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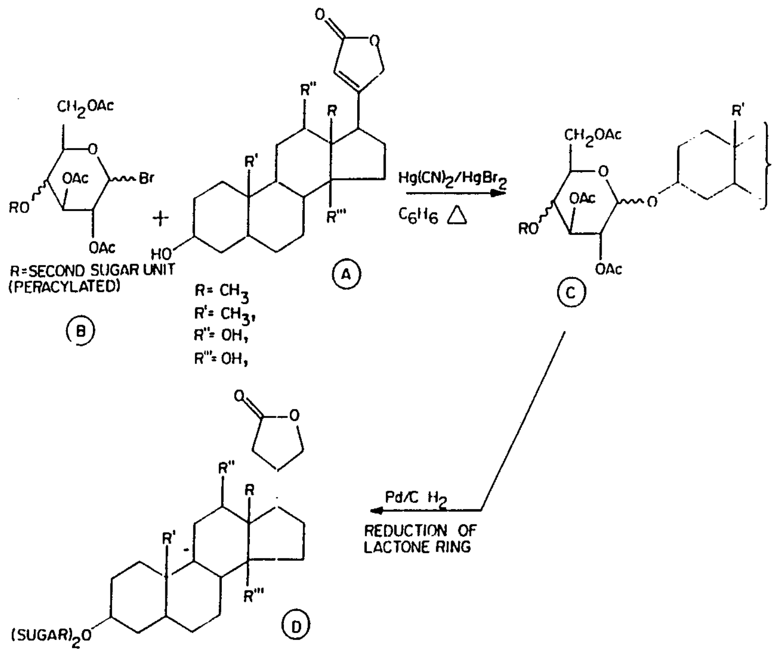
(54) Title: NOVEL STEROID GLYCOSIDES USEFUL IN THE EARLY DETECTION OF HYPERTENSION



(57) Abstract

The present invention discloses compounds useful for the screening or monitoring of hypertensive individuals, for correction of interference in the measurement of digoxin levels in patients, and as pressor agents, potassium sparing diuretics, natriuretics, or agents that alter cellular calcium levels. Methods for detecting the presence or amount of the compounds are also provided.

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Description

NOVEL STEROID GLYCOSIDES USEFUL IN THE EARLY DETECTION OF HYPERTENSION

Technical Field

The present invention relates generally to the purification, characterization, derivatization and uses of a series of compounds. This invention is more particularly related to the detection of a compound, e.g., to screen for or monitor hypertensive individuals or to correct for interference in the measurement of digoxin levels in patients, and to the use of a compound or a derivative thereof as a pressor agent, potassium sparing diuretic, natriuretic or agent that alters cellular calcium levels.

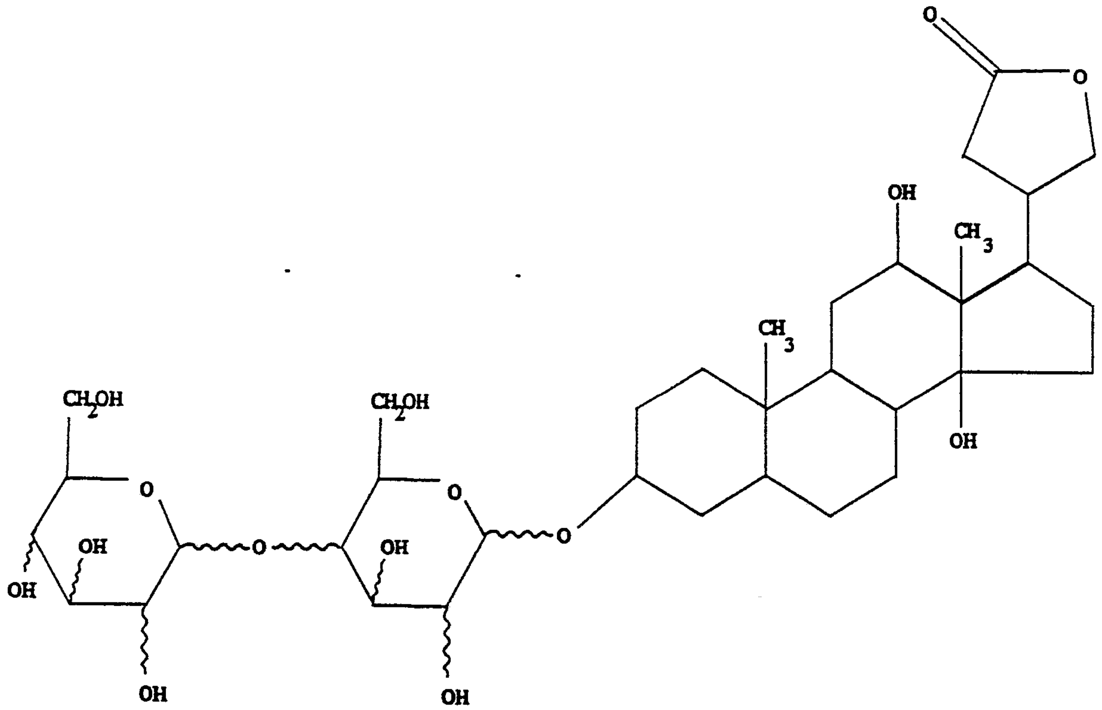
Background of the Invention

Essential hypertension with its associated risks of stroke and congestive heart failure contributes significantly to the morbidity and mortality rates in the United States and other countries. For example, it is estimated that over sixty million Americans alone are diagnosed as hypertensives. The current diagnostic procedure for hypertension focuses on taking blood pressure measurements which indicate the results of a physiological disease process already in progress. There does not currently exist a clinically effective method to identify these patients prior to the appearance of clinical symptoms. Identification of an individual with essential hypertension or at risk to develop essential hypertension could lead to an earlier initiation of dietary and pharmacological therapies to control the factors responsible for the disease process, thereby resulting in reduced morbidity and mortality.

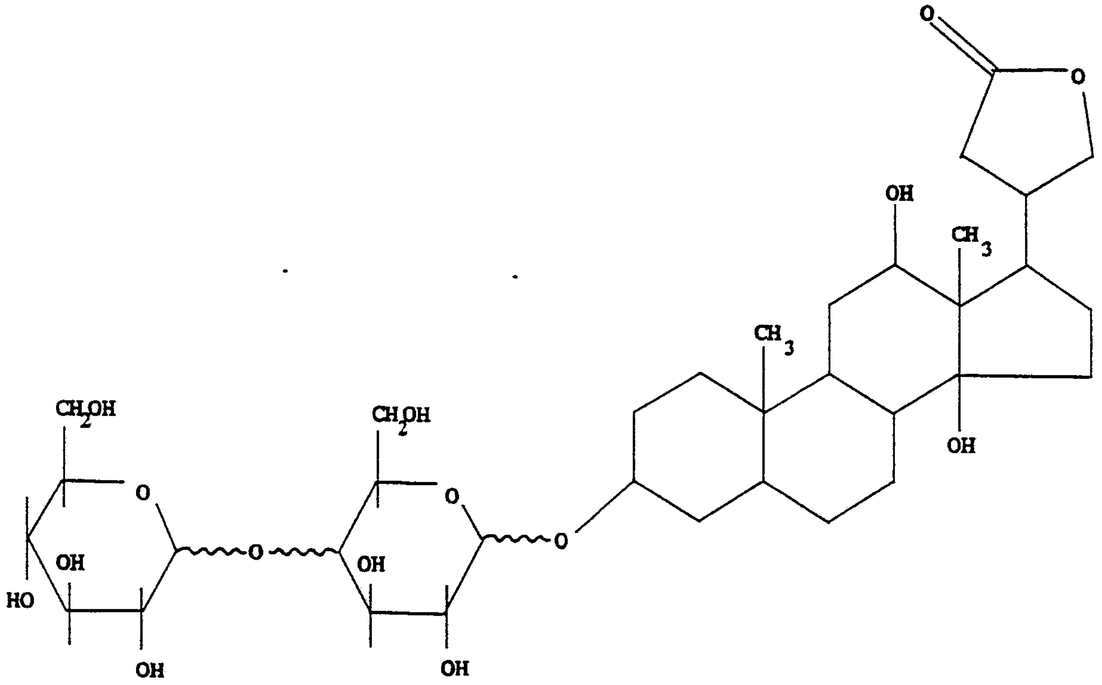
Thus, there is a need in the art for a method to detect hypertensive individuals prior to the appearance of clinical symptoms. The present invention fulfills this need and further provides other related advantages.

Summary of the Invention

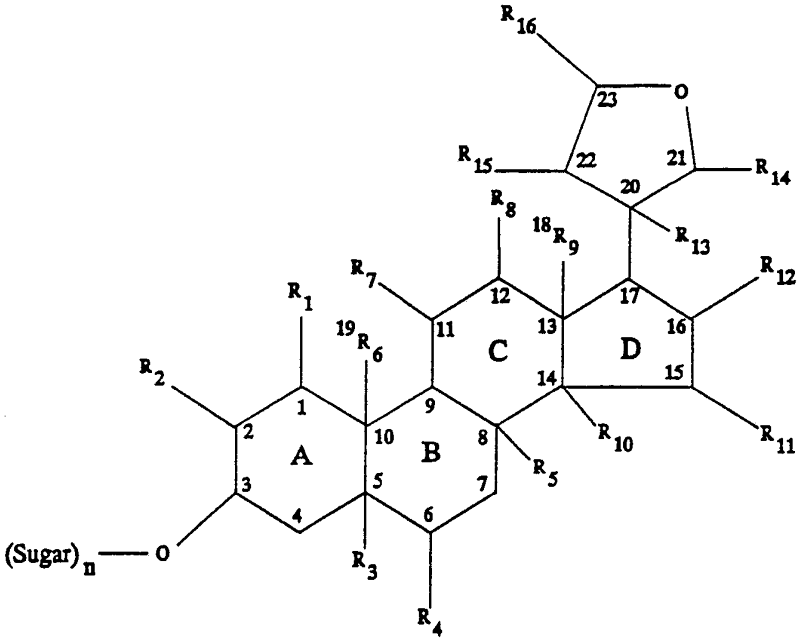
Briefly stated, the present invention provides compounds having the formula:



In one embodiment, a compound has the formula:



In another aspect, derivatives are provided having the formula:

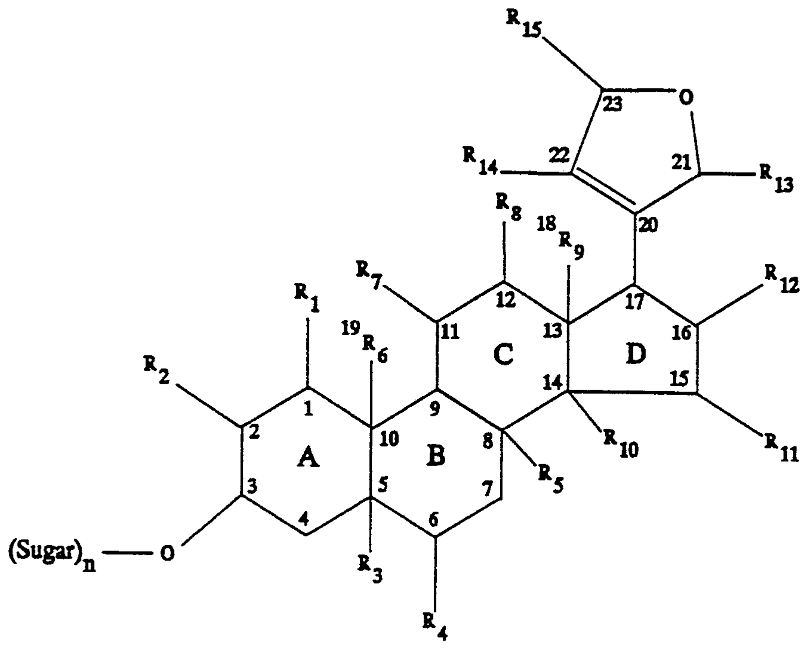


wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is X or O-(Sugar)$_n$; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{15} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{16} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from pentoses, hexoses or combinations thereof; and n is an integer from 1 to 5; with the provisos that when R_1, R_3 and R_7 are H or OH, R_2, R_4, R_5, R_{11}, R_{13}-R_{15} are all H, R_6 is CH_3, CH_2OH or CHO, R_9 is CH_3, R_8 and R_{10} are OH, R_{12} is H or OCOZ, R_{16} is a keto group and rings B, C and D are saturated, and (a) when n=1, the sugar is not digitoxose, acovenose, arabinose, cymarose, oleandrose, rhamnose, thevetose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (b) when n=2, the sugars are not two digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (c) when n=3, the sugars are not three digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, and

(d) when n=4, the sugars are not three digitoxoses and one glucose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups.

In another embodiment, derivatives have the formula depicted immediately above, wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is H; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{15} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{16} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from hexoses wherein at least one of the sugars is a glucose, a mannose, a fructose or a galactose; and n is an integer from 1 to 5.

In another embodiment, derivatives are provided having the formula:



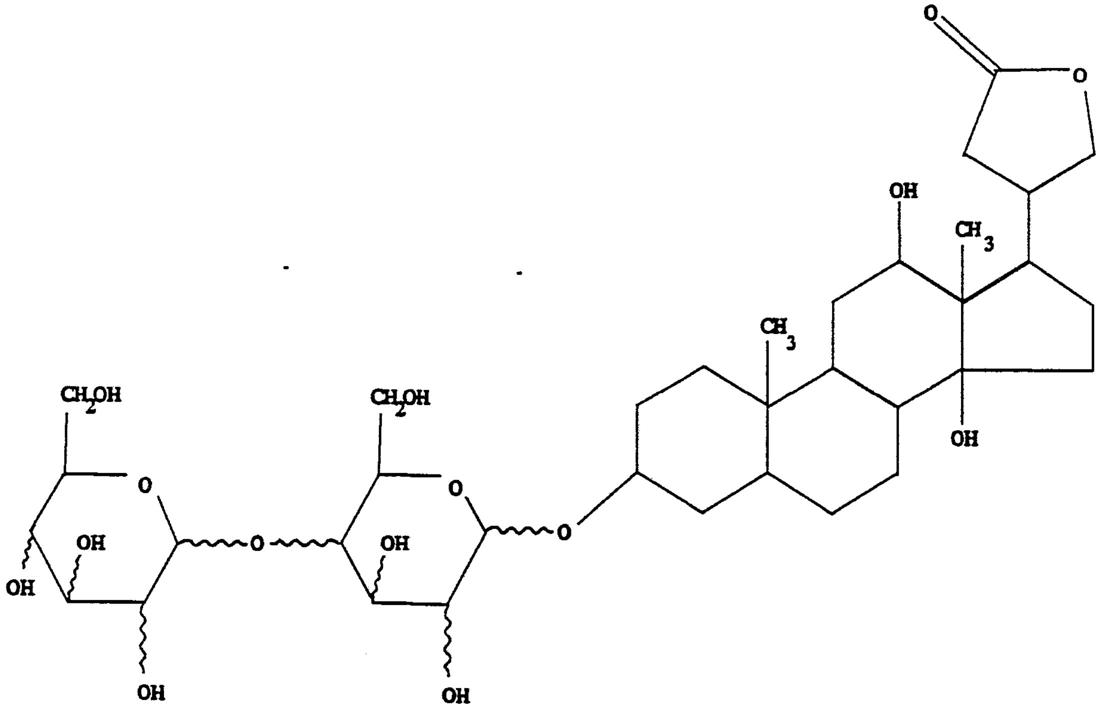
wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is X; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{14} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from

1 to 6 carbon atoms; R_{15} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from pentoses, hexoses or combinations thereof; and n is an integer from 1 to 5; with the provisos that when R_1, R_3 and R_7 are H or OH, R_2, R_4, R_5, R_{11}, R_{13}-R_{14} are all H, R_6 is CH_3, CH_2OH or CHO, R_9 is CH_3, R_8 and R_{10} are OH, R_{12} is H or OCOZ, R_{15} is a keto group and rings B, C and D are saturated, and (a) when n=1, the sugar is not digitoxose, acovenose, arabinose, cymarose, oleandrose, rhamnose, thevetose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, or when R_1, R_3, R_7, R_{12} are all H, R_6 is CH_3, and the other terms are according to the proviso definitions the sugar is not glucose or xylose, (b) when n=2, the sugars are not two digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (c) when n=3, the sugars are not three digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, or when R_1, R_3, R_7, R_{12} are all H, R_6 is CH_3, and the other terms are according to the proviso definitions the sugars are not three digitoxoses wherein the hydroxyl groups of the terminal digitoxose are alkanyoyl or carboxylic acid ester derivatives, and (d) when n=4, the sugars are not three digitoxoses and one glucose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups.

In another embodiment, derivatives have the formula depicted immediately above, wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is H; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{14} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{15} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from glucose, mannose, fructose and galactose; and n is an integer from 2 to 5.

In another embodiment, derivatives also have the formula depicted immediately above, wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is O-(Sugar)$_n$; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{14} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{15} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from hexoses wherein at least one of the sugars attached to C_3 is a glucose, a mannose, a fructose or a galactose; and n is an integer from 2 to 5.

The present invention is also directed toward methods for detecting the presence or amount of a compound having the following structure:



The methods comprise the step of: testing a biological fluid sample for the presence or amount of a compound described immediately above. In one embodiment, the step of testing comprises the steps of: (a) contacting a biological fluid sample with an antibody specific for a compound described immediately above under conditions and for a time sufficient to allow immunocomplexes to form therebetween; and (b) detecting the presence or amount of one or more immunocomplexes formed between the antibody and the compound, thereby determining the presence or amount of the compound.

In another embodiment, the step of testing comprises the steps of: (a) contacting a biological fluid sample with at least two antibodies, the combination of which are specific for a compound described immediately above under conditions and for a time sufficient to allow immunocomplexes to form therebetween; and (b) detecting the presence or amount of one or more immunocomplexes formed between each of the antibodies and the compound, thereby determining the presence or amount of the compound.

In another embodiment, the step of testing comprises the steps of: (a) contacting an aliquot of a biological fluid sample with a Ca^{2+}-ATPase preparation; (b) detecting the presence or amount of specific binding of a substance in the biological fluid sample to the Ca^{2+}-ATPase; (c) contacting another aliquot of the biological fluid sample with a Na^+, K^+-ATPase preparation; (d) detecting the presence or amount of specific binding of a substance in the biological fluid sample to the Na^+, K^+-ATPase; and (e) determining the presence or amount of a compound described immediately above by the presence or amount of specific binding to both the Ca^{2+}-ATPase and the Na^+, K^+-ATPase.

In yet another embodiment, the step of testing comprises the steps of: (a) contacting an aliquot of a biological fluid sample with a Ca^{2+}-ATPase preparation and substrates permitting enzymatic activity by the Ca^{2+}-ATPase; (b) detecting the presence or amount of specific inhibition of the Ca^{2+}-ATPase enzymatic activity; (c) contacting another aliquot of the biological fluid sample with a Na^+, K^+-ATPase preparation and substrates permitting enzymatic activity by the Na^+, K^+-ATPase; (d) detecting the presence or amount of specific inhibition of the Na^+, K^+-ATPase enzymatic activity; and (e) determining the presence or amount of a compound described immediately above by the presence or amount of specific inhibition of both the Ca^{2+}-ATPase and the Na^+, K^+-ATPase enzymatic activities.

Another method provided by the present invention for detecting the presence or amount of a compound described immediately above comprises the steps of: (a) contacting a biological fluid sample with a binding partner specific for the compound under conditions and for a time sufficient to allow complexes to form therebetween; and (b) detecting the presence or amount of one or more complexes formed between the binding partner and the compound, thereby determining the presence or amount of the compound. Additional methods provided for detecting the presence or amount of a compound described immediately above involve specific inhibition of Ca^{2+}-ATPase enzymatic activity or specific binding to the Ca^{2+}-ATPase. In one embodiment, the method comprises the steps of: (a) contacting a biological fluid sample with a Ca^{2+}-ATPase preparation and substrates permitting enzymatic activity by the Ca^{2+}-

ATPase; and (b) detecting the presence or amount of specific inhibition of the Ca^{2+}-ATPase enzymatic activity, thereby determining the presence or amount of the compound. In another embodiment, the method comprises the steps of: (a) contacting a biological fluid sample with a Ca^{2+}-ATPase preparation; and (b) detecting the presence or amount of specific binding of a substrate in the biological fluid sample to the Ca^{2+}-ATPase, thereby determining the presence or amount of the compound.

The present invention also provides methods for detecting the predisposition of a warm-blooded animal to hypertension. The methods comprise detecting the presence or amount of a compound described immediately above in a biological fluid sample of a warm-blooded animal, and therefrom determining the predisposition of the warm-blooded animal to hypertension.

The present invention is also directed toward methods for correcting for interference in the measurement of digoxin levels in patients. In one embodiment, the method comprises detecting the amount of a compound described immediately above in a biological fluid sample of a warm-blooded animal administered digoxin.

Within a related aspect of the present invention, a compound disclosed above may be used as an active therapeutic substance or in a composition by combination with a pharmaceutically acceptable carrier or diluent. These compositions may be used in the manufacture of a medicament for elevating blood pressure, for increasing diuresis, for increasing natriuresis, or for altering cellular calcium levels.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 depicts a synthetic scheme to produce a compound of the present invention and certain derivatives thereof.

Figure 2 depicts an alternative synthetic scheme to produce a compound of the present invention and certain derivatives thereof.

Figure 3 graphically illustrates a dose-response relationship for the inhibition of calmodulin-free Ca^{2+}-ATPase by a compound of the present invention. The percentage of inhibition of calmodulin-free Ca^{2+}-ATPase activity is shown as a function of the compound concentration. The specific activity of the

Ca^{2+}-ATPase in the absence of the compound was 10.5 nmole/min/mg protein. The IC_{50} of the compound was calculated as 20 ng/ml.

Figure 4 graphically illustrates a dose-response relationship for the inhibition of calmodulin-activated Ca^{2+}-ATPase by a compound of the present invention. The percentage of inhibition of calmodulin-activated Ca^{2+}-ATPase activity is shown as a function of the compound concentration. The specific activity of the calmodulin-activated Ca^{2+}-ATPase in the absence of the compound was 59.3 nmole/min/mg protein. The IC_{50} of the compound was approximately 20 ng/ml.

Figure 5 graphically illustrates a dose-response relationship for the inhibition of Na^+, K^+-ATPase by a compound of the present invention. The percentage of inhibition of Na^+,K^+-ATPase activity is shown as a function of the compound concentration. The specific activity of the Na^+, K^+-ATPase in the absence of the compound was 5.8 nmole/min/mg protein. The IC_{50} of the compound was approximately 20 ng/ml.

Figure 6 shows a dose-response curve for inhibition of an isolated Ca^{2+}-ATPase preparation as a function of the concentration of a compound of the present invention. The enzyme was isolated by calmodulin affinity chromatography and stabilized with BHT. Ca^{2+}-ATPase activity was measured by release of inorganic phosphate. The IC_{50} for inhibition of Ca^{2+}-ATPase activity by the compound was calculated to be 51 nM.

Detailed Description of the Invention

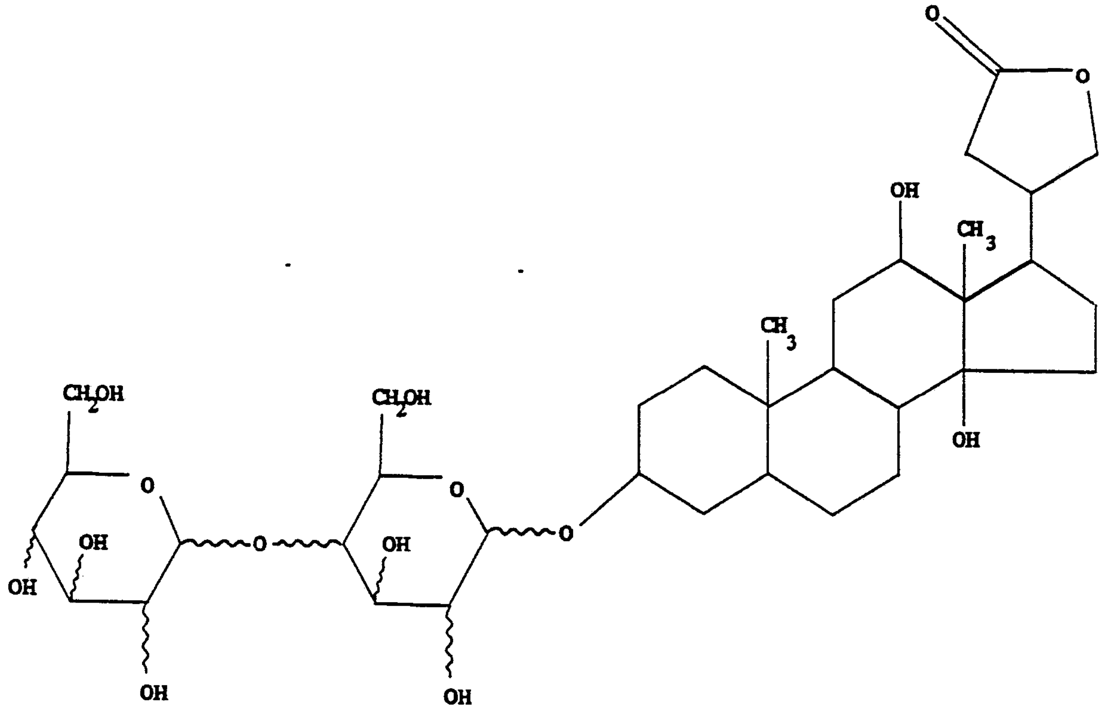
Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Sugar - as used herein, includes naturally occurring monosaccharides and derivatives, such as replacement of one or more hydroxyl groups with acetate groups. The sugar may exist in a pyranose or furanose form and may be linked by α or β, 1-4 or 1-6 ether linkages.

Antibody - as used herein, includes both polyclonal and monoclonal antibodies and may be an intact molecule, a fragment thereof, or a functional equivalent thereof. The antibody may be genetically engineered. Examples of antibody fragments include $F(ab')_2$ Fab', Fab and Fv.

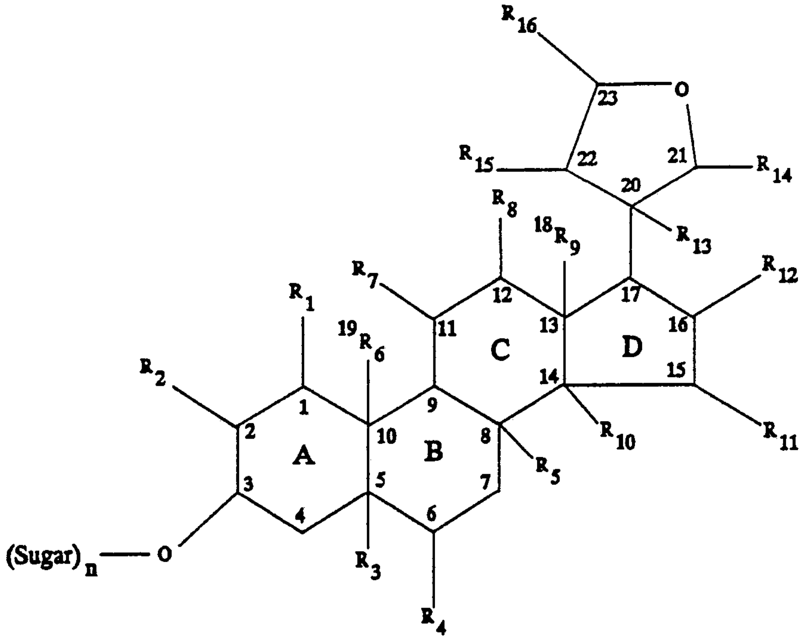
As noted above, a compound has been isolated from humans and purified. Suitable sources of starting material for such purification include a wide variety of biological fluids, e.g., blood, plasma, serum, plasma ultrafiltrate, urine, saliva, sweat and tears. Briefly, a biological fluid sample is subjected to ultrafiltration to isolate the low molecular weight compounds. After adjusting the pH to pH 8.6-8.8 and saturating with salt, the ultrafiltrate is extracted with benzene. The aqueous phase is extracted with ether:acetone (5:7), the extraction repeated two or more times, and the organic layers pooled. The solvent is removed, the residue reconstituted in an aqueous chloroform methanol solution, and fractionated by silicic acid column chromatography. Selected fractions are pooled, solvent removed, HPLC solvent added, applied to a C18 reverse phase column and eluted by gradient. The C18-HPLC step is repeated to yield a compound of the present invention. Given the teachings provided herein, it would be evident to those skilled in the art that modifications of this procedure would be suitable.

As disclosed within the present invention, a purified compound was determined by structural analysis to be a glycoside having a molecular weight of 716, a molecular formula of $C_{35}H_{56}O_{15}$, and the following formula:



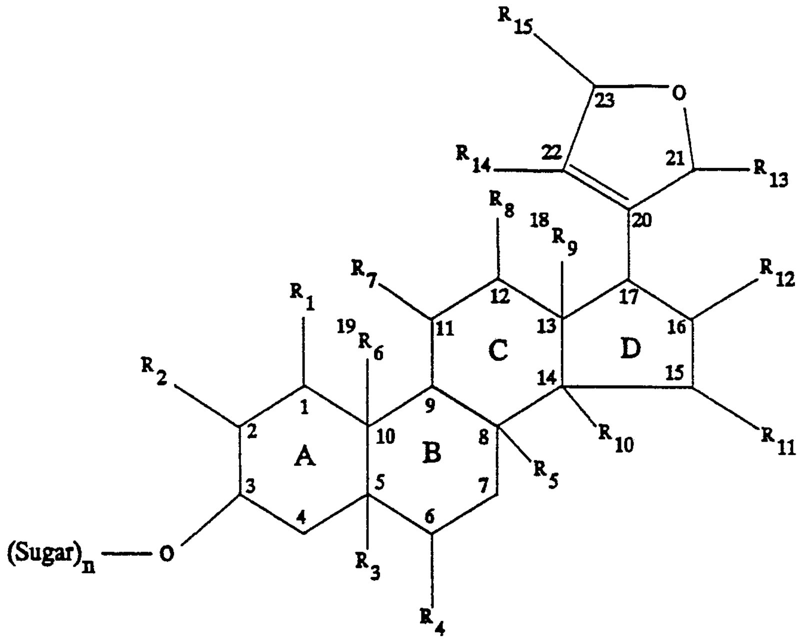
Structural elucidation permits the preparation of synthetic compounds by standard techniques. It will be evident to those skilled in the art that a variety of derivatives may also be prepared. For example, the sugar portion of a compound described immediately above may be replaced or joined with one or more pentoses or other hexoses or combinations of pentoses and hexoses. The sugar residues need not be identical, i.e., each sugar residue may be independently selected from pentoses and hexoses. Suitable hexoses include glucose, mannose, fructose and galactose. Typically, the number of sugar residues will be from 1 to 5. Particularly preferred are two galactoses, two glucoses, or one glucose and one galactose.

In addition to modification of the number or type of sugar residues found in a purified compound, the aglycone portion provides sites for substitution. In one embodiment, derivatives are provided having the formula:



A variety of substituents may be incorporated at the sites for substitution, designated as R_1-R_{16}. Suitable substituents include H, CH_3, CHO, keto, alkyl or haloalkyl from 1 to 6 carbon atoms, O-$(sugar)_n$ where n is typically from 1 to 5, and X and CH_2X where X is typically OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ where Z is typically an alkyl group from 1 to 6 carbon atoms. When an R_{16} substituent is attached to the lactone ring by a carbon-carbon bond, C_{23} may be sp^3 or sp^2 (i.e., a single or double bond between R_{16} and C_{23}). The four rings, identified above as A-D, may be saturated or unsaturated. For example, typical locations of double bonds are C_5-C_6, C_8-C_{14}, C_{14}-C_{15}, and C_{15}-C_{16}. Where any of R_1-R_{16} is attached to a ring carbon not participating in a double bond, there will be two substituents attached to the ring carbon. For example, OH and H may be attached to C_{12}, and CH_3 and H attached to C_{13}.

In another embodiment, derivatives are provided in which the lactone ring is unsaturated at C_{20}-C_{22}. Such derivatives have the formula:



A variety of substituents may be incorporated at the sites for substitution, designated as R_1-R_{15}. Suitable substituents include H, CH_3, CHO, keto, alkyl or haloalkyl from 1 to 6 carbon atoms, O-$(sugar)_n$ where n is typically from 1 to 5, and X and CH_2X where X is typically OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ where Z is typically an alkyl group from 1 to 6 carbon atoms. When an R_{15} substituent is attached to the lactone ring by a carbon-carbon bond, C_{23} may be sp^3 or sp^2 (i.e., a single or double bond between R_{15} and C_{23}). The four rings, identified above as A-D, may be saturated or unsaturated. For example, typical locations of double bonds are C_5-C_6, C_8-C_{14}, C_{14}-C_{15}, and C_{15}-C_{16}. Where any of R_1-R_{15} is attached to a ring carbon not participating in a double bond, there will be two substituents attached to the ring carbon. For example, OH and H may be attached to C_{12}, and CH_3 and H attached to C_{13}.

As noted above, compounds of the present invention may be used for numerous diagnostic and therapeutic purposes. Representative diagnostic uses include screening, or monitoring, for essential hypertension and correcting for interference by the compound in the measurement of digoxin levels. As described above, essential hypertension is a widespread problem and there exists a need for improved methods for the identification of hypertensive individuals. The present invention discloses that a compound is associated with essential hypertension and thus individuals may be screened for hypertension by testing for the presence or amount of this compound. In addition, hypertensive or pre-hypertensive individuals, receiving or not receiving dietary or pharmacological therapies, may be monitored by testing for this compound.

Digoxin is widely used in the treatment of cardiac irregularities. The narrow therapeutic index for digoxin necessitates an accurate and reliable method for the measurement of serum digoxin concentration. The disclosure of the present invention shows that a purified compound of the present invention binds anti-digoxin antibodies and therefore interferes with the measurement of digoxin concentrations in the serum. A compound of the present invention may be used to screen for anti-digoxin antibodies possessing minimal or no cross-reactivity with the compound. For example, a compound may be attached to a solid support and anti-digoxin sera contacted with the derivatized support to remove antibodies showing cross-reactivity. Alternatively, detection of the amount of a compound in a biological fluid, such as serum, permits the correction for its interference with the measurement of digoxin concentrations.

Compounds of the present invention may be detected in a variety of ways, including chemical, immunological and receptor-based assays. Such assays may involve the use of binding partners such as antibodies or receptors. Detection of the compound may be qualitative or quantitative, and a biological fluid sample may be tested *in vitro* or *in vivo*. Suitable biological fluid samples from a warm-blooded animal, such as a human, include those described above. In one embodiment, a biological fluid sample is tested for the presence of a compound through the use of an antibody specific for the compound (i.e., with a binding affinity of about 10^7 liters/mol or higher) or the use of two or more antibodies, the combination of which are specific for the compound. The antibodies may be polyclonal or monoclonal antibodies. Briefly, polyclonal antibodies may be produced by immunization of an animal and subsequent collection of its sera. Immunization is accomplished, for example, by a systemic administration, such as by subcutaneous, intrasplenic or intramuscular injection, into a rabbit, rat or mouse. It is generally preferred to follow the initial immunization with one or more booster immunizations prior to sera collection. Such methodology is well known and described in a number of references.

While polyclonal antibodies which are specific for a compound described above may be employed in the present invention, monoclonal antibodies (MAbs) are preferred. MAbs may be generally produced by the method of Kohler and Milstein (Nature 256:495-497, 1975; Eur. J. Immunol.

6:511-519, 1976). Briefly, cells of lymph nodes and/or spleens of an animal immunized with a compound described above are fused with myeloma cells to form hybrid cell lines ("hybridomas" or "clones"). Each hybridoma secretes a single type of immunoglobulin specific for the compound, and, like the myeloma cells, has the potential for indefinite cell division. Suitable MAbs include those of murine or human origin, or chimeric antibodies such as those which combine portions of both human and murine antibodies (i.e., antigen binding region of murine antibody plus constant regions of human antibody). Human and chimeric antibodies may be produced using methods well known by those skilled in the art. Human antibodies and chimeric human-mouse antibodies are advantageous for *in vivo* uses because they are less likely than murine antibodies to cause the production of anti-antibodies when administered clinically. An alternative to the production of MAbs via hybridomas is the creation of MAb expression libraries using bacteriophage and bacteria (e.g., Sastry et al., Proc. Natl. Acad. Sci. USA 86:5728-5732, 1989; Huse et al., Science 246:1275-1281, 1989).

Compounds of the present invention are preferably conjugated before administration as an antigen in order to enhance elicitation of an antibody response in the host animal and/or direct antibody production to particular epitopes. When the desired epitope for antibody binding is on the sugar portion of a compound (as opposed to the aglycone portion) the lactone ring on the compound may be first opened, e.g., by reductive ozonolysis. This modified compound with an open lactone ring is coupled to a methylated or alkylated carrier molecule, such as methylated bovine serum albumin (BSA). The methodology for the preparation of carrier-glycoside conjugates has been extensively described, e.g., U.S. Patent No. 4,767,720 to Lingwood (this patent and those disclosed below are herein incorporated by reference). Thus, a conjugate is formed in which a carrier is covalently bound to a compound through the opened lactone ring. Such a conjugate leaves the sugar portion of a compound the most exposed to elicit an antibody response. The antibodies generated will have minimal cross reactivity with digoxin because the sugar groups of the compounds described above are different from the sugar groups of digoxin.

Alternatively, a carrier can be bound to a compound by a sugar group to leave the aglycone portion, and particularly the ketone ring end, exposed as an epitope to elicit an antibody response. The conjugation may be accomplished by oxidizing a sugar group (e.g., U.S. Patent No. 4,671,958 to Rodwell et al.) of a compound to generate an active functional group. The activated sugar moiety is then coupled to a carrier, such as methylated BSA. Other suitable carriers include thyroglobulin.

Detection of the presence or amount of immunocomplexes formed between a compound described above and antibodies specific for the compound may be accomplished by a variety of known techniques, such as radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). The detection may be qualitative or quantitative, i.e., the number of immunocomplexes is measured. The level of compound in a sample may be determined from the number of immunocomplexes measured by comparison to values obtained for known concentrations of the compound.

Suitable immunoassays include the double monoclonal antibody sandwich immunoassay technique of David et al. (U.S. Patent 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide et al., in Kirkham and Hunter, eds., Radioimmunoassay Methods, E. and S. Livingstone, Edinburgh, 1970); the "western blot" method of Gordon et al. (U.S. Patent 4,452,901); immunoprecipitation of labeled ligand (Brown et al., J. Biol. Chem. 255:4980-4983, 1980); enzyme-linked immunosorbent assays as described by, for example, Raines and Ross (J. Biol. Chem. 257:5154-5160, 1982); immunocytochemical techniques, including the use of fluorochromes (Brooks et al., Clin. Exp. Immunol. 39: 477, 1980); and neutralization of activity [Bowen-Pope et al., Proc. Natl. Acad. Sci. USA 81:2396-2400 (1984)], all of which are hereby incorporated by reference. In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos.: 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

For detection purposes, the antibodies may either be labeled or unlabeled. When unlabeled, the antibodies find use in agglutination assays. In addition, unlabeled antibodies can be used in combination with labeled molecules that are reactive with immunocomplexes, or in combination with labeled antibodies (second antibodies) that are reactive with the antibody directed against the compound, such as antibodies specific for immunoglobulin. Alternatively, the antibodies can be directly labeled. Where they are labeled, the reporter group can include radioisotopes, fluorophores, enzymes, luminescers, or dye particles. These and other labels are well known in the art and are described, for example, in the following U.S. patents: 3,766,162; 3,791,932; 3,817,837; 3,996,345; and 4,233,402.

In one preferred embodiment for detecting immunocomplexes, a reporter group is bound to the antibody. The step of detecting immunocomplexes involves removing substantially any unbound antibody and then detecting the presence or amount of the reporter group. Unbound antibody is antibody which has not bound to the compound.

In another preferred embodiment, a reporter group is bound to a second antibody capable of binding to the antibodies specific for the compound. The step of detecting immunocomplexes involves (a) removing substantially any unbound antibody (i.e., antibody not bound to the compound), (b) adding the second antibody, (c) removing substantially any unbound second antibody and then (d) detecting the presence or amount of the reporter group. Where the antibody specific for the compound is derived from a mouse, the second antibody is an anti-murine antibody.

In a third preferred embodiment for detecting immunocomplexes, a reporter group is bound to a molecule capable of binding to the immunocomplexes. The step of detecting involves (a) adding the molecule, (b) removing substantially any unbound molecule, and then (c) detecting the presence or amount of the reporter group. An example of a molecule capable of binding to the immunocomplexes is protein A.

An alternative to the use of labeled antibodies, labeled second antibodies or labeled molecules reactive with immunocomplexes generally, is an immunoassay employing a labeled compound. In such an assay, a compound present in a sample will compete with labeled compound for the antibodies. Examples of compound labels include fluorescent labels, radioisotopes, and chemiluminescent labels. Chemiluminescent labels include lucigenin, acridinium derivatives (e.g., 10,10'-dimethyl-9,9'-biacridinium dinitrate), luminol, isoluminol and pyrogallol. A chemiluminescent form of a compound may be used in a luminescent immunoassay, a luminescent enzyme immunoassay, a luminescent cofactor immunoassay and a luminescent enzyme-multiplied immunoassay technique.

In a luminescent immunoassay (LIA) technique, a luminescent label essentially replaces the radioisotopic label of the typical RIA-type assay. A compound having been labeled with luminol, for example, competes with the compound in a sample for a limited amount of antibody specific for the compound. By attaching the antibody to a solid support such as plastic or agarose, antibody-bound luminol can be detected by the addition of hydrogen peroxide and a catalyst. Other oxidation systems can be used, including hydrogen peroxide-microperoxidase, hydrogen peroxide-hematin, hydrogen peroxide-lactoperoxidase, and persulfate.

In a luminescent enzyme immunoassay (LEIA), a compound may be labeled with an enzyme that can either catalyze a luminescent reaction (e.g., peroxidase or luciferase) or produce a substrate for a luminescent reaction. The second approach can increase assay sensitivity and has been called an amplified bioluminescent immunoassay.

A luminescent cofactor immunoassay (LCIA) uses a compound in the form of a conjugate with a cofactor. The cofactor is typically ATP. The amount of antibody bound cofactor can be assayed by using either the bacterial or firefly luciferase reactions. It is important that unbound compound-cofactor be removed so that only compound-cofactor bound to antibody is measured.

It will be evident to those skilled in the art that a variety of methods for detecting immunocomplexes may be employed within the present invention. Reporter groups suitable for use in any of the methods include radioisotopes, fluorosphores, enzymes, luminescers, and dye particles.

In another embodiment for detecting a compound of the present invention, receptor-based assays may be used. As disclosed within the present invention, certain compounds affect the enzyme activity of the Ca^{2+}-ATPase (calcium pump) and the Na^{+},K^{+}-ATPase (sodium pump). In particular, the compounds show specific inhibition of the Ca^{2+}-ATPase (with and without calmodulin activation) and Na^{+},K^{+}-ATPase, in intact cells, isolated plasma membranes and purified preparations of either enzyme. Thus, a biological fluid sample may be tested for the presence or amount of a compound described above by detecting the presence or amount of specific inhibition (i.e., an IC_{50} of about 10^{-7} M or more potent) or specific binding (i.e., about 10^{7} liters/mol or higher) to the Ca^{2+}-ATPase, or Ca^{2+}-ATPase and Na^{+},K^{+}-ATPase.

Inhibition of the Ca^{2+}-ATPase or Na^{+},K^{+}-ATPase by a sample containing a compound described above may be measured in a variety of assay formats. For example, the sample may be contacted with an active enzyme present in intact cells, isolated membranes, or purified form. Purified enzymes may be derived from tissue or genetically engineered. Activities of either enzyme may be measured in a number of ways, e.g., by the amount of inorganic phosphate liberated from ATP. The basal activity of the Ca^{2+}-ATPase can be used or, for increased sensitivity, the enzyme can be activated by the addition of calmodulin at an appropriate concentration, such as about 30 nM. For any of the assay formats, as the concentration of a compound of the present invention increases, the activities of the Ca^{2+}-ATPase and Na^{+},K^{+}-ATPase will decrease.

Binding of a compound to the Ca^{2+}-ATPase or Na^+,K^+-ATPase may also be measured in a variety of assay formats. As discussed above, suitable preparations of either enzyme include intact cells, isolated membranes and purified forms of the enzymes. Furthermore, it is intended that reference herein to either enzyme also includes a portion of each enzyme which is capable of binding a compound described above. Such portions (i.e., peptides or polypeptides) may be isolated from native enzyme or constructed by a variety of known techniques. A representative method for detecting the presence or amount of a compound in a sample is to measure the binding of a known quantity of the compound (which has been labeled with a reporter group) to either enzyme in the presence of a sample containing the compound. As the concentration of the compound in the sample increases, the amount of binding of the compound containing reporter group decreases which is reflected by a decrease in the amount of reporter group associated with the enzyme preparation. Suitable reporter groups include those described above, such as chemiluminescent labels.

As noted above, the compounds of the present invention may also be used for numerous therapeutic purposes. Representative therapeutic uses include as a pressor agent, a potassium sparing diuretic, and an agent that alters cellular calcium levels (e.g., as a cardiotonic agent). A compound is generally combined with a pharmaceutically acceptable carrier or diluent. Pharmaceutically acceptable carriers and diluents include water, physiological saline, alcohols, dimethyl sulfoxide (DMSO) and mixtures thereof. The composition may be administered by a variety of routes, including oral, parenteral and transdermal administration. For oral administration, the composition may be in pill, capsule or liquid form. For administration by injection, physiological saline is a preferred diluent. For transdermal administration, DMSO is a preferred carrier.

As disclosed within the present invention, the compounds described above have pharmacological effects which lead to vasoconstriction and thus are useful as pressor agents, i.e., as a substance for increasing blood pressure. Although vasoconstriction and the resulting higher blood pressure are typically undesirable, there are situations, such as shock, where vasoconstriction and higher blood pressure are desirable. A compound may be administered to treat shock and associated low blood pressure by causing vasoconstriction and elevation of blood pressure. Dangerously low blood pressure is an emergency condition frequently encountered in hospital and other settings.

The present invention also discloses that the compounds described above can function as a potassium sparing diuretic. Loss of potassium is a significant problem for patients taking conventional diuretics. Administration of a compound of the present invention increases the urinary excretion of water (diuresis) and sodium (natriuresis), without causing an increased loss of potassium.

As disclosed within the present invention, additional therapeutic uses of the compounds described above include the treatment of imbalances in calcium and hydrogen. Calcium imbalances have been associated with heart disorders, mood disorders and various bone diseases, such as osteoporosis. As described above, Ca^{2+}-ATPase activity may be inhibited and thus by altering cellular calcium levels is capable of treating a wide variety of disorders are capable of being treated.

In addition, warm-blooded animals possessing an excessive endogenous level of the compound may be treated by the administration of an antagonist to a compound described above. Suitable antagonists are those which block the effects of an endogenous compound and include agents which block the ATPase receptors for the compound as well as agents which bind to the compound itself. The ATPase receptors for the compound may be blocked using agents (e.g., derivatives of the compound) that bind to the receptors, but are incapable of significantly stimulating or inhibiting the activity of the receptor. Alternatively, the compound may be directly blocked by an agent which binds to the compound. Such agents include antibodies to the compound.

To summarize the examples which follow, Example 1 provides the purification and chemical characterization of a compound of the present invention; Example 2 provides the chemical synthesis and radiolabeling of a compound; Example 3 discloses the inhibition of Ca^{2+}-ATPase and Na^+, K^+-ATPase; Example 4 describes vasoconstrictive and natriuretic properties; and Example 5 provides assays for antagonists.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

PURIFICATION AND CHEMICAL CHARACTERIZATION OF A COMPOUND

A. Purification

1. From tissues:

Fresh tissue, e.g., kidney, removed in surgery or at autopsy, or cultured tissues, may be processed immediately or stored at -70°C. Tissue is thawed, minced, and mixed with 2 equal volumes of physiological saline and 0.001% BHT. It is then homogenized in the cold with short bursts of rotary curring blades. Sediment is removed by centrifugation. The supernate is subjected to the purification steps described below. Tissue culture media are treated like plasma samples.

2. From blood or urine:

Fresh whole blood or urine is filtered through an ultrafilter to separate the low molecular weight compounds. Alternatively, the plasma or serum can be separated from the blood prior to ultrafiltration. In samples for a screening clinical assay, the ultrafiltration step can be omitted. Ultrafilters with cutoffs from 50,000 to 2,000 molecular weight (2,000 cutoff is preferred) can be used, e.g., YM-2 diaflo membranes (Amicon Corp.). Supelco syringe filter tips and filters for renal patient hemodialysis (cuprophane) are also appropriate.

When the compound is being prepared in bulk, urease is added to the blood ultrafiltrate or to urine to eliminate urea. The specific activity of the enzyme and the urea content of the filtrate are taken into account so that this step, which takes place at about 25°C, is of short duration. This step can be omitted, but separation is then less reproducible. As the pH rises with NH_3 production, it is titrated to pH 7.0 by the addition of 1 M HCl. This step is desirable when the urea content exceeds 15 mg/dL. Urease treatment may be omitted in the clinical serum screening assay.

For bulk preparations using a fluid matrix with low compound content, the volume is reduced by lyophylizing the sample, e.g., an ultrafiltrate of blood. The dried residue is then reconstituted in H_2O so that the final volume in mL is three times the weight of residue in grams, including the volume of 50% NH_4OH used to titrate to pH 8.7. Dry NaCl is added to saturate the solution, which facilitates extraction. An antioxidant such as 0.01%-0.001% BHT should also be added.

For a clinical urine or serum assay, the pH is adjusted to 8.6 with 1 to 3 M ammonium acetate. If tris or other buffers are used, ammonium salts or ammonium hydroxide should be added. The dilution should not exceed 3-fold for optimum recovery. Ammonium ions are needed for maximum extraction. The pH can vary from 7.2 to 9.2, but maximum recovery is obtained at 8.6 to 8.8.

Solvent extraction of bulk preparations is accomplished in two steps. In the first, non-polar lipids are removed with benzene in a volume equaling the sample volume. The organic layer is discarded. The second extraction is to isolate the compound. The purest preparations are accomplished with ether:acetone (1:1), used in equal volume with the sample. This is a time-dependent step; recoveries increase with longer solvent exposure. One to twenty hours mixing before separation is satisfactory, with one hour adequate for the extraction for clinical screening serum tests. Ether:acetone (5:7) may be used for bulk work. Other solvent systems will extract the compound and different proportions of contaminants. The extraction is repeated two to three times, and the organic layers pooled. Centrifugation is needed to separate layers. Unless an antioxidant is used, the solvents should be evaporated under N_2 as rapidly as possible. Lyophylization is needed to dry the sample. From this step forward, exposure to light is restricted.

For a clinical screening assay, the residue of the organic phase is reconstituted in physiological saline or H_2O to the original sample volume, or less if required by the sensitivity limits of the immunoassay or receptor assay in use. Resolubilization requires at least 30 minutes and adequate vortexing. For a clinical serum or urine quantitative assay, the organic residue is reconstituted in HPLC solvent, e.g., methanol:acetonitrile:water (15:15:70).

For bulk preparation, an additional extraction step is added. The residue is dissolved in a minimal amount of chloroform:methanol:H_2O (60:35:5). The insoluble residue is separated by centrifugation and discarded after washing. The solvent is removed under N_2 and lyophylization as necessary. The residue is then dissolved in silicic acid chromatography solvent.

The sample is then subject to silicic acid column chromatography (e.g., with Biosil A, 100-200 mesh, Bio-Rad Laboratories, Inc., Richmond, Cal.). A column (2 x 27 cm) is prepared in chloroform:methanol:H_2O (60:35:2) at about 25°C. The sample is applied in this solvent and eluted with it or a gradient to a chloroform:methanol:H_2O mixture of 60:35:3. The compound elutes before most of the contaminating lipids and near the void volume of the column. Fraction content is conveniently monitored by thin layer chromatography (TLC), enzyme inhibition or immunoassay. As in all extraction steps, this solvent is removed under N_2 and/or vacuum, in the dark, and at temperatures less than 50°C. The residue, which may be oily in appearance, should not be dried excessively in the lyophylizer.

High pressure liquid chromatography is used to isolate the compound. HPLC grade solvents were used through the protocol. Water was deionized. When reducing solvent volume after HPLC purification, it is important to avoid lyophylizing the sample to dryness, as a loss may be encountered at this stage. Affinity chromatography with anti-digoxin antibody or Na^+,K^+-ATPase may be used to recover the compound after solvent (e.g., salt, alcohol, and acetonitrile) removal under nitrogen as an alternate to the lyophylization step. A C18 reverse phase column (7.8 mm x 30 cm semi-preparation, Waters, Milford, Mass.), coupled to a spectrophotometric detector set at 223 nm, is used. The column is thoroughly washed with ethanol then preconditioned with a preparation prepared from the pooled lipids which are eluted from the bulk extract under these chromatographic conditions and having retentions >16 minutes. Isolation of the compound on HPLC is a two-step process using a gradient elution system.

From zero to six minutes, the solvent mix is 90% solvent A (H_2O, MeOH, IsopropylOH; 70:15:15) and 10% solvent B (MeOH). At six minutes a gradient is initiated whereby solvent A is slowly decreased and solvent B is slowly increased until at the time of sixteen minutes, the ratio of solvent is 10% A and 90% B. This solvent mix is continued until the run termination time of thirty minutes. The flow rate throughout the run is 2 ml/min. Fractions are collected every thirty seconds from fifteen to twenty minutes of the run time. The fractions are analyzed by radioimmunoassay for digoxin. The compound appears most frequently in fractions 17-18 minutes of run time.

Once all crude material has been processed in this manner, all fractions indicating the presence of the compound are then pooled into one sample and concentrated by a stream of nitrogen at room temperature until the pool is of manageable volume. This material is then processed again on HPLC as just described. However, the fractions are manually collected at the proper run time in order to ensure the least contamination from neighboring peaks. Fractions are assayed, pooled and concentrated as described above.

A preferred isolation procedure for use in connection with immunoassays is as follows. At room temperature, 0.3 mL serum is added to each test tube (15 mL polypropylene conical centrifuge with screw caps). Ammonium acetate buffer (0.3 ml of 1.0 M at pH 8.6) and benzene (3.0 ml) are added into each test tube and vortexed for 30 seconds. The test tubes are then centrifuged for 10 minutes at room temperature, at about 1,500 x g.

The benzene is removed with a pipet, discarded, and the remaining organic layer and approximately 50% of the aqueous layer evaporated using a N_2 stream in 44°C water bath. Ether:acetone (6 ml) is added to the test tubes, which are then vortexed gently. The test tubes are placed on a rocker for 1.5 hours in the dark. The ether:acetone solvent is removed with pipette and saved in the dark at 4°C. Each test tube is rinsed with 0.5 mL ether:acetone mixture, vortexed, and the solvent removed with a pipette and added to the solvent stored in the dark. The combined solvent is evaporated to dryness with N_2 at 44°C. The residue is reconstituted in 0.3 mL physiological saline or H_2O. The tube is vortexed well, allowing at least a 30 minute (preferably a 120 minute) solvation time. Alternately, the residue can be dissolved in minimal volumes of ethanol:methanol (1:1 by volume) which is then diluted to $<1\%$ with buffer or saline.

B. Chemical Characterization

The structure of the purified compound was determined by fast atom bombardment (FAB) mass spectral studies. FAB mass spectra were recorded on VG-70 SEQ at 8 Kv acceleration voltage.

The FAB mass spectral data indicated the presence of a molecular ion at m/z (mass to charge ratio) of 716 (717 M+H, glycerol-HCl matrix). Several peaks in the FAB mass spectra indicated the presence of a cardiac glycoside-like compound, with a digoxigenin-type nucleus (aglycone). The characteristic peaks were obtained at m/z 393, 391, 375, 373, 357, 355, 339, and 337 (see, e.g., Greenwood et al., "Mass Spectrometry in Drug Metabolism," Frigerio and Ghisalberti, eds., pp. 174-189, Plenum Press, NY, 1976). The peaks at m/z 393, 391, 375, 357, and 339 indicated the presence of digoxigenin-type nucleus with a saturated lactone ring. The removal of a hydroxyl group from carbon-3 of the genin resulted in the peak observed at m/z 375. Further loss of a water molecule from this fragment resulted in a peak occurring at m/z 357.

The peaks occurring at m/z 163 and 325 indicated the presence of two hexose sugar units. The peak at m/z 163 is probably due to the loss of one hydroxyl group from the terminal sugar group. The peak at m/z 325 was attributed to the loss of one hydroxyl group from the two sugar units (see, e.g., Isobe et al., Biomedical & Environmental Mass Spectrometry 13:585-94, 1986).

The identity of the molecular ion occurring at m/z 716 was further established by running the FAB mass spectra of the sodium and potassium derivatives of the purified compound. The sodium adduct of the compound indicated the presence of a distinct peak at m/z 739 (M+Na). While the potassium adduct resulted in an intense peak at m/z 755 (M+K). Furthermore, in order to establish the presence of a digoxigenin-type nucleus in the purified compound, FAB mass spectra of standard digoxigenin, dihydrodigoxigenin, digoxin, and dihydrodigoxin were obtained and compared with the FAB mass spectra of the purified compound. As a result of the FAB mass spectral studies of the molecular ion and the fragment ions of the purified compound, the molecular formula was deduced as: $C_{35}H_{56}O_{15}$.

EXAMPLE 2

SYNTHESIS AND RADIOLABELING

A. Synthetic Protocol

The reaction pathway is shown in Figure 1. 5*β*, 20(22)-Cardenolide-3*β*, 12*β*, 14-triol (78 mg), A, was dissolved in benzene (28 ml) with heating. $Hg(CN)_2$ (50 mg) was added along with $HgBr_2$ (50 mg) and Bromohepta-O-acetyl-4-O-α-D-Glucopyranosyl-D-glucose (45 mg), B, with constant stirring. The solution was boiled for two hours. After two hours of boiling, $Hg(CN)_2$ (50 mg) was added with Bromohepta-O-acetyl-4-O-α-D-Glucopyranosyl-D-glucose (45 mg). The reaction mixture was boiled with constant stirring for 45 minutes and $Hg(CN)_2$ (50 mg) was added along with $HgBr_2$ (25 mg) and Bromohepta-O-acetyl-4-O-α-D-Glucopyranosyl-D-glucose (45 mg). The solution was boiled for 20 hours.

After 20 hours of boiling, the reaction mixture was diluted with chloroform and washed several times with a saturated solution of NaCl. The chloroform-soluble fraction was dried with sodium sulfate (anhydrous), filtered and purified by preparative thin-layer chromatography (TLC) using chloroform : methanol (9.4 : 0.6) as the solvent system. This resulted in compound C (50 mg). The compound C thus obtained was reduced under hydrogen gas with Pd/C (10%) (4 hours). This resulted in the formation of compound D. The hydroxyl groups of the sugar units in compound D are acetylated and can be hydrolyzed to hydroxyl groups by a variety of methods. The most common method for deacetylation involves the use of NaOMe.

B. Alternate Synthetic Protocol

The reaction pathway is shown in Figure 2. Fetizon's reagent was prepared as follows. Celite (2 g) was added to a stirred solution of silver nitrate (2.26 g) in distilled water (13.5 ml). A solution of potassium bicarbonate (1.4 g) in distilled water (20 ml) was added slowly to the resulting homogenous suspension. After the addition, the stirring was continued for 15 minutes at room temperature. The yellow green precipitate thus formed was filtered and washed several times with diethyl ether. The resulting precipitates were dried in a light-shielded desiccator due to the reagent's sensitivity to light and air.

5β, 20(22)-Cardenolide-3β, 12β, 14-triol (50 mg), A, was dissolved in a mixture of dichloromethane and dioxane 9 : 1, 15 ml) with slight heating. Freshly prepared Fetizon's reagent (1.25 g) was added with care, the solution was stirred at room temperature and $Hg(CN)_2$ (65 mg) was added along with $HgBr_2$ (65 mg) and Bromohepta-O-acetyl-4-O-α-D-Glucopyranosyl-D-glucose (160 mg), B. The reaction mixture was stirred at room temperature for 3 hours, filtered through celite and diluted with chloroform (50 ml). The solution was washed several times with a saturated solution of sodium chloride. The organic phase was dried with sodium sulfate (anhydrous) and subjected to open column chromatography using silica gel (6.5 g) with the following eluent system:

a) chloroform 20 ml
b) chloroform : ethylacetate (8 : 2) 100 ml
c) chloroform : ethylacetate (7 : 3) 50 ml
d) chloroform : ethylacetate (6 : 4) 100 ml
e) chloroform : ethylacetate (2.5 : 7.5) 80 ml
f) ethylacetate 50 ml

Ninety six fractions were collected. Compound C was eluted in fractions 43-58 and was further purified (21 mg) by preparative thin layer chromatography (TLC) using 5% methanol in chloroform. The compound C thus obtained was reduced, as described in section A above, to yield compound D. The hydroxyl groups of the sugar units in compound D are acetylated and can be hydrolyzed to hydroxyl groups by a variety of methods. The most common method for deacetylation involves the use of NaOMe.

C. Radiolabeling Protocol

Synthetic glycoside, after deacetylation, was dissolved in methanol and reduced with tritium gas using 5% Pd/C. The reaction was run for three hours and left overnight under hydrogen to reduce any unreacted compound remaining. The reaction mixture was then filtered through celite and purified by open column chromatography using silica gel (60-200 mesh) and 2.5% methanol in chloroform. Thin layer chromatography was performed using 10% methanol in chloroform. Sulfuric acid (5%) in ethanol was used as a spray reagent. Radioactivity was measured using Packard liquid scintillation analyzer model 2500TR.

EXAMPLE 3

Inhibition of Ca-ATPase and Na,K-ATPase Activities

A compound of the present invention was isolated as described in Example 1. The compound was quantified by the Rianen ^{125}I-digoxin radioimmunoassay kit (New England Nuclear) with the modification of an addition of 1.8 mg/ml of bovine globulin fraction to facilitate precipitation. The assay was linear from 0.1 ng/ml to 8 ng/ml digoxin equivalents while the imprecision was 0.28 ± 0.04 ng digoxin equivalents/ml (X ± SD).

The ATPases assayed were the Ca^{2+}-ATPase and Na^+,K^+-ATPase. The ATPase assays were carried out in a total volume of 0.1 ml in flat bottom 96 well plates. Purified compound in 85% aqueous methanol was evaporated in the test wells under a stream of ultra-pure N_2 at room temperature. ATP (3 mM) was added at the start of the reaction. The reaction was carried out for one hour at 37°C. The reaction was terminated by the addition of sodium dodecyl sulfate (SDS) (0.83%).

Inorganic phosphate was determined colorimetrically in the plates. Ca^{2+}-ATPase was measured in the basal and calmodulin activated states. Basal calcium ATPase activity was defined in the presence of ouabain and with added calcium (0.2 mM added and 0.1 mM in excess of EGTA). Calmodulin-activated Ca^{2+}-ATPase activity was defined by the addition of calmodulin (30 nM).

Purified Ca^{2+}-ATPase preparations are prepared by standard methodology. For example, plasma membranes from human red blood cells are isolated and solubilized with Triton X-100. The enzyme is separated from insoluble proteins by centrifugation. The resulting supernate is made 0.1% with azolecithin to stabilize the enzyme. The solution is passed over a calmodulin column in the presence of calcium to bind the enzyme. To remove extraneous proteins, the column is washed while the enzyme is still bound. The enzyme is released from the column by the addition of EDTA.

The results of the assays are presented in Figures 3-6. These data show a dose-response relationship for a purified compound inhibiting the Ca^{2+}-ATPase (both basal and calmodulin-activated) and Na^+,K^+-ATPase. The compound is a potent inhibitor of both enzymes with an IC_{50} of about 10^{-7}-10^{-8} M (expressed as digoxin equivalents).

EXAMPLE 4

VASOCONSTRICTIVE AND NATRIURETIC PROPERTIES

Female sheep weighing approximately 50 Kg are used for the experiments. An arteriovenous cannula is surgically implanted in the carotid artery and jugular vein in the neck. At least 1 week is allowed to pass before beginning the experiments. For each experiment, a standing sheep is placed in a gentle neck restraint in a cage. The arteriovenous cannula is clamped and separated in the middle. A Swan-Ganz cardiac catheter is passed into the pulmonary artery through the venous cannula. After verifying proper positioning, the catheter is left *in situ* to measure cardiac output by a thermal dilution method. The mean arterial pressure is measured through the carotid artery cannula. An infusion line is connected to the venous cannula and 72.5 mmol/L of NaCl is delivered at a constant rate with an infusion pump to replace normal losses. An indwelling catheter is passed into the urinary bladder. The bladder is emptied completely and base line collections initialized.

Urine, blood and hemodynamic data (i.e., cardiac output and mean arterial pressure) are collected every 15 minutes for at least 1 hour to establish control conditions. After 1 hour of base line study, a compound is injected into the jugular vein. Urine, blood and hemodynamic data are collected at 5 minutes following the dose of the compound, and then at every 15 minutes thereafter for a period of 2 hours. The systemic vascular resistance is calculated from the cardiac output and the mean arterial pressure values. Glomerular filtration rate (GFR) is calculated as creatinine clearance and para-aminohippurate (PAH) clearance is used to measure effective renal plasma flow. Each sheep has at least a one week resting period between experiments. Typically, a total of six studies are conducted on two different sheep. The dose of the compound generally ranges from 90 to 200 ng of digoxin equivalent (DE).

The plasma and urine electrolytes, urea nitrogen, creatinine and glucose determinations are made on an Astra automated critical laboratory analyzer (Beckman Instruments, Fullerton, Calif.). PAH is measured with a spectrophotometric Bratton-Marshall method.

EXAMPLE 5

Assays for Antagonists

An antagonist (antibody or otherwise) to a compound of the present invention may be verified by *in vitro* and/or *in vivo* assays. For example, a competitive assay may be used in which radiolabeled compound is used to compete for binding sites on washed erythrocyte membranes. The presence of an antagonist in such an assay system results in a reduced proportion of radiolabeled compound binding to the membranes. An alternative *in vitro* assay is to measure the amount of inhibition of Ca^{2+}-ATPase by a compound in the presence and absence of an antagonist. The presence of an antagonist in such an assay system results in an increase in enzyme activity (i.e., reduction in inhibition of enzyme activity) relative to samples in which the antagonist is absent. Alternatively, or in combination with an *in vitro* assay, an *in vivo* assay may be used.

A. *In Vitro* Assay

Human erythrocyte membranes are prepared by modification of the method previously described by Li et al. (Proc. West. Pharmacol. Sci. 33:143, 1990), which is a minor modification of the method of Farrance et al. (Biochim. Biophys. Acta 471:49, 1977). The present modifications are as follows. A free radical scavenger is added to the buffers used to wash the membranes. When the membranes are lysed, they are protected from calpain and other lytic enzymes by the addition of aprotinin (0.07 mg/ml added to the saline). A sulfhydryl protective agent, e.g., 1 mM dithiothreitol, is added to the membrane suspension before storage. The pH of the buffers is adjusted in the cold. The protein content is determined according to the Gornall modification for the biuret method as adapted for the PARAMAX automated chemistry analyzer.

Washed membranes are used in a colorimetric *in vitro* assay where the reaction tubes are microtiter plates. The colorimetric assay is based on phosphate quantitation after molybdic acid complex formation yielding a blue color. The estimation of enzyme inhibition is based on the calculations as described in Li et al. (1990).

In this competitive assay, the presence of an antagonist blocks inhibition observed by a compound alone and gives results relatively closer to the color developed when water is the sample and all other reagents are used. This water sample may be taken as 100% and all test samples compared to it. The greater the antagonist concentration, the greater the percentage of color relative to water will be seen.

As described by Li et al. (1990), microtiter plates are set up to assay for Mg, Na/K, Ca or calmodulin Ca ATPases, or all ATPases simultaneously if desired. The appropriate blanks and inhibition, or inhibition blocking profiles, may be derived from these data to characterize the antagonist more completely than a single assay of the antagonist activity.

For the microtiter reaction mixture, the contents per tube are those described by Li et al. (1990). Ultra pure water must be used in all reagent preparation. The volumes used in the microtiter plate protocol are: 30 µl sample (H_2O for 100% color controls), 10 µl compound, 2-8 ng/well, depending on the potency of the antagonist to compound and the preincubation time selected. (Omit from H_2O controls) 20 µl buffer, 10 µl ouabain (if used), 10 µl water or calmodulin and 20 µl washed membranes. All of these cold reagents are added to the cooled plates and are protected from light.

The antagonist and the compound membrane mixture are preincubated with shaking at 37° as long as 90 minutes to allow complete competition for the membrane binding sites and also for complete temperature equilibration. The reaction is initiated by adding 10 µl ATP. The plates are then incubated for 60 minutes at 37° before 20 µl of 5% SDS is added to all wells with ATPase reactions.

Inorganic phosphate standards and H_2O for the zero phosphate standard are added to the plate at this step, being placed in the empty wells carried around the perimeter. As is good practice, the empty plate should be prescreened for spurious transmission from any scratched or dirty wells and these blank values used or ignored as is appropriate.

Finally, 130 µl of color reagent is added to all wells on the plate. This reagent is made of 3 ml water, 6.5 ml molybdic acid, 7.9 ml 5% SDS, and 6.5 ml ascorbic acid which is 9% in water. This is a color reagent known to one skilled in the art and has been described in Raess and Vincenzi (J. Pharmacol. Methods 4:391, 1980). The color is allowed to develop for at least 20 minutes before measurement in a spectrometric plate reader.

The data calculated from the optical densities are µMoles phosphate formed/hour. The added compound concentration selected should allow 70-90%

inhibition when H_2O is substituted for the antagonist. The assay loses sensitivity at greater concentrations.

The reagent concentrations used in this competitive assay are adapted from the ATPase assay as described by Raess and Vincenzi (1980) to give the same reaction mixture concentrations, but diluted by 10%. The membranes are used at 0.375 to .7 μg/ml concentration in the added reagent, which also contains saponin (.02%). The calmodulin stock is 2.45 mg/ml which is prediluted to 0.059 mg/ml in a working stock and then diluted to 2 μm/ml in the working reagent. Dilutions are made in water.

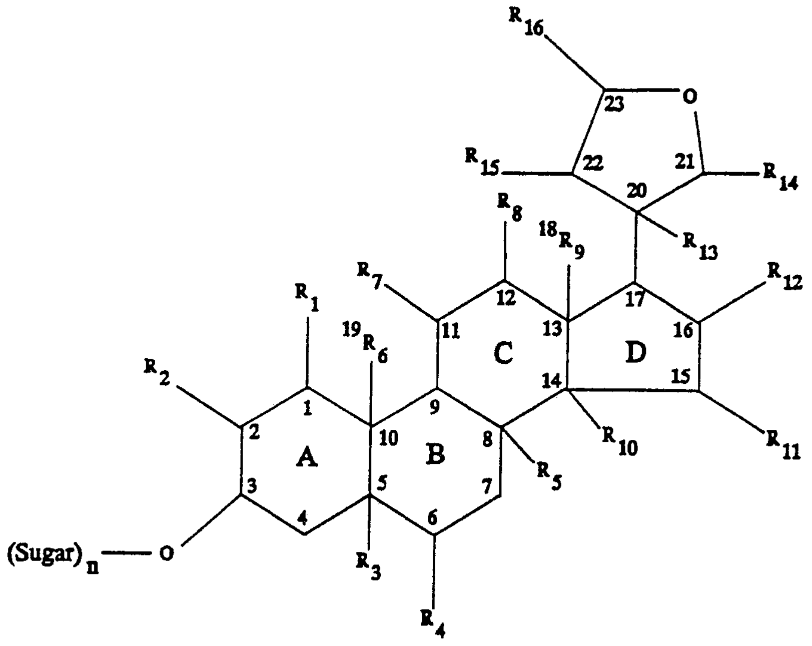
B. *In Vivo* Assay

The sheep model described in Example 4 may also be used to assess antagonists. Sheep are loaded with salt (in excess of 200-300 mmol of Na^+) in order to stimulate the production of glycoside. In this state, the basal renal excretion of various substances such as sodium and potassium and urine flow rates are obtained. The hemodynamic studies, including vascular resistance and systemic arterial pressure, are also measured. After obtaining these baseline values, an antagonist is administered and the above-mentioned studies are repeated. An effective antagonist leads to a drop in sodium excretion and urine flow rates, increase in potassium excretion, and a reduction in blood pressure and vascular resistance.

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

Claims

1. A compound having the formula:



wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is X or O-(Sugar)$_n$; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{15} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{16} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from pentoses, hexoses or combinations thereof; and n is an integer from 1 to 5; with the provisos that when R_1, R_3 and R_7 are H or OH, R_2, R_4, R_5, R_{11}, R_{13}-R_{15} are all H, R_6 is CH_3, CH_2OH or CHO, R_9 is CH_3, R_8 and R_{10} are OH, R_{12} is H or OCOZ, R_{16} is a keto group and rings B, C and D are saturated, and (a) when n=1, the sugar is not digitoxose, acovenose, arabinose, cymarose, oleandrose, rhamnose, thevetose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (b) when n=2, the sugars are not two digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (c) when n=3, the sugars are not three digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, and (d) when n=4, the sugars are not three digitoxoses and one glucose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups.

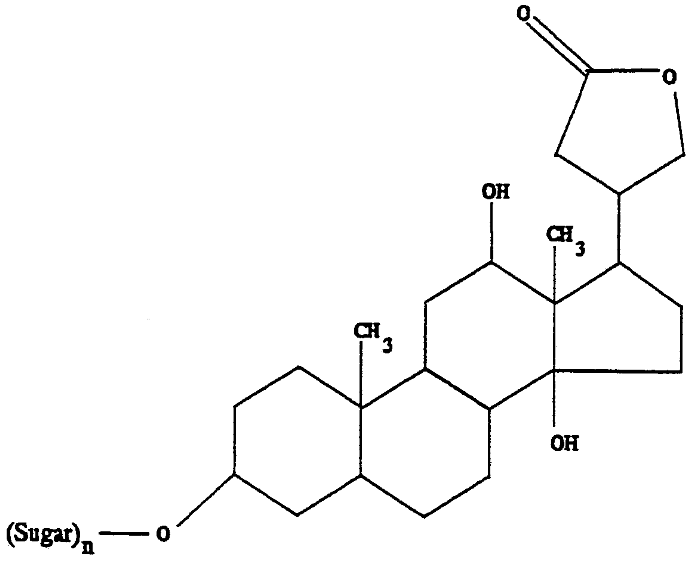
2. A compound according to claim 1 wherein sugar is independently selected from hexoses.

3. A compound according to claim 1 wherein at least one of the sugars is a glucose, a mannose, a fructose or a galactose.

4. A compound according to claim 3 wherein n is 2.

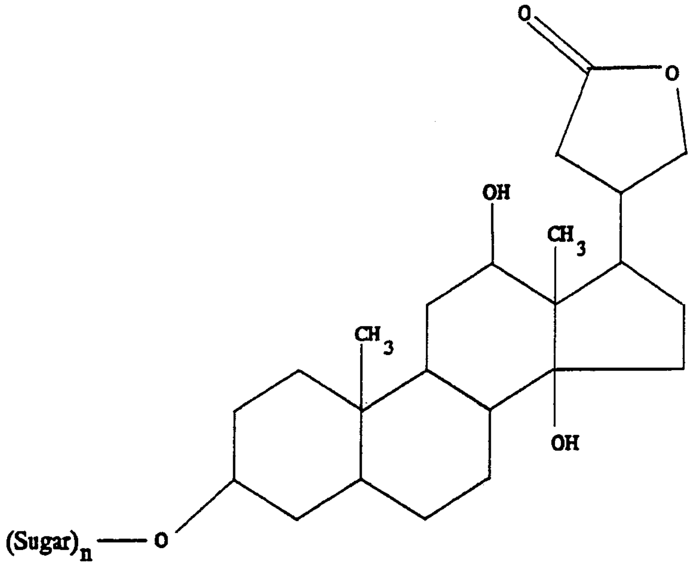
5. A compound according to claim 1 wherein n is 2 and the sugars are two glucoses, two galactoses, or one glucose and one galactose.

6. A compound having the formula:



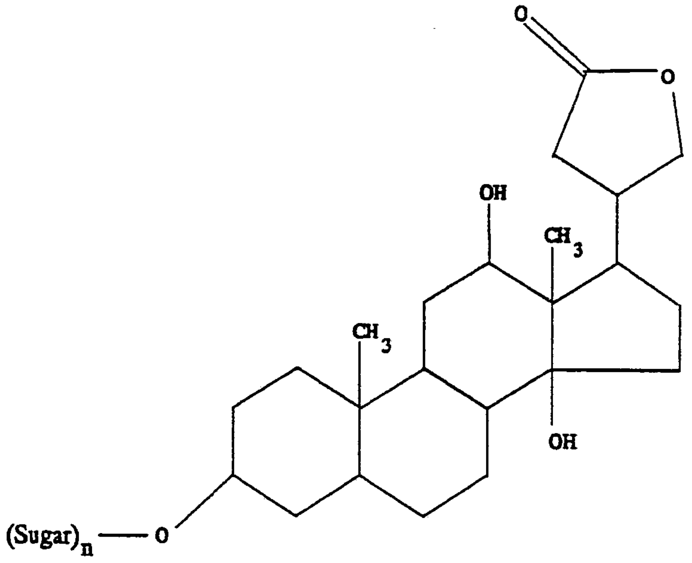
wherein sugar is independently selected from pentoses, hexoses, or combinations thereof; and n is an integer from 1 to 5; with the provisos that (a) when n=1, the sugar is not digitoxose, acovenose, arabinose, cymarose, oleandrose, rhamnose, thevetose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (b) when n=2, the sugars are not two digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (c) when n=3, the sugars are not three digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, and (d) when n=4, the sugars are not three digitoxoses and one glucose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups.

7. A compound having the formula:



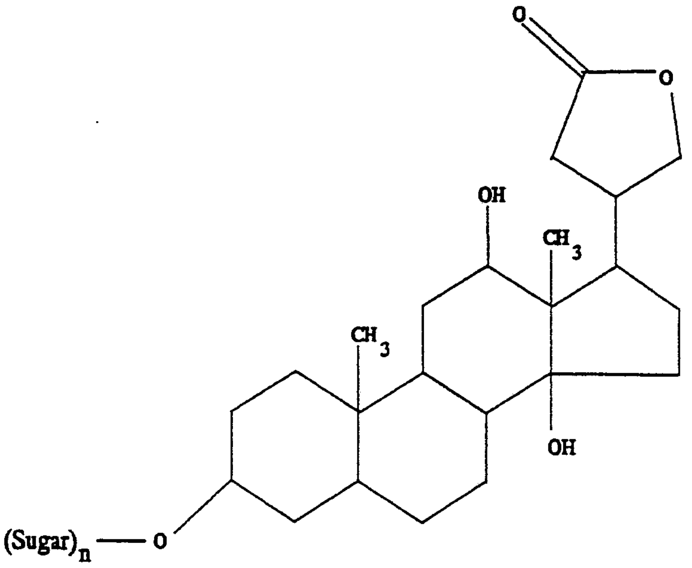
wherein sugar is independently selected from hexoses; and n is an integer from 1 to 5; with the provisos that (a) when n=1, the sugar is not digitoxose, acovenose, arabinose, cymarose, oleandrose, rhamnose, thevetose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (b) when n=2, the sugars are not two digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (c) when n=3, the sugars are not three digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, and (d) when n=4, the sugars are not three digitoxoses and one glucose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups.

8. A compound having the formula:



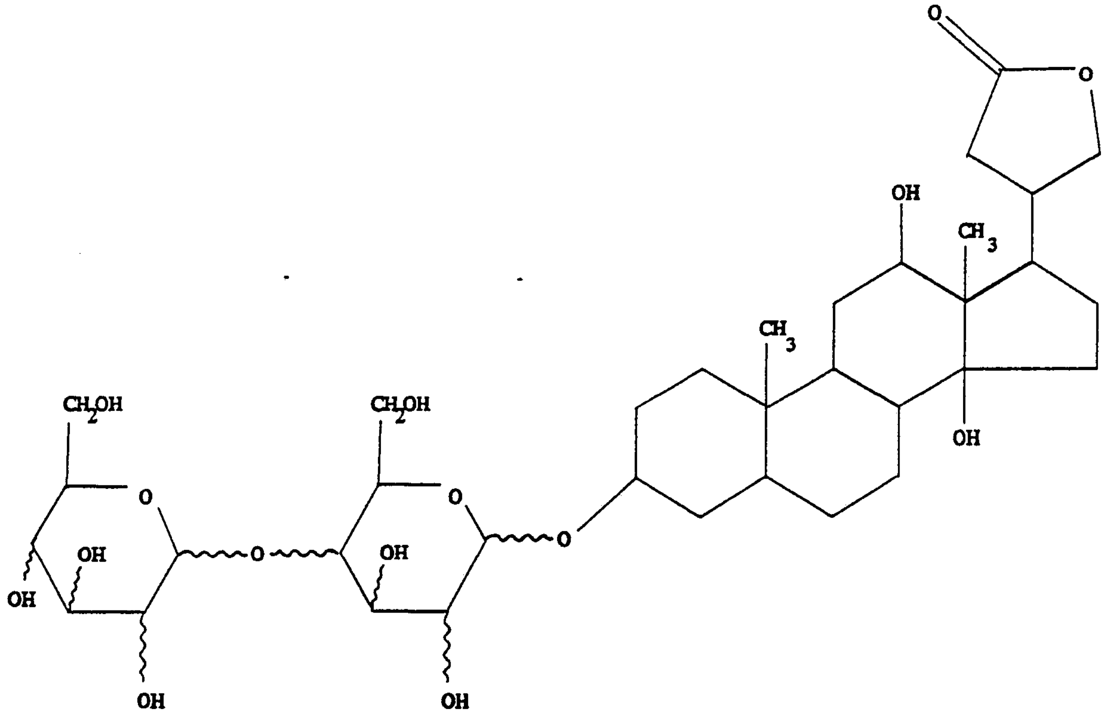
wherein at least one of the sugars is a glucose, a mannose, a fructose or a galactose; and n is an integer from 1 to 5; with the proviso that when n=4 the sugars are not three digitoxoses and one glucose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups.

9. A compound having the formula:

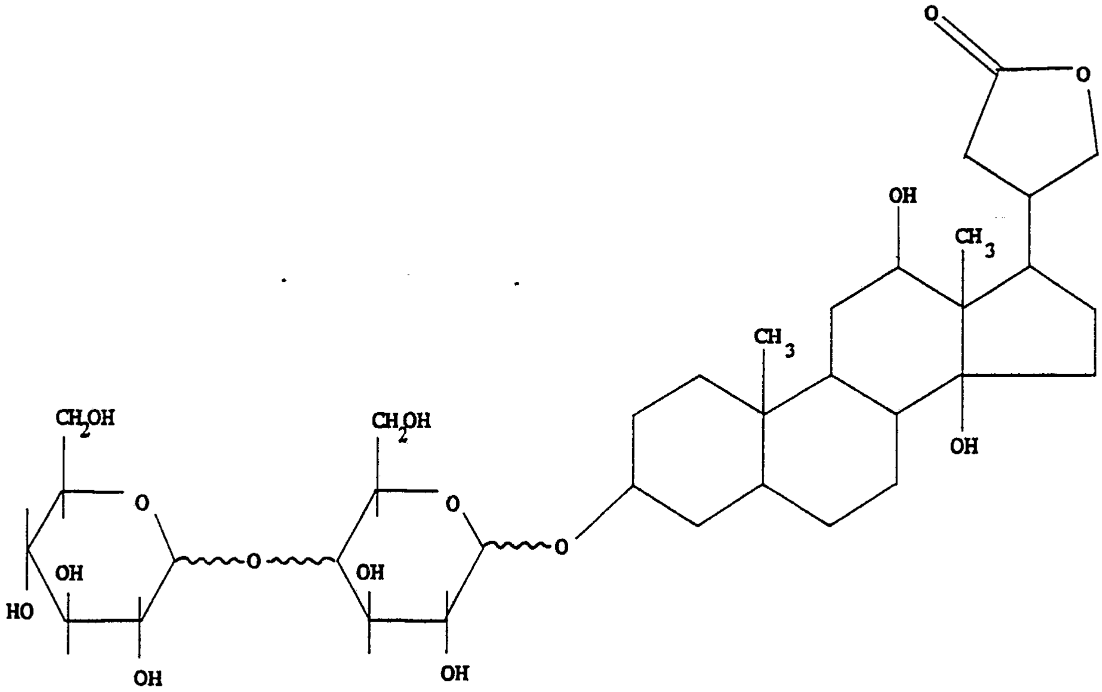


wherein at least one of the sugars is a glucose, a mannose, a fructose or a galactose; and n is 2.

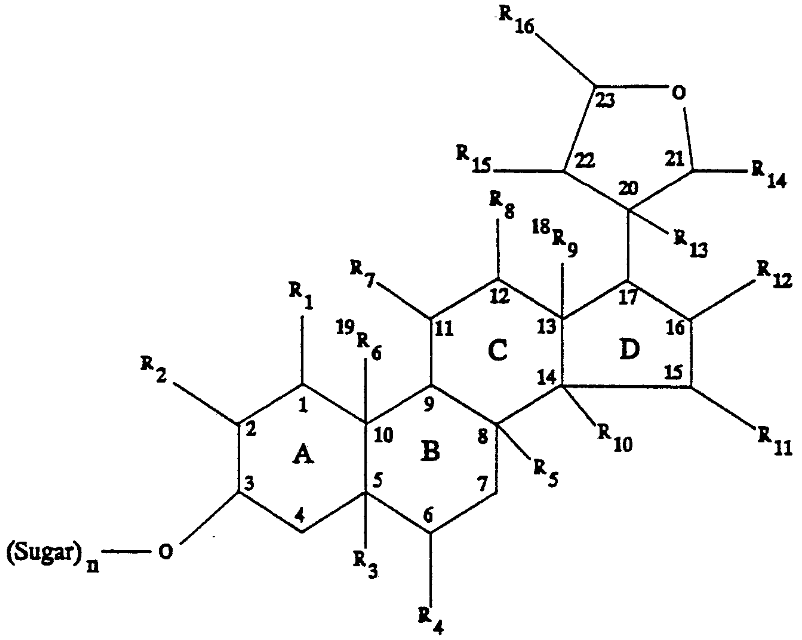
10. A compound having the formula:



11. A compound having the formula:

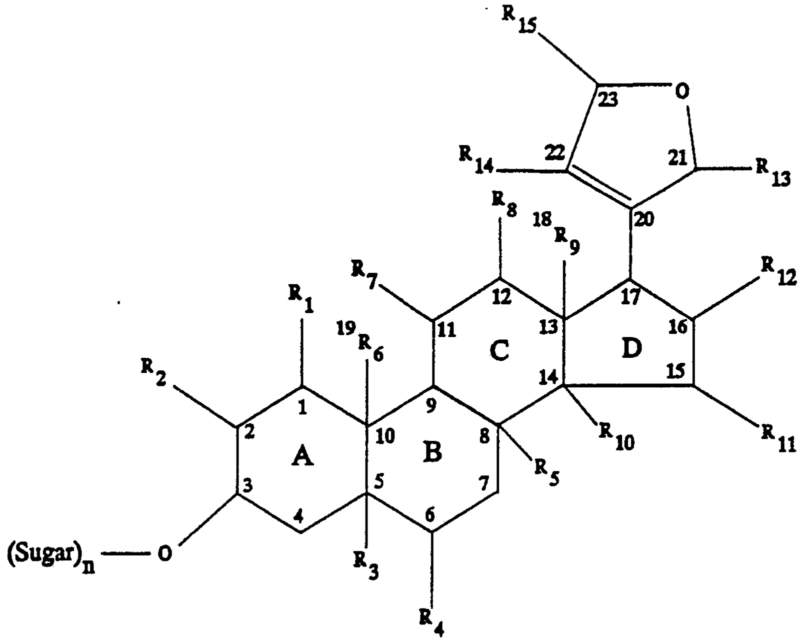


12. A compound having the formula:



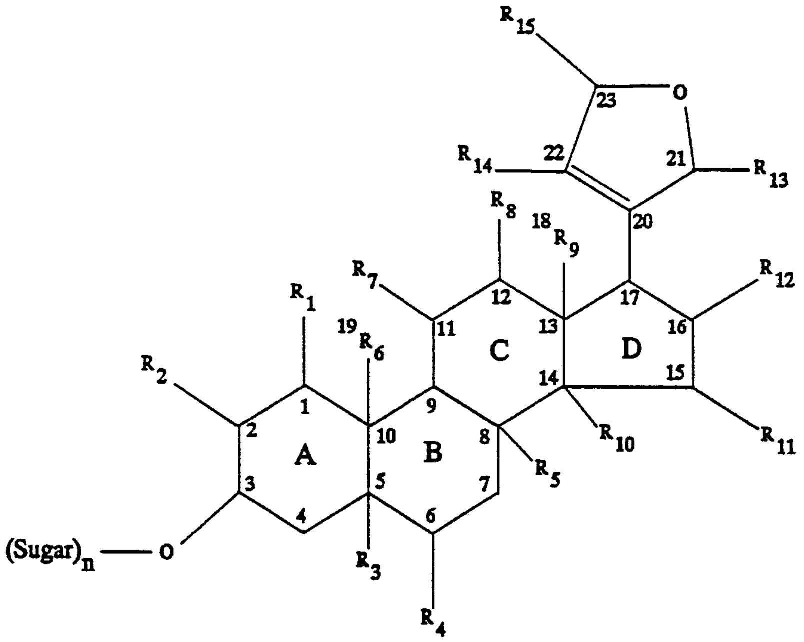
wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is H; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{15} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{16} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from hexoses wherein at least one of the sugars is a glucose, a mannose, a fructose or a galactose; and n is an integer from 1 to 5.

13. A compound having the formula:



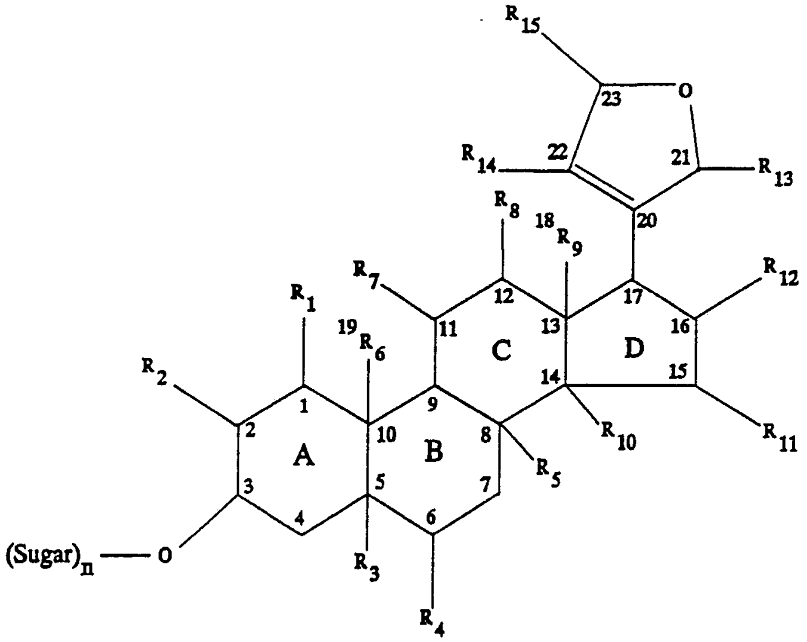
wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is X; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{14} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{15} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from pentoses, hexoses or combinations thereof; and n is an integer from 1 to 5; with the provisos that when R_1, R_3 and R_7 are H or OH, R_2, R_4, R_5, R_{11}, R_{13}-R_{14} are all H, R_6 is CH_3, CH_2OH or CHO, R_9 is CH_3, R_8 and R_{10} are OH, R_{12} is H or OCOZ, R_{15} is a keto group and rings B, C and D are saturated, and (a) when n=1, the sugar is not digitoxose, acovenose, arabinose, cymarose, oleandrose, rhamnose, thevetose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, or when R_1, R_3, R_7, R_{12} are all H, R_6 is CH_3, and the other terms are according to the proviso definitions the sugar is not glucose or xylose, (b) when n=2, the sugars are not two digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, (c) when n=3, the sugars are not three digitoxoses, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups, or when R_1, R_3, R_7, R_{12} are all H, R_6 is CH_3, and the other terms are according to the proviso definitions the sugars are not three digitoxoses wherein the hydroxyl groups of the terminal digitoxose are alkanyoyl or carboxylic acid ester derivatives, and (d) when n=4, the sugars are not three digitoxoses and one glucose, or derivatives thereof wherein hydroxyl groups are substituted by alkyl or acyl groups.

14. A compound having the formula:



wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is H; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{14} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{15} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from glucose, mannose, fructose and galactose; and n is an integer from 2 to 5.

15. A compound having the formula:



wherein R_1-R_5 are independently selected from H or X, where X is selected from the group consisting of OH, F, Cl, Br, I, NH_2, CN, OZ and OCOZ, where Z is an alkyl group from 1 to 6 carbon atoms; R_6 is H, X, CH_3, CHO or CH_2X; R_7 is H or X; R_8 is O-$(Sugar)_n$; R_9 is CH_3; R_{10}-R_{12} are independently selected from H or X; R_{13}-R_{14} are independently selected from H, F, Cl, Br, I, alkyl or haloalkyl group from 1 to 6 carbon atoms; R_{15} is a keto, OH, alkyl or haloalkyl group from 1 to 6 carbon atoms and C_{23} is sp^2 or sp^3; ring B is saturated or unsaturated at C_5-C_6; ring C is saturated or unsaturated at C_8-C_{14}; ring D is saturated or unsaturated at C_{14}-C_{15} or C_{15}-C_{16}; sugar is independently selected from hexoses wherein at least one of the sugars attached to C_3 is a glucose, a mannose, a fructose or a galactose; and n is an integer from 2 to 5.

16. A compound according to any one of claims 1-15 for use as an active therapeutic substance.

17. A pharmaceutical composition comprising a compound according to any one of claims 1-15, in combination with a pharmaceutically acceptable carrier or diluent.

18. A method for detecting the presence or amount of a compound according to claim 10, comprising the step of:

testing a biological fluid sample for the presence or amount of said compound.

19. The method of claim 18 wherein the step of testing comprises the steps of:

contacting said biological fluid sample with an antibody specific for said compound under conditions and for a time sufficient to allow immunocomplexes to form therebetween; and

detecting the presence or amount of one or more immunocomplexes formed between said antibody and said compound, thereby determining the presence or amount of said compound.

20. The method of claim 18 wherein the step of testing comprises the steps of:

contacting said biological fluid sample with at least two antibodies, the combination of which are specific for said compound, under conditions and for a time sufficient to allow immunocomplexes to form therebetween; and

detecting the presence or amount of one or more immunocomplexes formed between each of said antibodies and said compound, thereby determining the presence or amount of said compound.

21. The method of claim 18 wherein the step of testing comprises the steps of:

contacting an aliquot of said biological fluid sample with a Ca^{2+}-ATPase preparation;

detecting the presence or amount of specific binding of a substance in said biological fluid sample to said Ca^{2+}-ATPase;

contacting another aliquot of said biological fluid sample with a Na^+, K^+-ATPase preparation;

detecting the presence or amount of specific binding of a substance in said biological fluid sample to said Na^+, K^+-ATPase; and

determining the presence or amount of said compound by the presence or amount of specific binding to both said Ca^{2+}-ATPase and said Na^+, K^+-ATPase.

22. The method of claim 18 wherein the step of testing comprises the steps of:

contacting an aliquot of said biological fluid sample with a Ca^{2+}-ATPase preparation and substrates permitting enzymatic activity by said Ca^{2+}-ATPase;

detecting the presence or amount of specific inhibition of said Ca^{2+}-ATPase enzymatic activity;

contacting another aliquot of said biological fluid sample with a Na^+, K^+-ATPase preparation and substrates permitting enzymatic activity by said Na^+, K^+-ATPase;

detecting the presence or amount of specific inhibition of said Na^+, K^+-ATPase enzymatic activity; and

determining the presence or amount of said compound by the presence or amount of specific inhibition of both said Ca^{2+}-ATPase and said Na^+, K^+-ATPase enzymatic activities.

23. A method for detecting the presence or amount of a compound according to claim 10, comprising the steps of:

contacting a biological fluid sample with a binding partner specific for said compound under conditions and for a time sufficient to allow complexes to form therebetween; and

detecting the presence or amount of one or more complexes formed between said binding partner and said compound, thereby determining the presence or amount of said compound.

24. A method for detecting the presence or amount of a compound according to claim 10, comprising the steps of:

contacting a biological fluid sample with a Ca^{2+}-ATPase preparation and substrates permitting enzymatic activity by said Ca^{2+}-ATPase; and

detecting the presence or amount of specific inhibition of said Ca^{2+}-ATPase enzymatic activity, thereby determining the presence or amount of said compound.

25. A method for detecting the presence or amount of a compound according to claim 10, comprising the steps of:

contacting a biological fluid sample with a Ca^{2+}-ATPase preparation; and

detecting the presence or amount of specific binding of a substrate in said biological fluid sample to said Ca^{2+}-ATPase, thereby determining the presence or amount of said compound.

26. A method for detecting the predisposition of a warm-blooded animal to hypertension, comprising:

detecting the presence or amount of a compound according to claim 10 in a biological fluid sample of a warm-blooded animal, and therefrom determining the predisposition of said warm-blooded animal to hypertension.

27. A method for detecting the amount of a compound according to claim 10 to correct for interference by the compound in the measurement of digoxin, comprising:

detecting the amount of a compound according to claim 10 in a biological fluid sample of a warm-blooded animal administered digoxin.

28. A composition according to claim 17 for use in the manufacture of a medicament for elevating blood pressure.

29. A composition according to claim 17 for use in the manufacture of a medicament for increasing diuresis.

30. A composition according to claim 17 for use in the manufacture of a medicament for increasing natriuresis.

31. A composition according to claim 17 for use in the manufacture of a medicament for altering cellular calcium levels.

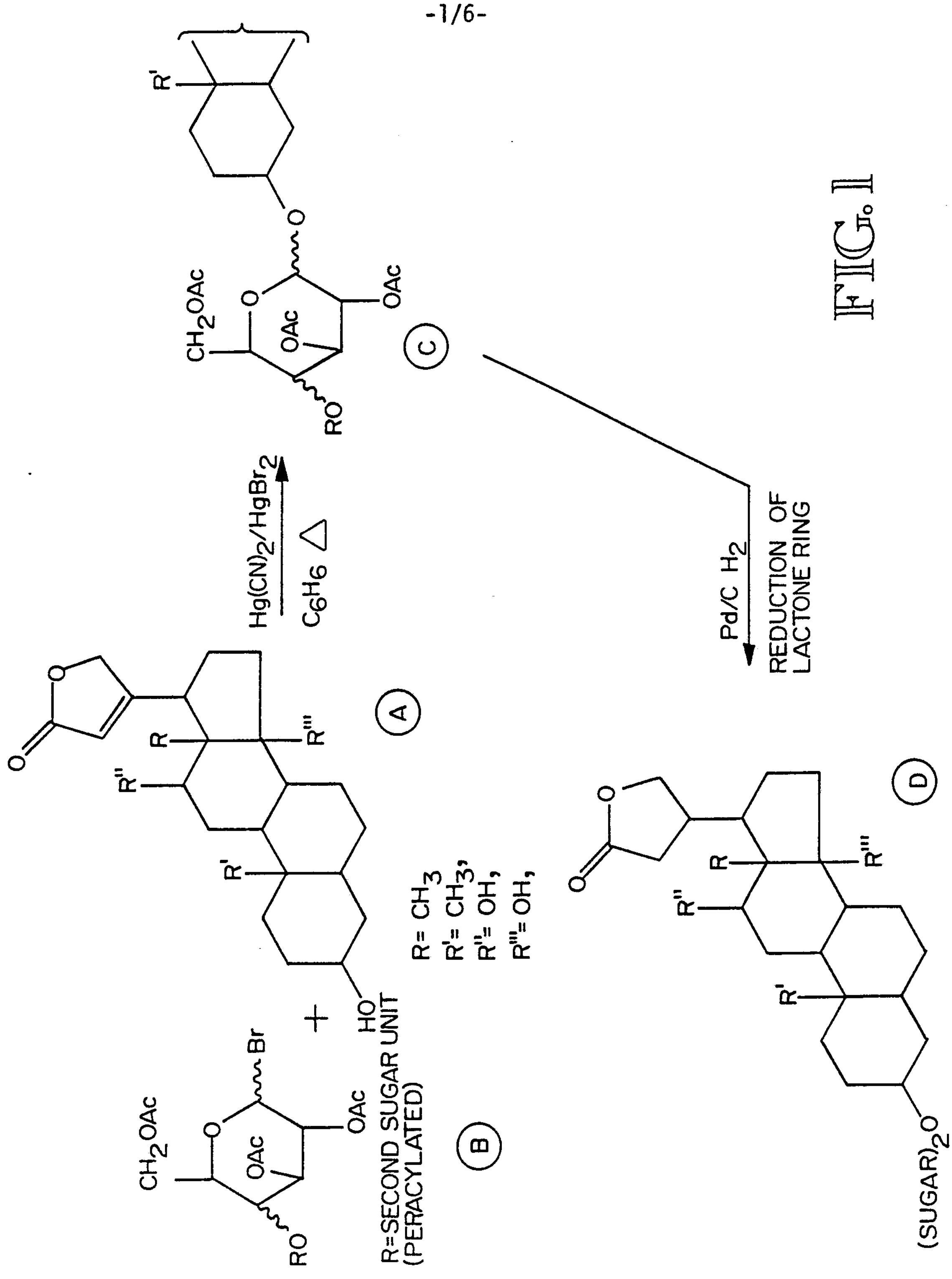

CH2OAc
OAc
OAc
Br
RO
B
R=SECOND SUGAR UNIT
(PERACYLATED)
+
HO
R'
R''
R
R'''
A
R= CH3
R'= CH3,
R''= OH,
R'''= OH,
Hg(CN)2/HgBr2
C6H6 △
CH2OAc
OAc
OAc
O
RO
C
R'
Pd/C H2
REDUCTION OF
LACTONE RING
(SUGAR)2O
R'
R''
R
R'''
D

FIG. 1

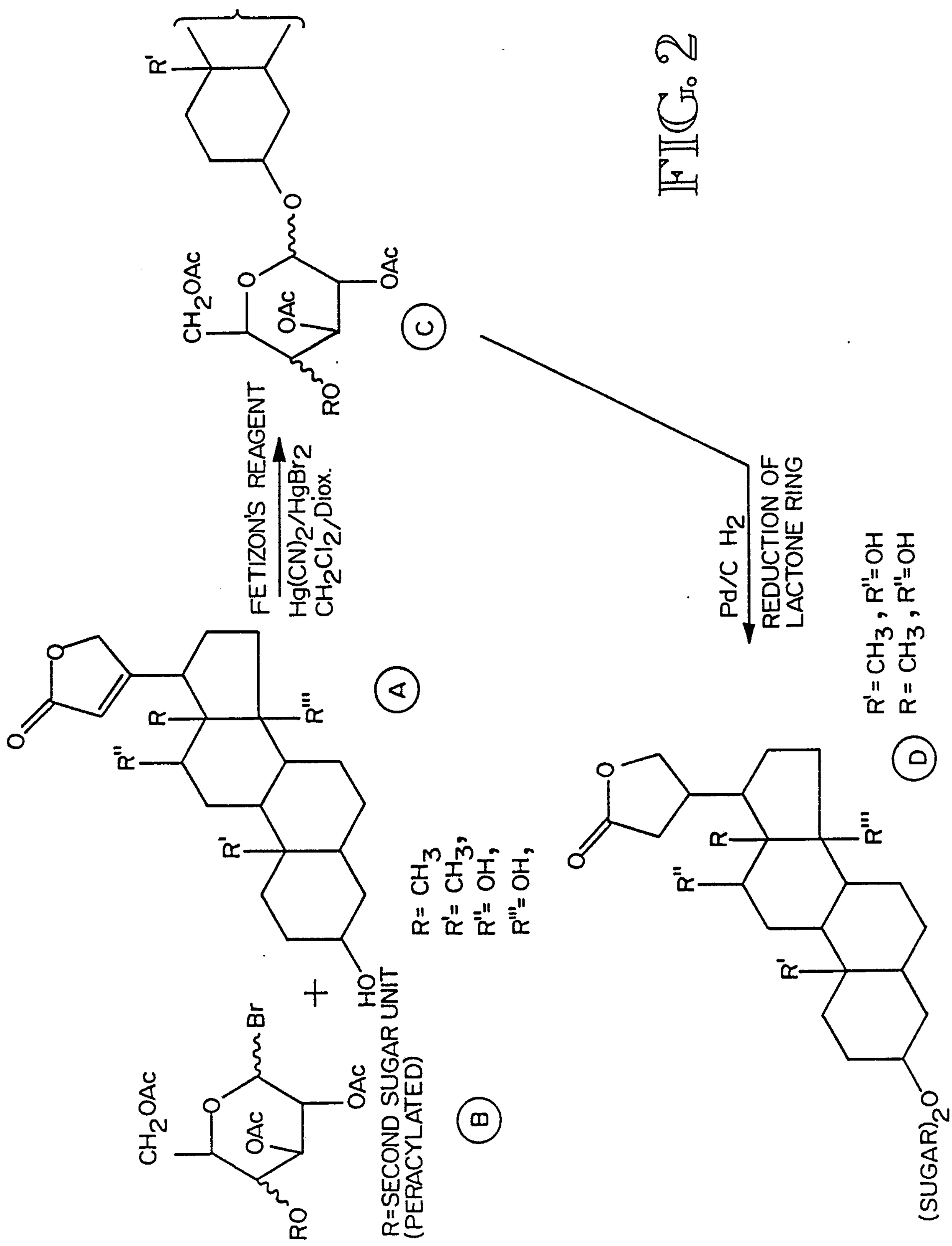

CH2OAc
OAc
OAc
RO
Br
R=SECOND SUGAR UNIT
(PERACYLATED)
B
+
HO
R'
R''
R
R'''
A
R= CH3
R'= CH3,
R''= OH,
R'''= OH,
FETIZON'S REAGENT
Hg(CN)2/HgBr2
CH2Cl2/Diox.
CH2OAc
OAc
OAc
RO
R'
C
Pd/C H2
REDUCTION OF
LACTONE RING
(SUGAR)2O
R'
R''
R
R'''
D
R' = CH3 , R'' = OH
R = CH3 , R'' = OH

FIG. 2

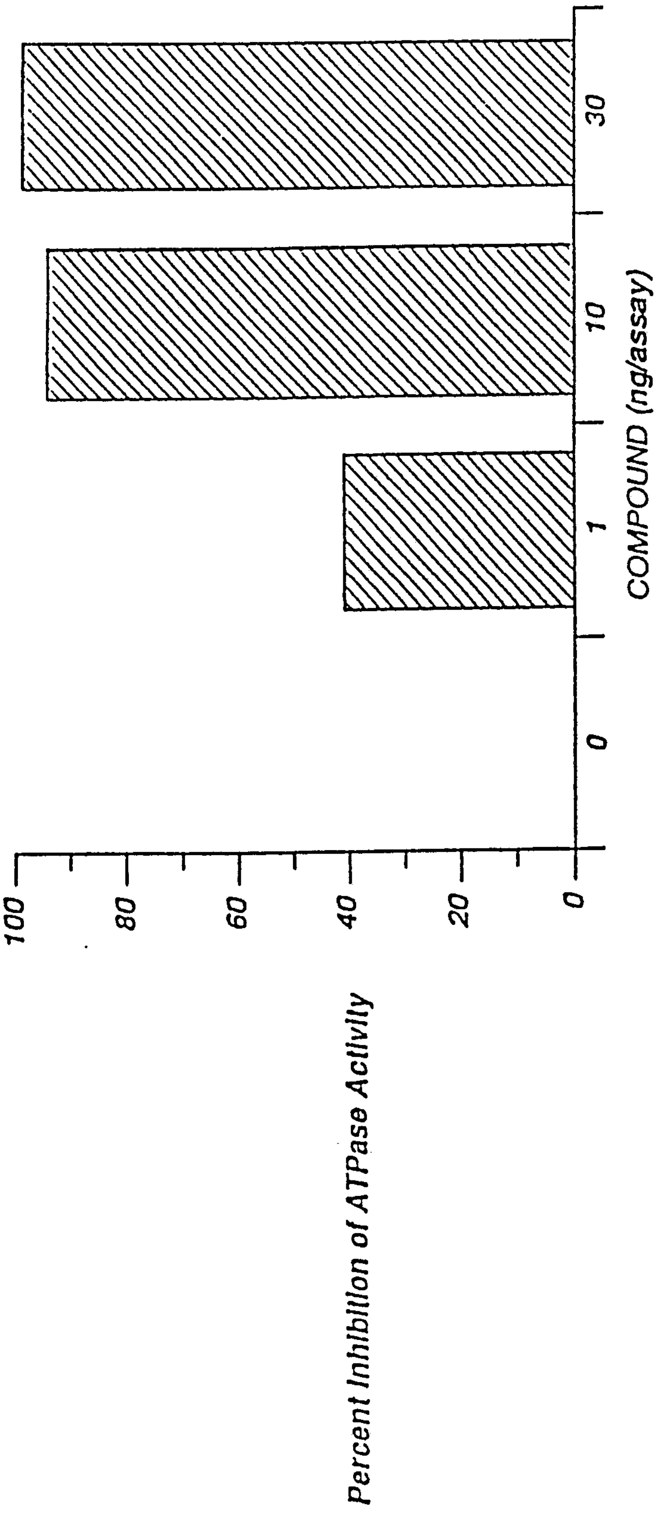

100
80
60
40
20
0
Percent Inhibition of ATPase Activity
0
1
10
30
COMPOUND (ng/assay)

Figure 3

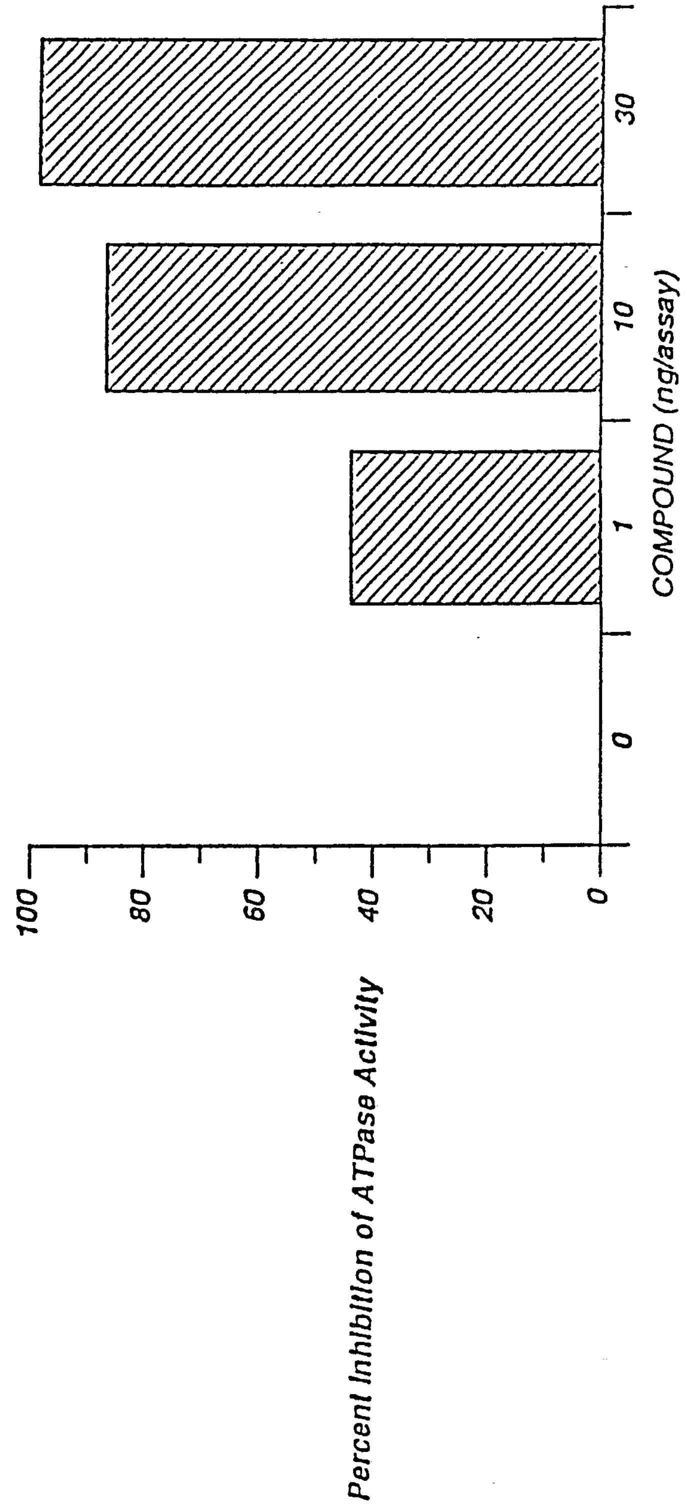

Percent Inhibition of ATPase Activity
100
80
60
40
20
0
0
1
10
30
COMPOUND (ng/assay)

Figure 4

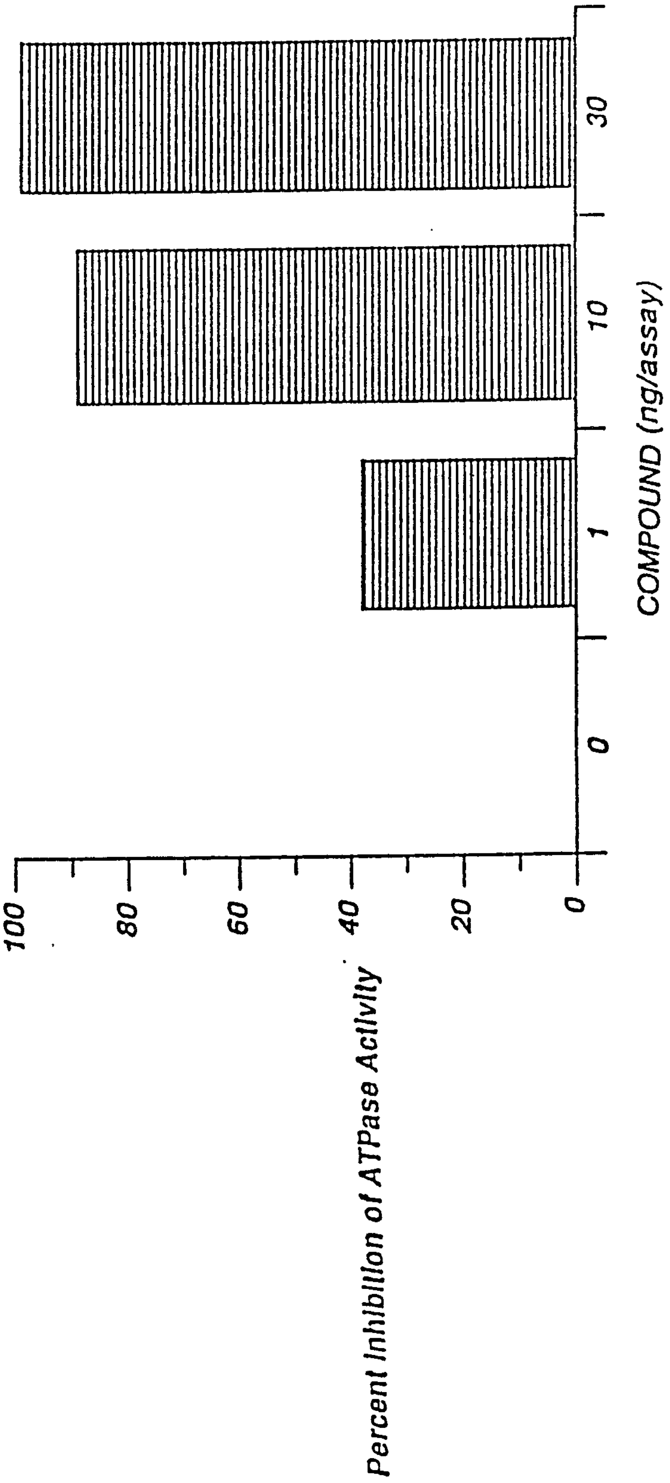

Percent Inhibition of ATPase Activity
100
80
60
40
20
0
0
1
10
30
COMPOUND (ng/assay)

Figure 5

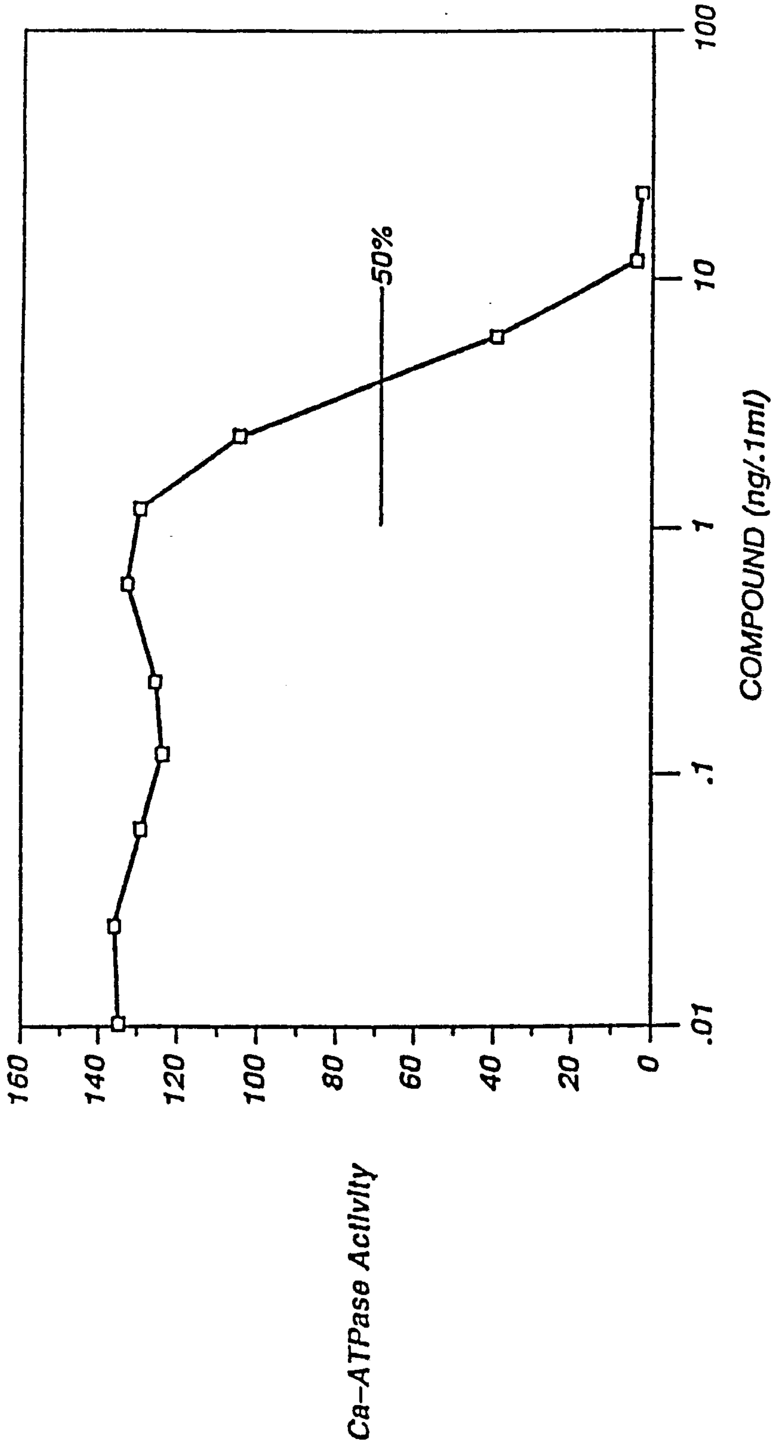

Ca-ATPase Activity
160
140
120
100
80
60
40
20
0
.01
.1
1
10
100
COMPOUND (ng/.1ml)
50%

Figure 6