B)}}$ is greater than $1 \text{ cm/s} \times 10^{-6}$ and less than $10 \text{ cm/s} \times 10^{-6}$, the compound will be considered as moderately permeability; and
- if the efflux ratio of test article is greater than or equal to 2, the test article will be considered as substrate of efflux transporter.

[00248] Acceptance Criteria for Control Compounds

[00249] The historical ranges of the control compounds, metoprolol, erythromycin and cimetidine are listed below.

Control compound	Papp (A→B) (cm/s × 10 ⁻⁶)
Metoprolol	18.6 – 29.5
Erythromycin	0.0603 – 0.397
Cimetidine	0.467 – 1.78

[00250] Results

[00251] The P_{app(A→B)} values for cimetidine, erythromycin and metoprolol incubated with Caco-2 cell monolayers were 1.14, 0.100 and 21.6, respectively. These results were within the respective historical range. Therefore, the acceptance criteria were met, and the assay results for C4 Deoxy 2FA and S-V1 were acceptable. The results are shown in Table 2.

[00252] P_{app(A→B)} values for C4 Deoxy 2FA and S-V1 at a concentration of 10 μM were 0.770 and 0.253 cm/s × 10⁻⁶ with efflux ratios of 1.05 and 1.36, respectively. Permeability results for C4 Deoxy 2FA and S-V1 in Caco-2 cell monolayers are shown in Table 2.

Table 2: Permeability results for test and control compounds in Caco=2 cell monolayers (n=2)

Compound and Conc.	Replicate	P _{app} (cm/s × 10 ⁻⁶)		Percentage recovery (%)		Efflux ratio
		A→B	B→A	A→B	B→A	
C4 Deoxy 2FA-10 μM	Replicate 1	0.831	0.864	96.5	97.1	1.04
	Replicate 2	0.708	0.754	100	102	1.06
	Mean	0.770	0.809	98.3	99.7	1.05
S-V1-10 μM	Replicate 1	0.252	0.332	97.6	104	1.32
	Replicate 2	0.255	0.358	108	109	1.41
	Mean	0.253	0.345	103	106	1.36
Metoprolol-10 μM	Replicate 1	22.3	20.0	105	103	0.898
	Replicate 2	20.9	19.8	99.3	98.8	0.951
	Mean	21.6	19.9	102	101	0.924
Erythromycin-10 μM	Replicate 1	0.0950	6.34	90.5	96.4	66.7
	Replicate 2	0.105	6.54	92.2	88.9	62.2
	Mean	0.100	6.44	91.4	92.6	64.5
Cimetidine-10 μM	Replicate 1	1.16	5.38	105	97.9	4.65
	Replicate 2	1.13	5.54	103	103	4.91

Compound and Conc.	Replicate	P _{app} (cm/s × 10 ⁻⁶)		Percentage recovery (%)		Efflux ratio
		A→B	B→A	A→B	B→A	
	Mean	1.14	5.46	104	101	4.78

[00253] The Lucifer yellow leakage values for C4 Deoxy 2FA and S-V1 and control compounds are shown in Table 3.

Table 3: Lucifer yellow leakage values for test compounds and control compounds (n=2)

Compound	Percentage LY leakage (%)			
	A→B		B→A	
C4 Deoxy 2FA (10 μM)	0.102	0.139	0.107	0.120
S-V1 (10 μM)	0.115	0.125	0.102	0.118
Metoprolol (10 μM)	0.129	0.135	0.104	0.106
Erythromycin (5 μM)	0.162	0.185	0.104	0.109
Cimetidine (10 μM)	0.131	0.156	0.101	0.0971

[00254] Conclusions

[00255] The results of this study demonstrated that C4 Deoxy 2FA and S-V1 were low permeability compounds and were not substrates of efflux transporters.

[00256] Metabolite Identification in Mouse and Human Liver Microsomes

[00257] The objective of this study was to identify the potential metabolites of C4 Deoxy 2FA and to predict its possible metabolic pathways in mouse and human liver microsomes.

[00258] Experiment Procedure

[00259] C4 Deoxy 2FA was incubated in singlicate in mouse or human liver microsomes (1 mg/mL) suspended in 0.2 M phosphate buffer at pH 7.4. These incubations were carried out at a final test concentration of 10 μM over a total incubation period of 60 minutes. The concentration of NADPH was 2 mM. Samples were taken at 0, 30 and 60 min and the reaction was terminated by addition of 400 μL of acetonitrile. The supernatants

were transferred and placed in the evaporator under steady stream of nitrogen at room temperature until dry. The dried residues were reconstituted with diluents. The control compound verapamil was included in the experiment under the same incubation conditions with the time points taken at 0 min and 60 min. The percentage of verapamil remaining at 60 minutes was used to monitor the metabolic activity of liver microsomes. The samples were analyzed by UPLC-MS/MS.

[00260] Bioanalytical Method

[00261] UPLC-MS/MS was used for sample analysis.

[00262] Study Results

[00263] The percentage remaining for positive control verapamil in mouse and human liver microsomes at 60 min were 8.86% and 10.33%, respectively, which were all within the historical range. Therefore, the acceptance criteria were met and assay results for the C4 Deoxy 2FA were acceptable. The percentage remaining for verapamil in mouse and human liver microsomes at 60 min is shown in Table 4.

Table 4: Percentage remaining of verapamil in mouse and human liver microsomes at 60 min

Species	Percentage remaining (%)
Mouse	8.86
Human	10.33

[00264] The percentage remaining for C4 Deoxy 2FA in mouse and human liver microsomes at 60 min is shown in Table 5.

Table 5: Percentage remaining of M-03055 in mouse and human liver microsomes at 60 min

Species	Percentage remaining (%)
Mouse	99.79
Human	95.91

[00265] The metabolite information of C4-Deoxy-2FA is shown in Table 6.

Table 6: Metabolite information of C4 Deoxy 2FA in mouse and human liver microsomes

Peak No.	R.T. (min)	Meas. m/z [M+H] ⁺	Mass shift (Da)	Mass Error (ppm)	Biotransformation	Percentage peak area at 60 min	
						Mouse	Human
1 (M1)	6.15	290.10294	15.99491	-1.7	Oxidation	2.45E+06	-
2 (M2)	7.90	290.10306	15.99491	-1.3	Oxidation	9.49E+05	8.96E+05
P (P)	8.17	274.10831	0.00000	-0.8	Parent Drug	4.23E+08	4.26E+08

Note: m/z acquired from MS Full Scan spectrum of liver microsomes samples. "-": not observed

[00266] Conclusions

[00267] Under the experimental conditions described herein, a total of two metabolites of C4-Deoxy-2FA were detected in mouse and human liver microsomes samples; both of which were detected in mouse liver microsomes samples and only one of which was detected in human liver microsomes samples.

[00268] The peak area percentages of parent drug and each metabolite were determined by semi-quantitative estimation using the extracted ion chromatogram peak areas of all detected drug-related material in the sample. Metabolites with percentage of peak area $\geq 10\%$ are proposed as major metabolites of C4-Deoxy-2FA. All metabolites observed were less than 10% of the drug-related material in the mouse and human liver microsomes samples.

[00269] EXAMPLE 7: Structural Attributes of Compounds Affecting IRI Activity

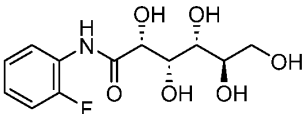
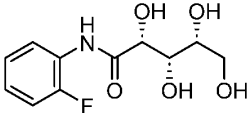
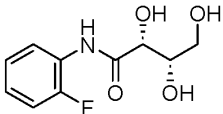
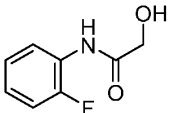
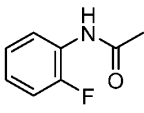
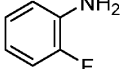
[00270] In this study a modified splat cooling assay was employed to assess IRI activity of compounds of the present application. Ice crystal images were used together with IC_{50} values as a measure of IRI activity.

[00271] *N*-(2-Fluorophenyl)-*D*-gluconamide (2FA) was discovered in 2015 and has been used successfully in cryopreservation of numerous cell types in conjunction with DMSO. This compound has become a standard for modern IRI development, and has an IC_{50} of 3 mM.

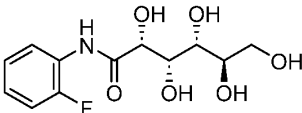
[00272] *N*-(octyl)-*D*-gluconamide (NOG) was discovered in 2012 and is known to be one of the most potent small molecule carbohydrate IRIs identified, having an IC_{50} of 0.8 mM. However, cellular applications are severely limited due to extreme solubility limits.

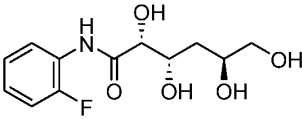
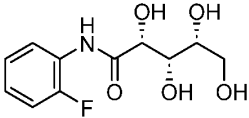
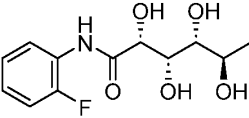
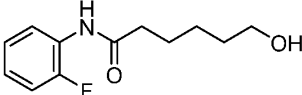
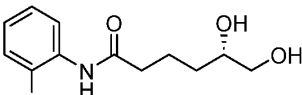
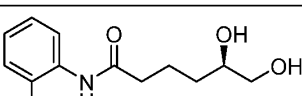
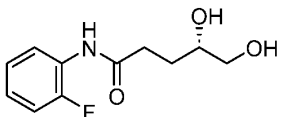
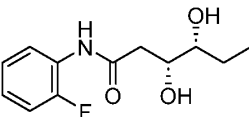
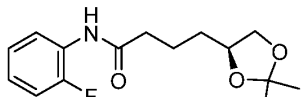
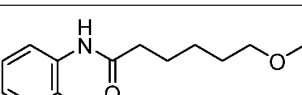
[00273] In considering the present compounds of Formula A, which are IRI compounds that incorporate features of one or both of 2FA and NOG. This study provides insight into the structural components that are required or beneficial for IRI activity.

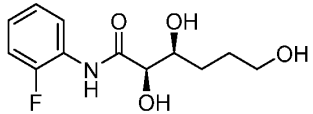
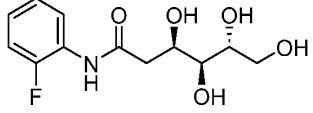
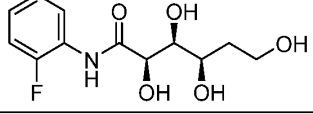
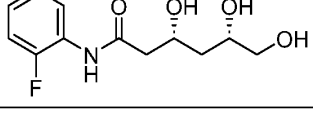
[00274] Aryl-*D*-gluconamide was used as a starting point for compounds having a truncated gluconamide portion. The results are summarized below:

Compound structure	IC ₅₀ (mM)
 2FA	3
	27
	43
	47
	>60
	>60

[00275] The above results demonstrate that the first three derivatives of 2FA have good IRI activity. However, there is a loss in IRI activity as the gluconamide portion of the compound is truncated. To further demonstrate whether this decrease in activity is resulting from the chain length or the loss of hydroxyl groups, deoxy-derivatives of 2FA were synthesized and evaluated. The results are summarized below:

Compound structure	IC ₅₀ (mM)
 2FA	3

Compound structure	IC ₅₀ (mM)
 C4-Deoxy-2FA	8
	27
	27
	23
	25
	27
	83
	>65
	N/A
	>30

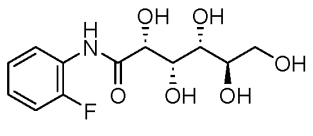
Compound structure	IC ₅₀ (mM)
	4
	8
	11
	>10

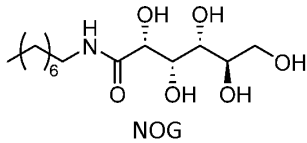
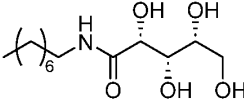
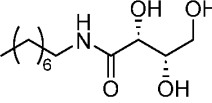
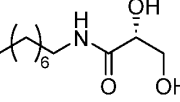
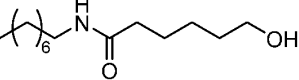
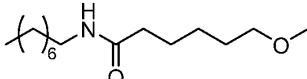
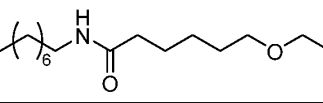
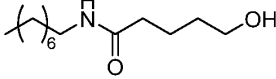
N/A = not active as an IRI

[00276] The above results suggest that an -OH group at the C6 position contributes significantly to IRI activity. However, the presence of the C6-OH group is not the sole source of activity and removal of hydroxyls can provide an order of magnitude increase in solubility, which is beneficial for use in cryopreservation compositions.

[00277] Further, in considering the lack of IRI activity observed with the last two compounds in the above table, it appears that both the position of the C6-OH in space and also the H-bond donor functionality is beneficial for IRI activity.

[00278] As noted above, NOG is known to be one of the most potent small molecule carbohydrate IRIs identified, but it suffers from solubility issues. In view of the finding that removing hydroxyls from the gluconamide portion of 2FA can improve solubility, compounds including an n-octyl with similarly modified gluconamide chains were studied. The results are summarized below:

Compound structure	IC ₅₀ (mM)
 2FA	3

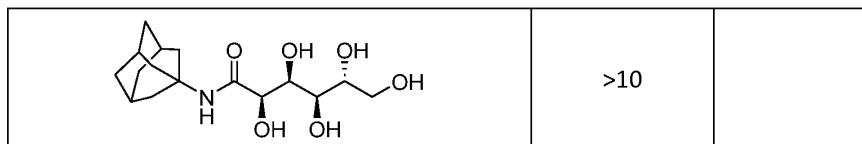
Compound structure	IC ₅₀ (mM)
 NOG	0.8
	10.2
	5.5
	12.4
	0.76 (LD ₅₀ = 1.1 mM)
	1.2
	3.2
	Similar to above compound

[00279] As illustrated by the data above, removal of all but the terminal OH from the gluconamide portion of NOG did not have any effect on IRI activity. However, there was an observed order of magnitude increase in solubility in buffer. The above results further demonstrate that replacement of the terminal OH group with an alkoxy group can be tolerated; with good IRI activity being retained.

[00280] The toxicity of the C2, C3, C4, C5-tetradecoxy NOG derivative was tested in HepG2 cells using the Resazurin viability assay with 24 hour incubation. This derivative was found to have low toxicity, with an LD₅₀ of 1.1 mM.

[00281] Based on the above data showing that NOG derivatives retain IRI activity while having improved solubility characteristics, compounds comprising alternative N-alkyl substituents were studied. The results are summarized below:

Compound structure	IC ₅₀ (mM)	LD ₅₀ (mM)
	3.2	6.1
	1.7	4.7
	>80	
	68.5	
	27.2	
	13.5	
	>65	
	24.8	
	17.2	
	>10	



[00282] As illustrated above, inclusion of cyclic and bulky alkyl groups in place of the n-octyl group of NOG, allows retention of IRI activity in these compounds.

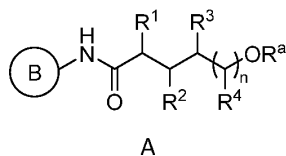
[00283] In summary, the results of this study demonstrate that a range of derivatives of 2FA and NOG retain IRI activity. The presence of a C6-OH group is beneficial to IRI activity in both the aryl-gluconamide derivatives and the alkyl-gluconamide derivatives. However, selective dihydroxylation is useful to improve solubility in both types of N-functionalized gluconamides.

[00284] All publications, patents and patent applications mentioned in this Specification are indicative of the level of skill of those skilled in the art to which this invention pertains and are herein incorporated by reference to the same extent as if each individual publication, patent, or patent applications was specifically and individually indicated to be incorporated by reference.

[00285] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

We Claim:

1. A compound of Formula A



where:

B is phenyl; phenyl mono- or di-substituted with C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F); or an alkyl;

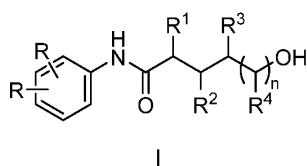
R^a is H when B is phenyl or substituted phenyl, and H or alkyl (e.g., a C₁-C₄ alkyl) when B is alkyl;

R¹, R², R³ and each R⁴ are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein at least one of the R¹, R², R³ and R⁴ substituents is not OH;

n is an integer from 1 to 7, wherein when n is greater than 1 each R⁴ is the same or different; and

each R⁵ – R¹² is independently H, alkyl or aryl.

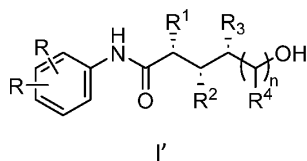
2. The compound of claim 1, which is compound of Formula I



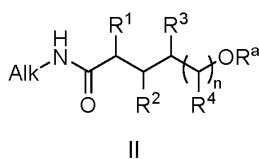
wherein:

each R is independently hydrogen, C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F).

3. The compound of claim 2, wherein the compound has the structure of Formula I'



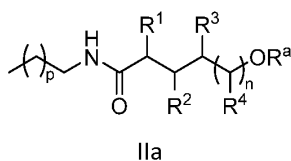
4. The compound of claim 1, which is a compound of Formula II



wherein:

Alk is a C₃ – C₉ linear or branched alkyl or a C₃ – C₁₀ mono- or poly-cyclic alkyl.

5. The compound of claim 4, which is a compound of Formula IIa

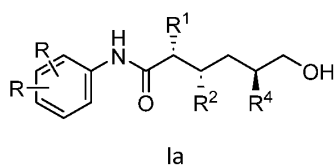


where:

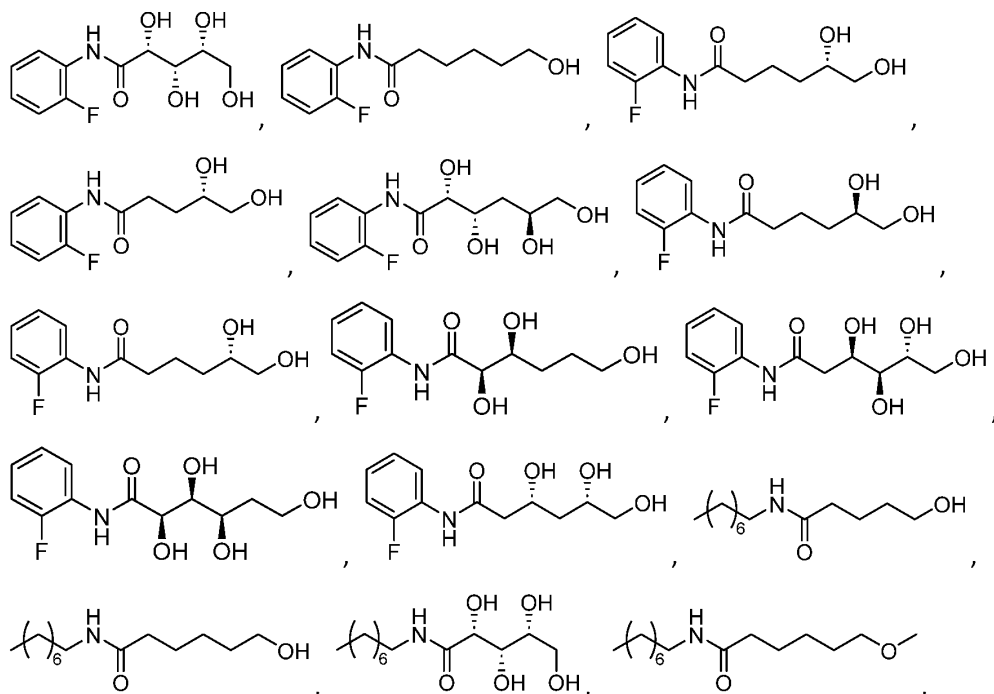
p is an integer from 1 to 7, or from 5 to 7, or p is 6.

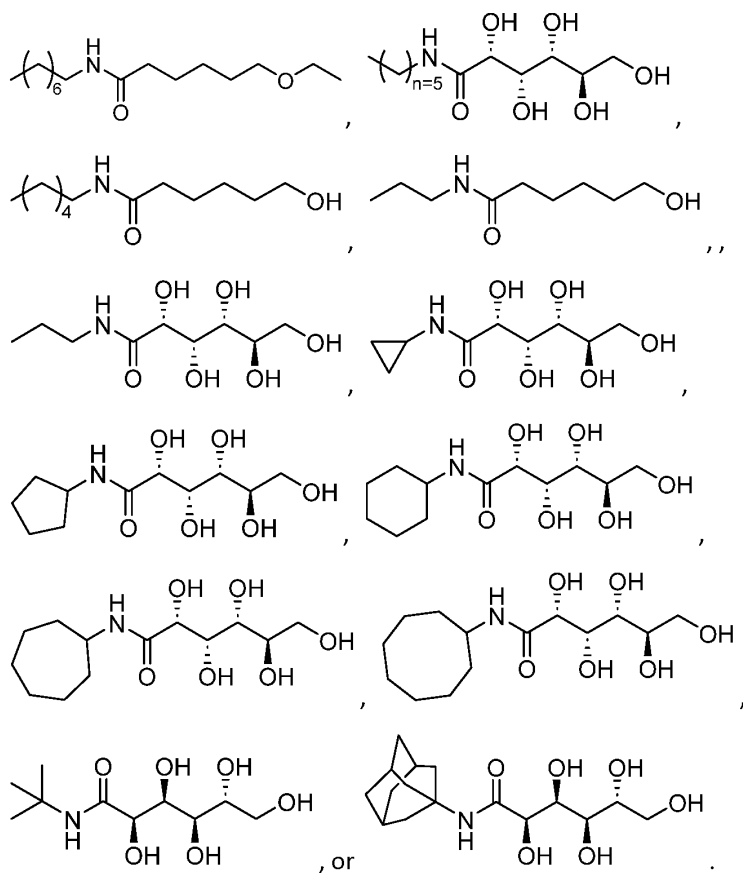
6. The compound of claim 4, wherein Alk is a C₃-C₈ straight alkyl, a C₃-C₈ branched alkyl (e.g., *t*-butyl), a C₃-C₈ cycloalkyl, or adamantyl.
7. The compound of any one of claims 1 to 6, wherein R¹, R², R³ and each R⁴ are independently H, -OH, -OR⁹.
8. The compound of any one of claims 1 to 6, wherein at least one of R¹, R², R³ and each R⁴ is H and/or at least one of R¹, R², R³ and each R⁴ is OH.
9. The compound of any one of claims 1 to 8, wherein R³ is H.

10. The compound of any one of claims 1 to 9, wherein at least one of R^1 , R^2 , R^3 and each R^4 are OH.
11. The compound of any one of claims 1 to 10, wherein n is 1, 2 or 3.
12. The compound of claim 11, wherein the compound has the structure of Formula Ia

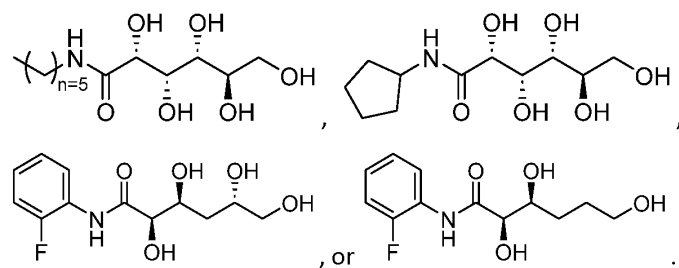


13. The compound of claim 11 or 12, wherein each of R^1 and R^2 are OH, and optionally wherein one R is H and one R is F.
14. The compound of claim 1, having the structure of:





15. The compound of claim 1, having the structure of:



16. The compound of any one of claims 1 to 3, wherein at least one of R^1 , R^2 , R^3 and the R^4 groups is $-\text{NHR}^{10}$, $-\text{[NH}_2\text{R}^{11}]^+$, or $-\text{[NH(R}^{12})_2]^+$.

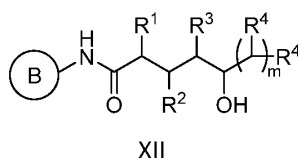
17. The compound of claim 16, wherein at least two of R^1 , R^2 , R^3 and R^4 groups are independently $-NHR^{10}$, $-[NH_2R^{11}]^+$, or $-[NH(R^{12})_2]^+$.
18. A composition comprising the compound of any one of claims 1 – 17 and a solvent (e.g., water) or a buffer.
19. The composition of claim 18, further comprising a cryopreservation agent.
20. The composition of claim 19, wherein the cryopreservation agent comprises DMSO, lactobionate, glycerol, polyvinylalcohol, hydroxyethyl starch (HES), ethylene glycol (EG), propylene glycol (PG), trehalose, mannitol, or a combination of two or more thereof.
21. The composition of any one of claims 18 to 20, further comprising a biological material from a human, an animal, a plant, a fungus or a microorganism.
22. The composition of claim 21, wherein the biological material is an organ, a tissue, cells or platelets.
23. The composition of claim 22, wherein the cells comprise stem cells, T cells (including CAR-T cells), peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells (including natural killer (NK) cells), endothelial cells, pancreatic cells (e.g., pancreatic islet cells), dendritic cells, fibroblasts or cells from a cell line, preferably the cells comprise stem cells, T cells, red blood cells, progenitor cells, or liver cells.
24. The composition of any one of claims 18 to 23, further comprising a cell culture medium.
25. The composition of claim 24, wherein the cell culture medium is phosphate buffered saline optionally comprising albumin, Eagle's minimum essential medium, Dulbecco's modified Eagle's medium, RPMI, fetal bovine serum, fetal calf serum, Ham's F-10,

Ham's F-12, Medium 199, Hank's Buffered Salt Solution, Hank's Buffered Salt Solution and dextrose, or a combination thereof.

26. A method for cryopreserving a biological material comprising:
 - a) combining the biological material with a solution comprising at least one compound according to any one of claims 1 to 17 t;
 - b) cooling the combination from step a) to a storage temperature at or below the freezing point of the solution; and
 - c) storing the cooled combination at the storage temperature.
27. The method of claim 26, wherein the biological material is from a human, an animal, a plant, a fungus or a microorganism and/or the biological material is food.
28. The method of claim 27, wherein the biological material is an organ, a tissue, cells or platelets.
29. The method of claim 28, wherein the cells comprise stem cells, T cells (including CAR-T cells), peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells (including natural killer (NK) cells), endothelial cells, pancreatic cells (e.g., pancreatic islet cells), dendritic cells, fibroblasts or cells from a cell line, preferably the cells comprise stem cells, T cells, red blood cells, progenitor cells, or liver cells.
30. The method of any one of claims 26 to 29, wherein the cooling comprises performing a rate-controlled cooling of about 1°C per minute over about 16 hours.
31. The method of any one of claims 26 to 30, wherein the biological material is cells and the combining step comprises suspending the cells in the solution and the combination is a suspension of the cells in the solution.
32. Use of the compound of any one of claims 1 to 17 to:

- a) reduce toxicity during cryopreservation in comparison to cryopreservation in the absence of the compound of any one of claims 1 to 17;
- b) improve viability and/or functionality of biological material following cryopreservation in comparison to cryopreservation in the absence of the compound of any one of claims 1 to 17;
- c) improve stability of biological material during temperature cycling in cryopreservation in comparison to temperature cycling in cryopreservation in the absence of the compound of any one of claims 1 to 17; and/or
- d) facilitate cryopreservation at a warmer temperature than possible in the absence of the compound of any one of claims 1 to 17.

33. A method for synthesizing a compound of Formula XII



wherein:

B is phenyl; phenyl mono- or di-substituted with C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F); or an alkyl;

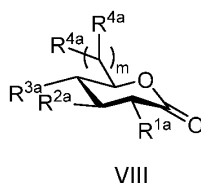
R¹, R², R³ and each R⁴ are each independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺ or -[NH(R¹²)₂]⁺ where each R¹² is the same or different, wherein the terminal R⁴ is OH;

m is an integer from 1 to 6; and

each R⁵ – R¹² is independently H, alkyl or aryl,

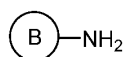
said method comprising:

(a) providing a lactone of Formula VIII



wherein R^{1a} , R^{2a} , R^{3a} and each R^{4a} are independently H, -OH, -OR⁹, -NHR¹⁰, -[NH₂R¹¹]⁺, -[NH(R¹²)₂]⁺ where each R^{12} is the same or different, a protected alcohol, or a protected amine, wherein the terminal R^{4a} is OH;

- (b) ring opening the compound of Formula VIII with an aryl or alkyl amine of Formula VII

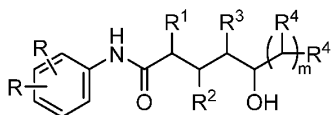


VII ; and

- (c) optionally, deprotecting the product of step (b).

34. The method according to claim 33, wherein:

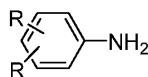
- a) the compound of Formula XII has the structure of Formula XIIa:



XIIa

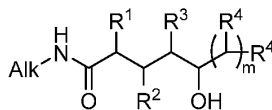
wherein:

each R is independently hydrogen, C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R^6 is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO, haloalkyl or halogen (e.g., Cl, Br, I or F), and each $R^8 - R^{12}$ is independently H, alkyl or aryl; and wherein the compound of Formula VII is an aryl amine having the structure of Formula VIIa:



VIIa; or

- b) the compound of Formula XII has the structure of Formula XIIb:

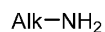


XIIb

wherein:

Alk is a C₃ - C₉ linear or branched alkyl or a C₃ - C₁₀ mono- or poly-cyclic alkyl; and

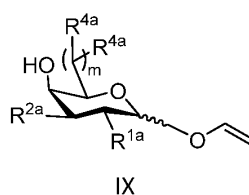
wherein the compound Formula VII is an alkyl amine having the structure of Formula VIIb:



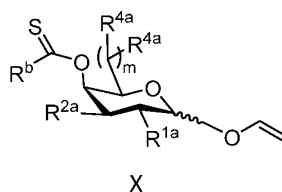
VIIb.

35. The method synthesizing a compound of Formula III according to claim 33 or 34, wherein R^3 is H, and wherein prior to step (a), a compound of Formula VIII, in which R^{3a} is H, is first produced by steps comprising:

- (i) providing a protected O-allyl pyranose of Formula IX

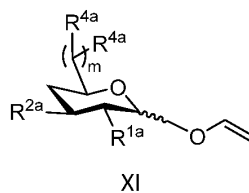


- (ii) converting the protected O-allyl pyranose of Formula IX to a thiocarbonyl derivative of Formula X



wherein R^b is H, CH_3 , SCH_3 , OCH_3 , phenyl (Ph), OPh or imidazolyl, preferably imidazolyl;

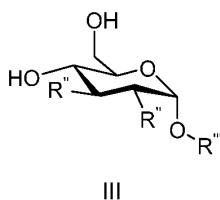
- (iii) treating the thiocarbonyl derivative of Formula X with a radical reducing agent to produce a C4-deoxy pyranose derivative of Formula XI



- (iv) converting the C1 allyl group in the compound of Formula XI to a C1 hydroxyl group; and

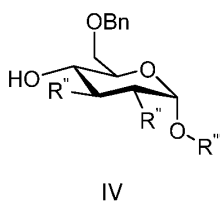
(v) oxidizing the C1 hydroxyl group to form the lactone of Formula VIII.

36. The method of any one of claims 33 to 35, wherein R^{1a} , R^{2a} , R^{3a} and each R^{4a} are independently H or a protected alcohol and/or wherein m is 1.
37. A method for synthesizing a compound of Formula I, I as defined in claim 2, wherein n is 2, R^3 is H and R^4 is OH, comprising the steps of:
- (i) converting a protected D-pyranose of Formula III to a benzylidene acetal



where each R'' is independently H or OH and $O-R'''$ is a hydroxyl protecting group, such as methoxy or silyl ether (e.g., a bulky silyl ether);

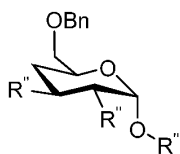
- (ii) when one or both of the R'' groups are OH, protecting the OH group or groups, and subsequently performing a selective ring opening of the benzylidene acetal to form a C4 acceptor compound of Formula IV



wherein each R'' is independently H or OPr^1 , where Pr^1 is a hydroxyl protecting group (such as, but not limited to, a benzyl group);

- (iii) converting the C4 hydroxyl group of the compound of Formula IV into a triflate and displacing the triflate to produce a C4 deoxy pyranose derivative of Formula V

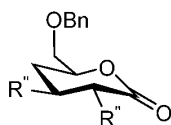
81



V

;

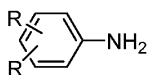
- (iv) deprotecting the anomeric protected group and oxidizing the resultant hydroxyl to produce the lactone of Formula VI



VI

; and

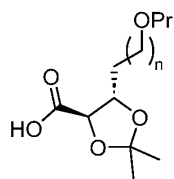
- (v) ring opening the lactone of Formula VI with an aryl amine of Formula VIIa



VIIa

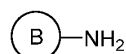
wherein each R is independently hydrogen, C₁-C₄-alkyl, C₁-C₄-alkoxy, -NH₂, -NR⁵H, -N(R⁶)₂ where each R⁶ is the same or different, -C(O)R⁷, -COOH, -COOR⁸, -CHO or halogen (e.g., Cl, Br, I or F), and removing the hydroxyl protecting group(s) to form the compound of Formula I as defined above, wherein n is 2, R³ is H and R⁴ is OH.

38. A method of synthesizing a compound of Formula I, as defined in claim 1, wherein R¹ and R² are both OH, R³ and R⁴ are H and R^a is H, said method comprising the steps of:
- (a) providing a carboxylic acid of Formula XX



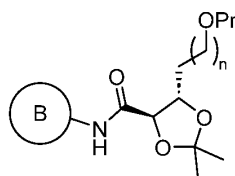
XX ;

- (b) coupling the carboxylic acid of Formula XX with an alkyl or aryl amine of Formula VII



VII

in the presence of a coupling agent (e.g., EDC) to form a protected amide of Formula XXI



XXI ; and

- (c) deprotecting of the protected amide to form the compound of Formula I in which R^1 and R^2 are both OH, R^3 and R^4 are H and R^a is H.