Pladienolide Pyridine Compounds and Methods of Use

BACKGROUND

The present invention provides novel organic compounds and pharmaceutical compositions containing such compounds. These compounds may be useful in the treatment of cancer, particularly cancers in which agents that target the spliceosome and mutations therein are known to be useful.

In eukaryote organisms, newly synthesized messenger RNAs typically have multiple introns, which are excised to provide the mature mRNA. The spliceosome is a multisubunit complex that accomplishes this task. The spliceosome consists of five small nuclear RNAs (snRNAs; U1-6) in combination with a variety of proteins. Mutations in spliceosome genes have been found in various types of cancers.

For example, mutations in the splicing factor 3B subunit 1 (SF3B1) of the spliceosome exist in a number of cancers and comprise a target for anticancer agents. Such cancers include, but are not limited to, myelodysplastic syndrome (MDS), leukemia such as chronic lymphocytic leukemia (CLL), chronic myelomonocytic leukemia (CMML), and acute myeloid leukemia (AML), and solid tumors such as breast cancer and uveal melanoma.

Compounds isolated from the bacteria *Streptomyces platensis* (Sakai, Takashi; Sameshima, Tomohiro; Matsufuji, Motoko; Kawamura, Naoto; Dobashi, Kazuyuki; Mizui, Yoshiharu. Pladienolides, New Substances from Culture of *Streptomyces platensis* Mer-11107. I. Taxonomy, Fermentation, Isolation and Screening. *The Journal of Antibiotics*. 2004, Vol. 57, No.3.), termed pladienolides and discovered while screening for inhibitors of the vascular endothelial growth factor (VEGF) promoter, inhibit expression of a reporter gene controlled by human VEGF promoter, which inhibition is known to be a useful mechanism of action for anticancer agents.

These compounds also inhibit proliferation of U251 human glioma cells *in vitro*. The most potent of these compounds, Pladienolide B, inhibits VEGF-promoted gene expression with an IC_{50} of 1.8 nM, and inhibits glioma cell proliferation with an IC_{50} of 3.5 nM. The structure of pladienolide B is known, (Sakai, Takashi; Sameshima, Tomohiro; Matsufuji, Motoko; Kawamura, Naoto; Dobashi, Kazuyuki; Mizui, Yoshiharu. Pladienolides, New Substances from Culture of *Streptomyces platensis* Mer-11107. II. Physico-chemical Properties and Structure Elucidation. *The Journal of Antibiotics*. Vol. 57, No.3. (2004)) and pladienolide B is known to target the SF3b spliceosome to inhibit splicing and alter the pattern of gene expression (Kotake

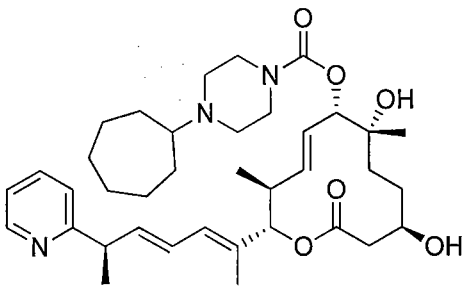
et al., "Splicing factor SF3b as a target of the antitumor natural product pladienolide", Nature Chemical Biology 2007, 3, 570-575).

Certain pladienolide B compounds, as well as other pladienolide compounds, are likewise known, as disclosed the following patent applications: WO 2002/060890; WO 2004/011459; WO 2004/011661; WO 2004/050890; WO 2005/052152; WO 2006/009276; and WO 2008/126918. For example, a pladienolide compound, (8E,12E,14E)-7-((4-Cycloheptylpiperazin-1-yl)carbonyl)oxy-3,6,16,21-tetrahydroxy-6,10,12,16,20-pentamethyl-18,19-epoxytricosa-8,12,14-trien-11-olide, also known as E7107, is a semisynthetic derivative of the natural product pladienolide D, and the results of its Phase I study have been reported.

However, additional agents useful in the treatment of cancer, particularly cancers in which agents that target the spliceosome and mutations therein are known to be useful, are needed.

SUMMARY OF THE INVENTION

The purpose of the present invention is to provide a compound of formula 1 ("Compound 1"), a compound of formula 2 ("Compound 2"), a compound of formula 3 ("Compound 3"), and a compound of formula 4 ("Compound 4"):



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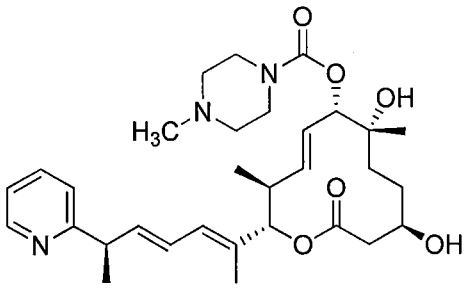

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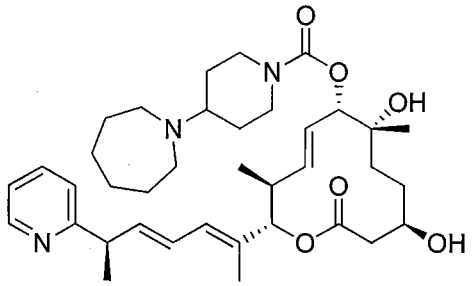

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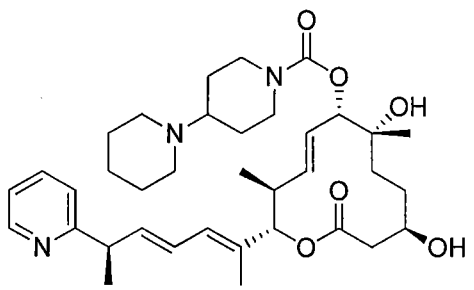

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and pharmaceutically acceptable salts thereof.

A further purpose of the present invention is to provide pharmaceutical compositions comprising Compound 1, Compound 2, Compound 3, Compound 4, or a pharmaceutically acceptable salt thereof. Such pharmaceutical compositions may be formulated with one or more pharmaceutically acceptable carriers. Such compositions are formulated for use through various conventional routes of administration, including intravenous, oral, subcutaneous, or intramuscular administration.

The present invention may also relate to a method of treating a subject with cancer comprising administering to the subject an amount of Compound 1, Compound 2, Compound 3, Compound 4, or a pharmaceutically acceptable salt thereof, effective to produce a therapeutically beneficial response. The cancer may be myelodysplastic syndrome, leukemia such as chronic lymphocytic leukemia, acute lymphoblastic leukemia, chronic myelomonocytic leukemia, or acute myeloid leukemia, or a solid tumor such as colon cancer, pancreatic cancer, endometrial cancer, ovarian cancer, breast cancer, uveal melanoma, gastric cancer, cholangiocarcinoma, lung cancer, or any subset thereof. The cancer may test positive for one or more mutations in a spliceosome gene or protein, such as those listed in Table 1.

The present invention may also relate to the use of Compound 1, Compound 2, Compound 3, Compound 4, or a pharmaceutically acceptable salt thereof, in a method of therapeutic treatment, *e.g.*, treatment for a cancer. The cancer may be myelodysplastic syndrome, leukemia such as chronic lymphocytic leukemia, acute lymphoblastic leukemia, chronic myelomonocytic leukemia, or acute myeloid leukemia, or a solid tumor such as colon cancer, pancreatic cancer, endometrial cancer, ovarian cancer, breast cancer, uveal melanoma, gastric cancer, cholangiocarcinoma, lung cancer, or any subset thereof. The cancer may test positive for one or more mutations in a spliceosome gene or protein, such as those listed in Table 1.

The present invention may also relate to the use of Compound 1, Compound 2, Compound 3, Compound 4, or a pharmaceutically acceptable salt thereof, in the preparation of a medicament. In particular, the medicament may be for the treatment of cancer. The cancer may be myelodysplastic syndrome, leukemia such as chronic lymphocytic leukemia, acute lymphoblastic leukemia, chronic myelomonocytic leukemia, or acute myeloid leukemia, or a solid tumor such as colon cancer, pancreatic cancer, endometrial cancer, ovarian cancer, breast cancer, uveal melanoma, gastric cancer, cholangiocarcinoma, lung cancer, or any subset thereof. The cancer may test positive for one or more mutations in a spliceosome gene or protein, such as those listed in Table 1.

The present invention further may relate to the use of Compound 1, Compound 2, Compound 3, Compound 4, or a pharmaceutically acceptable salt thereof, to target the spliceosome, *e.g.*, subunit 1 of the SF3B spliceosome.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the results of a pharmacokinetic (PK) study in CD-1 mice administered Compound **2** at doses of 5 mg/kg intravenous (IV) or 10 mg/kg oral adminstration (PO).

FIG. 2 shows the efficacy of Compound **2** in a Nalm-6 (human pre B-cell line) mouse xenograft model with an engineered $SF3B1^{K700E}$ mutation. Mice were administered 2.5, 5, or 10 mg/kg Compound **2** once daily (QD) for 14 days, and tumor volume was measured over a 40 day period.

FIG. 3 shows pharmacokinetic and pharmacodynamic analysis of Compound **2** in a Nalm-6 (human pre B-cell line) xenograft model with an engineered $SF3B1^{K700E}$ mutation. Mice were administered a 10 mg/kg PO dose of Compound **2,** and tumor concentration (μg/g) and fold change in expression of Pre-EIF4A1 (the pre-mRNA of the EIF4A1 transcript) and SLC25A19 (the mature mRNA of the SLC25A19 transcript) relative to vehicle were determined.

FIG. 4 shows the results of a cellular viability assay with Compound **2** in the PANC0504 cancer cell line ($SF3B1^{MUT}$) (mutant PANC 05.04) compared with wild-type SF3B1 pancreatic cancer cell lines BXPC3, HPAFII, PANC0403, PANC1005, CFPAC1 and MIAPACA2.

FIG. 5 shows the modulation of alternative splicing for E7107 and Compound **2** (cmpd **2**) based on Nanostring analysis. The "+" and "-" indicate positive or negative values, respectively, in the shade key, which indicates the varying levels of expression of the different splice junctions.

FIG. 6 shows the results of a PK study in CD-1 mice administered Compound **1** at doses of 5 mg/kg intravenous (IV) or 12 mg/kg oral administration (PO).

FIG. 7 shows the efficacy of Compound **1** in a Nalm-6 mouse xenograft model with an engineered $SF3B1^{K700E}$ mutation. Mice were administered 7.5 or 10 mg/kg Compound **1** once daily (QD) for 14 days, and tumor volume was measured over a 30 day period.

FIG. 8 shows pharmacokinetic and pharmacodynamic analysis of Compound **1** in a Nalm-6 xenograft model with an engineered $SF3B1^{K700E}$ mutation. Mice were administered a single PO dose of Compound **1,** and tumor concentration (μg/g) and fold change in expression of Pre-EIF4A1 (the pre-mRNA of the EIF4A1 transcript) and SLC25A19 (the mature mRNA of the SLC25A19 transcript) relative to vehicle were determined.

FIG. 9 shows the results of a PK study in CD-1 mice administered Compound **3** at doses of 5.964 mg/kg intravenous (IV) or 13.307 mg/kg oral administration (PO).

FIG. 10 shows the results of a PK study in CD-1 mice administered Compound **4** at doses of 5 mg/kg intravenous (IV) or 10 mg/kg oral administration (PO).

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

A. Definitions

As used herein, the following definitions shall apply unless otherwise indicated.

"Isomers" refers to compounds having the same number and kind of atoms, and hence the same molecular weight, but differing with respect to the arrangement or configuration of the atoms. "Stereoisomers" refers to compounds that have the same atomic connectivity but different arrangements of their atoms in space. "Diastereoisomers" or "diastereomers" refers to stereoisomers that are not enantiomers. "Enantiomers" refers to stereoisomers that are non-superimposable mirror images of one another. "Geometric isomers" refers to cis-trans isomers having different positions of groups with respect to a double bond or ring or central atom.

Enantiomers taught herein may include "enantiomerically pure" isomers that comprise substantially a single enantiomer, for example, greater than or equal to 90%, 92%, 95%, 98%, or 99%, or equal to 100% of a single enantiomer, at a particular asymmetric center or centers. An "asymmetric center" or "chiral center" refers to a tetrahedral carbon atom that comprises four different substituents.

"Stereomerically pure" as used herein means a compound or composition thereof that comprises one stereoisomer of a compound and is substantially free of other stereoisomers of

that compound. For example, a stereomerically pure composition of a compound having one chiral center will be substantially free of the opposite enantiomer of the compound. A stereomerically pure composition of a compound having two chiral centers will be substantially free of diastereomers, and substantially free of the opposite enantiomer, of the compound. A typical stereomerically pure compound comprises greater than about 80% by weight of one stereoisomer of the compound and less than about 20% by weight of the other stereoisomers of the compound, more preferably greater than about 90% by weight of one stereoisomer of the compound and less than about 10% by weight of the other stereoisomers of the compound, even more preferably greater than about 95% by weight of one stereoisomer of the compound and less than about 5% by weight of the other stereoisomers of the compound, and most preferably greater than about 97% by weight of one stereoisomer of the compound and less than about 3% by weight of the other stereoisomers of the compound. *See, e.g.,* US Patent No. 7,189,715.

"R" and "S" as terms describing isomers are descriptors of the stereochemical configuration at an asymmetrically substituted carbon atom. The designation of an asymmetrically substituted carbon atom as "R" or "S" is done by application of the Cahn-Ingold-Prelog priority rules, as are well known to those skilled in the art, and described in the International Union of Pure and Applied Chemistry (IUPAC) Rules for the Nomenclature of Organic Chemistry. Section E, Stereochemistry.

"Treatment," "treat," or "treating" cancer refers to reversing (*e.g.*, overcoming a differentiation blockage of the cells), alleviating (*e.g.*, alleviating one or more symptoms, such as fatigue from anemia, low blood counts, etc.), and/or delaying the progression of (*e.g.*, delaying the progression of the condition such as transformation to AML) a cancer as described herein.

"Subject", as used herein, means an animal subject, preferably a mammalian subject, and particularly human beings.

"Pharmaceutically acceptable carrier" as used herein refers to a nontoxic carrier, adjuvant, or vehicle that does not destroy the pharmacological activity of the compound with which it is formulated. Pharmaceutically acceptable carriers, adjuvants or vehicles that may be used in the compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol,

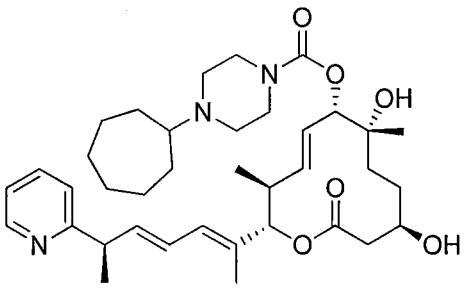
cyclodextrins, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

A "pharmaceutically acceptable salt" is a salt that retains the desired biological activity of the parent compound and does not impart undesired toxicological effects. Examples of such salts are: (a) acid addition salts formed with inorganic acids, for example, hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; and salts formed with organic acids, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (b) salts formed from elemental anions such as chlorine, bromine, and iodine. *See, e.g.*, Haynes et al., "Commentary: Occurrence of Pharmaceutically Acceptable Anions and Cations in the Cambridge Structural Database," J. Pharmaceutical Sciences, vol. 94, no. 10 (2005), and Berge et al., "Pharmaceutical Salts", J. Pharmaceutical Sciences, vol. 66, no. 1 (1977), which are incorporated by reference herein.

B. Compounds

Unless otherwise stated, compounds depicted herein may include mixtures of the compound depicted herein, and any of enantiomeric, diastereomeric, and geometric (or conformational) forms of the structure; for example, the R and S configurations for each asymmetric center, (Z) and (E) double bond isomers, and (Z) and (E) conformational isomers. Unless otherwise stated, compounds depicted herein coexisting with tautomeric forms are within the scope of the invention. Additionally, unless otherwise stated, structures depicted herein are also meant to include compounds that differ only in the presence of one or more isotopically enriched atoms. For example, compounds having the present structures except for the replacement of hydrogen by deuterium or tritium, or the replacement of a carbon by a ^{13}C- or ^{14}C-enriched carbon are within the scope of this invention. Such compounds may be useful, for example, as analytical tools or probes in biological assays.

Provided herein according to some embodiments is a compound of formula 1 ("Compound 1"), a compound of formula 2 ("Compound 2"), a compound of formula 3 ("Compound 3"), and a compound of formula 4 ("Compound 4"):



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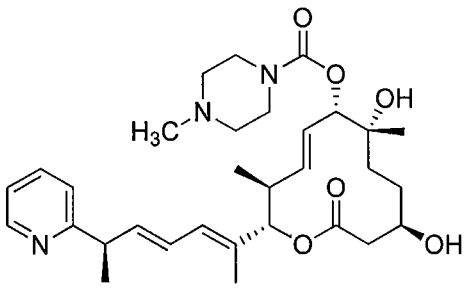

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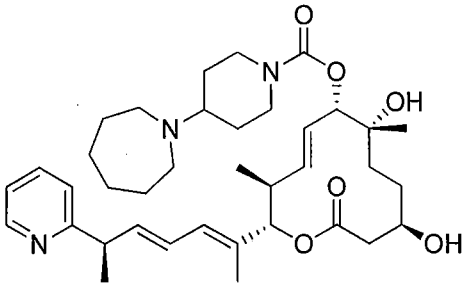

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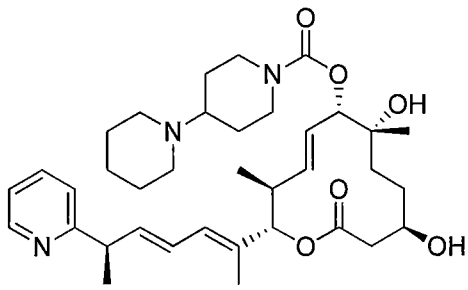

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and pharmaceutically acceptable salts thereof.

C. Pharmaceutical formulations

Compounds of the present invention can be combined with a pharmaceutically acceptable carrier to provide pharmaceutical formulations thereof. The particular choice of carrier and formulation will depend upon the particular route of administration for which the composition is intended.

The pharmaceutical compositions of the present invention may be suitably formulated for parenteral, oral, inhalation spray, topical, rectal, nasal, buccal, vaginal or implanted reservoir administration, *etc.* The term "parenteral" as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques. In particular embodiments, the compounds are administered intravenously, orally, subcutaneously, or via intramuscular administration. Sterile injectable forms of the compositions of this invention may be aqueous or oleaginous suspension. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or

solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium.

For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as carboxymethyl cellulose or similar dispersing agents that are commonly used in the formulation of pharmaceutically acceptable dosage forms including emulsions and suspensions. Other commonly used surfactants, such as Tweens, Spans and other emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms, may also be used for the purposes of formulation.

For oral administration, a compound may be provided in an acceptable oral dosage form, including, but not limited to, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, may also be added. For oral administration in a capsule form, useful diluents include lactose and dried cornstarch. When aqueous suspensions are required for oral use, the active ingredient is combined with an emulsifying and/or suspending agent. If desired, certain sweetening, flavoring or coloring agents may also be added.

D. Subjects and methods of use

A compound of the present invention may be used to treat various types of cancers, including those responsive to agents that target SF3B1. As noted above, the anti-tumor activity of pladienolide B is reported as being connected to its targeting of the SF3b complex, inhibiting splicing and altering the pattern of gene expression (Kotake et al., "Splicing factor SF3b as a target of the antitumor natural product pladienolide," Nature Chemical Biology 2007, 3, 570-575). Mutations in spliceosome genes such as the Splicing factor 3B subunit 1 (SF3B1) protein are known to be implicated in a number of cancers, such as hematologic malignancies and solid tumors. Scott et al., "Acquired mutations that affect pre-mRNA splicing in hematologic malignancies and solid tumors," JNCI 105, 20, 1540-1549.

Hematological malignancies may include cancers of the blood (leukemia) or cancers of the lymph nodes (lymphomas). Leukemias may include acute lymphoblastic leukemia (ALL),

acute myleogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), Chronic myelomonocytic leukemia (CMML), acute monocytic leukemia (AMoL), etc. Lymphomas may include Hodgkin's lymphoma and non-Hodgkin's lymphoma. Other hematologic malignancies may include myelodysplastic syndrome (MDS).

Solid tumors may include carcinomas such as adenocarcinoma, *e.g.*, breast cancer, pancreatic cancer, prostate cancer, colon or colorectal cancer, lung cancer, gastric cancer, cervical cancer, endometrial cancer, ovarian cancer, cholangiocarcinoma, glioma, melanoma, etc.

A compound of the present invention may also be used to treat cancers that may be responsive to agents that target a spliceosome gene or protein other than SF3B1. The following examples are illustrative of some of the various cancers that may be responsive to agents that target the spliceosome, and are not meant to limit the scope of the invention in any way. Thus, compounds of the present invention may be administered to subjects to treat a variety of such cancers or conditions, such as patients or subjects afflicted with:

a) Myelodysplastic syndrome (MDS): *See, e.g.*, "SF3B1 mutations in myelodysplastic syndromes: clinical associations and prognostic implications," Damm F. et al. Leukemia, 2011, 1-4; "Frequent pathway mutations in splicing machinery in myelodysplasia," Yoshida K. et al, Nature, 2011, 478, 64-69; "Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms," Malcovati L. et al., Blood, 2011, 118, 24, 6239-6246; "Mutations in the spliceosome machinery, a novel and ubiquitous pathway in leukemogenesis," Makishima et al, Blood, 2012, 119, 3203-3210; "Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts," Pappaemannuil, E. et al, New England J. Med. 2011, DOI 10.1056/NEJMoa1103283.

b) Chronic lymphocytic leukemia (CLL): *See, e.g.*, "Defects in the spliceosomal machinery: a new pathway of leukaemogenesis," Maciejewski, J.P., Padgett, R.A., Br. J. Haematology, 2012, 1-9; "Mutations in the SF3B1 splicing factor in chronic lymphocytic leukemia: associations with progression and fludarabine-refractoriness," Rossi et al, Blood, 2011, 118, 6904-6908; "Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia," Quesada et al, Nature Genetics, 2011, 44, 47-52.

c) Chronic myelomonocytic leukemia (CMML): *See, e.g.*, Yoshida et al, Nature 2011; "Spliceosomal gene mutations are frequent events in the diverse mutational spectrum of chronic myelomonocytic leukemia but largely absent in juvenile myelomonocytic leukemia," Kar S.A. et al, Haematologia, 2012, DOI: 10.3324/haematol.2012.064048; DeBoever et al., "Transcriptome

sequencing reveals potential mechanism of cryptic 3' splice site selection in SF3B1-mutated cancers," PLOS Computational Biology, 2013, DOI: 10.1371/journal.pcbi.1004105.

d) Acute myeloid leukemia (AML): *See, e.g.*, Malcovati et al., Blood 2011; Yoshida et al, Nature 2011.

e) Breast cancer: *See, e.g.*, "Whole genome analysis informs breast cancer response to aromatase inhibition," Ellis et al., Nature, 2012, 486, 353-360; DeBoever et al., "Transcriptome sequencing reveals potential mechanism of cryptic 3' splice site selection in SF3B1-mutated cancers," PLOS Computational Biology, 2013, DOI: 10.1371/journal.pcbi.1004105; Maguire et al., "SF3B1 mutations constitute a novel therapeutic target in breast cancer," J Pathol 2015, 235, 571-580.

f) Uveal melanoma: *See, e.g.*,"SF3B1 mutations are associated with alternative splicing in uveal melanoma," Furney et al., Cancer Disc. 2013, 10, 1122-1129; DeBoever et al., "Transcriptome sequencing reveals potential mechanism of cryptic 3' splice site selection in SF3B1-mutated cancers," PLOS Computational Biology, 2013, DOI: 10.1371/journal.pcbi.1004105.

g) Endometrial cancer: *See, e.g.*, Tefferi et al., "Myelodysplastic syndromes." N Engl J Med. 2009; 361:1872–85.

h) Gastric cancer: *See, e.g.*, Int J Cancer. 2013 Jul;133(1):260-5, "Mutational analysis of splicing machinery genes SF3B1, U2AF1 and SRSF2 in myelodysplasia and other common tumors." Je et al.

i) Ovarian cancer: *See, e.g.*, Int J Cancer. 2013 Jul;133(1):260-5, "Mutational analysis of splicing machinery genes SF3B1, U2AF1 and SRSF2 in myelodysplasia and other common tumors." Je et al.

j) Biliary Tract cancers such as Cholangiocarcinoma and Pancreatic cancer: *See, e.g.*, Biankin et al., "Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes," Nature 2012, 491, 399-405.

k) Lung cancer: *See, e.g.*, "Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia," Quesada et al., Nature Genetics 44, 47–52 (2012); Scott et al., "Acquired mutations that affect pre-mRNA splicing in hematologic malignancies and solid tumors," JNCI 105, 20, 1540-1549.

In addition, the Catalogue of somatic mutations in cancer (COSMIC) (Wellcome Trust Sanger Institute, Genome Research Limited, England) reports SF3B1 mutations have been found in various types of cancer samples.

A compound of the present invention may be administered to a subject in a treatment effective or therapeutically effective amount. The amount of a compound of the present invention that may be combined with a carrier material to produce a composition in a single dosage form will vary depending upon the subject treated and the particular route of administration. Preferably, the compositions should be formulated so that a dosage of between 0.01 - 100 mg/kg body weight/day of the active agent can be administered to a subject receiving these compositions. In certain embodiments, the compositions of the present invention provide a dosage of from 0.01 mg to 50 mg. In other embodiments, a dosage of from 0.1 mg to 25 mg or from 5 mg to 40 mg is provided.

It should also be understood that a specific dosage and treatment regimen for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, the judgment of the treating physician, and the severity of the particular disease being treated. The amount of active agent of the present invention in the composition will also depend upon the particular compound/salt in the composition.

In some embodiments, the cancer is tested for and/or is positive for one or more mutations in a spliceosome gene or protein, wherein the presence of the mutation(s) ("positive") may indicate the subject's cancer is responsive to a method of treatment comprising administration of a compound targeting this protein and/or the spliceosome. Examples of such spliceosome genes include, but are not limited to, those presented in **Table 1**.

Table 1: Spliceosome genes and potential diseases affected

Spliceosome gene	**Disease(s)**
Splicing factor 3B subunit 1 (SF3B1)	see listings above
U2 small nuclear RNA auxiliary factor 1	MDS, AML, CMML, LUAD, UCEC
(U2AF1)	CMML, MDS, PMF, AML
Serine/arginine-rich splicing factor 2 (SRSF2)	MDS
Zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2 (ZRSR2)	Retinitis Pigmentosa
Pre-mRNA-processing-splicing factor 8 (PRPF8)	Myeloid neoplasms
U2 Small Nuclear RNA Auxiliary Factor 2 (U2AF2)	MDS, PRAD, COAD
Splicing Factor 1 (SF1)	myeloid neoplasms, OV, COAD
Splicing factor 3a subunit 1 (SF3A1)	MDS
PRP40 pre-mRNA processing factor 40 homolog B (PRPF40B)	LUAD

RNA Binding Motif Protein 10 (RBM10)	COAD
Poly(rC) binding protein 1 (PCBP1)	SKCM
Crooked neck pre-mRNA splicing factor 1 (CRNKL1)	LUSC
DEAH (Asp-Glu-Ala-His) box helicase 9 (DHX9)	STAD
Peptidyl-prolyl cis-trans isomerase-like 2 (PPIL2)	SKCM
RNA binding motif protein 22 (RBM22)	LUAD
Small nuclear ribonucleoprotein Sm D3 (SNRPD3)	GBM, LGG
Probable ATP-dependent RNA helicase DDX5 (DDX5)	LUAD
Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 (DHX15)	DLBCL
Polyadenylate-binding protein 1 (PABPC1)	myeloid neoplasms

Key:

MDS = Myelodysplastic syndrome
AML = Acute Myeloid Leukemia
CMML = chronic myelomonocytic leukemia
LUAD = Lung adenocarcinoma
UCEC = Uterine Corpus Endometrial Carcinoma
PMF = Progressive Massive Fibrosis
PRAD = Prostate adenocarcinoma
COAD = Colon adenocarcinoma
OV = Ovarian serous cystadenocarcinoma
SKCM = Skin Cutaneous Melanoma
LUSC = Lung squamous cell carcinoma
STAD = Stomach adenocarcinoma
GBM = Glioblastoma multiforme
LGG = Brain Lower Grade Glioma
DLBCL = Diffuse Large B-Cell Lymphoma

In some embodiments, the subject's cancer may be responsive to a method of treatment comprising administration of a compound targeting this protein and/or the spliceosome even in the absence of such mutations in a spliceosome gene or protein.

Screening or testing for the mutations may be carried out by any known means, for example, genotyping, phenotyping, etc., by way of nucleic acid amplification, electrophoresis, microarrays, blot, functional assays, immunoassays, etc. Methods of screening may include, for example, collecting a biological sample from said subject containing the cancerous cells/tissue.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting this invention in any manner.

EXAMPLES

Preparation of Compounds 1, 2, 3, and 4

General:

Microwave heating was done using a Biotage Emrys Liberator or Initiator microwave. Column chromatography was carried out using an Isco Rf200d. Solvent removal was carried out using either a Büchi rotary evaporator or a Genevac centrifugal evaporator. Preparative LC/MS was conducted using a Waters autopurifier and 19 x 100mm XTerra 5 micron MS C18 column under acidic mobile phase condition. NMR spectra were recorded using a Varian 400MHz spectrometer.

When the term "inerted" is used to describe a reactor (*e.g.*, a reaction vessel, flask, glass reactor, and the like) it is meant that the air in the reactor has been replaced with an essentially moisture-free or dry, inert gas (such as nitrogen, argon, and the like).

General methods and experimentals for preparing compounds of the present invention are set forth below.

The following abbreviations are used herein:

MeOH: Methanol

DMF: Dimethylformamide

KHMDS: Potassium bis(trimethylsilyl)amide

LCMS: Liquid chromatography – mass spectrometry

TBSCl: tert-Butyldimethylsilyl chloride

THF: Tetrahydrofuran

TLC: Thin-layer chromatography

Materials: The following compounds are commercially available and/or can be prepared in a number of ways well known to one skilled in the art of organic synthesis. More specifically,

disclosed compounds can be prepared using the reactions and techniques described herein. In the description of the synthetic methods described below, it is to be understood that all proposed reaction conditions, including choice of solvent, reaction atmosphere, reaction temperature, duration of the experiment, and workup procedures, can be chosen to be the conditions standard for that reaction, unless otherwise indicated. It is understood by one skilled in the art of organic synthesis that the functionality present on various portions of the molecule should be compatible with the reagents and reactions proposed. Substituents not compatible with the reaction conditions are apparent to one skilled in the art, and alternate methods are therefore indicated. The starting materials for the examples are either commercially available or are readily prepared by standard methods from known materials.

LCMS information

Mobile phases: A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile).

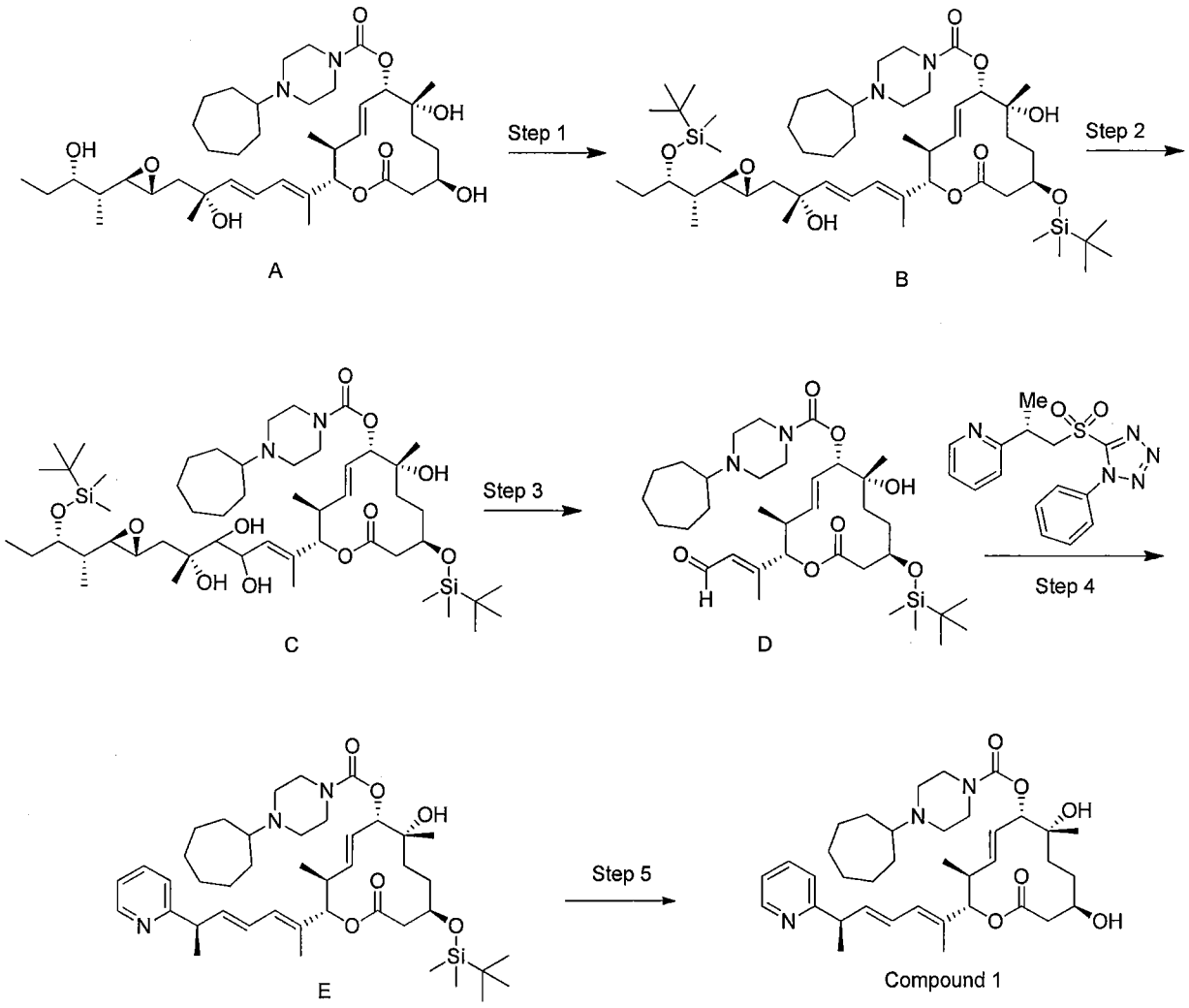
Gradient: B 5% → 95% in 1.8 minutes.

Column: Acquity BEH C18 column (1.7 um, 2.1 x 50 mm).

U.S. Patent Nos. 7,884,128 and 7,816,401, both entitled: Process for Total Synthesis of Pladienolide B and Pladienolide D, describe methods known in the art for synthesis of Pladienolide B and D. Synthesis of Pladienolide B and D may also be performed using methods known in the art and described in Kanada et al., "Total Synthesis of the Potent Antitumor Macrolides Pladienolide B and D," *Angew. Chem. Int. Ed.* 46:4350-4355 (2007). Kanada et al. and PCT application publication WO 2003/099813, entitled: Novel Physiologically Active Substances, describe methods known in the art for the synthesis of E7107 (Compound 45 of WO '813) from Pladienolide D (11107D of WO '813). A corresponding U.S. Patent is 7,550,503 to Kotake et al.

EXAMPLARY SYNTHESIS OF COMPOUNDS

Synthesis of Compound 1



Scheme I

Step 1: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy)-((R,2E,4E)-7-((2R,3R)-3-((2S,3S)-3-((*tert*-butyldimethylsilyl)oxy)pentan-2-yl)-6-hydroxy-6-methylhepta-2,4-dien-2-yl)-7-hydroxy-3,7-dimethyl-12-oxooxacyclododec-4-en-6-yl 4-cycloheptylpiperazine-1-carboxylate. A solution of E7107 (**A**, 3.7 g, 5.1 mmol, 1.0 equiv.) under nitrogen in DMF (100 mL, 0.05M) at 0 °C was treated with imidazole (2.5 g, 36.1 mmol, 7.0 equiv.) and TBSCl (3.9 g, 25.7 mmol, 5.0 equiv.) was added. The reaction was allowed to warm to room temperature and stirred for 20 hours or until the reaction was determined to be complete by LCMS or TLC. The reaction was diluted with ethyl acetate and the organic layer was washed with brine, dried over sodium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (hexanes/ethyl acetate as eluant) to afford the desired product (**B**, 4.7 g, 5.0 mmol, 96 %).

Step 2: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy)-2-((6R,E)-7-((2R,3R)-3-((2S,3S)-3-((*tert*-butyldimethylsilyl)oxy)pentan-2-yl)oxiran-2-yl)-4,5,6-trihydroxy-6-methylhept-2-en-2-yl)-7-hydroxy-3,7-dimethyl-12-oxooxacyclododec-4-en-6-yl 4-cycloheptylpiperazine-1-carboxylate. To a solution of olefin **B** (4.7 g, 5.0 mmol, 1.0 equiv.) in THF:H_2O (10:1, 133 mL:13 mL, 0.03M) under nitrogen at 0 °C was added osmium tetroxide (12.4 mL, 1.0 mmol, 0.2 equiv., 2.5% solution) followed by N-methylmorpholine N-oxide (1.16 g, 9.9 mmol, 2.0 equiv.). The reaction was allowed to warm to room temperature and stirred for 13 hours or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with sodium sulfite, diluted with ethyl acetate, and the organic layer was washed with water, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (dichloromethane/methanol as eluent) to afford the desired product (**C**, 4.8 g, 4.9 mmol, 99%).

Step 3: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy-7-hydroxy-3,7-dimethyl-12-oxo-2-((E)-4-oxobut-2-en-2-yl)oxacyclododec-4-en-6-yl 4-cycloheptylpiperazine-1-carboxylate. To a solution of diol **C** (4.4 g, 4.5 mmol, 1.0 equiv.) in benzene (100 mL, 0.05M) under nitrogen at room temperature was added lead tetraacetate (4.0 g, 9.0 mmol, 2.0 equiv.). The reaction was stirred for 30 minutes, or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with sodium sulfite and diluted with dichloromethane. The organic layer was washed with water, dried over sodium sulfate, filtered, and concentrated in vacuo. The desired product (**D**, 1.5 g, 2.3 mmol, 52 %) was advanced crude.

Step 4: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy-7-hydroxy-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl 4-cycloheptylpiperazine-1-carboxylate.

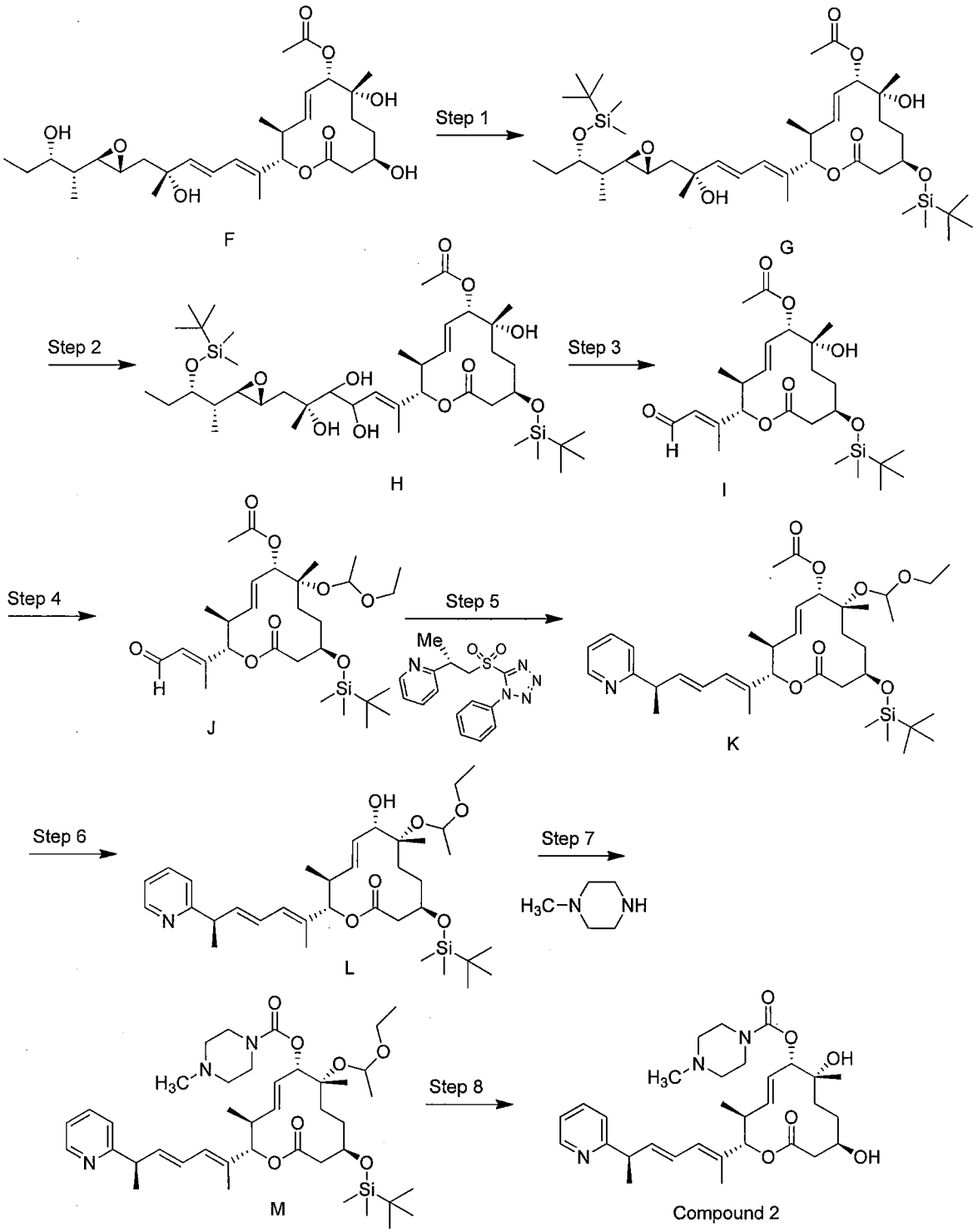
Note: The synthesis of (S)-2-(1-((1-phenyl-1H-tetrazol-5-yl)sulfonyl)propan-2-yl)pyridine is described below and depicted in Scheme V.

To (S)-2-(1-((1-phenyl-1H-tetrazol-5-yl)sulfonyl)propan-2-yl)pyridine (1.67 g, 5.08 mmol, 2.5 equiv.) in dry THF (30.0 mL, 0.05M) under nitrogen at -78 °C was added KHMDS (8.53 ml, 4.265 mmol, 2.1 equiv.) dropwise and the reaction was stirred for 10 minutes. Then aldehyde **D** (2S,3S,6S,7R,10R,E)-10-((tert-butyldimethylsilyl)oxy)-7-hydroxy-3,7-dimethyl-12-oxo-2-((E)-4-oxobut-2-en-2-yl)oxacyclo-dodec-4-en-6-yl 4-cycloheptylpiperazine-1-carboxylate (1.318 g, 2.031 mmol, 1.0 equiv.) in THF (10 mL) was added dropwise. The reaction was stirred at -78 °C for one hour and then allowed to warm to room temperature overnight. The reaction

was quenched with water and diluted with ethyl acetate. The organic layer was washed with water and brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (hexane/ethyl acetate as eluent) to afford the desired product (**E**, 1.20 g, 2.03 mmol, 79%).

Step 5: Synthesis of (2S,3S,6S,7R,10R,E)-7,10-dihydroxy-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl 4-cycloheptylpiperazine-1-carboxylate (**compound 1**). A solution of silyl ether **E** (1.80 g, 2.39 mmol, 1.0 equiv.) in MeOH (10.0 mL, 0.24M) under nitrogen at room temperature was treated with pTsOH (1.14 g, 5.98 mmol, 2.5 equiv.). The reaction was stirred for 2 hours, or until the reaction was determined to be complete by LCMS or TLC. The reaction was then diluted with ethyl acetate and washed with brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by preparative TLC (dichloromethane/methanol as eluant) to afford the desired product (**compound 1,** 1.19 g, 1.83 mmol, 76 %). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 0.88 (d, *J*=6.65 Hz, 6 H) 1.23 (s, 3 H) 1.34 - 1.78 (m, 12 H) 1.44 (d, *J*=7.03 Hz, 3 H) 1.73 (s, 3 H) 2.28 - 2.39 (m, 1 H) 2.45 - 2.66 (m, 8 H) 3.48 (br. s., 5 H) 3.72 (m, 2 H) 5.01 (d, *J*=9.54 Hz, 1 H) 5.14 (d, *J*=10.67 Hz, 1 H) 5.55 - 5.72 (m, 2 H) 6.00 (dd, *J*=15.00, 7.47 Hz, 1 H) 6.11 (d, *J*=11.29 Hz, 1 H) 6.28 - 6.35 (m, 1 H) 7.12 (ddd, *J*=7.47, 4.89, 1.07 Hz, 1 H) 7.16 (d, *J*=7.78 Hz, 1 H) 7.61 (t, *J*=7.65 Hz, 1 H) 8.55 (d, *J*=4.91 Hz, 1 H). MS (ES+) = 638.4 $[M+H]^+$.

Synthesis of Compound 2



Scheme II

Step 1: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy)-2-((R,2E,4E)-7-((2R,3R)-3-((2S,3S)-3-((*tert*-butyldimethylsilyl)oxy)pentan-2-yl)oxiran-2-yl)-6-hydroxy-6-methylhepta-2,4-dien-2-yl)-7-hydroxy-3,7-dimethyl-12-oxooxacyclododec-4-en-6-yl acetate. A solution of pladienolide D (**F**, 5.3 g, 9.7 mmol, 1.0 equiv.) under nitrogen in DMF (80 mL, 0.1M) at 0 °C was treated with imidazole (4.6 g, 67.8 mmol, 7.0 equiv.) and TBSCl (7.3 g, 48.4 mmol, 5.0 equiv.). The reaction was allowed to warm to room temperature and stirred for 20 hours, or until the reaction was determined to be complete by LCMS or TLC. The reaction was extracted with ethyl acetate and the organic layer was washed with brine, dried over sodium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (hexanes/ethyl acetate as eluant) to afford the desired product (**G**, 7.5 g, 9.6 mmol, 99%).

Step 2: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy)-2-((6R,E)-7-((2R,3S)-3-((*tert*-butyldimethylsilyl)oxy)pentan-2-yl)oxiran-2-yl)-4,5,6-trihydrox-6-methylhept-2-en-2-yl)-7-hydroxy-3,7-dimethyl-12-oxooxacyclododec-4-en-6-yl acetate. To a solution of olefin **G** (7.6 g, 9.7 mmol, 1.0 equiv.) in degassed THF:H_2O (210 mL:21 mL, 0.01M) under nitrogen at 0 °C was added osmium tetroxide (24.4 mL, 1.9 mmol, 0.2 equiv., 2.5% solution in *tert*-butanol) followed by N-methylmorpholine N-oxide (2.3 g, 19.5 mmol, 2.0 equiv.). The reaction was allowed to warm to room temperature and stirred for 13 hours, or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with sodium sulfite, diluted with ethyl acetate, and the organic layer was washed with water, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (dichloromethane/methanol as eluent) to afford the desired product (**H**, 6.8 g, 8.3 mmol, 86%).

Step 3: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy)-7-hydroxy-3,7-dimethyl-12-oxo-2-((E)-4-oxobut-2-en-2-yl)oxacyclododec-4-en-6-yl acetate. To a solution of diol **H** (7.9 g, 9.7 mmol, 1.0 equiv.) in benzene (350 mL, 0.03M) under nitrogen at room temperature was added lead tetraacetate (8.6 g, 19.4 mmol, 2.0 equiv.). The reaction was stirred for 30 minutes, or until the reaction was determined to be complete by LCMS or TLC. The reaction was concentrated and purified by silica gel column chromatography (hexane/ethyl acetate as eluent) to afford the desired product (**I**, 2.5 g, 5.26 mmol, 54%).

Step 4: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy)-7-(1-ethoxyethoxy)-3,7-dimethyl-12-oxo-2-((E)-4-oxobut-2-en-2-yl)oxacyclododec-4-en-6-yl acetate. To a solution of aldehyde **I** (1.4 g, 2.9 mmol, 1.0 equiv.) in THF (9.5 mL, 0.5M) was added

ethoxyethene (11.1 mL, 40.0 equiv.) and pyridinium p-toluenesulfonate (0.07 g, 0.3 mmol, 0.1 equiv.) at room temperature. The reaction was stirred for 24 hours, or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with sodium bicarbonate and diluted with ethyl acetate. The ethyl acetate was washed with water, brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (hexane/ethyl acetate as eluent) to afford the desired product (**J**, 1.2 g, 2.2 mmol, 75%).

Step 5: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy)-7-(1-ethoxyethoxy)-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl) acetate. To a solution of (S)-2-(1-((1-phenyl-1H-tetrazol-5-yl)sulfonyl)propan-2-yl)pyridine (695.0 mg, 2.1 mmol, 1.5 equiv.) in THF (20 mL, 0.06M) under nitrogen at -78 °C was added KHMDS (4.2 mL, 2.1 mmol, 1.5 equiv.) dropwise and the reaction was stirred for 20 minutes. Then aldehyde **J** (780.0 mg, 1.4 mmol, 1.0 equiv.) in THF (1.0 mL) was added dropwise. The reaction was stirred at -78 °C for 90 minutes and then allowed to warm to -20 °C for 1 hour. The reaction was quenched with ammonium chloride, diluted with ethyl acetate, and warmed to room temperature. The organic layer was washed with water, brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (hexane/ethyl acetate as eluent) to afford the desired Julia product (**K**, 490 mg, 0.7 mmol, 53%).

Step 6: Synthesis of (4R,7R,8S,11S,E)-4-((*tert*-butyldimethylsilyl)oxy)-7-(1-ethoxyethoxy)-8-hydroxy-7,11-dimethyl-12-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-9-en-2-one. To a solution of acetate **K** (490 mg, 0.7 mmol, 1.0 equiv.) in methanol (15 mL, 0.05M) at room temperature was added potassium carbonate (155 mg, 0.4 mmol, 1.5 equiv.). The reaction was run for 24 hours, or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with water, diluted with ethyl acetate, washed with brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting foamy solid (**L**, 459 mg, 0.7 mmol, 100%) was advanced into the next step without additional purification.

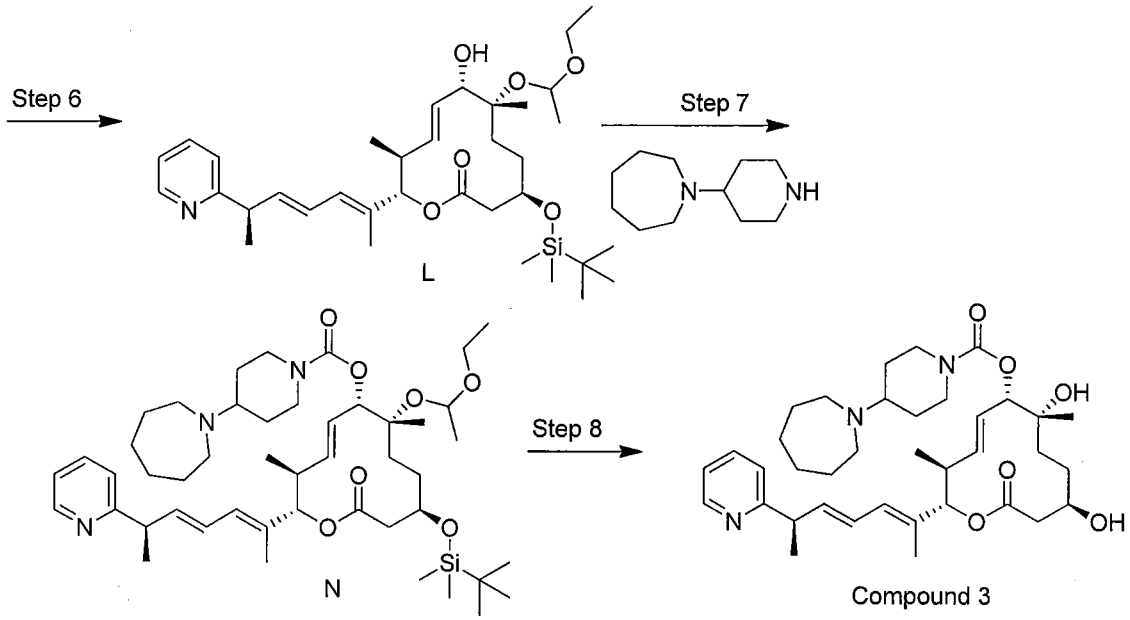
Step 7: Synthesis of (2S,3S,6S,7R,10R,E)-10-((*tert*-butyldimethylsilyl)oxy)-7-(1-ethoxyethoxy)-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl 4-methylpiperazine-1-carboxylate. To a solution of alcohol **L** (459 mg, 0.7 mmol, 1.0 equiv.) in dichloromethane (0.5 mL, 0.1M) at room temperature was added N,N-dimethylaminopyridine (27.3 mg, 0.2 mmol, 0.3 equiv.) and triethylamine (1.0 mL, 7.4

mmol, 10.0 equiv.) followed by 4-nitrophenyl chloroformate (451 mg, 02.2 mmol, 3.0 equiv.). The reaction was stirred at room temperature for three hours. Next, *N*-methyl-piperazine (299 mg, 2.98 mmol, 4.0 equiv.) was added at room temperature. After stirring for one hour, the reaction was quenched with water and diluted with dichloromethane. The organic layer was washed with 1N sodium hydroxide solution, and the organic layer was concentrated. The resulting oil was purified by silica gel column chromatography (hexanes/ethyl acetate as eluant) to afford the desired product (**M**, 553 mg, 0.75 mmol, 100 %).

Step 8: Synthesis of (2S,3S,6S,7R,10R,E)-7,10-dihydroxy-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl 4-methylpiperazine-1-carboxylate (**compound 2**). To a solution of silyl ether (**M,** 553 mg, 0.74 mmol, 1.0 equiv.) in methanol (20 mL, 0.04M) at room temperature was added p-methoxytoluenesulfonic acid (425 mg, 2.2 mmol, 3.0 equiv.). The reaction was stirred for 3 hours, or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with sodium bicarbonate and diluted with ethyl acetate. The organic layer was washed with water, brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (hexane/ethyl acetate as eluent) to afford the desired product (**compound 2**, 184 mg, 0.33 mmol, 44%). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 0.82-1.00 (m, 3H) 1.22-1.48 (m, 8H) 1.50-1.63 (m, 1H) 1.66-1.83 (m, 4H) 1.97 (s, 1H) 2.07 (s, 1H) 2.33 (s, 3H) 2.40 (br. s., 3H) 2.45-2.68 (m, 3H) 3.44-3.61 (m, 5H) 3.74 (dd, *J*=14.2, 7.2 Hz, 2H) 5.04 (d, *J*=9.3 Hz, 1H) 5.17 (d, *J*=10.5 Hz, 1H) 5.57-5.76 (m, 2H) 6.02 (dd, *J*=15.1, 7.5 Hz, 1H) 6.13 (d, *J*=10.8 Hz, 1H) 6.34 (ddd, *J*=15.1, 10.7, 1.0 Hz, 1H) 7.14 (t, *J*=6.2 Hz, 1H) 7.18 (d, *J*=7.4 Hz, 1H) 7.63 (t, *J*=7.3 Hz, 1H) 8.57 (d, *J*=5.1 Hz, 1H). MS (ES+) = 556.4 [M+H].

Synthesis of Compound 3

Steps 1 – 6 are as provided above in the synthesis of Compound 2, to give alcohol **L**.



Scheme III

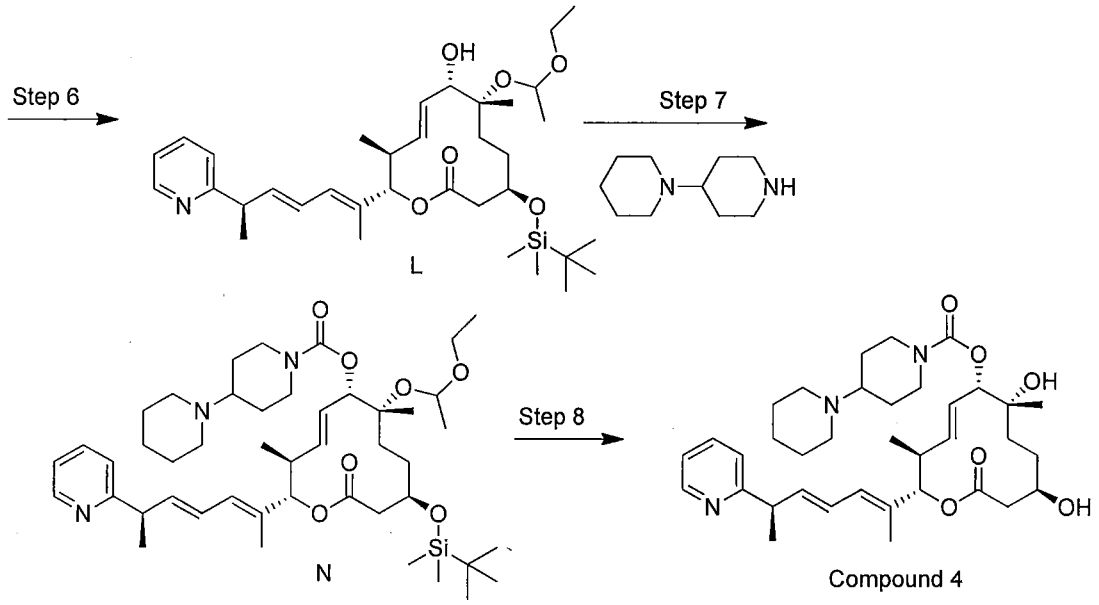
Step 7: Synthesis of (2S,3S,6S,7R,10R,E)-10-((tert-butyldimethylsilyl)oxy)-7-(1-ethoxyethoxy)-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl 4-(azepan-1-yl)piperidine-1-carboxylate. To a solution of alcohol **L** (300 mg, 0.49 mmol, 1.0 equiv.) in dichloromethane (3.0 mL, 0.15M) at room temperature was added N,N-dimethylaminopyridine (71.4 mg, 0.58 mmol, 1.2 equiv.) and triethylamine (0.27 mL, 1.95 mmol, 4.0 equiv.) followed by 4-nitrophenyl chloroformate (196 mg, 0.97 mmol, 2.0 equiv.). The reaction was stirred at room temperature for three hours. Next, 1-(piperidin-4-yl)azepane (265 mg, 1.46 mmol, 3.0 equiv.) was added at room temperature. After stirring for one hour, the reaction was quenched with water and diluted with dichloromethane. The organic layer was washed with 1N sodium hydroxide solution, and the organic layer was concentrated. The resulting oil was purified by silica gel column chromatography (hexanes/ethyl acetate as eluant) to afford the desired product (**N**, 400 mg, 0.48 mmol, 100 %).

Step 8: Synthesis of (2S,3S,6S,7R,10R,E)-7,10-dihydroxy-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl 4-(azepan-1-yl)piperidine-1-carboxylate (**compound 3**). To a solution of silyl ether (**N,** 400 mg, 0.48 mmol,

1.0 equiv.) in methanol (4.0 mL, 0.1M) at room temperature was added p-methoxytoluenesulfonic acid (231 mg, 1.2 mmol, 2.5 equiv.). The reaction was stirred for 3 hours, or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with sodium bicarbonate and diluted with ethyl acetate. The organic layer was washed with water, brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (hexane/ethyl acetate as eluent) to afford the desired product (**compound 3**, 226 mg, 0.35 mmol, 73%). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 0.88 (d, *J*=6.53 Hz, 3 H) 1.20 - 1.28 (m, 4 H) 1.35 (s, 3 H) 1.45 (d, *J*=7.03 Hz, 4 H) 1.59 (br. s., 10 H) 1.74 (d, *J*=0.75 Hz, 3 H) 1.75 - 1.83 (m, 2 H) 1.99 (s, 1 H) 2.46 - 2.62 (m, 3 H) 2.62 - 2.71 (m, 4 H) 2.79 (br. s., 2 H) 3.51 (d, *J*=9.79 Hz, 1 H) 3.63 - 3.82 (m, 2 H) 4.03 - 4.26 (m, 2 H) 5.01 (d, *J*=9.54 Hz, 1 H) 5.16 (d, *J*=10.79 Hz, 1 H) 5.54 - 5.64 (m, 1 H) 5.65 - 5.75 (m, 1 H) 6.01 (dd, *J*=15.06, 7.53 Hz, 1 H) 6.12 (d, *J*=11.04 Hz, 1 H) 6.25 - 6.39 (m, 1 H) 7.12 (ddd,*J*=7.47, 4.83, 1.25 Hz, 1 H) 7.17 (dt, *J*=8.03, 1.00 Hz, 1 H) 7.62 (td, *J*=7.65, 1.76 Hz, 1 H) 8.56 (ddd, *J*=4.96, 1.82, 1.00 Hz, 1 H). MS (ES+) = 638.6 [M+H].

Synthesis of Compound 4

Steps 1 – 6 are as provided above in the synthesis of **Compound 2**, to give alcohol **L**.

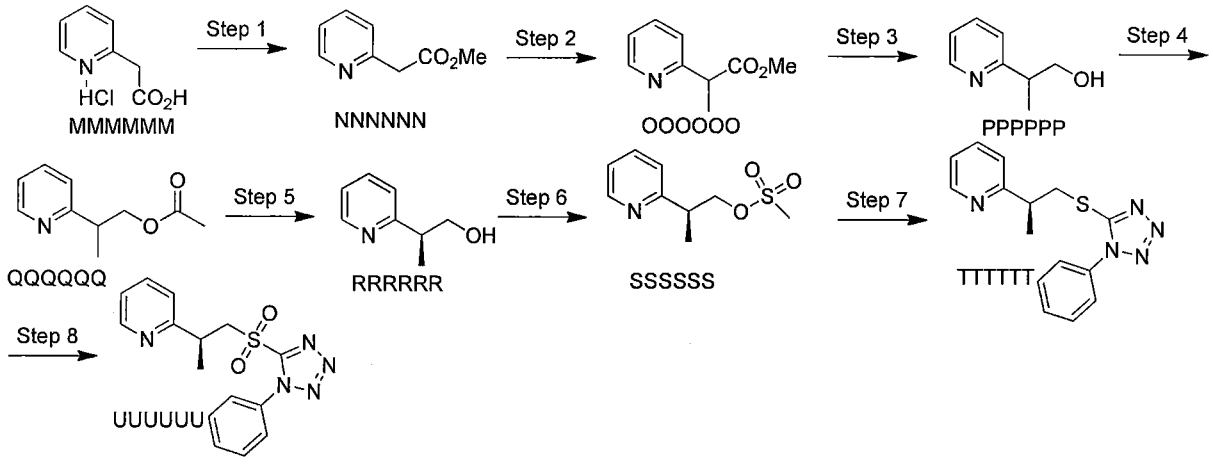


Scheme IV

Step 7: Synthesis of (2S,3S,6S,7R,10R,E)-10-((tert-butyldimethylsilyl)oxy)-7-(1-ethoxyethoxy)-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl [1,4'-bipiperidine]-1'-carboxylate. To a solution of alcohol **L** (20 mg, 0.032 mmol, 1.0 equiv.) in dichloromethane (0.3 mL, 0.1M) at room temperature was added N,N-dimethylaminopyridine (4.8 mg, 0.04 mmol, 1.2 equiv.) and triethylamine (0.02 mL, 0.13 mmol, 4.0 equiv.) followed by 4-nitrophenyl chloroformate (13.1 mg, 0.065 mmol, 2.0 equiv.). The reaction was stirred at room temperature for three hours. Next, 1,4'-bipiperidine (16.4 mg, 0.97 mmol, 3.0 equiv.) was added at room temperature. After stirring for one hour, the reaction was quenched with water and diluted with dichloromethane. The organic layer was washed with 1N sodium hydroxide solution, and the organic layer was concentrated. The resulting oil was purified by silica gel column chromatography (hexanes/ethyl acetate as eluant) to afford the desired product (**N**, 18 mg, 0.22 mmol, 68.4 %).

Step 8: Synthesis of (2S,3S,6S,7R,10R,E)-7,10-dihydroxy-3,7-dimethyl-12-oxo-2-((R,2E,4E)-6-(pyridin-2-yl)hepta-2,4-dien-2-yl)oxacyclododec-4-en-6-yl [1,4'-bipiperidine]-1'-carboxylate (**compound 4**). To a solution of silyl ether (**N,** 18 mg, 0.022 mmol, 1.0 equiv.) in methanol (0.5 mL, 0.04M) at room temperature was added p-methoxytoluenesulfonic acid (10.6 mg, 0.56 mmol, 2.5 equiv.). The reaction was stirred for 3 hours, or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with sodium bicarbonate and diluted with ethyl acetate. The organic layer was washed with water, brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified by silica gel column chromatography (hexane/ethyl acetate as eluent) to afford the desired product (**compound 4**, 4.0 mg, 0.006 mmol, 29%). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 0.90 (d, *J*=6.8 Hz, 3H) 1.17-1.42 (m, 5H) 1.46 (d, *J*=7.0 Hz, 6H) 1.51-1.65 (m, 6H) 1.65-1.78 (m, 5H) 1.85 (d, *J*=11.5 Hz, 2H) 2.44 (d, *J*=11.3 Hz, 2H) 2.49-2.66 (m, 6H) 2.80 (br. s., 2H) 3.42-3.62 (m, 1H) 3.63-3.82 (m, 2H) 4.18 (br. s., 2H) 5.02 (d, *J*=9.5 Hz, 1H) 5.17 (d, *J*=10.8 Hz, 1H) 5.57-5.75 (m, 2H) 6.02 (dd, *J*=15.2, 7.4 Hz, 1H) 6.14 (d, *J*=11.0 Hz, 1H) 6.34 (ddd, *J*=15.1, 10.8, 1.0 Hz, 1H) 7.14 (t, *J*=6.1 Hz, 1H) 7.18 (d, *J*=7.5 Hz, 1H) 7.29 (s, 2H) 7.63 (td, *J*=7.7, 1.9 Hz, 1H) 8.57 (d, *J*=5.1 Hz, 1H). MS (ES+) = 624.6 [M+H].

Synthesis of (S)-2-(1-((1-phenyl-1H-tetrazol-5-yl)sulfonyl)propan-2-yl)pyridine



Scheme V

Step 1: To a solution of 2-(pyridin-2-yl)acetic acid hydrochloride salt **MMMMMM** (50.0 g, 288.0 mmol, 1.0 equiv.) in methanol (500 mL, 0.5M) at 0 °C was added thionyl chloride (31.5 mL, 432.0 mmol, 1.5 equiv.) dropwise. The reaction was stirred at 0 °C for 60 minutes or until the reaction was determined to be complete by LCMS or TLC. The reaction was carefully quenched with sodium carbonate and the aqueous layer extracted with ethyl acetate. The combined organic layers were washed with water, brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting product (**NNNNNN**, 41.5 g, 275.0 mmol, 95%) was used in the next step without further purification.

Step 2: To a solution of ester **NNNNNN** (41.5 g, 275.0 mmol, 1.0 equiv.) in THF (1500 mL, 0.2M) at 0 °C was added sodium 2-methylpropan-2-olate (28.6 g, 288.3 mmol, 1.05 equiv.) and the reaction mixture was stirred for 30 minutes at 0 °C before addition of iodomethane (34.3 mL, 549.1 mmol, 2.0 equiv.). The reaction was stirred at room temperature for 1 hour or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with ammonium chloride and the excess of solvent was removed in vacuo. The crude material was then extracted with ethyl acetate. The combined organic layers were washed with brine, and dried over magnesium sulfate. After filtration, the mixture was concentrated in vacuo. The resulting methyl ester (**OOOOOO**, 41.3 g, 250 mmol, 91 %) was advanced without purification.

Step 3: To a solution of methyl ester **OOOOOO** (43.0 g, 260.3 mmol, 1.0 equiv.) in THF (1500 mL, 0.1M) at 0 °C was added lithium aluminum hydride (312 mL, 312.4 mmol, 1.2 equiv., solution in THF) dropwise. The reaction was allowed to warm gradually to 0 °C for 30 minutes

and then to room temperature for 1 hour or until the reaction was determined to be complete by LCMS or TLC. The reaction was carefully quenched with water, sodium hydroxide and water. After stirring the mixture for 30 minutes, the white precipitate was filtered off and the solvent was removed in vacuo. The reaction was then extracted with diethyl ether and the combined organic fractions were washed with water, brine, dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting alcohol (**PPPPPP**, 30.0 g, 219.0 mmol, 84%) was advanced without purification.

Step 4: To a solution of alcohol **PPPPPP** (30.0 g, 219.0 mmol, 1.0 equiv.) in dichloromethane (700 mL, 0.3M) at 0 °C was added triethylamine (61.5 mL, 437.4 mmol, 2.0 equiv), and DMAP (2.7 g, 21.9 mmol, 0.1 equiv.). Acetic anhydride (24.8 mL, 262.4 mmol, 1.2 equiv.) was added and the reaction mixture was stirred for 30 minutes or until the reaction was determined to be complete by LCMS or TLC. The reaction was quenched with ammonium chloride, the organic layer was washed with brine, dried over magnesium sulfate and filtered. The resulting solution was then evaporated and the crude acetate (**QQQQQQ**, 37.0 g, 206.0 mmol, 94%) was used in the following step without further purification.

Step 5: A solution of acetate **QQQQQQ** (39.4 g, 219.8 mmol, 1.0 equiv.) was dissolved in diethyl ether (100 mL) and then 118 g of silica gel was added. The excess of ether was removed in vacuo and the crude solid was then diluted in pH 7 aqueous buffer (1970 mL, 0.1M) (sodium hydroxyde / sodium phosphate monobasic / water). Porcine pancreatic lipase type II (3.3 g, (15mg/mmol)) was added and the reaction was stirred at 37 °C for four hours or until determined to be complete by TLC or LCMS. (After four hours, conversion reached 40% according to ELSD and the enantiomeric excess was determined by chiral SFC, and showed an enantiomeric ratio of 13:1 S:R). (SFC condition: SFC Investigator (Waters/Thar), software: Chromscope v1.2, method: Isocratic 15% co-solvent 95:5 Heptane:IPA +0.1% DEA over 10 minutes, Column: Lux-Amylose-2, 4.6x250mm, 5μm, Total Flow: 4ml/min (3.80 ml from CO_2 pump, 0.20 ml from modifier pump), Oven temp set to 35 °C and system pressure set to 100 bar, Retention Times: desired and major (S)-enantiomer 6.9 min, minor (R)-enantiomer 8.4 min). The silica gel was filtered off and the aqueous layer was extracted with ethyl acetate three times. The combined organic layers were washed with brine, dried over magnesium sulfate and concentrated. The product was purified by silica gel column chromatography (hexanes:ethyl acetate as eluant) to afford the desired alcohol (**RRRRRR**, 12.5 g, 91 mmol, 41%).

Step 6: To a solution of alcohol **RRRRRR** (12.5 g, 91.0 mmol, 1.00 equiv.) in dichloromethane (570 mL, 0.16M) at room temperature was added triethylamine (13.9 mL,

100.1 mmol, 1.1 equiv). The reaction was cooled down to 0 °C and then methanesulfonyl chloride (7.44 mL, 95.5 mmol, 1.05 equiv) was added. The reaction was stirred at 0 °C for 30 minutes or until determined to be complete by TLC or LCMS. The reaction was quenched with sodium bicarbonate and the layers were separated. The aqueous layer was then extracted with dichloromethane. The combined organic layers were washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The resulting sulfonate **SSSSSS** (19.2 g, 89 mmol, 98%) was advanced without additional purification.

Step 7: To a solution of sulfonate **SSSSSS** (19.2 g, 89 mmol, 1.0 equiv.) in DMF (120 mL, 0.1M) at room temperature was added cesium carbonate (40.7 g, 125.0 mmol, 1.4 equiv.) and 1-phenyl-1H-tetrazole-5-thiol (19.1 g, 107.1 mmol, 1.2 equiv.). The resulting mixture was stirred at 50 °C for 48 hours, or until determined to be complete by TLC or LCMS. After cooling the mixture to room temperature, brine was added and the aqueous layer was extracted three times with diethyl ether. The combined organic layers were washed with water, brine, and dried over magnesium sulfate. After filtration, the solvent was removed in vacuo and the residue was purified using silica gel column chromatography (hexanes/ethyl acetate) to give the desired product (**TTTTTT,** 28.9 g, 88 mmol, 99%).

Step 8: To a solution of sulfide **TTTTTT** (31.5 g, 105.9 mmol, 1.0 equiv.) in EtOH (700 mL, 0.1M) at -10 °C was added ammonium molybdate tetrahydrate (6.5 g, 5.3 mmol, 0.05 equiv.) and hydrogen peroxide (108 mL, 1060 mmol, 5.0 equiv., 33% aqueous solution). The reaction was stirred at -10 °C for four hours or until determined to be complete by TLC or LCMS. The reaction was quenched with water and sodium metabisulfite solution. The crude product was collected by filtration and was purified by silica gel column chromatography (hexanes:ethyl acetate as eluant) to afford the desired product (**UUUUUU**, 23.2 g, 70.4 mmol, 66 %). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 1.50 (d, *J*=7.03 Hz, 3 H) 1.66 (br. s., 1 H) 3.75 (m, 1 H) 3.94 (dd, *J*=14.81, 5.02Hz, 1 H) 4.55 (dd, *J*=14.68, 7.91 Hz, 1 H) 7.14 - 7.22 (m, 2 H) 7.29 (s, 1 H) 7.57 - 7.70 (m, 6 H) 8.44 - 8.49 (m, 1 H).

The colorless oil was then recrystallized using toluene/heptane (1/1) (1 mL of toluene and 1 mL of heptane per 100 mg of compound. Heat gently the mixture to mix the two solvents. Let the mixture cool down to room temperature for 12h. (If no recrystallization is observed, add one crystal to the solution. The crystal will help to get crystals via seeding process.) The crystals formed slowly over time. They could be isolated via filtration or removing liquid layer via pipette. The crystals were then washed with heptane and then quickly with toluene. The *er* of the sulfone was analized before and after recrystallization. (SFC conditions: SFC condition: SFC

Investigator (Waters/Thar), software: Chromscope v1.2, method: Isocratic 10% co-solvent MeOH over 10 minutes, Column: ChiralPak IC, 4.6x250mm, 5um, Total Flow: 4ml/min (3.80ml from $C0_2$ pump, 0.20 ml from modifier pump), Oven temp set to 35 °C and system pressure set to 100 bar, Retention Times: desired and major (S)-enantiomer 3.5 min, minor (R)-enantiomer 3.8 min).

pH Stability Measurements

Compounds were provided in 96-well plates and tested in triplicate. Four microliters of a 10 mM stock solution of compound in DMSO were distributed into each of three wells. The plate was stored at or below -20 °C until the day of the analysis. Methanol (HPLC grade) and 0.1 N HCl (EMD catalog HX0603A-6) were used for dilutions. Acetonitrile (HPLC grade), water (Milli-Q filtered), trifluoroacetic acid (spectral grade) and 0.2M phosphate buffer (Wako, catalog no.163-14471) were used to prepare the mobile phase for the two analyses.

Stability data was obtained using a Waters Acquity UPLC equipped with a UV detector (Waters TUV) and single quadrupole MS detector (Waters SQD). The 96-well plate containing the compound(s) of interest was(were) removed from the freezer and allowed to warm to room temperature for one hour. The UPLC was primed, equilibrated and the system performance was verified by injecting a standard. After 1 hour, each of the three wells was diluted with 266 µL 0.1 N HCl to give pH = 1. The plate was covered and placed on a shaker (Eppindorf Thermomixer R) for 45 minutes at 600 rpm. The plate was removed from the shaker, and the contents of each well were filtered through a filtration plate (Millipore catalog no. MSSLBPC50) by vacuum and injected into the UPLC. After approximately 24 hours, the contents of the wells were re-injected into the UPLC.

UPLC Instrument Parameters (Solubility and Stability Measurement)

Column	Acquity HSS T3 column, 2.1 x 100 mm, 1.8 µm	
Mobile Phase	A=0.05%TFA in Water B= 0.05%TFA in CH3CN	
Elution Gradient	Time	Mobile Phase A:B
	0.0	90/10

	0.75 1.0 1.01 1.5	3/97 3/97 90/10 90/10
Column temperature	50°C	
Detection	UV @ 220 nm	
Injection volume	1 µL	
Flow rate	0.9 mL/minute	

Stability in the various buffers was measured by comparing the peak area-% of the analyte in methanol versus the peak area-% of the analyte at the same retention time in 0.1 N HCl buffer in the 24 hr time point injection. The stability assays reported in **Table 2** show that compounds 1-4 have greater stability at pH 1 than compound E7107 over a 24-hour period.

Table 2: Stability Assay Results

Compound	% parent remaining (pH = 1, 24 hours)
E7107	44%
Compound 1	91%
Compound 2	96%
Compound 3	98%
Compound 4	99%

Biological Assays

Cell viability assay protocol

Cells (WiDr and Panc05.04 obtained from ATCC) were seeded in 96-well plates, with 2000 cells/100µL/well, and incubated overnight. Spent media was removed, and fresh media containing 9 different concentrations of compound (100µL/well) were added, with DMSO concentration from compound stock solution adjusted to be 0.1%. Each compound treatment was done in duplicate or triplicate at each concentration.

Another plate with cells seeded was dedicated as a time zero (Tz) plate, to which was added 0.1% DMSO in media (100µL/well) followed by CellTiter-Glo® reagent (Promega

Corporation, Madison, Wisconsin) (50μL/well) for ATP measurement as a surrogate of cell viability. Average value from measurement of multiple wells of this plate is used as Tz.

Compound-treated plates were incubated for 72hr at 37°C. Then, CellTiter-Glo® reagent (50μL/well) was added and ATP was measured. Average value from measurement of the duplicate or triplicate compound-treated wells is used as Ti, and seeded plates with medium having 0.1% DMSO without compound is used as control growth (C).

Percentage growth inhibition/Percentage viability was calculated as:

[(Ti-Tz)/(C-Tz)] x 100 for concentrations for which Ti>/=Tz

[(Ti-Tz)/Tz] x 100 for concentrations for which Ti<Tz.

*time zero (Tz), control growth (C), and test growth in the presence of compound (Ti)

Percentage growth inhibition/Percentage viability are plotted versus compound concentration to determine E_{max}.

Growth inhibition of 50% (GI_{50}) was calculated from [(Ti-Tz)/(C-Tz)] x 100 = 50, which is the drug concentration resulting in a 50% reduction in the net increase of ATP in control growth (C) during the compound treatment.

In vitro splicing (biochemical) assay protocol

Biotin-labeled pre-mRNA of an adenovirus type 2 construct with a deletion of intervening sequence (Ad2) (Berg, M.G., et al. 2012 Mol. Cell Bio., 32(7):1271-83) was prepared by *in vitro* transcription. The Ad2 construct containing Exon 1 (41 nucleotides), Intron (231 nucleotides), and Exon 2 (72 nucleotides) was generated by gene synthesis and cloned into the EcoRI and XbaI sites of pGEM®-3Z vector (Promega) by Genewiz® (South Plainfield, New Jersey). The plasmid was then linearized by XbaI digestion and purified. *In vitro* transcription and purification of transcribed pre-mRNA were performed using the MEGAscript® T7 transcription kit (Invitrogen™, Life Technologies™, Grand Island, New York) and MEGAclear™ transcription clean-up kit (Invitrogen™, Life Technologies™, Grand Island, New York), respectively, following the manufacturer's instructions. The ratio of biotin-16-UTP (Roche Diagnostics Corporation, Indianapolis, Indiana) to cold UTP was 1:13 to incorporate approximately two biotin molecules per spliced Ad2 mRNA.

In vitro splicing assay was performed at 30°C in 25μL reaction mixtures containing 95μg HeLa nuclear extract (Promega Corporation, Madison, Wisconsin), 47nM Ad2 pre-mRNA, 25U RNasin RNase inhibitor (Promega Corporation, Madison, Wisconsin), 1X SP buffer (0.5 mM

ATP, 20mM creatine phosphate, 1.6 mM $MgCl_2$), and compounds in DMSO (with 1% final concentration of DMSO). After 90 min of incubation, the reaction was stopped by addition of 18μL of 5M NaCl, and the mixtures were incubated with 10μL of M-280 streptavidin-coated magnetic beads (Invitrogen™, Life Technologies™, Grand Island, New York) for 30 min at room temperature to capture Ad2 pre- and spliced mRNA. The beads were washed twice with 100uL buffer containing 10mM Tris pH=7.5, 1mM EDTA and 2M NaCl, and then incubated in RNA gel loading buffer containing 95% formamide at 70°C for 10 min to elute the RNAs. Ad2 RNAs were resolved by 6% TBE-UREA gel, transferred to a nylon membrane, UV cross-linked, and probed with an IRDye® labeled streptavidin (LI-COR, Lincoln, Nebraska). The amount of spliced RNA was quantified by measuring the band fluorescent intensity using LI-COR Image Studio software.

Results

Data are reported in **Table 3** below. E_{max} refers to the maximum achievable response to a compound in a tested dose range, with a negative value indicating cellular lethality. A larger negative E_{max} value indicates greater cellular lethality for a particular compound. For example, in Panc 05.04 cells, a mutant SF3B1 cell line, the larger negative E_{max} value indicates that Compound 1 had greater cellular lethality than Compound 2.

WiDr-R cells are colon cancer cells which have a chemically-induced R1074H mutation and have been shown to be resistant to pladienolide B in terms of growth inhibition (Yokoi, A., et al., 2011 FEBS Journal, 278:4870-4880). The counter-screening of compounds in this viability assay with a "resistant" WiDr-R cell line may indicate whether these compounds have off-target effect(s). Compounds that lack growth inhibitory (GI_{50}) activity in the resistant WiDr-R cell line but maintain activity in the parental WiDr cell line suggests that on-mechanism splicing modulation is responsible for the growth inhibition which is observed in the parental WiDr cell line.

The *in vitro* splicing (IVS) assay described above is a biochemical assay that monitors inhibition of the splicing of an exemplary pre-mRNA into an mRNA. This biochemical assay enables researchers to assess at what compound concentration splicing of this particular transcipt is inhibited in a non-cellular context and is used to demonstrate mechanistic splicing inhibitory activity.

Table 3: Biological Activity of Compounds 1, 2, 3 and 4

Compound number	Panc 05.04 (mt SF3B1 cells) E_{max} (%)	Panc 05.04 (mt SF3B1 cells), GI_{50} (nM)	WiDr GI_{50} (nM)	WiDr-R GI_{50} (nM)	In vitro splicing (IVS) assay (nM)
1	-86.85	19.48	15.85	>1000	188.00
2	-66.09	32.72	31.78	>1000	1330.00
3	-90.81	20.75	26.29	>1000	770.00
4	-86.16	11.81	12.98	>1000	89.00

Key

Panc 05.04 cells: Pancreatic cancer cells, mutant SF3B1 cell line (Q699H and K700E mutations in SF3B1)

WiDr cells: Colon cancer cells (wildtype SF3B1)

WiDr-R cells: Colon cancer cells (chemically-induced SF3B1 mutant which is resistant to E7107 (R1074H mutation))

ADDITIONAL TESTING OF COMPOUNDS

Mouse Pharmacokinetic (PK) Study

Compound **2** was dosed at 5 mg/kg IV (intravenous) or 10 mg/kg PO (oral administration) to CD-1 mice. Following administration, blood samples were collected at pre-determined time points from five mice via serial bleeding of the tail vein. Blood was collected at 0.083 (0.167 PO only), 0.5, 1, 2, 4, 6, 8, and 24 hours post administration. The blood samples were centrifuged at 5000 RPM for 5 minutes to collect plasma within 30 minutes of blood collection. After extraction, samples were assayed using LCMS. PK parameters were calculated using non-compartmental analysis in WinNonlin v6.3.

The data indicated that Compound **2** shows oral bioavailability and favorable pharmacokinetic properties in the mouse model (**FIG. 1, Table 4**).

TABLE 4

Pharmacokinetic Property	Dose	
	5 mg/kg IV	10 mg/kg PO
C_{max} (ng/mL)	NA	840.73
C_{max}/D (ng/mL/D)	NA	84.07
t_{max} (h)	NA	1.00
$t_{1/2}$ (h)	3.88	4.04
AUC_{0-t} (ng•h/mL)	3156.95	2513.35
AUC_{0-inf} (ng•h/mL)	3163.56	2544.86
AUC_{0-inf}/D (ng•h/mL/D)	632.71	254.49
CL (L/kg/h)	1.58	NA
Vss (L/kg)	2.37	NA
%F	NA	40.22

Mouse Xenograft Model

The efficacy of Compound **2** was tested in a mouse xenograft model. Nalm-6 $SF3B1^{K700E}$ isogenic cells (human pre B-cell line, $10x10^6$ cells) were subcutaneously implanted into the flank of female CB17-SCID mice. Mice were treated with Compound **2** (10% ethanol, 5% TWEEN-80, 85% saline) or vehicle control. The animals were orally dosed daily for 14 days (QDx14 PO) at the amounts indicated in **FIG.2** and were monitored until they reached either of the following

endpoints: 1) excessive tumor volume measured three times a week (tumor volume calculated by using the ellipsoid formula: (length × width^2)/2); or 2) development of any health problems such as paralysis or excessive body weight loss. All animal studies were carried out according to the H3 Biomedicine Guide for the Care and Use of Laboratory Animals.

The results indicated that Compound **2** was efficacious when administered via the oral route and reduced tumor growth in the xenograft mouse model (**FIG. 2**).

PK/PD Testing in Mouse Xenograft Model

Pharmacokinetics (PK)/pharmacodynamics (PD) of Compound **2** were also analyzed in the Nalm-6 mouse xenograft model. Nalm-6 SF3B1^{K700E} isogenic cells (human pre B-cell line, 10x10^6 cells) were subcutaneously implanted into the flank of female CB17-SCID mice. Mice were administered a single oral dose of 10 mg/kg Compound **2** (10% ethanol, 5% TWEEN-80, 85% saline), and the tumors were collected at the indicated times post administration for analysis.

RNA was isolated using RiboPure™ RNA purification kit (Ambion®) and used for qPCR analysis. The RNA was retrotranscribed according to the instructions of the SuperScript® VILO™ cDNA synthesis kit (Invitrogen™), and 0.04 µl of cDNA was used for quantitative PCR (qPCR). qPCR for pre-mRNA EIF4A1 and mature mRNA SLC24A19 and PK evaluation were performed as reported previously (Eskens, F. A. et al. Phase I pharmacokinetic and pharmacodynamic study of the first-in-class spliceosome inhibitor E7107 in patients with advanced solid tumors. *Clin Cancer Res.* 19, 6296-6304, doi:10.1158/1078-0432.CCR-13-0485 (2013)). All animal studies were carried out according to the H3 Biomedicine Guide for the Care and Use of Laboratory Animals.

The results shown in **FIG. 3** indicated that Compound **2** showed PD responses at a tolerated dose via the oral route of administration.

Cellular Viability Assay

To assess the viability of Panc 05.04 cancer cells (SF3B1^{MUT}) (Q699H and K700E mutations in SF3B1) in the presence of Compound **2**, cells were seeded at 750 cells per well in a 384-well plate and treated with Compound **2** at the concentrations indicated in **FIG. 4** for 72 hours at 37°C. The relative number of viable or apoptotic cells were measured by luminescence using CELLTITER-GLO® luminescent Cell Viability Assay (Promega).

The results indicated differential cellular lethality in the mutant SF3B1 pancreatic cancer cell line over wild-type SF3B1 pancreatic cancer cell lines (**FIG. 4**).

Comparison of Alternative Splicing for E7107 and Compound **2**

The modulation of alternative splicing for E7107 and Compound **2** was determined using the nCounter® analysis system (NanoString Techologies, Inc., Seattle, Washington). Nalm-6 isogenic cells were treated with Compound **2** or E7107 (obtained from Eisai, Inc.) at 10 x GI_{50} for 6 hours. RNA was isolated using RiboPure™ RNA purification kit (Ambion®) and used for analysis. The RNA was retrotranscribed according to the instructions of the SuperScript® VILO™ cDNA synthesis kit (Invitrogen™) and 0.04 µl of cDNA was used for qPCR.

Results shown in **FIG. 5** indicated that the splicing modulation profile for Compound **2** is distinct from the profile of E7107.

Mouse Pharmacokinetic (PK) Study of Compound **1**

Compound **1** was dosed at 5 mg/kg IV or 12 mg/kg PO to CD-1 mice. Following administration, blood samples were collected at pre-determined time points from five mice via serial bleeding from the tail vein. Blood was collected at 0.083 (0.167 PO only), 0.5, 1, 2, 4, 6, 8, and 24 hours post-dose. The blood samples were centrifuged at 5000 RPM for 5 minutes to collect plasma within 30 minutes of blood collection. After extraction, samples were assayed using LCMS. PK parameters were calculated using non-compartmental analysis in WinNonlin v6.3.

The data indicated that Compound **1** showed oral bioavailability and favorable pharmacokinetic properties in the mouse model (**FIG. 6, Table 5**).

TABLE 5

Pharmacokinetic Property	Dose	
	5 mg/kg IV	12 mg/kg PO
C_{max} (ng/mL)	NA	1810.81
C_{max}/D (ng/mL/D)	NA	141.47
t_{max} (h)	NA	0.50
$t_{1/2}$ (h)	2.54	3.00
AUC_{0-t} (ng·h/mL)	4453.75	6206.20
AUC_{0-inf} (ng·h/mL)	4670.80	6234.38
AUC_{0-inf}/D (ng·h/mL/D)	934.15	487.06
CL (L/kg/h)	1.07	NA
Vss (L/kg)	2.10	NA

%F	NA	52.14

Efficacy of Compound **1** in a Mouse Xenograft Model

The efficacy of Compound **1** was tested in a mouse xenograft model. Nalm-6 SF3B1^{K700E} isogenic cells (human pre B-cell line, $10x10^6$ cells) were subcutaneously implanted into the flank of female CB17-SCID mice. Mice were treated with Compound **1** (10% ethanol, 5% TWEEN-80, 85% saline) or vehicle control. The animals were orally dosed daily for 14 days (QDx14 PO) with 7.5 mg/kg or 10 mg/kg Compound **1** or vehicle and were monitored until they reached either of the following endpoints: 1) excessive tumor volume measured three times a week (tumor volume calculated by using the ellipsoid formula: (length × width^2)/2); or 2) development of any health problems such as paralysis or excessive body weight loss. All animal studies were carried out according to the H3 Biomedicine Guide for the Care and Use of Laboratory Animals.

The results indicated that Compound **1** was efficacious when administered via the oral route and reduced tumor growth in the xenograft mouse model (**FIG. 7**).

PK/PD Testing of Compound **1** in Mouse Xenograft Model

Pharmacokinetics (PK)/pharmacodynamics (PD) of Compound **1** were also analyzed in the Nalm-6 mouse xenograft model. Nalm-6 SF3B1^{K700E} isogenic cells (human pre B-cell line, $10x10^6$ cells) were subcutaneously implanted into the flank of female CB17-SCID mice. Mice were administered a single oral dose of Compound **1** (10% ethanol, 5% TWEEN-80, 85% saline), and the tumors were collected at the indicated times post administration for analysis.

RNA was isolated using RiboPure™ RNA purification kit (Ambion®) and used for qPCR analysis. The RNA was retrotranscribed according to the instructions of the SuperScript® VILO™ cDNA synthesis kit (Invitrogen™), and 0.04 µl of cDNA was used for quantitative PCR (qPCR). qPCR for pre-mRNA EIF4A1 and mature mRNA SLC24A19 and PK evaluation were performed as reported previously (Eskens, F. A. et al. Phase I pharmacokinetic and pharmacodynamic study of the first-in-class spliceosome inhibitor E7107 in patients with advanced solid tumors. *Clin Cancer Res.* 19, 6296-6304, doi:10.1158/1078-0432.CCR-13-0485 (2013)). All animal studies were carried out according to the H3 Biomedicine Guide for the Care and Use of Laboratory Animals.

The results shown in **FIG. 8** indicated that Compound **1** showed PD responses at a tolerated dose via the oral route of administration.

Mouse Pharmacokinetic (PK) Study of Compound **3**

Compound **3** was dosed at 5.964 mg/kg IV or 13.307 mg/kg PO to CD-1 mice. Following administration, blood samples were collected at pre-determined time points from five mice via serial bleeding from the tail vein. Blood was collected at 0.083 (0.167 PO only), 0.5, 1, 2, 4, 6, 8, and 24 hours post-dose. The blood samples were centrifuged at 5000 RPM for 5 minutes to collect plasma within 30 minutes of blood collection. After extraction, samples were assayed using LCMS. PK parameters were calculated using non-compartmental analysis in WinNonlin v6.3.

The data indicated that Compound **3** showed oral bioavailability and favorable pharmacokinetic properties in the mouse model (**FIG. 9, Table 6**).

TABLE 6

Pharmacokinetic Property	Dose	
	5.964 mg/kg IV	**13.307 mg/kg PO**
C_{max} (µg/mL)	NA	1.567
t_{max} (h)	NA	2.000
$t_{1/2}$ (h)	3.660	3.322
AUC_{finite} (µg/mL*hr)	6.210	8.136
$AUC_{0\text{-}inf}$ (µg/mL*hr)	6.245	8.188
$AUC_{0\text{-}inf/D}$ (µg/mL*hr/D)	1.047	0.615
V_{ss} (mL/kg)	2975.561	NA
CL_{tot} (mL/hr/kg)	954.975	NA
BA (%)	NA	58.758

Mouse Pharmacokinetic (PK) Study of Compound **4**

Compound **4** was dosed at 5 mg/kg IV or 10 mg/kg PO to CD-1 mice. Following administration, blood samples were collected at pre-determined time-points from five mice via serial bleeding from the tail vein. Blood was collected at 0.083 (0.167 PO only), 0.5, 1, 2, 4, 6, 8, and 24 hours post-dose. The blood samples will be centrifuged at 5000 RPM for 5 minutes to collect plasma within 30 minutes of blood collection. After extraction, samples were assayed using LCMS. PK parameters were calculated using non-compartmental analysis in WinNonlin v6.3.

The data indicated that Compound **4** showed oral bioavailability and favorable pharmacokinetic properties in the mouse model (**FIG. 10, Table 7**).

TABLE 7

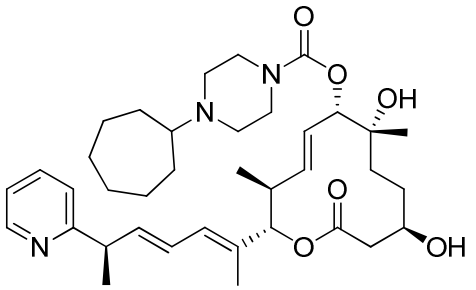
Pharmacokinetic Property	Dose	
	5 mg/kg IV	**10 mg/kg PO**
C_{max} (µg/mL)	NA	1.252
t_{max} (h)	NA	2.000
$t_{1/2}$ (h)	3.013	2.975
AUC_{0-t} (ug/mL*hr)	6.610	7.911
AUC_{0-inf} (ug/mL*hr)	6.623	7.951
AUC_{0-inf}/D (ug/mL*hr/D)	1.325	0.795
V_{ss} (mL/kg)	1893.683	NA
CL_{tot} (mL/hr/kg)	754.924	NA
BA (%)	NA	60.024

The results presented above demonstrate that compounds 1, 2, 3 and 4 each possess oral bioavailability and favorable pharmacokinetic properties. This is an improvement over E7107, which has been administered to the patients as intravenous infusion due to its insufficient oral bioavailability (Hong et al. (2014), Invest New Drugs 32, 436–444).

We claim:

1. A compound selected from

a compound of formula 1:



1

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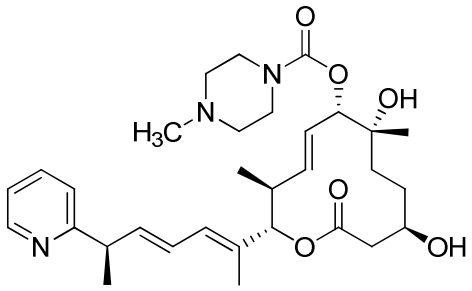
a compound of formula 2:



2

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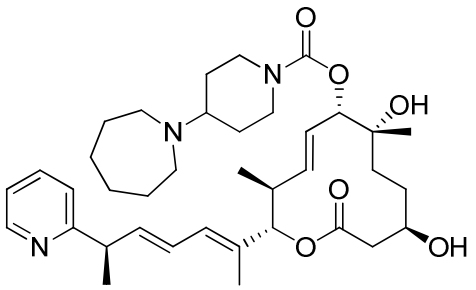
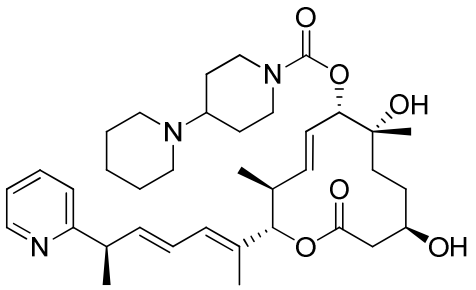
a compound of formula 3:



3

,

a compound of formula 4:



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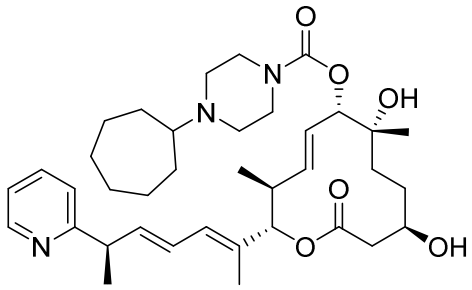
and pharmaceutically acceptable salts thereof.

2. A compound selected from a compound of formula 1:



1

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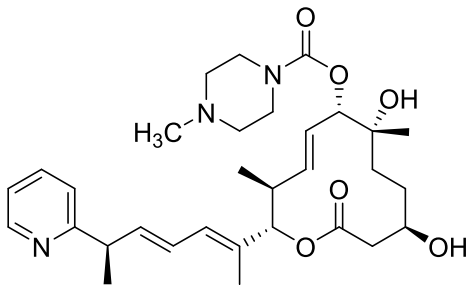
and pharmaceutically acceptable salts thereof.

3. A compound selected from a compound of formula 2:



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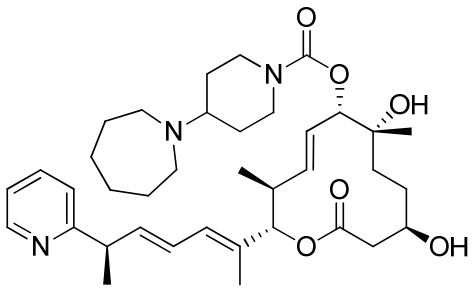
and pharmaceutically acceptable salts thereof.

4. A compound selected from a compound of formula 3:



3

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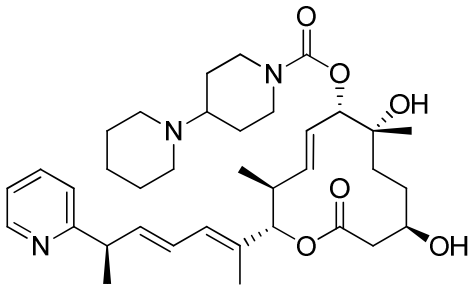
and pharmaceutically acceptable salts thereof.

5. A compound selected from a compound of formula 4:



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and pharmaceutically acceptable salts thereof.

6. The compound of claim 1, wherein said compound is stereomerically pure.

7. The compound of claim 1, wherein said compound comprises greater than about 80% by weight of one stereoisomer of the compound.

8. The compound of claim 1, wherein said compound comprises greater than about 90% by weight of one stereoisomer of the compound.

9. The compound of claim 1, wherein said compound comprises greater than about 95% by weight of one stereoisomer of the compound.

10. The compound of claim 1, wherein said compound comprises greater than about 97% by weight of one stereoisomer of the compound.

11. A pharmaceutical composition comprising a compound and/or pharmaceutically acceptable salt thereof according to any one of claims 1-10.

12. The pharmaceutical composition of claim 11, wherein said composition is formulated for intravenous, oral, subcutaneous, or intramuscular administration.

13. The pharmaceutical composition of claim 12, wherein said composition is formulated for oral administration.

14. The use of the compound or pharmaceutically acceptable salt thereof according to any one of claims 1-10, or the pharmaceutical composition according to any one of claims 11-13, in the preparation of a medicament for the treatment of a cancer selected from myelodysplastic syndrome, chronic lymphocytic leukemia, acute lymphoblastic leukemia, chronic myelomonocytic leukemia, acute myeloid leukemia, colon cancer, pancreatic cancer, endometrial cancer, ovarian cancer, breast cancer, uveal melanoma, gastric cancer, cholangiocarcinoma, and lung cancer.

15. The use of claim 14, wherein said cancer is colon cancer.

16. The use of claim 14, wherein said cancer is pancreatic cancer.

17. The use of claim 14, wherein said cancer is selected from myelodysplastic syndrome, chronic lymphocytic leukemia, acute lymphoblastic leukemia, chronic myelomonocytic leukemia, and acute myeloid leukemia.

18. The use of claim 14, wherein said cancer is acute myeloid leukemia.

19. The use of claim 14, wherein said cancer is myelodysplastic syndrome.

20. The use of claim 14, wherein said cancer is chronic lymphocytic leukemia.

21. The use of claim 14, wherein said cancer is acute lymphoblastic leukemia.

22. The use of claim 14, wherein said cancer is chronic myelomonocytic leukemia.

23. The use of claim 14, wherein said cancer is endometrial cancer.

24. The use of claim 14, wherein said cancer is ovarian cancer.

25. The use of claim 14, wherein said cancer is breast cancer.

26. The use of claim 14, wherein said cancer is uveal melanoma.

27. The use of claim 14, wherein said cancer is gastric cancer.

28. The use of claim 14, wherein said cancer is cholangiocarcinoma.

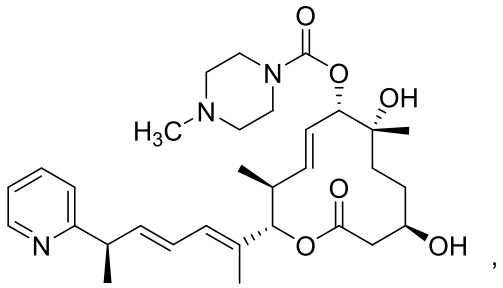
29. The use of claim 14, wherein said cancer is lung cancer.

30. The use of any one of claims 14-29, wherein said cancer is positive for one or more mutations in a spliceosome gene or protein.

31. The use of claim 30, wherein said spliceosome gene or protein is selected from splicing factor 3B subunit 1 (SF3B1), U2 small nuclear RNA auxiliary factor 1 (U2AF1), serine/arginine-rich splicing factor 2 (SRSF2), zinc finger (CCCH type) RNA-binding motif and serine/arginine rich 2 (ZRSR2), pre-mRNA-processing-splicing factor 8 (PRPF8), U2 small nuclear RNA auxiliary factor 2 (U2AF2), splicing factor 1 (SF1), splicing factor 3a subunit 1 (SF3A1), PRP40 pre-mRNA processing factor 40 homolog B (PRPF40B), RNA binding motif protein 10 (RBM10), poly(rC) binding protein 1 (PCBP1), crooked neck pre-mRNA splicing factor 1 (CRNKL1), DEAH (Asp-Glu-Ala-His) box helicase 9 (DHX9), peptidyl-prolyl cis-trans isomerase-like 2 (PPIL2), RNA binding motif protein 22 (RBM22), small nuclear ribonucleoprotein Sm D3 (SNRPD3), probable ATP-dependent RNA helicase DDX5 (DDX5), pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 (DHX15), and polyadenylate-binding protein 1 (PABPC1).

32. The use of claim 31, wherein said spliceosome gene or protein is splicing factor 3B subunit 1 (SF3B1).

33. A pharmaceutical composition comprising a compound selected from a compound of formula 2:



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and pharmaceutically acceptable salts thereof.

34. The pharmaceutical composition of claim 33, wherein said composition is formulated for intravenous, oral, subcutaneous, or intramuscular administration.

35. The pharmaceutical composition of claim 34, wherein said composition is formulated for oral administration.

36. The use of a pharmaceutical composition of any one of claims 33-35 in the preparation of a medicament for the treatment of a cancer selected from myelodysplastic syndrome, chronic lymphocytic leukemia, acute lymphoblastic leukemia, chronic myelomonocytic leukemia, acute myeloid leukemia, colon cancer, pancreatic cancer, endometrial cancer, ovarian cancer, breast cancer, uveal melanoma, gastric cancer, cholangiocarcinoma, and lung cancer.

37. The use of claim 36, wherein said cancer is colon cancer.

38. The use of claim 36, wherein said cancer is pancreatic cancer.

39. The use of claim 36, wherein said cancer is selected from myelodysplastic syndrome, chronic lymphocytic leukemia, acute lymphoblastic leukemia, chronic myelomonocytic leukemia, and acute myeloid leukemia.

40. The use of claim 36, wherein said cancer is acute myeloid leukemia.

41. The use of claim 36, wherein said cancer is myelodysplastic syndrome.

42. The use of claim 36, wherein said cancer is chronic lymphocytic leukemia.

43. The use of claim 36, wherein said cancer is acute lymphoblastic leukemia.

44. The use of claim 36, wherein said cancer is chronic myelomonocytic leukemia.

45. The use of claim 36, wherein said cancer is endometrial cancer.

46. The use of claim 36, wherein said cancer is ovarian cancer.

47. The use of claim 36, wherein said cancer is breast cancer.

48. The use of claim 36, wherein said cancer is uveal melanoma.

49. The use of claim 36, wherein said cancer is gastric cancer.

50. The use of claim 36, wherein said cancer is cholangiocarcinoma.

51. The use of claim 36, wherein said cancer is lung cancer.

52. The use of any one of claims 36-51, wherein said cancer is positive for one or more mutations in a spliceosome gene or protein.

53. The use of claim 52, wherein said spliceosome gene or protein is selected from splicing factor 3B subunit 1 (SF3B1), U2 small nuclear RNA auxiliary factor 1 (U2AF1), serine/arginine-rich splicing factor 2 (SRSF2), zinc finger (CCCH type) RNA-binding motif and serine/arginine rich 2 (ZRSR2), pre-mRNA-processing-splicing factor 8 (PRPF8), U2 small nuclear RNA auxiliary factor 2 (U2AF2), splicing factor 1 (SF1), splicing factor 3a subunit 1 (SF3A1), PRP40 pre-mRNA processing factor 40 homolog B (PRPF40B), RNA binding motif protein 10 (RBM10), poly(rC) binding protein 1 (PCBP1), crooked neck pre-mRNA splicing factor 1 (CRNKL1), DEAH (Asp-Glu-Ala-His) box helicase 9 (DHX9), peptidyl-prolyl cis-trans isomerase-like 2 (PPIL2), RNA binding motif protein 22 (RBM22), small nuclear ribonucleoprotein Sm D3 (SNRPD3), probable ATP-dependent RNA helicase DDX5 (DDX5), pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 (DHX15), and polyadenylate-binding protein 1 (PABPC1).

54. The use of claim 53, wherein said spliceosome gene or protein is splicing factor 3B subunit 1 (SF3B1).