Selective (α)-L-Rhamnosylation and Neuroprotective Activity Exploration of Cardiotonic Steroids

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ABSTRACT: This work describes the studies on the direct C3-glycosylation of the C19-hydroxylated cardiotonic steroids strophanthidol, *anhydro*-ouabagenin and ouabagenin using a strategy based on *in situ* protection of the C5 and C19 hydroxyl groups with boronic acids. While this strategy resulted in a successful one-pot C3-selective glycosylation of strophanthidol and *anhydro*-ouabagenin, it failed to provide ouabain from ouabagenin. The neuroprotective activity of the synthetic and natural glycosides against LPS-induced neuroinflammation was explored in neonatal mice primary glia cells. Co-administration of natural and synthetic C3-glycosides at 200 nM concentrations resulted in the significant reduction of the LPS-induced neuroinflammatory markers IL-6, IL-1, TNFα, and IKBKE, with the *anhydro*-ouabagenin-3-(α)-*L*-rhamnoside (*anhydro*-ouabain) showing the most significant effect. At the same time, unglycosylated *anhydro*-ouabagenin enhanced rather than suppressed LPS-induced neuroinflammation.

Keywords: Cardiotonic Steroids, Site-selective glycosylation, Anti-inflammatory activity, Glia cells

Cardenolides represent a broad family of glycosylated steroids found in animals and plants such as oleander, foxglove, lily of the valley, red squill, dogbane. etc.[1,2] For centuries, herbal remedies containing cardenolides as active ingredients have been used to treat various conditions such as edema or "dropsy".[3] Cardenolides are known to serve as inotropic agents and they are known to reversibly inhibit the Na,K-ATPase pump, which increases intracellular sodium and decreases intracellular potassium levels, which, in turn, results in intracellular accumulation of calcium. Due to this effect, cardiotonic steroids digoxin and digitoxin have been essential therapeutics for the treatment of heart failure and arrhythmia.[2,4–7] However, the biological activity of cardenolides extends well beyond their cardiotonic activity. Among numerous other applications, cardiotonic steroids have been explored as potential therapeutic agents for the treatment of cancer,[8–10] viral infections,[11,12] neurodegenerative diseases,[13,14] stroke,[15–17] and as anti-inflammatory,[12,18,19] and senolytic[20] agents. Particularly interesting are the recent reports indicating that cardiotonic steroids such as ouabain and digoxin may influence the peripheral and central nervous systems. Ouabain is an endogenous cardiotonic steroid produced by mammalians in subnanomolar concentrations by the adrenal gland, hypothalamus, and pituitary.[21–24] Several recent *in vivo* studies demonstrated ouabain has an anti-inflammatory effect in the rat hippocampus and that ouabain demonstrated neuroprotection against liposaccharide (LPS) induced inflammation.[25–28] This neuroprotective effect was attributed to the ability of ouabain to promote membrane lipid remodeling and increase the expression of glutamate transporter EAAT4, which helps to mitigate the oxidative stress induced by LPS. This effect is not limited to ouabain as cardiotonic steroids oleandrin and digoxin demonstrated neuroprotection for oxygen and glucose deprivation in ischemia models.[15–17]

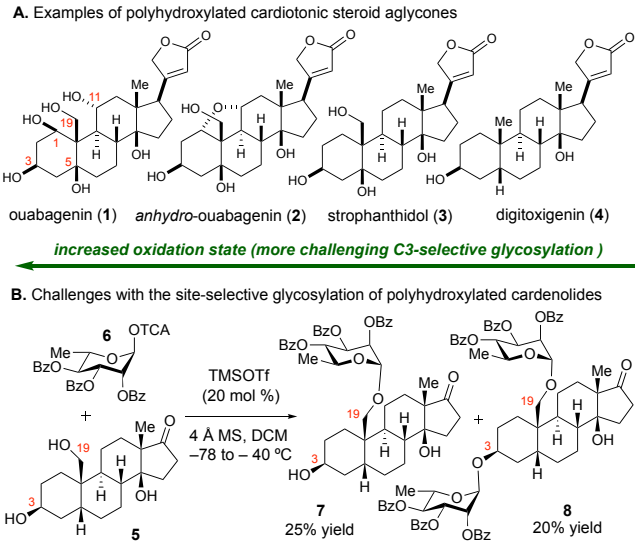


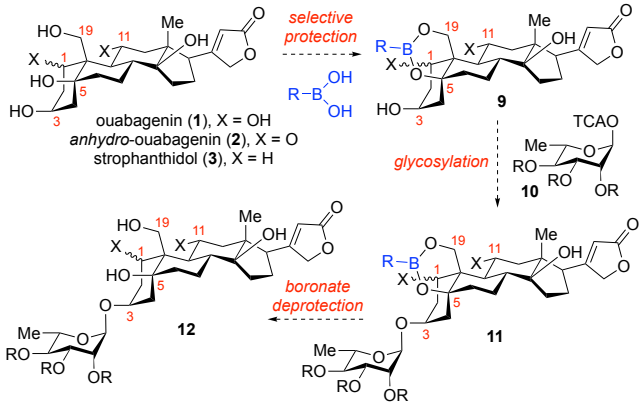
Figure 1. Polyhydroxylated cardiotonic steroid aglycones and challenges with site-selective glycosylation

While these prior examples suggest that cardenolides may exhibit neuroprotective properties, the specific structural features that are essential for these effects are unknown. It has been observed that both the carbohydrate structure and skeletal oxidation (Figure 1A) may impact the biological activity of cardenolides; however, no comparative study evaluating neuroprotective properties of cardenolides across a group of cardiotonic steroids with different oxidation patterns and glycosylation sites has been carried to date. This manuscript describes selective (α)-*L*-rhamnosylation and subsequent exploration of C19-hydroxylated cardenolides with different oxidation patterns (**1-3**). The potential of the synthetic and natural steroids to reduce LPS-caused neuroinflammation was investigated at 200 nM concentration using primary glia cells derived from neonatal 1-2 days old mice. The effect of steroids was monitored using the gene expression of inflammation markers IL-1, IL-6, TNFα, and IKBKE, and several significantly differing responses were observed. The synthetic and natural 3-(α)-*L*-rhamnosides of **1-3** as well as digitoxigenin were found to sig-

nificantly reduce the LPS-induced inflammation with the synthetic analog *anhydro*-ouabagenin 3-(α)-*L*-rhamnoside (**20**) exhibiting the most significant effect. At the same time, the aglycone of **20**, *anhydro*-ouabagenin (**2**) significantly enhanced the inflammation levels.

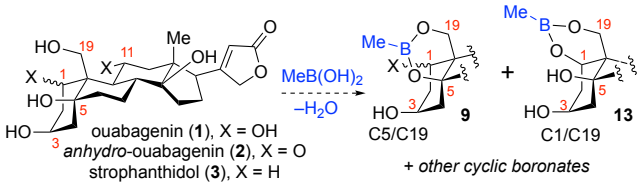
The Nagorny group has long-standing interests in streamlining the medicinal chemistry exploration of cardiotonic steroids by developing new strategies for the aglycone synthesis and their selective glycosylation.[29–32] Previously we described strategies for the installation of native (α)-*L*-rhamnoside moiety at the C3-position of various cardiotonic steroids.[33–35] It was observed that cardiotonic steroids such as **5** containing C3 and C19 oxidation undergo competitive C19 glycosylation that produces a complex mixture of products such as **7** and **8** (*cf.* Figure 1B).[33] While this challenge could be overcome by selective introduction of a C19-protecting group such as methoxyacetate,[33] this may require significant protecting group optimization and might be problematic for the substrates that have additional hydroxyl groups such as **1–3** (Figure 1A).[36–38]

Scheme 1. Traceless boronic acid protecting group for the selective protection of the C19 position of cardiotonic steroids.



In the context of their studies focused on regioselective glycosylation of 6-deoxyerythronolide B,[39] we utilized boronic acid esters as the traceless protecting groups.[40–43] *In situ* protection of the 1,3-diol moiety of 2-deoxyerythronolide B (6-dEB) was followed by the addition of glycosyl trichloroacetimidate and TMSOTf as the promoter to accomplish selective glycosylation. The basic reaction work up was used to remove the boronic acid and produce the desired glycoside as the single regioisomer. This concept could be applied to highly oxidized cardiotonic steroids **1–3** containing 1,3,5-hydroxyl groups (Scheme 1).[34] However, unlike the previous glycosylation developed for 6-dEB, the presence of multiple hydroxyl groups may lead to several regioisomeric protection products in addition to the desired boronic acid

Table 1. Computed relative stabilities of methyl boronic acid esters derived from steroids 1–3 (DFT, B3LYP, 6-31+G*)

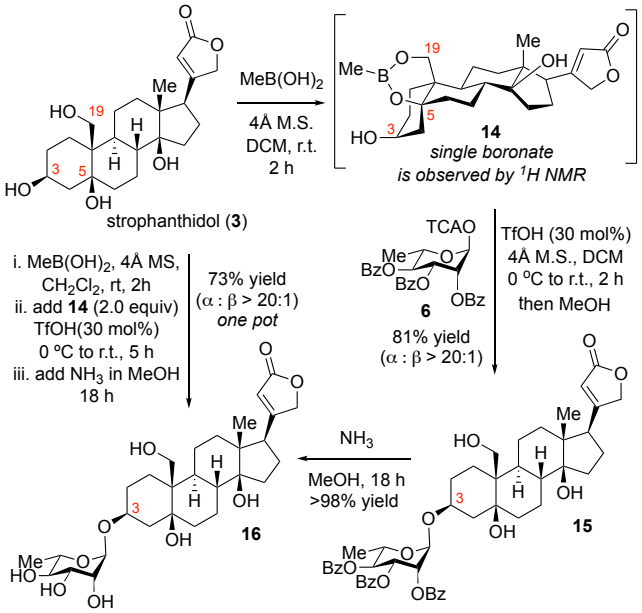


scaffold	realtive energy for the regioisomeric boronates (DFT, B3LYP, 6-31+G*) (kcal/mol)							
	C1/C3	C1/C5	C1/C11	C1/C19	C3/C5	C3/C19	C5/C19	C11/C19
1[a]	+8.3	+8.9	+7.6	0	+7.8	+18.3	+2.3	+7.6
2[b]	–	–	–	–	+1.9	+22.2	0	–
3[c]	–	–	–	–	+1.7	+17.2	0	–

[a]Referenced to the energy of cyclic C3/C5 boronate derived of **1**. [b]Referenced to the energy of cyclic C5/C19 boronate of **2**. [c]Referenced to the energy of cyclic C5/C19 boronate derived from **3**.

ester **9**. This would lead to multiple regioisomeric glycosylation products in addition to **9**. Assuming that boronic acid formation is a reversible process and the most thermodynamically stable boronate is formed under the protection conditions, the regioselectivity of boronic acid ester formation could be assessed computationally. The results of these computations (DFT, B3LYP, 6-31+G*) are summarized in Table 1. Ouabagenin (**1**) contains 5 hydroxyl groups that may participate in boronic acid ester formation and may form 8 potential products. The C1/C19 boronic acid ester **13** was found to have the lowest energy with 2.3 kcal/mol higher stability than the second most stable C5/C19 boronate **9**. Only 3 cyclic boronates are possible in the case of *anhydro*-ouabagenin (**2**) and strophanthidol (**3**). In both cases, the most stable ester is formed by the

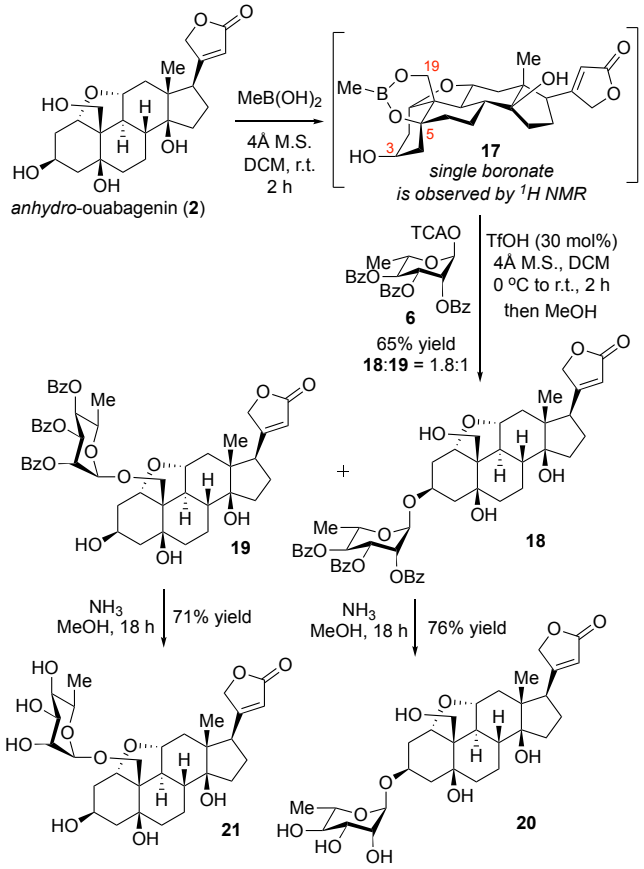
Scheme 2. C3-selective (α)-*L*-rhamnosylation of strophanthidol (3)



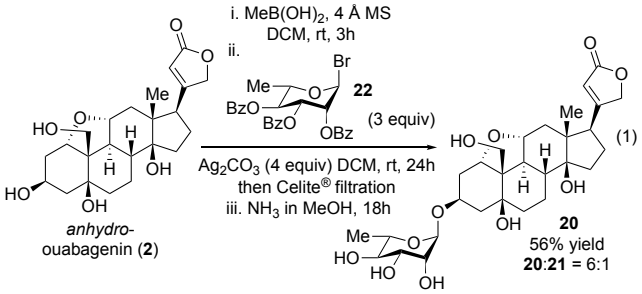
complexation of the C5/C19 sites, and the formation of the second most stable C3/C5 boronate would be disfavored by 1.9 kcal/mol (**2**) or 1.7 kcal/mol (**1**). The results in Table 1 suggest that the reaction of methyl boronic acid with **2** and **3** should lead to clean protection of the C5 and C19 positions and only the C3

alcohols would be available to react. At the same time, the reaction of ouabagenin will result in a selective protection of the C1 and C19 alcohols, and the C3 and C11 alcohols can be potentially glycosylated. Indeed, when strophanthidol (**3**) was treated with methyl boronic acid in the presence of 4Å MS, exclusive formation of boronate **14** was observed by NMR (Scheme 2). The reaction of **14** with trichloroacetimidate **6** required optimization.[34] Under the optimized conditions, that included using 2 equivalents of **6**, activation with triflic acid (30 mol%), 4 Å MS at 0 ºC to r.t. resulted in the efficient formation of the glycosylated product. The subsequent removal of the boronic acid ester with methanol resulted in 81% yield of the desired α-product **15**. The benzoyl protecting groups present on the rhamnose moiety of **15** were selectively removed with saturated solution of ammonia in methanol (18 h) to provide the desired deprotection product in the quantitative yield. This procedure was subsequently optimized to be performed as a one-pot telescoped operation to provide **α-16** directly from **3** in 73% overall yield.

Scheme 3. Initial studies on C3-selective (α)-*L*-rhamnosylation of anhydro-ouabagenin (2)

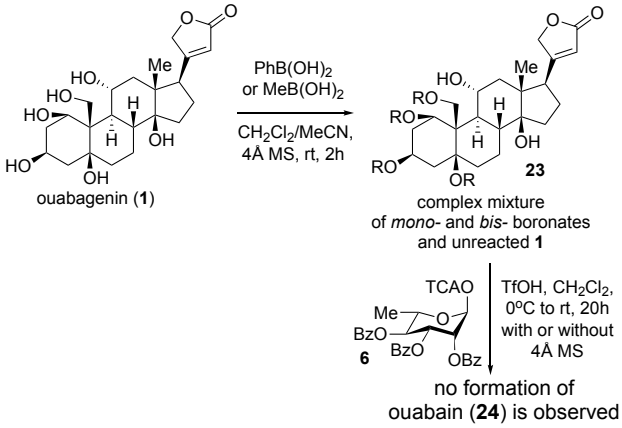


Our subsequent studies focused on investigating the C3-selective (α)-*L*-rhamnosylation of *anhydro*-ouabagenin (**2**) (Scheme 3). *Anhydro*-ouabagenin (**2**) is a known degradation product of ouabain.[44–46] While this compound carries all the major structural features present in ouabagenin (**1**) and strophanthidol (**3**), the glycosylated variants of these compound have not been previously synthesized and evaluated. As in the case of **3**, computational studies (Table 1), predicted selective formation of the C5/C19 boronate. Indeed, upon exposure to methylboronic acid, single boronate **17** was observed by ^{1}H NMR. However, subjecting **17** to reaction with **6** under previously developed conditions resulted in 1.8:1 mixture of the C3- and C19-glycosylated products **18** and **19** in 65% yield. This eroded selectivity could be attributed to *in situ* boronate isomerization under the acidic conditions, which is more favored for the conformationally restricted **2**.



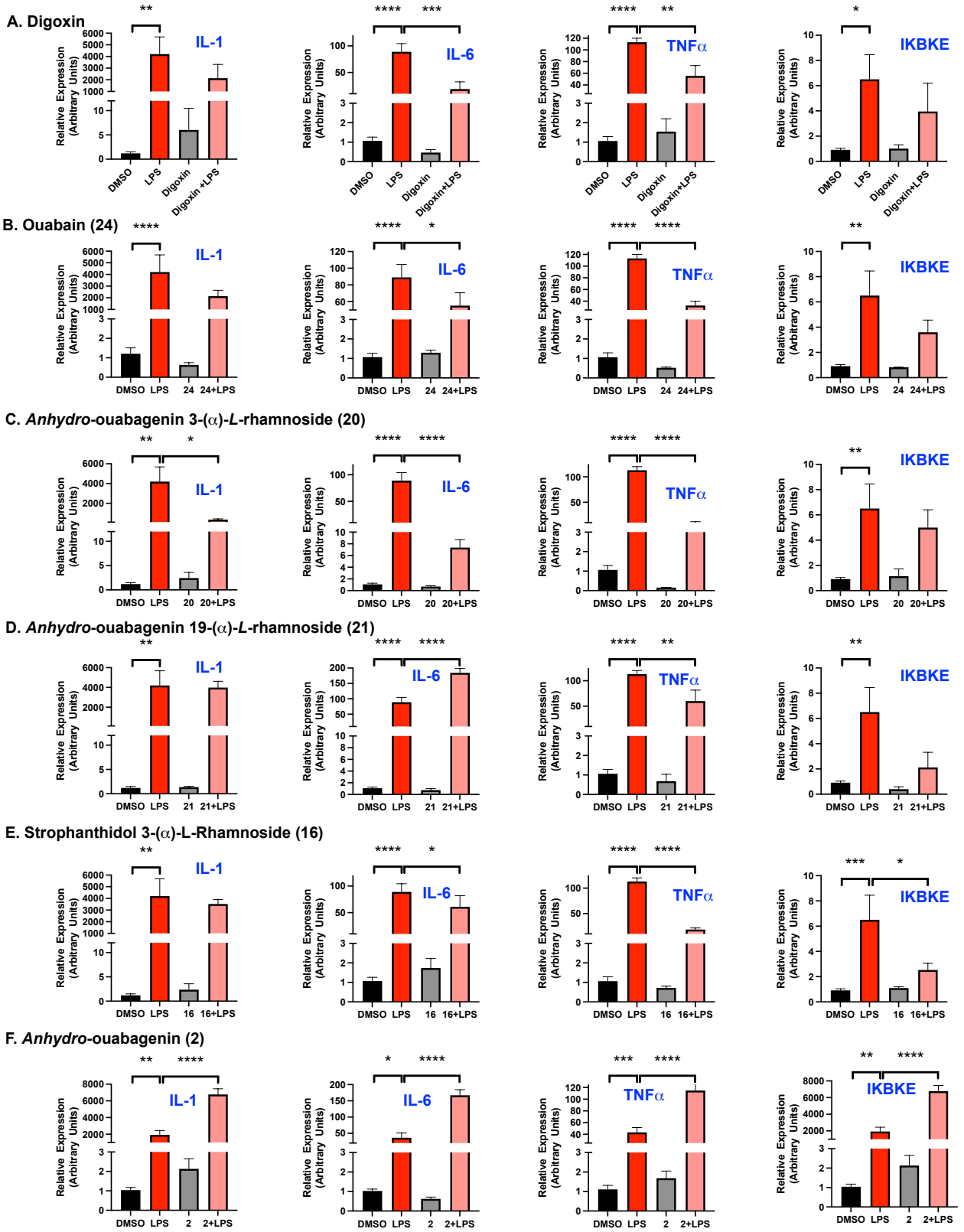
Both **18** and **19** were separated and subsequently deprotected to provide regioisomeric glycosides **20** and **21**. To avoid the isomerization of **17**, a base promoted glycosylation was investigated as a one-pot transformation (Eq. 1). Thus, cyclic boronate **17** was formed *in situ* and the resultant crude reaction mixture was subjected to glycosylation with glycosyl bromide **22** in the presence of silver carbonate. The resultant mixture was filtered through celite to remove excess of Ag(I) salts and treated with ammonia in methanol to provide 6:1 mixture of **20** and **21** in 56% yield.

Scheme 4. Attempts to accomplish the C3-selective glycosylation of ouabagenin (1)



Unlike **2** and **3**, ouabagenin (**1**) is expected to form a C1/C19 boronate (*cf.* Table 1). However, low solubility of **1** in dichloromethane prevented this reaction (Scheme 4). The addition of acetonitrile as a co-solvent seemed to aid the formation of boronic acid esters; however, complexation with either methyl or phenyl boronic acids resulted in the formation of multiple *mono*- and *bis*-protected esters. Subjecting these mixtures to the acid-catalyzed glycosylation with **6** did not lead to the formation of ouabain (**24**), and multiple decomposition products were isolated along with unreacted **1**.

Figure 2. Evaluation of anti-inflammatory activity of 200 nM solutions of (A) Digoxin; (B) ouabain (**1**); (C) *anhydro*-ouabagenin-3-(α)-*L*-rhamnoside (**20**); (D) *anhydro*-ouabagenin-19-(α)-*L*-rhamnoside (**21**); (E) strophanthidol-3-(α)-*L*-rhamnoside (**16**); and (F) *anhydro*-ouabagenin (**2**) during 24 hours in the primary glial cell culture. The gene expression of IL-1, IL-6, TNFα, and IKBKE was stimulated using LPS (250 ng/ml), and DMSO was used as a negative control. Data were expressed as the mean ± SEM and analyzed by two-way ANOVA followed by Newman-Keuls post hoc analysis. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).



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With the access to the synthetic aglycones **2** and **3**, and their (α)-*L*-rhamnosides **16**, **20** and **21**, our subsequent studies focused on exploring neuroprotective properties of these derivatives along with natural cardenolides digoxin and ouabain (Figure 2).

A primary glial cell culture extracted from the whole brain was used to assess the potential neuroprotective properties of cardiotonic steroids. The whole brain from 1-2 day-old C57BL/6J mice neonatal mice was collected, without the meninges, placed in HBSS modified medium without calcium or magnesium, and processed following the standard protocol to separate the oligodendrocytes and neurons from the glial cell culture.[47] The primary glial cells were treated with 200 nM solution of cardiotonic steroids and stimulated with LPS (250 ng/mL), to induce inflammatory cytokines gene expression. DMSO was used as a negative control. The *IL-1, IL-6, TNF-α, and IKBKE* gene expression were determined using qPCR.

The findings from these experiments are presented in Figure 2. In all cases, LPS stimulation triggered substantial increase in the expression of the inflammatory IL-1, IL-6, TNF-α, and IKBKE genes relative to the control treatment with DMSO. In contrast, applying 200 nM solutions of digoxin, and compounds **2**, **16**, **20**, **21**, and **24** did not trigger significant inflammatory gene expression. When cells were sequentially treated with LPS and then with 200 nM solutions of **16**, **24, 20** and digoxin, a significant reduction in the expression of all four inflammation genes was observed. The unnatural ouabain analog **20** demonstrated the strongest *anti*-inflammatory activity. In contrast, the use of its isomer, C19-glycoside **21**, enhanced the expression of IL-6, but reduced the production of TNFα as well as IKBKE markers. Finally, aglycone **2** enhanced the inflammation relative to the LPS treatment alone, and all four genes were expressed at significantly higher levels.

The results above suggest that both the glycosylation and skeletal substitution are important to the *anti*-inflammatory activity of cardenolides. The C3-glycosylation was found to have the most significant impact as the unglycosylated *anhydro*-ouabagenin (**2**) enhanced rather than reduced the levels of neuroinflammation. Similarly, altering the glycosylation site from the C3- to C19-position resulted in reduced activity of **21** in comparison to **20**. The side-by-side comparison of cardenolides **16**, **20** and **24** containing 3-*L*-(α)-rhamnosylation indicates that changes in skeletal substitution may lead to the enhancement of the *anti*-inflammatory activity. The unnatural analog *anhydro*-ouabain (**20**) represents a hybrid of **16** and **24** featuring the C1/C11 ether bridge that locks the conformation of otherwise flexible *cis*-AB rings. The direct comparison of **16**, *anhydro*-ouabain (**20**), and ouabain (**24**) indicates that **20** has the highest levels of *anti*-inflammatory activity across all four inflammatory genes.

In summary, this manuscript describes a direct strategy for achieving C3-(α)-L-rhamnosylation of common polyhydroxylated cardenolide aglycones **1–3** containing a primary C19-hydroxyl group. Based on the computational predictions suggesting that the reaction of **1–3** with boronic acids would favor the formation of the cyclic C5/C19 or C1/C19 esters, we have developed one pot glycosylation protocols that directly lead to strophanthidol-3-(α)-*L*-rhamnoside (**16**), and unnatural steroid *anhydro*-ouabagenin-3-(α)-*L*-rhamnoside (**20**). Despite the promising computational predictions, the attempts to achieve selective glycosylation of ouabagenin (**1**) failed due to its low solubility in organic solvents coupled with the propensity to form *bis*-boronates.

The potential of 200 nM solutions of synthetic C3-(α)-*L*-rhamnosides **16** and **20**, C19-(α)-*L*-rhamnoside **21**, *anhydro*-ouabagenin (**2**), ouabain (**24**) and digoxin to reduce LPS-induced neuroinflammation was subsequently investigated using primary murine glial cells. While the C3-glycosylated steroids reduced the expression of inflammatory IL-1, IL-6, TNFα and IKBKE, the co-administration of unnatural C19-glycoside **21** or aglycone **2** resulted in enhanced inflammation. In addition to glycosylation, the oxidation of the cardenolide skeleton was found to play a significant role in determining the extent of the *anti*-inflammatory activity exhibited by the steroids. Surprisingly, the previously unexplored unnatural *anhydro*-ouabagenin-3-(α)-*L*-rhamnoside (**20**) was found to exhibit the highest levels of *anti*-inflammatory activity. Further exploration of *anhydro*-ouabagenin glycosides and related synthetic derivatives of ouabain may lead to the analogs with enhanced *anti*-inflammatory properties and is currently the subject of ongoing studies in our laboratories.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information that includes the experimental procedures and ^{1}H and ^{13}C NMR spectra for **2**, **16**, **20** and **21** as well as detailed description of the biological studies is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

IL-1 refers to interleukin-1 cytokine; IL-6 refers to interleukin-6 cytokine; TNF-α refers to tumor necrosis factor alpha cytokine; IKBKE refers to the inhibitor of nuclear kappa B kinase subunit epsilon.

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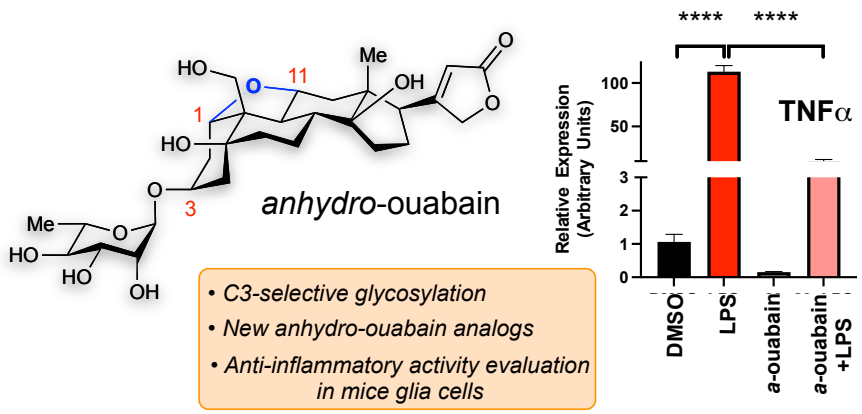
TOC Graphic



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