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(54) Title: NEOGLYCORANDOMIZATION AND DIGITOXIN ANALOGS

(57) Abstract: The present invention provides methods of producing libraries of compounds with enhanced desirable properties and diminished side effects as well as the compounds produced by the methods. In preferred embodiments, methods of the present invention use a universal chemical glycosylation method that employs reducing sugars and requires no protection or activation. In a preferred embodiment, the invention provides a library of neoglycoside digitoxin analogs that includes compounds with significantly enhanced cytotoxic potency toward human cancer cells and tumor-specificity, but are less potent Na^+/K^+-ATPase inhibitors in a human cell line than digitoxin.

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NEOGLYCORANDOMIZATION AND DIGITOXIN ANALOGS

GOVERNMENT RIGHTS

This invention was made with United States government support awarded by the following agency: NIH AI052218. The United States has certain rights in this invention.

RELATED APPLICATIONS

The present application claims the benefit of U.S. provisional patent application Serial No. 60/521,721, filed on June 24th, 2004, which application is incorporated herein by reference for all purposes.

FIELD OF THE INVENTION

The present invention generally relates to glycosylated secondary metabolites. Specifically, the present invention relates to methods, techniques and uses of neoglycorandomization, especially as applied to digitoxin, indolocarbazole and anthracyline analogs.

BACKGROUND OF THE INVENTION

The natural product pool, which contains many glycosylated secondary metabolites, is the source of over half the world's drug leads. Carbohydrate appendages often play a key role in drug-target interactions. Therefore, alteration of glycosylation patterns on secondary metabolites is a potential strategy for the generation of novel therapeutics.

Carbohydrates mediate many essential biological processes. For example, the saccharide-containing macromolecules that decorate cell surfaces are vital to a variety of cellular functions including cell-cell recognition, apoptosis, differentiation, and tumor metastasis. In a similar fashion, glycosylated natural products contain sugar attachments essential for their activity and continue to serve as reliable platforms for the development of many of existing front-line drugs (Clardy, J.; Walsh, C. (2004) Nature 432, 829-837; Thorson, J.S., et al., (2001) Curr. Org. Chem. 5, 139-150). While the diverse chemical space accessible by carbohydrates contributes to a

remarkably vast array of biological function (Dobson, C.M. (2004) Nature 432, 824-865), a precise understanding of the relationship between sugars and biological activity remains limited by the availability of convenient and effective glycosylation tools (Langenhan, J.M.; Thorson, J.S. (2005) Curr. Org. Synth. 2, 59-8).

Digitoxin (**1**) is a glycosylated natural product with numerous actions and therapeutic uses. In addition to its well-known cardiac activity, which is mediated by inhibition of the plasma membrane Na^+/K^+-ATPase (Paula, S.,et al., (2005) Biochemistry 44, 498-510), digitoxin has demonstrated *in vitro* anti-cancer properties (Johansson, S., et al., (2001) *Anti-Cancer Drugs* **12**, 475-483) and patient profiling suggests the survival rate of cancer patients taking digitoxin is statistically enhanced (Stenkvist, B. (2001) Anti-Cancer Drugs 12, 635-636; Haux, J., et al., (2001) BMC Cancer 1, 11). Cardiac glycosides were also recently noted to inhibit the expression of four genes that are overexpressed in prostate cancer cells, including transcription factors and the apoptosis inhibitor survivin (Johnson, P.H., et al., (2002) Molecular Cancer Therapeutics 1, 1293-1304), and to provide protective effects against polyglutamine-based diseases (Piccioni, F., et al., (2004) *Hum. Mol. Genet.* **13**, 437-446). Digitoxin also inhibits activation of the NF-κB signaling pathway in cystic fibrosis (CF) cells, suppressing hypersecretion of IL-8, a protein implicated in lung inflammation, from CF lung epithelial cells (Srivastava, M., et. al. (2004) Proc. Natl. Acad. Sci. 101, 7693-7698). Given that the attached sugars are implicated as mediators of the unique spectrum of biological properties exhibited by cardiac glycosides (Rathore, H., et al., (1986) *J. Med. Chem.* **29**, 1945-1952), digitoxin provides an excellent model to examine the general utility of neoglycosylation to efficiently construct a glycorandomized library and to directly assess the biological impact of varying the sugars attached to a given natural product-based drug.

SUMMARY OF THE INVENTION

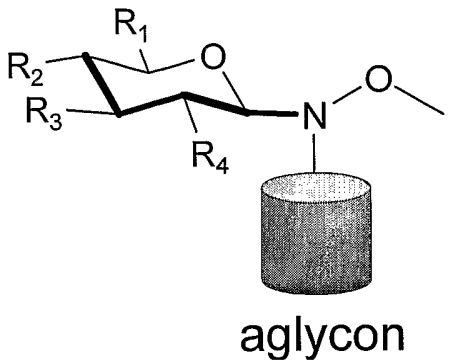
The present invention provides methods of producing libraries of compounds with enhanced desirable properties and diminished side effects as well as the libraries and compounds produced by the methods. In preferred embodiments, methods of the present invention use a universal chemical glycosylation method that employs reducing sugars and requires no protection or activation. In a preferred embodiment,

the invention provides a library of neoglycoside digitoxin analogs that includes compounds with significantly enhanced cytotoxic potency toward human cancer cells and tumor-specificity, but are less potent Na^+/K^+-ATPase inhibitors in a human cell line than digitoxin.

In general, the present invention provides neoglycosides produced by the reaction of an aglycon having a secondary alkoxylamine and a reducing sugar selected from the group consisting of L-sugars, D-sugars, deoxy-sugars, dideoxy-sugars, glucose epimers, substituted sugars, uronic acids and oligosaccharides. Suitable aglycons include digitoxin analogs, indolocarbazoles, anthracylines, macrolides, peptides, including ribosomal peptides as well as non-ribosomal peptides such as vancomycin, and alkaloids, such as colchicine. Suitable reducing sugars include L-ribose, D-ribose, L-fucose, D-fucose, 2-deoxy-D-galactose, 3-deoxy-D-glucose, 6-deoxy-D-glucose, 2-deoxy-2-fluoro-D-glucose, 6-deoxy-6-fluoro-D-glucose, L-lyxose, D-lyxose, L-rhamnose, L-allose, D-allose, L-altrose, D-altrose, L-galactose, D-galactose, L-xylose, D-xylose, D-gulose, L-mannose, D-mannose, L-idose, D-idose, L-mycarose, 6-keto-D-galactose, L-arabinose, D-arabinose, *N*-acetyl-D-galactosaminose, melibiose, lactose, maltose, D-galacturonose, L-talose, D-talose, 6-deoxy-6-azo-D-mannose, L-glucose, D-glucose, *O*-D-glucose, *R*-C(3)aglycon, *S*-C(3) aglycon.

In another embodiment, the present invention comprises neoglycosides produced by the reaction of an aglycon having a secondary alkoxylamine and a reducing sugar from the group consisting of L-riboside, D-riboside, L-fucoside, D-fucoside, 2-deoxy-D-galactoside, 3-deoxy-D-glucoside, 6-deoxy-D-glucoside, 2-deoxy-2-fluoro-D-glucoside, 6-deoxy-6-fluoro-D-glucoside, L-lyxoside, D-lyxoside, L-rhamnoside, L-alloside, D-alloside, L-altroside, D-altroside, L-galactoside, D-galactoside, L-xyloside, D-xyloside, D-guloside, L-mannoside, D-mannoside, L-idoside, D-idoside, L-mycaroside, 6-keto-D-galactoside, L-arabinoside, D-arabinoside, *N*-acetyl-D-galactosaminoside, melibioside, lactoside, maltoside, D-galacturonoside, L-taloside, D-taloside, 6-deoxy-6-azido-D-mannoside, L-glucoside, D-glucoside, *O*-D-glucoside, *R*-C(3)aglycon and *S*-C(3) aglycon.

In preferred embodiments, the invention provides neoglycosides of the formula:



In a preferred embodiment, the invention provides a library of comprising a plurality of neoglycosides selected from the group consisting of L-riboside (5β), D-riboside (6β), L-fucoside (7β), D-fucoside (8β), 2-deoxy-D-galactoside (9β), 3-deoxy-D-glucoside (10β), 6-deoxy-D-glucoside (11β), 2-deoxy-2-fluoro-D-glucoside (12β), 6-deoxy-6-fluoro-D-glucoside (13β), L-lyxoside (14β), D-lyxoside (15β), L-rhamnoside (16β), L-alloside (17β), D-alloside (18β), L-altroside (19β), D-altroside (20β), L-galactoside (21β), D-galactoside (22β), L-xyloside (23β), D-xyloside (24β), D-guloside (25β), L-mannoside (26β), D-mannoside (27β), L-idoside (28β), D-idoside (29β), L-mycaroside (30β), 6-keto-D-galactoside (31β), L-arabinoside (32β), D-arabinoside (33β), *N*-acetyl-D-galactosaminoside (34β), melibioside (35β), lactoside (36β), maltoside (37β), D-galacturonoside (38β), L-taloside (39β), D-taloside (40β), 6-deoxy-6-azido-D-mannoside (41β), L-glucoside (42β), D-glucoside (4β), *O*-D-glucoside (43β), *R*-C(3)aglycon (3β), *S*-C(3) aglycon (3α), L-riboside (5α), D-riboside (6α), L-fucoside (7α), D-fucoside (8α), 2-deoxy-D-galactoside (9α), 3-deoxy-D-glucoside (10α), 6-deoxy-D –glucoside (11α), 2-deoxy-2-fluoro-D-glucoside (12α), 6-deoxy-6-fluoro-D-glucoside (13α), L-lyxoside (14α), D-lyxoside (15α), L-rhamnoside (16α), L-alloside (17α), D-alloside (18α), L-altroside (19α), D-altroside (20α), L-galactoside (21α), D-galactoside (22α), L-xyloside (23α), D-xyloside (24α), D-guloside (25α), L-mannoside (26α), D-mannoside (27α), L-idoside (28α), D-idoside (29α), L-mycaroside (30α), 6-keto-D-galactoside (31α), L-arabinoside (32α), D-arabinoside (33α), *N*-acetyl-D-galactosaminoside (34α), melibioside (35α), lactoside

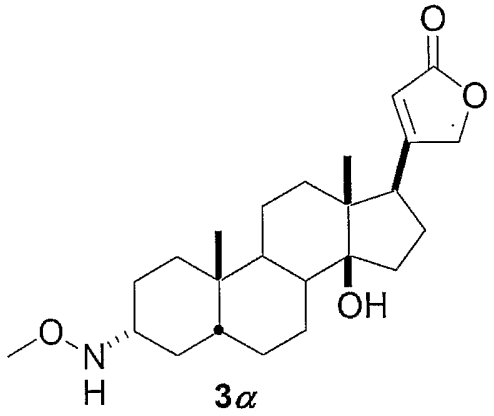
(36α), maltoside (37α), D-galacturonoside (38α), L-taloside (39α), D-taloside (40α), 6-deoxy-6-azido-D-mannoside (41α), L-glucoside (42α) and D-glucoside (4α).

In one preferred embodiment, the invention provides a library of neoglycosides comprising a plurality of neoglycosides selected from the group consisting of L-riboside (5β), D-riboside (6β), L-fucoside (7β), D-fucoside (8β), 2-deoxy-D-galactoside (9β), 3-deoxy-D-glucoside (10β), 6-deoxy-D-glucoside (11β), 2-deoxy-2-fluoro-D-glucoside (12β), 6-deoxy-6-fluoro-D-glucoside (13β), L-lyxoside (14β), D-lyxoside (15β), L-rhamnoside (16β), L-alloside (17β), D-alloside (18β), L-altroside (19β), D-altroside (20β), L-galactoside (21β), D-galactoside (22β), L-xyloside (23β), D-xyloside (24β), D-guloside (25β), L-mannoside (26β), D-mannoside (27β), L-idoside (28β), D-idoside (29β), L-mycaroside (30β), 6-keto-D-galactoside (31β), L-arabinoside (32β), D-arabinoside (33β), *N*-acetyl-D-galactosaminoside (34β), melibioside (35β), lactoside (36β), maltoside (37β), D-galacturonoside (38β), L-taloside (39β), D-taloside (40β), 6-deoxy-6-azido-D-mannoside (41β), L-glucoside (42β), D-glucoside (4β), *O*-D-glucoside (43β), *R*-C(3)aglycon (3β), *S*-C(3) aglycon (3α), L-riboside (5α), D-riboside (6α), L-fucoside (7α), D-fucoside (8α), 2-deoxy-D-galactoside (9α), 3-deoxy-D-glucoside (10α), 6-deoxy-D –glucoside (11α), 2-deoxy-2-fluoro-D-glucoside (12α), 6-deoxy-6-fluoro-D-glucoside (13α), L-lyxoside (14α), D-lyxoside (15α), L-rhamnoside (16α), L-alloside (17α), D-alloside (18α), L-altroside (19α), D-altroside (20α), L-galactoside (21α), D-galactoside (22α), L-xyloside (23α), D-xyloside (24α), D-guloside (25α), L-mannoside (26α), D-mannoside (27α), L-idoside (28α), D-idoside (29α), L-mycaroside (30α), 6-keto-D-galactoside (31α), L-arabinoside (32α), D-arabinoside (33α), *N*-acetyl-D-galactosaminoside (34α), melibioside (35α), lactoside (36α), maltoside (37α), D-galacturonoside (38α), L-taloside (39α), D-taloside (40α), 6-deoxy-6-azido-D-mannoside (41α), L-glucoside (42α) and D-glucoside (4α).

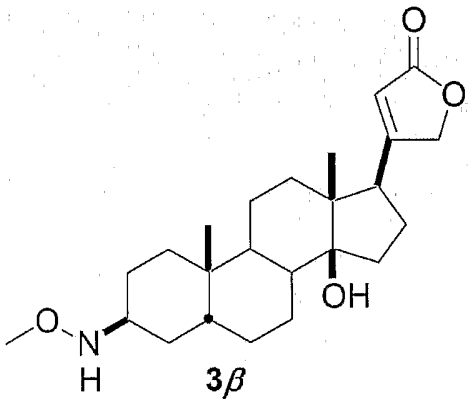
In one embodiment, the invention comprises a library comprising a plurality of neoglycosides produced by the reaction of an aglycon having a secondary alkoxylamine and at least one reducing sugar selected from the group consisting of L-sugars, D-sugars, deoxy-sugars, dideoxy-sugars, glucose epimers, substituted sugars and oligosaccharides. In another embodiment, the invention comprises a collection

comprising at least two compounds produced by the reaction of an aglycon having a secondary alkoxylamine and a reducing sugar from the group consisting of L-ribose, D-ribose, L-fucose, D-fucose, 2-deoxy-D-galactose, 3-deoxy-D-glucose, 6-deoxy-D-glucose, 2-deoxy-2-fluoro-D-glucose, 6-deoxy-6-fluoro-D-glucose, L-lyxose, D-lyxose, L-rhamnose, L-allose, D-allose, L-altrose, D-altrose, L-galactose, D-galactose, L-xylose, D-xylose, D-gulose, L-mannose, D-mannose, L-idose, D-idose, L-mycarose, 6-keto-D-galactose, L-arabinose, D-arabinose, *N*-acetyl-D-galactosaminose, melibiose, lactose, maltose, D-galacturonose, L-talose, D-talose, 6-deoxy-6-azo-D-mannose, L-glucose, D-glucose, *O*-D-glucose, *R*-C(3)aglycon, *S*-C(3) aglycon.

In some embodiments, the aglycon having a secondary alkoxylamine is selected from digitoxin analogs, indolocarbazoles, anthracylines, macrolides, peptides and alkaloids. In preferred embodiments, the aglycon having a secondary alkoxylamine is selected from the group consisting of 3α, 3β and mixtures thereof.

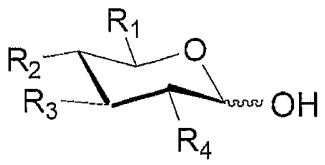


3*α*



3*β*

In certain embodiments, the present invention provides a method of making a library comprising a plurality of neoglycosides comprising the steps of providing at least two reducing sugars of the formula:



where R_1, R_2, R_3, and R_4 are independently selected from –H, -OH, $-N_3$, $-NH_2$, $-CH_3$, $-CH_2OH$, $-CN_3$, $-CH_2NH$, $-CH_2SH$, $-CNH_2$, $-CH_2N_3$, -COOH, $-COCH_3$, $-CXH_2$, $-CX_2H$, and where X is Cl, Br, F, or I; and

contacting the reducing sugars with at least one aglycon having a secondary alkoxylamine to form a neoglycoside. In preferred embodiments, the aglycon having a secondary alkoxylamine is a digitoxin methoxylamine. In some embodiments, the aglycon having a secondary alkoxylamine is selected from digitoxin analogs, indolocarbazoles, anthracylines, macrolides, peptides and alkaloids. In certain embodiments, the step of contacting is performed at a temperature from about 40 to about 60 degrees Celsius. In certain embodiments, the step of contacting is performed in the presence of a 3:1 mixture of DMF and AcOH.

In another aspect, the present invention provides a pharmaceutical composition of a neoglycoside of the present invention, a pharmaceutically acceptable ester, salt or prodrug thereof combined with a pharmaceutically acceptable carrier. In a further embodiment, the present invention provides a method of treating a subject having cancer cells comprising the step of contacting the cancer cells with an effective amount of the neoglyoside of the present invention, or pharmaceutically acceptable ester, salt or prodrug thereof. Preferred neoglycosides include L-riboside (5β), D-lyxoside (15β), L-xyloside (23β), D-mannoside (27β), D-arabinoside (33β), D-taloside (40β) and a mixture thereof. Also provided is the use of a neoglycoside of the present invention for the manufacture of a medicament for the treatment of cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a schematic illustration of the method of neoglycorandomization of the present invention that involves the chemoselective formation of glycosidic bonds between reducing sugars and a secondary alkoxylamine to form a library of neoglycosides compared to chemoenzymatic glycorandomization. Neoglycorandomization ("path A") is only limited by the ease of installation of the reactive secondary alkoxylamine group onto a complex natural product aglycon. In contrast, chemoenzymatic glycorandomization ("path B") uses nucleotide sugar activation enzymes ("E_1" and "E_2") and glycosyltransferase enzymes ("GlyT") that display natural or engineered promiscuity to glycosylate secondary metabolites, and is thus limited to natural products for which promiscuous glycosylation machinery is available. FIG. 1B is a schematic illustration showing that secondary alkoxylamines react to form closed-ring neoglycosides while primary alkoxylamines react with reducing sugars to form open-chain oximes.

FIG. 2A is a schematic illustration showing that aglycons having a secondary alkoxylamine, **3*β***, and its C(3) epimer **3*α***, were generated in three simple steps from the parent natural product digitoxin. FIG. 2B is a schematic illustration showing the reaction of aglycons **3*β*** and **3*α*** with D-glucose (2 equiv.) in 3:1 DMF/acetic acid at 60 °C to form neoglycosides **4*β*** and **4*α*** respectively, in > 70 % yield by ^{1}H NMR.

FIG. 3A is a schematic illustration of the solid state structure of **4*β*** shown with 50 % thermal probability ellipsoids. Hydrogen atoms are omitted for clarity. FIG. 3B is a schematic illustration of the solid state structure of **4*β*** (red) superimposed on the solid state structure of a homologous *O*-glucoside, actodigin. FIG. 3C is a Newman projection along the C(2)-C(3)-N(3)-C(1') torsion of neoglycoside **4*β*** and the corresponding torsion of 23 known cardiac glycosides showing that the neoglycoside torsion falls within the range of torsions displayed in the solid state by the known cardiac glycosides. FIG. 3D is a Newman projection along the C(3)-N(3)-C(1')-C(2') torsion of **4*β*** and the corresponding torsions of 23 known cardiac glycosides reveals that the neoglycoside torsion falls on the periphery of the narrow range of orientations displayed by natural *O*-glycosides. The crystallographic information displayed came from the following sources: actogenin (Fullerton, D.S., et al., From, A.H.L.; Ahmed,

K. Mol. Pharmacol. 1980,17, 43), oleandrin (Kartha, G.; Go, K. Cryst. Struct. Commun. 1981, 10, 1323), digoxigenin monodigitoxoside monohydrate (Go, K.; Kartha, G. Cryst. Struct. Commun. 1982, 11, 279), digoxigenin bis(digitoxoside) (Go, K.; Kartha, G. Cryst. Struct. Commun. 1982, 11, 285), digoxigenin bis(digitoxoside) tetrahydrate (Go, K.; Bhandary, K.K. Acta Crystallogr., Sect. B: Struct. Sci. 1989, 45, 306), (3β,5β,14β,20E)-methyl-3-((2,6-dideoxy-β- ribohexopyranosyl) oxy)-14-hydroxypregn-20-ene-21-carboxylate (*S7*), (3β,5β,14β,20E)-methyl-3-((2,6-dideoxy-3,4-O-(1-methylethylidene)-β-D-ribo-hexopyranosyl)oxy)-14-hydroxypregn-20-ene-21-carboxylate (Kihara, M., et al., Tetrahedron 1984, 40, 1121), (3β,5β,14β)-methyl-3-((2,6-dideoxy-3,4-O-(1-methylethylidene)-β-ribo-hexopyranosyl)oxy)-14-hydroxy-21-methylene-(pregane-21-carboxylate) (Kihara, M., et al., 1984), (20S)-20,22-dihydrodigitoxigenin-3-(2,6-dideoxy-3,4-O-(1-methylethylidene)-β-D-ribo-hexopyranoside) (Kihara, M., et al., 1984), digoxin (Go, K.; Kartha, G.; Chen, J.P. Cryst. Struct. Commun. 1979, 8, 149; Go, K.; Kartha, G.; Chen, J.P. Acta Crystallogr., Sect.B: Struct. Crystallogr. Cryst. Chem.1980, 36, 1811), gitoxigenin bisdigitoxoside ethyl acetate solvate (Go, K.; Bhandary, K.K. Acta Crystallogr., Sect. B: Struct. Sci. 1989, 45, 306), gitoxin (Go, K.; Kartha, G. Acta Crystallogr., Sect.B: Struct. Crystallogr. Cryst. Chem. 1980, 36, 3034.), cerleaside-A monohydrate (Go, K.; Kartha, G. Acta Crystallogr., Sect.B: Struct. Crystallogr. Cryst. Chem. 1980, 36, 3034), digitoxigenin bisdigitoxoside ethyl acetate solvate (Go, K.; Bhandary, K.K. Acta Crystallogr., Sect.B: Struct. Sci. 1989, 45, 306), ouabain octahydrate (Messerschmidt, A. Cryst. Struct. Commun. 1980, 9, 1185), 14β-hydroxy-3β-O-(L-thevetosyl)-5β-card-20(22)-enolide chloroform solvate (Fun, H.-K., et al.,. Acta Crystallogr., Sect. E: Struct. Rep. Online 2003, 59, o1694), (3β,5β,14β)-3-((2',6'-dideoxy-3',4'-O-(1'-methylethylidene)-β-D-ribo-hexopyranosyl)oxy)-14-hydroxycard-20(22)-enolide (Kihara, M., et al., 1984), (3β,5β,14β)-3-((2',6'-dideoxy-3',4'-O-(1'-methylethylidene)-β-D-ribo-hexopyranosyl)oxy)-14-hydroxycard-20(22)-enolide (Rohrer, D.C.; et al., From, A.H.L. From, Fullerton, D.S. 1984, 106, 8269), digitoxigenin bisdigitoxoside ethyl acetate hydrate (Go, K.; Bhandary, K.K. Acta Crystallogr., Sect.B: Struct. Sci. 1989, 45, 306), 3β-O-(2',3'-O-isopropylidene-α-L-rhamnopyranosyl)-digitoxigenin (Pfeiffer, D.; Reck, G.; Weiland, J. Cryst. Res. and Technol. 1986, 21, 223), 14β-hydroxy-3β-O-(L-thevetosyl)-5β-card-20(22)-enolide

methanol solvate hemihydrate (Chantrapromma, S., et al., Acta Crystallogr., Sect. C: Cryst. Struct. Commun. 2003, 59, o68), 3β-O-(L-2'-O-acetylhevetosyl)-14β-hydroxy-5β-card-20(22)-enolide (Chantrapromma, S., et al., Acta Crystallogr., Sect. C: Cryst. Struct. Commun. 2003, 59, o68.).

FIG. 4 is a graphic representation of the results of studies on the hydrolytic stability of neoglycoside 4α. The chemical stability of the neoglycosidic linkage was examined by monitoring the hydrolytic degradation of neoglycoside **4*α*** in a 3 mM solution of 1:1 DMSO/buffer. Three buffers were used, 50 mM acetate buffer (pH 5), 50 mM phosphate buffer (pH 7), and 50 mM Tris buffer (pH 9). Neoglycoside degradation was monitored by reverse phase HPLC on an Agilent Zorbax Eclipse XDB-C8 column (4.6 x 150 mm) with a flow rate of 0.8 mL min^{-1} and a linear gradient of 49 % CH_3OH/H_2O to 89 % CH_3OH/H_2O over 20 min. At $t = 0$, neoglycoside **4*α*** in 500 μL DMSO was added to 500 μL buffer, and the resulting solution was vortexed for 40 sec, then immediately injected onto the HPLC. Peak areas at 220 nm were used to estimate the neoglycoside/aglycon ratio, which is reported as "percent neoglycoside remaining" [Aneoglycoside/(Aneoglycoside+Aaglycon)] for each of the three buffer systems.

FIG. 5A and FIG. 5B are graphical representation of the results of a high-throughput assays of the cytotoxicity against cancer cell lines of members of a neoglycoside library of one embodiment of the present invention. In the assay, live cells were distinguished by the presence of a ubiquitous intracellular enzymatic activity which converts the non-fluorescent cell-permeable molecule calcein AM to the intensely fluorescent molecule calcein, which is retained within live cells. The IC_{50} value for each library member represents at least six replicates of dose-response experiments conducted over five concentrations using two-fold dilutions. For the entire panel of 81 compounds in 10 cell lines, the average error was 17 %. IC_{50} Reciprocal IC_{50} values as a function of library member and cell line. Standard errors are depicted with error bars. Numerical values and corresponding error values can be found in Table 4. Du145: human colon carcinoma; MCF7: human breast carcinoma; HCT-116: human colon carcinoma, Hep3B: human liver carcinoma; SF-268: human CNS glioblastoma; SK-OV-3: human ovary adenocarcinoma; NCI-H460: human lung

carcinoma; A549: human lung adenocarcinoma; NCI/ADR-RES: human breast carcinoma; NmuMG: mouse mammary normal epithelial.

FIG. 6. is a graphical summary the results of the high-throughput cytotoxicity assay, displaying data IC_{50} data from Table 4 and FIG. A and FIG. 5B. Reciprocal IC_{50} values are displayed for clarity showing an IC_{50} range of 18 nM (5 β, HCT-116) to > 25 μM (*e.g.* 35 β, all cell lines). Six library member "hits" are depicted in pyranose $^4C_1$ conformations to facilitate structural comparisons.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Glycosylated natural products are reliable platforms for the development of many front-line drugs, yet our understanding of the relationship between attached sugars and biological activity is limited by the availability of convenient glycosylation methods. Glycorandomization is a tool used to convert a single aglycon molecule into a library of analogs with a diverse array of sugar attachments.

Unless otherwise stated, the following terms used in this application, including the specification and claims, have the definitions given below. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

"Subject" means mammals and non-mammals. "Mammals" means any member of the class Mammalia including, but not limited to, humans, non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, and swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice, and guinea pigs; and the like. Examples of non-mammals include, but are not limited to, birds, and the like. The term "subject" does not denote a particular age or sex.

"Pharmaceutically acceptable" means that which is useful in preparing a pharmaceutical composition that is generally safe, non-toxic, and neither biologically nor otherwise undesirable and includes that which is acceptable for veterinary as well as human pharmaceutical use.

A "pharmaceutically acceptable carrier" as used herein means a chemical composition with which a biologically active ingredient can be combined and which, following the combination, can be used to administer the active ingredient to a subject.

A " pharmaceutically acceptable" ester or salt as used herein means an ester or salt form of the active ingredient which is compatible with any other ingredients of the pharmaceutical composition and which is not deleterious to the subject to which the composition is to be administered. The terms “pharmaceutically acceptable salts” or “prodrugs” includes the salts and prodrugs of compounds that are, within the scope of sound medical judgment, suitable for use with patients without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds.

"Pro-drug" means a pharmacologically inactive form of a compound which must be metabolized in vivo by a subject after administration into a pharmacologically active form of the compound in order to produce the desired pharmacological effect. After administration to the subject, the pharmacologically inactive form of the compound is converted in vivo under the influence of biological fluids or enzymes into a pharmacologically active form of the compound. Although metabolism occurs for many compounds primarily in the liver, almost all other tissues and organs, especially the lung, are able to carry out varying degrees of metabolism. For example, metabolism of the pro-drug may take place by hydrolysis in blood. Pro-drug forms of compounds may be utilized, for example, to improve bioavailability, mask unpleasant characteristics such as bitter taste, alter solubility for intravenous use, or to provide site-specific delivery of the compound. Reference to a compound herein includes pro-drug forms of a compound.

A discussion of the use of pro-drugs is provided by T. Higuchi and W. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987. For example, if a compound contains a carboxylic acid functional group, a pro-drug can comprise an ester formed

by the replacement of the hydrogen atom of the acid group with a group such as (C_1-C_8)alkyl, (C_2-C_{12})alkanoyloxymethyl, 1-(alkanoyloxy)ethyl having from 4 to 9 carbon atoms, 1-methyl-1-(alkanoyloxy)-ethyl having from 5 to 10 carbon atoms, alkoxycarbonyloxymethyl having from 3 to 6 carbon atoms, 1-(alkoxycarbonyloxy)ethyl having from 4 to 7 carbon atoms, 1-methyl-1-(alkoxycarbonyloxy)ethyl having from 5 to 8 carbon atoms, N-(alkoxycarbonyl) aminomethyl having from 3 to 9 carbon atoms, 1-(N-(alkoxycarbonyl)amino)ethyl having from 4 to 10 carbon atoms, 3-phthalidyl, 4-crotonolactonyl, gamma-butyrolacton-4-yl, di-N,N-(C_1-C_2)alkylamino(C_2-C_3)alkyl (such as β-dimethylaminoethyl), carbamoyl-(C_1-C_2)alkyl, N,N-di(C_1-C_2)alkylcarbamoyl-(C_1-C_2)alkyl and piperidino-, pyrrolidino- or morpholino(C_2-C_3)alkyl.

Similarly, if a compound comprises an alcohol functional group, a pro-drug can be formed by the replacement of the hydrogen atom of the alcohol group with a group such as (C_1-C_6)alkanoyloxymethyl, 1-(C_1-C_6)alkanoyloxy)ethyl, 1-methyl-1-(C_1-C_6)alkan- oyloxy)ethyl, (C_1-C_6)alkoxycarbonyloxymethyl, N-(C_1-C_6)alkoxycarbonylaminomethyl, succinoyl, (C_1-C_6)alkanoyl, α-amino(C_1-C_4)alkanoyl, arylacyl and alpha-aminoacyl, or alpha-aminoacyl- alpha-aminoacyl, where each alpha-aminoacyl group is independently selected from the naturally occurring L-amino acids, $P(O)(OH)_2$, --$P(O)(O(C_1$-$C_6)alkyl)_2$ or glycosyl (the radical resulting from the removal of a hydroxyl group of the hemiacetal form of a carbohydrate).

If a compound comprises an amine functional group, a pro-drug can be formed by the replacement of a hydrogen atom in the amine group with a group such as R-carbonyl, RO-carbonyl, NRR'-carbonyl where R and R' are each independently (C_1-C_{10})alkyl, (C_3-C_7)cycloalkyl, benzyl, or R-carbonyl is a natural alpha-aminoacyl or natural alpha-aminoacyl-, -C(OH)C(O)OY wherein Y is H, (C_1-C_6)alkyl or benzyl, -$C(OY_0)Y_1$ wherein Y_0 is (C_1-C_4) alkyl and Y_1 is ((C_1-C_6)alkyl, carboxy(C_1-C_6)alkyl, amino(C_1-C_4)alkyl or mono-N- or di-N,N-(C_1-C_6)alkylaminoalkyl, -$C(Y_2)$ Y_3 wherein Y_2 is H or methyl and Y_3 is mono-N- or di-N,N-(C_1-C_6)- alkylamino, morpholino, piperidin-1-yl or pyrrolidin-1-yl.

The term "salts" refers to inorganic and organic salts of compounds. These salts can be prepared in situ during the final isolation and purification of a compound,

or by separately reacting a purified compound with a suitable organic or inorganic acid or base, as appropriate, and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, nitrate, acetate, oxalate, palmitiate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, besylate, esylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts, and the like. These may include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium, calcium, magnesium, and the like, as well as non-toxic ammonium, quaternary ammonium, and amine cations including, but not limited to, ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. Compounds having N-oxides of amino groups, such as produced by reaction with hydrogen peroxide, are also encompassed.

A "therapeutically effective amount" means an amount of a compound that, when administered to a subject for treating a disease, is sufficient to effect such treatment for the disease. The "therapeutically effective amount" will vary depending on the compound, the disease state being treated, the severity or the disease treated, the age and relative health of the subject, the route and form of administration, the judgment of the attending medical or veterinary practitioner, and other factors.

For purposes of the present invention, “treating” or “treatment” describes the management and care of a patient for the purpose of combating the disease, condition, or disorder. The terms embrace both preventative, i.e., prophylactic, and palliative treatment. Treating includes the administration of a compound of present invention to prevent the onset of the symptoms or complications, alleviating the symptoms or complications, or eliminating the disease, condition, or disorder.

The form in which the active compound is administered to the cell is not critical; the active compound need only reach the cell, directly or indirectly. The invention encompasses preparation and use of medicaments and pharmaceutical compositions comprising a compound described herein as an active ingredient.

A neoglycoside is administered to a patient in a therapeutically effective amount. A neoglycoside can be administered alone or as part of a pharmaceutically acceptable composition. In addition, a compound or composition can be administered all at once, as for example, by a bolus injection, multiple times, such as by a series of tablets, or delivered substantially uniformly over a period of time, as for example, using transdermal delivery. It is also noted that the dose of the compound can be varied over time. A neoglycoside can be administered using an immediate release formulation, a controlled release formulation, or combinations thereof. The term "controlled release" includes sustained release, delayed release, and combinations thereof.

A pharmaceutical composition of the invention can be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient that would be administered to a patient or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the human treated and further depending upon the route by which the composition is to be administered. By way of example, the composition can comprise between 0.1% and 100% (w/w) active ingredient. A unit dose of a pharmaceutical composition of the invention will generally comprise from about 100 milligrams to about 2 grams of the active ingredient, and preferably comprises from about 200 milligrams to about 1.0 gram of the active ingredient.

In addition, a neoglycoside can be administered alone, in combination with other neoglycosides, or with other pharmaceutically active compounds. The other pharmaceutically active compounds can be selected to treat the same disease as the neoglycoside or a different disease. If the patient is to receive or is receiving multiple pharmaceutically active compounds, the compounds can be administered

simultaneously or sequentially in any order. For example, in the case of tablets, the active compounds may be found in one tablet or in separate tablets, which can be administered at once or sequentially in any order. In addition, it should be recognized that the compositions can be different forms. For example, one or more compounds may be delivered via a tablet, while another is administered via injection or orally as a syrup.

Another aspect of the invention relates to a kit comprising a pharmaceutical composition of the invention and instructional material. Instructional material includes a publication, a recording, a diagram, or any other medium of expression which is used to communicate the usefulness of the pharmaceutical composition of the invention for one of the purposes set forth herein in a human. The instructional material can also, for example, describe an appropriate dose of the pharmaceutical composition of the invention. The instructional material of the kit of the invention can, for example, be affixed to a container which contains a pharmaceutical composition of the invention or be shipped together with a container which contains the pharmaceutical composition. Alternatively, the instructional material can be shipped separately from the container with the intention that the instructional material and the pharmaceutical composition be used cooperatively by the recipient.

The invention also includes a kit comprising a pharmaceutical composition of the invention and a delivery device for delivering the composition to a human. By way of example, the delivery device can be a squeezable spray bottle, a metered-dose spray bottle, an aerosol spray device, an atomizer, a dry powder delivery device, a self-propelling solvent/powder-dispensing device, a syringe, a needle, a tampon, or a dosage- measuring container. The kit can further comprise an instructional material as described herein. For example, a kit may comprise two separate pharmaceutical compositions comprising respectively a first composition comprising a neoglycoside or a neoglycoside agonist and a pharmaceutically acceptable carrier; and composition comprising second pharmaceutically active compound and a pharmaceutically acceptable carrier. The kit also comprises a container for the separate compositions, such as a divided bottle or a divided foil packet. Additional examples of containers include syringes, boxes, bags, and the like. Typically, a kit comprises directions for

the administration of the separate components. The kit form is particularly advantageous when the separate components are preferably administered in different dosage forms (e.g., oral and parenteral), are administered at different dosage intervals, or when titration of the individual components of the combination is desired by the prescribing physician.

An example of a kit is a blister pack. Blister packs are well known in the packaging industry and are being widely used for the packaging of pharmaceutical unit dosage forms (tablets, capsules, and the like). Blister packs generally consist of a sheet of relatively stiff material covered with a foil of a preferably transparent plastic material. During the packaging process recesses are formed in the plastic foil. The recesses have the size and shape of the tablets or capsules to be packed. Next, the tablets or capsules are placed in the recesses and a sheet of relatively stiff material is sealed against the plastic foil at the face of the foil which is opposite from the direction in which the recesses were formed. As a result, the tablets or capsules are sealed in the recesses between the plastic foil and the sheet. Preferably the strength of the sheet is such that the tablets or capsules can be removed from the blister pack by manually applying pressure on the recesses whereby an opening is formed in the sheet at the place of the recess. The tablet or capsule can then be removed via said opening.

It may be desirable to provide a memory aid on the kit, e.g., in the form of numbers next to the tablets or capsules whereby the numbers correspond with the days of the regimen that the tablets or capsules so specified should be ingested. Another example of such a memory aid is a calendar printed on the card, e.g., as follows "First Week, Monday, Tuesday, . . . etc. . . . Second Week, Monday, Tuesday," etc. Other variations of memory aids will be readily apparent. A "daily dose" can be a single tablet or capsule or several pills or capsules to be taken on a given day. Also, a daily dose of a neoglycoside composition can consist of one tablet or capsule, while a daily dose of the second compound can consist of several tablets or capsules and vice versa. The memory aid should reflect this and assist in correct administration.

In another embodiment of the present invention, a dispenser designed to dispense the daily doses one at a time in the order of their intended use is provided. Preferably, the dispenser is equipped with a memory aid, so as to further facilitate

compliance with the dosage regimen. An example of such a memory aid is a mechanical counter, which indicates the number of daily doses that have been dispensed. Another example of such a memory aid is a battery-powered micro-chip memory coupled with a liquid crystal readout, or audible reminder signal which, for example, reads out the date that the last daily dose has been taken and/or reminds one when the next dose is to be taken.

A neoglycoside composition, optionally comprising other pharmaceutically active compounds, can be administered to a patient either orally, rectally, parenterally, (for example, intravenously, intramuscularly, or subcutaneously) intracisternally, intravaginally, intraperitoneally, intravesically, locally (for example, powders, ointments or drops), or as a buccal or nasal spray. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

Parenteral administration of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a human and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration includes subcutaneous, intraperitoneal, intravenous, intraarterial, intramuscular, or intrasternal injection and intravenous, intraarterial, or kidney dialytic infusion techniques.

Compositions suitable for parenteral injection comprise the active ingredient combined with a pharmaceutically acceptable carrier such as physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions, or emulsions, or may comprise sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents, or vehicles include water, isotonic saline, ethanol, polyols (propylene glycol, polyethylene glycol, glycerol, and the like), suitable mixtures thereof, triglycerides, including vegetable oils such as olive oil, or injectable organic

esters such as ethyl oleate. 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/or by the use of surfactants. Such formulations can be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations can be prepared, packaged, or sold in unit dosage form, such as in ampules, in multi-dose containers containing a preservative, or in single-use devices for auto-injection or injection by a medical practitioner.

Formulations for parenteral administration include suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations can further comprise one or more additional ingredients including suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e. powder or granular) form for reconstitution with a suitable vehicle (e.g. sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition. The pharmaceutical compositions can be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution can be formulated according to the known art, and can comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations can be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butanediol, for example. Other acceptable diluents and solvents include Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation can comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

These compositions may also contain adjuvants such as preserving, wetting, emulsifying, and/or dispersing agents. Prevention of microorganism contamination of the compositions can be accomplished by the addition of various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of injectable pharmaceutical compositions can be brought about by the use of agents capable of delaying absorption, for example, aluminum monostearate and/or gelatin.

Dosage forms can include solid or injectable implants or depots. In preferred embodiments, the implant comprises an effective amount of an active agent selected from the group consisting of a neoglycoside, a neoglycoside agonist and a neoglycoside antagonist and a biodegradable polymer. In preferred embodiments, a suitable biodegradable polymer can be selected from the group consisting of a polyaspartate, polyglutamate, poly(L-lactide), a poly(D,L-lactide), a poly(lactide-co-glycolide), a poly(ε-caprolactone), a polyanhydride, a poly(beta-hydroxy butyrate), a poly(ortho ester) and a polyphosphazene. In other embodiments, the implant comprises an effective amount of active agent and a silastic polymer. The implant provides the release of an effective amount of active agent for an extended period of about one week to several years.

Solid dosage forms for oral administration include capsules, tablets, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, mannitol, or silicic acid; (b) binders, as for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, or acacia; (c) humectants, as for example, glycerol; (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates, or sodium carbonate; (e) solution retarders, as for example, paraffin; (f) absorption accelerators, as for example, quaternary ammonium compounds; (g) wetting agents, as for example, cetyl alcohol or glycerol monostearate; (h) adsorbents, as for example, kaolin or bentonite; and/or (i) lubricants, as for example, talc, calcium stearate, magnesium

stearate, solid polyethylene glycols, sodium lauryl sulfate, or mixtures thereof. In the case of capsules and tablets, the dosage forms may also comprise buffering agents.

A tablet comprising the active ingredient can, for example, be made by compressing or molding the active ingredient, optionally with one or more additional ingredients. Compressed tablets can be prepared by compressing, in a suitable device, the active ingredient in a free-flowing form such as a powder or granular preparation, optionally mixed with one or more of a binder, a lubricant, an excipient, a surface active agent, and a dispersing agent. Molded tablets can be made by molding, in a suitable device, a mixture of the active ingredient, a pharmaceutically acceptable carrier, and at least sufficient liquid to moisten the mixture. Pharmaceutically acceptable excipients used in the manufacture of tablets include inert diluents, granulating and disintegrating agents, binding agents, and lubricating agents. Known dispersing agents include potato starch and sodium starch glycolate. Known surface active agents include sodium lauryl sulfate. Known diluents include calcium carbonate, sodium carbonate, lactose, microcrystalline cellulose, calcium phosphate, calcium hydrogen phosphate, and sodium phosphate. Known granulating and disintegrating agents include corn starch and alginic acid. Known binding agents include gelatin, acacia, pre-gelatinized maize starch, polyvinylpyrrolidone, and hydroxypropyl methylcellulose. Known lubricating agents include magnesium stearate, stearic acid, silica, and talc.

Tablets can be non-coated or they can be coated using known methods to achieve delayed disintegration in the gastrointestinal tract of a human, thereby providing sustained release and absorption of the active ingredient. By way of example, a material such as glyceryl monostearate or glyceryl distearate can be used to coat tablets. Further by way of example, tablets can be coated using methods described in U.S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874 to form osmotically-controlled release tablets. Tablets can further comprise a sweetening agent, a flavoring agent, a coloring agent, a preservative, or some combination of these in order to provide pharmaceutically elegant and palatable preparation.

Solid dosage forms such as tablets, dragees, capsules, and granules can be prepared with coatings or shells, such as enteric coatings and others well known in the

art. They may also contain opacifying agents, and can also be of such composition that they release the active compound or compounds in a delayed manner. Examples of embedding compositions that can be used are polymeric substances and waxes. The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Solid compositions of a similar type may also be used as fillers in soft or hard filled gelatin capsules using such excipients as lactose or milk sugar, as well as high molecular weight polyethylene glycols, and the like. Hard capsules comprising the active ingredient can be made using a physiologically degradable composition, such as gelatin. Such hard capsules comprise the active ingredient, and can further comprise additional ingredients including, for example, an inert solid diluent such as calcium carbonate, calcium phosphate, or kaolin. Soft gelatin capsules comprising the active ingredient can be made using a physiologically degradable composition, such as gelatin. Such soft capsules comprise the active ingredient, which can be mixed with water or an oil medium such as peanut oil, liquid paraffin, or olive oil.

Oral compositions can be made, using known technology, which specifically release orally-administered agents in the small or large intestines of a human patient. For example, formulations for delivery to the gastrointestinal system, including the colon, include enteric coated systems, based, e.g., on methacrylate copolymers such as poly(methacrylic acid, methyl methacrylate), which are only soluble at pH 6 and above, so that the polymer only begins to dissolve on entry into the small intestine. The site where such polymer formulations disintegrate is dependent on the rate of intestinal transit and the amount of polymer present. For example, a relatively thick polymer coating is used for delivery to the proximal colon (Hardy et al., 1987 Aliment. Pharmacol. Therap. 1:273-280). Polymers capable of providing site-specific colonic delivery can also be used, wherein the polymer relies on the bacterial flora of the large bowel to provide enzymatic degradation of the polymer coat and hence release of the drug. For example, azopolymers (U.S. Pat. No. 4,663,308), glycosides (Friend et al., 1984, J. Med. Chem. 27:261-268) and a variety of naturally available and modified polysaccharides (see PCT application PCT/GB89/00581) can be used in such formulations.

Pulsed release technology such as that described in U.S. Pat. No. 4,777,049 can also be used to administer the active agent to a specific location within the gastrointestinal tract. Such systems permit drug delivery at a predetermined time and can be used to deliver the active agent, optionally together with other additives that my alter the local microenvironment to promote agent stability and uptake, directly to the colon, without relying on external conditions other than the presence of water to provide in vivo release.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs. In addition to the active compounds, the liquid dosage form may contain inert diluents commonly used in the art, such as water or other solvents, isotonic saline, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils, in particular, almond oil, arachis oil, coconut oil, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil, sesame seed oil, MIGLYOL™, glycerol, fractionated vegetable oils, mineral oils such as liquid paraffin, tetrahydrofurfuryl alcohol, polyethylene glycols, fatty acid esters of sorbitan, or mixtures of these substances, and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, demulcents, preservatives, buffers, salts, sweetening, flavoring, coloring and perfuming agents. Suspensions, in addition to the active compound, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol or sorbitan esters, microcrystalline cellulose, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, agar-agar, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, aluminum metahydroxide, bentonite, or mixtures of these substances, and the like. Liquid formulations of a pharmaceutical composition of the invention that are suitable for oral administration can be prepared, packaged, and sold either in liquid form or in the form of a dry product intended for reconstitution with water or another suitable vehicle prior to use.

Known dispersing or wetting agents include naturally-occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g. polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include lecithin and acacia. Known preservatives include methyl, ethyl, or n-propyl-para-hydroxybenzoates, ascorbic acid, and sorbic acid. Known sweetening agents include, for example, glycerol, propylene glycol, sorbitol, sucrose, and saccharin. Known thickening agents for oily suspensions include, for example, beeswax, hard paraffin, and cetyl alcohol.

Liquid solutions of the active ingredient in aqueous or oily solvents can be prepared in substantially the same manner as liquid suspensions, the primary difference being that the active ingredient is dissolved, rather than suspended in the solvent. Liquid solutions of the pharmaceutical composition of the invention can comprise each of the components described with regard to liquid suspensions, it being understood that suspending agents will not necessarily aid dissolution of the active ingredient in the solvent. Aqueous solvents include, for example, water and isotonic saline. Oily solvents include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin.

In other embodiments, the pharmaceutical composition can be prepared as a nutraceutical, i.e., in the form of, or added to, a food (e.g., a processed item intended for direct consumption) or a foodstuff (e.g., an edible ingredient intended for incorporation into a food prior to ingestion). Examples of suitable foods include candies such as lollipops, baked goods such as crackers, breads, cookies, and snack cakes, whole, pureed, or mashed fruits and vegetables, beverages, and processed meat products. Examples of suitable foodstuffs include milled grains and sugars, spices and other seasonings, and syrups. The polypeptide compositions described herein are preferably not exposed to high cooking temperatures for extended periods of time, in order to minimize degradation of the compounds.

Compositions for rectal or vaginal administration can be prepared by mixing a neoglycoside and any additional compounds with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax, which are solid at ordinary room temperature, but liquid at body temperature, and therefore, melt in the rectum or vaginal cavity and release the neoglycoside. Such a composition can be in the form of, for example, a suppository, a retention enema preparation, and a solution for rectal or colonic irrigation. Suppository formulations can further comprise various additional ingredients including antioxidants and preservatives. Retention enema preparations or solutions for rectal or colonic irrigation can be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is known in the art, enema preparations can be administered using, and can be packaged within, a delivery device adapted to the rectal anatomy of a human. Enema preparations can further comprise various additional ingredients including antioxidants and preservatives.

A pharmaceutical composition of the invention can be prepared, packaged, or sold in a formulation suitable for vaginal administration. Such a composition can be in the form of, for example, a suppository, an impregnated or coated vaginally-insertable material such as a tampon, a douche preparation, or a solution for vaginal irrigation.

Dosage forms for topical administration of a neoglycoside include ointments, powders, sprays and inhalants. The compounds are admixed under sterile conditions with a physiologically acceptable carrier, and any preservatives, buffers, and/or propellants that may be required. Formulations suitable for topical administration include liquid or semi-liquid preparations such as liniments, lotions, oil-in-water or water-in-oil emulsions such as creams, ointments or pastes, and solutions or suspensions. Topically-administrable formulations can, for example, comprise from about 0.1% to about 10% (w/w) active ingredient, although the concentration of the active ingredient can be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration can further comprise one or more of the additional ingredients described herein.

Ophthalmic formulations, eye ointments, powders, and solutions are also contemplated as being within the scope of this invention. Such formulations can, for

example, be in the form of eye drops including, for example, a 0.1-1.0% (w/w) solution or suspension of the active ingredient in an aqueous or oily liquid carrier. Such drops can further comprise buffering agents, salts, or one or more other of the additional ingredients described herein. In other embodiments, ophthalmalmically administrable formulations comprise the active ingredient in microcrystalline form or in a liposomal preparation.

A pharmaceutical composition of the invention can be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation can comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 to about 7 nanometers, and preferably from about 1 to about 6 nanometers. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant can be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient dissolved or suspended in a low-boiling propellant in a sealed container. Preferably, such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. More preferably, at least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. Dry powder compositions preferably include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

Low boiling propellants generally include liquid propellants having a boiling point below 65 degrees F. at atmospheric pressure. Generally the propellant can constitute 50 to 99.9% (w/w) of the composition, and the active ingredient can constitute 0.1 to 20% (w/w) of the composition. The propellant can further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid diluent (preferably having a particle size of the same order as particles comprising the active ingredient).

Pharmaceutical compositions of the invention formulated for pulmonary delivery can also provide the active ingredient in the form of droplets of a solution or

suspension. Such formulations can be prepared, packaged, or sold as aqueous or dilute alcoholic solutions or suspensions, optionally sterile, comprising the active ingredient, and can conveniently be administered using any nebulization or atomization device. Such formulations can further comprise one or more additional ingredients including a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a preservative such as methylhydroxybenzoate. The droplets provided by this route of administration preferably have an average diameter in the range from about 0.1 to about 200 nanometers.

The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a pharmaceutical composition of the invention. Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 to 500 micrometers. Such a formulation is administered in the manner in which snuff is taken i.e. by rapid inhalation through the nasal passage from a container of the powder held close to the nares. Formulations suitable for nasal administration can, for example, comprise from about as little as 0. 1% (w/w) and as much as 100% (w/w) of the active ingredient, and can further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention can be prepared, packaged, or sold in a formulation suitable for buccal administration. Such formulations can, for example, be in the form of tablets or lozenges made using conventional methods, and can, for example, comprise 0.1 to 20% (w/w) active ingredient, the balance comprising an orally dissolvable or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration can comprise a powder or an aerosolized or atomized solution or suspension comprising the active ingredient. Such powdered, aerosolized, or atomized formulations, when dispersed, preferably have an average particle or droplet size in the range from about 0.1 to about 200 nanometers, and can further comprise one or more of the additional ingredients described herein.

For parenteral administration in non-human animals, the compound or compounds may be prepared in the form of a paste or a pellet and administered as an

implant, usually under the skin of the head or ear of the animal. Paste formulations can be prepared by dispersing a compound or compounds in pharmaceutically acceptable oil such as peanut oil, sesame oil, corn oil or the like. Pellets containing a therapeutically effective amount of a compound or compounds can be prepared by admixing the compound with a diluent such as a carbowax, carnauba wax, and the like, and a lubricant, such as magnesium or calcium stearate, can be added to improve the pelleting process. It is, of course, recognized that more than one pellet may be administered to an animal to achieve the desired dose level. Moreover, it has been found that such implants may also be administered periodically during the animal treatment period in order to maintain the proper active agent level in the animal's body.

The neoglycoside of the present invention, the stereoisomers and prodrugs thereof, and the pharmaceutically acceptable salts of the peptides, stereoisomers, and prodrugs, can be administered to a patient at dosage levels in the range of from about 0.01 to about 1,000 mg per day. For a normal adult human having a body weight of about 70 kg, a dosage in the range of from about 0.01 to about 300 mg is typically sufficient. However, some variability in the general dosage range may be required depending upon the age and weight of the subject being treated, the intended route of administration, the particular agent being administered and the like. The determination of dosage ranges and optimal dosages for a particular patient is well within the ability of one of ordinary skill in the art having the benefit of the instant disclosure. It is also noted that the compounds of the present invention can be used in sustained release, controlled release, and delayed release formulations, which forms are also well known to one of ordinary skill in the art.

It is not critical whether the compound is administered directly to the cell, to a tissue comprising the cell, a body fluid that contacts the cell, or a body location from which the compound can diffuse or be transported to the cell. It is sufficient that the compound is administered to the patient in an amount and by a route whereby an amount of the compound sufficient to mobilize lipids in the cell arrives, directly or indirectly at the cell. The minimum amount varies with the identity of the neoglycoside. In some embodiments, the minimum amount is generally in the range

from 10^{-9} to 10^{-5} molar. In other embodiments, the minimum amount is typically in the range from 10^{-7} to 10^{-5} molar.

In preferred embodiments, a pharmaceutical composition comprising a neoglycoside can be administered to a patient at dosage levels in the range of about 0.1 to about 7,000 mg per day. A preferred dosage range is about 1 to about 100 mg per day. In other embodiments, a pharmaceutical composition comprising a neoglycoside can be administered to deliver a dose of between 1 nanogram per day per kilogram body weight and 100 milligrams per day per kilogram body weight, preferably from about 0.1 to about 10 mg/kg body weight of the individual per day, and preferably to deliver of between 100 milligrams and 2 grams, to a human patient.

The specific dosage and dosage range that can be used depends on a number of factors, including the requirements of the patient, the severity of the condition or disease being treated, and the pharmacological activity of the compound being administered. The determination of dosage ranges and optimal dosages for a particular patient is well within the ordinary skill of one in the art in view of this disclosure. It is understood that the ordinarily skilled physician or veterinarian will readily determine and prescribe an effective amount of the compound to mobilize lipid stores, induce weight loss, or inhibit appetite in the patient. In so proceeding, the physician or veterinarian can, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. It is further understood, however, that the specific dose level for any particular human will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the human, the time of administration, the route of administration, the rate of excretion, any drug combination, and the severity of any disorder being treated.

In some embodiments, a neoglycoside of the present invention, a stereoisomer or prodrug thereof, or a pharmaceutically acceptable salt of the stereoisomer or prodrug; is administered to a subject in need of treatment therewith, preferably in the form of a pharmaceutical composition. It is generally preferred that such administration be oral or pulmonary. However, if the subject being treated is unable

to swallow, or oral administration is otherwise impaired or undesirable, parenteral or transdermal administration will be appropriate.

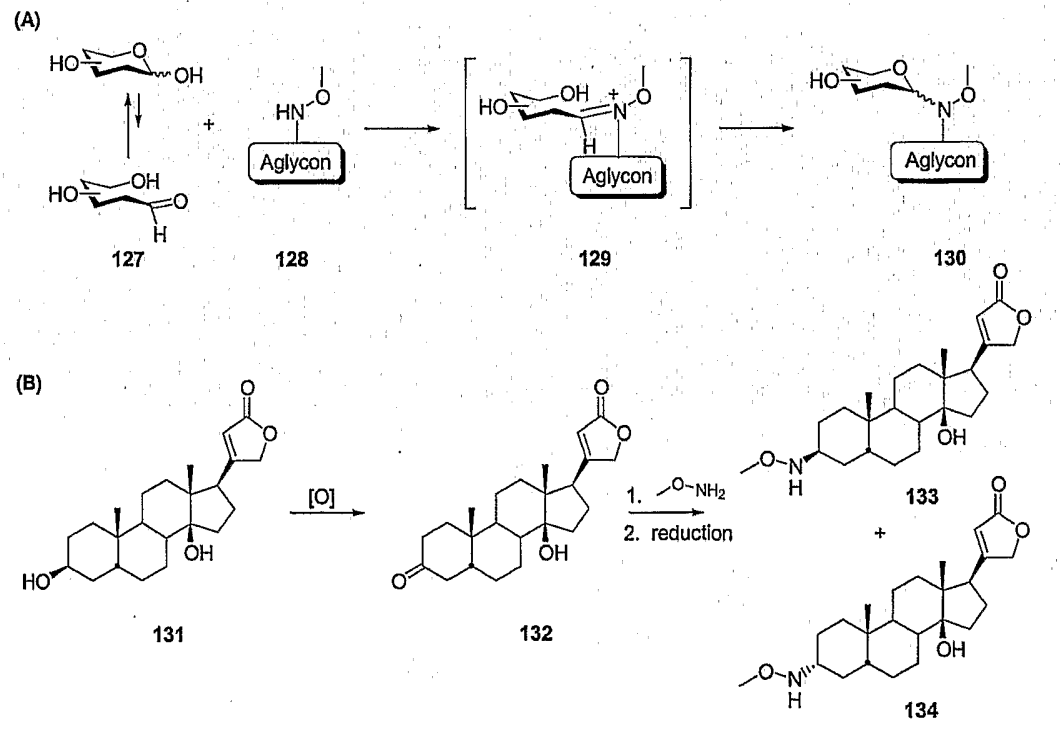
Chemoselective ligation reactions are means for expanding natural product sugar diversity. This approach is particularly attractive to complex natural products as chemoselective ligation offer advantages similar to those of enzymatic reactions (efficiency, regio- and stereospecificity), with the advantage of a much broader range of coupling partners. See Hang, H.C.; Bertozzi, C.R. Chemoselective approaches to glycoprotein assembly. Acc. Chem. Res. 2001, 34, 727-736; Kolb, H.C., et al.,. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. Angew. Chem. Int. Ed. 2001, 40, 2004-2021; Langenhan, J.M.; Fu, X.; Thorson, J.S. Chemoselective ligation reactions for in vitro glycorandomization. Curr. Org. Syn. 2004, manuscript in press.

In the context of sugars (*e.g.* an aldose, **127**, see below), one well known reaction is that between a free aldose and an aminooxy functionalized molecule to specifically provide the sugar oxime without the requirement of protecting groups or anomeric activation. This reaction, when using a reacting unit bearing a 'primary' –O-NH_2 group, leads to the open-chain sugar oxime. However, it was recently reported that the cyclic form of the sugar is restored when a 'secondary' hydroxylamino group (R-O-NH-R') is used (*e.g.* **128** to **130**). See Langenhan, J.M., et al., 2004; Peri, F.; Nicotra, F. Chemoselective ligation in glycochemistry. Chem. Comm. 2004, 623. This strategy has been employed in coupling simple sugars to peptides (termed 'neoglycopeptides'). See Peri, F.; Dumy, P.; Mutter, M. Chemo- and stereoselective glycosylation of hydroxylamino derivatives: a versatile approach to glycoconjugates. Tetrahedron 1998, 54, 12269; Carrasco, M.R. , et. al., Synthesis of neoglycopeptides by chemoselective reaction of carbohydrates with peptides containing a novel N'-methyl-aminooxy amino acid. Tetrahedron Lett. 2002, 43, 5727; Carrasco, M.R. , et. al., Synthesis of N-Fmoc-O-(N'-Boc-N'-methyl)-aminohomoserine, an amino acid for the facile preparation of neoglycopeptides. J. Org. Chem. 2003, 68, 195; Carrasco, M.R.; Brown, R.T. A versatile set of aminooxy amino acids for the synthesis of neoglycopeptides. J. Org. Chem. 2003, 68, 8853. More recently, the approach has

been used to generate a small set of di- and trisaccharides (termed 'neoglycosides'). Peri, F., et. al., Solution and solid-phase chemoselective synthesis of 1-6-amino(methoxy) di- and trisaccharide analogues. Chem. Comm. 2002, 1504; Peri, F.; Nicotra, F. Chemoselective ligation in glycochemistry. Chem. Comm. 2004, 623.

In examples using Glc or GlcNAc as the sugar donors, the product was found to favor the β-neogycoside (α:β= 7:1) and characterization/modeling via NMR spectroscopy, *ab initio*, molecular mechanics and molecular dynamics methods revealed the neoglycosides to exhibit only slight conformational distortion in comparison to the corresponding O-glycosides (Peri, F., et. al.,Synthesis and conformational analysis of novel N(OCH3)-linked disaccharide analogues. Chem. Eur. J. 2004, 10, 1433.) As a compliment to the existing glycorandomization platforms, the ease of neoglycoconjugate formation coupled with the structural similarities to parent O-glycosides suggest this method as another attractive route to the rapid exploration of sugar variation upon various natural product scaffolds.

Scheme 1



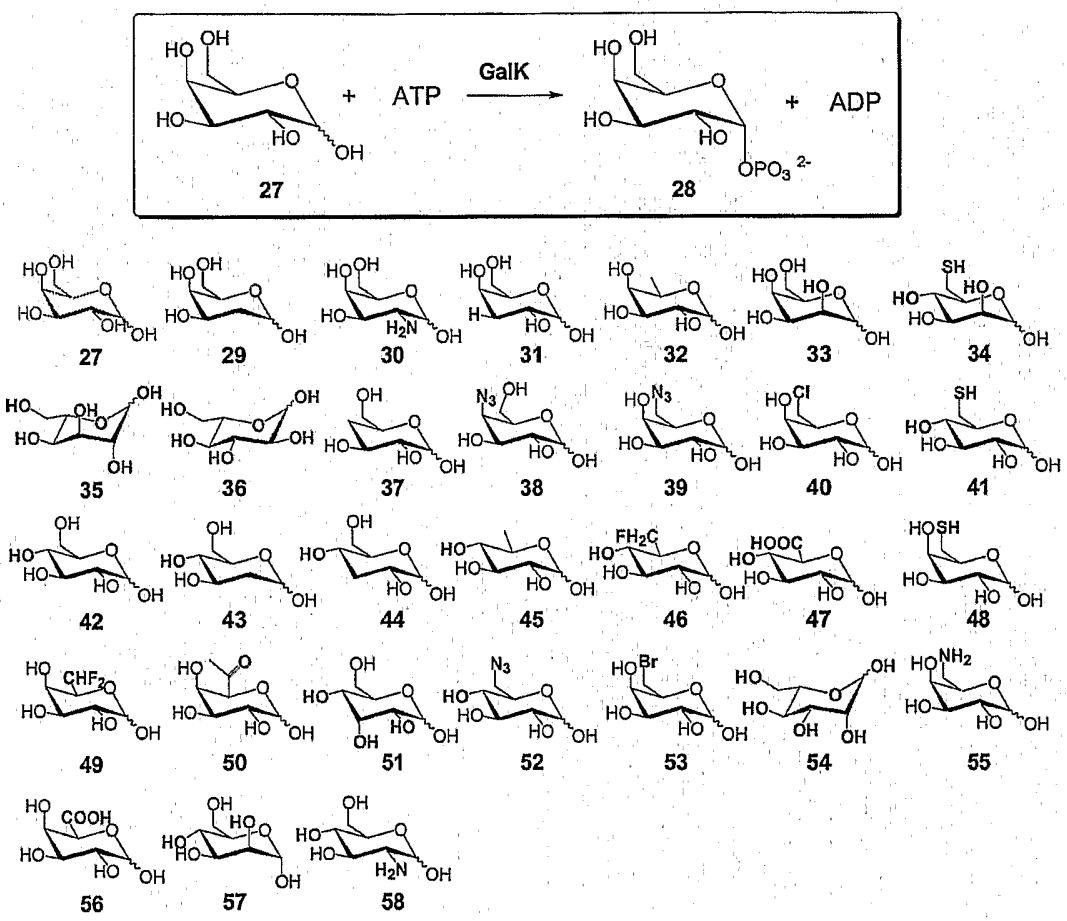
Digitoxin Neoglycorandomization.

To assess the application of this strategy toward natural product 'neoglycorandomization' (the term based upon an extension of existing nomenclature), the simple model natural product aglycon digitoxigenin was selected. Digitalis (mainly digitoxin and digoxin, extracts from *Digitalis purpurea* and *Digitalis lanata* used clinically, respectively) has been used as a cardiac drug for more than 200 years. The cardiac glycosides contain a steroid nucleus and unsaturated lactone (together referred to as the aglycon) substituted with a carbohydrate(s) – the general role of the latter of which is attributed primarily to absorption and pharmacokinetics. In addition, digitalis is known to block cell proliferation, induce apoptosis in different malignant cell lines, signal through the pathways of epidermal growth factor receptor (EGFR), and these compelling links to anticancer activities can, in part, be modulated by saccharide substitutions.

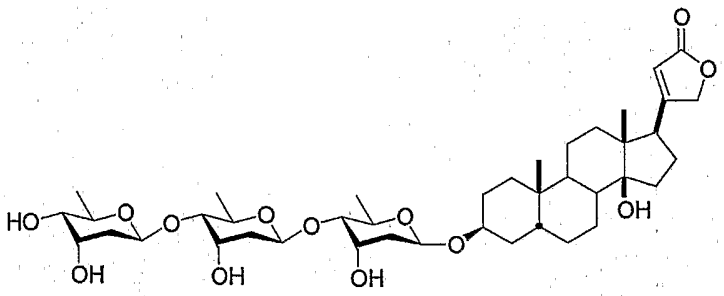
Commercially available digitoxigenin **131** was converted to ketone **132**, as shown above (81% yield) using Jones oxidation (reaction conditions: 0.742 mmol **131**, 31 mL acetone, cool to 0 °C, Jones agent added dropwise until orange color persisted, quenched with MeOH after 20 min).[234] Ketone **132** was subsequently reacted with methoxylamine in the presence of pyridine in methanolic solution to afford a mixture of E and Z oximes in quantitative yield (reaction conditions: 0.219 mmol **132** dissolved in 0.5 mL MeOH, 2.2. eq. pyridine, then $MeONH_2 \cdot HCl$ added, 30 min).[235] A variety of reducing agents were examined for oxime reduction, including K-selectride, $NaBH_3CN$, pyridine-borane complex and *t*-butylamine-borane complex, with the latter providing the desired methoxylamines **133** and **134** in the desired quantitative production of a 50:50 ratio (reaction conditions: 0.159 mm oxime suspended in 0.23 mL EtOH, 1 mL dioxane, cooled to 0 °C, added 3.3. eq. borane complex, then 0.43 mL 10% aq. HCl solution, 1 hr). Diastereomers **133** and **134** were easily separated using standard chromatography (EtOAc:hexane 3:2 followed by 100% EtOAc for the second product). As a test for neoglycorandomization, methoxylamine **133** was subsequently reacted with sugars **27-58**, as shown below, (0.1 mmol 133, 2 eq. aldose, DMSO, 50 °C, 12 hr) to provide > 70% product yield for

25 of the 31 sugars examined based upon LC-MS.[236] Of the 31 cardiac glycoside variants generated in this proof of concept demonstration, those deriving from **38**, **39** and **52** present the opportunity for further diversification via Huisgen 1,3-dipolar cycloaddition, while those deriving from **34**, **41** and **48** present handles for rapid alkylation.

Scheme 2

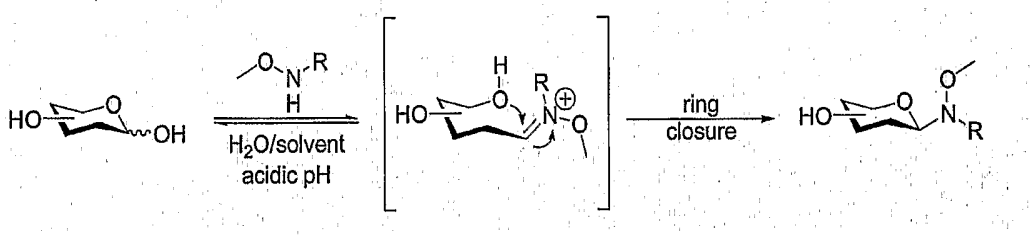


For example, the simple structure of digitoxin allows the easy installation of a reactive chemical handle, as shown below:



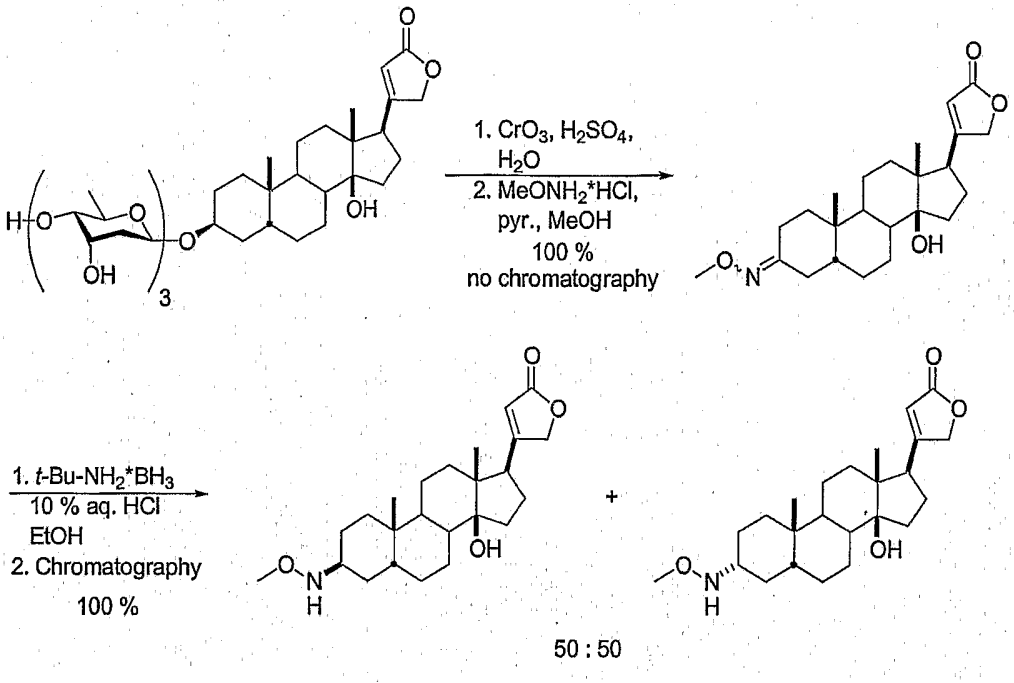
Studies have suggested that digitoxin and/or carbohydrate-altered digitoxin derivatives may display anti-cancer activities (Haux, J. Med. Hypotheses 2002, 59, 781; Stenkvist, B. Anti-Cancer Drugs 2002, 12, 635). In order to obtain these digitoxin derivatives, chemoselective ligation is done using methoxyamino group. The methoxyamino groups are reacted with free sugars to form closed-ring neoglycosides (unlike R-ONH_2 groups which form predominately open-chain sugar oximes). The ligations are high yielding and often stereoselective.

Scheme 3

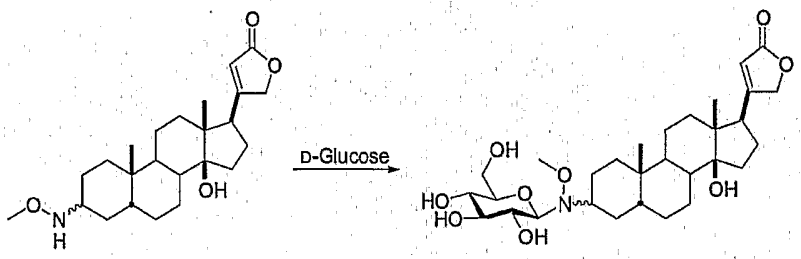


Installation of methoxyamino group occurs quantitatively. Isomers may be assigned via x-ray crystallography. The one-step digitoxin to digitoxigenone conversion (step 1) as shown below:

Scheme 4



The effect of different ligation conditions were evaluated for D-glucose as shown below:

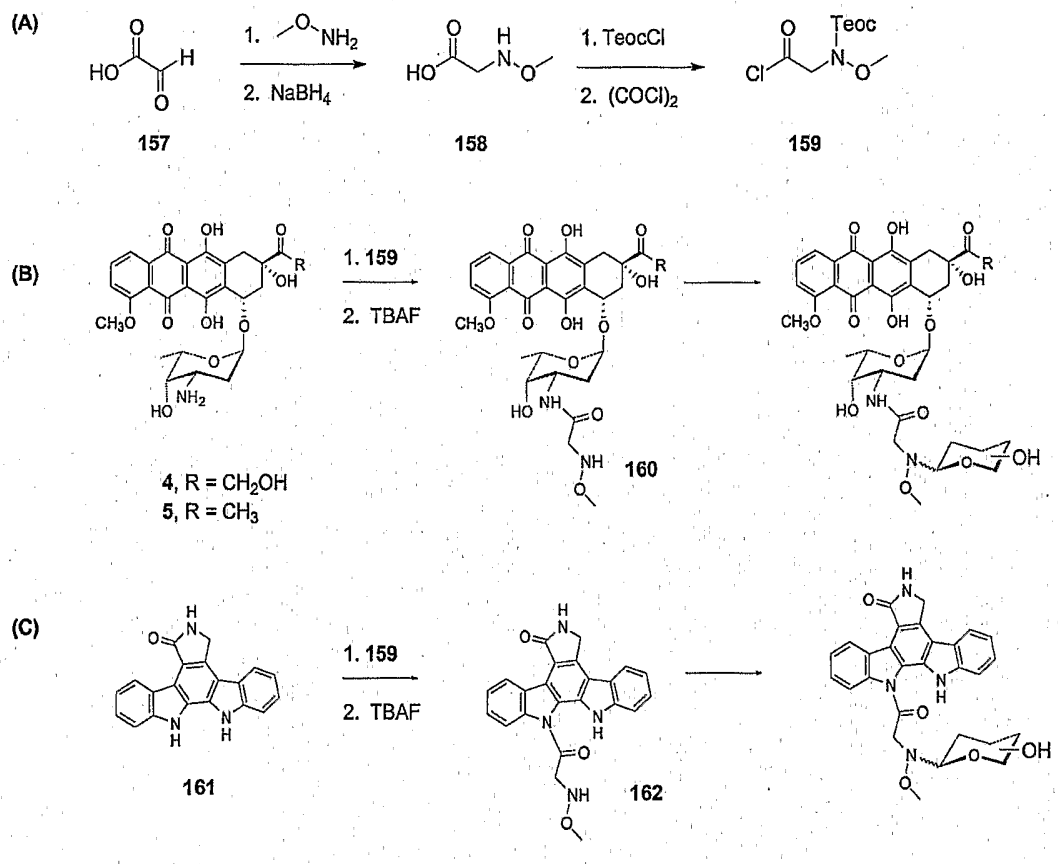


isomer	DMF/AcOH ratio	eq. glucose	temp (°C)	agitation method	% conv. by NMR
R	3:1	1.05	60	stirring	70 %
S	3:1	1.05	60	stirring	74 %
S	3:1	2	rm temp	shaking	65 %
S	3:1	2	60	shaking	71 %
S	1:1	2	60	shaking	~24 %

5

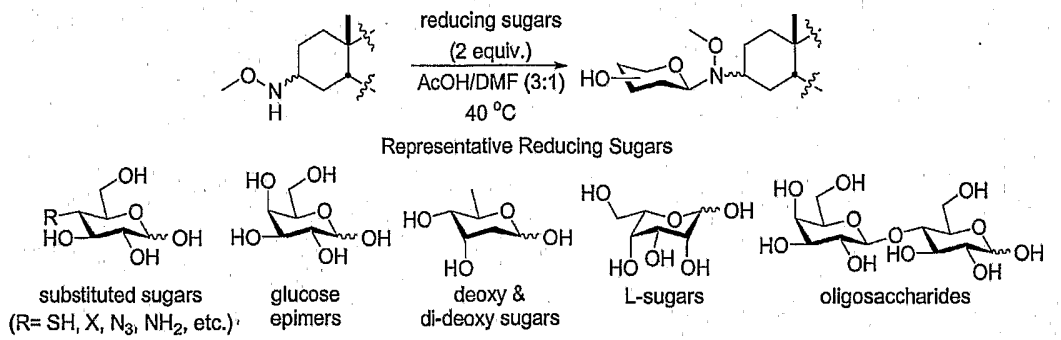
Neoglycorandomization Strategies – Amine Modification (Indolocarbazoles and Anthracyclines). As an expansion of neoglycorandomization, a second complimentary handle was installed to target amines within natural products. Specifically, amine-handle **159,** as shown below was synthesized as shown below and used to acylate the daunosamine sugar within **4** and **5** as well as the indole nitrogen(s) in various indolocarbazole aglycons (e.g. exemplified by the staurosporine aglycon **161**). Literature precendent exists for acylation of both daunosamine (Ingallinella, P., et. al., A New Method for Chemoselective Conjugation of Unprotected Peptides to Dauno- and Doxorubicin. Bioorg. Med. Chem. Lett. 2001, 11, 1343-1346 and indolocarbazole nitrogens[16] with diacylation, in some cases of the latter, observed in the presence of excess acylating agent. The predicted outcome for this approach is identical to that described for carbonyl installation with the exception that the parent methoxylamino-installed natural product (e.g. **160** and **162**) consists as a single species (versus diastereomers from oxime reduction). Thus, initial library size is estimated at ~150 derivatives for each indolocarbazole and anthracycline input.

Scheme 5



The methoxyamino-aglycon may be reacted with a collection of commercially available and synthetic reducing saccharides. (See below) Some of these sugars may contain orthogonal chemoselective ligation handles allowing further diversification. Such reactions may be run and purified in parallel.

Scheme 6



There are numerous advantages to practicing this invention, including potential automated synthesis of glycoconjugates, especially since this methodology is very systematic and high yielding.

Further, the invention has potential to be coupled to a solid support for further automation. Such solid supports and mechanism for using these supports are well known in the art. For example, a conjugate with protected handle-bearing carbohydrate may be deprotected such that a next carbohydrate may be added. This process may allow iterative cycles between deprotection, extension and congugation, and could be applied to any glycoconjugate (peptides, proteins, oligosaccharides, nucleic acids, small molecules, etc.).

This deprotection, extension and congugation iterative cycle could also be used specifically to extend natural product glyconjugates thereby generating oligosaccharide-substituted natural products (*i.e.* small molecules, metabolites, etc). In addition, approaches toward oligosaccharide-based bioactive secondary metabolites (*i.e.* natural products, small molecules) would benefit by this chemistry – notably, aminoglycosides, orthosomycins (evernimicin, avilamycin) and saccharomicins as representative examples.

Further, since this chemistry is amenable to certain physiological conditions (*e.g.* acidic tissues and/or cellular locales) 'scavenging' of carbohydrates *in vivo* may be possible. In placing the handle upon an appropriate carrier aglycon (*i.e.* natural

product, metabolite, small molecule), one of ordinary skill in the art can potentially selectively starve certain cells of energy.

The chemistry is not limited to only the digitoxin, indolocarbazole or anthracycline compounds – all carbonyls, amines and potentially hydroxyls are accessible, and contemplated to be within the scope of the present invention. Furthermore, this chemistry is not only limited to those carbohydrates explicitly shown herein but any reducing sugar. Especially given the selectivity towards reducing sugars, this chemistry is amenable to assaying reducing sugar concentrations and therefore, amenable to assaying any sugar-utilizing enzyme/system in which reducing sugar concentrations change.

In an effort to explore the contribution of the sugar constituents of pharmaceutically relevant glycosylated natural products, chemoenzymatic "glycorandomization" methods have been developed (FIG. 1A, path B) to rapidly convert a single aglycon structure into a library of analogs with a broad array of sugar attachments (9, 10). Despite these advances, chemoenzymatic glycorandomization currently excludes a number of essential glycoconjugates since it is limited to natural products for which promiscuous glycosyltransferases are available and can operate *in vitro*. This complementary robust chemical approach–referred to as "neoglycorandomization"– accomplishes a one step sugar ligation which does not require any prior sugar protection or activation (FIG. 1A, path A). Using digitoxin as a simple pharmaceutically-relevant model, neoglycorandomization leads to the discovery of digitoxin analogs that are much more potent and/or tumor-specific cytotoxins, but less potent Na^+/K^+-ATPase inhibitors, relative to the parent natural product. Thus, neoglycosylation is useful as a general tool for glycobiology and drug discovery. The studies also highlight a potentially divergent relationship between Na^+/K^+-ATPase inhibition and cytotoxicity of cardiac glycosides.

Neoglycorandomization is based upon the chemoselective formation of glycosidic bonds between reducing sugars and a secondary alkoxylamine-containing aglycon to form "neoglycosides" (FIG. 1A, path A). The notable advantage of this approach is, unlike most traditional chemical glycosylation reactions, unprotected and non-activated reducing sugars are used as sugar donors in the reaction under mild

conditions (Van Vranken, D.L.; Chisolm, J.D. (2000) J. Org. Chem. 65, 7541-7553). In early examples of this chemoselective reaction, sugars and peptides that contain secondary alkoxylamines were reacted with D-glucose, D-mannose, D-galactose, lactose, and D-*N*-acetylglucosamine to generate oligosaccharide and glycopeptide mimics, respectively (Peri, F., et al., (2002) Chem. Comm. 1504-1505; Carrasco, M.R.; Brown, R.T. (2003) J. Org. Chem. 68, 8853-8858). These pioneering studies revealed that, unlike primary alkoxylamines which provide open-chain oxime isomers (Cervigni, S.E., et al., (1996) *Angew. Chem. Int. Ed.* **35**, 1230-1232), secondary alkoxylamines react to form closed-ring neoglycosides (Fig. 1B). Although the stability of these model neoglycosides was not examined, the distribution of pyranose, and occasionally furanose, anomers in neoglycosides was found to be dependant on the identity of the sugar (Peri, F., et al., (1998) Tetrahedron 54, 12269-12278) and, equilibration between the product isomers is sometimes observed. Closed-ring neoglycosides were found to display conformational behavior similar to natural *O*-glycosides by NMR studies, molecular dynamics simulations, and *ab initio* calculations (Peri, F., et al., (2004) *Chem. Eur. J.* **10**, 1433-1444).

Aglycon Synthesis. Compounds **2a,b**, **3β**, and **3α** were synthesized according to procedures described below.

Digitoxigenone oximes (2a,b). Jones reagent was prepared by mixing CrO_3 (62.4 g), H_2SO_4 (55.2 mL), and water (170 mL). This reagent was slowly added to an Erlynmeyer flask containing digitoxin (29.67 g, 38.8 mmol) suspended in acetone (1300 mL) at 0 °C. The resulting mixture was mechanically stirred for 3 h at rm temp. The mixture was then cooled to 0 °C, quenched with ~ 100 mL MeOH, stirred for 20 min, and 100 mL water was added. Volatile solvents were removed under reduced pressure, and the aqueous mixture was extracted with chloroform (4 x 200 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$, 2 times with water, dried over Na_2SO_4, filtered, then concentrated. The product ketone digitoxigenone (9.48 g, 66 % yield), obtained as a white foam (TLC $R_f = 0.23$ in 3:2 EtOAc/hexane), was used without further purification. ^1H NMR ($CDCl_3$, 400 MHz) δ 5.89 (s, 1H), 5.03 (A of ABX, 1H, $J = 18.2, 1.5$), 4.94 (B of ABX, 1H, $J = 18.2, 1.7$), 2.82 (m, 1H), 2.65 (dd, 1H, $J = 14.5$), 2.37 (td, 1H, $J = 14.8, 5.4$), 2.17 (m, 4H), 2.04 (m, 2H), 1.96-1.73

(m, 6H), 1.67 (m, 1H), 1.61-1.23 (m, 7H), 1.02 (s, 3H), 0.92 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 12.9, 175.0, 174.7, 117.5, 85.0, 77.4, 73.6, 50.8, 49.7, 43.7, 42.1, 41.4, 39.7, 37.1, 36.7, 36.5, 35.2, 33.0, 26.9, 26.5, 22.5, 21.2, 20.9, 15.8; Electrospray ionization-MS *m/z* (M + H) calculated for $C_{23}H_{33}O_4$ 373.5, observed 373.2. Digitoxigenone (9.48 g, 25.5 mmol) was dissolved in methanol (57 mL) and pyridine (4.5 mL, 55.9 mmol). Methoxylamine hydrochloride (3.40 g, 0.7 mmol) was added, and the solution was stirred for 30 min then concentrated. The resulting residue was dissolved in CH_2Cl_2 and washed with 1 M HCl, brine, dried over MgSO4, filtered, and then concentrated. The desired mixture of oxime diastereomers **2a,b** (TLC R_f = 0.49 and 0.39 in 3:2 EtOAc/hexane), obtained as a white crust (9.30 g, 91 % yield), was used without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.88 (br t, 1H), 5.03-4.90 (m, 1H), 4.85-4.80 (m, 1H), 3.82 (s, 1. 3H), 3.81 (s, 1.7H), 3.01 (br d, 0.6H, *J* = 14.9), 2.87-2.77 (m, 1.5H), 2.45 (t, 0.6H, *J* = 13.9), 2.19-1.11 (m, 23H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 174.8, 174.7, 160.4, 60.3, 117.7, 85.4, 77.4, 73.6, 61.1, 50.9, 49.7, 43.5, 41.8, 41.7, 39.9, 36.8, 36.5, 36.2, 35.8, 35.7, 35.6, 33.1, 32.0, 27.0, 26.9, 26.5, 25.6, 23.0, 22.9, 21.2, 21.1, 20.5, 15.8; Electrospray ionization-MS *m/z* (M + H) calculated for $C_{24}H_{36}NO_4$ 402.5, observed 402.3.

Aglycons 3*β* and 3*α*. Oximes **2a,b** (539 mg, 1.34 mmol) were suspended in ethanol (1.9 mL) and dioxane (5 mL), then cooled to 0 °C. Borane *tert*-butylamine complex (385 mg, 4.43 mmol) was added, followed by the dropwise addition 10 % aq. HCl (3.6 mL). The reaction mixture was stirred at 0 °C for 2.5 hours. After this time, Na_2CO_3 was added until gas evolution ceased, and the mixture was partitioned between sat. aq. $NaHCO_3$ and CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude reaction mixture was purified via SiO_2 column chromatography eluting with 3:2 EtOAc/hexane to elute **3*β*** (TLC R_f = 0.33 in 3:2 EtOAc/hexane) and then with 100 % EtOAc to elute **3*α*** (TLC R_f = 0.09 in 3:2 EtOAc/hexane). Aglycon **3*β*** was obtained as a foam (137 mg, 25 % yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.88 (s, 1H), 4.99 (A of ABX, 1H, *J* = 18.0, 1.6), 4.81 (B of ABX, 1H, *J* = 18.0, 1.5), 3.55 (s, 3H), 3.26 (br s, 1H), 2.79 (m, 1H), 2.15 (m, 2H), 1.85 (m, 3H), 1.74-1.22 (m, 17H), 0.94 (s, 3H), 0.87 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 174.7, 174.6, 117.8, 85.7, 73.6, 62.6, 55.1, 51.1, 49.7, 42.0, 40.1, 36.7, 35.8, 35.7, 33.3, 30.5, 28.8, 27.0, 26.7, 23.9, 22.9, 21.3, 21.2, 15.9; Electrospray ionization-

MS *m/z* (M + H) calculated for $C_{24}H_{38}NO_4$ 404.6, observed 404.4. Aglycon **3*α*** was obtained as a white powder (227 mg, 44 % yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.86 (s, 1H), 4.98 (A of ABX, 1H, *J* = 18.1, 1.4), 4.80 (B of ABX, 1H, *J* = 18.1, 1.7), 3.55 (s, 3H), 2.91 (tt, 1H, *J* = 11.1, 3.9), 2.76 (m, 1H), 2.15 (m, 2H), 1.84 (m, 3H), 1.86-1.17 (m, 17H), 0.93 (s, 3H), 0.87 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 175.2, 174.6, 117.3, 85.1, 73.5, 62.5, 60.3, 50.8, 49.6, 41.8, 41.5, 39.8, 36.1, 35.3, 35.0, 33.0, 30.9, 27.0, 26.8, 25.3, 23.4, 21.5, 20.8, 15.7; Electrospray ionization-MS *m/z* (M + H) calculated for $C_{24}H_{38}NO_4$ 404.6, observed 404.4.

Neoglycoside 4*β*. Aglycon **3*β*** (18.4 mg, 45.6 μmol) and D-glucose (8.6 mg, 47.9 μmol) were dissolved in 3:1 DMF/AcOH (500 μL) and stirred at 60 °C for 48 h. The crude reaction mixture was concentrated, and examined by ^{1}H NMR to reveal a **4*β*:3*β*** ratio of 7:3 (70 % crude yield). Neoglycoside **4*β*:** (TLC R_f = 0.27 in 20 % EtOH/$CHCl_3$) ^{1}H NMR (CD_3OD, 500 MHz) δ 5.90 (s, 1H), 5.03 (A of ABX, 1H, *J* = 18.3, 1.1), 4.92 (B of ABX, 1H, *J* = 18.3, 1.4), 4.09 (d, 1H, *J* = 8.8), 3.81 (A of ABX, 0.5H, *J* = 12.0, 1.9), 3.72 (s, 3H), 3.66 (B of ABX, 0.5H, *J* = 12.0, 5.1), 3.59 (t, 1H, *J* = 9.0), 3.44 (br s, 1H), 3.40-3.30 (m, 2H), 3.15 (m, 1H), 2.84 (m, 1H), 2.19 (m, 2H), 2.01 (td, 1H, *J* = 14.8, 2.8), 1.88-1.44 (m, 15H), 1.27 (m, 5H), 1.00 (s, 3H), 0.89 (s, 3H); Electrospray ionization-MS *m/z* (M + H) calculated for $C_{30}H_{48}NO_9$ 566.7, observed 566.4.

Neoglycoside 4*α*. Aglycon **3*α*** (34.0 mg, 84.3 μmol) and D-glucose (15.9 mg, 88.5 μmol) were dissolved in 3:1 DMF/AcOH (940 μL) and stirred at 60 °C for 48 h. The crude reaction mixture was concentrated, and examined by ^{1}H NMR to reveal a **4*α*:3*α*** ratio of 37:50 (74 % crude yield).

Neoglycoside **4*α*:** (TLC $_{Rf}$ = 0.09 in 10 % EtOH/$CHCl_3$) ^{1}H NMR (CD_3OD, 500 MHz) δ 5.90 (br t, 1H, *J* = 1.6), 5.04 (A of ABX, 1H, *J* = 18.4, 1.4), 4.92 (B of ABX, 1H, *J* = 18.4, 1.7), 4.17 (d, 1H, *J* = 8.7), 3.81 (A of ABX, 0.5H, *J* = 12.1, 2.0), 3.70 (s, 3H), 3.66 (B of ABX, 0.5H, *J* = 12.1, 5.2), 3.57 (t, 1H, *J* = 8.2), 3.39-3.30 (m, 2H), 3.18 (m, 2H), 2.84 (m, 2H), 2.21 (m, 2H), 1.99-1.34 (m, 19H), 1.07 (td, 1H, *J* = 14.0, 3.5), 0.95 (s, 3H), 0.89 (s, 3H) ; Electrospray ionization-MS *m/z* (M + H) calculated for $C_{30}H_{48}NO_9$ 566.7, observed 566.4.

Aglycon 3β Data Collection. X-ray quality crystals of **3β** were obtained via slow evaporation from chloroform. A colorless crystal with approximate dimensions 0.41 x 0.36 x 0.35 mm^3 was selected under oil in ambient conditions and attached to the tip of a nylon loop. The crystal was mounted in a stream of cold nitrogen at 100°K and manually centered in the X-ray beam while visualizing via video camera. The crystal evaluation and data collection were performed on a Bruker CCD-1000 diffractometer with Mo Kα (λ = 0.71073 Å) radiation and a diffractometer to crystal distance of 4.9 cm. The initial cell constants were obtained from three series of ω scans at different starting angles. Each series consisted of 20 frames collected at intervals of 0.3° in a 6° range about ω with the exposure time of 10 sec per frame. A total of 54 reflections were obtained. The reflections were successfully indexed by an automated indexing routine built in the SMART program (*S1*). The final cell constants were calculated from a set of 6432 strong reflections from the actual data collection. The data were collected by using the hemisphere data collection routine. The reciprocal space was surveyed to the extent of a full sphere to a resolution of 0.80 Å. A total of 8852 data were harvested by collecting three sets of frames with 0.25° scans in ω with an exposure time 30 sec per frame. These redundant datasets were corrected for Lorentz and polarization effects. The absorption correction was based on fitting a function to the empirical transmission surface as sampled by multiple equivalent measurements.

Aglycon 3β Structure Refinement. The systematic absences in the diffraction data were consistent for the space groups $P\bar{1}$ and $P1$. The *E*-statistics were inconclusive and only the non-centrosymmetric space group $P1$ yielded chemically reasonable and computationally stable results of refinement (*S1*). A successful solution by the direct methods provided most on hydrogen atoms from the *E*-map. The remaining non-hydrogen atoms were located in an alternating series of least-squares cycles and difference Fourier maps. All non-hydrogen atoms were refined with anisotropic displacement coefficients. All hydrogen atoms were included in the structure factor calculation at idealized positions and were allowed to ride on the neighboring atoms with relative isotropic displacement coefficients. There are two symmetry independent molecules of **3β** in the asymmetric unit (and incidentally the unit cell) with essentially identical geometries. The absolute configuration could not

be unequivocally established from the experimental data but was assigned from synthesis. There is also one solvate molecule of water per two molecules of **3β** in the unit cell. The final least-squares refinement of 552 parameters against 7847 data resulted in residuals *R* (based on F^2 for $I \geq 2\sigma$) and *wR* (based on F^2 for all data) of 0.0392 and 0.1023, respectively. The final difference Fourier map was featureless. The ORTEP diagrams are drawn with 50% probability ellipsoids.

Table 1
Crystal data and structure refinement for 3β

Empirical formula	$C_{24}H_{37}NO_4 \cdot \frac{1}{2} H_2O$	
Formula weight	412.55	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	P1	
Unit cell dimensions	a = 7.7964(5) Å	α= 95.2600(10)°
	b = 7.8330(5) Å	β= 95.5760(10)°
	c = 17.9391(13) Å	γ = 99.3590(10)°
Volume	1069.37(12) $Å^3$	
Z	2	
Density (calculated)	1.281 Mg/m^3	
Absorption coefficient	0.087 mm^{-1}	
F(000)	450	
Crystal size	0.41 x 0.36 x 0.35 mm^3	
Theta range for data collection	2.30 to 26.39°.	
Index ranges	-9≤h≤9, -9≤k≤9, -22≤l≤22	
Reflections collected	8852	
Independent reflections	7847 [R(int) = 0.0103]	
Completeness to theta = 26.39°	98.7 %	
Absorption correction	Multi-scan with SADABS	
Max. and min. transmission	0.9702 and 0.9652	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	7847 / 7 / 552	
Goodness-of-fit on F^2	1.028	
Final R indices [I>2sigma(I)]	R1 = 0.0392, wR2 = 0.1000	
R indices (all data)	R1 = 0.0412, wR2 = 0.1023	
Absolute structure parameter	N/A – assigned from synthesis	
Largest diff. peak and hole	0.319 and -0.353 $e.Å^{-3}$	

Neoglycoside 4β Data Collection. X-ray quality crystals of **4β** was obtained by dissolving the neoglycoside in EtOH (~ 40 mg mL^{-1}) and slowly crystallizing via vapor diffusion using hexanes. A colorless crystal with approximate dimensions 0.43 x 0.31 x 0.15 mm^3 was selected under oil in ambient conditions and attached to the tip

of a nylon loop. The crystal was mounted in a stream of cold nitrogen at 100°K and manually centered in the X-ray beam while visualizing via video camera. The crystal evaluation and data collection were performed on a Bruker CCD-1000 diffractometer with Mo K_α ($\lambda = 0.71073$ Å) radiation and the diffractometer to crystal distance of 7.36 cm. The initial cell constants were obtained from three series of ω scans at different starting angles. Each series consisted of 40 frames collected at intervals of 0.3° in a 6° range about ω with the exposure time of 15 sec per frame. A total of 149 reflections were obtained. The reflections were successfully indexed by an automated indexing routine built in the SMART program (*S1*). The final cell constants were calculated from a set of 2244 strong reflections from the actual data collection. The data were collected by using the multi-run data collection routine. The reciprocal space was surveyed to the extent of a full sphere to a resolution of 0.80 Å. A total of 14014 data were harvested by collecting six sets of frames with 0.30° scans in ω with an exposure time 14 sec per frame. These highly redundant datasets were corrected for Lorentz and polarization effects. The absorption correction was based on fitting a function to the empirical transmission surface as sampled by multiple equivalent measurements.

Neoglycoside 4*β* Structure Solution and Refinement. The systematic absences in the diffraction data were consistent for the space groups $P\bar{1}$ and $P1$. The *E*-statistics strongly suggested the centrosymmetric space group $P1$ that yielded chemically reasonable and computationally stable results of refinement (*S1*). A successful solution by the direct methods provided all non-hydrogen atoms from the *E*-map. All non-hydrogen atoms were refined with anisotropic displacement coefficients. Soft restraints were applied to thermal displacement coefficients of atom C(6′). All hydrogen atoms were included in the structure factor calculation at idealized positions and were allowed to ride on the neighboring atoms with relative isotropic displacement coefficients. The absolute configurations of the chiral atoms were assigned from the known synthetic procedure. The crystal proved to be a twin with a 2:1 component ratio; the components are related about a 179.8° rotation about the [1, -1, 0] vector in real space. There are two independent molecules of the chiral compound and one molecule of solvated ethanol in the unit cell. The final least-squares refinement of 767 parameters against 14014 data resulted in residuals *R*

(based on F^2 for $I \geq 2\sigma$) and *wR* (based on F^2 for all data) of 0.0639 and 0.1583, respectively.

Table 2
Crystal data and structure refinement for 4β.

Empirical formula	$C_{30}H_{47}NO_9 \bullet$ ½CH_3CH_2OH	
Formula weight	588.72	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	P1	
Unit cell dimensions	a = 10.1923(5) Å	α= 82.533(2)°
	b = 10.2765(5) Å	β= 75.932(2)°
	c = 16.1912(8) Å	γ = 64.9970(10)°
Volume	1490.15(13) $Å^3$	
Z	2	
Density (calculated)	1.312 Mg/m^3	
Absorption coefficient	0.096 mm^{-1}	
F(000)	638	
Crystal size	0.43 x 0.31 x 0.15 mm^3	
Theta range for data collection	2.25 to 26.39°.	
Index ranges	-12≤h≤12, -12≤k≤12, -20≤l≤20	
Reflections collected	14014	
Independent reflections	14014 [R(int) = 0.0000]	
Completeness to theta = 26.39°	92.2 %	
Max. and min. transmission	0.9857 and 0.9598	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	14017 / 9 / 767	
Goodness-of-fit on F^2	0.995	
Final R indices [I>2sigma(I)]	R1 = 0.0639, wR2 = 0.1470	
R indices (all data)	R1 = 0.0898, wR2 = 0.1583	
Absolute structure parameter	-0.2(10)	
Largest diff. peak and hole	1.187 and -0.431 $e.Å^{-3}$	

Hydrolytic Stability of 4α. The chemical stability of the neoglycosidic linkage was examined by monitoring the hydrolytic degradation of neoglycoside **4*α*** in a 3 mM solution of 1:1 DMSO/buffer. Three buffers were used, 50 mM acetate buffer (pH 5), 50 mM phosphate buffer (pH 7), and 50 mM Tris buffer (pH 9). Neoglycoside degradation was monitored by reverse phase HPLC on an Agilent Zorbax Eclipse XDB-C8 column (4.6 x 150 mm) with a flow rate of 0.8 mL min^{-1} and a linear gradient of 49 % CH_3OH/H_2O to 89 % CH_3OH/H_2O over 20 min. At t = 0, neoglycoside **4*α*** in 500 μL DMSO was added to 500 μL buffer, and the resulting solution was vortexed for 40 sec, then immediately injected onto the HPLC. Peak areas at 220 nm were used to estimate the neoglycoside/aglycon ratio, which is

reported as "percent neoglycoside remaining" [$A_{neoglycoside}/(A_{neoglycoside}+A_{aglycon})$] for each of the three buffer systems (see FIG. 4).

Library Synthesis and Purification. Aglycon **3*β*** or **3*α*** (~ 40 μmol) was added to 4 mL glass vials equipped with stirring fleas. The appropriate sugar (2 eq.) was added to each vial, followed by 3:1 DMF/AcOH (final concentration of aglycon = 90 mM). The reaction mixtures were stirred at 40 °C using a stir plate equipped with a 48-well reaction block and a contact thermometer. After 2 days, the reaction mixtures were concentrated via Speed-Vac and suspended in 5 % EtOH/$CHCl_3$. The crude suspensions were purified in parallel on disposable SiO_2 solid phase extraction columns using a 24-port vacuum manifold. Library members (**β** and **α**) **5-16**, **23**, **24**, **30-36**, and **41** were purified on 1000 mg columns eluting first with 5 mL 5 % EtOH/$CHCl_3$ to remove the remaining aglycon and second with 5 mL 15 % EtOH/$CHCl_3$ to collect the product neoglycosides. Library members (**β** and **α**) **4**, **17-22**, **25-29**, **37-40**, and **42** were purified on 500 mg columns eluting first with 4 mL 5 % EtOH/$CHCl_3$ to remove the remaining aglycon and second with 5 mL 25 % EtOH/$CHCl_3$ to collect the product neoglycosides. The product solutions were concentrated via Speed-Vac, weighed, and dissolved in DMSO to make 30 mM or 20 mM stock solutions. The stock solutions were characterized by LCMS using reverse phase HPLC on an Agilent Zorbax Eclipse XDB-C8 column (4.6 x 150 mm) with a flow rate of 0.8 mL/min and a linear gradient of 45 % CH_3OH/H_2O to 85 % CH_3OH/H_2O over 20 min and electrospray ionization. Library member purities were estimated by dividing the sum of the peak areas at 220 nm of peaks corresponding to the desired product mass by the total area of all peaks. For mass information, the purity of specific library members, and a tabulation of which members display greater than 90 % of a single product isomer as judged by LCMS, see Table 3, below. Average library purity was 91 %.

Table 3
LCMS information for neoglycoside library

Neo-glycoside	Calculated Mass	Observed Mass	Percent Purity	90 % Isomeric Purity	Neo-glycoside	Calculated Mass	Observed Mass	Percent Purity	90 % Isomeric Purity
4β	566.7	566.7	97	+	4α	566.7	566.7	98	+
5β	536.6	536.6	98	--	5α	536.6	536.6	100	--
6β	536.6	536.6	99	--	6α	536.6	536.6	99	+
7β	550.7	550.7	97	+	7α	550.7	550.7	96	--
8β	550.7	550.7	100	+	8α	550.7	550.7	99	--
9β	550.7	550.7	21	--	9α	550.7	550.7	0	--
10β	550.7	550.7	100	--	10α	550.7	550.7	97	--
11β	548.7	548.7	100	+	11α	548.7	548.7	100	+
12β	568.6	568.6	88	--	12α	568.6	568.6	95	+
13β	568.6	568.6	100	+	13α	568.6	568.6	100	+
14β	536.6	536.6	97	+	14α	536.6	536.6	99	+
15β	536.6	536.6	95	+	15α	536.6	536.6	99	+
16β	568.5	568.5	91	--	16α	568.5	568.5	99	--
17β	566.7	566.7	100	--	17α	566.7	566.7	95	--
18β	566.7	566.7	98	+	18α	566.7	566.7	98	+
19β	566.7	566.7	65	--	19α	566.7	566.7	94	--
20β	566.7	566.7	88	--	20α	566.7	566.7	91	--
21β	566.7	566.7	96	--	21α	566.7	566.7	91	--
22β	566.7	566.7	93	--	22α	566.7	566.7	92	--
23β	536.6	536.6	99	+	23α	536.6	536.6	100	+
24β	536.6	536.6	99	+	24α	536.6	536.6	97	+
25β	566.7	566.7	89	+	25α	566.7	566.7	93	+
26β	566.7	566.7	90	--	26α	566.7	566.7	91	--
27β	566.7	566.7	88	--	27α	566.7	566.7	91	--
28β	566.7	566.7	52	--	28α	566.7	566.7	61	--
29β	566.7	566.7	61	--	29α	566.7	566.7	34	--
30β	548.7	548.7	98	+	30α	548.7	548.7	97	+
31β	578.7	578.7	62	--	31α	578.7	578.7	86	--
32β	536.6	536.6	96	+	32α	536.6	536.6	98	--
33β	354.6	354.6	98	+	33α	354.6	354.6	99	--
34β	607.7	607.7	97	--	34α	607.7	607.7	99	--
35β	728.8	728.8	92	+	35α	728.8	728.8	88	+
36β	728.8	728.8	64	+	36α	728.8	728.8	89	+
37β	728.8	728.8	96	+	37α	728.8	728.8	97	+
38β	580.7	580.7	70	--	38α	580.7	580.7	22	--
39β	566.7	566.7	89	+	39α	566.7	566.7	96	+
40β	566.7	566.7	96	+	40α	566.7	566.7	97	+
41β	562.7	562.7	87	--	41α	562.7	562.7	83	--
42β	566.7	566.7	89	+	42α	566.7	566.7	98	+

Cell Culture: All cell lines except NmuMG were maintained in RPMI 1640 medium from InVitrogen (Cat No. 11875-085) supplemented with 10 % w/v fetal bovine serum (FBS) from ICN (Cat No. 2916154) and penicillin-streptomycin (PS) (100 U/mL and 100 μg/mL) from InVitrogen (Cat No. 15140-122). NmuMG cells

were maintained in DMEM medium from InVitrogen (Cat No. 11965-084) supplemented with 10 % w/v fetal bovine serum (FBS) from ICN (Cat No. 2916154), 10 μg/ml insulin (InVitrogen Cat No 12585-014), and penicillin-streptomycin (PS) (100 U/mL and 100 μg/mL) from InVitrogen (Cat No. 15140-122). Cells were harvested by trypsinization using 0.25 % w/v trypsin and 0.1 % w/v EDTA from InVitrogen (Cat No. 15-050- 057) and then counted in a hemocytometer in duplicate with better than 10 % agreement in field counts. Cells were plated at a cell density of 10,000 -15,000 cells/well of each Corning Costar 96-well black tissue culture treated microtiter plate (Fisher Cat No. 07-200-627). Cells were grown for 1 h at 37 °C, with 5 % CO_2 in a humidified incubator to allow cell attachment to occur before compound addition.

Library Member Handling and Preparation for Cytotoxicity Assays: Library members were stored at -20 °C under dessicating conditions before the assay. Library member stocks (100X) were prepared in Corning Costar polypropylene 96-well V-bottom polypropylene microtiter plates (Fisher Cat No. 07-200-695). Five serial 1:2 dilutions were made with anhydrous DMSO at 100X the final concentration used in the assay.

Library Member Addition: The library member-containing plates were diluted 1:10 with complete cell culture media. The 10X stocks (10 μL) were added to the attached cells using a Biomek FX liquid handler (Beckman-Coulter). Library member stocks (10 μL) were added to 90 μL of cells in each plate to insure full mixing of stocks with culture media using a Beckman FX liquid handler with 96-well head.

Determination of Cytotoxicity: Cells were incubated with the library members for 72 h before fluorescence reading. Test plates were removed from the incubator and washed 1X in sterile PBS to remove serum containing calcium esterases. Calcein AM reagent (30 μL, 1 M) was added and the cells were incubated for 30 min at 37 °C. Plates were read for emission using a fluorescein filter (excitation 485 nm, emission 535 nm).

IC_{50} Calculation: For each library member, at least six dose response experiments were conducted. Within each experiment, percent inhibition values at each concentration were expressed as a percentage of the maximum fluorescence emission signal observed for a 0 nM control. To calculate IC_{50}, percent inhibitions were plotted as a function of log[concentration] and then fit to a four-parameter logistic model that allowed for a variable Hill slope using XL*fit* 4.1.

Cytotoxicity Assays. All cell lines except NmuMG were maintained in RPMI 1640 medium supplemented with 10 % w/v fetal bovine serum (FBS) and penicillin-streptomycin (PS) (100 U/mL and 100 μg/mL). NmuMG cells were maintained in DMEM medium supplemented with 10 % w/v fetal bovine serum (FBS), 10 μg/ml insulin, and penicillin-streptomycin (PS) (100 U/mL and 100 μg/mL). Cells were harvested by trypsinization using 0.25 % w/v trypsin and 0.1 % w/v EDTA and then counted in a hemocytometer in duplicate with better than 10 % agreement in field counts. Cells were plated at a cell density of 10,000 -15,000 cells/well of each 96-well black tissue culture treated microtiter plate. Cells were grown for 1 h at 37 °C, with 5 % CO_2 in a humidified incubator to allow cell attachment to occur before compound addition. Library members were stored at -20 °C under dessicating conditions before the assay. Library member stocks (100X) were prepared in polypropylene 96-well V-bottom polypropylene microtiter plates. Five serial 1:2 dilutions were made with anhydrous DMSO at 100X the final concentration used in the assay. The library member-containing plates were diluted 1:10 with complete cell culture media. The 10X stocks (10 μL) were added to the attached cells using a Biomek FX liquid handler. Library member stocks (10 μL) were added to 90 μL of cells in each plate to insure full mixing of stocks with culture media using a Beckman FX liquid handler with 96-well head. Cells were incubated with the library members for 72 h before fluorescence reading. Test plates were removed from the incubator and washed 1X in sterile PBS to remove serum containing calcium esterases. Calcein AM reagent (30 μL, 1 M) was added and the cells were incubated for 30 min at 37 °C. Plates were read for emission using a fluorescein filter (excitation 485 nm, emission 535 nm).

IC_{50} Calculations. For each library member, at least six dose response experiments were conducted. Within each experiment, percent inhibition values at each concentration were expressed as a percentage of the maximum fluorescence emission signal observed for a 0 nM control. To calculate IC_{50}, percent inhibitions were plotted as a function of log[concentration] and then fit to a four-parameter logistic model that allowed for a variable Hill slope using XL*fit* 4.1.

Na/K-ATPase Assays. Inhibition of Na^+/K^+-ATPase on HEK-293 cells and CHO-K1 cells by the library hits was determined by Aurora Biomed, Inc. using a high-throughput non-radioactive rubidium ion uptake assay. Experiments were conducted in duplicate using three different concentrations. Within each experiment, percent inhibition values at the three concentrations were expressed as the percent reduction of the maximum absorption signal observed for a 0 nM control. IC_{50} values were determined using the following formula: IC_{50} = [(50 - low %)/(high % - low %)] X (high conc.- low conc.) + low conc.

As highlighted in FIG. 3, the requisite methoxylamine functional group was installed at the C(3) of digitoxin (the natural position of sugar attachment) in three simple chemical steps. Specifically, digitoxin was oxidized under acidic conditions to simultaneously hydrolyze the *O*-glycoside and provide digitoxigenone which was then converted to the corresponding set of oxime diastereomers (**2a,b**). Treatment of **2a,b** with *tert*-butylamine borane resulted in a 1:1 mixture of stereoisomers which were easily resolved via standard column chromatography and assigned as **3*β*** and **3*α*** via X-ray crystallography. The accessibility of both digitoxigenin-like isomers **3*β*** and **3*α*** set the stage to explore the importance of the C(3) stereochemistry on biological activity. Pilot reactions of aglycons **3*β*** and **3*α*** with D-glucose were first explored in an attempt to generate the corresponding neoglycosides (FIG. 2B). Aglycons **3*β*** and **3*α*** reacted with D-glucose in DMF/acetic acid to form neoglycosides **4*β*** and **4*α*** in good yields (> 70 %). Both reactions proceeded stereoselectively, providing the β-anomer exclusively as determined by ^1H NMR. An X-ray crystal structure of neoglycoside **4*β*** was obtained (FIG. 3A) and compared to the crystal structures of related *O*-glycosides available from the Cambridge Crystal Database (FIG. 3B - FIG.3D). The observed orientations about the C(2)-C(3)-N(3)-C(1') torsion (FIG. 3C)

and the C(3)-N(3)-C(1')-C(2') torsion in the neoglycoside structure (FIG. 3D) fall on the periphery of the narrow range of orientations displayed in the solid state structures of 23 known cardiac *O*-glycosides.

A library of 78 digitoxin derivatives was synthesized in parallel from 39 reducing sugars and aglycons **3*β*** and **3*α***. The reaction mixtures were stirred for two days at 40 °C, concentrated, and then submitted to solid phase extraction in parallel to remove unreacted aglycon and sugar. The concentrated products were characterized by LCMS to assess purity and to confirm product identity. Even though a diverse array of reducing sugars were used—including L-sugars, deoxy sugars, dideoxy sugars, disaccharides, and uronic acids—in every case neoglycosides were successfully generated. The average purity of the library members was 91 %, and the LC chromatograms suggested that ~50 % of the library members contained greater than 90 % of a single product isomer. While combinatorial methods have been extensively applied to steroidal derivatives and cardenolides in particular (26, 27), the results reported herein represent the largest and most diverse glycorandomized library generated to date.

The chemical stability of the neoglycosidic linkage was examined by monitoring the hydrolytic degradation of neoglycoside **4*α*** in a 3 mM solution of 1:1 DMSO/buffer using buffers at three different pHs. Compound **4*α*** was completely stable over the period of one month under neutral or basic conditions but slowly hydrolyzed under acidic conditions over this same time period. Using identical acidic conditions, aglycon **3*α*** and D-glucose did not react to form neoglycoside **4*α***, ruling out equilibrium as a complicating factor in this analysis. Library member **27*β***, derived from aglycon **3*β***, also displayed no hydrolytic degradation under the same conditions at neutral and basic pHs, demonstrating that aglycon C(3) stereochemistry does not significantly influence neoglycoside stability. In conjunction with the neoglycoside structural analyses and the previously reported NMR and molecular dynamics studies, these hydrolytic studies suggest the neoglycoside nitrogen to be predominately charge-neutral at physiological pH.

Cytotoxicity

The activity of the library members was assessed using a high-throughput cytotoxicity assay on nine human cancer cell lines representing a broad range of carcinomas including breast, colon, CNS, liver, lung, and ovary, and a mouse mammary normal epithelial control line. The cytotoxicities of digitoxin and aglycons **3β** and **3α** were also examined. Digitoxin was a modest cytotoxin toward the nine human cancer cell lines (average $IC_{50} \sim 440$ nM) but was non-specific since it affected these cancer cells with similar potency. One library member (**33β**) closely mimicked this activity. Several hits identified from the neoglycoside library exhibited enhanced activities relative to the parent natural product digitoxin (**1**), both in terms of potency and specificity.

The two most significant hits, library members **5β** and **27β**, displayed striking potency and excellent selectivity, respectively. Specifically, library member **5β** was a potent cytotoxin against six cancer cell lines (18 ± 2 nM in the case of HCT-116, greater than nine-fold more potent than digitoxin), and also was modestly selective since three out of the nine cancer cell lines tested were much less affected.

In contrast, library member **27β** was a less potent cytotoxin than **5β** but **27β** exhibited dramatic selectivity since it was four times more cytotoxic toward NCI/ADR-RES cells ($IC_{50} = 100 \pm 10$ nM) than any other cell line. This result is especially significant since NCI/ADR-RES is a multi-drug resistant line that contains high levels of MDR-1 and P-glycoprotein expression (Fairchild, C.R., et al., (1987) *Cancer Res.* **47**, 5141-5148; Scudiero, D.A.; Monks, A.; Sausville, E.A. (1998) *J. Natl. Cancer Inst.* **90**, 862). Given that cardiac glycosides are substrates for P-glycoprotein (Tanigawara, Y., et al., (1992) J. Pharmacol. Exp. Ther. 263, 840-845), such tumor specificity suggests **27β** may no longer serve as a P-glycoprotein substrate or may be interacting with a unique target.

Other neoglycoside library members, while not as potent as **5β** or as selective as **27β**, also were significantly active. For example, library member **40β** exhibited notable selectivity, with modest cytotoxicity toward only Du145 and Hep3B cells ($IC_{50} = 200$ nM ± 30 and 180 nM ± 30, respectively) while library members **15β** and

23β were significantly more potent than digitoxin against some cell lines, but were somewhat non-selective like digitoxin.

Table 4
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-riboside (5β)	D-riboside (6β)	L-fucoside (7β)	D-fucoside (8β)	2-deoxy-D-galactoside (9β)	3-deoxy-D-glucoside (10β)	6-deoxy-D-glucoside (11β)	2-deoxy-2-fluoro-D-glucoside (12β)	6-deoxy-6-fluoro-D-glucoside (13β)
Du145	IC50	0.30	0.72	0.7	3.9	7	0.8	6	9	5
	Std Err	0.03	0.04	0.1	0.6	1	0.2	1	2	2
MCF7	IC50	0.19	3.0	1.3	11	5.1	0.46	1.9	8	6.2
	Std Err	0.03	0.4	0.4	2	0.8	0.09	0.2	1	0.9
HCT-116	IC50	0.018	1.3	1.2	7	10	1.8	2.3	8	4.8
	Std Err	0.002	0.6	0.3	1	1	0.4	0.4	2	0.8
Hep 3B	IC50	0.059	0.9	0.35	4.9	3.6	1.7	4.4	8	4.6
	Std Err	0.006	0.1	0.04	0.3	0.8	0.2	0.4	1	1.0
SF-268	IC50	0.23	1.8	0.90	10	8	0.77	2.5	7	4.7
	Std Err	0.02	0.2	0.23	1	1	0.09	0.6	1	0.7
SK-OV-3	IC50	0.045	1.7	0.5	5	4	2.5	4	9	3.4
	Std Err	0.006	0.3	0.1	1	1	0.3	1	2	1.0
NCI-H460	IC50	0.053	0.90	0.6	1.3	2.0	1.6	1.0	2.9	1.4
	Std Err	0.009	0.05	0.2	0.3	0.3	0.1	0.1	0.4	0.2
A549	IC50	0.033	1.02	0.30	1.8	1.3	1.60	1.2	3.7	2.0
	Std Err	0.004	0.03	0.03	0.2	0.2	0.07	0.1	0.3	0.2
NCI/ADR-RES	IC50	0.032	0.66	0.25	1.5	0.9	0.44	0.95	2.6	1.1
	Std Err	0.002	0.04	0.04	0.2	0.2	0.04	0.10	0.4	0.2
NmuMG	IC50	0.031	0.48	> 25	> 25	> 25	0.94	> 25	> 25	> 25
	Std Err	0.007	0.09				0.08			

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-riboside (5β)	D-riboside (6β)	L-fucoside (7β)	D-fucoside (8β)	2-deoxy-D-galactoside (9β)	3-deoxy-D-glucoside (10β)	6-deoxy-D-glucoside (11β)	2-deoxy-2-fluoro-D-glucoside (12β)	6-deoxy-6-fluoro-D-glucoside (13β)
Du145	IC50	0.30	0.72	0.7	3.9	7	0.8	6	9	5
	Std Err	0.03	0.04	0.1	0.6	1	0.2	1	2	2
MCF7	IC50	0.19	3.0	1.3	11	5.1	0.46	1.9	8	6.2
	Std Err	0.03	0.4	0.4	2	0.8	0.09	0.2	1	0.9
HCT-116	IC50	0.018	1.3	1.2	7	10	1.8	2.3	8	4.8
	Std Err	0.002	0.6	0.3	1	1	0.4	0.4	2	0.8
Hep 3B	IC50	0.059	0.9	0.35	4.9	3.6	1.7	4.4	8	4.6
	Std Err	0.006	0.1	0.04	0.3	0.8	0.2	0.4	1	1.0
SF-268	IC50	0.23	1.8	0.90	10	8	0.77	2.5	7	4.7
	Std Err	0.02	0.2	0.23	1	1	0.09	0.6	1	0.7
SK-OV-3	IC50	0.045	1.7	0.5	5	4	2.5	4	9	3.4
	Std Err	0.006	0.3	0.1	1	1	0.3	1	2	1.0
NCI-H460	IC50	0.053	0.90	0.6	1.3	2.0	1.6	1.0	2.9	1.4
	Std Err	0.009	0.05	0.2	0.3	0.3	0.1	0.1	0.4	0.2
A549	IC50	0.033	1.02	0.30	1.8	1.3	1.60	1.2	3.7	2.0
	Std Err	0.004	0.03	0.03	0.2	0.2	0.07	0.1	0.3	0.2
NCI/ADR-RES	IC50	0.032	0.66	0.25	1.5	0.9	0.44	0.95	2.6	1.1
	Std Err	0.002	0.04	0.04	0.2	0.2	0.04	0.10	0.4	0.2
NmuMG	IC50	0.031	0.48	> 25	> 25	> 25	0.94	> 25	> 25	> 25
	Std Err	0.007	0.09				0.08			

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-lyxoside (14β)	D-lyxoside (15β)	L-rhamnoside (16β)	L-alloside (17β)	D-alloside (18β)	L-altroside (19β)	D-altroside (20β)	L-galactoside (21β)	D-galactoside (22β)
Du145	IC50	2.4	0.07	0.17	0.79	2.2	2.1	1.2	2.1	1.5
	Std Err	0.5	0.02	0.03	0.07	0.2	0.3	0.3	0.2	0.1
MCF7	IC50	4.5	0.23	1.8	0.9	6.8	1.3	1.5	1.8	3.0
	Std Err	0.9	0.02	0.2	0.1	0.8	0.3	0.2	0.3	0.4
HCT-116	IC50	8	0.17	0.9	0.5	6	1.1	1.1	2.1	1.8
	Std Err	2	0.03	0.3	0.2	1	0.3	0.2	0.3	0.5
Hep 3B	IC50	0.6	0.09	0.54	0.8	3.3	2.0	0.35	1.3	0.6
	Std Err	0.1	0.01	0.07	0.1	0.6	0.4	0.04	0.2	0.3
SF-268	IC50	1.1	0.09	3.3	0.4	3.5	1.2	1.1	1.4	1.2
	Std Err	0.4	0.01	0.5	0.1	0.6	0.2	0.2	0.2	0.2
SK-OV-3	IC50	4.2	0.16	4	0.7	6.4	1.0	1.1	1.6	1.5
	Std Err	0.8	0.08	1	0.1	1.0	0.2	0.2	0.5	0.5
NCI-H460	IC50	5	0.09	0.33	0.69	3.2	1.6	0.62	2.6	1.6
	Std Err	1	0.01	0.05	0.09	0.2	0.2	0.09	0.2	0.2
A549	IC50	0.70	0.14	0.7	0.24	2.0	0.76	0.54	0.9	0.97
	Std Err	0.06	0.02	0.1	0.02	0.2	0.09	0.04	0.2	0.09
NCI/ADR-RES	IC50	0.73	0.075	0.35	0.16	0.8	0.41	0.9	1.6	0.8
	Std Err	0.04	0.005	0.02	0.01	0.2	0.05	0.1	0.5	0.1
NmuMG	IC50	0.9	0.069	> 25	0.33	3.1	1.0	0.9	1.0	1.5
	Std Err	0.1	0.005		0.04	0.5	0.2	0.2	0.2	0.2

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-xyloside (23β)	D-xyloside (24β)	D-guloside (25β)	L-mannoside (26β)	D-mannoside (27β)	L-idoside (28β)	D-idoside (29β)	L-mycaroside (30β)	6-keto-D-galactosidle (31β)
Du145	IC50	0.62	0.82	5.7	2.0	1.3	1.9	1.3	1.1	1.5
	Std Err	0.07	0.06	0.8	0.4	0.3	0.4	0.2	0.2	0.3
MCF7	IC50	0.23	1.8	0.9	3.0	1.7	2.6	0.44	1.8	9
	Std Err	0.02	0.6	0.3	0.4	0.2	0.4	0.07	0.3	2
HCT-116	IC50	0.10	1.8	2.1	4.2	0.8	3.2	2.3	0.6	3.1
	Std Err	0.03	0.3	0.4	0.7	0.2	0.6	0.3	0.1	0.8
Hep 3B	IC50	0.50	1.2	1.9	2.0	0.4	1.0	0.3	0.6	3.1
	Std Err	0.08	0.1	0.2	0.3	0.1	0.1	0.1	0.2	0.6
SF-268	IC50	0.49	2.1	2.0	2.1	2.2	2.1	1.3	1.1	8
	Std Err	0.09	0.4	0.5	0.4	0.4	0.3	0.3	0.2	3
SK-OV-3	IC50	0.22	1.8	2.2	3.1	0.8	2.4	1.5	1.1	2.9
	Std Err	0.04	0.4	1.0	0.8	0.2	0.5	0.3	0.2	0.3
NCI-H460	IC50	0.08	1.8	2.6	1.6	0.7	1.3	1.3	0.19	2.4
	Std Err	0.02	0.1	0.2	0.2	0.1	0.2	0.2	0.02	0.6
A549	IC50	0.079	1.1	2.0	1.7	0.63	1.14	0.37	0.6	2.3
	Std Err	0.005	0.2	0.4	0.2	0.07	0.08	0.02	0.1	0.9
NCI/ADR-RES	IC50	0.138	0.54	1.1	4.8	0.10	0.61	1.5	0.35	1.0
	Std Err	0.009	0.04	0.3	0.5	0.01	0.06	0.4	0.03	0.1
NmuMG	IC50	0.057	0.9	1.8	1.3	1.0	1.8	> 25	> 25	> 25
	Std Err	0.007	0.1	0.3	0.2	0.1	0.5			

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-arabinoside (32β)	D-arabinoside (33β)	*N*-acetyl-D-galactosaminoside (34β)	melibioside (35β)	lactoside (36β)	maltoside (37β)	D-galacturonoside (38β)	L-taloside (39β)
Du145	IC50	1.1	0.17	> 25	27	13	4.4	1.9	0.76
	Std Err	0.3	0.03		4	3	1.0	0.4	0.07
MCF7	IC50	2.2	0.30	> 25	> 25	> 25	> 25	5	1.8
	Std Err	0.3	0.04					2	0.3
HCT-116	IC50	0.3	0.36	> 25	> 25	> 25	24	2.7	2.3
	Std Err	0.1	0.07				6	0.6	0.4
Hep 3B	IC50	1.2	0.26	17	26	26	7	2.1	0.96
	Std Err	0.1	0.04	2	6	4	2	0.3	0.07
SF-268	IC50	1.0	0.16	> 25	> 25	> 25	6	2.2	1.4
	Std Err	0.3	0.03				1	0.7	0.2
SK-OV-3	IC50	3.0	0.47	17	15	19	5	3.1	1.5
	Std Err	0.5	0.09	4	2	4	1	0.9	0.5
NCI-H460	IC50	0.72	0.33	20	29	> 25	6.1	1.4	1.1
	Std Err	0.07	0.04	2	8		0.9	0.2	0.1
A549	IC50	1.7	0.34	> 25	> 25	24	4.4	2.2	0.89
	Std Err	0.3	0.05			4	0.2	0.2	0.07
NCI/ADR-RES	IC50	0.65	0.124	6.4	16	13	4.4	1.6	1.0
	Std Err	0.05	0.007	0.5	1	2	0.4	0.1	0.3
NmuMG	IC50	0.4	0.12	> 25	> 25	> 25	> 25	> 25	0.5
	Std Err	0.1	0.02						0.2

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Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		D-taloside (40β)	6-deoxy-6-azido-D-mannoside (41β)	L-glucoside (42β)	D-glucoside (4β)	*O*-D-glucoside (43β)	*R*-C(3) aglycon (3β)	*S*-C(3) aglycon (3α)	digitoxin (1)
Du145	IC50	0.20	2.0	1.3	8.2	0.22	1.3	2.9	0.26
	Std Err	0.03	0.7	0.3	0.9	0.04	0.3	0.9	0.03
MCF7	IC50	1.4	3.3	0.6	10	0.7	1.8	13	0.32
	Std Err	0.3	0.6	0.3	1	0.1	0.3	2	0.03
HCT-116	IC50	1.9	6	0.9	3.8	0.6	1.2	9	0.17
	Std Err	0.2	1	0.4	0.7	0.1	0.2	2	0.04
Hep 3B	IC50	0.18	1.8	0.9	2.4	0.21	1.4	3.5	0.22
	Std Err	0.03	0.3	0.2	0.3	0.03	0.1	0.3	0.01
SF-268	IC50	1.3	2.5	1.9	4.5	0.22	1.0	3.5	1.5
	Std Err	0.1	0.6	0.3	0.8	0.08	0.2	0.5	0.2
SK-OV-3	IC50	0.49	2.1	1.0	6	0.40	0.9	3	0.7
	Std Err	0.09	0.3	0.4	1	0.08	0.2	1	0.1
NCI-H460	IC50	0.80	2.7	1.3	3.5	0.39	0.7	2.4	0.28
	Std Err	0.06	0.3	0.1	0.5	0.05	0.1	0.2	0.03
A549	IC50	0.41	1.7	0.8	2.9	0.29	1.2	8.3	0.28
	Std Err	0.03	0.1	0.2	0.3	0.02	0.1	0.5	0.04
NCI/ADR-RES	IC50	1.9	0.84	0.55	1.9	0.19	0.71	5.1	0.22
	Std Err	0.5	0.09	0.05	0.5	0.02	0.05	0.6	0.03
NmuMG	IC50	0.48	> 25	1.2	3.1	> 25	> 25	> 25	> 25
	Std Err	0.06		0.1	0.2				

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-riboside (5α)	D-riboside (6α)	L-fucoside (7α)	D-fucoside (8α)	2-deoxy-D-galactoside (9α)	3-deoxy-D-glucoside (10α)	6-deoxy-D-glucoside (11α)	2-deoxy-2-fluoro-D-glucoside (12α)	6-deoxy-6-fluoro-D-glucoside (13α)
Du145	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
MCF7	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
HCT-116	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
Hep 3B	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
SF-268	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
SK-OV-3	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NCI-H460	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
A549	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NCI/ADR-RES	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NmuMG	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-lyxoside (14α)	D-lyxoside (15α)	L-rhamnoside (16α)	L-alloside (17α)	D-alloside (18α)	L-altroside (19α)	D-altroside (20α)	L-galactoside (21α)	D-galactoside (22α)
Du145	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
MCF7	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
HCT-116	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
Hep 3B	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
SF-268	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
SK-OV-3	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NCI-H460	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
A549	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NCI/ADR-RES	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NmuMG	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-xyloside (23α)	D-xyloside (24α)	D-guloside (25α)	L-mannoside (26α)	D-mannoside (27α)	L-idoside (28α)	D-idoside (29α)	L-mycaroside (30α)	6-keto-D-galactoside (31α)
Du145	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
MCF7	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
HCT-116	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
Hep 3B	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
SF-268	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
SK-OV-3	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NCI-H460	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
A549	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NCI/ADR-RES	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									
NmuMG	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err									

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		L-arabinoside (32α)	D-arabinoside (33α)	*N*-acetyl-D-galactosaminoside (34α)	melibioside (35α)	lactoside (36α)	maltoside (37α)	D-galacturonoside (38α)	L-taloside (39α)
Du145	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
MCF7	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
HCT-116	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
Hep 3B	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
SF-268	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
SK-OV-3	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
NCI-H460	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
A549	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
NCI/ADR-RES	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								
NmuMG	IC50	> 25	> 25	> 25	> 25	> 25	> 25	> 25	> 25
	Std Err								

Table 4 (Continued)
Cancer Cell Cytoxicity
Library member IC_{50} values (μM) and standard errors.

Sugar Name		D-taloside (40α)	6-deoxy-6-azido-D-mannoside (41α)	L-glucoside (42α)	D-glucoside (4α)
Du145	IC50	> 25	> 25	> 25	> 25
	Std Err				
MCF7	IC50	> 25	> 25	> 25	> 25
	Std Err				
HCT-116	IC50	> 25	> 25	> 25	> 25
	Std Err				
Hep 3B	IC50	> 25	> 25	> 25	> 25
	Std Err				
SF-268	IC50	> 25	> 25	> 25	> 25
	Std Err				
SK-OV-3	IC50	> 25	> 25	> 25	> 25
	Std Err				
NCI-H460	IC50	> 25	> 25	> 25	> 25
	Std Err				
A549	IC50	> 25	> 25	> 25	> 25
	Std Err				
NCI/ADR-RES	IC50	> 25	> 25	> 25	> 25
	Std Err				
NmuMG	IC50	> 25	> 25	> 25	> 25
	Std Err				

The library members showed stereospecificity in the cytotoxic actions. In contrast to the **3β**-derived analogs, the 38 neoglycosides derived from aglycon **3α** uniformly displayed low cytotoxicities in the assay (IC_{50} > 25 μM), as did aglycon **3α** itself, establishing the importance of the natural β configuration of the C(3) stereocenter. Aglycon **3β** was only weakly cytotoxic against the cell lines (average IC_{50} ~ 1.10 μM), consistent with the influence sugars have upon library member cytotoxicity. Interestingly, the six hits described above all contain sugars with a common structural feature, an *S*-configured C(2′) sugar stereocenter. This C(2′) stereochemistry appears to be of critical importance for compound activity. For example, the C(2′) epimer of the extremely potent library member **5β** (L-arabinose-containing member **32β**) was relatively inactive toward the ten cell lines examined.

Likewise, D-glucose-containing library member **4*β*** was relatively inactive and displayed none of the cell line specificity observed for its C(2′) epimer **27*β***.

Neoglycosides with sugars containing reactive handles were successfully generated. For example, while not active under these assay conditions, **41*β*** contains the C(2′) stereochemistry shared by the library hits and a reactive azido group which is amenable to further diversification via Huisgen 1,3-dipolar cycloaddition (Fu, X., et al., (2003) Nat. Biotechnol. 21, 1467-1469). Such library members can this serve as the starting point for the development of compounds with enhanced cytotoxicity.

To assess how these structural modifications impact the ability of library members to inhibit Na^+/K^+-ATPases, a fundamental activity of cardiac glycosides (Paula, S., et al., (2005) Biochemistry 44, 498-510), library hits **5*β***, **15*β***, **23*β***, **27*β***, **33*β***, **40*β***, and digitoxin (**1**) were submitted to a non-radioactive rubidium uptake assay to gauge Na^+/K^+-ATPase inhibition in both HEK-239 human embryonic kidney cells and CHO-K1 hamster ovary cells (Gill, S., et al., . (2004) ASSAY and Drug Development Technologies 2, 535-542). In HEK-239 cells, digitoxin displayed an IC_{50} of 75.4 ± 0.5 μM, while none of the library hits showed 50 % inhibition even at the highest concentration tested (300 μM for **5*β***, **15*β***, **23*β***, and **33*β***; 200 μM for **27*β*** and **40*β***). A similar trend was observed in the CHO-K1 cells. Thus, hits identified from the neoglycoside library not only displayed enhanced cytotoxic properties toward human cancer cells, but the rubidium uptake assays reveal these six neoglycosides to be less potent Na^+/K^+-ATPase inhibitors in a human cell line than digitoxin.

Table 5.
Na^+/K^+-ATPase Inhibition
IC_{50} values (μM) and standard deviations.

	Library Member	digitoxin (1)	L-riboside (5β)	D-lyxoside (15β)	L-xyloside (23β)	D-mannoside (27β)	D-arabinoside (33β)	D-taloside (40β)
HEK-298	IC50	75.4	> 300	> 300	> 300	> 200	> 300	> 200
	Std Dev	0.5						
CHO-K1	IC50	77	270	200	> 500	> 300	170	180
	Std Dev	2	20	10			50	30

The growing body of epidemiological (Johnson, P.H., et al., (2002) Molecular Cancer Therapeutics 1, 1293-1304), *in vitro* (Johansson, S., et al., (2001) Anti-Cancer Drugs 12, 475-483) and *in vivo* (Svensson, A., et al., (2005) Anticancer Res. 25, 207-212) evidence supporting the anti-cancer benefits of cardinolides has prompted the search for non-cardioactive analogs which still retain anticancer activity. The specific mechanism of cardenolide-induced cytotoxicity remains controversial. For example, a preferred ligand for cardenolides, the Na^+/K^+-ATPase, belongs to the 'Na^+/K^+-ATPase signalosome' the activation of which by certain cardenolides can lead to NF-κB pathway inactivation (Dmitrieva, R.I.; Doris, P.A. (2002) *Exp. Biol. Med.* **227**, 561-569). Constitutive activation of the NF-κB pathway protects a large group of cancer cells against apoptosis while suppression of this transcription factor can restore normal levels of apoptosis in cancer cells and also potentially block tumorigenesis and inflammation (Quanquebeke E.V. et al. (2005) J. Med. Chem. 48, 849-856; Sreenivasan, Y., et al.,(2003) Biochem. Pharmacol. 66, 2223-2239). Yet, digitoxin-mediated inhibition of the NF-κB signaling pathway in CF lung epithelial cells has been demonstrated to be mechanistically distinct from Na^+/K^+-ATPase inhibition (Srivastava, M., *et. al.* (2004)). With respect to other implicated cellular players, the same nonlethal cardenolide concentrations that inhibit breast cancer cell proliferation also activates Src kinase, stimulates the interaction between Na^+/K^+-ATPase, the activated Src kinase and epidermal growth factor (EGFR), and leads to the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and subsequent cell cycle arrest caused by increased levels of $p21^{Cip1}$ (Kometiani, P., et al., (2005) Mol. Pharmacol. 67, 929-936). Cardiac glycosides have also been demonstrated to initiate apoptosis via the classical caspase-dependent pathways in malignant T lymphoblasts (Daniel, D.;., et al., (2003) Internatl. Immunopharmacol. 3, 1791-1801) and prostrate cancer cells (Lin, H.;, et al., (2004) J. Biol. Chem. 279, 29302-29307) and, in the latter, also inhibit testosterone production *in vivo* (Lin, H. et al. (1998) Br. J. Pharmacol. 125, 1635-1640). Thus, while the cytotoxicity of certain cardiac glycosides may correlate with Na^+/K^+-ATPase inhibition, the present study reveals a new class of desirable non-cardioactive tumor-specific and potent cytotoxins, the mechanism of which remains to be elucidated.

The neoglycorandomization of digitoxin illustrates the remarkable ease by which the influence a sugar has on a natural product scaffold can be quickly scanned via this simple, mild, and robust reaction with unprotected and non-activated reducing sugars. In this prototype example, we show that subtle sugar modifications can dramatically, and independently, modulate both the cytotoxic properties and the Na^+/K^+-ATPase inhibitory properties of cardiac glycosides. The potential of neoglycorandomization is further augmented by its compatibility with chemical handles (*e.g.*, azido groups) for additional elaboration. Neoglycorandomization is solely limited by the efficiency and specificity of alkoxylamine handle installation and the availability of reducing sugar donors; thus, these studies highlight the unique potential of neoglycosylation and/or neoglycorandomization as a universally powerful tool for glycobiology and drug discovery. Moreover, a wide range of reducing sugars are available commercially or via elegant transformations from simple precursors, presenting broad access to the only building blocks essential to this approach .

While, the present invention has been described in what is perceived to be the most practical and preferred embodiments and examples, it is to be understood that the invention is not intended to be limited to the specific embodiments set forth above. Rather, it is recognized that modifications may be made by one of skill in the art of the invention without departing from the spirit or intent of the invention and, therefore, the invention is to be taken as including all reasonable equivalents to the subject matter of the appended claims. All references cited herein are incorporated by reference for all purposes.

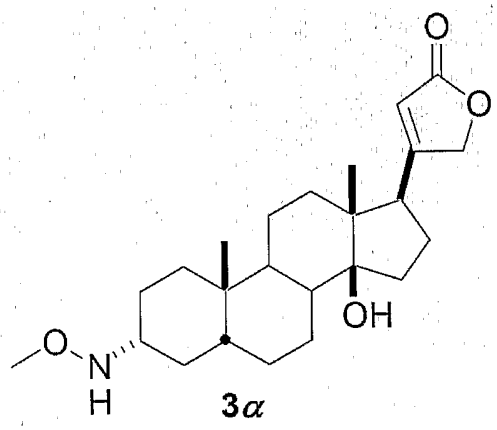
CLAIMS

We claim:

1. A neoglycoside produced by the reaction of an aglycon having a secondary alkoxylamine and a reducing sugar selected from the group consisting of a L-sugar, a D-sugar, a deoxy-sugar, a dideoxy-sugar, a glucose epimer, a substituted sugar, a uronic acid, an oligosaccharide and mixtures thereof and where the aglycon is a digitoxin analog, an indolocarbazole, an anthracyline, a macrolide, a peptide or an alkaloid.

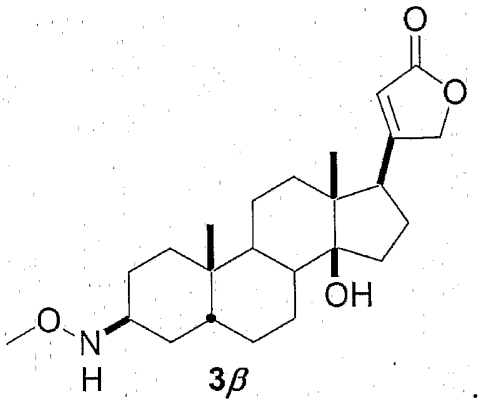
2. The neoglycoside of claim 1 wherein the reducing sugar is selected from the group consisting of L-ribose, D-ribose, L-fucose, D-fucose, 2-deoxy-D-galactose, 3-deoxy-D-glucose, 6-deoxy-D-glucose, 2-deoxy-2-fluoro-D-glucose, 6-deoxy-6-fluoro-D-glucose, L-lyxose, D-lyxose, L-rhamnose, L-allose, D-allose, L-altrose, D-altrose, L-galactose, D-galactose, L-xylose, D-xylose, D-gulose, L-mannose, D-mannose, L-idose, D-idose, L-mycarose, 6-keto-D-galactose, L-arabinose, D-arabinose, *N*-acetyl-D-galactosaminose, melibiose, lactose, maltose, D-galacturonose, L-talose, D-talose, 6-deoxy-6-azo-D-mannose, L-glucose, D-glucose, *O*-D-glucose, *R*-C(3)aglycon, *S*-C(3) aglycon and mixtures thereof.

3. The neoglycoside of claim 1 wherein the aglycon is



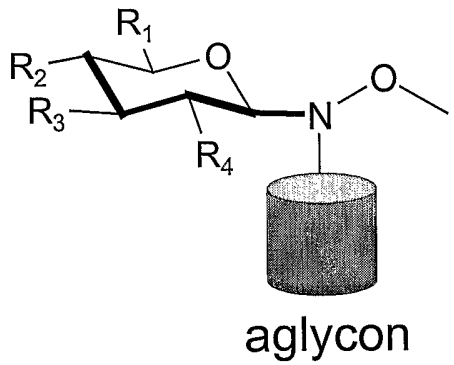
3α

or



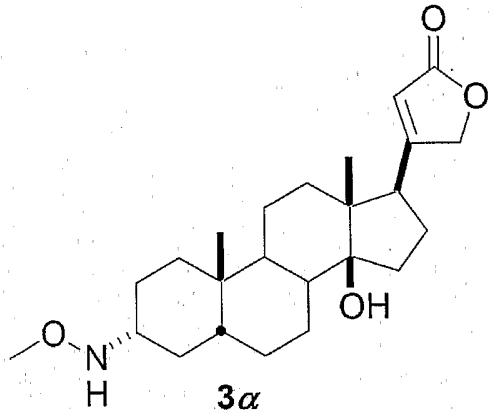
4. A composition comprising the neoglycoside of claim 1, pharmaceutically acceptable ester, salt or prodrug thereof combined with a pharmaceutically acceptable carrier.

5. A neoglycoside of the formula:



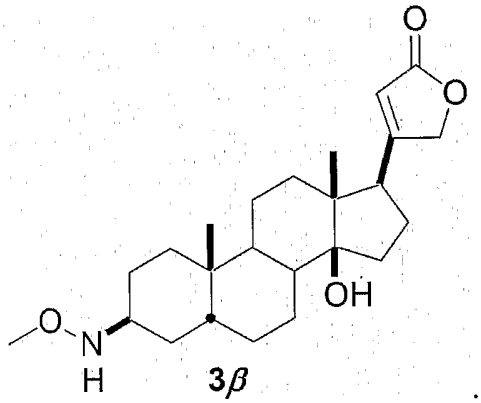
where R_1, R_2, R_3, and R_4 are independently selected from –H, -OH, $-N_3$, $-NH_2$, $-CH_3$, $-CH_2OH$, $-CN_3$, $-CH_2NH$, $-CH_2SH$, $-CNH_2$, $-CH_2N_3$, -COOH, $-COCH_3$, $-CXH_2$, $-CX_2H$, and where X is Cl, Br, F, or I and the aglycon is a digitoxin analog, an indolocarbazole, an anthracyline, a macrolide, a peptide or an alkaloid.

6. The neoglycoside of claim 5 wherein the aglycon is



3α

or



3β

7. A composition comprising the neoglycoside of claim 5, pharmaceutically acceptable ester, salt or prodrug thereof combined with a pharmaceutically acceptable carrier.

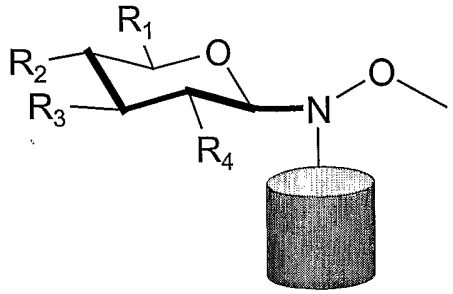
8. A neoglycoside selected from the group consisting of L-riboside (5β), D-riboside (6β), L-fucoside (7β), D-fucoside (8β), 2-deoxy-D-galactoside (9β), 3-deoxy-D-glucoside (10β), 6-deoxy-D-glucoside (11β), 2-deoxy-2-fluoro-D-glucoside (12β), 6-deoxy-6-fluoro-D-glucoside (13β), L-lyxoside (14β), D-lyxoside (15β), L-rhamnoside (16β), L-alloside (17β), D-alloside (18β), L-altroside (19β), D-altroside (20β), L-galactoside (21β), D-galactoside (22β), L-xyloside (23β), D-xyloside (24β), D-guloside (25β), L-mannoside (26β), D-mannoside (27β), L-idoside (28β), D-idoside (29β), L-mycaroside (30β), 6-keto-D-galactoside (31β), L-arabinoside (32β), D-arabinoside (33β), *N*-acetyl-

D-galactosaminoside (34β), melibioside (35β), lactoside (36β), maltoside (37β), D-galacturonoside (38β), L-taloside (39β), D-taloside (40β), 6-deoxy-6-azido-D-mannoside (41β), L-glucoside (42β), D-glucoside (4β), *O*-D-glucoside (43β), *R*-C(3)aglycon (3β), *S*-C(3) aglycon (3α), L-riboside (5α), D-riboside (6α), L-fucoside (7α), D-fucoside (8α), 2-deoxy-D-galactoside (9α), 3-deoxy-D-glucoside (10α), 6-deoxy-D –glucoside (11α), 2-deoxy-2-fluoro-D-glucoside (12α), 6-deoxy-6-fluoro-D-glucoside (13α), L-lyxoside (14α), D-lyxoside (15α), L-rhamnoside (16α), L-alloside (17α), D-alloside (18α), L-altroside (19α), D-altroside (20α), L-galactoside (21α), D-galactoside (22α), L-xyloside (23α), D-xyloside (24α), D-guloside (25α), L-mannoside (26α), D-mannoside (27α), L-idoside (28α), D-idoside (29α), L-mycaroside (30α), 6-keto-D-galactoside (31α), L-arabinoside (32α), D-arabinoside (33α), *N*-acetyl-D-galactosaminoside (34α), melibioside (35α), lactoside (36α), maltoside (37α), D-galacturonoside (38α), L-taloside (39α), D-taloside (40α), 6-deoxy-6-azido-D-mannoside (41α), L-glucoside (42α) and D-glucoside (4α).

9. A composition comprising the neoglycoside of claim 8, pharmaceutically acceptable ester, salt or prodrug thereof combined with a pharmaceutically acceptable carrier.

10. A library of neoglycosides comprising a plurality of neoglycosides produced by the reaction of an aglycon having a secondary alkoxylamine and a reducing sugar selected from the group consisting of a L-sugar, a D-sugar, a deoxy-sugar, a dideoxy-sugar, a glucose epimer, a substituted sugar, a uronic acid, and an oligosaccharide.

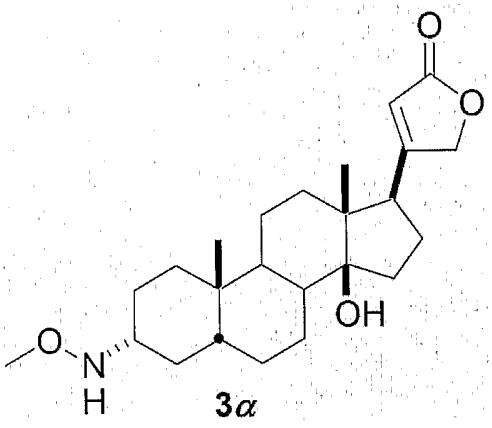
11. The library of claim 10 wherein the neoglycosides each have the structure of the formula:



aglycon

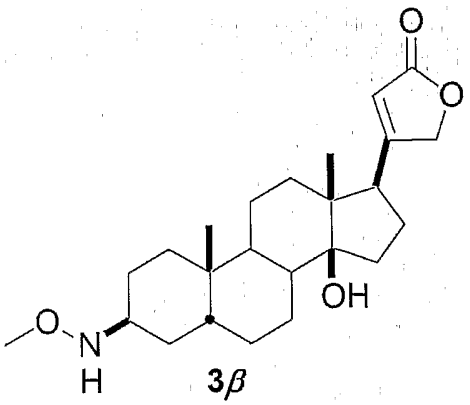
where R_1, R_2, R_3, and R_4 are independently selected from –H, -OH, $-N_3$, $-NH_2$, $-CH_3$, $-CH_2OH$, $-CN_3$, $-CH_2NH$, $-CH_2SH$, $-CNH_2$, $-CH_2N_3$, -COOH, $-COCH_3$, $-CXH_2$, $-CX_2H$, and where X is Cl, Br, F, or I and the aglycon is a digitoxin analog, an indolocarbazole, an anthracyline, a macrolide, a peptide or an alkaloid.

12. The library of claim 10 wherein the aglycon is



3α

or



3β

13. The library of claim 10 wherein the reducing sugar is selected from the group consisting of L-ribose, D-ribose, L-fucose, D-fucose, 2-deoxy-D-galactose, 3-deoxy-D-glucose, 6-deoxy-D-glucose, 2-deoxy-2-fluoro-D-glucose, 6-deoxy-6-fluoro-D-glucose, L-lyxose, D-lyxose, L-rhamnose, L-allose, D-allose, L-altrose, D-altrose, L-galactose, D-galactose, L-xylose, D-xylose, D-gulose, L-mannose, D-mannose, L-idose, D-idose, L-mycarose, 6-keto-D-galactose, L-arabinose, D-arabinose, *N*-acetyl-D-galactosaminose, melibiose, lactose, maltose, D-galacturonose, L-talose, D-talose, 6-deoxy-6-azo-D-mannose, L-glucose, D-glucose, *O*-D-glucose, *R*-C(3)aglycon, *S*-C(3) aglycon and mixtures thereof.

14. The library of claim 10 wherein the neoglycosides are selected from the group consisting of L-riboside (5β), D-riboside (6β), L-fucoside (7β), D-fucoside (8β), 2-deoxy-D-galactoside (9β), 3-deoxy-D-glucoside (10β), 6-deoxy-D-glucoside (11β), 2-deoxy-2-fluoro-D-glucoside (12β), 6-deoxy-6-fluoro-D-glucoside (13β), L-lyxoside (14β), D-lyxoside (15β), L-rhamnoside (16β), L-alloside (17β), D-alloside (18β), L-altroside (19β), D-altroside (20β), L-galactoside (21β), D-galactoside (22β), L-xyloside (23β), D-xyloside (24β), D-guloside (25β), L-mannoside (26β), D-mannoside (27β), L-idoside (28β), D-idoside (29β), L-mycaroside (30β), 6-keto-D-galactoside (31β), L-arabinoside (32β), D-arabinoside (33β), *N*-acetyl-D-galactosaminoside (34β), melibioside (35β), lactoside (36β), maltoside (37β), D-galacturonoside (38β), L-taloside (39β), D-taloside (40β), 6-deoxy-6-azido-D-mannoside (41β), L-glucoside (42β), D-glucoside (4β), *O*-D-glucoside (43β), *R*-C(3)aglycon (3β), *S*-C(3) aglycon (3α), L-riboside (5α), D-riboside (6α), L-fucoside (7α), D-fucoside (8α), 2-deoxy-D-galactoside (9α), 3-deoxy-D-glucoside (10α), 6-deoxy-D – glucoside (11α), 2-deoxy-2-fluoro-D-glucoside (12α), 6-deoxy-6-fluoro-D-glucoside (13α), L-lyxoside (14α), D-lyxoside (15α), L-rhamnoside (16α), L-alloside (17α), D-alloside (18α), L-altroside (19α), D-altroside (20α), L-galactoside (21α), D-galactoside (22α), L-xyloside (23α), D-xyloside (24α), D-guloside (25α), L-mannoside (26α), D-mannoside (27α), L-idoside (28α), D-

idoside (29α), L-mycaroside (30α), 6-keto-D-galactoside (31α), L-arabinoside (32α), D-arabinoside (33α), *N*-acetyl-D-galactosaminoside (34α), melibioside (35α), lactoside (36α), maltoside (37α), D-galacturonoside (38α), L-taloside (39α), D-taloside (40α), 6-deoxy-6-azido-D-mannoside (41α), L-glucoside (42α) and D-glucoside (4α).

15. A method of treating a subject having cancer cells comprising the step of contacting the cancer cells with an effective amount of the neoglyoside of claim 8 or pharmaceutically acceptable ester, salt or prodrug thereof.

16. The method of claim 15 wherein the neoglycoside is selected from the group consisting of L-riboside (5β), D-lyxoside (15β), L-xyloside (23β), D-mannoside (27β), D-arabinoside (33β), D-taloside (40β) and a mixture thereof.

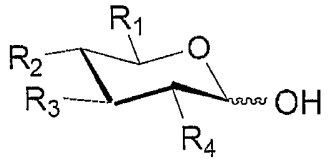
17. A method of making a neoglycoside library comprising the steps of:

providing a plurality of reducing sugars selected from the group consisting of a L-sugar, a D-sugar, a deoxy-sugar, a dideoxy-sugar, a glucose epimer, a substituted sugar, a uronic acid, an oligosaccharide and mixtures thereof; and

contacting the reducing sugars with at least one aglycon having a secondary alkoxylamine to form a plurality of neoglycosides.

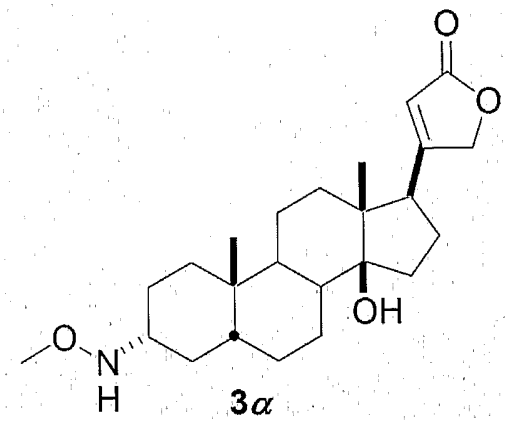
18. The method of claim 17 wherein the aglycon is a digitoxin analog, an indolocarbazole, an anthracyline, a macrolide, a peptide or an alkaloid.

19. The method of claim 17 wherein the reducing sugar is of the formula:



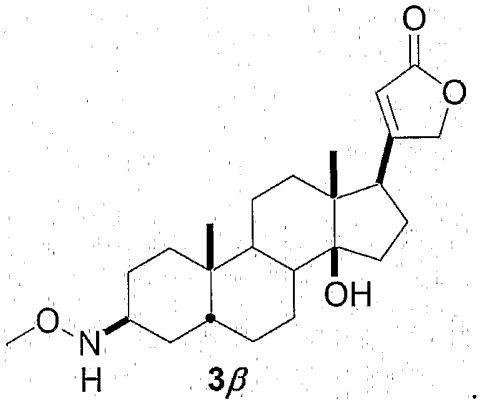
where R_1, R_2, R_3, and R_4 are independently selected from –H, -OH, $-N_3$, $-NH_2$, $-CH_3$, $-CH_2OH$, $-CN_3$, $-CH_2NH$, $-CH_2SH$, $-CNH_2$, $-CH_2N_3$, -COOH, $-COCH_3$, $-CXH_2$, $-CX_2H$, and where X is Cl, Br, F, or I.

20. The method of claim 17 wherein the aglycon is



3α

or



3β

21. The method of claim 17 wherein the step of contacting is performed at a temperature from about 40 to about 60 degrees Celsius.

22. The method of claim 17 wherein the step of contacting is performed in the presence of a 3:1 mixture of DMF and AcOH.

23. The method of claim 17 wherein the neoglycosides are selected from the group consisting of L-riboside (5β), D-riboside (6β), L-fucoside (7β), D-fucoside

(8β), 2-deoxy-D-galactoside (9β), 3-deoxy-D-glucoside (10β), 6-deoxy-D-glucoside (11β), 2-deoxy-2-fluoro-D-glucoside (12β), 6-deoxy-6-fluoro-D-glucoside (13β), L-lyxoside (14β), D-lyxoside (15β), L-rhamnoside (16β), L-alloside (17β), D-alloside (18β), L-altroside (19β), D-altroside (20β), L-galactoside (21β), D-galactoside (22β), L-xyloside (23β), D-xyloside (24β), D-guloside (25β), L-mannoside (26β), D-mannoside (27β), L-idoside (28β), D-idoside (29β), L-mycaroside (30β), 6-keto-D-galactoside (31β), L-arabinoside (32β), D-arabinoside (33β), *N*-acetyl-D-galactosaminoside (34β), melibioside (35β), lactoside (36β), maltoside (37β), D-galacturonoside (38β), L-taloside (39β), D-taloside (40β), 6-deoxy-6-azido-D-mannoside (41β), L-glucoside (42β), D-glucoside (4β), *O*-D-glucoside (43β), *R*-C(3)aglycon (3β), *S*-C(3) aglycon (3α), L-riboside (5α), D-riboside (6α), L-fucoside (7α), D-fucoside (8α), 2-deoxy-D-galactoside (9α), 3-deoxy-D-glucoside (10α), 6-deoxy-D – glucoside (11α), 2-deoxy-2-fluoro-D-glucoside (12α), 6-deoxy-6-fluoro-D-glucoside (13α), L-lyxoside (14α), D-lyxoside (15α), L-rhamnoside (16α), L-alloside (17α), D-alloside (18α), L-altroside (19α), D-altroside (20α), L-galactoside (21α), D-galactoside (22α), L-xyloside (23α), D-xyloside (24α), D-guloside (25α), L-mannoside (26α), D-mannoside (27α), L-idoside (28α), D-idoside (29α), L-mycaroside (30α), 6-keto-D-galactoside (31α), L-arabinoside (32α), D-arabinoside (33α), *N*-acetyl-D-galactosaminoside (34α), melibioside (35α), lactoside (36α), maltoside (37α), D-galacturonoside (38α), L-taloside (39α), D-taloside (40α), 6-deoxy-6-azido-D-mannoside (41α), L-glucoside (42α) and D-glucoside (4α).

24. The use of the neoglycoside of any one of claims 1, 2, 3, 5, 6 or 8 for the manufacture of a medicament for the treatment of cancer.

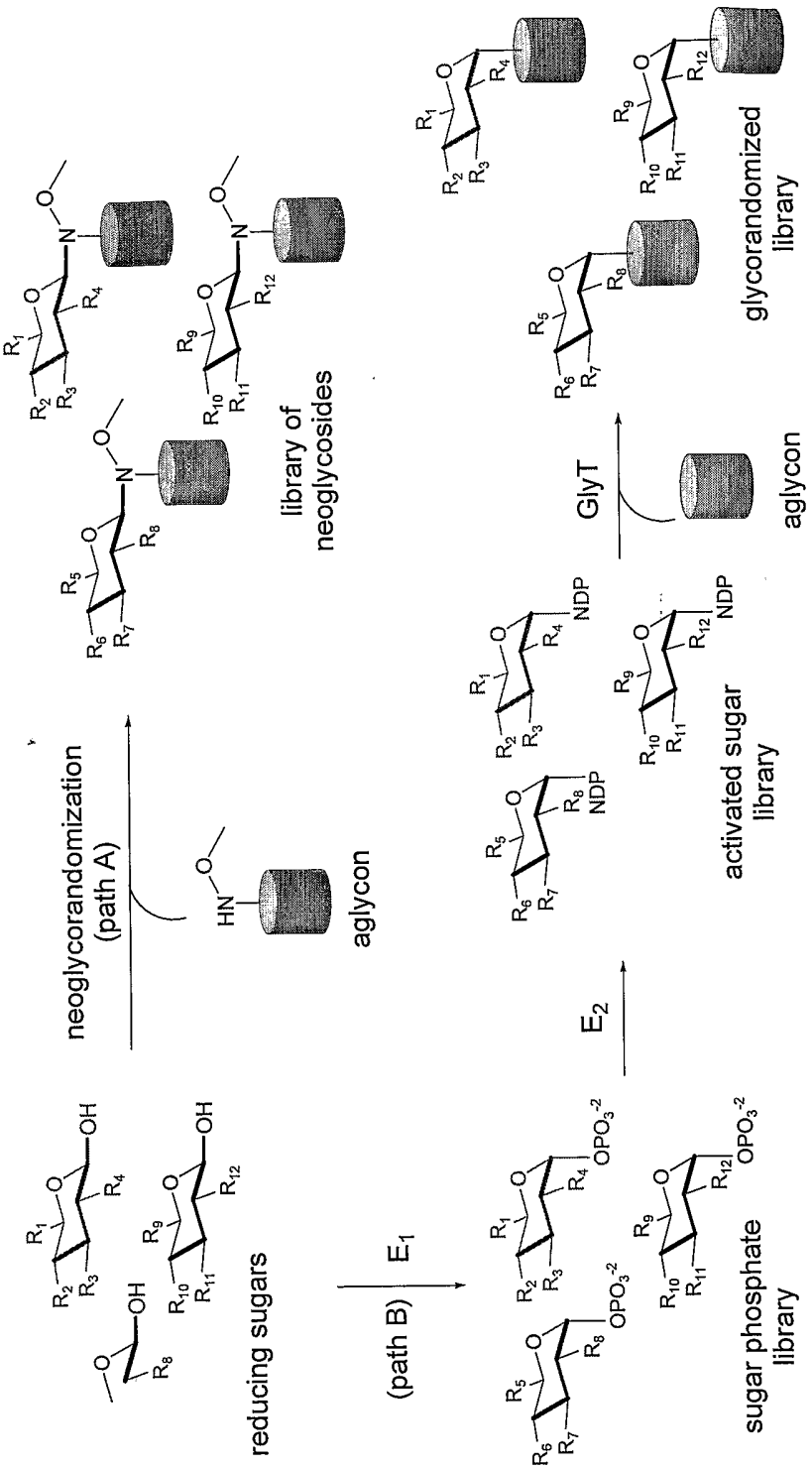

neoglycorandomization
(path A)
aglycon
library of
neoglycosides
reducing sugars
(path B)
E1
E2
sugar phosphate
library
activated sugar
library
GlyT
aglycon
glycorandomized
library

FIG. 1A

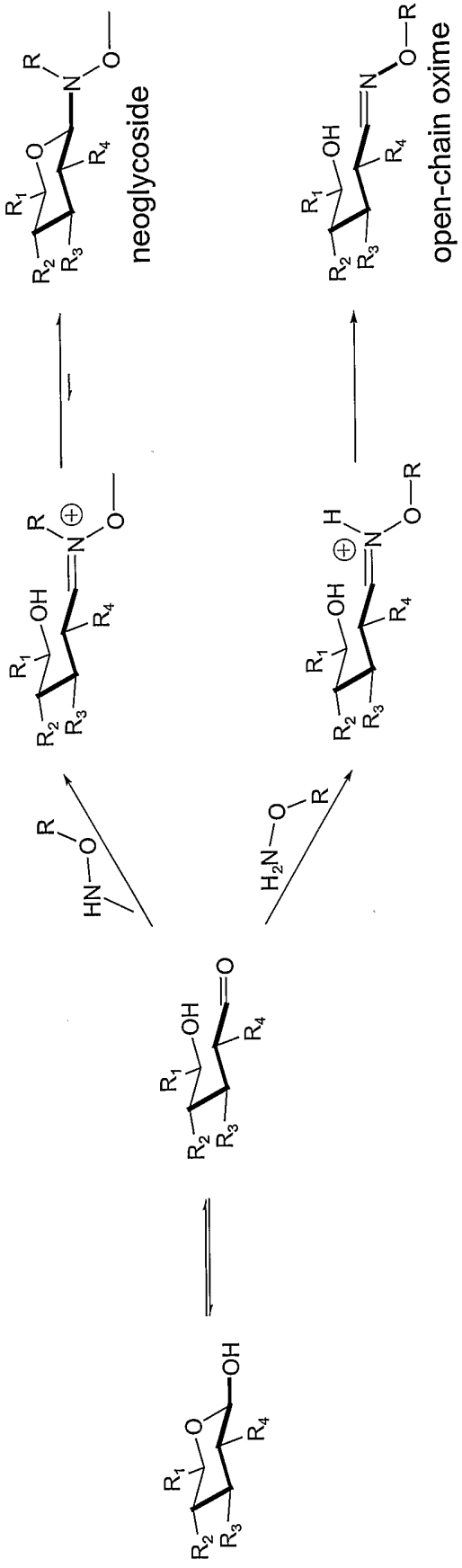


Fig. 1B

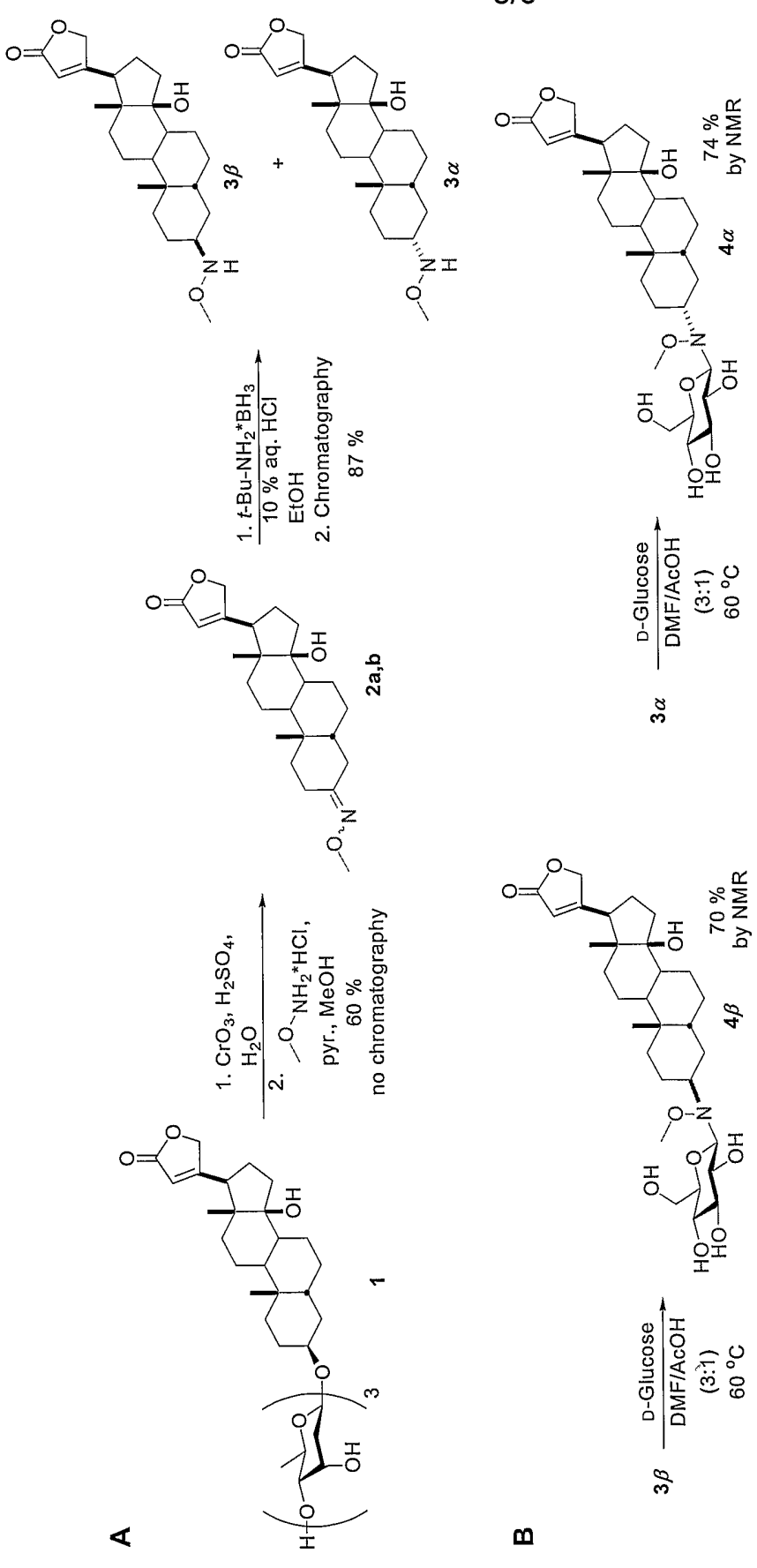

A
1
3
1. CrO_3, H_2SO_4, H_2O
2. NH_2*HCl, pyr., MeOH
60 %
no chromatography
2a,b
1. t-Bu-NH_2*BH_3
10 % aq. HCl
EtOH
2. Chromatography
87 %
3β
+
3α
B
3β
D-Glucose
DMF/AcOH
(3:1)
60 °C
4β
70 %
by NMR
3α
D-Glucose
DMF/AcOH
(3:1)
60 °C
4α
74 %
by NMR

FIG. 2

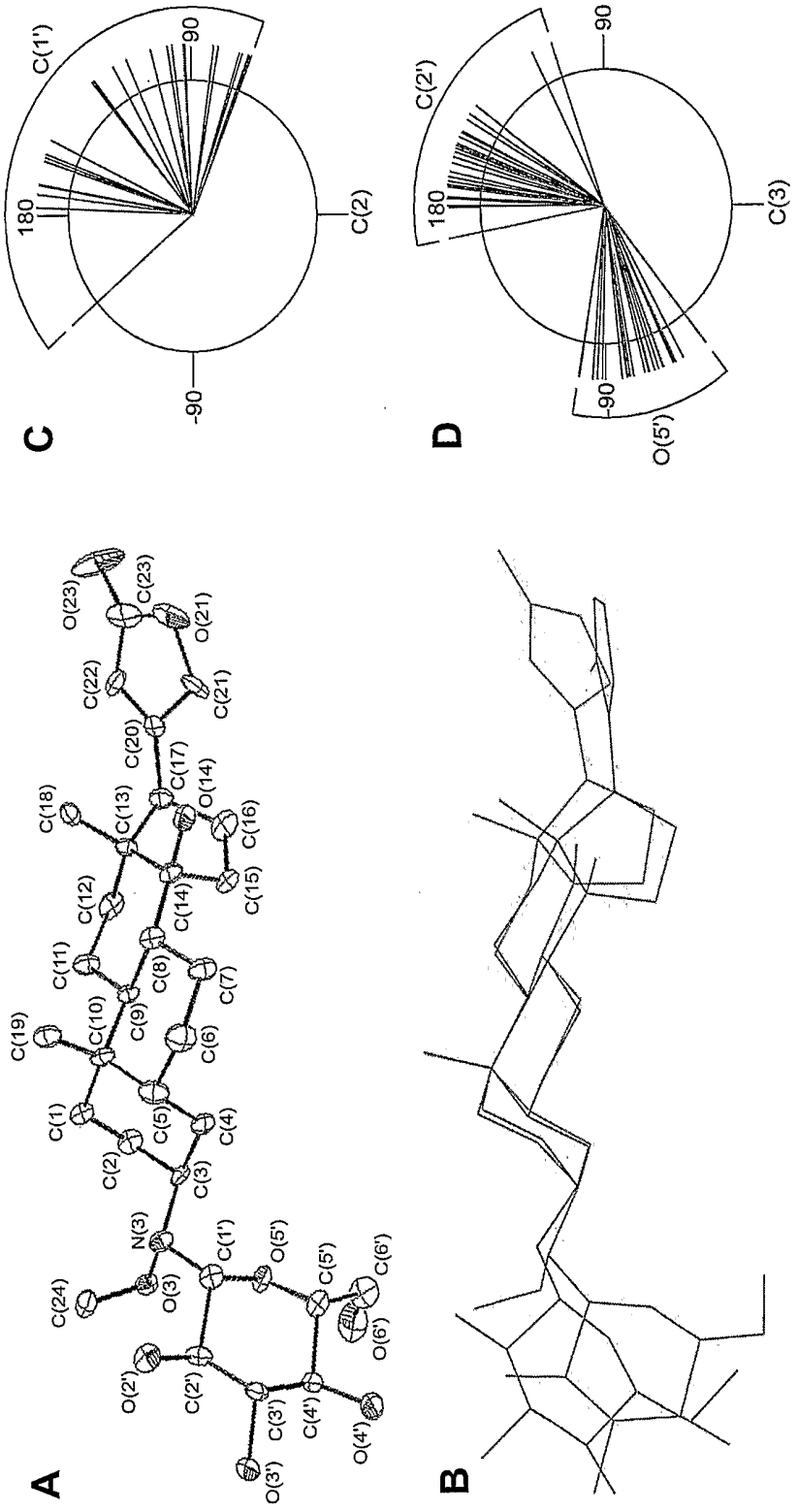

C
C(1')
90
180
C(2)
-90
D
C(2')
90
180
C(3)
-90
O(5')
A
O(23)
C(23)
O(21)
C(22)
C(21)
C(20)
C(17)
O(14)
C(18)
C(13)
C(16)
C(15)
C(12)
C(14)
C(11)
C(8)
C(7)
C(9)
C(19)
C(10)
C(6)
C(1)
C(5)
C(4)
C(2)
C(3)
N(3)
C(1')
O(5')
C(24)
O(3)
C(5')
C(6')
O(6')
O(2')
C(2')
C(3')
C(4')
O(4')
O(3')
B

FIG. 3

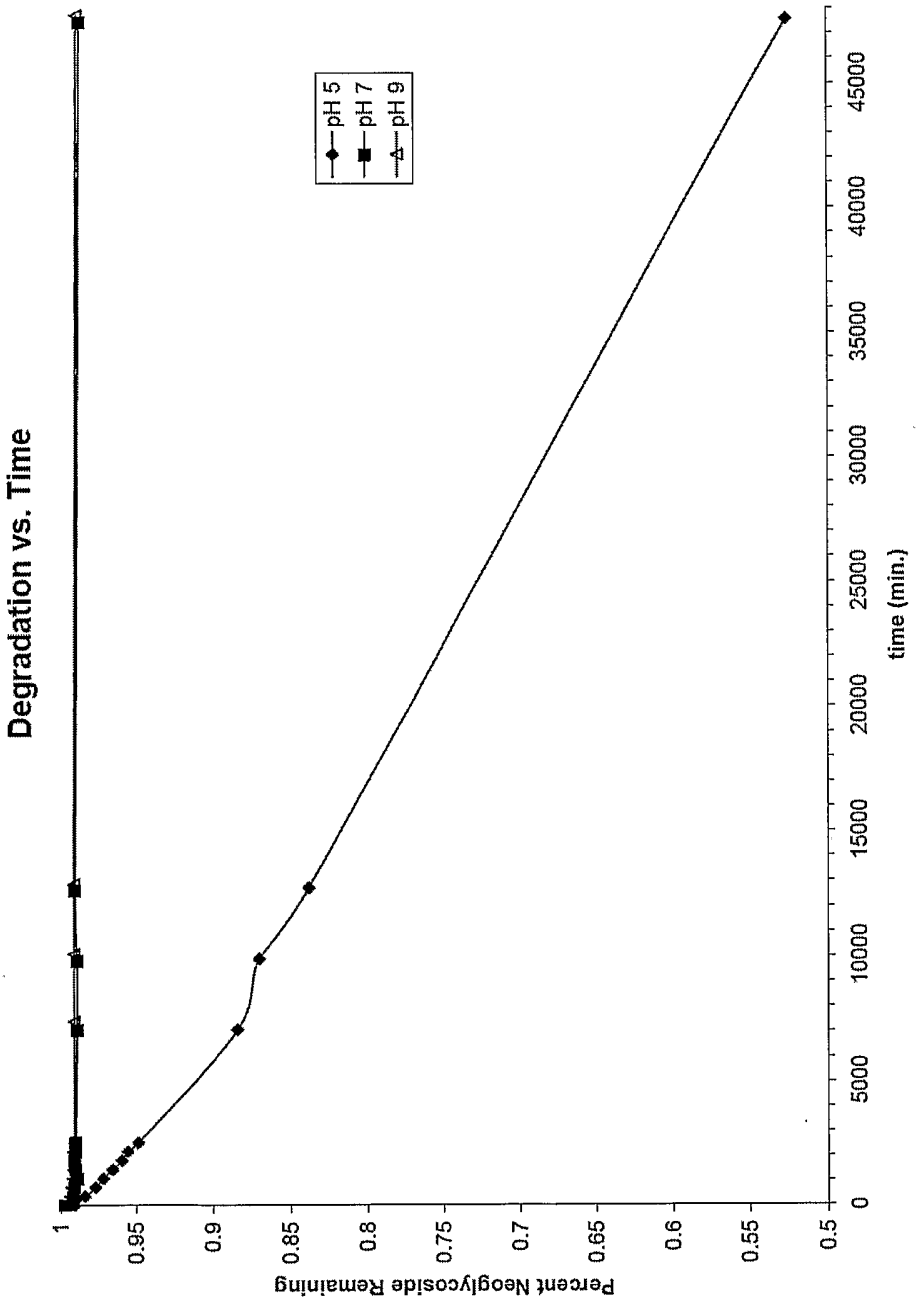

Degradation vs. Time
pH 5
pH 7
pH 9
Percent Neoglycoside Remaining
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
time (min.)

FIG. 4

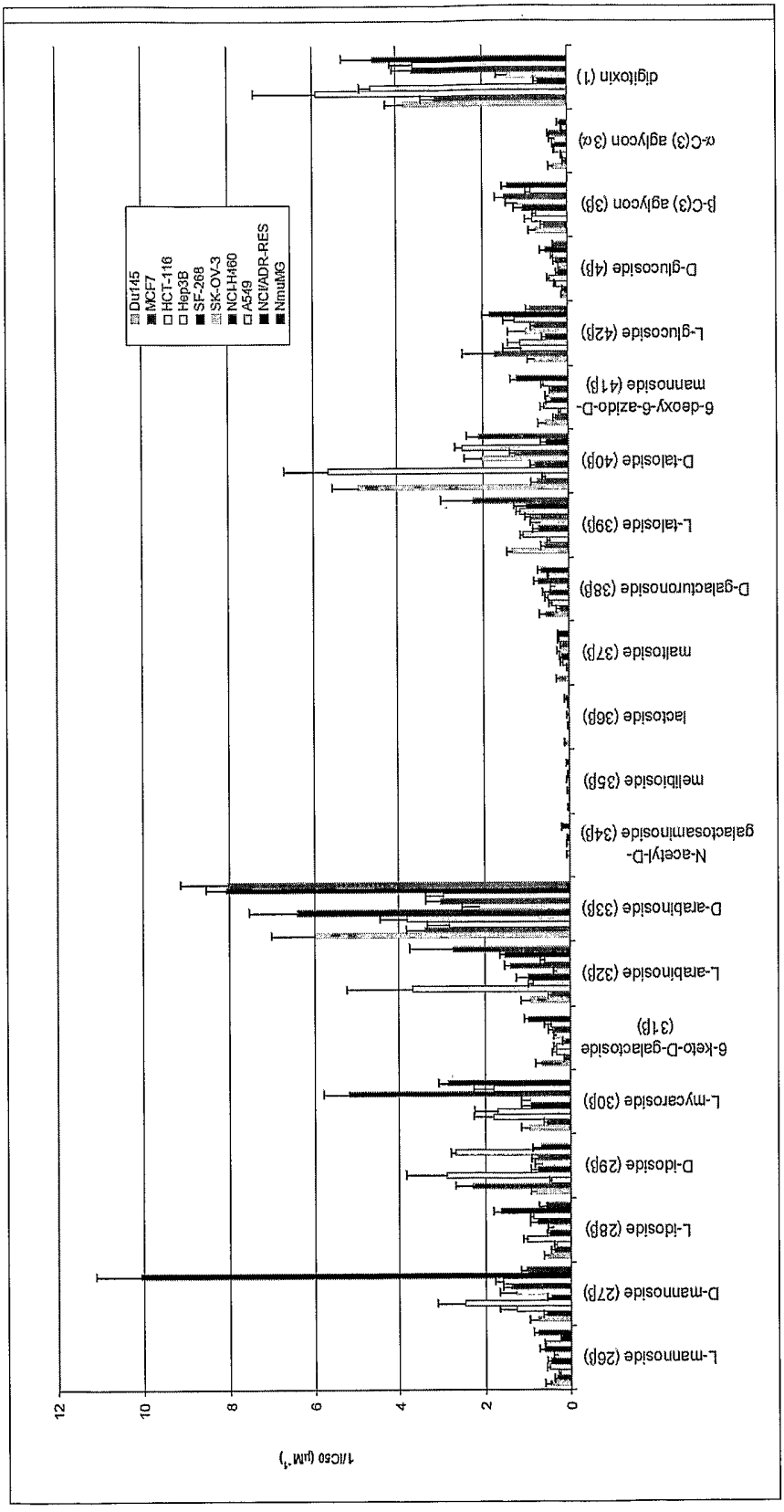

1/IC50 (μM⁻¹)
0
2
4
6
8
10
12
Dur145
MCF7
HCT-116
Hep3B
SF-268
SK-OV-3
NCI-H460
A549
NCI/ADR-RES
NmuMG
digitoxin (1)
α-C(3) aglycon (3α)
β-C(3) aglycon (3β)
D-glucoside (4β)
L-glucoside (42β)
6-deoxy-6-azido-D-mannoside (41β)
D-talοside (40β)
L-talοside (39β)
D-galacturonoside (38β)
maltoside (37β)
lactoside (36β)
melibioside (35β)
N-acetyl-D-galactosaminoside (34β)
D-arabinoside (33β)
L-arabinoside (32β)
6-keto-D-galactoside (31β)
L-mycaroside (30β)
D-lidoside (29β)
L-lidoside (28β)
D-mannoside (27β)
L-mannoside (26β)

FIG. 5A

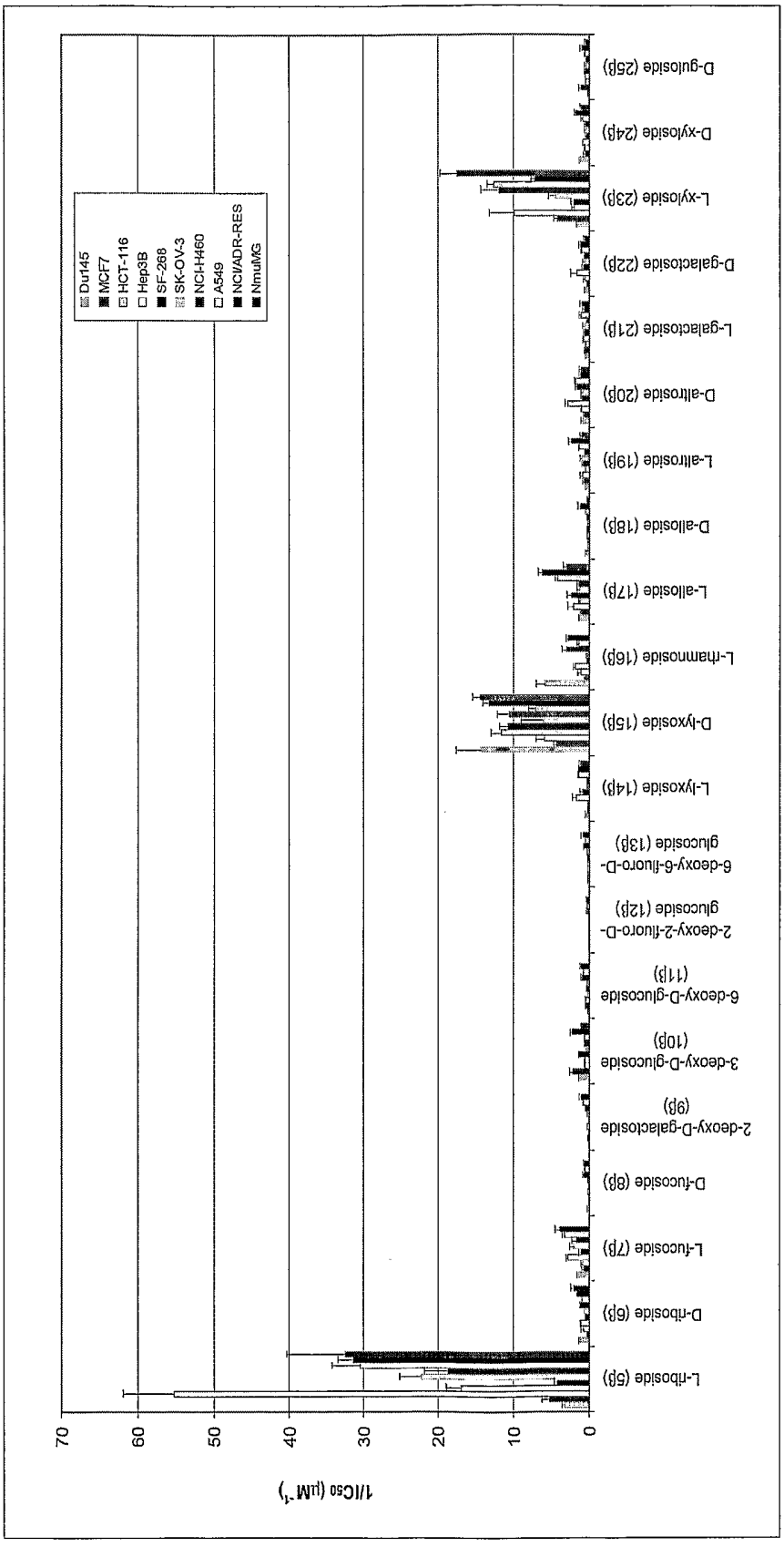

1/IC50 (μM-1)
70
60
50
40
30
20
10
0
Du145
MCF7
HCT-116
Hep3B
SF-268
SK-OV-3
NCI-H460
A549
NCI/ADR-RES
NmuMG
L-riboside (5β)
D-ribosde (6β)
L-fucoside (7β)
D-fucoside (8β)
2-deoxy-D-galactoside (9β)
3-deoxy-D-glucoside (10β)
6-deoxy-D-glucoside (11β)
2-deoxy-2-fluoro-D-glucoside (12β)
6-deoxy-6-fluoro-D-glucoside (13β)
L-lyxoside (14β)
D-lyxoside (15β)
L-rhamnoside (16β)
L-alloside (17β)
D-alloside (18β)
L-altroside (19β)
D-altroside (20β)
L-galactoside (21β)
D-galactoside (22β)
L-xyloside (23β)
D-xyloside (24β)
D-guloside (25β)

FIG. 5B

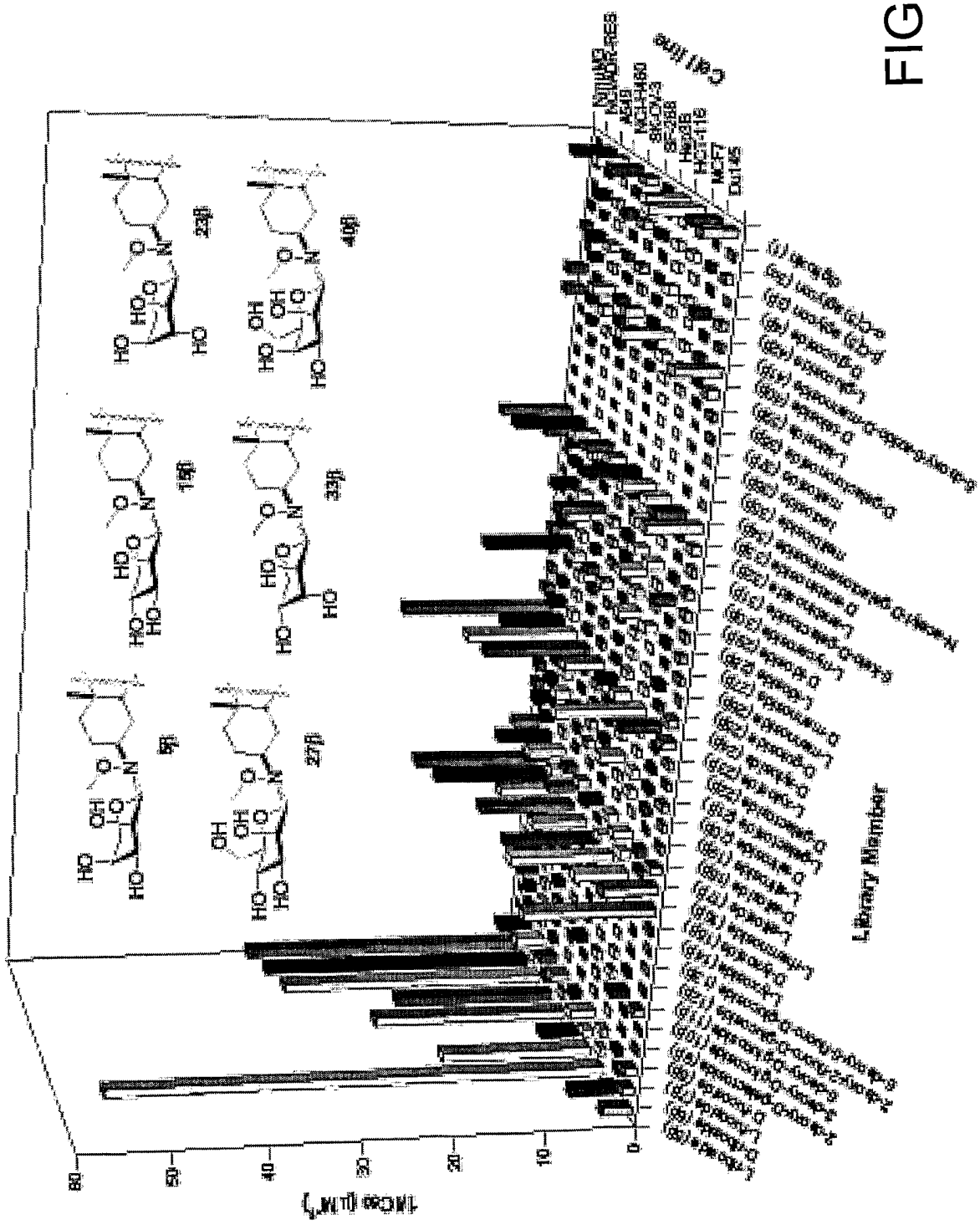


FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/022516

A. CLASSIFICATION OF SUBJECT MATTER
C07J41/00 A61K31/58 A61P35/00

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)
C07J A61K A61P

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, BEILSTEIN Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FILIRA, F. ET AL.: "Opioid peptides: synthesis and biological properties of '(N-glucosyl,N-methoxy)-alpha,gamma-diamino-(S)-butanoyl!4-deltorphin-1-neoglycopeptide and related analogues" ORGANIC & BIOMOLECULAR CHEMISTRY, vol. 1, 7 September 2003 (2003-09-07), pages 3059-3063, XP002354084 page 3059; compound F	1,2,4,5, 7,10,11, 13, 17-19, 21,22
Y	page 3059, column 1, lines 12-23	1-24

-/--

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

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 2005	29/11/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Matés Valdivielso, J

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2005/022516

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOHANSSON, S. ET AL.: "Cytotoxicity of digitoxin and related cardiac glycosides in human tumor cells" ANTI-CANCER DRUGS, vol. 12, June 2001 (2001-06), pages 475-483, XP008055680 cited in the application the whole document	1-24
A	PERI F ET AL: "Chemo- and Stereoselective Glycosylation of Hydroxylamino Derivatives: A Versatile Approach to Glycoconjugates" 1 October 1998 (1998-10-01), TETRAHEDRON, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, PAGE(S) 12269-12278 , XP004138800 ISSN: 0040-4020 cited in the application abstract	1-24

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US2005/022516

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 15 and 16 (in part)
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15 and 16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.