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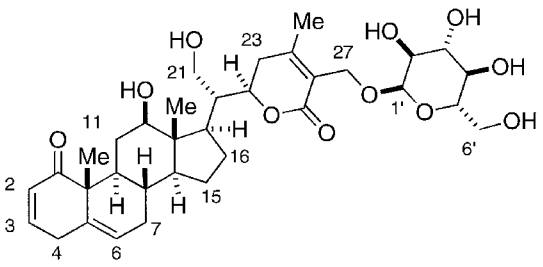
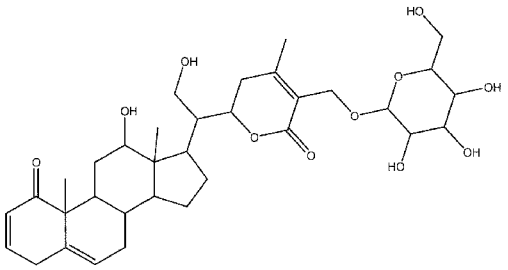
[Continued on next page]

(54) **Title:** *D. INNOXIA* WITHANOLIDES WITH SPECIFIC ANTI-CANCER ACTIVITIES

FIGURE 10





(57) Abstract: What is described is a withanolide, dinoxin B (12,21 -dihydroxy- 1 -oxowitha-2,6, 24- trienolide-27-0-β-D-glucopyranoside), and a method of isolating dinoxin B from a methanol extract of *Datura innoxia* Mill, leaves, using bioassay guided fractionation.. Extracts and the purified compound have cytotoxic activity towards a cancer cell lines, and sub-micromolar IC50 concentrations against several breast cancer cell lines. The structure of dinoxin B has the following formula:

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SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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D. INNOXIA WITHANOLIDES WITH SPECIFIC ANTI-CANCER ACTIVITIES

CROSS REFERENCE TO PRIOR PATENT APPLICATIONS

[0001] This application claims the benefit of the filing date of U.S. provisional application No. 61/316,295, filed March 22, 2010.

FEDERAL GOVERNMENT GRANTS

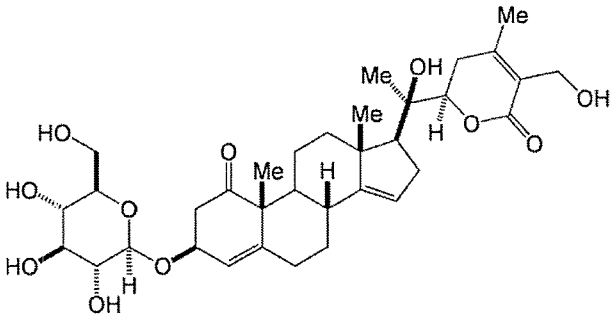
[0002] This description is a result of U.S. government support under NIH grant numbers NCI U56 CA96286 and U54 CA132383. The U.S. government has certain rights in the claimed invention.

BACKGROUND

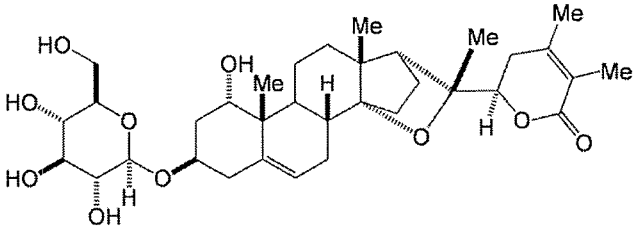
[0003] Secondary metabolites obtained from plants continue to be a major source of compounds with anticancer activity. Natural products represent 74% of the anticancer drugs developed from 1981-2002. Despite recent advances in combinatorial chemistry, natural products continue to be responsible for 50% or more of the small molecules developed for drugs for a wide array of diseases. Natural products are the source of greater than 70% of the small molecules in use as anticancer agents. These include vincristine, vinblastine, paclitaxel, docetaxel, topotecan, irinotecan, flavopiridol, scronyciline, bruceantin, and thalicarpine.

[0004] The genus *Datura* belongs to the family Solanaceae, commonly known as the nightshade family. Species within the Solanaceae are known for their toxic metabolites, commonly known to cause hallucinogenic effects. This genus contains many species indigenous to the American southwest including *Datura innoxia, D. stramonium,* and *D. ferox,* while the genus is found worldwide. Medicinally, *Datura* extracts have been used in folk medicine for their anesthetic, demulcent, expectorant, hypnotic, intoxicant, and sedative properties and are traditionally used to treat asthma, earache, and headache. Some folk remedies are associated for treating tumors. The genus *Datura* has been an important commercial source of alkaloids, particularly for its content of tropane alkaloids, including atropine.

[0005] Withanolides constitute a large family of plant triterpenes and triterpene glycosides typified by a fused tetracyclic cholestane core and a side-chain unsaturated δ-lactone. The basic structure is shown in the following formulae. The first shows a C28 ergostane skeleton consisting of a 6-membered lactone E ring. The second shows a representative withanolide as a glycoside.



FW 632.78 Formula: $C_{34}H_{48}O_{11}$

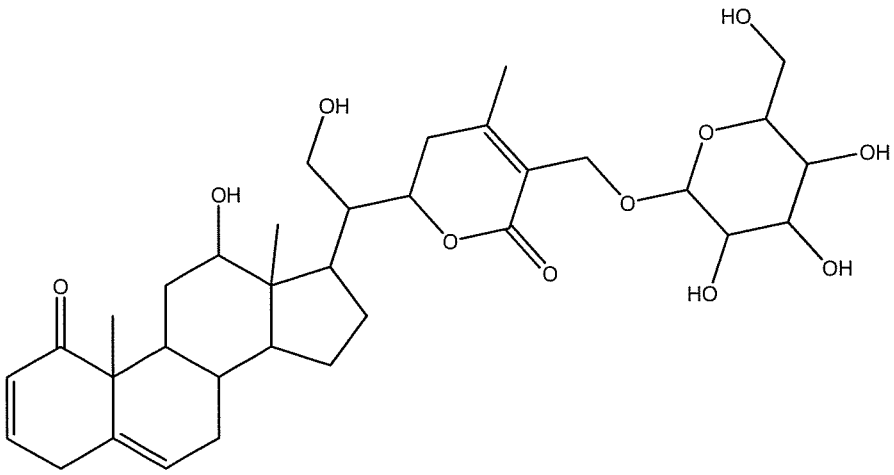


FW 632.78 Formula: $C_{34}H_{48}O_{11}$

[0006] Within the genus *Datura,* withanolides have been isolated from several species, including *D. stramonium, D.ferox, D. quercifolia, D. innoxia, D. metel,* and *D.fastuosa.* There have been very few in vitro bioassays using withanolides from *Datura spp.* Extracts from *D. metel* leaves were found to inhibit the growth of plant pathogens *in vitro,* the major abundant compound in this extract had a mass spectra that matched the published report for the withanolide, datruilin. Several withanolides from *D. metel* flowers were found cytotoxic at 10 μM or lower against three human cancer cell lines. A withanolide from *D.fastuos* was reported to reduce experimentally induced ulcers in rats.

SUMMARY

[0007] Methanolic extracts of *Datura innoxia* are subjected to a series of extraction, chromatographic and purification steps to yield *inter alia* Dinoxin B. The general formula of the compounds discussed herein is shown in the chemical formula:



[0008] In the process of isolation the leaves of *Datura innoxia* is extracted with sequentially with hexane, chloroform and 80% aqueous methanol and subjected to a series of extractions, partitions, and column fractionations shown and outlined in Figs 1-10 and discussed in greater detail herein below. The extract was tested for anti-cancer activity in vitro against cells derived from human breast cancer cell lines, including MCF7 and MD-MBA-468 and against a variety of cancer cells derived from other tissue types. Dinoxin B demonstrates significant anti-breast cancer activity at concentrations that are not cytotoxic against other cancers or normal cell types.

[0009] Dinoxin B is a light brown emulsion/amorphous solid.

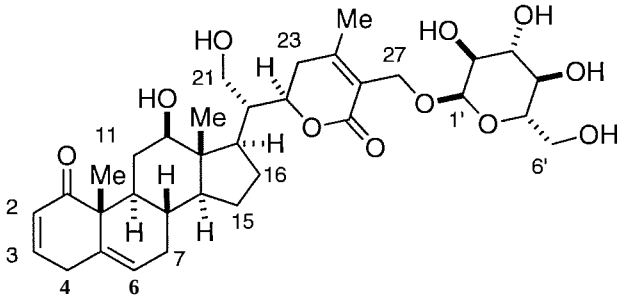
[0010] A UV spectrum of Dinoxin B in methanol showed λ™χ (log e), 230, 314 (3.60) nm.

[0011] A IR spectrum (KBr, disc) of Dinoxin B showed λ™χ (v cm^{-1}): 3409, 29 10, 1683, 1664(sh), 1395, 1077, 1050(sh), and 798 cm-1.

[0012] A HREIMS positive ion analysis of Dinoxin B showed m/z 655.3 146 $[M+Na]^+$ (calculated for $C_{34}H_{48}0nNa$, 655.3088); with major fragments m/z 471.2830 $[M\text{-glucose}+H]^+$ (calculated for $C_{28}H_{39}0_6$ 471.2741) and 453.2686 $[M\text{-glucose-water}+H]^+$ (calculated for $C_{28}H_{37}0_5$ 453.2635).

[0013] Based on the molecular formula determined and the NMR spectroscopic data obtained, dinoxin B was assigned the structure 12,21-dihydroxy-l-oxowitha-2,6,24-trienolide-27-0 -β-**D**-glucopyranoside. It can be alternatively named Ergosta-2,6,24-trien-26-oic acid, 27-(β-**D**-glucopyranosyloxy)-12,21,22-trihydroxy-l-oxo, δ-lactone. Dinoxin B can also be classified as a sitoindoside, which is a withanolide containing a glucose group at the C-27 position.

[0014] The stereochemistry at carbons 8, 9, 10, 12, 13, 14, 17, 20, 22, 1', 2', 3', 4' and 5' is as shown in the structure illustration.



One aspect of the description is process of producing dinoxin B, the sequential steps comprising:

(a) extracting *Datura innoxia* with an alkanols/aqueous solution, preferably methanol/water, most preferably 80% methanol

(b) partitioning the alkanols/aqueous extract against an organic solvent, preferably n-butanol, hexane, or ether;

(c) chromatographing the aqueous fraction by reverse phase chromatography; and

(d) collecting fractions that contain a compound that is cytotoxic to a cancer cell.

Additional steps may further comprise one of the following steps: collecting leaves from mature plants, and pulverizing the leaves with sand; extracting the pulverized leaves with hexane; extracting the pulverized leaves with chloroform.

Another additional step may be following the extraction in step (a), that the alkanol solution is dried *in vacuo.* Alternatively, following the extraction in step (a), an equal volume of water is added to the alkanol solution.

Another aspect of the description is a compound produced by the above purification process. Preferably, the compound consists of a light brown emulsion/amorphous solid. More preferably, the compound according has a UV spectrum in methanol with λ^{max} (log e) at 230, 314 (3.60) nm, an IR spectrum (KBr, disc) with λ^{max} (v cm^{-1}) at 3409, 2910, 1683, 1664(sh), 1395, 1077, 1050(sh), and 798 cm^{-1}, and/or a mass spectroscopic profile with a m/z 655 and major fragments at a m/z 471 and 453. Most preferably, the elemental composition of the compound consists of $C_{34}H_{48}0nNa$, $C_{28}H_{39}0_6$, or $C_{28}H_{37}0_5$.

Another aspect of the description is a compound produced by a process consisting of the following steps:

(a) extracting *Datura innoxia* with an alkanol,

(b) partitioning the alkanolic extract against and organic solvent;

(c) chromatographing the aqueous fraction by reverse phase chromatography; and

(d) collecting fractions that contain a compound that is cytotoxic to a cancer cell.

Another aspect of the description method of treating cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition, wherein the active component of the pharmaceutical composition consists of the above compound. Preferably, the pharmaceutical composition is suitable to be administered parenterally, transdermally, mucosally, nasally, buccally, sublingually, topically, or orally. Preferably, the subject is a cancer patient, more preferably a breast cancer patient.

Another aspect of the description is a pharmaceutical formulation comprising the above compound, together with a pharmaceutically acceptable carrier. Preferably, the active compound is specifically cytotoxic to breast cancer cells.

Another aspect of the description is a compound for use in a method for treating cancer, the method comprising a step of administering to a cancer patient an effective amount of the above compound, or a solvate, hydrate, or prodrug thereof, such that the cancer is treated. Preferably, the cancer is breast cancer. Preferably, the compound is administered parenterally, transdermally, mucosally, nasally, buccally, sublingually, topically, or orally.

Another aspect of the description is use of the above compound in the preparation of a medicament for the treatment of cancer in a suitable subject. Preferably, the medicament is capable of being administered parenterally, transdermally, mucosally, nasally, buccally, sublingually, topically, or orally. Preferably, the cancer is breast cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Fig. 1. Extraction and purification process for *D. innoxia* material, including specific activity of cytotoxic bioactivity against MCF7 breast cancer cells at various stages of the extraction process.

[0016] Fig. 2. Methanol, chloroform and hexane soxhlet extracts of *D. innoxia* leaf inhibit MCF7 breast cancer cell line. Cells were treated with 1% DMSO, (the control cell) or with the indicated extracts for four days. The cells were separated from the media and then incubated with growth medium supplemented with [methyl ^{3}H] thymidine, 2 μCi/mL. The plates were counted using a scintillation microplate reader. Results are shown as a percentage of growth of the control cell. Dashed red line indicates 50% inhibition **($IC5_0$)**.

[0017] Fig. 3. The activity in the methanol extract of Fig. 1 was fractionated on silica gel by flash chromatography. The eluant was separated into nine fractions, and fractions 2 through 8 were tested for biological specificity against four cancer cell lines from different tissue types: A549, HCTl 16, PC3 and MCF7. Cells were treated with 1%, v/v DMSO (control) or with

the indicated fractions from the silica column (at 1 mg/mL) for 4 days as in Fig. 1. Results are shown as a percentage of inhibition relative to growth of control cells.

[0018] Fig. 4. HPLC separation of butanol extracted material from the methanol fractions on a Waters Atlantis 5 micron C18 (10 mm x 100 mm) column; eluting in a 50 minute gradient from 80 % solution A (0.1% trifluroacetic acid (TFA)) in solution B (acetonitrile) to 100 % B; flow rate, 3.5 mL/min; detection at 230 nm.

[0019] Fig. 5. HPLC chromatograph of the active fraction (eluting at 26 minutes from the column shown in Fig. 3) chromatographed again on the same column, but in a 10 minute gradient.

[0020] Fig. 6. HPLC chromatograph of the each of the two peaks (A and B) from the column of Fig. 4, chromatographed separately on the same column in a 10 minute gradient.

[0021] Fig. 7 Biological activity of peaks A and B from Fig. 5 and semi-purified extract (active fraction 26 from Fig. 3) against (A) MCF-7 and (B) MDA-MB-468 breast cancer cell lines.

[0022] Fig. 8. Preparative HPLC of *D innoxia* extracts were chromatographed on a Atlantis C18 columns (29 x 10 mm) as in Fig. 3 in a 18 minute solvent gradient.

[0023] Fig. 9. The bioactive compound of the column in Fig. 7 (eluting at 7.65 min) was collected and analyzed by liquid chromatograph-mass spectroscopy (LC-MS). This figure shows the ESI mass spectral analysis of samples resolved on HPLC column with acetonitrile formic acid gradients. Upper panel is the total ion chromatogram, the lower panel is the MS of the compound eluting at 5.35 min.

[0024] Fig. 10. The structure of the bioactive compound of Figure 9.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0025] In the process of the present description as shown in Fig. 1, leaves of *D. innoxia*, are first infused with hexane, then chloroform, and then the bioactive compound is extracted with a lower alkanol, suitably methanol, most preferably 80% methanol. Preferably the infusion step involves zero to two extractions in hexane, two in chloroform and four in methanol. The methanol extracts combined and the solvent removed, suitably by evaporation to yield a residue. Alternatively, water may be added to the methanol extract. The addition of water to the methanol fraction was adequate to precipitate the chlorophyll. The volume of water added to the methanol extract preferably is between 50 and 67 percent.

The solid residue is then partitioned between n-butanol and water, or hexane and water. Alternatively, the methanolic extract is partitioned with an organic solvent, preferably n-butanol,

hexane or ether. The aqueous phase is retained, and applied directly to a chromatographic column. The organic phase can optionally be extracted again.

[0026] A variety of chromatographic matrices can be used to optimize for resolution and purification of the active compound, for example silica gel and HPLC column chromatography, dihydropyran resin (Ellman resin) for alcohol capture; polystyrene benzaldehyde (nucleophile scavenger), or MeOH/H?0 or CH_3CN/H_20 gradients on C_4, C_8, C_{18}, and cyano columns, preferably by using a CI8 HPLC column with a gradient of 20% to 60% (v/v) acetonitrile in 0.1% trifluoroacetic acid.

A slightly higher dilution of the methanolic fraction improves retention of the analyte on the C18 prep column. The water-diluted methanol extract is preferably run through the C18 column twice to increase efficiency.

[0027] Bioactivity was measured by culturing breast cancer cell lines, preferably MCF7 or MDA-MB-468, in the presence of purified material believed to contain the bioactive compound. Comparison may be made by using other cancer cell types, e.g., A549 (lung), HCTl 16 (colon), and PC3 (prostate), as controls. Inhibition of proliferation can be measured, for example, by uptake of tritiated thymidine or by ATP levels.

[0028] Fractions with biological activity can be further purified using any column, above, or preferably a C18 HPLC column with a gradient of 20% to 60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Two fractions can be separated and further purified on the same or a different column. When assayed for activity, IC_{50} values range from 10 ng/ml to 1000 ng/ml using tritiated thymidine, preferably at least 300 ng/ml, more preferably at least 30 ng/ml.

[0029] Elemental composition can be determined in a variety of ways, preferably by LC-MS, which shows a large positive ion m/z 655 $[M+Na]^+$ with major fragments m/z 471 $[M-glucose+H]^+$ and 453 $[M-glucose-water+H]^+$. The larger form of the compound predicts a molecular formula of $C_{34}H_{46}0nNa$. The lower molecular weight form of the compound predicts a molecular formula of $C_{28}H_{37}0_5$.

[0030] Dinoxin B is found to be a light brown emulsion/amorphous solid.

[0031] Its UV spectrum in methanol shows λ^{max} (log e), 230, 314 (3.60) nm.

[0032] Its IR spectrum (KBr, disc) shows λ^{TMx} (v cm^{-1}): 3409, 2910, 1683, 1664(sh), 1395, 1077, 1050(sh), and 798 cm-1.

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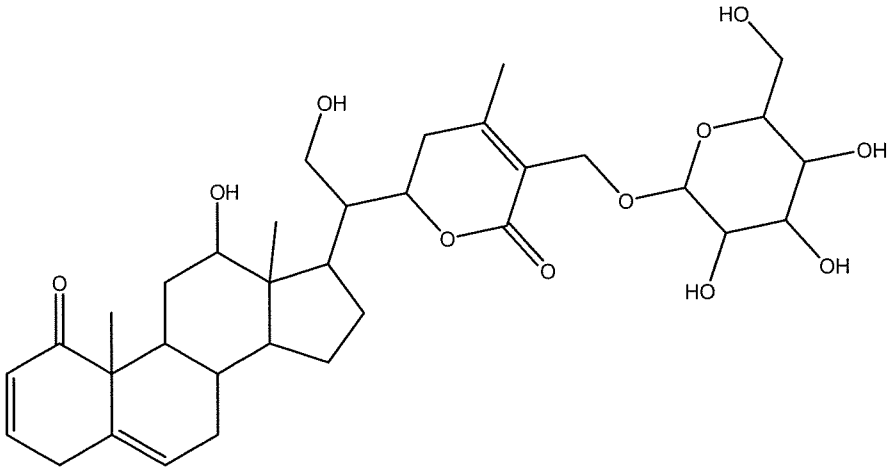
[0034] Based on the molecular formula determined and the NMR spectroscopic data obtained, dinoxin B was assigned the structure 12,21-dihydroxy-l-oxowitha-2,6,24-trienolide-27-0-p -**D**-glucopyranoside. It can be alternatively named Ergosta-2,6,24-trien-26-oic acid, 27-**(**β-**D**-glucopyranosyloxy)-12,21,22-trihydroxy-l-oxo, δ-lactone. Dinoxin B can also be classified as a sitoindoside, which is a withanolide containing a glucose group at the C-27 position, as shown in the chemical formula:



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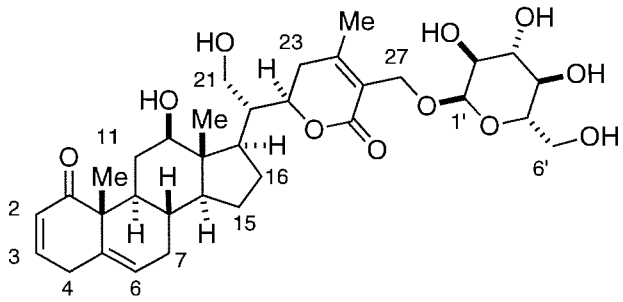


[0036] The modes contemplated by the inventor of carrying out the description include pharmaceutical compositions and processes of administration thereof.

[0037] Solutions of the principal active ingredient can be prepared in water or in water suitably diluted with, for example, ethanol, glycerin, edible polyols (for example, glycerine, polyethylene glycols, propylene glycol), and the like. Dispersions can be prepared in glycerol, liquid polyethylene glycols, and mixture thereof, and in oils.

[0038] Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0039] The pharmaceutical compositions can be in forms suited for injectable use which forms include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form

must be sterile and must be fluid to the extent that easy syringeability exists. It must be stable under the conditions or manufacture and storage must be preserved against the contaminating action of microorganism such as bacteria and fungi. The basic solvent or dispersion medium can contain water, ethanol, polyols (for example, glycerol, propylene, glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants (for example, a condensation product of ethylene oxide with fatty acids or fatty alcohols, partial esters of fatty acids and a hexitol anhydride, and polyoxethylene condensation products of the esters). The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, benzyl alcohol, phenol, sorbic acid, thermerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0040] Sterile injectable solutions are prepared by incorporating the principal active ingredient in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. Generally, dispersions are prepared by incorporating the previously sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above.

[0041] In the case for sterile powders for the preparation of sterile injectable solutions the preferred method of preparation is the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredients from a previously sterile-filtered solution thereof. The powders can also be sterilized by the use of a gas, for example, ethylene oxide and subsequently incorporated, with the required additional ingredients and in the proper particle size, into the basic powder for later reconstitution with the desired suspending liquid which, of course, itself must be sterile.

[0042] Supplementary active ingredients can be incorporated into the inventive compositions. These ingredients include for example, mechorethamine hydrochloride and 5-bis (2-chloroethyl) amino-uracil; triethylene melamine; actinomycin C; cycloheximide.

[0043] It is especially advantageous to formulate the inventive compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discrete units suited as unitary dosages for

the animal and human subjects to be treated, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specifications for the novel dosage unit forms of this description are dictated by and directly dependent of (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as disclosed in detail in this specification, these being features of the present description.

[0044] The dosage of the principal active ingredient for the treatment of the indicated condition depends on the age, weight, and condition of the subject being treated, the particular condition and its severity, the particular form of the active ingredient and the route of administration. A dose of from about 100 μg/kg or a daily total dose of from about 5 to about 20 mg. given singly or in individually smaller doses is deemed suitable.

[0045] The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore described. A unit dosage form can contain the principal active ingredient in amounts ranging from about 1 to about 5 mg. per unit. Expressed in proportions the active ingredient is present in from about 0.01 to about 0.1% w./v. of the liquid compositions.

[0046] The compounds of the present description are useful as a method of treating breast cancer. The compounds are administered as therapeutic or pharmaceutical compositions by any suitable route known to the skilled artisan including, for example, intravenous, subcutaneous, intramuscular, intradermal, transdermal, intrathecal, intracerebral, intraperitoneal, intransal, epidural, and oral routes. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulations. In addition, it can be desirable to introduce a compound into the target tissue by any suitable route, including intravenous and intrathecal injection. Pulmonary administration can also be employed, such as, for example, by use of an inhaler or nebulizer, and formulation of the compound with an aerosolizing agent. In certain embodiments, the compound is coadministered with an inhibitor of esterase activity to further stabilize the compound. Pharmaceutical compositions can also be administered orally in any orally acceptable dosage form including, but not limited to, capsules, tablets, caplets, lozenges, aqueous suspensions or solutions. In the case of tablets for oral use, carriers that are commonly used include lactose and corn starch. Lubricating aids, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are required, the agent

can be combined with emulsifying and suspending aids. If desired, certain sweeteners, flavorants, or colorants can also be used.

[0047] Further, the compounds of the present description can be combined with any other tumor and/or cancer therapy. The therapy can include, for example and not by way of limitation, surgery, radiation, and chemotherapy either individually or in any combination. Chemotherapy can include any current known or yet to be discovered chemotherapeutic agent including but are not limited to Aceglatone; Aclarubicin; Altretamine; Aminoglutethimide; 5-Aminogleavulinic Acid; Amsacrine; Anastrozole; Ancitabine Hydrochloride; 17-1 A Antibody; Antilymphocyte Immunoglobulins; Antineoplaston AIO; Asparaginase; Pegaspargase; Azacitidine; Azathioprine; Batimastat; Benzoporphyrin Derivative; Bicalutamide; Bisantrene Hydrochloride; Bleomycin Sulphate; Brequinar Sodium; Broxuridine; Busulphan; Campath-1H; Caracemide; Carbetimer; Carboplatin; Carboquone; Carmofur; Carmustine; Chlorambucil; Chlorozotocin; Chromomycin; Cisplatin; Cladribine; Corynebacterium parvum; Cyclophosphamide; Cyclosporin; Cytarabine; Dacarbazine; Dactinomycin; Daunorubicin Hydrochloride; Decitabine; Diaziquone; Dichlorodiethylsulphide; Didemnin B.; Docetaxel; Doxifluridine; Doxorubicin Hychloride; Droloxifene; Echinomycin; Edatrexate; Elliptinium; Elmustine; Enloplatin; Enocitabine; Epirubicin Hydrochloride; Estramustine Sodium Phosphate; Etanidazole; Ethoglucid; Etoposide; Fadrozole Hydrochloride; Fazarabine; Fenretinide; Floxuridine; Fludarabine Phosphate; Fluorouracil; Flutamide; Formestane; Fotemustine; Gallium Nitrate; Gencitabine; Gusperimus; Homoharringtonine; Hydroxyurea; Idarubicin Hydrochloride; Ifosfamide; Ilmofosine; Improsulfan Tosylate; Inolimomab; lnterleukin-2; Irinotecan; JM-216; Letrozole; Lithium Gamolenate; Lobaplatin; Lomustine; Lonidamine; Mafosfarnide; Meiphalan; Menogaril; Mercaptopurine; Methotrexate; Methotrexate Sodium; Miboplatin; Miltefosine; Misonidazole; Mitobronitol; Mitoguazone Dihydrochioride; Mitolactol; Mitomycin; Mitotane; Mitozanetrone Hydrochloride; Mizoribine; Mopidamol; Muitlaichilpeptide; Muromonab-CD3; Mustine Hydrochloride; Mycophenolic Acid; Mycophenolate Mofetil; Nedaplatin; Nilutamide; Nimustine Hydrochloride; Oxaliplatin; Paclitaxel; PCNU; Penostatin; Peplomycin Sulphate; Pipobroman; Pirarubicin; Piritrexim Isethionate; Piroxantrone Hydrochloride; Plicamycin; porfimer Sodium; Prednimustine; Procarbazine Hydrochloride; Raltitrexed; Ranimustine; Razoxane; Rogletimide; Roquinimex; Sebriplatin; Semustine; Sirolimus; Sizofiran; Sobuzoxane; Sodium Bromebrate; Sparfosic Acid; Sparfosate Sodium; Sreptozocin; Sulofenur; Tacrolimus; Tamoxifen; Tegafur; Teloxantrone Hydrochloride; Temozolomide; Teniposide; Testolactone; Tetrasodium Mesotetraphenylporphine-sulphonate; Thioguanine; Thioinosine; Thiotepa; Topotecan; Toremifene; Treosulfan; Trimetrexate; Trofosfamide; Tumor Necrosis Factor;

Ubenimex; Uramustine; Vinblastine Sulphate; Vincristine Sulphate; Vindesine Sulphate; Vinorelbine Tartrate; Vorozole; Zinostatin; Zolimomab Aritox; and Zorubicin Hydrochloride, and the like, either individually or in any combination. See, e.g., US Patent No. 7,071,158.

[0048] In some embodiments, the compounds of the present description can be administered locally to the area in need of treatment; this administration can be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application (e.g., in conjunction with a wound dressing after surgery), by injection, by means of a catheter, by means of a suppository, or by means of an implant, the implant being of a porous, non- porous, or gelatinous material, including membranes such as silastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

[0049] In another embodiment, the compounds of the description can be delivered in a vesicle, in particular a liposome (see, e.g., Langer, Science 249:1527-33, 1990; Treat et al, In Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-65, 1989; Lopez-Berestein, supra, pp. 317-27).

(All references cited herein are incorporated into this detailed description in their entirety.)

[0050] In yet another embodiment, the compounds of the description can be delivered in a controlled release system. In one embodiment, a pump can be used (see, e.g., Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201, 1987; Buchwald et ah, Surgery 88:507, 1980; Saudek et al., N. Engl. J. Med. 321 :574, 1989). In another embodiment, polymeric materials can be used (see, e.g., Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida, 1974; Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York, 1984; Ranger and Peppas, J. Macro mol. Sci. Rev. Macromol. Chem. 23:61, 1983; see also Levy et al, Science 228: 190, 1985; During et al, Ann. Neurol. 25:351, 1989; Howard et al, J. Neurosurg. 71: 105, 1989). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, Medical Applications of Controlled Release, supra, Vol. 2, pp. 115-138, 1984). Other controlled release systems are discussed in, for example, the review by Langer (Science 249: 1527-1533, 1990).

[0051] The present description also provides pharmaceutical compositions. Such compositions comprise a pharmaceutically acceptable carrier and a therapeutically effective amount of a compound of the description. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S.

Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more typically in humans. The term "carrier" refers to a diluent, adjuvant, excipient, stabilizer, vehicle, or any combination thereof, with which the agent is formulated for administration. Pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water is a typical carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. The composition, if desired- can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. Pharmaceutical compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations, and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. In addition, in certain embodiments, the pharmaceutical composition includes an inhibiter of esterase activity as a stabilizing agent.

[0052] Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. Examples of suitable pharmaceutical carriers are described in, for example, Remington 's Pharmaceutical Sciences, by E.W. Martin. Such compositions will contain a therapeutically effective amount of a compound of the description, typically in purified form, together with a suitable amount of carrier so as to provide a formulation proper for administration to the subject. The formulation should suit the mode of administration.

[0053] In one embodiment, the compound of the present description is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, pharmaceutical compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition can also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form. For example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered

by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

[0054] The compounds of the description can be formulated as neutral or salt forms. A "pharmaceutically acceptable salt" as used herein refers to a salt form of a compound permitting its use or formulation as a pharmaceutical and which retains the biological effectiveness of the free acid and base of the specified compound and that is not biologically or otherwise undesirable. Examples of such salts are described in *Handbook of Pharmaceutical Salts: Properties, Selection, and Use,* Wermuth, C.G. and Stahl, P.H. (eds.), Wiley-Verlag Helvetica Acta, Zurich, 2002. Examples of pharmaceutically acceptable salts, without limitation, include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, and the like, and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine and the like. Examples of salts also include sulfates, pyrosulfates, bisulfates, sulfites, bisulfltes, phosphates, monohydrogenphosphates, dihydrogen phosphates, metaphosphates, pyrophosphates, chlorides, bromides, iodides, acetates, propionates, decanoates, caprylates, acrylates, formates, isobutyrates, caproates, heptanoates, propiolates, oxalates, malonates, succinates, suberates, sebacates, fumarates, maleates, butyne-l,4-dioates, hexyne-l,6-dioates, benzoates, chlorobenzoates, methylbenzoates, dinitrobenzoates, hydroxybenzoates, methoxybenzoates, phthalates, xylenesulfonates, phenylacetates, phenylpropionates, phenylbutyrates, citrates, lactates, γ-hydroxybutyrates, glycollates, tartrates, methanesulfonates, ethane sulfonates, propanesulfonates, toluenesulfonates, naphthalene- 1-sulfonates, naphthalene-2-sulfonates, and mandelates. In some embodiments, pharmaceutically acceptable salt includes sodium, potassium, calcium, ammonium, trialkylarylammonium and tetraalkylammonium salts.

[0055] Furthermore, "pharmaceutically acceptable prodrugs" of the compounds may be used in embodiments of the description. Pharmaceutically acceptable prodrugs as used herein refers to those prodrugs of the active compounds of the present description which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, commensurate with a reasonable risk/benefit ratio, and effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds of the description. The term "prodrug" refers to compounds that are rapidly transformed in vivo to yield the parent compound of the above formula, for example, by hydrolysis in blood. A thorough discussion is provided in T. Higuchi and V. Stella, *Prodrugs as Novel delivery Systems,* Vol. 14 of the A.C.S. Symposium

Series and in Edward B. Roche, ed., *Bioreversible Carriers in Drug Design,* American Pharmaceutical Association and Pergamon Press, 1987, both of which are incorporated by reference herein. See also US Patent No. 6,680,299. Examples include a prodrug that is metabolized *in vivo* by a subject to an active drug having an activity of active compounds as described herein, wherein the prodrug is an ester of an alcohol or carboxylic acid group, if such a group is present in the compound; an acetal or ketal of an alcohol group, if such a group is present in the compound; an N-Mannich base or an imine of an amine group, if such a group is present in the compound; or a Schiff base, oxime, acetal, enol ester, oxazolidine, or thiazolidine of a carbonyl group, if such a group is present in the compound, such as described in US Patent No. 6,680,324 and US Patent No. 6,680,322.

[0056] The amount of the compound of the description that is combined with the carrier to produce a single dosage form will vary, depending upon the nature of that agent and the composition of the dosage form. It should be understood, however, that a specific dosage and treatment regime for any particular patient or disease state will depend upon a variety of factors, including 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 will also depend upon the specific activity of the compound and whether that agent is co-administered with any other therapeutic or prophylactic ingredients. Determination of therapeutically effective dosages is typically based on animal model studies and is guided by determining effective dosages and administration protocols that significantly reduce the occurrence or severity of the apoptosis- associated disease in model subjects (e.g., in the case of treatment of malignancies, a tumor xenograft model in mice can be used. For treatment of human subjects, such animal model studies are typically followed up by human clinical trials. A non-limiting range for a therapeutically effective amount of the compounds is about 0.001 mg/kg and about 100 mg/kg body weight per day, and in more specific embodiments between about 0.001 mg/kg and about 50 mg/kg, between about 0.01 mg/kg and about 20 mg/kg. between about 0.1 and about 10 mg/kg, or between about 0.1 mg/kg and about 5 mg/kg body weight per day.

[0057] The description also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the description. Optionally associated with such containers can be a notice, in the form prescribed by a governmental agency, regulating the manufacture, use, or sale of pharmaceuticals or biological products. The government notice should reflect approval by the agency of manufacture, use, or sale for human administration.

[0058] The following examples are provided merely as illustrative of various aspects of the description and shall not be construed to limit the description in any way.

EXAMPLES

Example 1

[0059] Plant Material. *D. innoxia* (Mill.) and *D. stramonium* were collected on the NMSU campus in Dona Ana county in southern New Mexico; *D. metel* plants were grown in a greenhouse on the NMSU campus in Las Cruces. A voucher specimen of *D. innoxia* was submitted to the Range Science Herbarium at New Mexico State University in Las Cruces, NM.

[0060] Dried, pulverized *D. innoxia* leaves were extracted in 10 g aliquots in an ASE 350. A 5 mL layer of sand was placed in each 35 mL cell, followed by a mixture of the crushed leaves and sufficient sand to fill the cell (ca 15 g). The samples were extracted three times with hexane (80°C, 8 min static extraction, 50% rinse volume, 60 sec purge), followed by three extractions with chloroform (same conditions, but 100 second purge). Finally, the sample was extracted four times with methanol (same conditions as for chloroform extractions). Extracts of hexane, chloroform and methanol were dried, solubilized in DMSO and assayed against MCF7 breast cancer cell line using tritiated thymidine uptake to measure the extent of proliferation. The yields of the hexane, chloroform and methanol extracts were 3.43%, 2.87%, and 19% of the leaf dry weight respectively (Fig. 1).

[0061] GC/MS analysis was performed to evaluate the chemical composition of the extracts for any known biological active compounds. Compounds were identified by comparing mass spectra with those in the NIST Library. The hexane fraction was rich in fats and waxes. This fraction had no inhibitory activity against MCF7 cell line. The psychoactive alkaloids, atropine and scopolamine, were extracted into the chloroform fraction. These compounds are known to have cytotoxic activities in vitro and are probably responsible for the inhibitory activity against MCF7 cells (LD50 = 4.96 µg/mL). The most potent activity was found in the methanol extract, LD50 = 1.54 µg/mL (Fig. 2).

[0062] The activity in the *Datura* leaf extract is not generally cytotoxic but specific for the breast cancer cell line MCF7. The bioactivity in the methanol extract was fractionated on silica gel by flash chromatography. The eluant was separated into nine fractions, and fractions 2 through 8 were tested for biological specificity against four cancer cell lines from different tissue types: A549, HCT1 16, PC3 and MCF7. Cells were treated by the fractions from the silica column (at 1 mg/mL) for 4 days as before. Fig. 2 shows results as a percentage of inhibition

relative to growth of control cells. The bioactive compound was most abundant in fraction 2, but the silica column did not fractionate the compound well (Fig. 3).

[0063] The compound was extracted from methanol fractions by butanol, and chromatographed on a Waters Atlantis 5 micron C18 (10 mm x 100 mm) column; eluting in a 50 minute gradient from 80 % solution A (0.1% trifluroacetic acid (TFA)) in solution B (acetonitrile) to 100 % B; flow rate, 3.5 mL/min; detection at 230 nm (Fig. 4). The fraction eluting at 26 minutes had the most bioactivity.

[0064] This bioactivity was chromatographed again on the same column, but in a steeper, 10 minute gradient. Two peaks were observed (Fig. 5), named A and B. A and B were separately further purified by HPLC chromatography as before to obtain pure A and B (Fig. 6). These were both potent against breast cancer cells. Fig. 7 shows bioactivity of peaks A and B compared to semi-purified extract (active fraction 26 from Fig. 4) when tested against MCF-7 (Fig. 7A) and MDA-MB-468 (Fig. 7B) breast cancer cell lines.

[0065] The relative abundance of dinoxin B in other organs of *D. innoxia* was investigated by monitoring methanol extracts by LC-MS for the presence of the indictor 655 ion. Extracts from leaves, stems, roots, and flowers were analyzed. The most abundant source of the dinoxin B was the leaves; this organ accumulated 10 fold greater concentrations of this compound than flowers or leaves from immature plants. None of the other organs, stems or roots, had any appreciable concentrations of dinoxin B. Leaves from two other *Datura* species, *D. metel* and *D. stramonium,* were collected and extracted. Only leaf extracts from *D. innoxia* contained dinoxin B; it was not detected in extracts from leaves of *D. metel* or *D. stramonium.*

Example 2 Preparative scale isolation

[0066] A preparative scale isolation using 700-1000 g of dried leaves collected from field grown *D. innoxia.* This material went through successive soxhlet refluxes, hexane, chloroform, and then the methanol soluble fraction was collected. The methanol soluble fraction was then extracted against butanol, resolved on silica gel flash chromatography, and finally separated to homogeneity on preparative HPLC.

[0067] The methanol extracts were diluted with an equal volume of water, then centrifuged. The supernatant was extracted with half a volume of hexane and pooled. The methanol phase in 250 mL aliquots, was loaded onto a 20 mL (5g) LC-18 column (Supelco). After washing the column extensively with 30% (v/v) methanol, the sample was eluted with 100% methanol. Four volumes of anhydrous ethyl ether were slowly added to the extract (with continuous stirring) at 0°C. The sample was centrifuged for 15 min (10,000 x g, 4°C). The

supernatant was collected, and the ether was removed under a stream of nitrogen at ~50°C. Semi-preparative HPLC was then performed using a Waters HPLC system: 10 x 100 mm Waters Atlantis Prep dC18 column (5μm), resolved with 6 mL/min linear gradient of 20% to 60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Following one minute of 20% acetonitrile, the gradient slope was 5% per minute. The column was run at 54°C and washed with 100% acetonitrile and equilibrated with 20% acetonitrile prior to each injection. Absorbance was monitored at 271 nm using a Waters 960 photodiode array with the major peak collected at approximately 7.35 minutes. (Fig. 8). This material was diluted with an equal part of water, then concentrated on an LC-18 column as before. This material was used for the bioassays.

[0068] The fraction with the strongest anti-cancer activity as determined by the bioassays was then purified to homogeneity by HPLC and examined by high resolution LC-MS. Examination of the sample by HPLC showed the elution of a single UV absorbing peak at several wavelengths.

[0069] Dinoxin B is found to be a light brown emulsion/amorphous solid.

[0070] Its UV spectrum in methanol shows λ^{max} (log e), 230, 314 (3.60) nm.

[0071] Its IR spectrum (KBr, disc) shows λ^{max} (v cm^{-1}): 3409, 2910, 1683, 1664(sh), 1395, 1077, 1050(sh), and 798 cm-1.

Example 3 Elemental composition

[0072] For structural analysis, a second purification using a Shimadzu (Columbia, MD) preparative HPLC system was used with dual 8A pumps, SIL lOvp autoinjector, SPD MIOAvp photodiode array detector, SCL lOAvp system controller all operating under the Shimadzu Class VP operating system. 1 mL of sample aliquots in methanol were injected on a Phenominex (Torrance, CA) Luna C I8(2) semi-preparative reverse-phase column (5u, 100A, 250 x 25 cm). The column was pre-equilibrated with 1% acetic acid, 20% methanol and 79% water at a flow rate of 10 mL per minute and the effluent was monitored at 280 nm. The column was developed to 100% methanol over 45 minutes. UV absorbing peaks were collected by peak absorption with a Shimadzu FRC-IOA fraction collector. The procedure was repeated to obtain sufficient purified material. Pooled material was allowed to evaporate to remove organic solvent, and then freeze-dried to recover the purified dinoxin B.

[0073] Using a calibrated infusion run on the Q-TOF the molecular formula of dinoxin B was determined to consist of two related forms, one with a mass of -453 and a second with a mass of -655 (Fig. 9). The large positive ion m/z 655 was determined to be $[M+Na]^+$ with major

fragments m/z 471 $[M\text{-glucose}+H]^+$ and 453 $[M\text{-glucose-water}+H]^+$. When the sample was run on LC-MS a m/z ion at 633 $[M+H]^+$ was associated with the single UV absorbing peak. The larger form of the compound has a mass of 655.3087, which predicts a molecular formula of $C_{34}H_{46}O_{11}Na$. The lower molecular weight form of the compound has a mass of 453.2651, which predicts a molecular formula of $C_{28}H_{37}O_5$. These two forms of the bioactive compound likely differ by the presence of a single hexose, so the smaller compound will be considered the aglycone of the larger form.

[0074] In summary, HREIMS positive ion analysis shows m/z 655.3146 $[M+Na]^+$ (calculated for $C_{34}H_{48}0nNa$, 655.3088); with major fragments m/z 471.2830 $[M\text{-glucose}+H]^+$ (calculated for $C_{28}H_{39}O_6$ 471.2741) and 453.2686 $[M\text{-glucose-water}+H]^+$ (calculated for $C_{28}H_{37}O_5$ 453.2635).

Example 4. Structural analysis.

[0075] For HR-MS analysis, samples were run on an Applied Biosystems/MDS Sciex QStar Elite Q-TOF mass spectrometer with a Turboionspray electrospray source, and a Agilent 1100 series HPLC system (G1379A degasser, G1357A binary capillary pump. G1389A autosampler, G1315B photodiode array detector, and a G1316A column oven) all running under Applied Biosystems Analyst 2.0 (build 1446) LC-MS software. The MS was calibrated at least once daily with a standard calibration mixture recommended by Applied Biosystems and the signal detection was optimized as needed. The data was acquired in the TOF MS mode, positive. The MS parameters were as follows: accumulation time - 1 sec, mass range 200 to 1000 daltons, source gas 1 - 50 units, source gas 2 - 35 units, curtain gas - 25 units, ion spray voltage 4500, source heater - 400 degrees, declustering potential -80, focusing potential - 265, declustering potential 2 - 15, ion release delay - 6, ion release width - 5. For HR-MS determination sample was mixed with a standard calibrant and infused directly into the MS. For LC-MS analysis, the column used was an Inertsil ODS-3 reverse phase C-18 column (3 µ, 150 x 3 mm, with a Metaguard column, from Varian). The initial conditions were 20% methanol and 0.2% acetic acid in water, at a flow rate of 0.25 ml per minute. The effluent was monitored at 280 nm on the PDA. After a delay of two minutes, the column was developed to 100% methanol with a linear gradient over 60 minutes.

[0076] All NMR spectra were collected at 27°C with a Bruker (Billerica, MA) Avance 500 using a 5mm BBO probe. The solvent was d6-DMSO. The data was processed with Bruker Topspin 1.3 and the chemical shift predictions made using ACD/ChemSketch (Toronto, Ontario, Canada) version 12.01. Chemical shifts are reported as parts per million from TMS based on the

lock solvent. For ^{1}H NMR (DMSO-*de*, 500 MHz) and ^{13}C NMR (DMSO-*d6*, 100 MHz) spectroscopic data, see Table 1.

Table 1. ^{1}H and ^{13}C NMR spectroscopic data for dinoxin B.

#	δ_C mult.	δ_H (*J* in Hz)	HMBC
1	203.9, C		
2	127.3, CH	5.81, dd (9.9, 2.7)	
3	147.4, CH	6.94, ddd (9.9, 4.9, 2.3)	4, 10
4	33.2, CH_2	3.30, 2.89, d (21.4) dd (21.4, 4.9)	1, 4, 5
5	136.4, C		
6	124.6, CH	5.59, d (5.9)	4, 7, 8, 10
7	30.5, CH_2	1.93, 1.49, dt (17.6, 4.9), m	5, 6, 8, 9
8	32.3, CH	1.29, m	1, 9, 10, 13
9	42.0, CH	1.58, m	1, 7, 8, 10, 11, 19
10	50.2, C		
11	33.4, CH_2	2.26, 1.39, dt (13.0, 4.1), q (12.1)	8, 9, 10, 12, 13
12	77.8, CH	3.42, dd (11.5, 4.1)	11, 13, 18
13	47.7, C		
14	54.2, CH	1.04, dd (11.1, 7.0)	8, 12, 13, 15, 18
15	24.0, CH_2	1.59, 1.19, m, m	8, 16
16	26.9, CH_2	1.75, 1.50, m, m	13
17	48.3, CH	1.73, m	13, 16, 20, 22
18	7.9, CH_3	0.71, s	12, 13, 14
19	18.9, CH_3	1.16, s	1, 5, 9, 10
20	45.3, CH	1.84, m	17, 22
21	59.6, CH_2	3.74, 3.67, dd (11.5, 3.7), dd (11.4, 1.4)	17, 22
22	77.7, CH	4.44, dt (12.8, 3.8)	20, 21, 24
23	33.0, CH_2	2.76, 2.32, dd (18.3, 13.0) dd (18.3, 2.9),	22, 24, 25, 28
24	158.7, C		
25	122.4, C		
26	165.6, C		
27	62.5, CH_2	4.51, 4.24, d (10.9), d (10.9)	24, 25, 26, 1’
28	20.7, CH_3	2.04, s	23, 24, 25, 26, 27
1'	103.2, CH	4.17, d (7.9)	27, 3’
2'	73.8, CH	2.93, t (8.3)	1’, 3’
3'	77.2, CH	3.13, t (8.6)	
4'	70.6, CH	3.04, m	5’
5'	77.4, CH	3.08, m	6’
6’	61.6, CH_2	3.67, 3.44, dd (11.5, 1.7), dd (11.9, 5.6)	4’, 5’

HMBC correlations, optimized for 10 Hz, are from proton(s) stated to the indicated carbon.

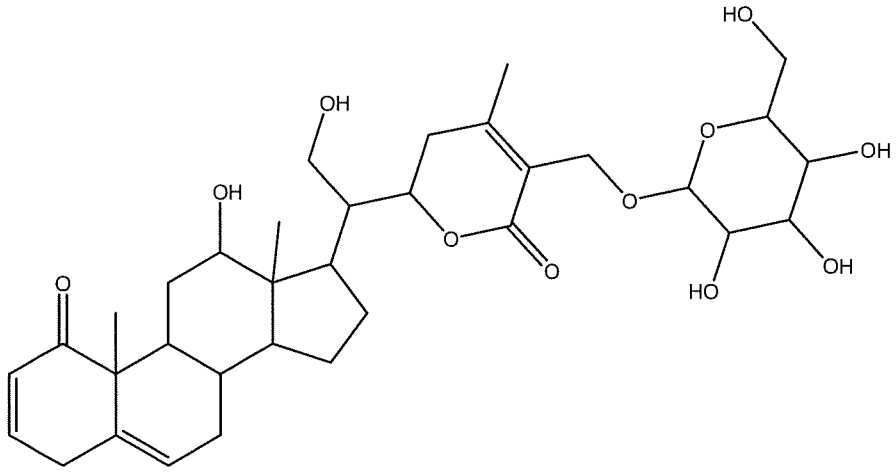
[0077] There are strong NOESY cross-peaks from H-1' to H-3' and H-1' to H-5', implying that H-1', H-3' and H-5' are on the same side of the glucose ring. This requires that the glucose have the beta conformation.

[0078] Strong NOESY cross-peaks from H-12 to H-9, H-1 la, H-14, H-17 and weak or absent cross-peaks from H-12 to H-8, H-llb, H-18 and H-19 imply the stereochemistry for most of the molecule. H-9, H-12, H-14 and H-17 are on one side of the molecule, while H-8, H-1 IB, H-18 and H-19 are on the other. Strong NOESY cross-peaks from H-18 to H-8, H-1 lb and H-19 and lack of cross-peaks from H-18 to H-9, H-1 la, H-12 and H-14 (all shown right) agree with the stereochemistry suggested above. The cross-peak from H-18 to H-20 does not imply any stereochemistry due to the free rotation around the C-17 - C-20 bond, as a strong NOESY signal would be present for any configuration at C-13, C-17 or C-20. Related 12-hydroxy compounds show both R and S stereochemistry are found in *Datura* spp. at C-12.

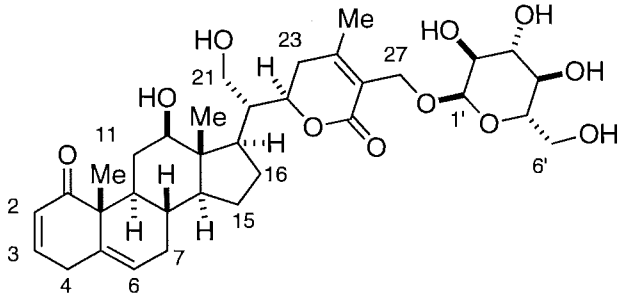
[0079] The diagnostic mass spec and NMR spectra suggested a steroidal lactone glucoside. ^{13}C and HSQC spectra showed the presence of a ketone, one carboxyl moiety and 6 olefinic carbons, 3 of which were protonated. HMBC and COSY spectra showed that all the double bonded carbons were isolated from each other. There are three methyls on unprotonated carbons.

[0080] Beginning with the ketone at position 1, it is possible to follow the connectivity of the *abed* rings with the HMBC and COSY spectra and assign the basic steroidal structure. COSY and HMBC both show the C-17 to C-20 connection as the branch point for the rest of the molecule. COSY then follows H-20 to H-22 to H-23, after which HMBC can assign the rest of the lactone ring. HMBC also shows the connection between methylene C-27 and C-Γ of the glucose anomeric carbon.

[0081] Based on the molecular formula determined and the NMR spectroscopic data obtained, dinoxin B was assigned the structure 12,21-dihydroxy-l-oxowitha-2,6,24-trienolide-27-0 -β-**D**-glucopyranoside. It can be alternatively named Ergosta-2,6,24-trien-26-oic acid, 27-**(**β-**D**-glucopyranosyloxy)-12,21,22-trihydroxy-l-oxo, δ-lactone. Dinoxin B can also be classified as a sitoindoside, which is a withanolide containing a glucose group at the C-27 position.. The structure of dinoxin B is shown in the chemical formula:



[0082] The stereochemistry at carbons 8, 9, 10, 12, 13, 14, 17, 20, 22, 1', 2', 3', 4' and 5' is as shown in the structure illustration.



[0083] Withametelinol and withametelin (and other related compounds) were shown to have C-22 stereochemistry of R based on a positive Cotton effect around 250 nm. Both researchers assign the stereochemistry at C-20 as R based on biogenetic arguments because all the reported withanolides unsubstituted at C-20 have the same configuration.

[0084] The structure of dinoxin B is similar to but not identical with the reported structure of withametelinol. Dinoxin-B differs from withametelinol in several aspects: the stereochemistry of C-12 is different between the two structures; the addition of a glucose moiety in dinoxin B; the position of the double bond is C-24 - C-25 in withametelinol, but C-25 - C-27 in dinoxin B; and the oxygen at C-21 is a bicyclic ether in withametelinol and an alcohol in dinoxin B.

Example 5 Biological Activity

[0085] The cell viability assay was carried out using the ATP-sensitive CellTiterGlo luminescent assays. Cells, typically 5,000-15,000 cells per well were plated in 96-well microtiter plates. Cells were allowed to adhere using standard growth culture media and conditions as recommended by the ATCC. After 24-hours, medium was replaced and dinoxin B was added with final DMSO concentration <0.1%. Cells were grown in the presence of dinoxin B for 48

hours at which time medium was replaced with drug-free medium. Cells were allowed to recover for 24 hours. The CellTiter Glo reagent was added directly to the medium and luminescence was measured using a Packard TopCount microtiter plate luminometer. Cytotoxicity determined in this method was highly consistent with the 3H-thymidine incorporation assay.

[0086] The breast cancer cell line MCF-7 was used throughout the initial extraction process in the bioassay guided fractionation of these extracts. The mass spectra of the active HPLC fraction against MCF7 was determined and this chemical signature was used to purify preparative amounts of dinoxin B.

[0087] Peaks A and B were purified and tested for anticancer activity against two different breast cancer cell lines (Fig. 7). HPLC-MS analysis of peaks A and B indicate these peaks are not pure compounds but still mixtures of relatively high molecular weight masses, 450 to 750 AMU. Peak A and peak B have very similar UV/Vis spectra with λmax of 228 nm for peak A and 233 for peak B. The bioactivities in these peaks are very specific and very potent (-40 nM); the IC_{50} against MDA-MB-468 was 20 ng/mL for peak A and 30 ng/mL for peak B.

[0088] Once purified, the specificity of dinoxin B was investigated by bioassays against a number of cell lines. Normal and cancer cell lines, organized by organ source, were treated with varying doses of dinoxin B to determine the IC_{50} using the CellTiter-Glo luminescent cell viability assay. The IC_{50} values for dinoxin B against 24 different cell lines is presented in Table 2.

[0089] These results shown that Dinoxin B exhibited the highest activity against breast cancer cell lines and was less active against other cancer cell lines or normal cells. The IC_{50} for dinoxin B against a sensitive breast cancer cell line, i.e. T47D, is 0.22 μM.

Table 2

Type	Cell line	IC_{50} (μM)
Normal	IEC6	3.25 +/- 0.05
	NHF177	1.23 +/- 0.06
	HUVEC	0.74 +/- 0.09
Colon	Colo205	2.24 +/- 0.12
	HCT116	1.00 +/- 0.07
	SW48	0.58 +/- 0.08
	RKO	0.36 +/- 0.02
Melanoma	SK-MEL28	2.27 +/- 0.05
	SK-MEL5	1.38 +/- 0.03
	UACC62	0.50 +/- 0.03
	SK-MEL2	0.36 +/- 0.05
Liver	HEPG2	1.50 +/- 0.04
	HUH7	1.48 +/- 0.02
	HEP3B	0.67 +/- 0.29
Lung	A549	3.39 +/- 0.02
	A427	0.87 +/- 0.03
Ovarian	OVCAR3	3.23 +/- 0.06
Breast	MDA-MB453	2.95 +/- 0.05
	MDA-MB231	1.00 +/- 0.07
	MCF7	0.61 +/- 0.05
	MDA-MB468	0.58 +/- 0.07
	HS578T	0.44 +/- 0.38
	T47D	0.22 +/- 0.06

To determine if the presence of the glucosyl group at C-27 of compound 1 influenced the biological activity, the aglycone compound 2 was generated and used in bioassays. The IC50 values for compound 2 against the T47D, MDA-MB468, and MCF-7 cell lines were 0.99+/- 0.09, 0.75+/- 0.05, and 0.87+/- 0.22, respectively. In each case, the most potent cytotoxic compound was compound 1; compound 2 was slightly less active. Compound 1 is unusual in that it has submicromolar cytotoxicity levels as a glucosylated form of a withanolide. Other withanolide glycosides have less cytotoxicity relative to their aglycone forms. This is in direct contrast to the bioactivities of other withanolides; the published IC50 values for withaferin A and its glycoside form against MCF-7 cells are 0.6 and 7.9 uM, respectively. In the case of withaferin A, unlike compound 1, the presence of a glucose group on the withanolide reduces the bioactivity 10-fold.

Example 6 Characterizing the novel withanolide from *D. innoxia* with anticancer activity

[0090] Analogues of the novel withanolide are synthesize by treating the natural product to generate purified forms of the glycoside and aglycone forms of the withanolide. Gram quantities of the novel withanolide/glycoside are isolated from *D. innoxia,* from 30-50 kilograms of dried leaves collected from field grown plants.

[0091] Initial extraction is optimized by comparing 80% methanol/water to extraction using petroleum ether, and ethyl acetate commonly applied to withanolides. The bioactive withanolide is unstable during isolation. Sequential soxhlet extractions of at least 140 g dried *D. innoxia* leaves with hexane, chloroform and finally 80% methanol are performed. The first two remove the waxes and alkaloids before the methanol extraction. The bioactivities are further separated with a butanol extraction against water. Purified samples of the glycoside form of the withanolide are acid hydrolyzed, and fractionated by solubility and/or by HPLC-MS. Fractions of the aglycone form of the withanolide are collected. Chemical confirmation of the predicted structures for these fractions are obtained using MS and NMR.

[0092] The chemical structure is analyzed by 1H-NMR and $^{1}{}^{3}$C-NMR, ^{2}D NMR, COSY, HSQC, and HMBC along with high-resolution MS. The bioactivity specificity will be confirmed as well using selected cancer lines as negative controls (AN3CA lung cancer line) as well as the expected responsive cell line, breast cancer cell lines M DA-MB 468 using the 3H-thymidine assay.

[0093] Four cell lines: MDA-MB-468, MCF-7, UACC-62 and OVCAR-3 are used for xenograft studies because of their sensitivity and resistance, and amenability to xenograft studies. These cell lines include a variety of genetic alterations. For example, the sensitive MCF-7 (breast) and resistant UACC-62 (melanoma) lines express wild type p53 while sensitive MDA-MB-468 (breast) and resistant OVCAR-3 (ovarian) express mutant p53. The two breast cancer cells lines represent estrogen receptor positive (MCF-7) and negative (MDA-MB-468) tumors. The in vitro LD50 values for each of these four cell lines are provided in Example 5. Specifically, the glycosidic form of the withanolide is likely the active compound most potent against MCF7 and MDA-MB-468 cell lines. The mechanism of action and related structural analogues are then developed based on in vivo studies on forms of the withanolide demonstrated to have bioactivity in vitro.

[0094] Example 7 Maximum tolerated doses and pharmacokinetic parameters of the withanolide and/or glycoside form in mice

[0095] The maximal tolerated dose (MTD) and pharmacokinetics of active principal and analogues in the mouse are determined. The purpose of the pharmacokinetic studies is to determine: 1) compound plasma half-life in order establish the optimal dosing intervals: 2) the bioavailability of the compound after intraperitoneal and oral administration to allow a more convenient route for drug administration; and 3) the predominant route of drug elimination.

[0096] The MTD and pharmacokinetic (PK) studies are measured for the *Datura* active principal (withanolide or the aglycone version as assessed by in vitro assays). In the initial toxicity studies in BalbC mice, the maximal dose of compound that is administered by tail vein injection is 100 mg/kg formulated as an aqueous solution, in ethanol or as an 80:10:10, PBS:ethanol:Cremaphor suspension. Other formulations, including those containing DMSO, PEG and cyclodextrin, are measured to increase the dose that can be administered.

[0097] Active principal and analogues are given in two fold increments by tail vein injection, intraperitoneal injection of oral gavage. MTD is defined as the maximum dose at which animals do not show acute distress. PK studies are conducted based on the MTD for a single dose administration. The pharmacokinetic studies determine compound blood half-life, the predominant pathways of elimination and the bioavailability of the active principal after intraperitoneal and oral administration relative to tail vein injections. Blood samples (50 μï per time point) are obtained from animals at 5 minutes, 30 minutes, lhour, 4 hours and 24 hours after active principal administration. Estimation of the half-life are calculated from the serial measurements of LC/MS signal or radioactivity in blood. The area under the serum concentration-time curve (AUC) are calculated by noncompartmental analysis using WinNonlin Version 4.0 (Pharsight, Mountain View, CA.). Mice are placed within a metabolic cage to allow collection of excreted material for 48-hour following drug administration. To estimate the oral bioavailability of active principal, the average AUC after mouse tail vein injection is compared to the average AUC after oral gavage.

[0098] In addition to MTD and basic PK parameters, tolerance for each compound is determined in a multiple dosing schedule for xenograft studies. Animals are administered compound at the MTD and frequency of dosing determined by the PK studies are monitored for toxicity including weight loss. These studies define the number of doses at the MTD that animals can tolerate.

[0099] Example 8. Xenograft models of human breast cancer

[0100] The activity of the *Datura* active principal in vivo using human xenografts in mice is determined. Inhibition of growth of tumor xenografts in mice demonstrate that the active principal exhibits antitumor activity at the doses that can be tolerated in vivo. Xenograft studies are used to evaluate growth inhibition of human breast cancer cell lines, or other cell lines that are identified as sensitive, in animals.

[0101] Results will demonstrate that administration of active principal to mice with palpable breast cancer cell xenografts leads to significant reduction of tumor growth (see below

for criteria). Breast cancer cell lines were chosen for the initial xenograft studies because a number of them showed sensitivity in vitro among a panel of human cancer and normal cell lines studied. Animals with small, palpable breast cancer xenografts are treated to demonstrate efficacy in blocking tumor growth. The xenografts are then allowed to achieve larger volumes prior to initiation of treatment to determine whether the active principal can induce regression of established tumors. NOD/SCID mice and other immunosuppressed mouse models are also used to establish breast cancer cell lines as xenografts. The xenograft studies use animals with small, established tumors using a multiple dosing schedule as determined in the MTD analysis.

Power calculation assume equal variance in control and treated groups and two-sided test (Table 3).

Table 3. Power calculation assuming equal variance in control and treated groups and two-sided test.

Ratio of Difference Over Standard Deviation	Sample Size in Each Group (Treated and Controls)	Significance Level	Power
0.75	29	0.05	0.80
1.00	17	0.05	0.80
1.25	11	0.05	0.80
1.50	8	0.05	0.80
1.75	7	0.05	0.80
2.00	5	0.05	0.80
2.25	5	0.05	0.80
2.50	4	0.05	0.80

[0102] Serial tumor measurements use calipers and tumor volumes calculated as described below. Animals are sacrificed if weight of the tumors approaches 10% of the body weight, if animals become moribund, exhibit signs of distress or lose more than 10% of the body weight. Statistical power calculation to determine the size of the control and experimental group is presented in Table 3. For example, assuming a standard deviation in the initial xenograft studies of approximately 35%, the use of 8 animals in each group would have 80% power to detect 52% tumor reduction (52%/35%=1.5 in column 1).

[0103] Tumor volume are evaluated using standard criteria: V = (W2 X L)/2 where V = volume; W = width (lesser dimension); L = length (greater dimension). Relative tumor volume is calculated as RTV = VT/VS where VT = tumor volume after treatment and VS = tumor volume at the start of treatment for each group (active principal and vehicle control).

[0104] The efficacy of the active principal treatment is calculated as: TGI = (T/V) X 100, where TGI is the percent tumor growth inhibition; T = mean RTV for the treatment arm;

and V = mean RTV for the vehicle arm. A minimum standard for efficacy set by the NCI (TGI>42%) will be used. When xenografts approach 10% of body weight or animals exhibit evidence of distress, mice are sacrificed and their tumors will be excised and weighed. In addition, the apoptotic fraction, the proliferative fraction and the viable fraction of cells are measured in each explanted tumor sample.

What is Claimed:

1. A process of producing dinoxin B, the sequential steps comprising:

   (a) extracting *Datura innoxia* with an alkanols/aqueous solution,

   (b) partitioning the alkanols/aqueous extract against an organic solvent;

   (c) chromatographing the aqueous fraction by reverse phase chromatography; and

   (d) collecting fractions that contain a compound that is cytotoxic to a cancer cell.

2. The process of claim 1, wherein step (a) further comprises at least one of the following steps:

   (i) collecting leaves from mature plants, and pulverizing the leaves with sand;

   (ii) extracting the pulverized leaves with hexane;

   (iii) extracting the pulverized leaves with chloroform;

3. The process of claim 1, wherein the alkanol in step (a) is methanol

4. The process of claim 3, wherein the methanol is a 4:1 (v:v) methanokwater solution.

5. The process of claim 1, wherein following the extraction in step (a), the alkanol solution is dried *in vacuo.*

6. The process of claim 1, wherein following the extraction in step (a), an equal volume of water is added to the alkanol solution.

7. The process of claim 1, wherein the organic solvent in step (b) is n-butanol, hexane, or ether.

8. The compound produced by the process according to any of claims 1-7.

9. The compound of claim 8, consisting of a light brown emulsion/amorphous solid.

10. The compound according to any of claims 1-9 having a UV spectrum in methanol with λ^{max} (log e) at 230, 314 (3.60) nm.

11. The compound according to any of claims 1-10 having an IR spectrum (KBr, disc) with λ^{max} (v cm^{-1}) at 3409, 2910, 1683, 1664(sh), 1395, 1077, 1050(sh), and 798 cm^{-1}.

12. The compound according to any of claims 1-1 1 having mass spectroscopic profile with a m/z 655 and major fragments at a m/z 471 and 453.

13. The compound according to any of claims 1-12 having elemental composition consisting of C_{34}¾ 80nNa

14. The compound according to any of claims 1-12 having elemental composition consisting ofC $_{28}H_{39}0_{6}$.

15. The compound according to any of claims 1-12 having elemental composition consisting ofC $_{28}H_{37}0_{5}$.

16. A compound produced by a process consisting of the following steps:

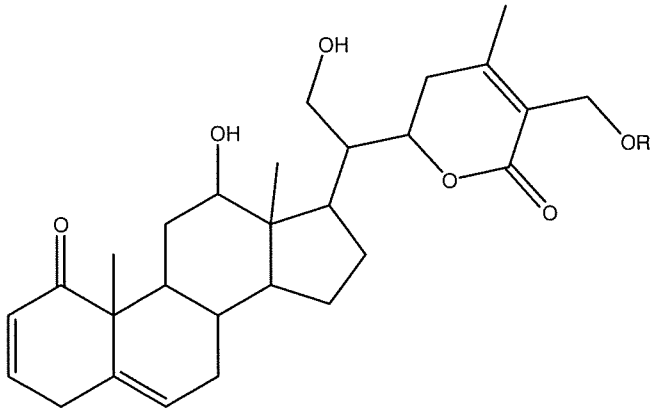
(a) extracting *Datura innoxia* with an alkanol,

(b) partitioning the alkanolic extract against and organic solvent;

(c) chromatographing the aqueous fraction by reverse phase chromatography; and

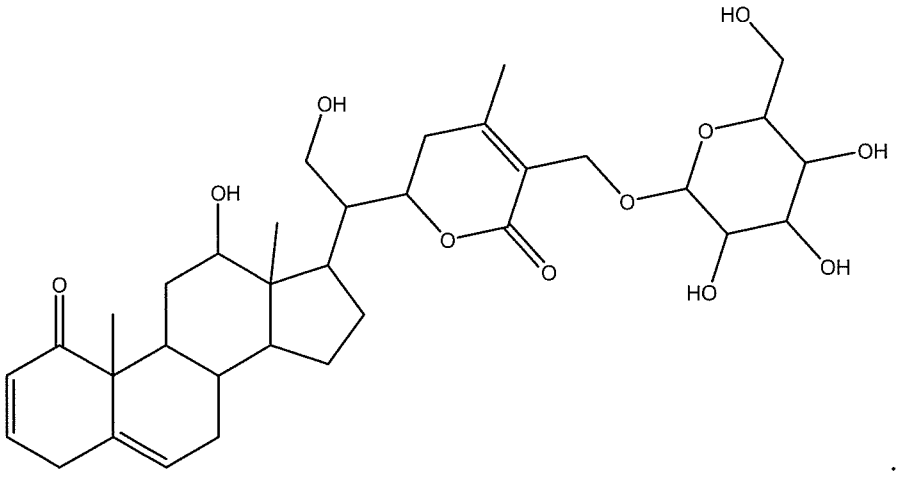
(d) collecting fractions that contain a compound that is cytotoxic to a cancer cell.

17. A compound having the following chemical structure:

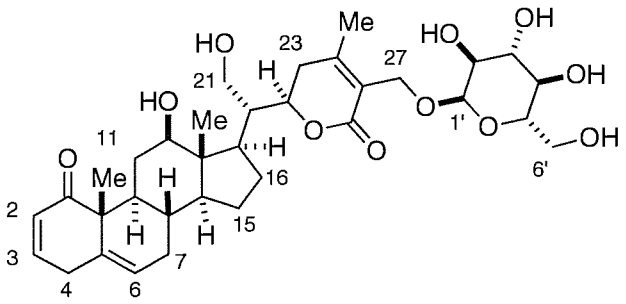


wherein R is a H or a sugar group.

18. The compound of claim 17 having the following chemical structure:



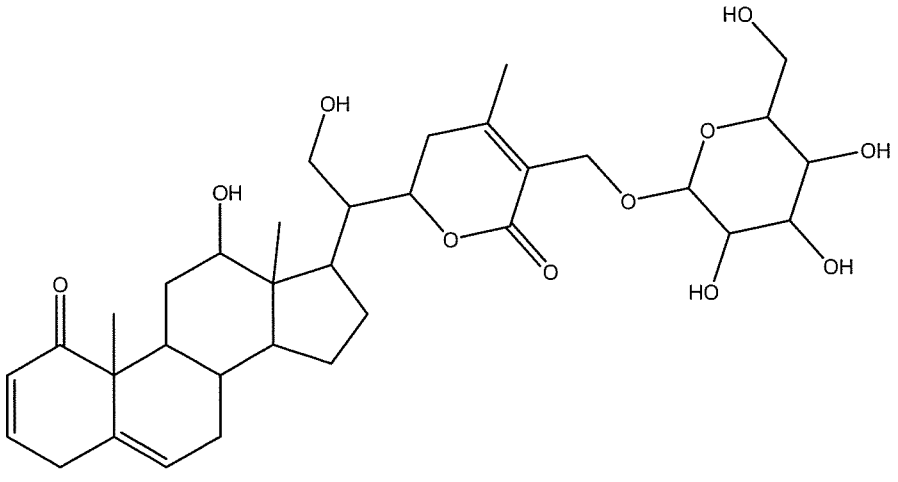
19. The compound according to claim 18 having the following stereochemistry at carbons 8, 9, 10, 12, 13, 14, 17, 20, 22, Γ, 2', 3', 4' and 5' as in the following structure:



20. A method of treating cancer in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition, wherein the active component of the pharmaceutical composition consists of the compound according to any of claims 8-19.

21. The method of treating cancer according to claim 20, wherein the pharmaceutical composition is administered parenterally, transdermally, mucosally, nasally, buccally, sublingually, topically, or orally.

22. The method of claim 21, wherein the compound of has the following chemical structure:

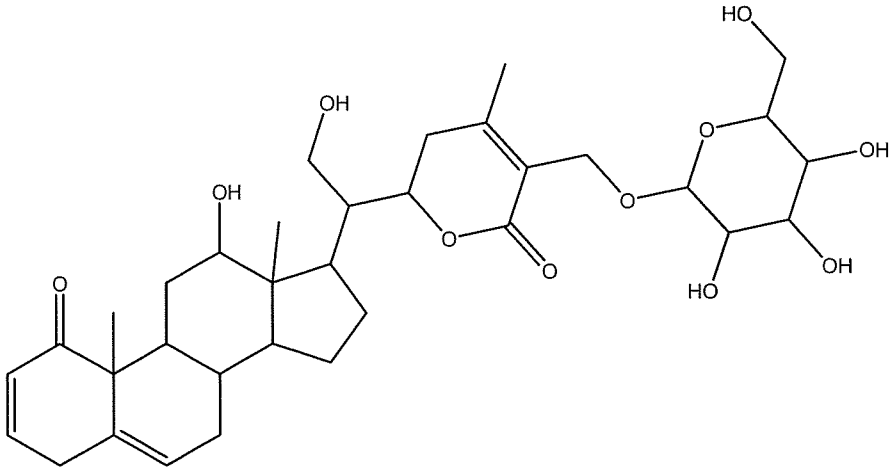


23. The method according to any of claims claim 20-22, wherein the subject is a cancer patient.

24. The method according to claim 23, wherein the subject is a breast cancer patient.

25. A pharmaceutical formulation comprising the compound according to any of claims 8-19, together with a pharmaceutically acceptable carrier.

26. The pharmaceutical formulation claim 25, wherein the compound has the following chemical structure:



27. The pharmaceutical formulation according to claim 25 or claim 26, wherein the active compound is specifically cytotoxic to breast cancer cells.

28. A compound for use in a method for treating cancer, the method comprising a step of administering to a cancer patient an effective amount of a compound according to any of claims 8-18, or a solvate, hydrate, or prodrug thereof, such that the cancer is treated.

29. The compound for use according to claim 28, wherein the cancer is breast cancer.

30. The compound for use according to claim 28, wherein the compound is administered parenterally, transdermally, mucosally, nasally, buccally, sublingually, topically, or orally.

31. Use of a compound according to any of claims 8-18 in the preparation of a medicament for the treatment of cancer in a suitable subject.

32. The use according to claim 31, where in the medicament is capable of being administered parenterally, transdermally, mucosally, nasally, buccally, sublingually, topically, or orally.

33. The use according to claim 31, wherein the cancer is breast cancer.

FIGURE 1

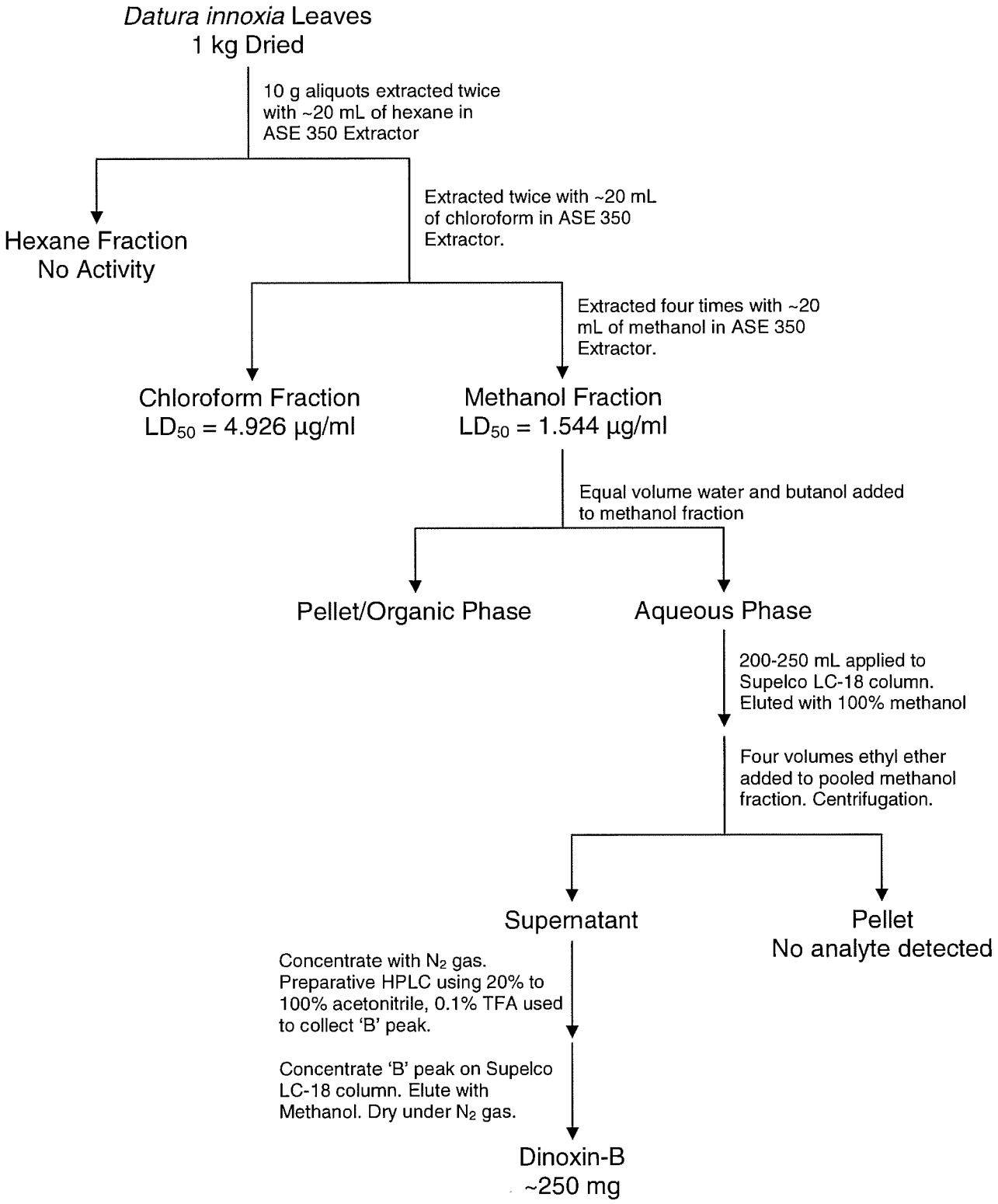

Datura innoxia Leaves
1 kg Dried
10 g aliquots extracted twice with ~20 mL of hexane in ASE 350 Extractor
Hexane Fraction
No Activity
Extracted twice with ~20 mL of chloroform in ASE 350 Extractor.
Extracted four times with ~20 mL of methanol in ASE 350 Extractor.
Chloroform Fraction
LD_{50} = 4.926 µg/ml
Methanol Fraction
LD_{50} = 1.544 µg/ml
Equal volume water and butanol added to methanol fraction
Pellet/Organic Phase
Aqueous Phase
200-250 mL applied to Supelco LC-18 column. Eluted with 100% methanol
Four volumes ethyl ether added to pooled methanol fraction. Centrifugation.
Supernatant
Pellet
No analyte detected
Concentrate with N_2 gas. Preparative HPLC using 20% to 100% acetonitrile, 0.1% TFA used to collect 'B' peak.
Concentrate 'B' peak on Supelco LC-18 column. Elute with Methanol. Dry under N_2 gas.
Dinoxin-B
~250 mg

FIGURE 2

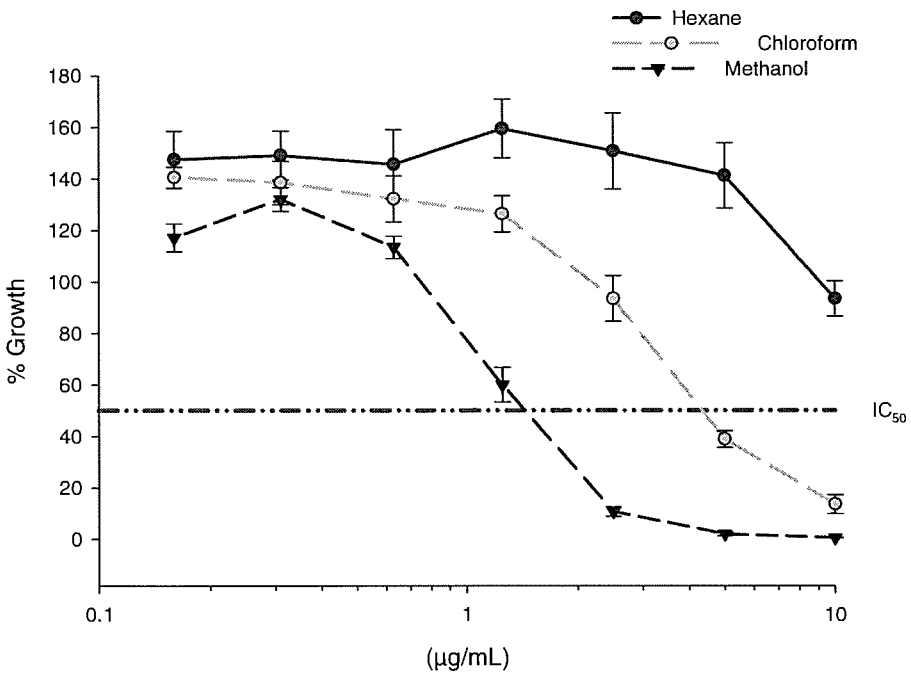

Hexane
Chloroform
Methanol
180
160
140
120
100
80
60
40
20
0
% Growth
IC50
0.1
1
10
(µg/mL)

FIGURE 3

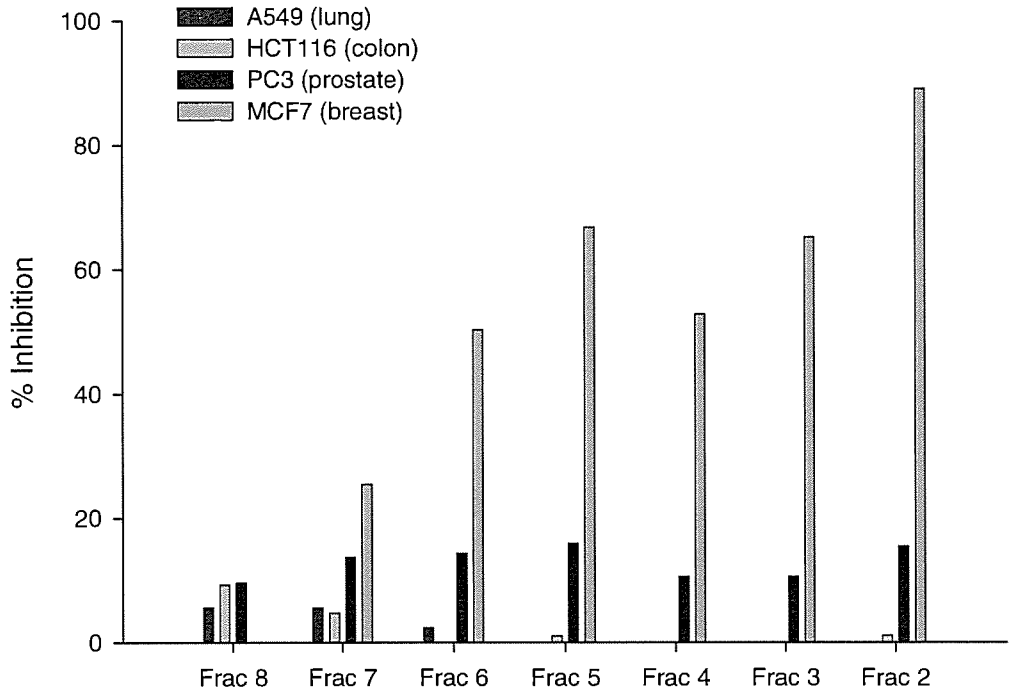

A549 (lung)
HCT116 (colon)
PC3 (prostate)
MCF7 (breast)
% Inhibition
100
80
60
40
20
0
Frac 8
Frac 7
Frac 6
Frac 5
Frac 4
Frac 3
Frac 2

FIGURE 4

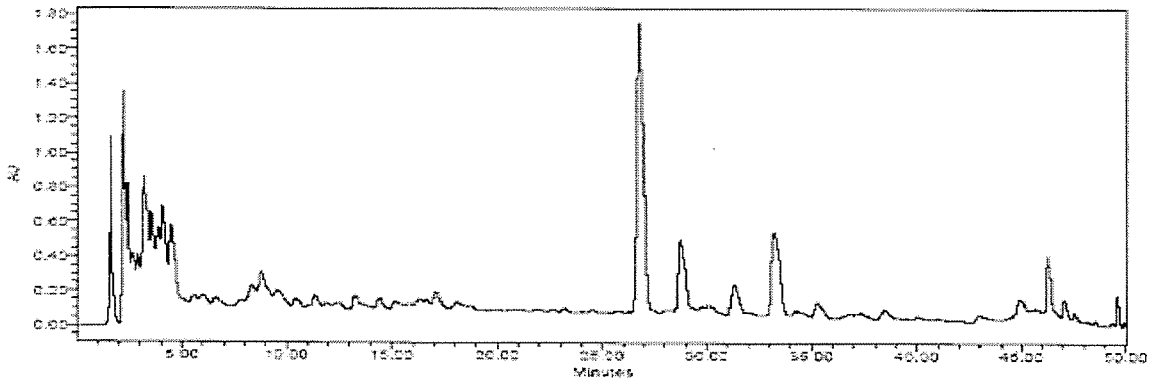


FIGURE 5

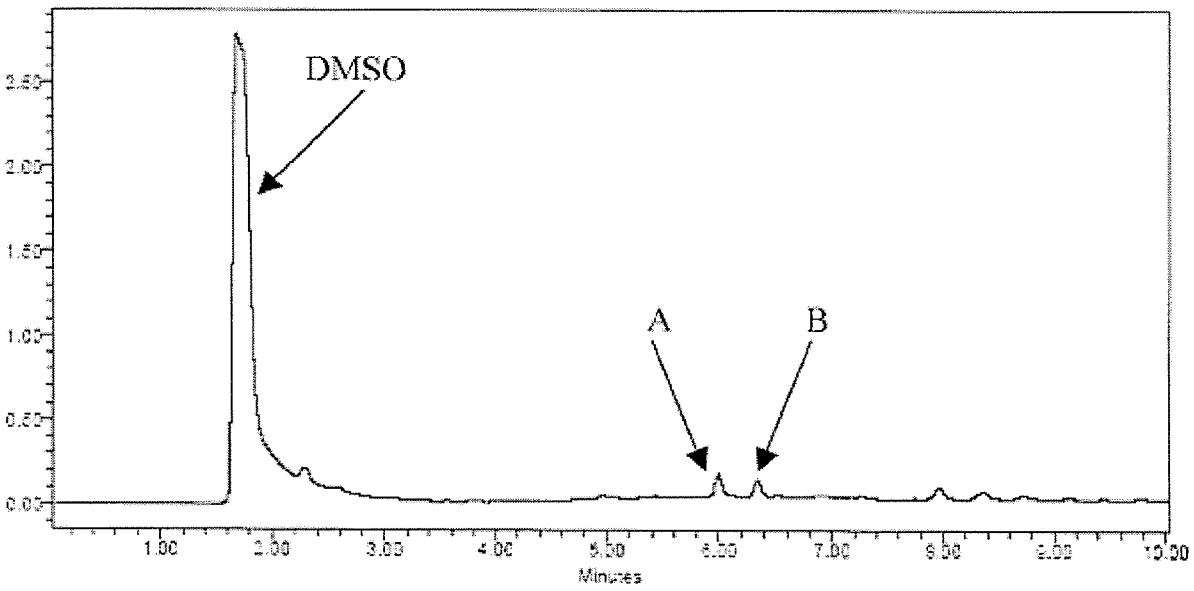
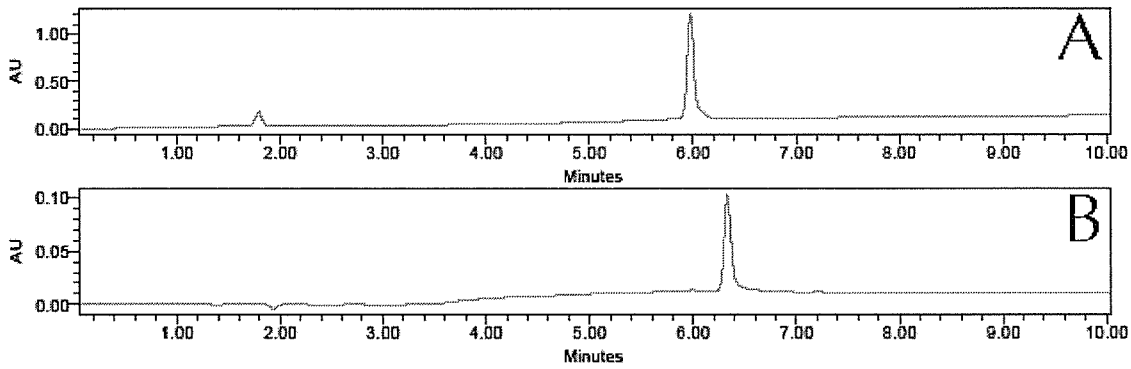


FIGURE 6


A
AU
1.00
0.50
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4.00
5.00
6.00
7.00
8.00
9.00
10.00
Minutes
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AU
0.10
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0.00
1.00
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5.00
6.00
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8.00
9.00
10.00
Minutes

FIGURE 7A

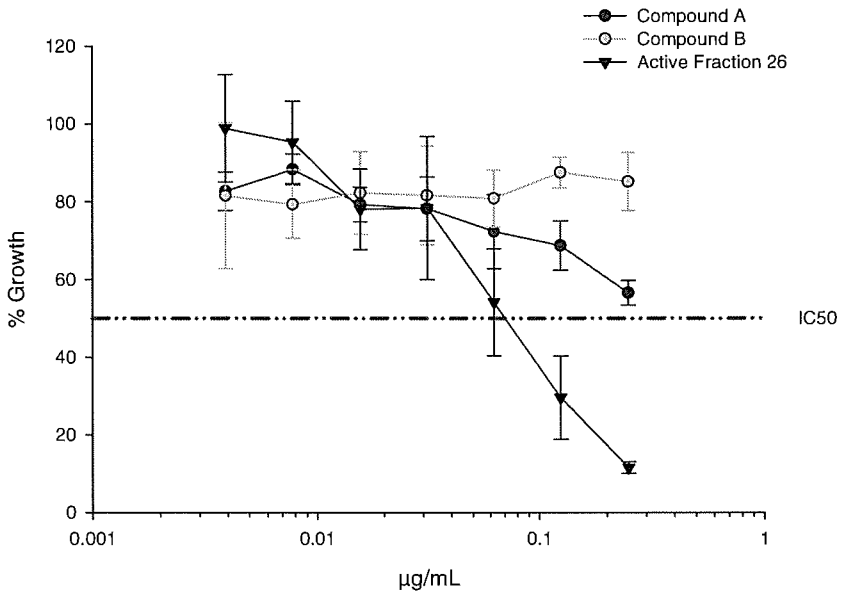


FIGURE 7B

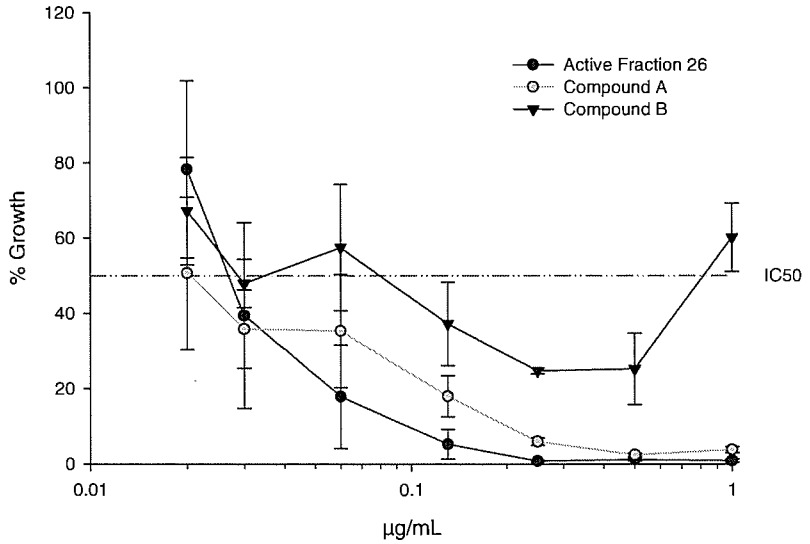


FIGURE 8

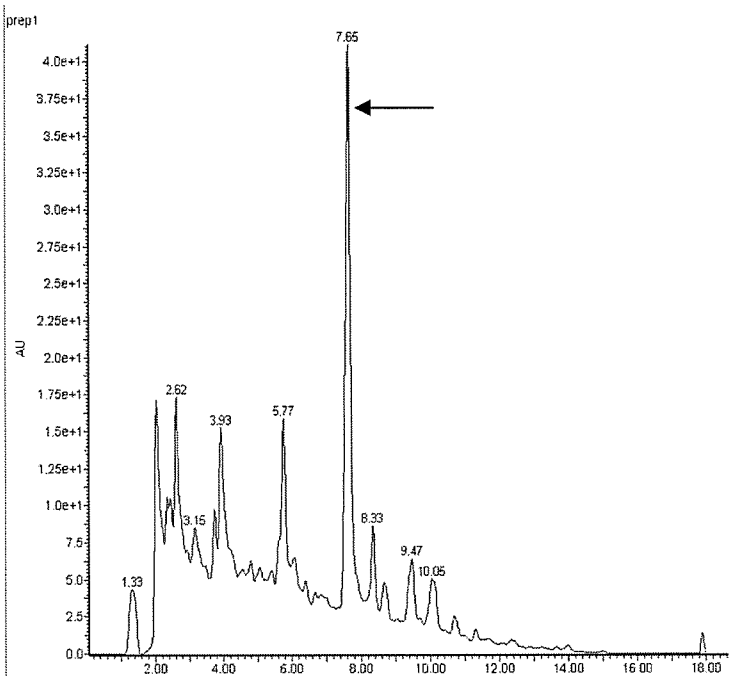

prep1
AU
4.0e+1
3.75e+1
3.5e+1
3.25e+1
3.0e+1
2.75e+1
2.5e+1
2.25e+1
2.0e+1
1.75e+1
1.5e+1
1.25e+1
1.0e+1
7.5
5.0
2.5
0.0
1.33
2.62
3.15
3.93
5.27
7.65
8.33
9.47
10.05
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00

FIGURE 9

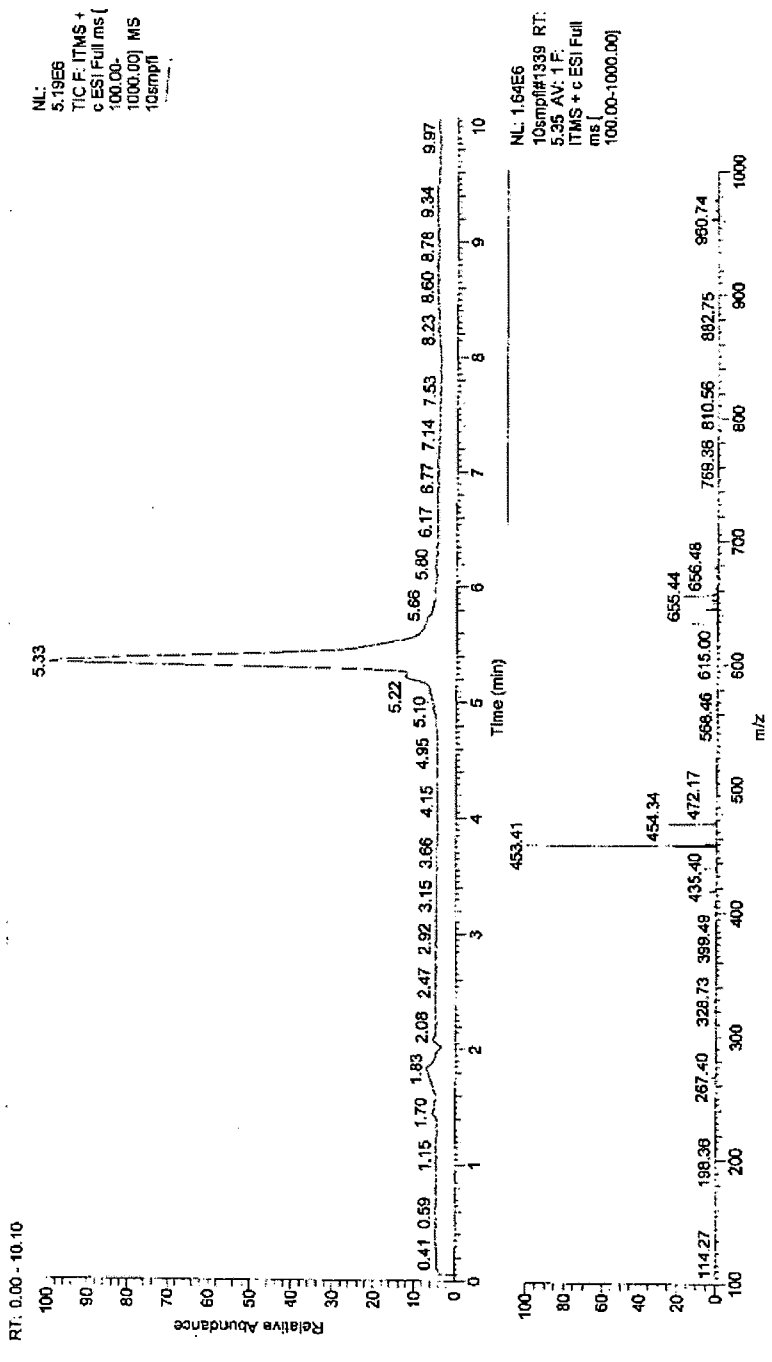
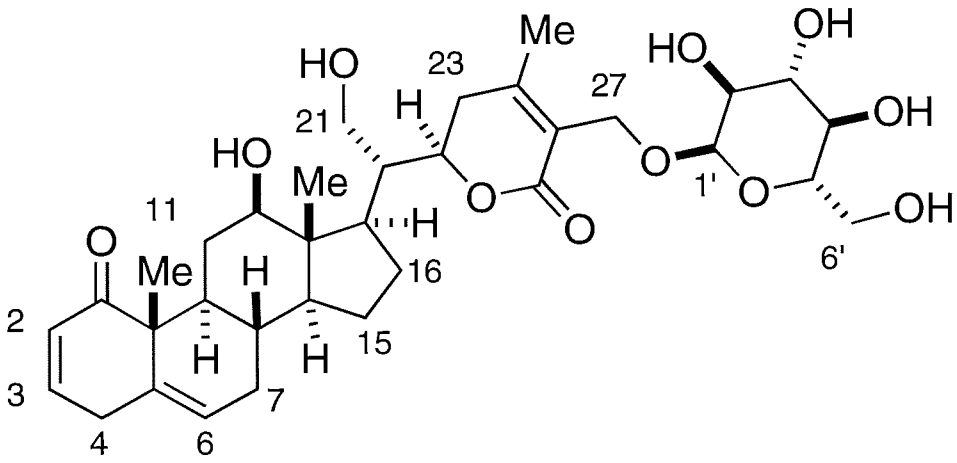

NL:
5.19E6
TIC F: ITMS + c ESI Full ms [100.00-1000.00] MS 10smpfl
RT: 0.00 - 10.10
Relative Abundance
Time (min)
NL: 1.64E6
10smpfl#1339 RT: 5.35 AV: 1 F: ITMS + c ESI Full ms [100.00-1000.00]
m/z

FIGURE 10



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/029455

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K36/81 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal , WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2006/188592 A1 (NAIR MURALEEDHARAN G [US] ET AL) 24 August 2006 (2006-08-24) *cf. abstract, page 1, section [0006], section [0017] on page 2*	1-33
Y	US 2009/088412 A1 (WU YANG-CHANG [TW] ET AL) 2 April 2009 (2009-04-02) *cf. abstract, pages 1/2, sections [0005] to [0010] , claims 1-6*	1-33
Y	US 2004/033273 A1 (PATWARDHAN BHUSHAN [IN] ET AL) 19 February 2004 (2004-02-19) *cf. abstract, page 2, sections [0015] and [0016], page 3, sections [0026], [0027] and [0029], claims 1-15, 24-29 and 69*	1-33

-/- .

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 May 2011	08/06/2011

Name and mailing address of the ISA/	Authorized officer
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1

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SIDDIQUI B S ET AL: "Daturacin, a new withanolide from [Daturainnoxia]", MEDICINAL & AROMATIC PLANTS ABSTRACTS, SCIENTIFIC PUBLISHERS, SCIENTIFIC PUBLISHERS, NEW DELHI - INDIA, vol. 27, no. 5, 1 October 2005 (2005-10-01), XP018005146, ISSN: 0250-4367 *cf. abstract no. 2005-05-2540*	1-33
A	EFTEKHAR F ET AL: "Antimicrobial activity of Daturainnoxia and Daturastramonium", FITOTERAPIA, IDB HOLDING, MILAN, IT, vol. 76, no. 1, 1 January 2005 (2005-01-01), pages 118-120, XP025264820, ISSN: 0367-326X, DOI: DOI: 10.1016/J.FITOTE.2004.10.004 [retrieved on 2005-01-01] *cf. abstract and conclusions on page 120*	1-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/029455

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