

Article

Regioselective Esterification of Cardiac Glycosides Catalyzed by Novozym 435 and Lipase PS in Organic Solvents

Ivan Bassanini ¹, Lucia Roncaglia ¹, Bruno Danieli ² and Sergio Riva ^{1,*}

¹ Istituto di Scienze e Tecnologie Chimiche “Giulio Natta” –SCITEC, Consiglio Nazionale delle Ricerche, via Mario Bianco 9, 20131 Milano, Italy; ivan.bassanini@cnr.it (I.B.)

² Dipartimento di Chimica, Università degli Studi di Milano, via Camillo Golgi 19, 20131 Milano, Italy

* Correspondence: sergio.riva@scitec.cnr.it; Tel.: +39-02-28500-024

Abstract: The enzymatic acetylation in the organic solvents of a number of the important bioactive cardiac glycosides was investigated. With the bufanolide proscillaridin A and the cardenolide lanatoside C, acylation, as expected, occurred at the secondary 4'-OH of the rhamnopyranosyl unit of the former (by the action of Novozym 435 lipase) and the primary 6'''-OH of the terminal glucopyranosyl unit of the latter (best results obtained by the action of the lipase PS). Only lipase PS was found to be able to acylate the cardenolides digitoxin and digoxin at the 4'''-OH of their terminal digitoxose unit. The corresponding monoacetyl derivatives, both of which are commercialized drugs, could be isolated with good yields. The investigation of the Novozym 435-catalyzed acetylation of free *D*-digitoxose provided a possible explanation for the inability of this lipase to acylate digitoxin and digoxin.

Keywords: cardiac glycosides; Novozym 435; Lipase ps; biocatalysis; regioselective esterification; organic solvents; *Digitalis* sp.

Citation: Bassanini, I.; Roncaglia, L.; Danieli, B.; Riva, S. Regioselective Esterification of Cardiac Glycosides Catalyzed by Novozym 435 and Lipase PS in Organic Solvents. *Catalysts* **2023**, *13*, 819. <https://doi.org/10.3390/catal13050819>

Academic Editor:
Roberto Fernandez-Lafuente

Received: 31 March 2023

Revised: 26 April 2023

Accepted: 27 April 2023

Published: 28 April 2023



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1. Introduction

Among pharmacologically active natural products, cardiotoxic glycosides (Figure 1) are of prominent interest due to their peculiar and diverse biological activities. Historically, they have been investigated for their ability to improve cardiac contractility in the treatment of congestive heart failure [1–5]. A particular focus was dedicated to their inhibitory activity against Na⁺/K⁺-ATPase, a cell-membrane-located enzyme that plays a key role in the active transport of monovalent cations across membranes [6–8]. More recently, the biological profile of this family of molecules has become attractive since the discovery of their ability to act as modulators of neuroinflammation and as anticancer agents [9–12].

Cardiac glycosides are isolated from many plant sources belonging to different families and are structurally characterized by the presence of a steroidal genine having a *cis* A/B junction and carrying a lactone ring at the 17β-position and a sugar chain at position 3β. When the lactone ring is a butanolide, they are called cardenolides, while if an α-pyrone ring is present, they are called bufadienolides. The saccharide chain can contain up to four monosaccharides belonging to the *D*- and *L*-series, joined either through 1–4 or 1–6 glycosidic linkages [13].

The prototypes of cardiotoxic glycosides (Figure 1) are digitoxin (1), isolated from *Digitalis purpurea* and made up of the genine digitoxigenine and a trisaccharide containing three molecules of the deoxysugar *D*-digitoxose, and digoxin (2), isolated from *Digitalis lanata*, made up of the same trisaccharide and the genine digixigenine. They are both used routinely for congestive heart failure and in the treatment of cardiac arrhythmias, in particular atrial fibrillation, under strictly controlled dosage because the therapeutic dose is very close to the toxic dose.

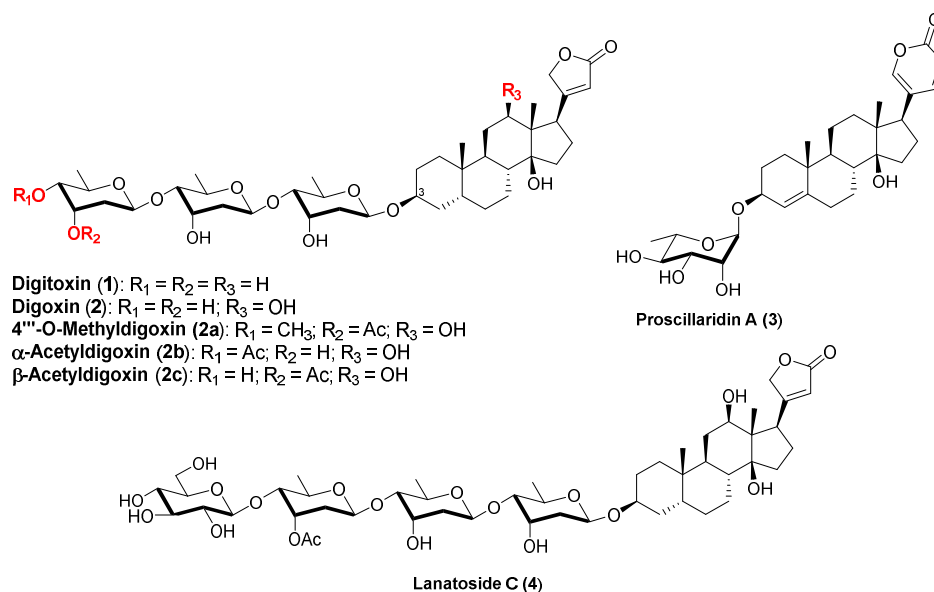


Figure 1. Chemical structures of the cardiac glycosides **1**, **2**, **3**, and **4** and their methylated (**2a**) and acetylated derivatives (**2b**—trade name: Novodigal and **2c**—trade names: Dioxanin, Lanatilin, and Sandolanid; both commercially available oral drugs).

The highly valuable pharmacological activity of these compounds depends on the aglycone moiety, but it is significantly modulated by the nature of the sugar chain. Therefore, with the aim of improving and modifying the therapeutic action and bioavailability compared to the parent compounds, extensive research has been conducted, mainly by pharmaceutical companies, to modify the sugar chain, as shown, i.e., by a long list of patents. For example, with digoxin, worthy of mention is the chemical methylation to give the 4'''-O-methyl derivative (**2a**) or acetylation to furnish the 3'''- and 4'''-O-acetyl derivatives (**2b**) and (**2c**), respectively, used as drugs with improved oral absorption for the treatment of heart failure.

The exploitation of biocatalytic approaches has also been reported. For instance, in the early 1980s, Satoh and colleagues described the use of β -galactosidases for the one-step selective glycosylation of the aglycone digitoxigenin [14,15]. More recently and more efficiently, the glycosylation of the same aglycone has been obtained via sequential glycosylation using a steroid glycosyltransferase UGT74AN3 from *Catharanthus roseus* and a cyclodextrin glycosyltransferase from *Bacillus licheniformis* [16].

More recently, organocatalytic protocols have been proposed for the selective acylation of structurally complex and fragile natural products [17], specifically polyols and carbohydrates [18,19]. For instance, Kawabata and colleagues reported the regioselective acylation of digitoxin (**1**) with different esters at its 4'''-OH with a monoacylation yield of up to 98% [20].

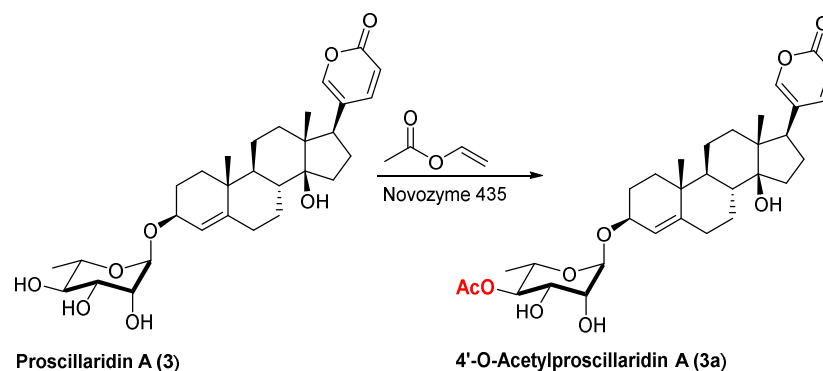
The esterification of OH's group under the catalysis of hydrolytic enzymes has proved to be a mild and selective methodology for the preparation of the specific derivatives of saccharides. We contributed to the development of this methodology by preparing the selected acyl derivatives of a large number of naturally occurring glycosides, ranging from flavonoid glycosides [21–23], colchicoside and thiocolchicoside [24], ginsenosides [25], stevioside [26], and others.

Cardiac glycosides appeared as attractive substrates to extend the application of this methodology to the substrates of pharmaceutical value and to highlight the importance of natural catalysts for their mild and selective derivatization. Here, we report the results obtained, in terms of product characterization and regioselectivity determination, in the enzymatic acetylation of digitoxin and digoxin (**1** and **2**) and of proscillaridin A and

lanatoside C (**3** and **4**), other examples of this important family of bioactive natural products.

2. Results and Discussion

Our investigation started with Proscillaridin A (**3**), a cardiac glycoside belonging to the bufenolide family that can be isolated from plants of the genus *Scilla*, such as *Urginea* (or *Drimia*) *Maritima* [27–30], and that, besides its cardiotoxic effects, possesses promising anticancer activity [31,32]. The sugar moiety of this compound, one of the most potent cardiac glycosides [33], is the simple monosaccharide L-rhamnose. From previous investigations [34,35], it was known that the lipase B from *Candida antarctica* (and specifically its immobilized form Novozym 435) is able to regioselectively acylate the hydroxyl group at position C-4 of a terminal α -rhamopyranoside unit. In addition, this was also the case with compound **3**, once dissolved in vinyl acetate, acting both as solvent and acylating agent, and stirred at 45 °C for 24 h in the presence of this immobilized lipase. (Reaction conditions and enzyme dosage (5 mg/mL) were based on our previous investigations.) [34,35]. The less polar product formed was purified by flash chromatography on silica gel (ca 50% isolated yield) and characterized as 4'-O-acetylproscillaridin A (**3a**, Scheme 1). Monoacetylation was confirmed by the expected value of molecular mass (HR-ESI-MS, m/z : 595.28834 [M + Na]⁺) and the regioselectivity indicated by the diagnostic downfield shift of 4'-H (a triplet, now at 4.94 ppm) and the downfield shift of C-4' (at 76.8 ppm from 73.3 ppm in the parent **3**) accompanied by the upfield shift of the adjacent C-3' and C-5'.



Scheme 1. Enzymatic acetylation of proscillaridin A catalyzed by Novozym 435.

The enzymatic acylation of the structurally more complex lanatoside C (**4**) was investigated next. Lanatoside C is a clinically used cardiac glycoside composed of the aglycon digoxigenin and a tetrasaccharide composed of three digitoxose units and a glucopyranoside at its non-reducing end [36]. Currently, medical research is devoted to the characterization of the oncological potential of the administration of this drug as an apoptosis inducer and/or as a partner in combinatorial therapies with other anticancer compounds [37–40]. With this compound too, our previous experience with aglycones carrying oligosaccharides [41,42] suggested that, possibly, enzymatic esterification could be effective on the terminal glucose moiety, with a preference for the primary OH. In this case too, the reaction conditions and enzymatic dosages were based on protocols proposed by us that highlighted the use of a mixed-solvent system, made of *t*-amyl alcohol and pyridine, as optimal to guarantee substrate solubility and efficient biotransformations [41,42].

In a preliminary screening, compound **4**, dissolved in a 3:1 mixture of *t*-amyl alcohol/pyridine containing an excess of vinyl acetate, was subjected to the action of different lipases and the protease subtilisin, following the reaction by TLC and HPLC (see Supplementary Materials). In detail, the lipases investigated were: *Candida antarctica*

lipase B, lipozyme IM20, lipase from porcine pancreas, lipase from *Humicola lanuginosa*, lipase PS from *Pseudomonas cepacea*, lipase from *Candida rugosa*, lipase AK, lipase AG, and lipase from *Chromobacterium viscosum*. The best results in terms of substrate acetylation (conversion of the starting material quantified by HPLC—ca 80%) and apparent regioselectivity (only one spot detected in TLC) were obtained with lipase PS supported on celite (0.3% *w/w*).

The reaction was scaled up, and a single product was isolated in 90% yield. Monoacylation was confirmed by its mass spectrum, and NMR analysis confirmed the expected acylation of the primary hydroxyl of the glucose moiety (**4a**, Figure 2). Specifically, the two dd due to the diastereotopic proton, H-6a'''' ($J_1 = 11.8$ Hz, $J_2 = 2.1$ Hz) and H-6b'''' ($J_1 = 11.8$ Hz, $J_2 = 5.5$ Hz), were downfield shifted at 4.36 and 4.16 ppm, respectively. Similarly, the signal due to the C-6'''' carbon was downfield shifted to 65.52 ppm.

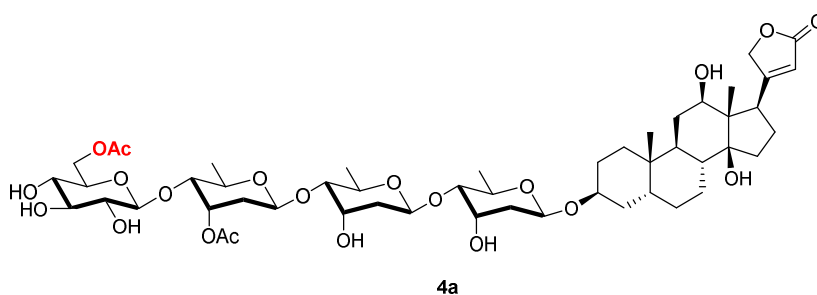


Figure 2. Acetylation product obtained by reacting lanatoside C (**4**) in the presence of vinyl acetate and lipase PS.

The other lipases were either inactive or, according to TLC analysis, formed the same product, albeit with a much lower yield. The protease subtilisin behaved differently: substrate acetylation was almost quantitative (as judged by TLC), but two additional less polar products could be detected. Based on our previous experience with glucosylated derivatives, we could hypothesize that the two additional products could be a C-3''''-acetylated monoester and a C-3''''- and C-6''''-acetylated diester [43,44]. However, this reaction was not further investigated.

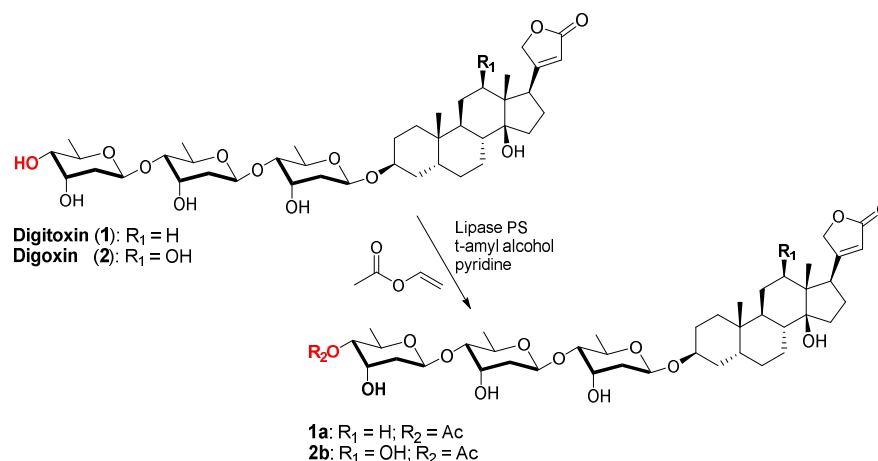
The results obtained in the enzymatic acylation of lanatoside C compare well with those obtained by Kawataba with their organocatalyst, as with their proposed protocols, mixtures of monoacylated derivatives mainly at C-3'''' OH and C-4'''' OH were always obtained, albeit in good yields [45].

The next step was to consider as substrates digitoxin (**1**) and its cognate digoxin (**2**), bufadienolide-based cardiac glycosides, whose acetylated derivatives are the drugs Novodigal (**2b**) and Dioxanin (**2c**) (Scheme 2). These two molecules differ only slightly in their aglycon moiety (the former contains a 12β -OH) and possess the same glycosidic structure, a trisaccharide formed by three units of *D*-digitoxose (**5**). As previously discussed [46], several examples of the enzymatic esterification of carbohydrates and natural glycosides have been reported in the literature, but, in the specific case of these compounds, to the best of our knowledge, there have been no previous examples of the enzymatic acylation of digitoxose or of its derivative.

Since digitoxin (**1**) is a poly-glycosylated digoxigenin natural compound such as lanatoside C (**4**), its acetylation was conducted exploiting the same reaction conditions described for **4**. Specifically, **1** was thus dissolved in a 3:1 mixture of *t*-amyl alcohol and pyridine containing vinyl acetate as an acyl donor and submitted to the action of a panel of lipases and of protease subtilisin. Only two lipases showed the formation of less polar products in TLC (see supporting information). With lipase “AK” and lipase from *Chromobacterium viscosum*, substrate acetylation was poor, while lipase PS showed the transformation of the starting material to a single acylation product that could be isolated

after chromatography (46% i.y.). The HR-MS-ESI spectrum of this product showed a peak at m/z : 829.43310 $[M + Na]^+$, indicating that the isolated product was a monoacetylated digitoxin. A careful inspection of the NMR spectra (1D and 2D) in comparison with those of **1** allowed the determination of the position of acetylation. In the $^1\text{H-NMR}$ spectrum, the signals due to the genine (aglycone) moiety were unaffected, whereas a considerable perturbation was observed in the region of the hydroxymethyne protons. A clean dd appeared downfield at 4.44 ppm, connected via a *trans*-diaxial coupling to a dq at 4.05 ppm and via an ax-eq coupling to an apparent q at 4.19 ppm. From these data, the downfield oxymethyne proton can be attributed only to the H-4''' and the other protons to the H-5''' and H-3'', respectively, thus establishing that the reaction product was a monoacetylated derivative of the terminal digitoxose unit and that it was 4'''-O-acetyldigitoxin (**1a**).

When the reaction was carried out on digoxin (**2**), using lipase PS as a biocatalyst and the same conditions described for the acetylation of **1**, the reaction product (51% i.y.) was also recognized as the now expected 4'''-O-acetyl derivative **2b**—the structure of the drug Novodigal—by HR-MS-ESI and NMR spectra, with signals analogous to those of **1a**.



Scheme 2. Acetylation products obtained by reacting digitoxin (**1**) and digoxin (**2**) in the presence of lipase PS and vinyl acetate in a 3 to 1 mixture of t-amyl alcohol and pyridine.

The identification of the signals due to the carbohydrate moieties of **1a** and **2b** (as well as of their parents **1** and **2**) was also supported by the investigation of the spectrum of the sugar digitoxose (**5**, Figure 3).

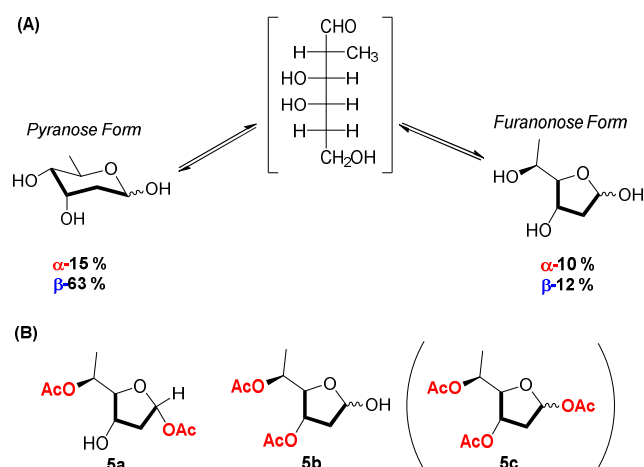


Figure 3. (A) Equilibrium of *D*-digitoxose α - and β -pyranose and furanose forms in solution; (B) isolated products from the Novozym-435-mediated acylation of digitoxose.

The structure of crystalline **5** is configured as a β -pyranose form, as evidenced by its $^1\text{H-NMR}$ spectrum recorded immediately after dissolution in CD_3OD . (The signals are reported in the Experimental section, and the spectra are in the Supplementary Materials). After equilibration in solution, **5** mutarotated via the open aldehyde (not detected) to afford a mixture of four different forms. The β -pyranose form was still predominant (ca 63%) accompanied by the anomeric α -pyranose form (ca 15%), which displayed H-1 at 5.11 ppm as a brd ($J = 3.2$ Hz). The two other forms were identified as the β -digitoxofuranose form (ca 12%) with H-1 as a triplet at 5.52 ppm ($J = 4.4$ Hz) and the anomeric α -furanose form (ca 10%) with H-1 as a dd at 5.46 ppm ($J_1 = 5.6$ Hz, $J_2 = 2.1$ Hz). Similar results had been reported by dissolving digitoxose in deuterated DMSO [47].

As we were quite surprised by the inability of Novozym 435 to acylate the two cardiac glycosides **1** and **2**, we decided to investigate the outcome of the same reaction (solvent *t*-amyl alcohol/pyridine 3:1, acylating agent vinyl acetate) using the free sugar digitoxose as the substrate. Indeed, this compound was converted into a mixture of at least three less polar products. Two of them could be isolated in sufficient amounts for their characterization and were identified as two diacetates in furanose form. Specifically, the isolated less polar spot in TLC (R_f 0.63) proved to be a single diacetylated compound and gave a clean $^1\text{H-NMR}$ spectrum characterized by the presence of an anomeric proton at 6.27 ppm as a dd ($J = 5.5$ and 1.0 Hz) and of a qd ($J = 6.5$ and 4.1 Hz) at 4.96 ppm due to H-5. From H-1 via H-2 at 2.40 and 2.05, H-3 was detected at 4.34 as a ddd ($J = 7.5, 3.2,$ and 2.1 Hz) and H-4 at 4.06 ppm as a dd ($J = 3.8$ and 3.5 Hz). These data are in agreement with an α -furanose form and suggest, for this product, the structure of 1,5-diacetyl- α -*D*-digitoxofuranose (**5a**). The more polar spot (R_f 0.54) was due to an inseparable mixture of two diacetylated products as emerged from the presence in the $^1\text{H-NMR}$ spectrum of two anomeric protons at 5.56 ppm ($t, J = 4.9$ Hz) and at 5.54 ppm ($J_1 = 5.4$ Hz and $J_2 = 1.1$ Hz). The first anomeric proton was correlated to the downfield signal at 5.32 ppm, a ddd due to H-3 ($J_1 = 6.9, J_2 = 4.3,$ and $J_3 = 3.2$ Hz), to the qt due to H-4 at 3.86 ppm ($J_1 = 6.6$ and $J_2 = 2.9$ Hz), and this in turn to H-5 in the region 4.95–5.05 ppm. The second anomeric proton at 5.54 ppm was correlated to H-3 at 5.21 ppm (ddd, $J_1 = 7.8, J_2 = 3.6,$ and $J_3 = 2.0$ Hz), and then to H-4 and H-5 in the region 4.95–5.05 ppm. To this compound, the structure of the epimeric mixture of 3,5-diacetate (**5b**) could be assigned. The third compound could not be isolated, but, being significantly less polar than **5a** and **5b**, it might likely have been the triacetate **5c** (Figure 3). The lack of products in the pyranose configuration might explain why Novozym 435 was not able to acylate digitoxin (**1**) and digoxin (**2**).

3. Materials and Methods

3.1. General Information

The ^1H and ^{13}C -NMR spectra were recorded in CD_3OD (unless otherwise stated) with a Bruker Avance AC400 instrument (400.13 MHz and 100.1 MHz, respectively). Chemical shifts are given on a δ (ppm) scale, and coupling constants J are given in Hz.

HR-ESI-MS were measured with a Fourier Transfer Ion Cyclotronic Resonance (FT-ICR) ApexTM II instrument (Bruker Daltonics, Billerica, Massachusetts, US) equipped with a 4.7 Tesla cryo-magnet (MagneX). Compounds were dissolved in $\text{CH}_3\text{OH}/\text{H}_2\text{O}$.

Thin-layer chromatography (TLC) was performed on silica plates (Merck 60 F254). Substrates and products were visualized by plate treatment with the molybdate reagent ($(\text{NH}_4)_6\text{MoO}_{24}\cdot 4\text{H}_2\text{O}$, 42 g; $\text{Ce}(\text{SO}_4)_2$, 2 g; H_2SO_4 , concentrated 62 mL; made up to 1 L with deionized water). Purifications were performed by flash chromatography on silica gel (Merck 60, 230–400 mesh).

Candida antarctica lipase B was from Novozyme (Novozym 435); lipozyme IM20, lipase PS from *Pseudomonas capacea*, lipase AK, lipase AG, and lipase CE5 from *Humicola lanuginosa* were from Amano; the protease subtilisin and the lipases from porcine pancreas, *Candida rugosa*, and *Chromobacterium viscosum* were from Sigma Aldrich (St. Louis, Missouri, US).

Lipase PS was immobilized by mixing the crude enzyme powder (3 g, ca 30 mg of biocatalyst) with 10 g of celite (Hyflo Super Cell). A total of 10 mL of PB (0.1 M, pH 7.0) was added, and the obtained solid–liquid combination was energetically mixed and then dried in vacuo for 24 h.

3.2. Synthesis

4'-O-acetyl-proscillaridin A (3a). Proscillaridin A (**3**, 90 mg) was dissolved in 10 mL of vinyl acetate and treated with 50 mg of Novozym 435. The suspension was stirred (220 rpm) at 45 °C for 24 h, then filtered, solvent evaporated, and the residue purified by flash column chromatography on silica gel using the eluent CHCl_3 –MeOH (25:2) to obtain 46 mg of pure **3a**.

Rf (CHCl_3 –MeOH– H_2O , 10:2:0.25): **3a**, 0.34; proscillaridin A, 0.14. ^1H -NMR (CDCl_3): selected data, δ : 8.01 (1H, dd, $J_1 = 9.7$ Hz, $J_2 = 2.5$ Hz, H-22); 7.45 (1H, bs, H-21); 6.30 (1H, d, $J = 9.7$ Hz, H-23); 5.36 (1H, bs, H-4); 4.94 (1H, t, $J = 9.4$ Hz, H-4'); 4.91 (1H, bs, H-1'); 4.15 (1H, m, H-3); 3.81 (3H, m, H-2', H-3', and H-5'); 2.57 (H-17); 2.10 (3H, s, CH_3CO); 1.15 (3H, d, $J = 5.9$ Hz, CH_3 -6'); 1.09 (3H, s, CH_3 -19); 0.76 (3H, s, CH_3 -18). ^{13}C -NMR (CDCl_3): δ : 173.49 (CH_3CO); 151.43 (C-21); 150.25 (C-22); 149.78 (C-5); 125.91 (C-20); 122.403 (C-4); 116.30 (C-23); 101.712 (C-1'); 86.66; 76.76 (C-4'); 76.19 (C-3); 73.79, 71.44, and 68.78 (C-2', C-3', and C-5'); 53.19 (C-17); 52.44; 44.41; 42.65; 39.59; 37.43; 34.44; 34.20; 30.96; 30.63; 29.04, 23.37; 21.87 (CH_3CO); 20.46 (CH_3 -19); 18.65 (CH_3 -6'); 18.12 (CH_3 -18). HR-ESI-MS, m/z : 595.28834 [$\text{M} + \text{Na}$]⁺, calc for $\text{C}_{32}\text{H}_{44}\text{O}_9\text{Na}$ 595.28775.

6'''-O-Acetyl-lanatoside C (4a). Lanatoside C (**4**, 90 mg) was dissolved in a 1:3 mixture (8 mL) of pyridine and *tert*-amyl alcohol (t-AmOH) and reacted with 2 mL of vinyl acetate and 1 g of lipase PS on celite at 45 °C (220 rpm) for 96 hr. After filtration and solvent evaporation, the residue was purified via flash column chromatography on silica gel using the eluent CHCl_3 –MeOH 15:1 to obtain 81 mg of pure **4a** as an amorphous powder.

Rf (CHCl_3 –MeOH– H_2O , 20:2:0.25): **4a**, 0.21; lanatoside C, 0.11. ^1H -NMR; selected data, δ : 5.93 (1H, s, H-22); 5.84 (1H, q, $J = 3.0$ Hz, H-3'''); 5.00 (1H, dd, $J_1 = 18.6$ Hz, $J_2 = 1.7$ Hz, H-21a); 4.92 (1H, dd, $J_1 = 18.6$ Hz, $J_2 = 1.8$ Hz, H-21b); 4.94, 4.92, and 4.89 (1H each, d each, $J = 7.8$ Hz, $J = 8.2$ Hz, and $J = 7.4$ Hz, H-1', H-1'', and H-1'''); 4.38 (1H, d, $J = 7.8$ Hz, H-1'''); 4.36 (1H, dd, $J_1 = 11.8$ Hz, $J_2 = 2.1$ Hz, H-6a'''); 4.28 and 4.26 (1H each, q each, $J = 2.9$ Hz, and $J = 3.0$ Hz, H-3', and H-3''); 4.16 (1H, dd, $J_1 = 11.8$ Hz, $J_2 = 5.5$ Hz, H-6b'''); 4.03 (1H, br m, H-3); 3.97 (1H, dq, $J_1 = 9.6$ Hz, $J_2 = 6.2$ Hz, H-5'''); 3.85 and 3.81 (1H each, dq each, $J_1 = 9.6$ Hz and $J_2 = 6.2$ Hz each, H-5', and H-5''); 3.45 (1H, ddd, $J_1 = 9.4$ Hz, $J_2 = 5.5$ Hz, $J_3 = 2.1$ Hz,

H-5''''); 3.41 (1H, dd, $J_1 = 9.5$ Hz, $J_2 = 3.2$ Hz, H-4''''); 3.37 (1H, t, $J = 9.2$ Hz, H-4''''); 3.29 (1H, t, $J = 8.9$ Hz, H-3''''); 3.29, and 3.24 (1H each, dd each, $J_1 = 9.6$ Hz, $J_2 = 2.9$ Hz and $J_1 = 9.5$ Hz, $J_2 = 2.9$ Hz, H-4', and H-4''); 3.17 (1H, t, $J = 8.2$ Hz, H-2''''); 2.09 and 2.06 (3H each, s each, 2 CH₃CO); 1.35 (3H, d, $J = 6.2$ Hz, CH₃-6'''); 1.22 and 1.21 (3H each, d each, $J = 6.2$ Hz each, CH₃-6', and CH₃-6''); 0.97 (3H, s, CH₃-19); 0.80 (3H, s, CH₃-18). ¹³C-NMR; δ : 179.34 and 178.16 (C-20 and C-23); 173.78 and 172.67 (2 CH₃CO); 118.62 (C-22); 106.46 (C-1''''); 101.35, 101.32, and 97.92 (C-1', C-1'', and C-1'''); 87.69 (C-14); 84.72 and 84.42 (C-4' and C-4''); 82.22; 78.84 (C-4''''); 76.64 (C-4'''); 76.34 (C-21); 76.05 (C-2'''' and C-5'''); 75.39 (C-3); 72.49 and 72.37 (C-3'''' and C-3'''); 71.55 (C-5'''); 70.40 (C-5' and C-5''); 69.30 and 69.12 (C-3' and C-3''); 65.52 (C-6'''); 58.22; 48.05 (C-17); 43.21; 39.80 and 39.38 (C-2' and C-2''); 37.80; 37.10; 34.63; 34.51; 32.34 (C-16); 32.01 (C-11); 31.80; 29.29; 28.70; 28.40; 25.10 (C-19); 23.63; 21.93 and 21.66 (2 CH₃CO); 19.51 (C-6'''); 19.35 (C-6' and C-6''); 10.76 (C-18). HR-ESI-MS m/z : 1049.49123 [M + Na]⁺, calculated for C₅₁H₇₈O₂₁ Na 1049.49278.

4''-O-Acetyl-digitoxin (1a). Digitoxin (1, 100 mg) was dissolved in a 1:3 mixture (8 mL) of pyridine and *t*-AmOH and reacted with 2 mL of vinyl acetate and 1 g of lipase PS at 45 °C (220 rpm) for 96 hr. After filtration and solvent evaporation, the residue was purified via flash column chromatography on silica gel using the eluent CHCl₃-MeOH 15:1 to obtain 48 mg of pure **1a** as an amorphous powder.

Rf (CHCl₃-MeOH-H₂O, 20:2:0.25): **1a**, 0.18; digitoxin, 0.06. ¹H-NMR; selected data, δ : 5.91 (1H, brs, H-22); 5.05 (1H, dd, $J_1 = 18.4$ Hz, $J_2 = 1.2$ Hz, H-21a); 5.00 (1H, dd, $J_1 = 9.6$ Hz, $J_2 = 1.6$ Hz, H-1'''); 4.94 and 4.92 (1H each, brd each, $J = 9.8$ Hz, and $J = 9.5$ Hz; H-1' and H-1''); 4.93 (1H, dd, $J_1 = 18.6$ Hz, $J_2 = 1.6$ Hz, H-21b); 4.44 (1H, dd, $J_1 = 10.0$ Hz, $J_2 = 2.8$ Hz, H-4'''); 4.27 (2H, m, H-3', and H-3''); 4.19 (1H, q, $J = 3.0$ Hz, H-3'''); 4.05 (1H, dq, $J_1 = 9.9$ Hz, $J_2 = 6.4$ Hz, H-5'''); 4.03 (1H, brm, H-3); 3.86 and 3.81 (1H each, dq each, $J_1 = 9.7$ Hz, $J_2 = 6.4$ Hz each, H-5', and H-5''); 3.28 and 3.24 (1H each, dd each, $J_1 = 9.6$ Hz, $J_2 = 3.2$ Hz, and $J_1 = 9.6$ Hz, $J_2 = 2.8$ Hz, H-4', and H-4''); 2.85 (1H, brdd, $J_1 = 8.2$ Hz, $J_2 = 5.6$ Hz, H-17); 2.10 (3H, s, CH₃CO); 1.24 and 1.22 (3H each, d each, $J = 6.4$ Hz each, H-6', and H-6''); 1.18 (3H, d, $J = 6.4$ Hz, H-6'''); 0.96 (3H, s, CH₃-19); 0.90 (3H, s, CH₃-18). ¹³C-NMR, δ : 177.04 and 175.15 (C-20 and C-23); 170.66 (CH₃CO) 116.38 (C-22); 99.30 (C-1'''); 99.11 and 95.51 (C-1' and C-1''); 85.04 (C-14); 82.39 and 82.22 (C-4' and C-4''); 75.21 (C-4'''); 73.94 (C-21); 73.10 (C-3); 68.07 (C-5' and C-5''); 67.07 (C-3' and C-3''); 66.99 (C-5'''); 64.91 (C-3'''); 50.75 (C-17); 49.67; 41.31; 39.58 (C-2'''); 37.82 and 37.52 (C-2' and C-2''); 37.16; 36.57; 35.48; 34.93; 32.00; 29.98; 29.62; 26.66; 26.47; 26.11; 22.87 (C-19); 21.17; 20.96; 19.49 (CH₃CO); 17.04 (C-6' and C-6''); 16.89 (C-6'''); 14.99 (C-18). HR-ESI-MS m/z : 829.43310 [M + Na]⁺, calculated for C₄₃H₆₆O₁₄Na 829.43448.

4''-O-Acetyldigoxin (2b). To a solution of 100 mg of digoxin (**2**) prepared in a 1:3 mixture (8 mL) of pyridine and *t*-AmOH, 2 mL of vinyl acetate and 1 g of lipase PS were added. The reaction mixture was held at 45 °C (220 rpm) for 96 h, then filtered, and solvents evaporated. The purification of the residue via flash column chromatography on silica gel using the eluent CHCl₃-MeOH 23:2 yielded 52 mg of pure **2b** as an amorphous powder.

Rf (CHCl₃-MeOH-H₂O, 20:2:0.25): **2b**, 0.20; digoxin 0.09. ¹H-NMR; selected data, δ : 5.93 (1H, brs, H-22); 5.00 (1H, dd, $J_1 = 18.6$ Hz, $J_2 = 1.6$ Hz, H-21a); 4.98, 4.96, and 4.92 (1H each, brd each, $J = 9.2$ Hz, $J = 9.3$ Hz, and $J = 9.0$ Hz; H-1', H-1'', and H-1'''); 4.94/(1H, dd, $J_1 = 18.6$ Hz, $J_2 = 1.6$ Hz, H-21b); 4.44 (1H, dd, $J_1 = 9.9$ Hz, $J_2 = 2.8$ Hz, H-4'''); 4.27 and 4.24 (1H each, dd, each; $J_1 = 9.9$ Hz, $J_2 = 2.8$ Hz; H-3', and H-3''); 4.20 (1H, q, $J = 3.1$ Hz, H-3'''); 4.05 (1H, dq, $J_1 = 9.9$ Hz, $J_2 = 6.4$ Hz, H-5'''); 4.03 (1H, brm, H-3); 3.84 and 3.81 (1H each, dq each, $J_1 = 9.7$ Hz, $J_2 = 6.4$ Hz each, H-5', and H-5''); 3.40 (1H, dd, $J_1 = 11.9$ Hz, $J_2 = 4.1$ Hz, H-12); 3.34 (1H, m, H-17); 3.28 and 3.24 (1H each, dd each, $J_1 = 9.6$ Hz, $J_2 = 3.2$ Hz, and $J_1 = 9.6$ Hz, $J_2 = 2.8$ Hz; H-4' and H-4''); 2.10 (3H, s, CH₃CO); 1.24 and 1.22 (3H each, d each, $J = 6.8$ Hz and $J = 6.7$ Hz; H-6' and H-6''); 1.18 (3H, d, $J = 6.2$ Hz, H-6'''); 0.97 (3H, s, CH₃-19); 0.81 (3H, s, CH₃-18). ¹³C-NMR; δ : 179.35 and 178.19 (C-20 and C-23); 172.98 (CH₃CO); 118.62 (C-22); 101.56 (C-1'''); 101.37 and 97.91 (C-1' and C-1''); 87.69 (C-14); 84.72 and 84.55 (C-4' and C-4''); 77.56 (C-4'''); 76.64 (C-12); 76.34 (C-21); 75.40 (C-3); 70.41 (C-5' and C-5''); 69.44

and 69.31 (C-3' and C-3''); 69.25 (C-5'''); 67.26 (C-3'''); 48.04 (C-17); 43.21; 40.18 (C-2'''); 39.79 and 39.44 (C-2' and C-2''); 38.90; 38.01; 37.11; 34.62; 34.49; 32.34 (C-16); 32.00 (C-11); 31.79; 29.28; 28.69; 28.39; 25.08 (C-19); 23.68; 21.76 (CH₃CO); 19.35 (C-6' and C-6''); 19.18 (C-6'''); 10.74 (C-18). HR-ESI-MS *m/z*: 845.42899 [M + Na]⁺, calculated for C₄₃H₆₆O₁₅Na 845.42939.

¹H-NMR of *D*-digitoxose (5).

(a) Immediately after dissolution in CD₃OD (β-pyranose conformation): δ: 5.08 (1H, dd, J₁ = 9.7 Hz, J₂ = 2.0 Hz, H-1); 4.02 (1H, app q, J = 3.1 Hz, H-3); 3.80 (1H, dd, J₁ = 9.5 Hz, J₂ = 6.2 Hz, H-5); 3.16 (1H, dd, J₁ = 9.5 Hz, J₂ = 3.1 Hz, H-4); 2.03 (1H, ddd, J₁ = 13.6 Hz, J₂ = 2.5 Hz, J₃ = 2.1 Hz, H-2eq); 1.64 (1H, ddd, J₁ = 13.7 Hz, J₂ = 9.8 Hz, J₃ = 2.8 Hz, H-2ax); 1.25 (3H, d, J = 6.3, CH₃-6).

(b) The equilibrated mixture of four different forms of β-D-digitoxose after 2–3 days of dissolution in CD₃OD: β-D-digitoxopyranose (62.9%): δ 5.09 (1H, dd, J₁ = 9.6 Hz, J₂ = 2.0 Hz, H-1); 4.02 (1H, app q, J = 2.8 Hz, H-3); 3.81 (1H, dd, J₁ = 9.6 Hz, J₂ = 6.2 Hz, H-5); 3.16 (1H, dd, J₁ = 9.6 Hz, J₂ = 3.2 Hz, H-4); 2.03 (1H, ddd, J₁ = 13.6 Hz, J₂ = 3.6 Hz, J₃ = 2.4 Hz, H-2eq); 1.64 (1H, ddd, J₁ = 13.6 Hz, J₂ = 10.0 Hz, J₃ = 2.8 Hz, H-2ax); 1.25 (3H, d, J = 6.2, CH₃-6). α-D-digitoxopyranose (15.2%): δ 5.11 (1H, brd, J = 3.2 Hz, H-1); 4.10 (1H, dq, J₁ = 9.6 Hz, J₂ = 6.4 Hz, H-5); 3.80–3.85 (1H, m, H-3); 3.18 (1H, dd, J₁ = 9.6 Hz, J₂ = 3.2 Hz, H-4); 2.05–2.08 (1H, m, H-2ax); 1.86 (1H, ddd, J₁ = 13.9 Hz, J₂ = 3.6 Hz, J₃ = 3.2 Hz, H-2eq); 1.27 (3H, d, J = 6.2, CH₃-6). β-D-digitoxofuranose (13.0%): δ 5.52 (1H, t, J = 4.4 Hz, H-1); 4.48 (1H, td, J₁ = 5.6 Hz, J₂ = 3.6 Hz, H-3); 3.80 (1H, m, H-5); 3.64 (1H, dd, J₁ = 7.6 Hz, J₂ = 4.8 Hz, H-4); 2.08–2.05 (2H, m, H-2eq and H-2ax); 1.21 (3H, d, J = 6.4, CH₃-6). α-D-digitoxofuranose (8.4%): δ 5.46 (1H, dd, J₁ = 5.6 Hz, J₂ = 2.1 Hz, H-1); 4.30 (1H, td, J₁ = 6.8 Hz, J₂ = 3.2 Hz, H-3); 3.89 (1H, dd, J₁ = 4.8 Hz, J₂ = 3.6 Hz, H-4); 3.75 (1H, qd, J₁ = 6.6 Hz, J₂ = 3.9 Hz, H-5); 2.23 (1H, ddd, J₁ = 13.6 Hz, J₂ = 7.2 Hz, J₃ = 5.2 Hz, H-2ax); 1.85 (1H, ddd, J₁ = 13.5 Hz, J₂ = 3.6 Hz, J₃ = 3.2 Hz, H-2 eq); 1.24 (3H, d, J = 6.4, CH₃-6).

Acetylation of *D*-digitoxose (5). To a solution of 70 mg of *D*-digitoxose (5) prepared in a 1:3 mixture (8 mL) of pyridine and *tert*-amyl alcohol (*t*-AmOH), 2 mL of vinyl acetate and 100 mg of Novozym 435 were added. The reaction mixture was held at 45 °C (220 rpm) for 7 h, then filtered, and solvents evaporated. Three new spots could be detected by TLC (eluent AcOEt–MeOH–H₂O, 7:1:0.5) with R_f 0.71 (5c), 0.63 (5b), and 0.54 (5a) (R_f 5, 0.37). Samples of almost pure 5a (40 mg, as an inseparable mixture of anomeric epimers) and 5b (5 mg) could be isolated by flash column chromatography on silica gel using the eluent AcOEt–MeOH 25: 1.

Selected ¹H-NMR signals (CD₃OD) are reported below for the isolated compounds:

5a (1,5-diacetyl-digitoxose, α-furanose conformation), δ 6.27 (1H, dd, J₁ = 5.5 Hz, J₂ = 1.0 Hz, H-1);

5b (3,5-diacetyl-digitoxose)

α-furanose conformation: δ 5.54 (1H, dd, J₁ = 5.4 Hz, J₂ = 1.1 Hz, H-1); 5.21 (1H, ddd, J₁ = 7.8 Hz, J₂ = 3.6 Hz, J₃ = 2.0 Hz, H-3); 5.00 (2H, m, H-5, and H-4); 2.15 (1H, m, H-2ax); 1.92 (1H, ddd, J₁ = 14.5 Hz, J₂ = 1.9 Hz, J₃ = 1.2, H-2eq); 2.05 and 2.03 (3H each, s, CH₃CO), 1.29 (3H, d, J = 6.5 Hz, CH₃-6);

β-Furanose conformation: δ 5.56 (1H, t, J = 5.56 Hz, H-1); 5.32 (1H, ddd, J₁ = 6.9 Hz, J₂ = 4.2 Hz, J₃ = 3.2 Hz, H-3); 5.00 (1H, m, H-5); 3.86 (1H, dd, J₁ = 6.6 Hz, J₂ = 2.9 Hz, H-4); 2.37 (1H, ddd, J₁ = 14.4 Hz, J₂ = 7.8 Hz, J₃ = 5.3, H-2ax); 2.15 (1H, m, H-2eq); 2.08 and 2.06 (3H each, s, CH₃CO), 1.25 (3H, d, J = 6.5 Hz, CH₃-6).

4. Conclusions

In light of the increasing interest in cardiac glycosides as potential immuno- and cancer-chemotherapeutics, this work has been focused on a selective, enzymatic entry to the mono-acetylated derivatives of selected cardiotonics isolated from *Digitalis* sp. These simple compounds are, in fact, known to possess improved pharmacokinetic profiles compared to their respective parental molecules. Accordingly, we took inspiration from the efficient regioselective esterification protocols developed by us and others in the late

1980s and early 1990s for the regioselective enzymatic acylation of polyhydroxylated natural compounds.

The acetylation of compounds carrying terminal *L*-rhamnopyranosyl or *D*-glucopyranosyl units was achieved by following previous literature reports on natural glycosides carrying the same sugar moieties and allowing the easy and selective preparation of mono-acetylated derivatives of lanatoside C and proscillaridin A. Conversely, the regioselective enzymatic acetylation of derivatives carrying a terminal *D*-digitoxose unit was achieved for the first time, identifying lipase PS as the catalyst of choice. This novel biocatalytic entry allowed the regioselective preparation of the drug Novodigal, a mono-acetylated derivative of digoxin.

These preliminary findings could be useful for the preparative-scale synthesis of new bioactive derivatives of these glycosides, either acetylated or carrying other acyl moieties on their oligosaccharidic units.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/catal13050819/s1>: Copies of the NMR and mass spectra of the starting and the obtained compounds as well as HPLC and TLC screening from lanatoside C and digitoxin acetylation, respectively.

Author Contributions: Conceptualization, S.R. and B.D.; methodology and experiment, S.R. and L.R.; formal analysis, B.D.; data curation, B.D. and L.R.; writing—original draft preparation, B.D.; writing—review and editing, I.B. and S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are available in Supplementary Material.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviation

AcOEt = ethyl acetate; *t*-AmOH = *tert*-amyl alcohol; MeOH = methanol; i.y. = isolated yield; brd = broad; s = singlet; d = doubled; t = triplet; ax = axial; eq = equatorial; PB = phosphate buffer.

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