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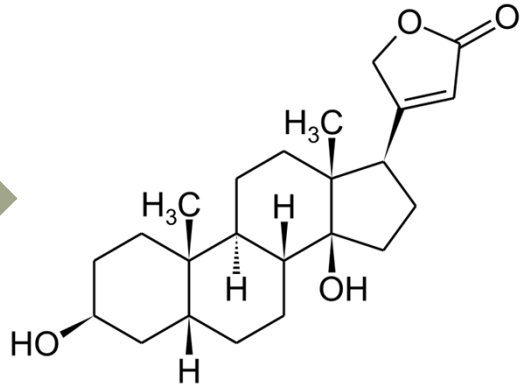
Graphical abstract

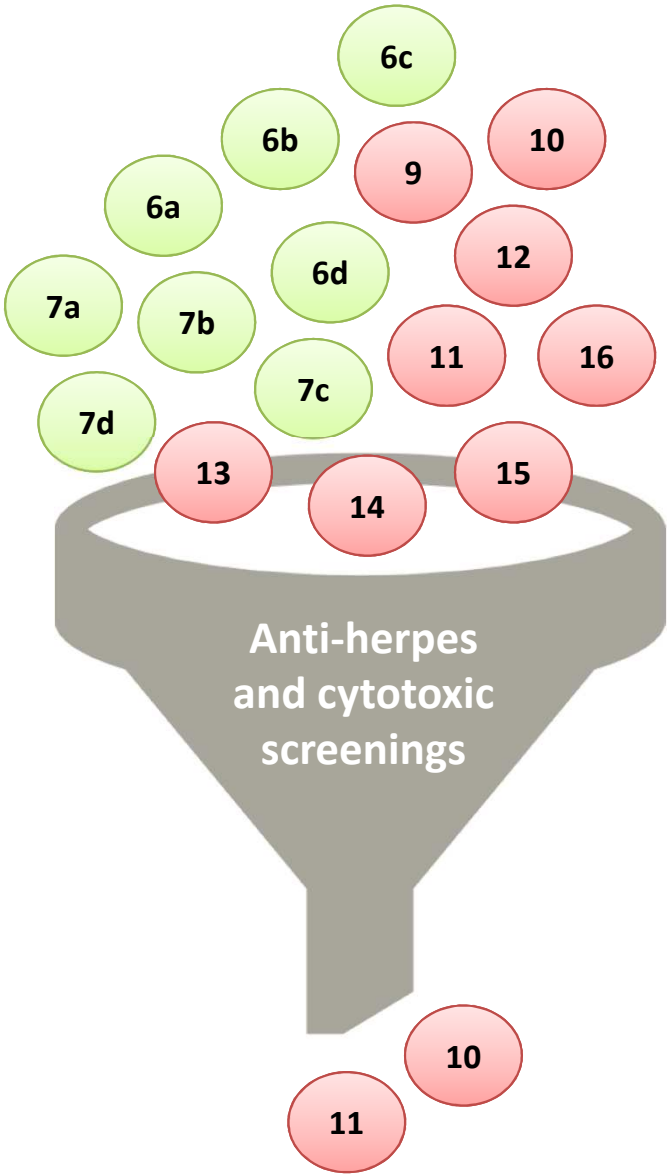
Digitalis lanata



Digitoxigenin

Series i

Series ii



Potential anti-herpes and cytotoxic action of novel semisynthetic digitoxigenin-derivatives

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Abstract

In recent years, new therapeutic possibilities were proposed for cardiac glycosides traditionally used to treat heart diseases, such as anticancer and antiviral activities. In this sense, this work aimed to synthesize the readily accessible 3β-azido-3-deoxydigitoxigenin **(5)** from digitoxigenin **(1)**. Two new series of compounds were obtained from derivative **(5)**: (i) glycosylated triazoles through click chemistry with propargyl glycosides; and (ii) compounds substituted in the alpha carbonyl position with different amines. All obtained derivatives have their chemical structures confirmed, and their anti-herpes (against HSV-types 1 and 2 replication) and cytotoxic (against PC3, A549, HCT-8 and LNCaP cell lines) activities evaluated. Compounds **10** and **11** exhibited the most promising results against HSV-1 (KOS and 29-R strains) and HSV-2 (333 strain) replication with SI values >1,000. Both compounds were also the most cytotoxic for the human cancer cell lines tested with IC_{50} values similar to those of paclitaxel. They also presented reduced toxicity toward non-cancerous cell lines (MRC-5 and HGF cells). All compounds were tested in regard to their ability to inhibit Na^+/K^+ ATPase. The inhibition rate correlates suitably with the bioactivity demonstrated by those both compounds against the different human cancer cells tested as well as against HSV replication. Moreover, the results showed that specific chemical features influenced the

bioactivities tested. In summary, it was possible to obtain novel digitoxigenin-derivatives with remarkable cytotoxic and anti-herpes activities as well as low toxicity and selectivity. In this way, they could be considered potential molecules for the development of new drugs.

Keywords

Cardenolides, digitoxigenin-derivatives, anti-herpes, cytotoxic.

List of Abbreviations

A549: non-small cell lung cancer

ACV: acyclovir

Ac_2O: acetic anhydride

AcOH: acetic acid

ACN: acetonitrile

$AgCO_3$: silver carbonate

AgOTf: silver triflate

$BF_3.Et_2O$: boron trifluoride diethyl etherate

CC_{50}: 50% cytotoxic concentration

$CHCl_3$: chloroform

CH_2Cl_2: dichloromethane

CMC: carboxymethylcellulose

CrO_3: chromium trioxide

$CuSO_4.5H_2O$: cupric sulfate

DIPEA: N,N-diisopropylethylamine

DMEM: Dulbecco's modified eagle's medium

DMF: N,N-dimethylformamide

DMSO: Dimethyl sulfoxide

EDTA: Ethylenediamine tetraacetic acid

ETA: ethanolamine

EtOAc: Ethyl acetate

H460: non-small cell lung cancer

H_2O: distilled water

H_2SO_4: sulfuric acid

HBr: hydrobromic acid

HCl: hydrochloric acid

HCT-8: human ileocecal adenocarcinoma

HGF: human gingival fibroblasts

HSV-1: *Herpes Simplex Virus* type 1

HSV-2: *Herpes Simplex Virus* type 2

IC_{50}: concentration that inhibited 50% of viral replication or cell viability

IR: infrared spectroscopy

K_2CO_3: potassium carbonate

KI: potassium iodide

$LiOH.H_2O$: lithium hydroxide monohydrate

LNCaP: androgen-sensitive human prostate adenocarcinoma cells

MEM: Eagle's minimum essential medium

MeOH: methanol

MRC-5: human fetal lung fibroblast cell line

Na_2CO_3: sodium carbonate

Na_2SO_4: sodium sulfate anhydrous

$NaBH_4$: sodium borohydride

$NaHCO_3$: sodium bicarbonate

NaN_3: sodium azide

NI: no inhibitory activity

NSCLC: non-small lung cancer cells

NT: not tested

NMR: nuclear magnetic resonance

PBS: phosphate-buffered saline

PC3: no hormone-sensitive human prostate adenocarcinoma cells

PFU: plaque-forming units

PI: propidium iodide

Ph_3P: triphenylphosphine

RPMI: Roswell Park Memorial Institute

THF: Tetrahydrofuran

TsCl: p-toluenesulfonyl chloride

TPP: triphenylphosphine

SD: Standard Deviation

SI: Selectivity Index

SRB: sulforhodamine B

1. Introduction

Bioactive compounds from natural sources have great relevance in the development of new drugs used to treat different diseases, including those from microbial and parasitic origins, different types of cancers, and for the control of blood lipid levels [1]. In addition, natural compounds are frequently used as templates for the total synthesis or semisynthesis of derivatives, an useful tool widely explored for drug development [2].

Cardiac glycosides (CGs), classified as cardenolides and bufadienolides, are natural compounds found in species of Apocynaceae (e.g. *Nerium oleander* L.) and Plantaginaceae (e.g. *Digitalis lanata* Ehrh. and *D. purpurea* L.) families [3] among others. They have been used to treat heart diseases for more than 200 years [4] and are characterized by their high specific and potent cardiotonic action. The mechanism of the cardiotonic effects occurs through the inhibition of Na^+/K^+ ATPase responsible for regulate the ions Na^+ and K^+ that promote cardiac muscle contraction [5].

Despite the widespread use of cardenolides as positive inotropic agents, the investigation of their effects on other pathological conditions has been intensified in recent years offering new therapeutic possibilities [6]. One of them is their anticancer action since several authors have already reported cytotoxic and antitumor effects [7-16] (recently reviewed by De et al. [17] and Schneider et al. [18], as well as their potential antiviral activity [19-28].

Cancer is a global disease that accounts for almost 13% of deaths worldwide. It is estimated that by 2020 there will be 15 to 17 million new cases every year [29]. The treatment of cancer depends on several factors, which is generally adapted to the stage of the disease and the characteristics of the tumor. Several chemotherapy drugs are currently available and, as previously mentioned, many are derived from natural products (e.g. paclitaxel from *Taxus brevifolia;* vinblastine and vincristine from *Catharanthus roseus;*

and camptothecin from *Camptotheca acuminata*) [2]. In this sense, several CGs showed potent effects *in vitro* in non-adherent and adherent cancer cell lines e.g. AMANTADIG [9, 10, 13], convallatoxin [12], digitoxigenin [30], ouabain [31], glucoevatromonoside [32, 33] and digoxin [34-36]. Also, some of them have been investigated for cancer treatment in phases I and II clinical trials (eg. extracts rich in different CGs: Anvirzel^{TM}, PBI-05204, HuaChanSu; and the cardenolides UNBS1450 and digoxin) [17, 18, 37].

Besides cancer, some important human diseases are from viral origin, such as those caused by *Herpes Simplex Virus* (HSV-1 and HSV-2), which cause oral, esophageal, genital and rectal lesions [38]. It is estimated that the majority of the population is infected by at least one of HSV. Acyclovir is the gold standard therapy for HSV infections [39-41]. Although this drug as well as other available ones are effective and selective, the emergence of resistant strains has hampered herpes infections treatment since most drugs share the same mechanism of action implying cross-resistance [42, 43]. In this context, natural products can provide an important source of bioactive compounds playing a key role in the research and development of novel anti-herpes products. Several CGs have been tested *in vitro* against HSV replication (eg. Digoxin [25], digitoxin [27], ouabain [44], evatromonoside [45], glucoevatromonoside [20]), and showed to be potent inhibitors of viral replication. Their powerful effects against DNA viruses were well correlated with the inhibition of sodium transport by Na^+/K^+ ATPase [46].

In view of the promising results obtained by the aforementioned researchers, this work aimed to synthesize the readily accessible 3β-azido-3-deoxydigitoxigenin **(5)** from digitoxigenin **(1**). Two new series of cardenolides were obtained from derivative **(5):** (i) glycosylated triazoles through click chemistry with propargyl glycosides; and (ii) compounds substituted in the alpha carbonyl position with different amines, which can be prepared from compound **5** by reduction to 3β-amino-3-deoxydigitoxigenin **(8)**, coupling with chloroacetyl chloride and subsequent substitution at alpha-position with different amines.

In the first series (i), the designed glycosides can be considered as analogues of glucoevatromonoside in which the D-digitoxose moiety was replaced by a triazole in order to facilitate synthesis and to evaluate the influence of the chosen carbohydrates and triazole ring in the activity of digitoxigenin. Besides improving aqueous solubility, the carbohydrate moiety can contribute to the interaction with the biological target (receptor), and can direct the bioactive molecule to cells in view of the presence of carbohydrate receptors in cells surface. For example, D-glucosides can be taken up by cancer cells over expressing D-glucose transporters in their cell surfaces. This lead to the accumulation of glucosylated

compounds inside the cells and therefore can contribute to enhance their activity [47]. D-galactose is a C-4 epimer of D-glucose, while D-mannose is a C-2 epimer, so the corresponding glycosides allow for the evaluation of the influence of sugar configuration on bioactivity. D-cellobiose is a disaccharide formed by two 1-4-β-linked D-glucose units presenting a higher number of hydroxyl groups affecting water solubility and offers the possibility to investigate the influence of an additional D-glucose residue on the biological response.

The second series (ii) was designed based on the structure of AMANTADIG, a potent cytotoxic cardenolide derivative [9, 10, 13]. The coupling with different hydrophobic, hydrophilic, or small mimic of 1-adamantyl-amine residues might help understanding the mechanism of action of bioactive compounds.

All obtained derivatives have their chemical structures confirmed unequivocally, and their anti-HSV-1, anti-HSV-2, and cytotoxic activities against different human cancer cell lines were evaluated.

2. Results and discussion

2.1 Chemistry

The propargyl glycosides of D-glucose, D-galactose, D-mannose and D-cellobiose used to synthesize the triazol glycosides derivatives of digitoxigenin **(1)** were prepared as shown in Scheme 1.

The peracetylated propargyl glycosides **IIa**, **IIb** and **IIc** were obtained by glycosylation of propargyl alcohol with the β anomer of peracetylated D-glucose, D-galactose and α anomer of peracetylated D-mannose, respectively, in dry dichloromethane using boron trifluoride etherate as catalyst, at room temperature [48]. The propargyl glycoside **III** was prepared by the Koenigs-Knorr method, which consisted in the glycosylation of propargyl alcohol with 2,3,6,2',3',4',6'-hepta-*O*-acetyl-α-D-cellobiosyl bromide (**IId**) in dry dichloromethane, using a mixture of silver carbonate and silver triflate as glycosylation promoters [49].

Compound **5** was prepared in four steps from digitoxigenin **(1)** following mainly a literature procedure (Scheme 2) [50]. Digitoxigenin was oxidized to the corresponding digitoxigenone **(2)** by the Jones reagent with 84% yield; reduction of **2** with sodium borohydride in aqueous dioxane at 5 °C furnished 3α-digitoxigenin (**3**) in a stereoselective manner with 94% yield on molar basis. Tosylation of **3** with tosyl chloride in dry pyridine gave the corresponding 3-α-tosylate **4** in 50% yield which, upon reaction with

sodium azide in *N*,*N*-dimethylformamide (DMF) at 75 °C, furnished 3β-azido-3-deoxydigitoxigenin **(5)** with a yield of 85% on molar basis [50].

The *click* reaction of the propargyl glycosides **IIa-c** and **III** with 3β-azido-3-deoxydigitoxigenin **(5)** in a mixture of tetrahydrofuran/water, in the presence of copper (II) sulfate and sodium ascorbate, gave the glycosylated triazol derivatives of digitoxigenin **6a-d** [51]. Deacetylation with lithium hydroxide in water/methanol afforded the corresponding deprotected derivatives **7a-d** [52, 53].

Reduction of compound **5** with triphenylphosphine, in a mixture of tetrahydrofuran/water (70 °C), furnished 3β-amino-3-deoxydigitoxigenin (**8**) with 60% yield [50, 54]. The catalytic reduction of derivative **5** was performed based on the patent WO2013000286 [54] and the Staudinger reaction; however, the extraction of 3β-amino-3-deoxydigitoxigenin **(8)** followed the procedure described by Sawlewicz et al. [50]. The reaction of compound **8** with chloroacetyl chloride, in tetrahydrofuran and potassium carbonate, gave 3-β-(chloroacetylamino)-3-deoxydigitoxigenin **(9)** [54, 55] (90% yield).

Compound **9** was used for synthesis of derivatives **10**-**14**, as shown in Scheme 2. Reaction of **9** with ethanolamine in tetrahydrofuran gave derivative **10** (85% yield). Finally, one-pot reactions or those occurring in two steps of derivative **9** with potassium iodide (KI) in a mixture of acetonitrile/water, gave the iodine derivative, which was used to obtain the amino- and aromatic-amino-digitoxigenin and hydroxyl derivatives **11**-**14**, through the reaction with the appropriate compound in acetonitrile using DIPEA as base [56, 57]. After chromatographic purification, all derivatives were obtained with yields ranging from 23% **(12)** and 25% **(11, 13)** to 46% **(14).**

The obtained compounds were characterized by IR, NMR and ESI-MS spectroscopy. The infrared spectra of the propargyl glycosides **IIa-c** and **III** showed bands in the region of 3255-3282 cm^{-1} (C-H, sp carbon), 2117-2119 cm^{-1} (C≡C), and 1732-1754 cm^{-1} (C=O). The ^{1}H NMR spectra of these compounds showed signals at δ_H 1.91-2.10 ppm ascribed to the CH_3CO groups, as well as signals at δ_H 2.41-2.44 ppm attributed to terminal acetylenic hydrogens. The duplets centered at δ_H 4.73, 4.67 and 4.67 ppm (*d*, *J*=7.9-8.0 Hz; 1H, H-1) were assigned to the anomeric hydrogens of the propargyl glycosides **IIa**, **IIb** and **III**, respectively. The *J*-coupling values are compatible with β-type glycosides (*trans*-diaxial coupling) [58]. The anomeric hydrogen of the propargyl glycoside **IIc** ressonates at δ_H 4.96 ppm (*d*, *J*=1.6 Hz; 1H, H-1), and its *J*-coupling indicates α-configuration (diequatorial coupling). The ^{13}C NMR spectra showed signals

at δ_C 54.9-55.9 ppm ascribed to the methylene group of the propargyl moiety, and δ_C 96.2-100.6 ppm attributed to the anomeric carbon.

The spectral data of digititoxigenin derivatives **2-5** are in accordance with their structures. The infrared spectrum of the key intermediate compound **5** showed a strong absorption band at 2097 cm^{-1} due to N=N=N stretching of the azido group. The ^{13}C resonance signal at δ_C 58.6 ppm (CH, C-3) was ascribed to the corresponding C-N_3 of this compound, thus confirming the structure.

The infrared spectra of the peracetylated glycosylated triazol derivatives of digitoxigenin **6a-d** showed bands at 3412-3468 cm^{-1} (OH, C-14), 1739-1746 cm^{-1} (C=O, ester) and 1218-1228 cm^{-1} (C-O, ester). The ^1H NMR spectra of these compounds showed signals at δ_H 0.82-0.94 ppm (methyl groups at C-18 and C-19 of the aglycone), δ_H 1.91-2.16 ppm (CH_3C=O), δ_H 3.50-5.50 ppm (pyranosidic protons), δ_H 5.81-5.89 ppm (*s*,1H, CH, H-22) and 7.60-7.66 ppm (hydrogen of the triazole ring). These structural features were confirmed by their ^{13}C NMR spectra, which showed signals at δ_C 15.6-15.9 ppm (CH_3, C-18), δ_C 20.4-20.8 ppm (CH_3, CH_3=O), δ_C 22.4-23.5 ppm (CH_3, C-19), δ_C 96.8-100.6 ppm (CH, C-1'), δ_C 117.4-117.5 ppm (CH, C-22), andδ_C 169.0-170.6 ppm (C=O, ester). The β-configuration at the anomeric carbon of glycosides **6a**, **6b** e **6d** was confirmed by the resonance signals at δ_C 99.8-100.6 (CH, C-1'). On its turn, the α-configuration at the anomeric carbon of glycoside **6c** was indicated by the signal at δ_C 96.8 ppm (CH, C-1').

The infrared spectra of the deprotected glycosylated triazol derivatives of digitoxigenin **7a-d** showed, as expected, absorption bands in the region of 3253-3397 cm^{-1}. This is due to OH stretching of the carbohydrate moiety, as well as absence of carbonyl absorption bands at *circa* 1750 cm^{-1}, found in the spectra of the peracetylated precursors. Similarly, the signals of hydrogen and carbon of the acetyl groups are absent in the ^1H and ^{13}C NMR spectra of compounds **7a-d**.

The infrared spectrum of compound **8** showed a band in 3356 cm^{-1} that is characteristic of NH_2 stretching, and a band in 3280 cm^{-1} due to OH stretching. The resonance signals at δ_H 4.05 ppm (tt, J =4.4 Hz, J =11.9 Hz, 1H, H-3) and at δ_C 52.6 ppm (C-3) in the ^1H and ^{13}C NMR spectra of derivative **8** allowed us to confirm the structure of this key intermediate.

The infrared spectrum of compound **9** showed bands at 3335-3455 cm^{-1} due to NH and C=O stretching of the amide group. The formation of the 2'-chloroacetamide derivative **9** was further confirmed by the NMR signals at δ_H 7.33 ppm (d, J =5.5 Hz, 1H, N-H) and δ_C 166.0 ppm (C-1').

In the same way, infrared data obtained for compound **10** showed bands at 3304 and 1643 cm^{-1} related, respectively, to NH and C=O stretching of the amide group. The resonance signals at δ_H 2.91 ppm (t, J =6.5 Hz, 1H, H3'), 3.73 (t, J =6.5 Hz, 1H, H4') and δ_C 52.2 (C-3') and 64.0 (C-4') further evidenced the structure of the 2'-hydroxyethyl derivative **10**.

The NMR data obtained for compound **11** were very similar to those of derivative **9**. The difference is owing to the substitution of the chloride atom at compound **9** for the more electronegative oxygen at compound **11**, which resulted in deshielding of the resonance signal of C-2' at derivative **11** in comparison to compound **9** (62.8 vs. 43.8 ppm, respectively).

The spectral data of derivatives **12** and **14** were in agreement with their structures and the infrared spectrum of compound **12** showed the characteristic C≡N stretching band at 2214 cm^{-1}, while the spectrum of derivative **14** exhibited the C-Cl bending at 820 cm^{-1}. The ^{1}H and ^{13}C NMR spectra of compounds **12** and **14** demonstrated the aromatic nature of the amine group bound at C-2' as well as the presence of the 4-phenylpiperidine group in compound **13**.

The purity of all synthesized compounds were ≥ 95%, and their mass spectra showed molecular weight values compatible with the proposed structures (Supplementary Data available).

This synthesis approach included in total 16 new cardenolide derivatives that were all derived from 3β-azido-3-deoxydigitoxigenin **(5),** and could be potentially cytotoxic similar to other structures based on the 3β-amino-3-deoxydigitoxigenin **(8)**, which were already tested and showed promising cytotoxic effects [eg. AMANTADIG (3β-[2-(1-amantadine)-1-on-ethylamine]-digitoxigenin) against leukemia (K562 and SEM), prostate cancer (PC-3, DU145, LNCaP) and renal tumor cells (A498, 786-O and Caki-1) [9, 10, 13]. Further, bufogenin derivatives containing similar residues also showed cell growth inhibition of different cancer cell lines, such as non-small lung (A549), breast (MCF-7), colon (LoVo) and prostate (PC3) [54]. Compound **(8)** was coupled to residues individually, which were chosen following [55] or presenting small mimics of the basic AMANTADIG structure. Moreover, we describe herein, for the first time, the synthesis of peracetylated and non-acetylated glycosylated triazol derivatives of digitoxigenin **(1)** as well their bioactive effects. Since the triazol group shifts the sugar moieties, its presence in the

steroid scaffold can also influences bioactivities. In summary, the newly synthesized compounds will help to identify functional groups that are important for their cytotoxicity and anti-herpes effects.

2.2 Biological activities

2.2.1 Anti-herpes in vitro activity

2.2.1.1 Plaque number reduction assay

According to the CC_{50} values (Table 1), only compounds **9** (83.98 ± 9.03 µM) and **15** (36.07 ± 6.58 µM) presented moderate toxic effects, while the other derivatives showed low toxicity on Vero cells ranging from 111.9 ± 7.01 to >300 µM. Regarding the plaque number reduction assay, compounds **10** and **11** exhibited the most promising results against HSV-1 (KOS and 29-R strains) and HSV-2 (333 strain) with SI values >1,000. Comparing to acyclovir (ACV), used as positive control, these results are similar for HSV-1(KOS strain) and much better for HSV-2. Concerning to the anti-HSV-1 activity (KOS strain), the obtained IC_{50} values of compounds **10** and **11** were 0.23 ± 0.01 and 0.24 ± 0.03 µM, respectively, almost six-folder more potent than ACV (1.38 ± 0.46 µM). On its turn, the obtained IC_{50} values of compounds **10** and **11** against HSV-2 replication were 0.27 ± 0.01 and 0.30 ± 0.04 µM, respectively, twelve and eleven-folder higher than ACV (3.23 ± 0.89 µM), respectively. Regarding the anti-HSV-1 activity (29-R strain, which is resistant to ACV), both compounds were still more active showing IC_{50} values of 0.18 ± 0.01 and 0.19 ± 0.02 µM, respectively. It is worth mentioning that compounds **7a**, **7b**, **7c**, **12**, **13** and **14** also showed encouraging results with SI values >100 for all viruses tested.

Anti-herpes activity of other cardenolides has been reported against HSV-1 and HSV-2 [20, 27, 46] replication. Results found for compounds **10** and **11** were similar to those of glucoevatromonoside (GEV) [20] and digitoxin [27]. On the other hand, regarding to cardenolides toxicity that show narrow therapeutic index [18], our compounds were less cytotoxic on Vero cells when compared to GEV [20] and digitoxin [27] which can be considered an advantage.

Herein, digitoxigenin **(1)** was used as scaffold for the semisynthesis of novel cardenolide derivatives, and then assayed for all viruses tested. Digitoxigenin **(1)** alone was one of the most cytotoxic compounds on Vero cells (27.54 ± 4.29 µM) (Table 1), while presented potent IC_{50} values against the three tested HSV strains. These results disclosed digitoxigenin **(1)** as the tested compound with the lowest SI values,

whereas its derivatives here described showed higher SI values. This reinforces the importance of the synthesis/semisynthesis of new derivatives more active and less cytotoxic.

2.2.2 Cytotoxic activity

2.2.2.1 Cell viability

For the cytotoxic screening, the 16 new cardenolide derivatives were tested on human A549, HCT-8, LNCaP and PC3 cell lines for 48 h, and stained with sulforhodamine B (SRB) that measure total protein mass, which is related to cell viability. Fig. 1 shows the color grid effects, where compounds labeled with shades of green were considered the most active and those with red shades were less active. Compounds **6c**, **7c**, **10**, **11**, **12**, **13**, **14** and **16** inhibited cell proliferation, wherein once again compounds **10** and **11** appeared to be the most cytotoxic, with IC_{50} values similar to those of the anti-cancer drug paclitaxel here used as a positive control. The remaining compounds were less cytotoxic showing IC_{50} values up to 29.46 µM after 48 h [Table S1 in Supplementary Data contains IC_{50} values and Standard Deviation (SD)].

During the initial cytotoxic screening, A549 cell line was the most sensitive for the tested cardenolide derivatives, with IC_{50} values ranging from 0.11 to 2.86 µM. For this reason, the most cytotoxic compounds on A549 cells were tested, at the same concentrations, on other NSCLC, the H460 cell line. Taken together (Table 2), these results showed that the most promising cardenolide derivatives were compounds **7c**, **10**, **11**, **12** and **16**. To verify the selectivity on non-tumoral cells, the selected compounds were tested on MRC-5 and HGF cells. They were all less cytotoxic for HGF cells than for A549 or H460 cells (Table 2). Digitoxigenin (**1),** the aglycone of digitoxin, was used as scaffold for the semisynthesis of the novel derivatives herein tested, and it showed a moderate activity for all human cancer cells assayed, when compared to their derivatives (shades of yellow, Fig. 1).

Our results are in line with previous investigations showing the ability for same CGs to induce cytotoxic effects mainly in lung cancer cells, as it has been showed for digoxin, ouabain [59], convallatoxin [60], and glucoevatromonoside [33]. Moreover, these potent cardenolides showed IC_{50} values at nM concentration range, as it was also identified herein for compounds **7c**, **10**, **11, 12** and **16**.

Additionally, the IC_{50} value of compound **10** on the non-tumor MRC-5 cells was similar to that of digoxin (~150 nM) [59] and even better for compound **11** (IC_{50} 250 nM). This could be an important finding since 16 clinical trials with digoxin are in progress to treat several cancer types, including lung cancer [18].

2.2.3 Effects on Na+/K+ ATPase

The most promising anti-herpes compounds were the same most cytotoxic (compounds **10** and **11**) suggesting that they share the same primary target (i.e. Na^+/K^+ ATPase). As recognized and explored in the literature, Na^+/K^+ ATPase plays essential roles in ionic homeostasis and is the main target of cardenolides through their binding to the alpha-subunit [61, 62]. All compounds evaluated for their cytotoxic activity were also tested in regard to their ability to inhibit Na^+/K^+ ATPase. The IC_{50} values found for the inhibition of this enzyme were ranging from 0.84 µM (**11**), 1.99 µM (**10**), 2.04 µM (**16**), 4.16 µM (**12**), 4.46 µM (**6c**) 5.47 µM (**7c**), 6.60 µM (**13**) to 10.82 µM (**14**) (Fig. S1 in Supplementary Data).

In most cases, it is possible to establish a correlation between the cytotoxicity of the individual CGs and their ability to bind to the alpha subunits and inhibit Na^+/K^+ ATPase even at in low concentrations [10]. Within our study, we only determined the inhibition rate of the new derivatives using a mixture of α1,2,3 subunits of Na^+/K^+ ATPase. Therefore, we cannot distinguish between the selective affinities of the derivatives on the individual α subunits. It has been previously demonstrated that different CGs and some derivatives have distinct affinities to the four individual α subunits. Several studies addressed the selective affinities of CGs, such ouabain towards α1 and α3 subunits or digoxin towards α2 subunit [63-65]. In addition, Clifford and Kaplan [66] demonstrated that malignant or oncogene-transfected cells are less sensitive than non-tumor breast cells to ouabain-mediated inhibition of proliferation and induction of apoptosis. A further study clearly demonstrated that proliferation and survival of malignant and non-tumor cell lines in the presence of ouabain are depending on the cell surface abundance and the expression rates of the different α subunits of the Na^+/K^+ ATPase, also influencing its selectivity index [67]. To evaluate the affinity of compounds **10** and **11** towards the individual α subunits will be an interesting and challenging further project.

The inhibition rate correlates suitably with the bioactivity demonstrated by compounds **10** and **11** that were the most actives against the different tested human cancer cell lines as well as against HSV replication. This correlation was also showed for other cardenolide derivates such as AMANTADIG [10, 13] whose bioactivity was not discussed only based on the cardenolide scaffold but also on the antiviral action of 1-adamantyl-amine residue [9].

2.3 Influence of specific chemical features on bioactivity of new digitoxigenin-derivatives

In this work, two series of digitoxigenin derivatives were obtained and evaluated against different cancer cell lines. Series (i) is formed by glycosylated triazole derivatives (compounds **6a-d** and **7a-d**) in which compounds **6c** and **7c** presented promising results for cytotoxicity and compounds **7b** and **7c** for anti-herpes activity. Compound **7c** was the best one showing a lower IC_{50} value than digitoxigenin **(1)** suggesting that groups bound to the position H-3β of digitoxigenin **(1)** may influence positively both activities. Furthermore, compound **7c** has a mannose bound to the tetrazolic nucleus. Thus, the configuration of the hydroxyl group at positions C-1 (alpha-configuration) and C-2 (beta-configuration) of mannose may influence positively the activities, when compared to compounds **7a, 7b** and **7d**, which do not present hydroxyl groups with these configurations. Moreover, the deacetylated glycosylated triazole derivatives **(7a-d)** were more active than the corresponding acetylated compounds **(6a-d)** indicating that the free hydroxyl group of the sugar may improve both activities. This finding is aligned with previous reports that demonstrated the influence of sugar residues attached to digitoxigenin scaffold on the antitumor activity [68, 69]. It is also noteworthy to mention that triazole derivatives possess an additional structural feature that may favor bioactivity: the presence of mannose receptor in cell membranes (Endo 180 - endocytic recycling glycoprotein) [70] that can facilitate absorption of those derivatives by the cells [71].

Derivatives of series (ii) (compounds **9-15)** are based on the bioactive semisynthetic cardenolide AMANTADIG (3β-[2-(1-amantadine)-1-on-ethylamine]-digitoxigenin. Compounds **10** and **11**, presenting a hydroxyl group and an ethanolamine group, both bound to the alpha-carbonyl position, were the most active ones relating the anti-herpes and cytotoxic activities. On the other hand, hindering groups bounding at an electronegative atom, as those found in compounds **12**, **13** and **14**, interfere negatively in both activities. Consequently, the presence of an electronegative atom at the alpha-carbonyl position of the amide group at the side chain at C-3 seems to be important to reduce the IC_{50} values in comparison to digitoxigenin **(1)**. In addition, lipophilicity of the compounds may interfere with the biological response, since the target of cardenolides is the membrane bound Na^+/K^+ ATPase enzyme. In order to investigate the relationship between lipophilicity and bioactivity, we calculated the LogP values [72] of some of the bioactive compounds of series (ii), and compared the obtained values with the ability of the derivatives to inhibit Na^+/K^+ ATPase. Lower LogP values (1.59 and 1.96) were obtained for the highly active cytotoxic compounds **10** and **11** in comparison to the values calculated for the less cytotoxic compounds **12** (3.78),

13 (5.16), **14** (4.53) and **15** (3.50). These results suggest that lipophilicity may influence the binding of the tested derivatives to the Na^+/K^+ ATPase and therefore their cytotoxic effects.

Furthermore, it was recently demonstrated that the IC_{50} values of the derivative AMANTADIG (3β-[2-(1-amantadine)-1-on-ethylamine]-digitoxigenin were 0.68 and 0.186 µM for PC-3 and LNCaP cell lines, respectively [9, 13]. Compounds **10** and **11** proved to be nearly as cytotoxic or even more cytotoxic for PC-3 or LNCaP prostate cancer cell lines. In this work, compound **11,** which contains the smallest and simplest hydroxyl group as residue, showed similar IC_{50} value for PC-3 cell line (0.42 µM). Compound **10** containing a N-(2-hydroxyetil)aminoacetyl residue at C-3 was even more cytotoxic for both cell lines (PC-3: 0.18 µM and LNCaP: 0.13 µM) than AMANTADIG, leading to the conclusion that the coupling of the cardenolide scaffold to the 1-adamantyl-amine residue is not essential for the reduction of cancer cells viability. This conclusion was strengthened by evaluating the 1-adamantyl-amine residue inhibition potency of the Na^+/K^+ ATPase and its bioactivity against two carcinoma cell lines [DU145 and 786-O (data not shown)]. These results indicated that the 1-adamantyl-amine residue neither has the ability to inhibit Na^+/K^+ ATPase nor to reduce the viability of both tested cell lines. However, AMANTADIG as well as the novel cardenolide derivatives reported here, whose advantage is to have a selective influence on cell viability of different cancer cell lines, are all very promising compounds. This was shown by the different IC_{50} values of the same compound obtained on different cell lines (Table 2 and Fig. S1).

3. Conclusions

Two new series of compounds were obtained from 3β-azido-3-deoxydigitoxigenin **(5)** from digitoxigenin **(1)**: (i) glycosylated triazoles through click chemistry with propargyl glycosides; and (ii) compounds substituted in the alpha carbonyl position with different amines. These synthesis approaches generated 16 new cardenolide derivatives, and regarding their anti-herpes and cytotoxic activities, the most potent compounds were **10** and **11.**

In relation to anti-herpes action, both compounds were very active against all tested HSV strains, but they were even more active against a resistant ACV strain (HSV-1, 29-R). It is well known that the emergence of resistant strains to ACV has hampered the treatment of herpes infections, since most available antiviral drugs share the same mechanism of action implying cross-resistance. In this way, the search for new alternatives is an important goal to follow, and our findings seem to be in this direction. Complementary approaches to better understand how they inhibit HSV replication cycle have to be performed.

Regarding the cytotoxic activity against different cancer cell lines, our findings suggest that lung cancer cells were more sensitive to compounds **10** and **11**, and the results obtained allow us to conclude that they could be seen as promising molecules as well, and deserve further studies to elucidate their mechanisms of action.

4. Experimental Section

4.1 General methods

4.1.1 TLC analysis

TLC plates (Merck, Silica gel 0.063-0.2 mm) were used and spots were detected under natural light, after straining with either anisaldehyde or Kedde reagent. As mobile phase 100% ethyl acetate (items 4.2.1 and 4.2.2), hexane: ethyl acetate (3:2, v/v; items 4.2.3 to 4.2.5) or methanol/ethyl acetate/triethylamine (4.99/4.99/0.02, v/v; items 4.2.10 and 4.2.11) and methanol/ethyl acetate (0.1/9.9, v/v, items 4.2.12 to 4.2.14) were used. Fifteen μL of samples and standards (digitoxigenin and digitoxin at 2 mg/ mL) were assayed.

4.2 Synthesis

*4.2.1 Hydrolysis of methanolic CGs extract to obtain digitoxigenin **(1)***

Digitoxigenin was obtained as described by Pádua et al. [73] with few modifications. Instead of digitoxin, 10g of *Digitalis lanata* methanolic extract enriched in A-series cardenolides were used for hydrolysis reaction. The dried residue (7 g) was dissolved in dichloromethane (100 mL) and filtrated under reduced pressure over silica gel (0.04-0.063 mm, Büchner funnel size 4). Then, silica gel was washed with the following solvents of different polarities (100% dichloromethane 3 × 100 mL → dichloromethane/ethyl acetate – 9:1, 15 × 50 mL → dichloromethane/ethyl acetate – 7:3, 15 × 50 mL→ dichloromethane/ethyl acetate – 1:1, 7 × 50 mL). Fractions 21 to 26 contained digitoxigenin, and the combined fractions resulted in 1.3 g of digitoxigenin.

*4.2.1.1 Digitoxigenin **(1)***

mp 233.5 – 240.3 ºC (Lit: 248-253 °C [74]). $[\alpha]_D^{27}$ +14.5 ° (*c* 0.55; MeOH). Lit:$[\alpha]_D^{25}$ +14.1 ° (*c* 0.55; MeOH [74]). IR: ῡ 3522 cm^{-1} (OH), 2864–2932 cm^{-1} (C-H sp^3), 1733 cm^{-1} (C=O), 1632 cm^{-1} (C=C), 1260 cm^{-1} (O-C=O), 1035 cm^{-1} (C-O). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.77 (s, 3H, CH_3, H-18), 0.87 (s, 3H,

CH_3, H-19), 1.05–1.79 (m, 19H, H-1, H-2, H-4, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-16), 1.98–2.08 (m, 2H, H-15), 2.72 (dd, *J* = 5.4 Hz, *J* = 9.0 Hz, 1H, H-17), 3.89 (s, 1H, H-3), 4.04 (s, 1H, OH), 4.16 (d, *J* = 2.6 Hz, 1H, OH), 4.86 (dd, *J* = 1.5 Hz, *J* = 18.4 Hz, 1H, H-21a), 4.96 (dd, *J* =1.4 Hz, *J* =18.4 Hz, 1H, H-21b), 5.89 (s, 1H, H-22). ^{13}C NMR (100 MHz, DMSO-d_6): 15.7 (CH_3, C-18), 20.8 (CH_2, C-7), 21.1 (CH_2, C-11), 23.7 (CH_3, C-19), 26.4 (CH_2, C-6), 26.5 (CH_2, C-16), 27.5 (CH_2, C-2), 29.5 (CH_2, C-15), 32.2 (CH_2, C-4), 33.1 (CH_2, C-1), 34.8 (CH, C-9), 35.0 (C_0, C-10), 35.7 (CH, C-5), 39.0 (CH_2, C-12), 40.9 (CH, C-8), 49.4 (C_0, C-13), 50.2 (CH, C-17), 64.6 (CH, C-3), 73.1 (CH_2, C-21), 83.8 (C_0, C-14), 116.2 (CH, C-22), 173.8 (C=O, C-23), 176.3 (C_0, C-20); HRMS-ESI: calcd for $C_{23}H_{34}O_4$ $[M+H]^+$ 375.5211, found: 375.45

*4.2.2 Oxidation of digitoxigenin **(1)** to digitoxigenone **(2)***

1 g of digitoxigenin **(1)** was dissolved in 112 mL acetone, and the solution was cooled down to 0 °C by stirring on ice. Then, 1.4 mL of Kiliani reagent were added drop by drop until a yellow/orange color was maintained. The reaction was stirred for 20 min on an ice bath. After adding 20 to 25 mL of methanol to remove surplus CrO_3, the reaction mixture was further stirred for 20 min at 0 °C. Next, adding 40 mL of water, the acetone was removed under *vacuo*. The reaction mixture was extracted with 4 × 30 mL dichloromethane, neutralized with 20 mL of 3% w/v Na_2CO_3 aqueous solution, and washed with 3 × 30 mL water. The organic layer was dried over anhydrous sodium sulfate, evaporated to dryness, and the reaction product was analyzed by TLC.

*4.2.2.1 Digitoxigenone **(2)***

Yield: 90%; mp 187.2 – 191.8 ºC (Lit:191-194 °C [75]). $[\alpha]_D^{30}$ +22 ° (*c* 1.00; MeOH). Lit: $[\alpha]_D^{20}$ +27.6 ° (*c* 1.0; MeOH [50]). IR: $\bar{\upsilon}$ 3486 cm^{-1} (OH), 2867–2941 cm^{-1} (C-H sp^3), 1704–1738 cm^{-1} (C=O), 1618 cm^{-1} (C=C), 1026–1283 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (s, 3H, CH_3, H-18), 0.98 (s, 3H, CH_3, H-19), 1.21–2.17 (m, 19H, H-1, H-2α, H-4α, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 2.30 (td, *J* = 5.4 Hz, *J* = 14.6 Hz, 1H, H-2β), 2.59 (t, *J* = 14.3 Hz, 1H, H-4β), 2.74–2.78 (m, 1H, H-17), 4.78 (dd, *J* = 1.3 Hz, *J* = 18.1 Hz, 1H, H-21a), 4.96 (d, *J* =18.0 Hz, 1H, H-21b), 5.84 (s, 1H, H-22). ^{13}C NMR (100 MHz, $CDCl_3$): 15.9 (CH_3, C-18), 21.0 (CH_2, C-7), 21.3 (CH_2, C-11), 22.6 (CH_3, C-19), 26.6 (CH_2, C-6), 27.0 (CH_2, C-16), 33.1 (CH_2, C-15), 35.3 (C_0, C-10), 36.7 (CH, C-9), 36.8 (CH_2, C-12), 37.2 (CH_2, C-2), 39.8 (CH_2, C-1), 41.6 (CH, C-8), 42.2 (CH_2, C-4), 43.8 (CH, C-5), 49.8 (C_0, C-13), 50.9 (CH, C-17), 73.7

(CH_2, C-21), 85.2 (C_0, C-14), 117.7 (CH, C-22), 174.7 (C=O, C-23), 174.9 (C_0, C-20), 212.8 (C_0, C-3); HRMS-ESI: calcd for $C_{23}H_{32}O_4$ $[M+H]^+$ 373.5052, found: 373.45

*4.2.3 Reduction of digitoxigenone **(2)** to 3α-digitoxigenin **(3)***

1 g of digitoxigenone **(2)** was dissolved in 40 mL dioxane and 10 mL water. Then, 365 mg $NaBH_4$ in 37.5 mL of 80% dioxane (30 mL dioxane + 7.5 mL H_2O) were added to the solution and stirred for 30 min at 20 °C. The reaction was neutralized with 30 mL of 5% acetic acid and the pH was adjusted to 5. Hence, the reaction color turned from yellow to white after adding the acid. For stopping the reaction and avoiding the formation of additional reaction products, the fast reducing of dioxane under *vacuo* is essential. The aqueous residue was extracted with 5 × 30 mL chloroform/ethanol (3:1) and the organic layer was washed 3 × 30 mL of water, and the organic layer was dried over anhydrous sodium sulfate and evaporated to dryness.

*4.2.3.1 3α-Digitoxigenin **(3)***

Yield: 60%; mp 266.4 – 269.5 °C (Lit: 268-270 °C [76]). $[\alpha]_D^{30}$ +5.7 ° (*c* 0.35; MeOH). Lit:$[\alpha]_D^{22}$ +26.8 ° (*c* 0.33; MeOH [77]). IR: ῡ 3419–3507 cm^{-1} (OH), 2859–2929 cm^{-1} (C-H sp^3), 1733 cm^{-1} (C=O), 1632 cm^{-1} (C=C), 1036 cm^{-1} (C-O). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.77 (s, 3H, CH_3, H-18), 0.85 (s, 3H, CH_3, H-19), 0.89–1.83 (m, 19H, H-1, H-2, H-4, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-16), 2.00–2.08 (m, 2H, H-15), 2.72–2.74 (m, 1H, H-17), 3.36–3.41 (m, 1H, H-3), 4.06 (s, 1H, OH), 4.43 (d, *J* = 4.4 Hz, 1H, OH), 4.87 (d, *J* = 18.6 Hz, 1H, H-21a), 4.97 (d, *J* =18.2 Hz, 1H, H-21b), 5.90 (s, 1H, H-22). ^{13}C NMR (100 MHz, DMSO-d_6): 15.7 (CH_3, C-18), 20.6 (CH_2, C-7), 21.2 (CH_2, C-11), 23.1 (CH_3, C-19), 26.4 (CH_2, C-6), 26.9 (CH_2, C-16), 30.4 (CH_2, C-2), 32.2 (CH_2, C-1), 34.5 (C_0, C-10), 34.9 (CH_2, C-15), 35.5 (CH, C-9), 36.2 (CH_2, C-4), 39.0 (CH_2, C-12), 41.0 (CH, C-5), 41.3 (CH, C-8), 49.4 (C_0, C-13), 50.2 (CH, C-17), 69.8 (CH, C-3), 73.1 (CH_2, C-21), 83.7 (C_0, C-14), 116.2 (CH, C-22), 173.8 (C=O, C-23), 176.3 (C_0, C-20); HRMS-ESI: calcd for $C_{23}H_{34}O_4$ $[M+H]^+$ 375.5211, found: 375.45

*4.2.4 Tosylation of 3α-digitoxigenin **(3)***

500 mg of 3α-digitoxigenin **(3)** were dissolved in 12.5 mL of dried pyridine. Then, 465 mg of tosylchloride in 3 mL pyridin were added, and the reaction mixture was stirred for 15 h at 20 °C. The pH was adjusted to 4 by adding 140 to 180 mL of 1 M HCl aqueous solution. The reaction mixture was sequentially extracted with chloroform (6 × 30 mL), neutralized with 3% w/v Na_2CO_3 aqueous solution (2

× 10 mL), and washed with water (3 × 30 mL). In the sequence, the organic layer was dried over anhydrous sodium sulfate and evaporated to dryness.

*4.2.4.1 3α-O-Tosyldigitoxigenin **(4)***

Yield: 90%; mp 107.0 – 110.2 °C (Lit: 155 – 156 °C [50]). $[\alpha]_D^{29}$ +28 ° (*c* 1,0; $CHCl_3$). Lit:$[\alpha]_D^{25}$ +35.0 ° ($CHCl_3$ [50]). IR: ῡ 3534 cm^{-1} (OH), 2939 cm^{-1} (C-H sp^3), 1753 cm^{-1} (C=O), 1628 cm^{-1} (C=C), 1167 e 1339 cm^{-1} (S=O), 911 cm^{-1} (S-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.85 (s, 3H, CH_3, H-18), 0.88 (s, 3H, CH_3, H-19), 0.99–1.80 (m, 19H, H-1, H-2, H-4, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-16), 2.14–2.17 (m, 2H, H-15), 2.45 (s, 3H, CH_3, H-7'), 2.75–2.77 (m, 1H, H-17), 4.45 (ddd, *J* = 4.8 Hz, *J* = 11.0 Hz, *J* = 16.0 Hz, 1H, H-3), 4.80 (d, *J* = 18.1 Hz, 1H, H-21a), 4.98 (d, *J* =18.9 Hz, 1H, H-21b), 5.86 (s, 1H, H-22), 7.34 (d, *J* =7.8 Hz, 2H, H-3'), 7.79 (d, *J* =8.0 Hz, 2H, H-2'). ^{13}C NMR (100 MHz, $CDCl_3$): 15.9 (CH_3, C-18), 21.1 (CH_2, C-7), 21.5 (CH_2, C-11), 21.8 (CH_3, C-7'), 23.1 (CH_3, C-19), 26.8 (CH_2, C-6), 27.0 (CH_2, C-2), 27.8 (CH_2, C-16), 33.2 (CH_2, C-15), 33.3 (CH_2, C-4), 34.8 (C_0, C-10), 34.9 (CH_2, C-1), 36.3 (CH, C-9), 40.0 (CH_2, C-12), 41.8 (CH, C-5), 42.0 (CH, C-8), 49.7 (C_0, C-13), 51.0 (CH, C-17), 73.6 (CH_2, C-21), 82.9 (CH, C-3), 85.5 (C_0, C-14), 117.8 (CH, C-22), 127.8 (2CH, C-2'), 130.0 (2CH, C-3'), 134.8 (C_0, C-4'), 144.7 (C_0, C-1'), 174.7 (C=O, C-23), 174.8 (C_0, C-20); HRMS-ESI: calcd for $C_{30}H_{40}O_6S$ $[M+H]^+$ 529.7074, found: 529.56

*4.2.5 Azidation of 3α-Tosyl-digitoxigenin **(4)** to 3β-azido-3-deoxydigitoxigenin **(5)***

500 mg of 3α-tosyl-digitoxigenin **(4)** were dissolved in 43 mL dimethylformamide (DMF) and 700 mg of NaN_3 were added. The reaction mixture was heated to 75 °C and stirred for 3 h. After 16 h at 20 °C, the remaining NaN_3 was removed by filtering twice through cotton, and three volumes of EtOAc were added to the clear filtration residue. The organic layer was extracted 8 × 20 mL of water, and the DMF remained in the water and the organic layer was dried over anhydrous sodium sulfate and evaporated to dryness.

*4.2.5.1 3β-azido-3-deoxydigitoxigenin **(5)***

Yield: 85%; mp 191.5 – 194.5 ºC (Lit: 213-217 °C [50]). $[\alpha]_D^{29}$ +18.0 ° (*c* 1.00; $CHCl_3$). Lit:$[\alpha]_D^{25}$ +22.0 ($CHCl_30$ [50]). IR: ῡ 3473 cm^{-1} (OH), 2930 cm^{-1} (C-H), 2097 cm^{-1} (N=N=N), 1737 cm^{-1} (C=O), 1620 cm^{-1} (C=C), 1025 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.81 (s, 3H, CH_3, H-18), 0.88 (s, 3H, CH_3, H-19), 1.17–1.85 (m, 19H, H-1, H-2, H-4, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-16), 2.01–2.11 (m, 2H, H-15), 2.69–2.71 (m, 1H, H-17), 3.89 (s, 1H, H-3), 4.74 (d, *J* = 18.1 Hz, 1H, H-21a), 4.93 (d, *J* =18.1 Hz,

1H, H-21b), 5.80 (s, 1H, H-22). ^{13}C NMR (400 MHz, $CDCl_3$): 15.9 (CH_3, C-18), 21.2 (CH_2, C-11), 21.5 (CH_2, C-7), 23.8 (CH_3, C-19), 24.9 (CH_2, C-6), 26.5 (CH_2, C-2), 27.0 (CH_2, C-16), 30.3 (CH_2, C-4), 30.5 (CH_2, C-1), 33.2 (CH_2, C-15), 35.4 (C_0, C-10), 36.0 (CH, C-8), 36.9 (CH, C-5), 40.1 (CH_2, C-12), 41.9 (CH, C-9), 49.8 (C_0, C-13), 51.1 (CH, C-17), 58.6 (CH, C-3), 73.7 (CH_2, C-21), 85.5 (C_0, C-14), 117.7 (CH, C-22), 174.8 (C=O, C-23), 175.0 (C_0, C-20); HRMS-ESI: calcd for $C_{23}H_{33}N_3O_3$ $[M+H]^+$ 400.5338, found: 400.49

*4.2.6 General procedure for the synthesis of peracetylated propargyl glycosides **(IIa-c)***

To a solution of peracetylated D-glucose, D-galactose or D-mannose (1.0 g, 2.56 mmol) in 20 mL of dry dichloromethane at 0 °C, 0.5 mL of BF_3, Et_2O 46% v/v and 0.6 mL (10.4 mmol) of propargyl alcohol were added in a stepwise manner. The reaction mixture was stirred at room temperature for 24 h, water (50 mL) was added and the mixture transferred to a separatory funnel. Next, the organic layer was separated and the aqueous phase was extracted with 3 × 50 mL of dichloromethane. The combined organic layers were washed with water (3 × 25 mL), dried over anhydrous sodium sulfate and evaporated to dryness. The crude propargyl glycosides obtained from D-glucose and D-mannose were purified by recrystallization from ethanol. The propargyl glycoside obtained from D-galactose was purified by column chromatography using hexane-ethyl acetate 7:3 as eluent.

*4.2.6.1 Propargyl 2,3,4-6-tetra-O-acetyl-β-D-glucopyranoside **(IIa)***

Yield: 60%; mp 105.7 – 107.6 °C (Lit. 102-104 ºC [78]). $[\alpha]_D^{28}$ -32.7 ° (*c* 1.10; $CHCl_3$). Lit. $[\alpha]_D^{20}$ -43.4 ° (*c* 0.9; $CHCl_3$ [79]). IR: $\bar{\upsilon}$ 3273 cm^{-1} (C-H sp), 1732–1754 cm^{-1} (C=O), 1366–1379 cm^{-1} (α-CH_3), 1207–1233 cm^{-1} (O-C=O), 1037 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): 1.96–2.04 (s, 12H, CH_3C=O), 2.44 (t, *J* = 2.3 Hz, 1H, H-9), 3.69 (ddd, *J* = 2.3 Hz, *J* = 4.5 Hz, *J* = 9.9 Hz, 1H, H-5), 4.10 (dd, *J* = 2.2 Hz, *J* = 12.3 Hz, 1H, H-6a), 4.22 (dd, *J* = 4.6 Hz, *J* = 12.3 Hz, 1H, H-6b), 4.32 (d, *J* = 2.3 Hz, 2H, H-7), 4.73 (d, *J* = 8.0 Hz, 1H, H-1), 4.96 (dd, *J* =7.9 Hz, *J* =9.6 Hz, 1H, H-2), 5.05 (t, *J* = 9.7 Hz, 1H, H-3), 5.19 (t, *J* = 9.4 Hz, 1H, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): 20.4–20.8 (4CH_3, CH_3C=O), 55.9 (CH_2, C-7), 61.8 (CH_2, C-6), 68.4 (CH, C-3), 69.8 (CH, C-9), 71.0 (CH, C-2), 72.0 (CH, C-4), 72.8 (CH, C-5), 78.1 (C_0, C-8), 98.2 (CH, C-1).

*4.2.6.2 Propargyl 2,3,4-6-tetra-O-acetyl-β-D-galactopyranoside **(IIb)***

Yield: 70%. $[\alpha]_D^{24}$ -32.0 ° (*c* 1.10; $CHCl_3$). Lit. $[\alpha]_D^{20}$ -23.0 ° (*c* 1.00; $CHCl_3$ [80]). IR: ῡ 3275 cm^{-1} (C-H, sp), 2981 cm^{-1} (C-H, sp^3), 2119 cm^{-1} (C≡C), 1740 cm^{-1} (C=O), 1368 cm^{-1} (α-CH_3), 1211 cm^{-1} (O-C=O), 1016–1043 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): 1.92–2.08 (s, 12H, CH_3C=O), 2.42 (t, *J* = 2.2 Hz, 1H, H-9), 3.88 (t, *J* = 6.4 Hz, 1H, H-5), 4.06 (dd, *J* =6.8 Hz, *J* =11.2 Hz, 1H, H-6a), 4.12 (dd, *J* =6.8 Hz, *J* =11.3 Hz, 1H, H-6b), 4.31 (d, *J* = 2.2 Hz, 2H, H-7), 4.67 (d, *J* = 8.0 Hz, 1H, H-1), 4.99 (dd, *J* = 3.4 Hz, *J* = 10.4 Hz, 1H, H-3), 5.14 (dd, *J* = 8.0 Hz, *J* = 10.3 Hz, 1H, H-2), 5.33 (d, *J* = 2.3 Hz, 1H, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): 20.5–20.7 (4CH_3, CH_3C=O), 55.9 (CH_2, C-7), 61.2 (CH_2, C-6), 67.0 (CH, C-4), 68.5 (CH, C-2), 70.8 (2CH, C-3, C-5), 75.4 (CH, C-9), 78.2 (C_0, C-8), 98.6 (CH, C-1).

4.2.6.3 Propargyl 2,3,4-6-tetra-O-acetyl-α-D-mannopyranoside ***(IIc)***

Yield: 50%; mp 101.5 – 103.1 °C (Lit. 103-104 ºC [81]). $[\alpha]_D^{29}$ +48.7 ° (*c* 1.15; $CHCl_3$). Lit. $[\alpha]_D^{20}$ +68.0 (*c* 1.00; $CHCl_3$ [81]). IR: ῡ 3255 cm^{-1} (C-H, sp), 2117 cm^{-1} (C≡C), 1739 cm^{-1} (C=O), 1367 cm^{-1} (α-CH_3), 1217–1231 cm^{-1} (O-C=O), 1056–1078 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): 1.92–2.10 (s, 12H, CH_3C=O), 2.43 (t, *J* = 2.4 Hz, 1H, H-9), 3.96 (ddd, *J* = 2.4 Hz, *J* = 5.2 Hz, *J* = 9.2 Hz, 1H, H-5), 4.05 (dd, *J* =2.4 Hz, *J* =12.3 Hz, 1H, H-6a), 4.21 (d, *J* = 2.4 Hz, 2H, H-7), 4.22 (dd, *J* =5.1 Hz, *J* =12.4 Hz, 1H, H-6b), 4.96 (d, *J* = 1.6 Hz, 1H, H-1), 5.20 (dd, *J* = 1.9 Hz, *J* = 3.1 Hz, 1H, H-2), 5.23–5.29 (m, 2H, H-3, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): 20.6–20.8 (4CH_3, CH_3C=O), 54.9 (CH_2, C-7), 62.3 (CH_2, C-6), 66.0 (CH, C-4), 68.9 (CH, C-2), 69.0 (CH, C-3), 69.4 (CH, C-5), 75.6 (CH, C-9), 77.9 (C_0, C-8), 96.2 (CH, C-1).

4.2.7 General procedure for the synthesis of peracetylated propargyl cellobioside ***(III)***

To a 100 mL round bottom flask containing activated 4A molecular sieves, Ag_2CO_3 (0.64 g, 2.30 mmol), AgOTf (0.06 g, 0.22 mmol) and dry dichloromethane (10 mL) were added. The flask was kept at -10 °C for 20 min. Then, propargyl alcohol (0.6 g, 5.0 mmol) was added; the mixture was allowed to reach room temperature and stirred for 30 min. Finally, hepta-*O*-acetyl-α-D-cellobiosyl bromide (0.5 g, 0.72 mmol) was added and the reaction mixture was stirred at room temperature for 24 h. The mixture was filtered through Celite, transferred to a separatory funnel, washed with saturated aqueous $NaHCO_3$ solution (3 × 30 mL) and brine (3 × 50 mL), dried over anhydrous sodium sulfate and evaporated to dryness. The crude propargyl cellobioside was recristallized from ethanol.

4.2.7.1 Propargyl 2,3,6,2´,3´,4´,6´-hepta-O-acetyl-β-D-cellobioside ***(III)***

Yield: 50%; mp 155.0 – 158.1 °C. $[\alpha]_D^{29}$ -44.0 ° (*c* 0.50; $CHCl_3$). Lit. $[\alpha]_D^{20}$ -52.2 ° (*c* 0.50; $CHCl_3$ [82]). IR: $\bar{\upsilon}$ 3282 cm^{-1} (C-H, sp), 2938 cm^{-1} (C-H, sp^3), 1742 cm^{-1} (C=O), 1366 cm^{-1} (α-CH_3), 1218 cm^{-1} (O-C=O), 1039 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): 1.91–2.10 (s, 21H, CH_3C=O), 2.41 (t, *J* = 2.2 Hz, 1H, H-15), 3.55–3.62 (m, 2H, H-5, H-11), 3.73 (t, *J* = 9.6 Hz, 1H, H-8), 3.98 (dd, *J* = 1.9 Hz, *J* = 12.4 Hz, 1H, H-6a), 4.04 (dd, *J* = 4.9 Hz, *J* = 12.0 Hz, 1H, H-6b), 4.26 (d, *J* = 2.3 Hz, 2H, H-13), 4.29 (dd, *J* = 4.3 Hz, *J* =12.5 Hz, 1H, H-12a), 4.46 (d, *J* = 8.2 Hz, 2H, H-7, H-12b), 4.67 (d, *J* =7.9 Hz, 1H, H-1), 4.85 (t, *J* =8.7 Hz, 2H, H-3, H-10), 4.99 (t, *J* =9.9 Hz, 1H, H-2), 5.08 (t, *J* = 9.3 Hz, 1H, H-4), 5.14 (t, *J* = 9.3 Hz, 1H, H-9). ^{13}C NMR (100 MHz, $CDCl_3$): 20.6–20.9 (7CH_3, CH_3C=O), 56.0 (CH_2, C-13), 61.7 (CH_2, C-6), 61.9 (CH_2, C-12), 68.1 (CH, C-2), 71.4 (CH, C-3), 71.8 (CH, C-10), 72.1 (CH, C-4), 72.6 (CH, C-5), 73.0 (CH, C-9), 73.1 (CH, C-11), 75.6 (CH, C-14), 76.4 (CH, C-8), 78.2 (CH, C-15), 98.1 (CH, C-1), 100.8 (CH, C-7), 169.1–170.8 (7C=O, CH_3C=O).

4.2.8 General procedure for the synthesis of peracetylated digitoxigenin triazolyl glycosides ***(6a-d)***

To a 50 mL round bottom flask, 3β-azido-3-deoxydigitoxigenin (**5**, 0.16 mmol) dissolved in 1 mL of tetrahydrofuran was added, followed by the appropriate propargyl glycoside (0.2 mmol) dissolved in 0.5 mL of tetrahydrofuran. Then, $CuSO_4.5H_2O$, 50% mol, dissolved in 0.5 mL of water and sodium ascorbate (60 % mol) was dissolved in 1 mL of water and added in a stepwise manner. The reaction mixture was stirred at room temperature for 4 h. After, the tetrahydrofuran was removed by distillation at reduced pressure. The reaction residues were solubilized in 50 mL CH_2Cl_2, washed with 2 × 50 mL H_2O and subsequently washed with 3 × 50 mL alkaline EDTA 20% w/v. The organic phase was dried over Na_2SO_4, filtered and removed by distillation at reduced pressure. The derivatives **3a-d** were added in Florisil and purified by silica column chromatography using CH_2Cl_2: ethyl acetate /4:6 as eluent.

4.2.8.1 3β-[4-[(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)oxymethyl]-1,2,3-triazol-1-yl]-3-deoxydigitoxigenin ***(6a)***

Yield: 65%; mp 106.2 – 108.5 °C. $[\alpha]_D^{26}$ -9.00 (*c* 2.00; CH_2Cl_2). IR: $\bar{\upsilon}$ 3412 cm^{-1} (OH), 2935 cm^{-1} (C-H sp^3), 1746 cm^{-1} (C=O), 1619 cm^{-1} (C=C), 1374 cm^{-1} (α-CH_3), 1228 cm^{-1} (O-C=O), 1038 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3, H-18), 0.92 (s, 3H, CH_3, H-19), 1.17–2.24 (m, 20H, H-1, H-2, H-4α, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 1.98–2.09 (s, 12H, CH_3C=O), 2.32 (td, *J* = 4.2 Hz, *J* = 14.2 Hz, 1H, H-4β), 2.79–2.82 (m, 1H, H-17), 3.76 (ddd, *J* = 2.1 Hz, *J* = 4.2 Hz, *J* = 9.8 Hz, 1H, H-5'), 4.15 (dd, *J* =1.9 Hz, *J* =12.4 Hz, 1H, H-6'a), 4.28 (dd, *J* =4.7 Hz, *J* =12.3 Hz, 1H, H-6'b), 4.71

(d, *J* = 7.9 Hz, 2H, H-1', H-3), 4.82 (d, *J* =16.2 Hz, 2H, H-7'a, H-21a), 4.93–5.03 (m, 3H, H-4', H-7'b, H-21b), 5.10 (t, *J* = 9.6 Hz, 1H, H-2'), 5.21 (t, *J* = 9.4 Hz, 1H, H-3'), 5.88 (s, 1H, H-22), 7.63 (s, 1H, H-9'). ^{13}C NMR (100 MHz, $CDCl_3$): 15.9 (CH_3, C-18), 20.7–20.8 (4CH_3, CH_3C=O), 21.2 (CH_2, C-7), 21.4 (CH_2, C-11), 23.8 (CH_3, C-19), 25.1 (CH_2, C-6), 26.5 (CH_2, C-16), 27.0 (CH_2, C-2), 30.0 (CH_2, C-4), 30.6 (CH_2, C-1), 33.2 (CH_2, C-15), 35.2 (C_0, C-10), 36.4 (CH, C-9), 36.9 (CH, C-5), 40.0 (CH_2, C-12), 41.9 (CH, C-8), 49.8 (C_0, C-13), 51.0 (CH, C-17), 56.7 (CH, C-3), 62.0 (CH_2, C-6'), 63.2 (CH_2, C-7'), 68.5 (CH, C-2'), 71.5 (CH, C-4'), 72.0 (CH, C-5'), 72.9 (CH, C-3'), 73.6 (CH_2, C-21), 85.4 (C_0, C-14), 100.1 (CH, C-1'), 117.8 (CH, C-22), 169.5–170.8 (4C=O, CH_3C=O), 174.7 (C=O, C-23), 174.8 (C_0, C-20); HRMS-ESI: calcd for $C_{40}H_{55}N_3O_{13}$ $[M+H]^+$ 786.8844, found: 787.45.

4.2.8.2 3β-[4-[(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)oxymethyl]-1,2,3-triazol-1-yl]-3-deoxydigitoxigenin ***(6b)***

Yield: 75%; mp 121.1 – 123.1 °C. $[\alpha]_D^{26}$ -6.00 (*c* 2.00; CH_2Cl_2). IR: ῡ 3455 cm^{-1} (OH), 2938 cm^{-1} (C-H sp^3), 1739 cm^{-1} (C=O), 1622 cm^{-1} (C=C), 1368 cm^{-1} (α-CH_3), 1218 cm^{-1} (O-C=O), 1045 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.82 (s, 3H, CH_3, H-18), 0.86 (s, 3H, CH_3, H-19), 1.12–2.17 (m, 20H, H-1, H-2, H-4α, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 1.91–2.10 (s, 12H, CH_3C=O), 2.27 (t, *J* = 12.7 Hz, 1H, H-4β), 2.72–2.75 (m, 1H, H-17), 3.91 (t, *J* = 5.8 Hz, 1H, H-5'), 4.09 (d, *J* = 5.0 Hz, 2H, H-6'), 4.62–4.98 (m, 3H, H-1', H-3, H-3', H-7', H-21), 5.15 (dd, *J* = 8.0 Hz, *J* = 9.9 Hz, 1H, H-2'), 5.33 (d, *J* = 2.7 Hz, 1H, H-4'), 5.81 (s, 1H, H-22), 7.60 (s, 1H, H-9'). ^{13}C NMR (100 MHz, $CDCl_3$): 15.9 (CH_3, C-18), 20.6–20.9 (4CH_3, CH_3C=O), 21.2 (CH_2, C-7), 21.4 (CH_2, C-11), 23.8 (CH_3, C-19), 25.0 (CH_2, C-6), 26.4 (CH_2, C-16), 27.0 (CH_2, C-2), 30.0 (CH_2, C-4), 30.6 (CH_2, C-1), 33.2 (CH_2, C-15), 35.2 (C_0, C-10), 36.4 (CH, C-9), 36.9 (CH, C-5), 40.0 (CH_2, C-12), 41.8 (CH, C-8), 49.8 (C_0, C-13), 51.0 (CH, C-17), 56.9 (CH, C-3), 61.4 (CH_2, C-6'), 63.2 (CH_2, C-7'), 67.2 (CH, C-4'), 69.0 (CH, C-2'), 70.9 (CH, C-5'), 71.0 (CH, C-3'), 73.6 (CH_2, C-21), 85.4 (C_0, C-14), 100.7 (CH, C-1'), 117.8 (CH, C-22), 169.6–170.5 (4C=O, CH_3C=O), 174.7 (C=O, C-23), 174.8 (C_0, C-20); HRMS-ESI: calcd for $C_{40}H_{55}N_3O_{13}$ $[M+H]^+$ 786.8844, found: 787.58.

4.2.8.3 3β-[4-[(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)oxymethyl]-1,2,3-triazol-1-yl]-3-deoxydigitoxigenin ***(6c)***

Yield: 68%; mp 114.9 – 117.5 °C. $\mathbf{[\alpha]_D^{26}}$ +29.3 (*c* 2.12; CH_2Cl_2). IR: ῡ 3458 cm^{-1} (OH), 2940 cm^{-1} (C-H sp^3), 1740 cm^{-1} (C=O), 1612 cm^{-1} (C=C), 1364 cm^{-1} (α-CH_3), 1220 cm^{-1} (O-C=O), 1044 cm^{-1} (C-O). ^{1}H

NMR (400 MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3, H-18), 0.94 (s, 3H, CH_3, H-19), 1.20–2.36 (m, 21H, H-1, H-2, H-4, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 1.99–2.16 (s, 12H, CH_3C=O), 2.81 (dd, *J* = 5.4 Hz, *J* = 8.8 Hz, 1H, H-17), 4.08–4.10 (m, 1H, H-5'), 4.12 (dd, *J* =2.3 Hz, *J* =12.3 Hz, 1H, H-6'a), 4.31 (dd, *J* =5.3 Hz, *J* =12.4 Hz, 1H, H-6'b), 4.67–5.03 (m, 6H, H-1', H-3, H-7', H-21), 5.24–5.34 (m, 3H, H-2', H-3', H-4'), 5.89 (s, 1H, H-22), 7.66 (s, 1H, H-9'). ^{13}C NMR (100 MHz, $CDCl_3$): 15.9 (CH_3, C-19), 20.8–20.9 (4CH_3, CH_3C=O), 21.2 (CH_2, C-7), 21.4 (CH_2, C-11), 23.7 (CH_3, C-19), 25.0 (CH_2, C-6), 26.4 (CH_2, C-16), 27.0 (CH_2, C-2), 29.9 (CH_2, C-4), 30.6 (CH_2, C-1), 33.2 (CH_2, C-15), 35.2 (C_0, C-10), 36.4 (CH, C-9), 36.8 (CH, C-5), 40.0 (CH_2, C-12), 41.8 (CH, C-8), 49.8 (C_0, C-13), 51.0 (CH, C-17), 57.0 (CH, C-3), 61.1 (CH_2, C-6'), 62.5 (CH_2, C-7'), 66.2 (CH, C-4'), 68.8 (CH, C-2'), 69.2 (CH, C-5'), 69.6 (CH, C-3'), 73.6 (CH_2, C-21), 85.4 (C_0, C-14), 97.0 (CH, C-1'), 117.7 (CH, C-22), 169.8–170.8 (4C=O, CH_3C=O), 174.7 (C=O, C-23), 174.8 (C_0, C-20); HRMS-ESI: calcd for $C_{40}H_{55}N_3O_{13}$ $[M+H]^+$ 786.8844, found: 787.58.

4.2.8.4 3β-[4-[[4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-2,3,6-tri-O-acetyl-β-D-glucopyranosyl]oxymethyl]-1,2,3-triazol-1-yl]-3-deoxydigitoxigenin ***(6d)***

Yield: 70%; mp 104.2 – 107.1 °C. $[\alpha]_D^{26}$ -4.05 (*c* 2.00; CH_2Cl_2). IR: ῡ 3468 cm^{-1} (OH), 2933 cm^{-1} (C-H sp^3), 1739 cm^{-1} (C=O), 1619 cm^{-1} (C=C), 1367 cm^{-1} (α-CH_3), 1219 cm^{-1} (O-C=O), 1034 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (s, 3H, CH_3, H-18), 0.92 (s, 3H, CH_3, H-19), 1.15–2.23 (m, 20H, H-1, H-2, H-4α, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 1.98–2.14 (s, 21H, CH_3C=O), 2.32 (td, *J* = 4.2 Hz, *J* = 14.3 Hz, 1H, H-4β), 2.79–2.83 (m, 1H, H-17), 3.63–3.70 (m, 2H, H-5', H-11'), 3.80 (t, *J* = 9.5 Hz, 1H, H-8'), 4.04 (dd, *J* =1.8 Hz, *J* =12.4 Hz, 1H, H-6'a), 4.12 (dd, *J* =4.8 Hz, *J* =12.0 Hz, 1H, H-6'b), 4.37 (dd, *J* =4.3 Hz, *J* =12.5 Hz, 1H, H-12'a), 4.52–5.19 (m, 13H, H-1', H-2', H-3', H-4', H-7', H-9', H-10', H-3; H-12'b, H-13', H-21'), 5.89 (s, 1H, H-22), 7.61 (s, 1H, H-15'). ^{13}C NMR (100 MHz, $CDCl_3$): 15.9 (CH_3, C-18), 20.6–21.0 (7CH_3, CH_3C=O), 21.2 (CH_2, C-7), 21.4 (CH_2, C-11), 23.8 (CH_3, C-19), 25.0 (CH_2, C-6), 26.4 (CH_2, C-16), 27.0 (CH_2, C-2), 29.9 (CH_2, C-4), 30.5 (CH_2, C-1), 33.2 (CH_2, C-15), 35.2 (C_0, C-10), 36.3 (CH, C-9), 36.8 (CH, C-5), 40.0 (CH_2, C-12), 41.8 (CH, C-8), 49.8 (C_0, C-13), 51.0 (CH, C-17), 56.7 (CH, C-3), 61.7 (CH_2, C-12'), 61.9 (CH_2, C-6'), 63.1 (CH_2, C-13'), 68.0 (CH, C-2'), 71.6 (CH, C-9'), 71.7 (CH, C-4'), 72.0 (CH, C-11'), 72.6 (CH, C-5'), 72.9 (CH, C-10'), 73.0 (CH, C-3'), 73.6 (CH_2, C-21), 76.4 (CH, C-8'), 85.4 (C_0, C-14), 99.8 (CH, C-7'), 100.8 (CH, C-1'), 117.7

(CH, C-22), 122.1 (CH, C-15'), 143.7 (C_0, C-14'), 169.2–170.6 (7C=O, CH_3C=O), 174.7 (C=O, C-23), 174.8 (C_0, C-20); HRMS-ESI: calcd for $C_{52}H_{71}N_3O_{21}$ $[M+H]^+$ 1075.1350, found: 1075.54.

*4.2.9 General procedure for the synthesis of deacetylated digitoxigenin triazoly glycosides **(7a-d)***

To a solution of 0.1 g of the appropriate digitoxigenin triazolyl glycoside (0.13 mmol) of compounds **6a-c** and 0.093 mmol of **6d** in 5 mL of methanol, $LiOH.H_2O$ (0.60 mmol) dissolved in 2 mL of water were added. The reaction mixture was stirred at 0 °C for 2 h, neutralized with resin Amberlite IRA 120 H^+, filtered, and the resin washed with methanol. Finally, the filtrate was concentrated in *vacuo* to furnish the desired glycosides.

*4.2.9.1 3β-[4-[(β-D-glucopyranosyl)oxymethyl]-1,2,3-triazol-1-yl]-3-deoxydigitoxigenin **(7a)***

Yield: 80%; mp >104 °C (decomp.). $[\alpha]_D^{25}$ -0.96 (*c* 2.08; MeOH). IR: ῡ 3363 cm^{-1} (OH), 2930 cm^{-1} (C-H sp^3), 1732 cm^{-1} (C=O), 1621 cm^{-1} (C=C), 1022 cm^{-1} (C-O). ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.89 (s, 3H, CH_3, H-18), 0.92 (s, 3H, CH_3, H-19), 1.05–2.28 (m, 20H, H-1, H-2, H-4α, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 2.44 (t, *J* = 13.8 Hz, 1H, H-4β), 2.85 (s, 1H, H-17), 3.21–3.38 (m, 4H, H-4', H-5', H-6'), 3.69 (d, *J* = 11.0 Hz, 1H, H-3'), 3.75–3.84 (m, 1H, OH), 3.90 (d, *J* = 11.6 Hz, 1H, H-2'), 4.40 (d, *J* = 7.1 Hz, 1H, H-1'), 4.48–5.12 (m, 7H, H-3, H-7', H-21, OH), 5.48 (s, 1H, OH), 5.91 (s, 1H, H-22), 8,12 (s, 1H, H-9'). ^{13}C NMR (100 MHz, MeOD-d_4): 16.5 (CH_3, C-18), 22.4 (CH_2, C-7), 22.6 (CH_2, C-11), 24.4 (CH_3, C-19), 25.8 (CH_2, C-6), 27.7 (CH_2, C-16), 28.2 (CH_2, C-2), 30.9 (CH_2, C-4), 31.8 (CH_2, C-1), 33.6 (CH_2, C-15), 36.4 (C_0, C-10), 37.4 (CH, C-9), 38.5 (CH, C-5), 41.0 (CH_2, C-12), 42.8 (CH, C-8), 51.2 (C_0, C-13), 52.2 (CH, C-17), 58.5 (CH, C-3), 62.9 (CH_2, C-6'), 63.3 (CH_2, C-7'), 71.8 (CH, C-2'), 75.1 (CH, C-4'), 75.5 (CH_2, C-21), 78.1 (CH, C-5'), 78.2 (CH, C-3'), 86.4 (C_0, C-14), 103.7 (CH, C-1'), 117.9 (CH, C-22), 124.8 (CH, C-9'), 177.4 (C=O, C-23), 178.6 (C_0, C-20); HRMS-ESI: calcd for $C_{32}H_{47}N_3O_9$ $[M+H]^+$ 618.7377, found: 618.43.

*4.2.9.2 3β-[4-[(β-D-galactopyranosyl)oxymethyl]-1,2,3-triazol-1-yl]-3-deoxydigitoxigenin **(7b)***

Yield: 88%; mp >138 °C (decomp.). $[\alpha]_D^{25}$ -0.94 (*c* 2.12; MeOH). IR: ῡ 3354 cm^{-1} (OH), 2932 cm^{-1} (C-H sp^3), 1732 cm^{-1} (C=O), 1621 cm^{-1} (C=C), 1024 cm^{-1} (C-O). ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.89 (s, 3H, CH_3, H-18), 0.92 (s, 3H, CH_3, H-19), 1.05–2.24 (m, 20H, H-1, H-2, H-4α, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 2.46 (dt, *J* = 3.2 Hz, *J* = 14.2 Hz, 1H, H-4β), 2.84–2.87 (m, 1H, H-17), 3.48 (dd, *J* = 2.9 Hz, *J* = 9.5 Hz, 1H, H-3') 3.55–3.59 (m, 2H, H-6'), 3.72–3.82 (m, 3H, H-2', H-5', OH), 3.85

(d, *J* = 2.3 Hz, 1H, H-4'), 4.36 (d, *J* = 8.9 Hz, 1H, H-1'), 4.50–5.07 (m, 8H, H-3, H-7'; H-21, OH), 5.91 (s, 1H, H-22), 8,12 (s, 1H, H-9'). ^{13}C NMR (100 MHz, MeOD-d_4): 16.5 (CH_3, C-18), 22.4 (CH_2, C-7), 22.6 (CH_2, C-11), 24.4 (CH_3, C-19), 25.8 (CH_2, C-6), 27.7 (CH_2, C-16), 28.2 (CH_2, C-2), 30.9 (CH_2, C-4), 31.8 (CH_2, C-1), 33.6 (CH_2, C-15), 36.4 (C_0, C-10), 37.4 (CH, C-9), 38.5 (CH, C-5), 41.0 (CH_2, C-12), 42.8 (CH, C-8), 51.2 (C_0, C-13), 52.2 (CH, C-17), 58.5 (CH, C-3), 62.7 (CH_2, C-6'), 63.3 (CH_2, C-7'), 70.4 (CH, C-2'), 72.6 (CH, C-4'), 75.0 (CH, C-5'), 75.5 (CH_2, C-21), 76.9 (CH, C-3'), 86.4 (C_0, C-14), 104.4 (CH, C-1'), 117.9 (CH, C-22), 177.3 (C=O, C-23), 178.5 (C_0, C-20); HRMS-ESI: calcd for $C_{32}H_{47}N_3O_9$ $[M+H]^+$ 618.7377, found: 618.62.

4.2.9.3 3β-[4-[(α-D-mannopyranosyl)oxymethyl]-1,2,3-triazol-1-yl]-3-deoxydigitoxigenin ***(7c)***

Yield: 82%; mp >169 °C (decomp.). $[\alpha]_D^{25}$ +21.82 (*c* 1.92; MeOH). IR: ῡ 3397 cm^{-1} (OH), 2932 cm^{-1} (C-H sp^3), 1729 cm^{-1} (C=O), 1626 cm^{-1} (C=C), 1026 cm^{-1} (C-O). ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.89 (s, 3H, CH_3, H-18), 0.92 (s, 3H, CH_3, H-19), 1.13–2.30 (m, 20H, H-1, H-2, H-4α, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 2.45 (t, *J* = 12.6 Hz, 1H, H-4β), 2.85–2.87 (m, 1H, H-17), 3.60–3.97 (m, 6H, H-2', H-3', H-4', H-5', H-6'), 4.52–4.87 (m, 4H, H-3, H-7', H-1'), 4.92 (d, *J* = 18.4 Hz, 1H, H-21a), 5.04 (d, *J* = 18.3 Hz, 1H, H-21b), 5.91 (s, 1H, H-22), 8.16 (s, 1H, H-9'). ^{13}C NMR (100 MHz, MeOD-d_4): 16.5 (CH_3, C-18), 22.4 (CH_2, C-7), 22.6 (CH_2, C-11), 24.4 (CH_3, C-19), 25.8 (CH_2, C-6), 27.7 (CH_2, C-16), 28.2 (CH_2, C-2), 30.9 (CH_2, C-4), 31.8 (CH_2, C-1), 33.6 (CH_2, C-15), 36.4 (C_0, C-10), 37.4 (CH, C-9), 38.5 (CH, C-5), 41.0 (CH_2, C-12), 42.8 (CH, C-8), 51.2 (C_0, C-13), 52.3 (CH, C-17), 58.6 (CH, C-3), 61.1 (CH_2, C-7'), 63.2 (CH_2, C-6'), 68.8 (CH, C-2'), 72.2 (CH, C-4'), 72.7 (CH, C-3'), 75.1 (CH, C-5'), 75.5 (CH_2, C-21), 86.5 (C_0, C-14), 101.0 (CH, C-1'), 118.0 (CH, C-22), 177.3 (C=O, C-23), 178.5 (C_0, C-20); HRMS-ESI: calcd for $C_{32}H_{47}N_3O_9$ $[M+H]^+$ 618.7377, found: 618.56.

4.2.9.4 3β-[4-[[4-O-(β-D-glucopyranosyl)-β-D-glucopyranosyl]oxymethyl]-1,2,3-triazol-1-yl]-3-deoxydigitoxigenin ***(7d)***

Yield: 82%; mp >163 °C (decomp.). $[\alpha]_D^{25}$ +2.10 (*c* 1.90; MeOH). IR: ῡ 3253 cm^{-1} (OH), 2853–2923 cm^{-1} (C-H sp^3), 1735 cm^{-1} (C=O), 1670 cm^{-1} (C=C), 1259 cm^{-1} (O-C-O), 1023 cm^{-1} (C-O). ^{1}H NMR (400 MHz, MeOD-d_4): δ 0.89 (s, 3H, CH_3, H-18), 0.92 (s, 3H, CH_3, H-19), 1.05–2.31 (m, 20H, H-1, H-2, H-4α, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15, H-16), 2.44 (td, *J* = 3.8 Hz, *J* = 14.2 Hz, 1H, H-4β), 2.85–2.87 (m, 1H, H-17), 3.23 (t, *J* = 8.4 Hz, 1H, H-11'), 3.33–3.60 (m, 8H, H-2', H-3', H-4', H-5', H-8', H-9', H-10', H-12'a), 3.66 (dd, *J* = 5.2 Hz, *J* = 11.8 Hz, 1H, H-6'a), 3.86–3.95 (m, 3H, H-3, H-6'b, H-

12'b), 4.43 (t, *J* = 7.9 Hz, 2H, H-1', H-7'), 4.90–5.07 (m, 4H, H-13', H-21), 5.91 (s, 1H, H-22), 8.13 (s, 1H, H-15'). ^{13}C NMR (100 MHz, MeOD-d_4): 16.5 (CH_3, C-18), 22.4 (CH_2, C-7), 22.6 (CH_2, C-11), 24.4 (CH_3, C-19), 25.8 (CH_2, C-6), 27.7 (CH_2, C-16), 28.2 (CH_2, C-2), 30.9 (CH_2, C-4), 31.8 (CH_2, C-1), 33.6 (CH_2, C-15), 36.4 (C_0, C-10), 37.4 (CH, C-9), 38.5 (CH, C-5), 41.0 (CH_2, C-12), 42.8 (CH, C-8), 51.2 (C_0, C-13), 52.3 (CH, C-17), 58.6 (CH, C-3), 62.0 (CH_2, C-12'), 62.6 (CH_2, C-7'), 63.4 (CH_2, C-6'), 71.5 (CH, C-2'), 74.9 (CH, C-9'), 75.1 (CH, C-4'), 75.5 (CH_2, C-21), 76.5 (CH, C-11'), 76.7 (CH, C-5'), 78.0 (CH, C-10'), 78.2 (CH, C-3'), 80.9 (CH, C-8'), 86.5 (C_0, C-14), 103.6 (CH, C-7'), 104.8 (CH, C-1'), 118.0 (CH, C-22), 177.4 (C=O, C-23), 178.6 (C_0, C-20); HRMS-ESI: calcd for $C_{38}H_{57}N_3O_{14}$ $[M+H]^+$ 780.8783, found: 780.41.

4.2.10 Synthesis of 3β-amino-3-deoxydigitoxigenin ***(8)***

300 mg of 3β-azido-3-deoxydigitoxigenin **(5)** (0.75 mmol, EQ 1) and 236 mg TPP (triphenylphosphine, 0.9 mmol, EQ 1.2) were dissolved in 5 mL of tetrahydrofuran. 1 mL of water was added and the reaction was kept under reflux at 70 °C overnight. After, the reaction was dissolved in 80 mL dichloromethane and the organic layer (CH_2Cl_2 I, containing mainly the intermediate ring product) was extracted with 4 × 100 mL with a mixture of 390 mL of H_2O and 10 mL 2 M HCl. The acid aqueous phases were extracted with 8 × 30 mL dichloromethane and the organic phase (CH_2Cl_2 II) was dried over anhydrous sodium sulfate and evaporated. Next, the aqueous phases were combined, pH adjusted to 8-9 (13 to 15 mL of 3% NH_3 water solution) and submitted to extraction with dichloromethane (8 × 30 mL). The organic layer was washed with 2 × 50 mL water (pH adjusted to 8 – 9 by 3% NH_3 water solution), dried over anhydrous sodium sulfate and evaporated to dryness furnish a yellow oil.

4.2.10.1 3β-amino-3-deoxydigitoxigenin ***(8)***

Yield: 60%; mp 207.0 – 209.5 ºC (Lit: 216-217 °C [50]). $[\alpha]_D^{26}$ +26.0 ° (*c* 1.00; acetone). Lit:$[\alpha]_D^{20}$ +17.0 ° ($CHCl_3$ [50]). IR: ῡ 3356 cm^{-1} (NH), 2862–2933 cm^{-1} (C-H), 1740–1783 cm^{-1} (C=O lactone), 1635 cm^{-1} (C=C), 1450 cm^{-1} (N-H), 1036 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (s, 3H, CH_3, H-19), 0.92 (s, 3H, CH_3, H-18), 1.09 (td, *J* =3.6 Hz, *J* =14.2 Hz, 1H, H-1a), 1.16–1.58 (m, 8H, H-5, H-6α, H-7β, H-8, H-11, H-12), 1.68–1.75 (m, 3H, H-2β, H-7α, H-9), 1.80–1.92 (m, 5H, H-1α, H-4α, H-6β, H-15α, H-16β), 2.03–2.06 (m, H, H-2α), 2.14–2.25 (m, 3H, H-4β, H-15β, H-16α), 2.79 (dd, *J* =6.0 Hz , *J* =8.6 Hz, 1H, H-17), 4,06 (tt, *J* =4.4 Hz, *J* =12.0 Hz, 1H, H-3), 4.81 (dd, *J* =1.6 Hz, *J* =18.0 Hz, 1H, H-21a), 4.99 (dd, *J* =1.2 Hz, *J* =18.0 Hz, 1H, H-21b), 5.88 (s, 1H, H-22). ^{13}C NMR (400 MHz, $CDCl_3$): 16.0 (CH_3, C-18),

21.1 (CH_2, C-11), 21.7 (CH_2, C-7), 23.5 (CH_3, C-19), 27.1 ($2CH_2$, C-6, C-16), 33.5 (CH_2, C-15), 33.6 (CH_2, C-2), 34.9 (C_0, C-10), 36.5 (CH, C-9), 37.9 (CH_2, C-1), 39.1 (CH_2, C-4), 40.1 (CH_2, C-12), 42.1 (CH, C-8), 44.8 (CH, C-5), 49.8 (C_0, C-13), 51.1 (CH, C-17), 52.8 (CH, C-3), 73.6 (CH_2, C-21), 85.6 (C_0, C-14), 117.9 (CH, C-22), 174.6 (C_0, C-20), 174.7 (C=O, C-23); HRMS-ESI: calcd for $C_{23}H_{35}NO_3$ $[M+H]^+$ 374.5363, found: 374.35

*4.2.11 Synthesis of 3β-(chloroacetyl)amino-3-deoxydigitoxigenin **(9)***

A suspension of 100 mg of 3β-amino-3-deoxydigitoxigenin **(8)** (0.27 mmol) in 2 mL tetrahydrofuran was added dropwise over 30 min to a stirred mixture of [48 µL chloroacetyl chloride (0.6 nmol) and 149 mg K_2CO_3 (1.08 mmol) in tetrahydrofuran (200 µL)] at room temperature. Next, the reaction mixture was stirred for 18 h at room temperature, filtrated through cotton to remove the K_2CO_3 and diluted with 80 mL CH_2Cl_2. Finally, the organic layer was washed 3 × 30 mL of water, dried over anhydrous sodium sulfate and evaporated.

*4.2.11.1 3β-(Chloroacetyl)amino-3-deoxydigitoxigenin **(9)***

Yield: 95%; mp 221.0-223.3 ºC. $[\alpha]_D^{26}$ +8.0 ° (*c* 0.50; acetone). IR: ῡ 3335–3455 cm^{-1} (N-H amide), 2865–2932 cm^{-1} (C-H), 1732 cm^{-1} (C=O), 1677 cm^{-1} (C=O amide), 1615 cm^{-1} (C=C), 1532 cm^{-1} (N-H), 1019 cm^{-1} (C-O), 779 cm^{-1} (C-Cl). ^{1}H NMR (400 MHz, acetone-d_6): δ 0.91 (s, 3H, CH_3, H-18), 0.96 (s, 3H, CH_3, H-19), 1.23–1.96 (m, 18H, H-1, H-2, H-4β, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15β, H-16β), 2.09–2.28 (m, 3H, H-4α, H-H-15α, H-16α), 2.85–2.87 (m, 1H, H-17), 3.26 (s, 1H, OH), 4.04 (s, 2H, H-2'), 4.13 (t, *J* =3.2 Hz, 1H, H-3), 4.85 (dd, *J* =1.7 Hz, *J* =18.1 Hz, 1H, H-21a), 5.01 (dd, *J* =1.3 Hz, *J* =18.1 Hz, 1H, H-21b), 5.86 (dd, *J* =1.2 Hz, *J* =1,6 Hz, 1H, H-22), 7.33 (d, *J* = 5.5 Hz, 1H, N-H). ^{13}C NMR (400 MHz, acetone-d_6): 16.3 (CH_3, C-18), 22.0 (CH_2, C-11), 22.2 (CH_2, C-7), 24.2 (CH_3, C-19), 25.4 (CH_2, C-2), 27.7 ($2CH_2$, C-6, C-16), 31.1 (CH_2, C-4), 31.6 (CH_2, C-1), 33.6 (CH_2, C-15), 36.2 (C_0, C-10), 36.3 (CH, C-9), 38.2 (CH, C-5), 40.5 (CH_2, C-12), 42.6 (CH, C-8), 43.8 (CH_2, C-2'), 46.7 (CH, C-3), 50.6 (C_0, C-13), 51.9 (CH, C-17), 74.0 (CH_2, C-21), 85.5 (C_0, C-14), 117.9 (CH, C-22), 166.0 (C=O, C-1'), 174.5 (C=O, C-23), 176.4 (C_0, C-20); HRMS-ESI: calcd for $C_{25}H_{36}ClNO_4$ $[M+H]^+$ 451.0180, found: 450.50 and 452.44

*4.2.12 Synthesis of 3β-[(N-(2-hydroxyetil)aminoacetyl]amino-3-deoxydigitoxigenin **(10)***

To a solution of 50 mg (0.11 mmol) of 3β-(Chloroacetyl)amino-3-deoxydigitoxigenin **(9)** in 5 mL of THF, 24 mg (0.39 mmol) ethanolamine were added and stirred for 72 h at room temperature. Then, the reaction mixture was diluted with 80 mL of CH_2Cl_2, washed with H_2O (3 ×10 mL), dried over anhydrous sodium sulfate and evaporated to residue under reduced pressure. The silica gel (0.04 – 0.63 mm; 15 g to reaction mixture) was washed with the following solvents of different polarities: ethyl acetate:hexane 7:3, (20 × 10 mL fractions), ethyl acetate (20 × 10 mL fractions) → ethyl acetate: acetone 8:2 (20 × 10 mL fractions) → ethyl acetate: acetone 1:1 (20 × 10 mL fractions) → ethyl acetate: acetone 2:8 (20 × 10 mL fractions) → acetone (20 × 10 mL fractions). Compound **10** eluted in fractions 80-105.

*4.2.12.1 3β-[(N-(2-hydroxyetil)aminoacetyl]amino-3-deoxydigitoxigenin **(10)***

Yield: 85%; mp 88.0-89.4 ºC. $[\alpha]_D^{25}$ +8.3 ° (*c* 0.24; acetone). IR: ῡ 3304 cm^{-1} (O-H), 2863–2940 cm^{-1} (C-H), 1719-1755 cm^{-1} (C=O), 1643 cm^{-1} (C=O amide), 1544 cm^{-1} (N-H), 1024-1067 cm^{-1} (C-O). ^{1}H NMR (400 MHz, acetone-d_6): δ 0.78 (s, 3H, CH_3, H-18), 0.85 (s, 3H, CH_3, H-19), 1.00–1.81 (m, 19H, H-1, H-2, H-4β, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15α, H-16β), 2.00–2.15 (m, 3H, H-4α, H-15β, H-16α), 2.70-2.74 (br, 1H, H-17), 2.91 (t, *J* =6.5 Hz, 1H, H-3'), 2.96 (s, 1H, H-2'), 3.12–3.15 (m, 2H, N-H), 3.41–3.46 (m, 2H, OH), 3.73 (t, *J* =6.5 Hz, 1H, H-4'), 3.98 (s, 1H, H-3), 4.72 (d, *J* =18.1 Hz, 1H, H-21a), 4.88 (d, *J* =18.1 Hz, 1H, H-21b), 5.73 (s, 1H, H-22). ^{13}C NMR (400 MHz, acetone-d_6): 16.3 (CH_3, C-18), 22.1 (CH_2, C-11), 22.3 (CH_2, C-7), 24.6 (CH_3, C-19), 25.8 (CH_2, C-6), 27.7 (CH_2, C-16), 27.8 (CH_2, C-2), 31.5 (CH_2, C-4), 32.1 (CH_2, C-1), 33.7 (CH_2, C-15), 36.3 (C_0, C-10), 36.4 (CH, C-9), 38.9 (CH, C-5), 40.5 (CH_2, C-12), 42.6 (CH, C-8), 45.4 (CH, C-3), 50.6 (C_0, C-13), 51.9 (CH, C-17), 52.2 (CH_2, C-3'), 54.1 (CH_2, C-2'), 64.0 (CH_2, C-4'), 74.0 (CH_2, C-21), 85.5 (C_0, C-14), 117.9 (CH, C-22), 169.6-169.7 (C=O, C-1'), 174.5 (C=O, C-23), 176.3 (C_0, C-20); HRMS-ESI: calcd for $C_{27}H_{42}N_2O_5$ $[M+H]^+$ 475.6402, found: 475.48

*4.2.13 Synthesis of 3β-(hydroxyacetyl)amino-3-deoxydigitoxigenin **(11)***

To a solution of 5 mg (0.011 mmol) of 3β-(Chloroacetyl)amino-3-deoxydigitoxigenin **(9)** in 500 µL acetonitrile, 50 mg KI (0.3 mmol) in 100 µL H_2O and 50 µL of DIPEA were added. The reaction was stirred for 120 h at 70 °C. Next, the reaction mixture was diluted with 80 mL of CH_2Cl_2, washed 3 × 10 mL 2M HCl and 3 × 10 mL H_2O, dried over anhydrous sodium sulfate and evaporated. Compound **11** was purified by flash column chromatography. The silica gel (0.04 – 0.63 mm; 10 g to 30 g reaction

mixture) was washed with the following solvents of different polarities: dichloromethane 100%, (3 × 20 mL fractions), dichlromethane: ethyl acetate 1:1 (10 × 10 mL fractions) → dichlromethane: ethyl acetate 2:8 (10 × 10 mL fractions) → ethyl acetate – 100% (10 × 10 mL fractions) ethyl acetate: acetone 1:1 (15 × 10 mL fractions). Compound **11** eluted in fractions 33-43.

*4.2.13.1 3β-(hydroxyacetyl)amino-3-deoxydigitoxigenin **(11)***

Yield: 25%; mp 253.0-255.3 ºC. $[\alpha]_D^{24}$ +17.8 ° (*c* 0.45; MeOH). IR: ῡ 3397 cm^{-1} (O-H), 2896 cm^{-1} (C-H), 1723 cm^{-1} (C=O), 1659 cm^{-1} (C=O amide), 1617 cm^{-1} (C=C), 1523 cm^{-1} (N-H), 1072 cm^{-1} (C-O). ^{1}H NMR (400 MHz, MeOD-d_4/acetone-d_6): δ 0.94 (s, 3H, CH_3, H-18), 1.05 (s, 3H, CH_3, H-19), 1.21–2.00 (m, 18H, H-1, H-2, H-4β, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15β, H-16α), 2.10–2.31 (m, 3H, H-4α, H-15α, H-16β), 2.87–2.91 (m, 1H, H-17), 4.01 (s, 2H, H-2'), 4.20 (s, 1H, H-3), 4.96 (dd, *J* =1.5 Hz, *J* =18.4 Hz, 1H, H-21a), 5.09 (dd, *J* =1.1 Hz, *J* =18.4 Hz, 1H, H-21b), 5.95 (s, 1H, H-22). ^{13}C NMR (400 MHz, MeOD-d_4/acetone-d_6): 16.5 (CH_3, C-18), 22.4 (CH_2, C-7), 22.6 (CH_2, C-11), 24.4 (CH_3, C-19), 25.9 (CH_2, C-6), 28.0 (CH_2, C-2), 28.2 (CH_2, C-16), 31.6 (CH_2, C-4), 32.1 (CH_2, C-1), 33.6 (CH_2, C-15), 36.6 (C_0, C-10), 36.9 (CH, C-9), 38.9 (CH, C-5), 41.0 (CH_2, C-12), 42.8 (CH, C-8), 46.5 (CH, C-3), 51.2 (C_0, C-13), 52.3 (CH, C-17), 62.8 (CH_2, C-2'), 75.4 (CH_2, C-21), 86.4 (C_0, C-14), 118.0 (CH, C-22), 174.0 (C=O, C-1'), 177.2 (C=O, C-23), 178.5 (C_0, C-20); HRMS-ESI: calcd for $C_{25}H_{37}NO_5$ $[M+H]^+$ 432.5724, found: 432.50

*4.2.14 General procedure for the synthesis of 3β-(2'-aminoacetyl)amino-3-deoxydigitoxigenin derivatives **(12-14)***

4.2.14.1 Synthesis of 3β-(iodoacetyl)amino-3-deoxydigitoxigenin

To a solution of 5 mg (0.011 mmol) of chloroacetyl-amin-digitoxigenin **(9)** in 500 µL acetonitrile, 50 mg KI (0.3 mmol) in 100 µL H_2O were added and the reaction was stirred for 24 h at 70 °C. Next, the reaction mixture was diluted with 10 mL H_2O, extracted 3 × 15 mL CH_2Cl_2, washed 3 × 10 mL H_2O, dried over anhydrous sodium sulfate and evaporated.

Coupling of different residues

Different residues were coupled to obtain the varying amino-digitoxigenin derivatives (ratio 4:1). Therefore, the different residues were dissolved in 200 µL of acetonitrile, 5 mg (9.25 nmol) of 3β-(iodoacetyl)amino-3-deoxydigitoxigenin and 12 µL of DIPEA . The reaction was stirred for 24 h to 48 h

at 70 °C, diluted with 80 mL CH_2Cl_2, washed 3 × 10 mL H_2O, dried over anhydrous sodium sulfate and evaporated. Then, the compounds were purified by flash column chromatography and the silica gel (0.04-0.63 mm; 10 g to 30 g reaction mixture) was washed with the following solvents of different polarities: dichloromethane 100%, (3 × 20 mL fractions), → dichlromethane: ethyl acetate 1:1 (10 × 10 mL fractions) → dichlromethane: ethyl acetate 2:8 (10 × 10 mL fractions) → ethyl acetate – 100% (10 ×10 mL fractions) → ethyl acetate: acetone 1:1 (15 ×10 mL fractions). Compound **12** eluted in fractions 21-31, compound **13** in fractions 21-36, and compound **14** in fractions 37-53 whereby double number of fractions and volume of solvents were used.

*4.2.14.2 3β-(p-cyanophenylaminoacetyl)amino-3-deoxydigitoxigenin **(12)***

Yield: 13%; mp 122.5-126.7 ºC. $[\alpha]_D^{26}$ +11.3 ° (*c* 0.71; CH_2Cl_2). IR: ῡ 3361 cm^{-1} (N-H amine), 2924 cm^{-1} (C-H), 2214 cm^{-1} (C≡N ciane), 1733 cm^{-1} (C=O lactone), 1659 cm^{-1} (C=O amide), 1606 cm^{-1} (C=C aromatic), 1523 cm^{-1} (N-H amide), 1173 cm^{-1} (C-O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.81 (s, 3H, CH_3, H-19), 0.86 (s, 3H, CH_3, H-18), 1.17–1.42 (m, 7 H, H-4β, H-5, H-6α, H-7β, H-11, H-12α), 1.49–1.70 (m, 9H, H-1, H-2, H-7α, H-8, H-9, H-12β, H-15β), 1.81-2.00 (m, 3H, H-4α, H-6β, H-16α), 2.10–2.17 (m, 2H, H-15α, H-16β), 2.75–2.78 (m, 1H, H-17), 3.84 (s, 2H, H-2'), 4.22 (s, 1H, H-3), 4.79 (dd, *J* =1.5 Hz, *J* =18.1 Hz, 1H, H-21a), 4.97 (dd, *J* =1.1 Hz, *J* =18.0 Hz, 1H, H-21b), 5.87 (s, 1H, H-22), 6.44 (d, *J* = 7.5 Hz, 1H, N-H amide), 6.63 (d, *J* = 8.7 Hz, 1H, H-4'), 7.47 (d, *J* = 8.7 Hz, 1H, H-5'). ^{13}C NMR (400 MHz, $CDCl_3$): 15.8 (CH_3, C-18), 21.1 (CH_2, C-11), 21.3 (CH_2, C-7), 23.9 (CH_3, C-19), 24.8 (CH_2, C-2), 26.5 (CH_2, C-6), 26.9 (CH_2, C-16), 30.4 (CH_2, C-4), 30.9 (CH_2, C-1), 33.2 (CH_2, C-15), 35.3 (C_0, C-10), 35.6 (CH, C-9), 37.5 (CH_2, C-5), 39.9 (CH, C-12), 41.8 (CH_2, C-8), 45.3 (CH, C-3), 47.4 (CH_2, C-2'), 49.6 (C_0, C-13), 50.9 (CH, C-17), 73.5 (CH_2, C-21), 85.4 (C_0, C-14), 101.0 (C_0, C-6'), 113.0 (CH, C-4'), 117.8 (CH, C-22), 119.8 (C_0, C-7' nitrile), 133.9 (CH, C-5'), 150.2 (C_0, C-3'), 167.8 (C=O, C-1'), 174.4 (C=O, C-23), 174.5 (C_0, C-20); HRMS-ESI: calcd for $C_{32}H_{41}N_3O_4$ $[M+H]^+$ 532.6930, found: 532.59

*4.2.14.3 3β-(phenylpiperidinoacetyl)amino-3-deoxydigitoxigenin **(13)***

Yield: 14%; mp 250.7-254.0 ºC. $[\alpha]_D^{22}$ +18.4 ° (*c* 0.76; CH_2Cl_2). IR: ῡ 3463 cm^{-1} (N-H amide), 3331 cm^{-1} (O-H), 2918 cm^{-1} (C-H), 1746 cm^{-1} (C=O lactone), 1671 cm^{-1} (C=O amide), 1508 cm^{-1} (N-H amide). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (s, 3H, CH_3, H-19), 0.99 (s, 3H, CH_3, H-18), 1.26–1.82 (m, 17 H, H-1, H-2, H-4β, H-4'a, H-5, H-6α, H-7, H-8, H-9, H-11, H-12, H-15α), 1.86–2.00 (m, 4H, H-4'b, H-4α, H-6β, H-16α), 2.11-2.22 (m, 2H, H-15β, H-16α), 2.34 (s, 1H, H-3'a), 2.55 (d, *J* = 11.9 Hz, 1H, H-5'),

2.76–2.80 (m, 1H, H-17), 2.99–3.03 (m, 2H, H-2', H-3'b), 4.23 (s, 1H, H-3), 4.80 (dd, *J* =1.5 Hz, *J* =18.0 Hz, 1H, H-21a), 4.99 (d, *J* =18.0 Hz, 1H, H-21b), 5.88 (s, 1H, H-22), 7.21–7.26 (m, 3H, H-7', H-9'), 7.30–7.34 (m, 2H, H-8'), 7.76 (d, *J* = 7.5 Hz, 1H, N-H amide). ^{13}C NMR (400 MHz, $CDCl_3$): 15.8 (CH_3, C-18), 21.2 (CH_2, C-11), 21.4 (CH_2, C-7), 24.3 (CH_3, C-19), 25.2 (CH_2, C-2), 26.7 (CH_2, C-6), 26.9 (CH_2, C-16), 30.7 (CH_2, C-4), 31.3 (CH_2, C-1), 33.3 (CH_2, C-15), 33.9 (CH_2, C-4'), 35.4 (C_0, C-10), 35.7 (CH, C-9), 37.9 (CH_2, C-5), 40.0 (CH, C-12), 41.8 (CH_2, C-8), 41.9 (CH_2, C-5'), 44.3 (CH, C-3), 49.6 (C_0, C-13), 50.9 (CH, C-17), 54.7 (CH_2, C-3'), 62.0 (CH_2, C-2'), 73.4 (CH_2, C-21), 85.5 (C_0, C-14), 117.8 (CH, C-22), 126.4 (CH, C-9'), 126.7 (CH, C-7'), 128.6 (CH, C-8'), 145.7 (C_0, C-6'), 174.4 (C=O, C-20, C-23), HRMS-ESI: calcd for $C_{36}H_{50}N_2O_4$ $[M+H]^+$ 575.8006, found: 575.51

4.2.14.4 3β-(p-chlorophenylaminoacetyl)amino-3-deoxydigitoxigenin ***(14)***

Yield: 46%; mp 242.8-245.2 ºC. $[\alpha]_D^{22}$ +7.8 ° (*c* 0.78; CH_2Cl_2). IR: $\bar{\upsilon}$ 3377 cm^{-1} (N-H amine), 2936 cm^{-1} (C-H), 1737 cm^{-1} (C=O lactone), 1671 cm^{-1} (C=O amide), 1496 cm^{-1} (N-H amide), 1063 cm^{-1} (C-O), 820 cm^{-1} (C-Cl).). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.78 (s, 3H, CH_3, H-19), 0.85 (s, 3H, CH_3, H-18), 1.13–1.94 (m, 19 H, H-1, H-2, H-4, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15α, H-16β), 2.10-2.17 (m, 2H, H-15β, H-16α), 2.63 (s, 1H, OH), 2.74–2.78 (m, 1H, H-17), 3.77 (s, 2H, H-2'), 4.20 (s, 1H, H-3), 4.79 (dd, *J* =1.4 Hz, *J* =18.0 Hz, 1H, H-21a), 4.97 (dd, *J* =1.1 Hz, *J* =18.1 Hz, 1H, H-21b), 5.86 (s, 1H, H-22), 6.57 (d, *J* = 8.8 Hz, 1H, H-4'), 6.82 (d, *J* = 7.6 Hz, 1H, N-H amide), 7.16 (d, *J* = 8.8 Hz, 1H, H-5'). ^{13}C NMR (400 MHz, $CDCl_3$): 15.8 (CH_3, C-18), 21.1 (CH_2, C-11), 21.3 (CH_2, C-7), 23.8 (CH_3, C-19), 24.9 (CH_2, C-2), 26.5 (CH_2, C-6), 26.9 (CH_2, C-16), 30.4 (CH_2, C-4), 30.9 (CH_2, C-1), 33.2 (CH_2, C-15), 35.2 (C_0, C-10), 35.6 (CH, C-9), 37.3 (CH_2, C-5), 39.9 (CH, C-12), 41.8 (CH_2, C-8), 45.0 (CH, C-3), 48.8 (CH_2, C-2'), 49.6 (C_0, C-13), 50.9 (CH, C-17), 73.4 (CH_2, C-21), 85.5 (C_0, C-14), 114.4 (CH, C-5'), 117.8 (CH, C-22), 124.1 (C_0, C-6'), 129.4 (CH, C-4'), 145.5 (C_0, C-3'), 168.9 (C=O, C-1'), 174.4 (C=O, C-20 C-23), HRMS-ESI: calcd for $C_{31}H_{41}ClN_2O_4$ $[M+H]^+$ 542.1286, found: 541.56 and 543.50

Compounds **15** and **16** were furnished by Prof. Dr. Wolfgang Kreis (Erlangen-Nuremberg University) and their chemical structures were unequivocally determined by spectroscopic methods (available as Supplementary Data).

4.2.15 3β-(Bromoacetyl)amino-3-deoxydigitoxigenin ***(15)***

mp 196.4-198.9 ºC. $[\alpha]_D^{22}$ +28.2 ° (*c* 0.21; CH_2Cl_2).IR: $\bar{\upsilon}$ 3327 cm^{-1} (N-H amide), 2936 cm^{-1} (C-H), 1732 cm^{-1} (C=O), 1652 cm^{-1} (C=O amide), 1531 cm^{-1} (N-H), 1446 cm^{-1} (-CH_2-), 1025 cm^{-1} (C-O). ^{1}H NMR

(400 MHz, $CDCl_3$): δ 0.88 (s, 3H, CH_3, H-18), 0.99 (s, 3H, CH_3, H-19), 1.11 (td, *J* =4.4 Hz, *J* =14.4 Hz, 1H, H-1β), 1.22–1.74 (m, 15H, H-1b, H-2β, H-4β, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15α), 1.86–2.02 (m, 3H, H-2α, H-4α H-16 β), 2.10–2.22 (m, 2H, H-15β, H-16α), 2.77–2.80 (m, 1H, H-17), 3,89 (s, 2H, H-2'),4.19 (t, *J* =3.3 Hz, 1H, H-3), 4.80 (dd, *J* =1.6 Hz, *J* =18.0 Hz, 1H, H-21a), 4.98 (dd, *J* =1.2 Hz, *J* =18.0 Hz, 1H, H-21b), 5.88 (s, 1H, H-22), 6.81 (d, *J* = 7.2 Hz, 1H, N-H). ^{13}C NMR (400 MHz, $CDCl_3$): 15.8 (CH_3, C-18), 21.2 (CH_2, C-11), 21.3 (CH_2, C-7), 24.0 (CH_3, C-19), 24.7 (CH_2, C-6), 26.6 (CH_2, C-2), 26.9 (CH_2, C-16), 29.8 (CH_2, C-2'), 30.2 (CH_2, C-4), 31.0 (CH_2, C-1), 33.2 (CH_2, C-15), 35.4 (C_0, C-10), 35.7 (CH, C-9), 37.6 (CH_2, C-5), 39.9 (CH, C-12), 41.8 (CH_2, C-8), 45.9 (CH, C-3), 49.6 (C_0, C-13), 50.9 (CH, C-17), 73.4 (CH_2, C-21), 85.5 (C_0, C-14), 117.8 (CH, C-22), 164.4 (C=O, C-1'), 174.3 (C=O, C-23), 174.4 (C_0, C-20); HRMS-ESI: calcd for $C_{25}H_{36}BrNO_4$ $[M+H]^+$ 495.4690, found: 494.39 and 496.45

4.2.16 (2'R,3'S,4'S)-6'-((3β-amino-3-deoxydigitoxigenin)amino)hexane-2',3',4'-triol ***(16)***

$[\alpha]_D^{22}$ +10.7 ° (*c* 0.37; MeOH). IR: ῡ 3382 cm^{-1} (N-H amine), 2852–2922 cm^{-1} (C-H), 1731–1754 cm^{-1} (C=O lactone), 1618 cm^{-1} (C=C), 1463 cm^{-1} (-CH_2-), 1034 cm^{-1} (C-O). ^{1}H NMR (400 MHz, DMSO-d_6/acetone-d_6): δ 0.88 (s, 3H, CH_3, H-19), 1.03 (s, 3H, CH_3, H-18), 1.17 (d, *J* =6.2 Hz, 1H, H-1'), 1.22–1.98 (m, 19H, H-1, H-2, H-4β, H-5, H-6, H-7, H-8, H-9, H-11, H-12, H-15α, H-16β, H-5`α), 2.09–2.28 (m, 4H, H-4α, H-15β, H-16α, H-5'β), 2.83 (dd, *J* =5.4 Hz, *J* =9.2 Hz, 1H, H-17), 3.14-3.20 (m, 3H, H-6', N-H), 3.30 (t, *J* =6.5 Hz, 1H, H-3'), 3.44 (s, H, H-3), 3.74 (quint, *J* =6.3 Hz, 1H, H-2'), 3.81–3.85 (m, 2H, H-4', OH-14), 4.53 (s, 1H, N-H), 4.86 (dd, *J* =1.3 Hz, *J* =18.2 Hz, 1H, H-21a), 5.02 (dd, *J* =1.0 Hz, *J* =18.1 Hz, 1H, H-21b), 5.88 (s, 1H, H-22). ^{13}C NMR (400 MHz, DMSO-d_6/acetone-d_6): 16.3 (CH_3, C-18), 20.0 (CH_3, C-1'), 21.8 (2CH_2, C-7, C-11), 22.2 (CH_2, C-6), 23.2 (CH_3, C-19), 27.2 (CH_2, C-2), 27.5 (CH_2, C-4), 27.9 (CH_2, C-16), 29.3 (CH_2, C-5`), 29.9 (CH_2, C-1), 33.3 (CH_2, C-15), 36.0 (C_0, C-10), 36.1 (CH_2, C-5), 36.3 (CH_2, C-9), 40.2 (CH, C-12), 42.2 (CH, C-8), 43.9 (CH, C-6'), 50.4 (C_0, C-13), 51.7 (CH, C-17), 55.5 (CH, C-3), 69.4 (CH_2, C-2'), 71.4 (CH_2, C-4'), 74.0 (CH_2, C-21), 78.4 (CH_2, C-3'), 85.0 (C_0, C-14), 117.6 (CH, C-22), 174.5 (C_0, C-20), 176.6 (C=O, C-23); HRMS-ESI: calcd for $C_{29}H_{47}NO_6$ $[M+H]^+$ 506,6940, found: 506.26

4.3 UPLC/MS analyses of cardenolide derivatives

UPLC/MS analyses were carried out using an ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) linked simultaneously to both a PDA 2996 photo diode array detector (Waters,

Milford, MA, USA) and an ACQUITY TQ Detector (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionization (ESI) source operating in positive mode. MassLynx™ software (version 4.1, Waters, Milford, MA, USA) was used to control the instruments, as well as for data acquisition and processing. The solutions of cardenolide derivatives (3 µL; 0.5 mg/mL) were injected into a reversed phase column (BEH_{C18}, 1.7 µm, 1 × 50 mm, Waters, Milford, MA), and maintained at 40 °C. The mobile phase consisted of solvent A (H_2O/0.1 HCOOH) and solvent B (acetonitrile/0.1 HCOOH) at a flow rate of 300 µL/min: T=0 min, 5% B; T=10 min, 95% B; T=11 min, 5% B; T=13 min, 5% B. The effluent was introduced into a PDA detector (scanning range 210–400 nm, resolution 1.2 nm) and subsequently into an electrospray source (source block temperature 120 °C, desolvation temperature 350 °C, capillary voltage 3.5 kV, cone voltage 30 V), and nitrogen was used as the desolvation gas (600 L/h). Then, mass chromatograms were recorded in the positive and negative ionization mode in the range from 100 to 1300 Da.

4.4 Infrared spectroscopy

Infrared spectrum was recorded on a Spectrum One, Perkin-Elmer ATR system.

4.5 NMR analysis

^{1}H NMR, ^{13}C NMR, DEPT-135, ^{1}H–^{1}H COSY, HSQC, HMBC and NOESY spectra were recorded on Bruker Avance DRX-400 and DPX-200 spectrometer (^{1}H 400/200 MHz and ^{13}C 100/50 MHz) in aceton-d_6, $CDCl_3$, DMSO-d_6 and CD_3OD at 300K using TMS as internal standard for both nuclei. Chemical shifts (δ) are given in ppm and *J* couplings in Hertz (Hz).

4.6 Biological activities

4.6.1 Viruses and cell lines

The cytotoxic screening was conducted on five human cancer cell lines: (1) non-small cell lung cancer cells (NSCLC, A549, ATCC: CCL185) grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco® Carlsbad, CA, USA); (2) no hormone-sensitive human prostate adenocarcinoma cells (PC3, DSMZ, ACC: 465, Braunschweig, Germany) grown in DMEM with no phenol red (Gibco); (3) androgen-sensitive human prostate adenocarcinoma cells (LNCaP, ATCC: CRL-1740) cultured in RPMI 1640 medium with no phenol red (Gibco); (4) human ileocecal adenocarcinoma (HCT-8, Texas A&M

University System); and (5) another NSCLC (H460, ATCC: HTB-177) both cultured in RPMI 1640 medium (Gibco).

To verify selectivity on non-tumor cells, human fetal lung fibroblast cell line (MRC-5 cells, ECACC: 05090501) and human gingival fibroblasts (HGF, obtained from human gingival primary cell culture/Experimental protocol approved by the Ethics Committee on Human Research, UFSC, authorization number 062, protocol number 21/09) grown in DMEM were used. MRC-5 and LNCaP cells were supplemented with 1% glutamine (Cultilab, Campinas, São Paulo, Brazil) and 1% non-essential amino acids (Gibco), and PC3 cells just with 1% glutamine (Cultilab). All cell lines were supplemented with 10% fetal bovine serum (FBS; Gibco) and maintained at 37 ºC and 5% CO_2 in a humidified atmosphere.

The anti-herpes screening was performed on fibroblasts of African green monkey kidneys (Vero cells, ATCC: CCL81) grown in Eagle's minimum essential medium (MEM; Cultilab) supplemented with 10% fetal bovine serum (Gibco) and maintained at 37 ºC and 5% CO_2 in a humidified atmosphere.

The HSV-1 (KOS and 29-R strains, which are sensitive and resistant to acyclovir, respectively; Faculty of Pharmacy, University of Rennes I, Rennes, France) and the HSV-2 (333 strain; Department of Clinical Virology, Göteborg University, Sweden) viral stocks were prepared, titrated based on plaque-forming units (PFU), counted by plaque assay as described by Burleson et al. [83], and stored at -80 ºC.

4.6.2 Anti-herpes in vitro activity

4.6.2.1 Plaque number reduction assay

Firstly, the cytotoxicity of the new cardenolide derivatives was determined by sulforhodamine B (SRB) assay [84]. In brief, Vero cells (2.5×10^4 cells per well) were exposed to different concentrations of the samples for 48 h. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced cell viability by 50% when compared to untreated controls.

Thereafter, the potential anti-herpes activity was screened by plaque number reduction assay as described previously by Boff et al. [85]. Briefly, confluent cell monolayers (2.5×10^5 cells per well) were infected with approximately 100 PFU of each virus strain for 1 h at 37 ºC. Treatments were performed by adding non-cytotoxic concentrations of the compounds for Vero cells after viral infection (post infection treatment). Cells were then washed with phosphate-buffered saline (PBS) and overlaid with MEM

containing 1.5% carboxymethylcellulose (CMC; Sigma-Aldrich) in the presence or absence of different concentrations of the compounds, and incubated for 48 h. Cells were fixed and stained with naphthol blue-black (Sigma-Aldrich) and viral plaques were counted by using a stereomicroscope. The concentration of each sample that inhibited viral replication by 50% (IC_{50}) when compared to untreated controls was estimated. The ratio between CC_{50} and IC_{50} values was calculated to obtain the selectivity index (SI) of each sample. Acyclovir (ACV, Sigma-Aldrich) was used as positive control to HSV-1 (KOS strain) and HSV-2 (333 strain) and as negative control to HSV-1 (29-R strain) replication.

4.6.3 Cytotoxic activity

4.6.3.1 Cytotoxic screening

It was performed by SRB assay, as described above. Briefly, the human cancer (PC3, A549, HCT-8, LNCaP and H460) and non-cancer cell lines (MRC-5 and HGF) were seeded in 96-well plates (2.5×10^4 cells per well) and exposed to different concentrations of the cardenolide derivatives for 48 h. Paclitaxel was used as positive control. After the incubation period, the 50% inhibitory concentration (IC_{50}) of each compound was defined as the concentration that inhibited cell viability by 50% when compared to untreated controls.

4.7 Na+/K+ ATPase assay

Enzymatic activity of the Na^+/K^+ ATPase α1, 2, 3 subunits of porcine cortex (Sigma-Aldrich) was assayed as described by Baykov et al. [86] and Nolte et al. [10].

4.8 Statistical analyses

The results were expressed as mean ± standard deviation (SD) of three independent experiments. For the determination of CC_{50} and IC_{50} values, nonlinear regression of concentration-response curves was used.

997 **Conflict of interest**

998 The authors declare that they have no conflict of interest.

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Figure and scheme caption list

Fig. 1. Cytotoxic potency of new cardenolide derivatives against four human cancer cell lines (PC3, A549, HCT-8 and LNCaP). The IC_{50} values are color scaled in a heatmap as shown (green: more active; red: less active). Their standard errors (SD) are available in Supplementary Data (Table S1). DGTN: digitoxigenin.

Scheme 1. Synthesis of the triazole glycoside derivatives of digitoxigenin. Reagents and conditions: **(a)** I_2, Ac_2O r.t., 1 h, [90-95%]; **(b)** $BF_3.Et_2O$, propargyl alcohol, CH_2Cl_2, 0 °C-r.t., 24 h, [50-70%]; **(c)** HBr/AcOH, CH_2Cl_2, 0 °C-r.t., 6h, [90%]; **(d)** $AgCO_3$, AgOTf, propargyl alcohol, CH_2Cl_2, r.t., 24 h, [50%]. r.t. = room temperature; yield = [ ]

Scheme 2. Synthesis of cardenolide derivatives **10-14**. Reagents and conditions: **(a)** CrO_3, H_2SO_4 (Jones reagent), acetone, 0 °C, 1 h, [95%]; **(b)** $NaBH_4$ dixoxane / H_2O (8:2), -5 °C, 1 h, [65%]; **(c)** TsCl, pyridine, r.t., 15 h, [90%]; **(d)** NaN_3, DMF, 75 °C, 8 h, [85%]; **(e)** peracetylated propargyl glycoside (IIa-c, III), $CuSO_4.5H_2O$; sodium ascorbate, THF / H_2O (1:1), r.t., [65-75%]; **(f)** $LiOH.H_2O$, MeOH / H_2O, 0 °C, 1.5 h, [80-88%]; **(g)** Ph_3P, THF / H_2O (5:1), 70 °C, overnight, [60%]; **(h)** chloroacetyl chloride, K_2CO_3, THF / H_2O, r.t., 5 h, [95%]; **(i)** ETA, THF, r.t., 72 h, [85%]; **(j)** KI, DIPEA, ACN / H_2O (5:1), 70 °C, 120 h, [25%]; **(k)** KI, R-NH_2, DIPEA, ACN, 70 °C, 24 h, [23-46%]. r.t. = room temperature; yields = [ ]

References

[1] M.S. Butler, D.J. Newman, Mother Nature's gifts to diseases of man: the impact of natural products on anti-infective, anticholestemics and anticancer drug discovery, Prog Drug Res. 65 (2008) 1, 3-44.

[2] D.J. Newman, G.M. Cragg, Natural Products as Sources of New Drugs from 1981 to 2014, J Nat Prod. 79 (2016) 629-661.

[3] W. Kreis, The Foxgloves (Digitalis) Revisited, Planta Med. 83 (2017) 962-976.

[4] H.A. Bessen, Therapeutic and toxic effects of digitalis: William Withering, 1785, J Emerg Med. 4 (1986) 243-248.

[5] J.D. Horisberger, Recent insights into the structure and mechanism of the sodium pump, Physiology (Bethesda). 19 (2004) 377-387.

[6] I. Prassas, E.P. Diamandis, Novel therapeutic applications of cardiac glycosides, Nat Rev Drug Discov. 7 (2008) 926-935.

[7] H.S. Chang, M.Y. Chiang, H.Y. Hsu, C.W. Yang, C.H. Lin, S.J. Lee, I.S. Chen, Cytotoxic cardenolide glycosides from the root of *Reevesia formosana*, Phytochemistry. 87 (2013) 86-95.

[8] T. Mijatovic, I. Roland, E. Van Quaquebeke, B. Nilsson, A. Mathieu, F. Van Vynckt, F. Darro, G. Blanco, V. Facchini, R. Kiss, The alpha1 subunit of the sodium pump could represent a novel target to combat non-small cell lung cancers, J Pathol. 212 (2007) 170-179.

[9] E. Nolte, A. Sobel, S. Wach, H. Hertlein, N. Ebert, F. Muller-Uri, R. Slany, H. Taubert, B. Wullich, W. Kreis, The New Semisynthetic Cardenolide Analog 3beta-[2-(1-Amantadine)-1-on-ethylamine]-digitoxigenin (AMANTADIG) Efficiently Suppresses Cell Growth in Human Leukemia and Urological Tumor Cell Lines, Anticancer Res. 35 (2015) 5271-5275.

[10] E. Nolte, S. Wach, I. Thais Silva, S. Lukat, A.B. Ekici, J. Munkert, F. Muller-Uri, W. Kreis, C.M.O. Simões, J. Vera, B. Wullich, H. Taubert, X. Lai, A new semisynthetic cardenolide analog 3beta-[2-(1-amantadine)- 1-on-ethylamine]-digitoxigenin (AMANTADIG) affects G2/M cell cycle arrest and miRNA expression profiles and enhances proapoptotic survivin-2B expression in renal cell carcinoma cell lines, Oncotarget. 8 (2017) 11676-11691.

[11] N.F. Schneider, F.C. Geller, L. Persich, L.L. Marostica, R.M. Pádua, W. Kreis, F.C. Braga, C.M. Simões, Inhibition of cell proliferation, invasion and migration by the cardenolides digitoxigenin monodigitoxoside and convallatoxin in human lung cancer cell line, Nat Prod Res. 30 (2016) 1327-1331.

[12] N.F. Schneider, I.T. Silva, L. Persich, A. de Carvalho, S.C. Rocha, L. Marostica, A.C. Ramos, A.G. Taranto, R.M. Pádua, W. Kreis, L.A. Barbosa, F.C. Braga, C.M. Simões, Cytotoxic effects of the cardenolide convallatoxin and its Na,K-ATPase regulation, Mol Cell Biochem. 428 (2017) 23-39.

[13] I.T. Silva, J. Munkert, E. Nolte, N.F.Z. Schneider, S.C. Rocha, A.C.P. Ramos, W. Kreis, F.C. Braga, R.M. de Padua, A.G. Taranto, V. Cortes, L.A. Barbosa, S. Wach, H. Taubert, C.M.O. Simões, Cytotoxicity of AMANTADIG - a semisynthetic digitoxigenin derivative - alone and in combination with docetaxel in human hormone-refractory prostate cancer cells and its effect on Na(+)/K(+)-ATPase inhibition, Biomed Pharmacother. 107 (2018) 464-474.

[14] L.Q. Sousa, K.D. Machado, S.F. Oliveira, L.D. Araujo, E.D. Moncao-Filho, A.A. Melo-Cavalcante, G.M. Vieira-Junior, P.M. Ferreira, Bufadienolides from amphibians: A promising source of anticancer prototypes for radical innovation, apoptosis triggering and Na+/K+-ATPase inhibition, Toxicon. 127 (2017) 63-76.

[15] D.M. Tian, H.Y. Cheng, M.M. Jiang, W.Z. Shen, J.S. Tang, X.S. Yao, Cardiac Glycosides from the Seeds of *Thevetia peruviana*, J Nat Prod. 79 (2016) 38-50.

[16] Zeino, R. Brenk, L. Gruber, M. Zehl, E. Urban, B. Kopp, T. Efferth, Cytotoxicity of cardiotonic steroids in sensitive and multidrug-resistant leukemia cells and the link with Na(+)/K(+)-ATPase, J Steroid Biochem Mol Biol. 150 (2015) 97-111.

[17] S. De, S. Banerjee, M. Niranjan Babu, B. Mohana Lakhmi, T.M. Suneel Babu, Review on cardiac glycosides in cancer research and cancer therapy, Indo Am J Pharm Res. 6 (2016) 5391-5400.

[18] N.F.Z. Schneider, C. Cerella, C.M.O. Simões, M. Diederich, Anticancer and Immunogenic Properties of Cardiac Glycosides, Molecules. 22 (2017).

[19] A.W. Ashbrook, A.J. Lentscher, P.F. Zamora, L.A. Silva, N.A. May, J.A. Bauer, T.E. Morrison, T.S. Dermody, Antagonism of the Sodium-Potassium ATPase Impairs Chikungunya Virus Infection, MBio. 7 (2016) e00693-00616.

[20] J.W. Bertol, C. Rigotto, R.M. de Pádua, W. Kreis, C.R. Barardi, F.C. Braga, C.M. Simões, Antiherpes activity of glucoevatromonoside, a cardenolide isolated from a Brazilian cultivar of *Digitalis lanata*, Antiviral Res. 92 (2011) 73-80.

[21] H. Cai, A. Kapoor, R. He, R. Venkatadri, M. Forman, G.H. Posner, R. Arav-Boger, In vitro combination of anti-cytomegalovirus compounds acting through different targets: role of the slope parameter and insights into mechanisms of Action, Antimicrob Agents Chemother. 58 (2014) 986-994.

[22] Y.Y. Cheung, K.C. Chen, H. Chen, E.K. Seng, J.J. Chu, Antiviral activity of lanatoside C against dengue virus infection, Antiviral Res. 111 (2014) 93-99.

[23] T. Cohen, J.D. Williams, T.J. Opperman, R. Sanchez, N.S. Lurain, D. Tortorella, Convallatoxin-Induced Reduction of Methionine Import Effectively Inhibits Human Cytomegalovirus Infection and Replication, J Virol. 90 (2016) 10715-10727.

[24] T.J. Gardner, T. Cohen, V. Redmann, Z. Lau, D. Felsenfeld, D. Tortorella, Development of a high-content screen for the identification of inhibitors directed against the early steps of the cytomegalovirus infectious cycle, Antiviral Res. 113 (2015) 49-61.

[25] C. Hartley, M. Hartley, I. Pardoe, A. Knight, Ionic Contra-Viral Therapy (ICVT); a new approach to the treatment of DNA virus infections, Arch Virol. 151 (2006) 2495-2501.

[26] S. Singh, S. Shenoy, P.N. Nehete, P. Yang, B. Nehete, D. Fontenot, G. Yang, R.A. Newman, K.J. Sastry, *Nerium oleander* derived cardiac glycoside oleandrin is a novel inhibitor of HIV infectivity, Fitoterapia. 84 (2013) 32-39.

[27] C.T. Su, J.T. Hsu, H.P. Hsieh, P.H. Lin, T.C. Chen, C.L. Kao, C.N. Lee, S.Y. Chang, Anti-HSV activity of digitoxin and its possible mechanisms, Antiviral Res. 79 (2008) 62-70.

[28] R.W. Wong, A. Balachandran, M.A. Ostrowski, A. Cochrane, Digoxin suppresses HIV-1 replication by altering viral RNA processing, PLoS Pathog. 9 (2013) e1003241.

[29] M. Lopez-Gomez, E. Malmierca, M. de Gorgolas, E. Casado, Cancer in developing countries: the next most preventable pandemic. The global problem of cancer, Crit Rev Oncol Hematol. 88 (2013) 117-122.

[30] N.F.Z. Schneider, L. Persich, S.C. Rocha, A.C.P. Ramos, V.F. Cortes, I.T. Silva, J. Munkert, R.M. Padua, W. Kreis, A.G. Taranto, L.A. Barbosa, F.C. Braga, C.M.O. Simões, Cytotoxic and cytostatic effects of digitoxigenin monodigitoxoside (DGX) in human lung cancer cells and its link to Na,K-ATPase, Biomed Pharmacother. 97 (2018) 684-696.

[31] W.H. Chou, K.L. Liu, Y.L. Shih, Y.Y. Chuang, J. Chou, H.F. Lu, H.W. Jair, M.Z. Lee, M.K. Au, J.G. Chung, Ouabain Induces Apoptotic Cell Death Through Caspase- and Mitochondria-dependent Pathways in Human Osteosarcoma U-2 OS Cells, Anticancer Res. 38 (2018) 169-178.

[32] J. Munkert, M. Santiago Franco, E. Nolte, I. Thais Silva, R. Oliveira Castilho, F. Melo Ottoni, N.F.Z. Schneider, M.C. Oliveira, H. Taubert, W. Bauer, S.F. Andrade, R.J. Alves, C.M.O. Simões, F.C. Braga, W. Kreis, R.M. de Padua, Production of the Cytotoxic Cardenolide Glucoevatromonoside by Semisynthesis and Biotransformation of Evatromonoside by a *Digitalis lanata* Cell Culture, Planta Med. 83 (2017) 1035-1043.

[33] N.F.Z. Schneider, C. Cerella, J.Y. Lee, A. Mazumder, K.R. Kim, A. de Carvalho, J. Munkert, R.M. Padua, W. Kreis, K.W. Kim, C. Christov, M. Dicato, H.J. Kim, B.W. Han, F.C. Braga, C.M.O. Simões, M. Diederich, Cardiac Glycoside Glucoevatromonoside Induces Cancer Type-Specific Cell Death, Front Pharmacol. 9 (2018) 70.

[34] J.Y. Lee, M.S. Kim, M.S. Lee, J.E. Ju, N. Chung, Y.K. Jeong, Digoxin enhances radiation response in radioresistant A549 cells by reducing protein phosphatase 2A, Biosci Rep. 37 (2017) pii: BSR20171257.

[35] T. Wang, P. Xu, F. Wang, D. Zhou, R. Wang, L. Meng, X. Wang, M. Zhou, B. Chen, J. Ouyang, Effects of digoxin on cell cycle, apoptosis and NF-kappaB pathway in Burkitt's lymphoma cells and animal model, Leuk Lymphoma. 58 (2017) 1673-1685.

[36] Y.T. Zhao, J.Y. Yan, X.C. Han, F.L. Niu, J.H. Zhang, W.N. Hu, Anti-proliferative effect of digoxin on breast cancer cells via inducing apoptosis, Eur Rev Med Pharmacol Sci. 21 (2017) 5837-5842.

[37] M. Slingerland, C. Cerella, H.J. Guchelaar, M. Diederich, H. Gelderblom, Cardiac glycosides in cancer therapy: from preclinical investigations towards clinical trials, Invest New Drugs. 31 (2013) 1087-1094.

[38] B. Roizman, D.M. Knipe, R.J. Whitley, Herpes Simplex Viruses, in: D.M. Knipe, P.M. Howley, J.I. Cohen, D.E. Griffin, R.A. Lamb, M.A. Martin, V.R. Racaniello, B. Roizman (Eds.) Fields Virology, Lippincott Willians & Wilkins, Philadelphia, 2013, pp. 1823-1897.

[39] E. De Clercq, Antivirals: past, present and future, Biochem Pharmacol. 85 (2013) 727-744.

[40] S.K. Mamidyala, S.M. Firestine, Advances in herpes simplex virus antiviral therapies, Expert Opinion on Therapeutic Patents. 16 (2006) 1463-1480.

[41] S.T. Rottinghaus, R.J. Whitley, Current non-AIDS antiviral chemotherapy, Expert Rev Anti Infect Ther. 5 (2007) 217-230.

[42] T.H. Bacon, M.J. Levin, J.J. Leary, R.T. Sarisky, D. Sutton, Herpes simplex virus resistance to acyclovir and penciclovir after two decades of antiviral therapy, Clin Microbiol Rev. 16 (2003) 114-128.

[43] Y.C. Jiang, H. Feng, Y.C. Lin, X.R. Guo, New strategies against drug resistance to herpes simplex virus, Int J Oral Sci. 8 (2016) 1-6.

[44] S. Milutinovic, S. Heynen-Genel, E. Chao, A. Dewing, R. Solano, L. Milan, N. Barron, M. He, P.W. Diaz, S. Matsuzawa, J.C. Reed, C.A. Hassig, Cardiac Glycosides Activate the Tumor Suppressor and Viral Restriction Factor Promyelocytic Leukemia Protein (PML), PLoS One. 11 (2016) e0152692.

[45] R. Wangteeraprasert, V. Lipipun, M. Gunaratnam, S. Neidle, S. Gibbons, K. Likhitwitayawuid, Bioactive compounds from *Carissa spinarum*, Phytother Res. 26 (2012) 1496-1499.

[46] A.W. Dodson, T.J. Taylor, D.M. Knipe, D.M. Coen, Inhibitors of the sodium potassium ATPase that impair herpes simplex virus replication identified via a chemical screening approach, Virology. 366 (2007) 340-348.

[47] E.C. Calvaresi, P.J. Hergenrother, Glucose conjugation for the specific targeting and treatment of cancer, Chem Sci. 4 (2013) 2319-2333.

[48] R. Yang, H. Ding, Y. Song, Q. Xiao, J. Wu, Synthesis of 5-(Carbohydrate-ethynyl)-2'-deoxyuridine Derivatives, Letters in Organic Chemistry. 5 (2008) 518-521.

[49] A. Yajima, Y. Oono, R. Nakagawa, T. Nukada, G. Yabuta, A simple synthesis of four stereoisomers of roseoside and their inhibitory activity on leukotriene release from mice bone marrow-derived cultured mast cells, Bioorg Med Chem. 17 (2009) 189-194.

[50] L. Sawlewicz, E. Weiss, H.H. Linde, K. Meyer, [245. 3-Alpha- and 3-beta-amino-3-deoxydigitoxigenin. Partial synthesis experiments in the series of heart poisons. 5], Helv Chim Acta. 55 (1972) 2452-2460.

[51] V.V. Rostovtsev, L.G. Green, V.V. Fokin, K.B. Sharpless, A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes, Angew Chem Int Ed Engl. 41 (2002) 2596-2599.

[52] T.M. Beale, M.S. Taylor, Synthesis of cardiac glycoside analogs by catalyst-controlled, regioselective glycosylation of digitoxin, Org Lett. 15 (2013) 1358-1361.

[53] M. Zhou, G.A. O'Doherty, A stereoselective synthesis of digitoxin and digitoxigen mono- and bisdigitoxoside from digitoxigenin via a palladium-catalyzed glycosylation, Org Lett. 8 (2006) 4339-4342.

[54] L. Hu, D. Guo, Z. Xiao, X. Liu, B. Ma, M. Lei, Bufogenin derivatives, preparation methods, compositions containing such derivatives and uses thereof, in: C.A.o.S. Shanghai Institute of Materia Medica (Ed.) Kingsound & Partners, Kingsound & Partners, Beijing, China, 2013.

[55] E.B. Villhauer, J.A. Brinkman, G.B. Naderi, B.F. Burkey, B.E. Dunning, K. Prasad, B.L. Mangold, M.E. Russell, T.E. Hughes, 1-[[(3-hydroxy-1-adamantyl)amino]acetyl]-2-cyano-(S)-pyrrolidine: a potent, selective, and orally bioavailable dipeptidyl peptidase IV inhibitor with antihyperglycemic properties, J Med Chem. 46 (2003) 2774-2789.

[56] H.B. Liu, H. Tang, D. Yang, Q. Deng, L.J. Yuan, Q.G. Ji, Synthesis and biological evaluation of novel N-acyl substituted quinolin-2(1H)-one derivatives as potential antimicrobial agents, Bioorg Med Chem Lett. 22 (2012) 5845-5848.

[57] S.P. Wu, K.J. Du, Y.M. Sung, Colorimetric sensing of Cu(II): Cu(II) induced deprotonation of an amide responsible for color changes, Dalton Trans. 39 (2010) 4363-4368.

[58] M. Karplus, Vicinal Proton Coupling in Nuclear Magnetic Resonance, J Am Chem Soc. 85 (1963) 2870-2871.

[59] Y. Wang, Q. Qiu, J.J. Shen, D.D. Li, X.J. Jiang, S.Y. Si, R.G. Shao, Z. Wang, Cardiac glycosides induce autophagy in human non-small cell lung cancer cells through regulation of dual signaling pathways, Int J Biochem Cell Biol. 44 (2012) 1813-1824.

[60] V. Kaushik, J.S. Yakisich, N. Azad, Y. Kulkarni, R. Venkatadri, C. Wright, Y. Rojanasakul, A.K.V. Iyer, Anti-Tumor Effects of Cardiac Glycosides on Human Lung Cancer Cells and Lung Tumorspheres, J Cell Physiol. 232 (2017) 2497-2507.

[61] M. Laursen, L. Yatime, P. Nissen, N.U. Fedosova, Crystal structure of the high-affinity Na+K+-ATPase-ouabain complex with Mg2+ bound in the cation binding site, Proc Natl Acad Sci U S A. 110 (2013) 10958-10963.

[62] R.A. Newman, P. Yang, A.D. Pawlus, K.I. Block, Cardiac glycosides as novel cancer therapeutic agents, Mol Interv. 8 (2008) 36-49.

[63] G. Crambert, U. Hasler, A.T. Beggah, C. Yu, N.N. Modyanov, J.D. Horisberger, L. Lelievre, K. Geering, Transport and pharmacological properties of nine different human Na, K-ATPase isozymes, J Biol Chem. 275 (2000) 1976-1986.

[64] A. Katz, Y. Lifshitz, E. Bab-Dinitz, E. Kapri-Pardes, R. Goldshleger, D.M. Tal, S.J. Karlish, Selectivity of digitalis glycosides for isoforms of human Na,K-ATPase, J Biol Chem. 285 (2010) 19582-19592.

[65] A. Katz, D.M. Tal, D. Heller, H. Haviv, B. Rabah, Y. Barkana, A.L. Marcovich, S.J. Karlish, Digoxin derivatives with enhanced selectivity for the alpha2 isoform of Na,K-ATPase: effects on intraocular pressure in rabbits, J Biol Chem. 289 (2014) 21153-21162.

[66] R.J. Clifford, J.H. Kaplan, Human breast tumor cells are more resistant to cardiac glycoside toxicity than non-tumorigenic breast cells, PLoS One. 8 (2013) e84306.

[67] M. Cherniavsky Lev, S.J. Karlish, H. Garty, Cardiac glycosides induced toxicity in human cells expressing alpha1-, alpha2-, or alpha3-isoforms of Na-K-ATPase, Am J Physiol Cell Physiol. 309 (2015) C126-135.

[68] H. Cai, H.Y. Wang, R. Venkatadri, D.X. Fu, M. Forman, S.O. Bajaj, H. Li, G.A. O'Doherty, R. Arav-Boger, Digitoxin analogues with improved anticytomegalovirus activity, ACS Med Chem Lett. 5 (2014) 395-399.

[69] H.Y. Wang, W. Xin, M. Zhou, T.A. Stueckle, Y. Rojanasakul, G.A. O'Doherty, Stereochemical survey of digitoxin monosaccharides: new anticancer analogues with enhanced apoptotic activity and growth inhibitory effect on human non-small cell lung cancer cell, ACS Med Chem Lett. 2 (2011) 73-78.

[70] H. Sheikh, H. Yarwood, A. Ashworth, C.M. Isacke, Endo180, an endocytic recycling glycoprotein related to the macrophage mannose receptor is expressed on fibroblasts, endothelial cells and macrophages and functions as a lectin receptor, J Cell Sci. 113 (Pt 6) (2000) 1021-1032.

[71] L. East, C.M. Isacke, The mannose receptor family, Biochim Biophys Acta. 1572 (2002) 364-386.

[72] Chemicalize (calculation module developed by ChemAxon), https://chemicalize.com/#/calculation, 2018.

[73] R.M. Pádua, A.B. Oliveira, J.D.F. Souza, G.J. Vieira, J.A. Takahashi, F.C. Braga, Biotransformation of digitoxigenin by *Fusarium ciliatum*, J Braz Chem Soc. 16 (2005) 614-619.

[74] Y.C. Kim, R. Higuchi, T. Komori, F. Abe, T. Yamauchi, Thermal degradation of glycosides, III. Degradation of cardenolide glycosides, Liebigs Ann. Chem. 1990 (1990) 943-947.

[75] I.F. Makarevich, Y.I. Gubin, R. Megges, Ketoimines of cardenolides, Chem. Nat. Compd. 41 (2005) 52-55.

[76] K. Kawaguchi, M. Hirotani, T. Furuya, Biotransformation of digitoxigenin by cell suspension cultures of *Strophanthus intermedius*, Phytochemistry. 28 (1989) 1093-1097.

[77] H.P. Sigg, C. Tamm, T. Reichstein, 3□Epi□digitoxigenin. Glykoside und Aglykone. 111. Mitteilung, Helv. Chim. Acta. 36 (1953) 985-989.

[78] R. Rajaganesh, P. Ravinder, V. Subramanian, T. Mohan Das, FACE-selective fluorogenic cycloaddition reaction between coumarin azides and sugar terminal alkynes: an experimental and computational study, Carbohydr Res. 346 (2011) 2327-2336.

[79] T.N. Hoheisel, H. Frauenrath, A convenient Negishi protocol for the synthesis of glycosylated oligo(ethynylene)s, Org Lett. 10 (2008) 4525-4528.

[80] H.B. Mereyala, S.R. Gurrala, A highly diastereoselective, practical synthesis of allyl, propargyl 2,3,4,6-tetra-O-acetyl-β-d-gluco, β-d-galactopyranosides and allyl, propargyl heptaacetyl-β-d-lactosides, Carbohydr Res. 307 (1998) 351-354.

[81] M. Polakova, M. Belanova, K. Mikusova, E. Lattova, H. Perreault, Synthesis of 1,2,3-triazolo-linked octyl (1-->6)-alpha-D-oligomannosides and their evaluation in mycobacterial mannosyltransferase assay, Bioconjug Chem. 22 (2011) 289-298.

[82] L. Moni, A. Marra, J.S. Skotnicki, F.E. Koehn, M. Abou-Gharbia, A. Dondoni, Synthesis of rapamycin glycoconjugates via a CuAAC-based approach, Tetrahedrom Lett. 54 (2013) 6999-7003.

[83] F.G. Burleson, T.M. Chambers, D.L. Wiedbrauk, Virology: a laboratory manual., San Diego, 1992.

[84] V. Vichai, K. Kirtikara, Sulforhodamine B colorimetric assay for cytotoxicity screening, Nat Protoc. 1 (2006) 1112-1116.

[85] L. Boff, I.T. Silva, D.F. Argenta, L.M. Farias, L.F. Alvarenga, R.M. Pádua, F.C. Braga, J.P. Leite, J.M. Kratz, C.M. Simões, *Strychnos pseudoquina* A. St. Hil.: a Brazilian medicinal plant with promising in vitro antiherpes activity, J Appl Microbiol. 121 (2016) 1519-1529.

[86] A.A. Baykov, O.A. Evtushenko, S.M. Avaeva, A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay, Anal Biochem. 171 (1988) 266-270.

Table 1. Anti-herpes activity of new cardenolide derivatives against HSV-1 (KOS and 29-R strains) and HSV-2 (333 strain) replication.

Samples	Vero cells	HSV-1 (KOS strain)		HSV-1 (29-R strain)		HSV-2 (333 strain)	
	CC_{50} (μM)	IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI
6a	285.5 ± 8.30	6.27 ± 0.94	45.53	2.11 ± 0.43	135.3	3.80 ± 0.54	75.13
6b[#]	216.2 ± 11.93	NT	NT	NT	NT	NT	NT
6c	393.2 ± 6.90	10.09 ± 1.48	38.97	6.01 ± 0.62	65.42	4.55 ± 0.63	86.42
6d	180.7 ± 3.96	26.33 ± 4.45	6.86	33.37 ± 4.92	5.42	9.75 ± 1.89	18.53
7a	>300	2.79 ± 0.10	>107.5	2.71 ± 0.06	>110.7	2.14 ± 0.06	>140.2
7b	289.6 ± 11.18	0.98 ± 0.12	295.5	0.92 ± 0.07	314.8	1.24 ± 0.20	233.5
7c	>300	0.55 ± 0.07	>545.5	0.37 ± 0.02	>810.8	0.41 ± 0.09	>731.7
7d	147.0 ± 11.52	4.29 ± 0.06	34.27	3.24 ± 0.24	45.37	4.18 ± 0.39	35.17
9	83.98 ± 9.03	0.69 ± 0.06	121.7	0.34 ± 0.05	247.0	1.37 ± 0.17	61.30
10	>300	0.23 ± 0.01	>1,304	0.18 ± 0.01	>1,667	0.27 ± 0.01	>1,111
11	>300	0.24 ± 0.03	>1,250	0.19 ± 0.02	>1,579	0.30 ± 0.04	>1,000
12	>300	0.60 ± 0.11	>500.0	0.42 ± 0.05	>714.3	1.00 ± 0.12	>300.0
13	111.9 ± 7.01	0.44 ± 0.03	254.3	0.20 ± 0.03	559.5	0.49 ± 0.07	228.4
14	313.7 ± 7.88	1.51 ± 0.14	207.7	1.80 ± 0.07	174.3	1.40 ± 0.10	224.1

15[#]	36.07 ± 6.58	NT	NT	NT	NT	NT	NT
16	>300	1.46 ± 0.33	>205.5	3.23 ± 0.27	>92.88	1.27 ± 0.07	>236.2
DGTN	27.54 ± 4.29	1.09 ± 0.02	25.27	1.02 ± 0.18	27.00	3.23 ± 0.66	8.53
ACV	>2.000	1.38 ± 0.46	>1,449	NI	-	3.23 ± 0.89	>619

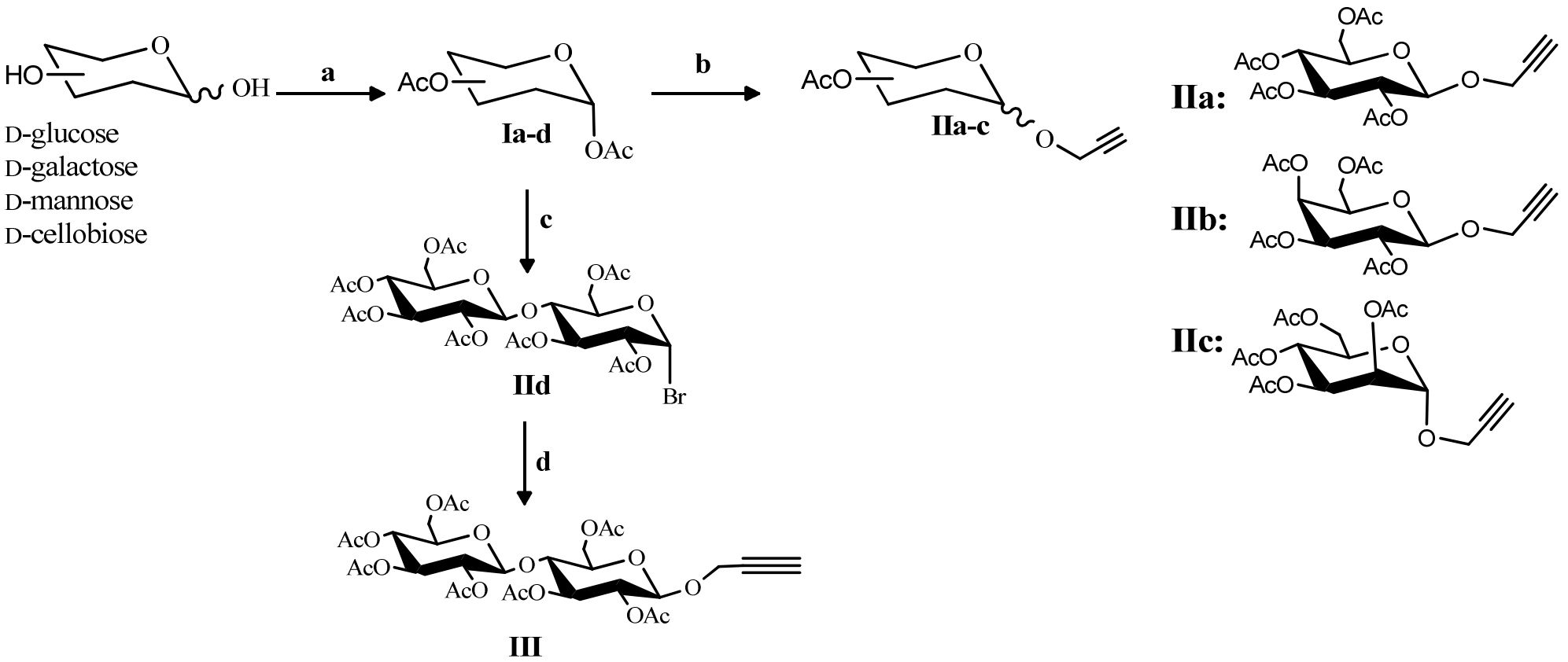
[#] These samples inhibited viral replication <30% in the preliminary screening (data not shown) and then were not tested (NT) to calculate their IC_{50} values.
CC_{50}: 50% cytotoxic concentration for Vero cells (µM); IC_{50}: 50% concentration that inhibited viral replication (µM). These values represent the mean ± SD of three independent experiments. SI: Selectivity index (SI = CC_{50}/IC_{50}); NI: no inhibitory activity; DGTN: digitoxigenin; ACV: acyclovir.

Table 2. Cytotoxic activity of some cardenolide derivatives[#] on A549 and H460 human lung cancer cell lines, and on MRC-5 and HGF human non-cancer cell lines.

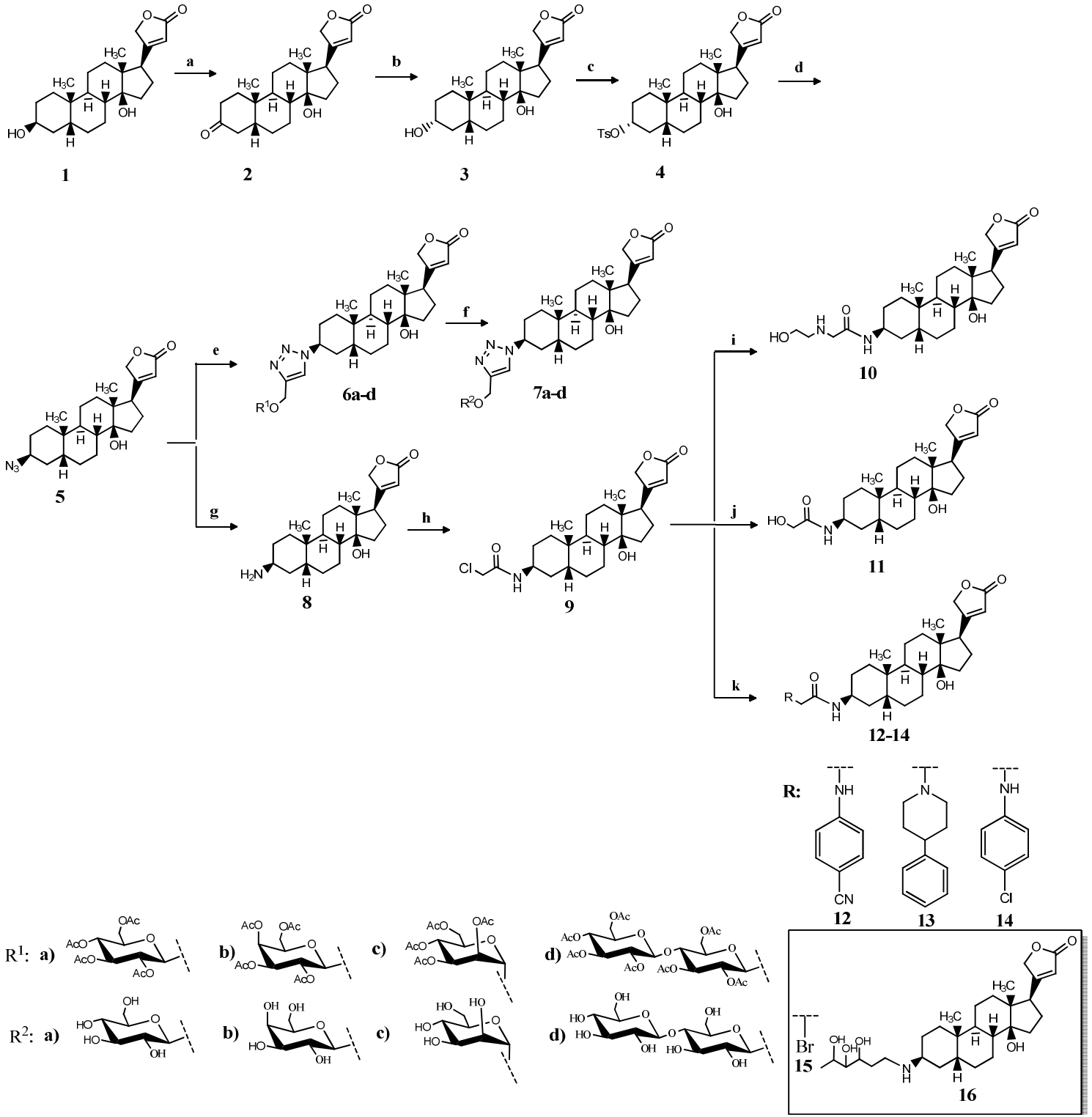
Samples	IC_{50} (µM)			
	A549 cells	**H460 cells**	**MRC-5 cells**	**HGF cells**
6c	1.57 ± 0.58	1.59 ± 0.09	NT	NT
7c	0.58 ± 0.15	0.24 ± 0.01	0.36 ± 0.01	1.41 ± 0.54
10	0.19 ± 0.03	0.07 ± 0.00	0.13 ± 0.01	0.66 ± 0.18
11	0.34 ± 0.03	0.26 ± 0.03	0.25 ± 0.03	1.67 ± 0.34
12	0.54 ± 0.25	0.25 ± 0.04	1.06 ± 0.22	2.09 ± 0.18
13	1.43 ± 0.36	0.32 ± 0.02	NT	NT
14	1.38 ± 0.43	1.14 ± 0.15	NT	NT
16	0.68 ± 0.13	0.37 ± 0.03	0.55 ± 0.09	2.55 ± 0.25
DGTN	1.68 ± 0.52	0.92 ± 0.02	1.16 ± 0.14	1.47 ± 0.19
Paclitaxel	0.11 ± 0.03	0.08 ± 0.01	>200.0	>200.0

[#] These are the most active cardenolide derivatives detected during the initial cytotoxic screening, see Fig. 1; IC_{50}: concentration that inhibited 50% of cell viability (µM). These values represent the mean ± SD of three independent experiments; NT: not tested; DGTN: digitoxigenin.

Scheme 1

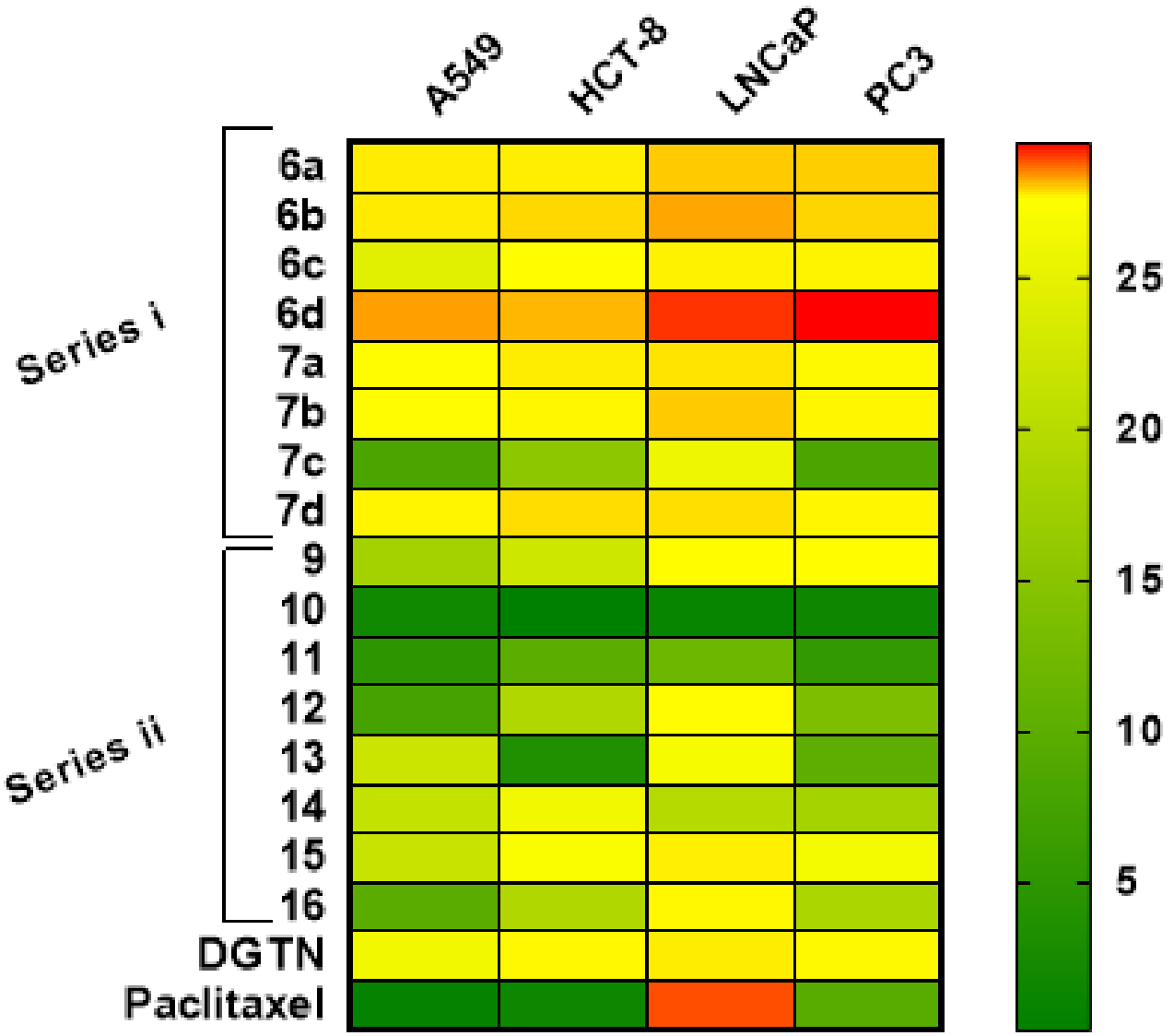

HO
OH
a
AcO
OAc
Ia-d
b
AcO
IIa-c
O
IIa:
OAc
AcO
AcO
AcO
IIb:
AcO
OAc
AcO
AcO
IIc:
AcO
OAc
AcO
AcO
c
IId
Br
d
III
D-glucose
D-galactose
D-mannose
D-cellobiose

Scheme 2



Derivative from the collection library of co-author Wolfgang Kreis

Fig. 1


A549
HCT-8
LNCaP
PC3
Series i
6a
6b
6c
6d
7a
7b
7c
7d
Series ii
9
10
11
12
13
14
15
16
DGTN
Paclitaxel
25
20
15
10
5

Highlights

1) New cardenolide derivatives (CDs) were synthetized by different approaches;

2) New CDs showed potent cytotoxicity against different human cancer cell lines;

3) New CDs showed potent anti-herpes action against different strains of HSV-1 and HSV-2;

4) Specific chemical features influenced the bioactivity of the new CDs.