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C5′-Alkyl Substitution Effects on Digitoxigenin α-L-Glycoside Cancer Cytotoxicity

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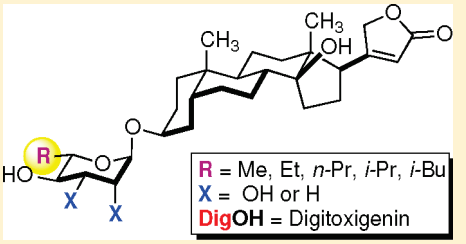
Supporting Information

ABSTRACT: A highly regio- and stereoselective asymmetric synthesis of various C5′-alkyl side chains of rhamnosyl- and amicetosyl-digitoxigenin analogues has been established via palladium-catalyzed glycosylation with postglycosylated dihydroxylation or diimide reduction. The C5′-methyl group in both α-L-rhamnose and α-L-amicetose digitoxin analogues displayed a steric directed apoptosis induction and tumor growth inhibition against nonsmall cell human lung cancer cells (NCI-H460). The antitumor activity is significantly reduced when the steric hindrance is increased at the C5′-stereocenter.



KEYWORDS: Digitoxin, rhamnose, amicetose, apoptosis, cancer, NCI-H460

For centuries, cardiac glycosides have been used for the treatment of congestive heart failure. The mechanism leading to this cardiotonic activity is the result of plasma membrane Na^+/K^+ ATPase inhibition.[1,2] The accumulated intracellular Na^+ concentration increases Ca^{2+} sequestration by the sarcoplasmic reticulum and enhances myocardial cell contractility via excitation−contraction coupling.[3] Digitoxin (**3**; Figure 1), a commonly used cardiac glycoside originally isolated from *Digitalis purpurea*, has been recognized for its positive inotropic activity, and more recently for its potential application in oncology.[4,5]

The cause of digitoxin cancer cell cytotoxicity has inevitably been associated with Na^+/K^+ ATPase inhibition and the resultant change in Ca^{2+} ion concentration. However, many research groups have found digitoxin induces apoptotic cell death at a subcardiotoxic concentration in plasma.[6−10] These findings suggest the possible role of digitoxin binding to other biological targets as an apoptotic inducing event. In fact, direct evidence correlating Na^+/K^+ ATPase inhibition anticancer effects is still lacking.

Digitoxin consists of digitoxigenin (pharmacophore) and the trisaccharide moiety, which are known to play a crucial role in both its cardiotoxicity and anticancer activity.[11] Because digitoxin's cardiotonic activity has been known for a much longer time, most structure activity relationship (SAR) studies have focused on the Na^+/K^+ ATPase inhibition and examined the effect of modifying the carbohydrate portion of the cardiac glycosides.[12−15] More recently, Karlish demonstrated that inhibition of the α2-isoform over the α1-Na^+/K^+ ATPase isoform could induce cardiac contraction with minimal Ca^{2+} overload and less cardiotoxicity.[16,17] These studies also suggest that an α2-selective cardiac glycoside could result from sugar modification, as the structural differences in these isoforms lie primarily in the extracellular carbohydrate binding loops. Thus, sugar modification could provide the potential for the discovery of new and safer digitoxin analogues with improved anticancer activity.

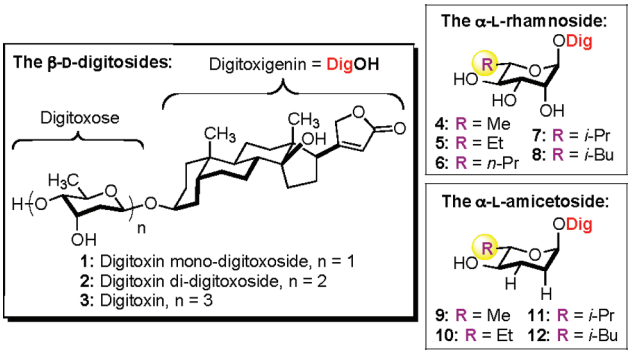


Figure 1. Digitoxin (**3**) and related carbohydrate analogues.

The first anticancer SAR study of digitoxin was conducted by Thorson in 2005, when they screened a neoglycoside library of digitoxin monosaccharide. Several β-L-sugar analogues were found to have improved cytotoxicity over digitoxin.[18] More recently, we have had success in performing a series of systematic SAR studies on the carbohydrate portion of digitoxin for apoptotic cytotoxicity in the human lung cancer (NCI-H460) cell line. These studies include a comparison of digitoxin *MeNO*-neo- and

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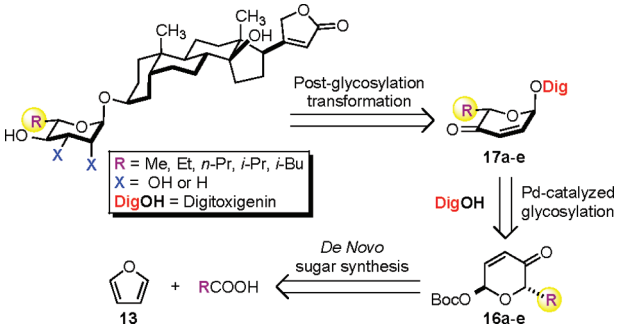
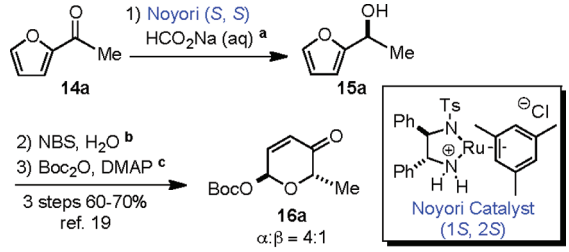
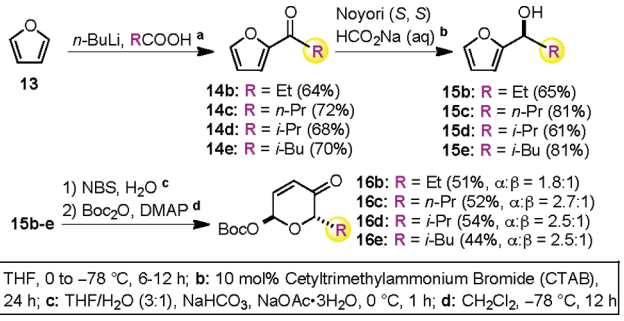
Scheme 1. Retrosynthetic Analysis of Digitoxin Analogues



Scheme 2. *De Novo* Approach to α-L-Boc-pyranone 16a



Scheme 3. *De Novo* Approach to the Pyranone Building Blocks 16b−e



O-glycosides (with *O*- better than *MeNO*-neo-glycosides),[8] a study of sugar-chain length (with mono- better than di- or trisaccharides),[10] and a stereochemical survey of digitoxin monosaccharides (with β-D-*digitoxo*-, α-L-*amiceto*-, and α-L-*rhamno*-glycosides being the most active).[9] From these studies, we concluded that *O*-glycosides were better than *MeNO*-neoglycosides[8] and that sugars with β-D-*digitoxo*-, α-L-*amiceto*-, and α-L-*rhamno*-stereochemistry were the most active.[9] Regardless of the glycosidic linkage (*O*-/neo- and/or α-/β-), the sugar stereochemistry of the monosaccharides was more effective than those of di- and trisaccharides.[10]

It is worth noting that these observations were stereospecific (i.e., α-D-*rhamno*- and α-D-*amiceto*-glycosides were significantly less active than their α-L-sugar diastereomers) and, thus, not attributable to nonspecific solubility and/or membrane transport properties. To further probe the specificity of the sugar portion of the α-L-digitoxin glycosides, we decided to test the SAR-importance of the C5′-alkyl position. Herein, we report our successful synthesis and cytotoxicity study of four digitoxigenin α-L-*rhamno*-glycosides (**5**−**8**) and three digitoxigenin α-L-*amiceto*-glycosides (**10**−**12**) using our *de novo* asymmetric approach to glycoside assembly (Figure 1).

Outlined in Scheme 1 is our retrosynthetic analysis for the preparation of various C5′-alkyl substituted digitoxin analogues. We envisioned that the desired *rhamno*- and *amiceto*-stereochemistries and functionalities could be installed by a diastereoselective ketone reduction and a dihydroxylation, or alternatively a diimide reduction, of the digitoxin pyranone precursors **17a**−**e**. These structurally diverse pyranones, in turn, could be prepared by a stereospecific palladium-catalyzed glycosylation to couple digitoxigenin (**DigOH**) with the corresponding α-L-Boc pyranones **16a**−**e**. The preparation of these desired glycosyl donors could be simply amended by the use of our *de novo* asymmetric carbohydrate synthesis from an achiral furan starting material with different α-substituted alkyl carboxylic acids.

As we have previously described, the synthesis of C6′-deoxy-α-L-Boc-pyranone **16a** was successfully achieved by a practical three-step sequence from acetylfuran **14a** (Scheme 2).[19] The absolute L-sugar stereochemistry was installed via an enantioselective Noyori asymmetric reduction.[20] The resulting furfural alcohol **15a** (>99% *ee*) was converted to the corresponding pyranone via an Achmatowicz oxidative rearrangement and a stereodivergent Boc-protection to yield an easily separable 4 to 1 ratio of α-/β-mixtures of Boc-pyranones in good overall yield (60−70%).[21]

The flexibility of this *de novo* Achmatowicz approach allowed us to further expand the diversity of C5′-alkyl substituted Boc-pyranone sugar building blocks (**16b**−**e**, Scheme 3). The required acetyl furans (**14b**−**e**) with various α-alkyl substitutions (i.e., ethyl, *n*-propyl, isopropyl, and isobutyl) could be prepared by a one-pot, two-step procedure.[22−24] Thus, the addition of furan (**13**) to a hexane solution of *n*-BuLi generated a solution of 2-lithiofuran, to which was added a THF solution of the appropriate substituted carboxylic acid (<0.5 equiv) to provide **14b**−**e** in good yields. Exposure of the acylfurans to our preferred Noyori asymmetric reduction phase transfer conditions (Noyori (*S*, *S*), HCO_2Na (aq), 10 mol % CTAB) provided good yields of the furan alcohols **15b**−**e** with excellent enantiomeric purity (>98% *ee*). The furan alcohols **15b**−**e** were then oxidized under the Achmatowicz conditions (NBS/H_2O), and the resulting pyranone intermediates were protected with Boc_2O to give the desired Boc-pyranones **16b**−**e** in good overall yields with modest α-selectivity.

With the desired C5′-alkyl substituted α-L-Boc-pyranone sugar building blocks **16a**−**e** in hand, we next employed them in a palladium-catalyzed glycosylation with digitoxigenin (**DigOH**) to provide α-L-digitoxin pyranones **17a**−**e** (82−98%) as a single diastereomer with complete stereocontrol at the anomeric center (Scheme 4). The desired C4′-hydroxyl group was stereoselectively installed via Luche reduction to give equatorial allylic alcohols **18a**−**e** (77−99%). The resulting C2′−C3′ double bonds in **18a**−**e** were readily dihydroxylated under Upjohn conditions to provide C5′-alkyl substituted rhamnosyl digitoxin analogues **4**−**8** (Condition **A**).[25] Alternatively, the double bonds were reduced to provide C5′-alkyl substituted amicetosyl digitoxin analogues **9**−**12** via a diimide reduction (Condition **B**).[26]

With the desired *rhamno*- and *amiceto*-glycosides in hand, we decided to first evaluate their apoptotic cytotoxicity against

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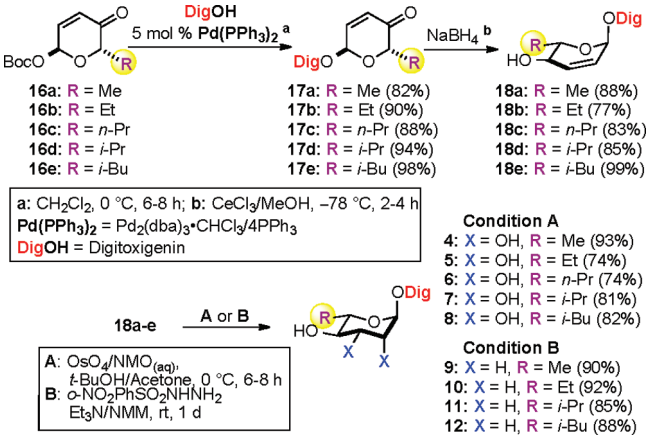
NCI-H460 human lung cancer cells, with α-L-rhamnose (**4**) and α-L-amicetose (**9**) serving as controls. Using our standard conditions, the nine compounds were tested at a single concentration (50 nM, 12 h), with the degree and the mechanism of cell death (apoptosis or necrosis) quantified using the Hoechst 33342 nuclear stain/propidium iodide method (Figure 2).[27] The blue Hoechst nuclear stained cells with condensed and fragmented nuclei are indicative of apoptosis, whereas the completely ruptured cells (indicative of necrosis) were stained red with propidium iodide (Figure 2C/D). While all the digitoxin analogues **4**−**12** displayed varying degrees of cytotoxicity, the C5′-Me analogues *rhamno*-**4** and *amiceto*-**9** were the most cytotoxic (Figure 2A and B). Regardless of potency, apoptosis was the predominant mechanism of cell death for all the analogues. Interestingly, the activity trend of apoptosis significantly decreased when the size of the C5′-alkyl substituent increased.

The cytotoxicity (apoptosis and necrosis) was further evaluated in a 12 h treatment of increasing concentration (10 nM to 10 μM) of drugs. These results were plotted in Figure 3, which showed that all the analogues induced apoptotic cell death in a dose-dependent manner. The degree of cytotoxicity resulting in α-L-*rhamno*-C5′-Me (**4**) and α-L-*amiceto*-C5′-Me (**9**) concur with the literature data from the previous study, where both analogues **4** and **9** showed at least 4-fold stronger potency than natural digitoxin.[9] The IC_{50} values were determined by a nonlinear regression analysis and recorded in Table 1. As in the single dose experiments, the IC_{50} values increased commensurately with the size of the C5′-substituent. This trend could be seen in both the rhamnosides (**4**−**8**, Figure 3A) and amicetosides (**9**−**12**, Figure 3B). Although demonstrably different, the potency of the C5′-Et-*rhamno*-analogue (**5**; IC_{50} 57 nM) was only slightly weaker than that of the C5′-Me-*rhamno*-analogue (**4**; IC_{50} 52 nM). The sensitivity to this negative steric effect greatly increased as subsequent methylene groups were added (e.g., C5′-*n*-Pr (**6**; IC_{50} 116 nM), C5′-*i*-Pr (**7**; IC_{50} 130 nM), and C5′-*i*-Bu

Scheme 4. Systematic Approach to α-L-Digitoxin C5′-Alkyl Analogues (4−12)



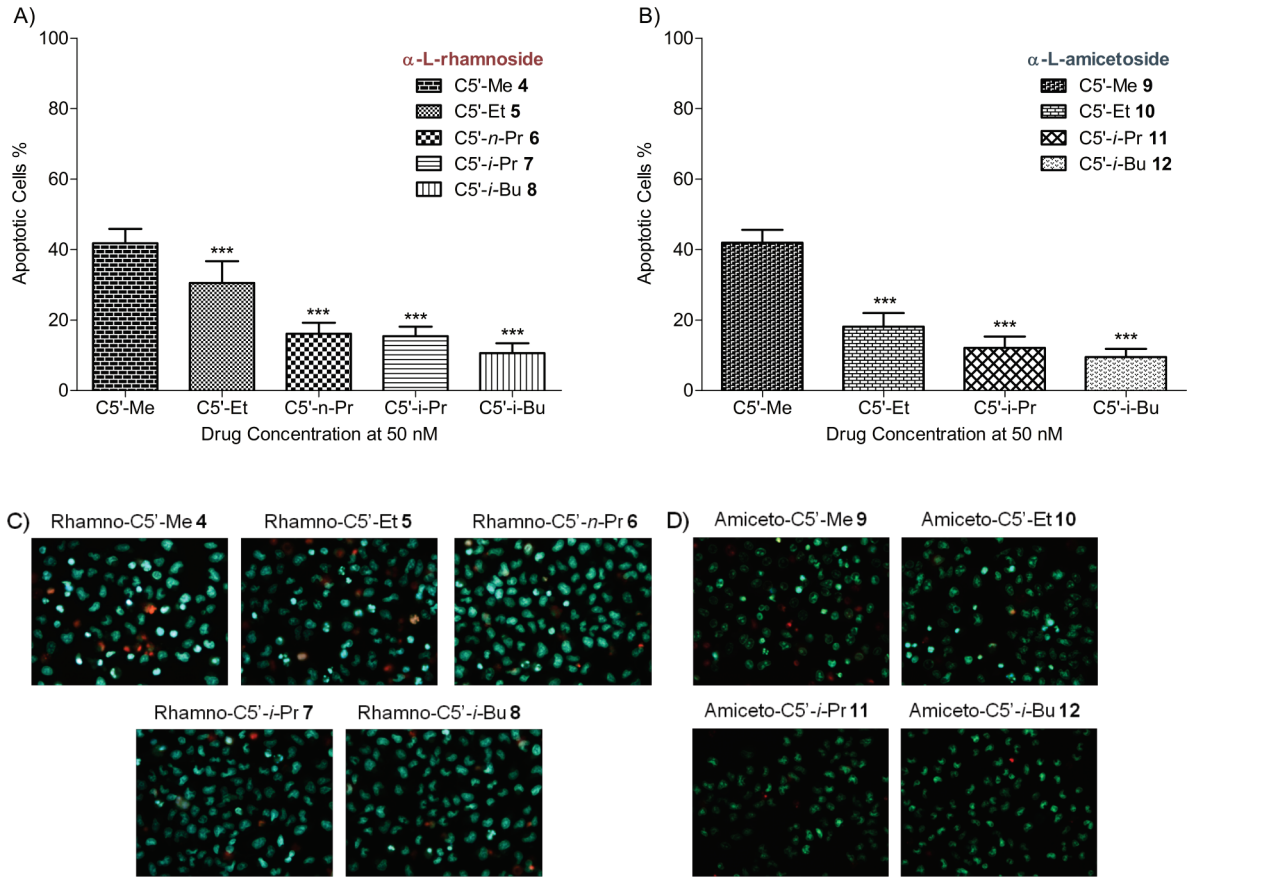


Figure 2. Apoptotic cell death and modification of C5′ bulk. (A and B) Apoptotic cell death (%) was compared for each C5′-alkyl substituted digitoxin rhamnoside and amicetoside at 50 nM concentration (one-way ANOVA; $N = 6$; ***, $P < 0.001$). (C and D) Hoechst 33342 stained apoptotic cells appear in blue and propidium iodide stained necrotic cells in red at 50 nM drug concentration.

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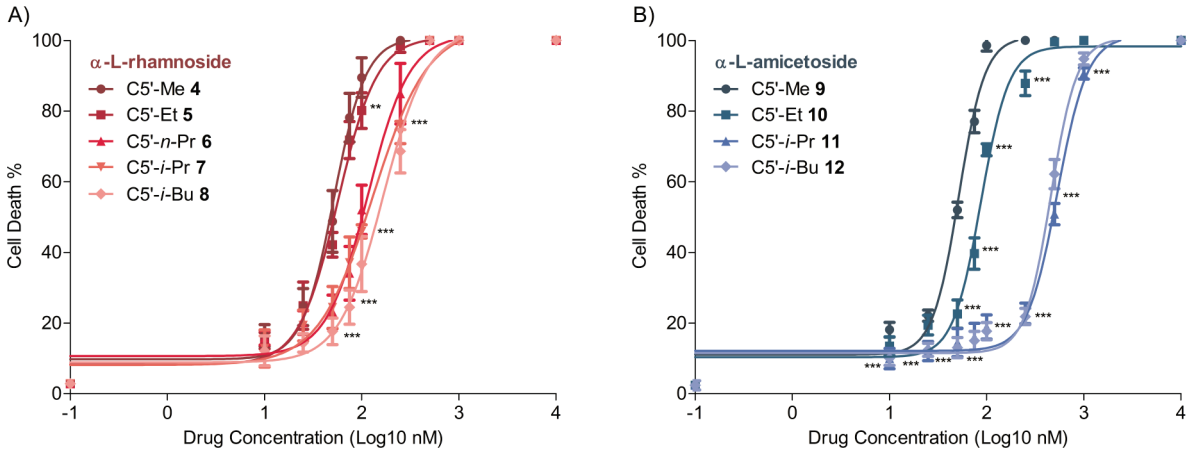

Figure 3. Cytotoxicity as a function of drug concentration in the comparison of C5′-alkyl substitution. Dose response curve of total cell death (apoptosis/necrosis) mediated by digitoxin analogues in a 12 h treatment at increasing concentrations (10 nM to 10 μM). All the data were analyzed by two-way ANOVA ($N = 6$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

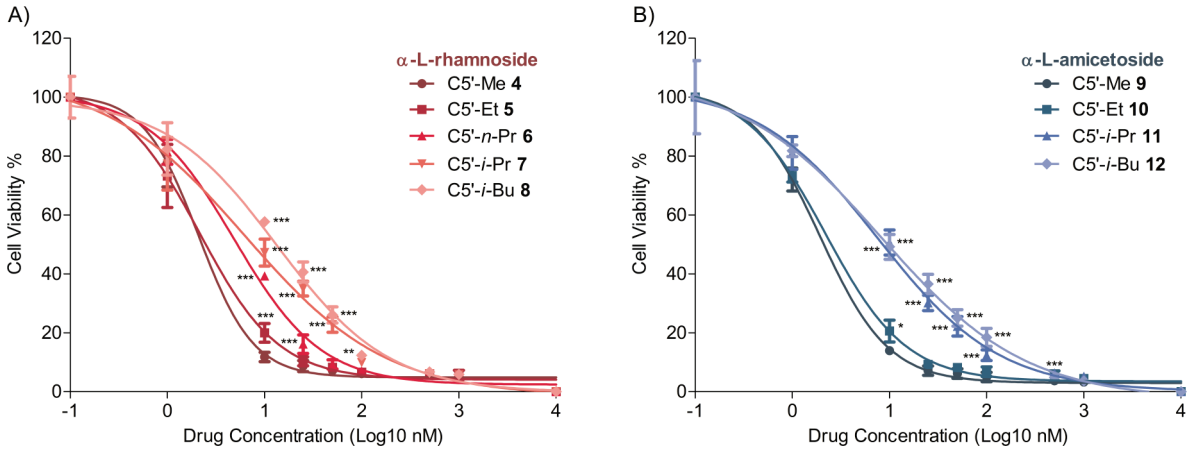

Figure 4. Cell viability as a function of drug concentration in the comparison of C5′-alkyl substitution. The dose response curve of cell viability in a 48 h treatment at increasing concentrations (1 nM to 10 μM). All the data were analyzed by two-way ANOVA ($N = 9$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(**8**; IC_{50} 180 nM)). Interestingly, this effect of the C5′-alkyl substitution appeared to be even more significant on the *amiceto*-series (**9**−**12**), where there was a more significant increase in the IC_{50} values with the addition of methylene groups (cf., C5′-Me (**9**; IC_{50} 52 nM), C5′-Et (**10**; IC_{50} 87 nM), C5′-*i*-Pr (**11**; IC_{50} 533 nM), and C5′-*i*-Bu (**12**; IC_{50} 458 nM)). It is worth noting that α-L-*amiceto*-C5′-Me (**9**) has shown consistently stronger potency in growth inhibition against the NCI-panel of 60 human cancer cell lines than *amiceto*-C5′-*i*-Pr (**11**) and C5′-*i*-Bu (**12**) (see Supporting Information).

To demonstrate that this steric-demand trend was not specific to apoptosis, we decided to measure the cytotoxicity of these digitoxin analogues with a different assay. We chose to use the MTT assay for this comparison, which evaluated the cell viability based on the measurement of mitochondrial enzymatic activity after 48 h of exposure.[28,27] These results (Table 1 and Figure 4) showed a dose-dependent cytotoxic activity for each analogue that was similar to that of the apoptosis assay. As seen earlier, a commensurate drop in cytotoxicity was observed with the increasing size of the C5′-substituent. This trend was consistent across both rhamnoside (Figure 4A) and amicetoside (Figure 4B) series of digitoxin analogues. It should be noted, however, that the greater steric sensitivity of the *amiceto*-series in the apoptosis assay was not observed in the MTT assay.

Table 1. Cytotoxicity Evaluation of Digitoxin Analogues on NCI-H460 Human Lung Cancer Epithelial Cells

compd	IC_{50}[a]	GI_{50}[b]	compd	IC_{50}[a]	GI_{50}[b]
α-L-rhamnoside			α-L-amicetoside		
C5′-Me-**4**	52 ± 1	2 ± 1	C5′-Me-**9**	52 ± 1	2 ± 1
C5′-Et-**5**	57 ± 1	2 ± 1	C5′-Et-**10**	87 ± 1	2 ± 1
C5′-*n*-Pr-**6**	116 ± 1	5 ± 1	C5′-*i*-Pr-**11**	533 ± 1	8 ± 1
C5′-*i*-Pr-**7**	130 ± 1	6 ± 1	C5′-*i*-Bu-**12**	458 ± 1	8 ± 1
C5′-*i*-Bu-**8**	180 ± 1	13 ± 1			

[a] The IC_{50} value was measured by a 12 h treatment in Hoechst and propidium iodide stain assays. [b] The GI_{50} value was measured by a 48 h treatment in an MTT assay. All values represent the standard error of the mean value of three independent experiments with duplicate determinations.

In summary, we have demonstrated the SAR-effects of C5′-alkyl substitution on digitoxin α-L-rhamnoside (**4**) and α-L-amicetoside (**9**). Our results suggest that both the *rhamno*- and *amiceto*-analogues occupy a similar binding site and orientation in its target

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with a small hydrophobic pocket for the C5′-Me. While these cytotoxicity trends are not inconsistent with the models for Na^+/K^+ ATPase inhibition, further study is needed. The efficiency with which this systematic SAR-study was conducted was enabled by the flexibility of this unique *de novo* approach to carbohydrate synthesis. Further efforts aimed at optimizing cytotoxicity and elucidation of mechanism are ongoing and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information. Assay protocols, statistical analysis data, synthetic procedures, characterization data, and NMR spectra. This information is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All the biological experimental work was performed by H.-Y.L.W. Analogues synthesis was primarily carried out by H.-Y.L.W., with significant contributions being made by B.W., Q.Z., and S.-W.K. The experimental design, data analysis and manuscript preparation was performed by all the authors.

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