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Production of the Cytotoxic Cardenolide Glucoevatromonoside by Semisynthesis and Biotransformation of Evatromonoside by a *Digitalis lanata* Cell Culture*

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ABSTRACT

Recent studies demonstrate that cardiac glycosides, known to inhibit Na^+/K^+-ATPase in humans, have increased susceptibility to cancer cells that can be used in tumor therapy. One of the most promising candidates identified so far is glucoevatromonoside, which can be isolated from the endangered species *Digitalis mariana* ssp. *heywoodii*. Due to its complex structure, glucoevatromonoside cannot be obtained economically by total chemical synthesis. Here we describe two methods for glucoevatromonoside production, both using evatromonoside obtained by chemical degradation of digitoxin as the precursor. 1) Catalyst-controlled, regioselective glycosylation of evatromonoside to glucoevatromonoside using 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide as the sugar donor and 2-aminoethyldiphenylborinate as the catalyst resulted in an overall 30% yield. 2) Biotransformation of evatromonoside using *Digitalis lanata* plant cell suspension cultures was less efficient and resulted only in an overall 18% pure product. Structural proof of products has been provided by extensive NMR data. Glucoevatromonoside and its non-natural 1–3 linked isomer neo-glucoevatromonoside obtained by semisynthesis were evaluated against renal cell carcinoma and prostate cancer cell lines.

Introduction

Cardiac glycosides (CGs) are used to treat congestive heart failure and arrhythmias, since they are known to bind and inhibit Na^+/K^+-ATPase, leading to a positive inotropic effect [1]. More recently, CGs came into focus for their antiviral [2–6] and anticancer activities as reviewed in [7–11]. CGs inhibit the Na^+/K^+-ATPase signalosome complex at nanomolar concentrations leading to the activation of various signaling pathways that provoke cell death. In this respect, digitoxigenin monodigitoxoside [evatromonoside

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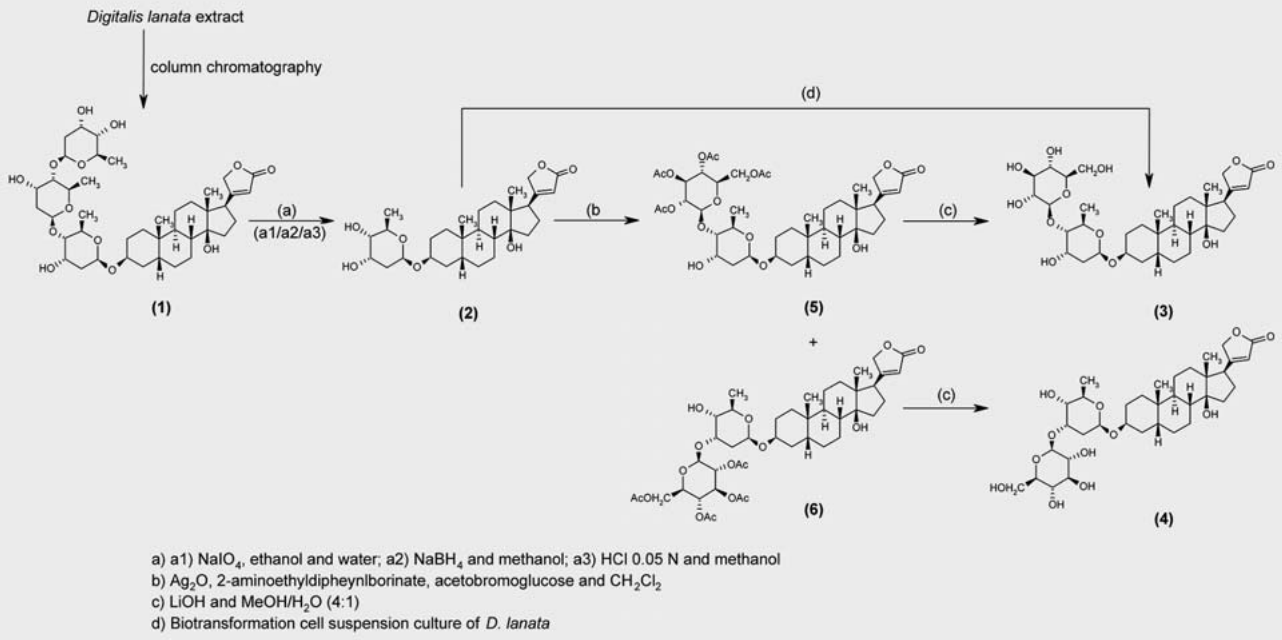
** These authors contributed equally to this manuscript.



▶ **Fig. 1** Schematic overview for obtaining GEV (**3**) by different processes. EV (**2**) is the common precursor for both approaches. The product GEV (**3**) will either be produced by chemical synthesis or biotransformation reaction with plant cell suspension cultures.

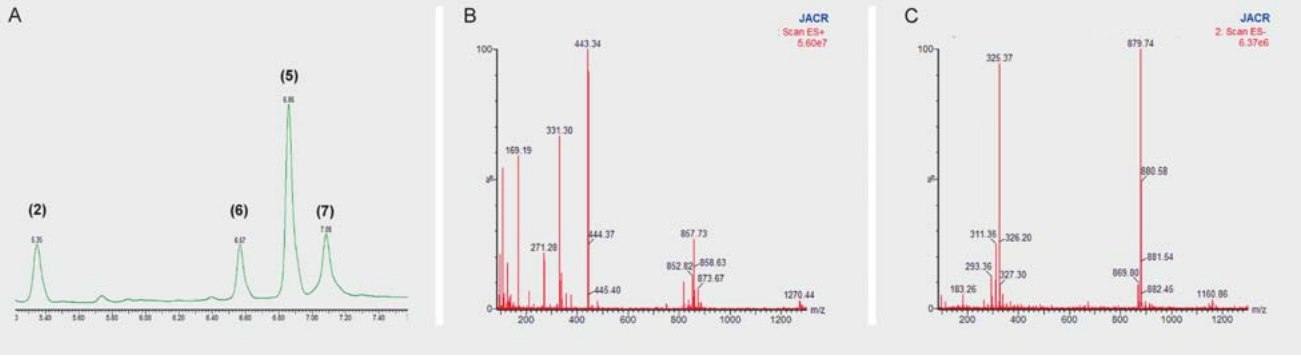
(EV) (**2**)] (▶ **Fig. 1**) turned out to be a very promising candidate, which was investigated by Schneider et al. [12] and Wang et al. [13]. Other targets of CGs are viral infections, such as those caused by human *Herpes Simplex Virus* (HSV). Out of 65 tested cardenolides, Bertol and coworkers [5] attributed the strongest antiherpes effects to EV and to its naturally 4′-glycosylated derivative, glucoevatromonoside [GEV (**3**)] (▶ **Fig. 1**). These compounds inhibited HSV-1 and HSV-2 replication at nanomolar concentrations and the mechanism of action of GEV was elucidated. Additionally, promising cytotoxic effects, including reduction of proliferation, migration and invasion in A549 (non-small cell lung cancer) and U937 cells (human leukemic monocyte lymphoma cell line) were detected for GEV [14].

Despite their potential anticancer and antiviral effects, CGs such as EV and GEV are still isolated from plant sources, since total synthesis is hampered by their structural complexity. GEV is not even commercially available and the sample employed in our previous studies [5, 14] was isolated from *Digitalis lanata* leaves [15]. Whereas GEV and EV are only minor CGs in this species, GEV is a major CG in *Digitalis heywoodii* (syn. *Digitalis mariana* ssp. *heywoodii*, Heywood's foxglove) [16, 17]. Therefore, this species is a promising source for large-scale production and isolation of GEV. Unfortunately, Heywood's foxglove is an endemic and endangered species growing only in a few places in the Iberian Peninsula. A recent study showed a high content of GEV in *D. mariana* ssp. *heywoodii* plants, which were regenerated from seed-derived shoot explants through *in vitro* propagation [17]. This approach is very labor-intensive and time-consuming, and significant amounts of GEV have so far only been demonstrated in green house cultivated plants, but not in field trials.

Two alternative approaches to obtain GEV and also EV are described here. For the production of EV, we propose the rather simple chemical degradation of digitoxin [DTG, (**1**)] (▶ **Fig. 1**), which can be isolated from any *Digitalis* extract enriched in A-series cardenolides. To produce GEV, we established a biotransformation process using suitable precursors and *D. lanata* plant cell suspension cultures, as well as a semisynthetic approach based on a combination of two previously described methods [18, 19] (▶ **Fig. 1**). Besides the desired GEV (**3**), the non-natural 3′-glycosylated derivative, neo-glucoevatromonoside [(N-GEV, (**4**)], was also obtained and both compounds had their activity evaluated against tumor cell lines.

Results and Discussion

DTG was used as the starting material in the production of EV. It was isolated from a *D. lanata* extract enriched in A-series cardenolides. The extract was fractionated by flash chromatography on a silica gel column and yielded 1 g of DTG per 4 g of extract. Fractions containing DTG were combined and used to prepare EV. The purity was assessed by UPLC-ESI-MS (**Fig. 1S**, Supporting Information). The chemical degradation of DTG to EV was carried out according to the method described in US Patent 3,856,944 (1974) [20] with small changes regarding solvents, the molar ratio of reactants, and reaction conditions. Oxidation of digitoxin using $NaIO_4$ resulted in dialdehyde I with a 95% yield. This was subsequently treated with $NaBH_4$ to produce dimethylol I (95% yield). This reaction was carried out in MeOH instead of EtOH to improve $NaBH_4$ solubility. The molar proportion of $NaBH_4$ was increased to



▶ **Fig. 2** Glycosylation reaction. **A** UPLC chromatogram of glycosylation reaction 1. Compound **2**: substrate EV; compound **6**: (2,3,4,6)-tetra-*O*-acetly-neo-glucoevatromonside, Rt = 6.57 min; compound **5**: (2,3,4,6)-tetra-*O*-acetly-glucoevatromonside, Rt = 6.86 min, detected by a PDA 2996 photo diode array detector. **B, C** MS spectrum of both products **6** and **5**, mass 834 *m/z*. (**7**) unidentified product of identical mass.

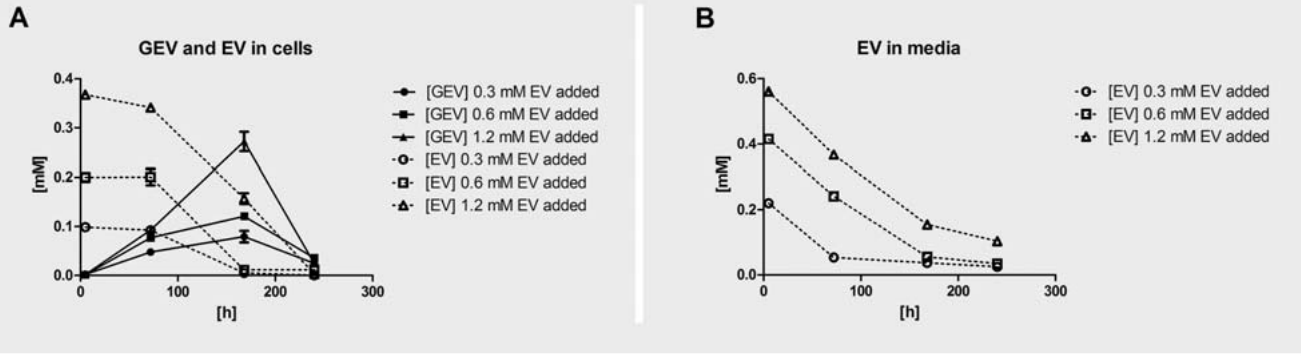
▶ **Table 1** Profile of the intermediated products acetylated GEV (**5**) and acetylated N-GEV (**6**) obtained in different conditions for the glycosylation of EV (**2**) calculated w/w.

Reaction	Reaction conditions	Substrate (2) [%]	Product (5) [%]	Product (6) [%]	Ratio 5/6
1	Literature conditions	32	54	14	80:20
2	Increased amounts of reagents	8	72	20	78:22
3	Dark	13	66	21	76:24
4	Optimized	7	72	21	77:23

10 instead of 4, which reduced the reaction time by 4 h. Dimethylol I was hydrolyzed using 0.05 N hydrochloric acid, resulting in digitoxigenin-bisdigitoxoside with a 90% yield. This compound was further oxidized to dialdehyde II (95% yield). The second reduction step afforded dimethylol II (88% yield), which was finally hydrolyzed to EV with an 84% yield. This technique resulted in an EV production yield of 70% overall on a molar basis. The identity of EV was confirmed by UPLC-ESI-MS and NMR analyses. LC/MS analysis disclosed the characteristic peak of the molecular ion at *m/z* 504 (**Fig. 2S**, Supporting Information). All ^{1}H and ^{13}C resonance signals in the NMR spectra of EV were assigned by a combination of 1D and 2D experiments (DEPT, HMQC, and HMBC). Moreover, the spectroscopic data allowed us to confirm the purity of the obtained EV (**Fig. 5S**, Supporting Information). An alternative approach to synthesize EV has been previously reported using palladium-catalyzed digitoxosylation of digitoxigenin [19]. This method also resulted in high yields of the final product. However, in addition to digitoxigenin, digitoxose had to be obtained and modified appropriately to carry out the reaction. Therefore, the chemical degradation of digitoxin described here offers a more efficient and alternative approach to produce EV that can be directly used as starting material for biotransformation experiments or chemical synthesis. The method also produced digitoxigenin-bisdigitoxoside, another bioactive cardenolide non-commercially available, and is thus more versatile than the palladium-catalyzed glycosylation.

O-Glycosylation of natural products is a valid strategy to modify the biopharmaceutical properties of bioactive compounds. In addition to chemical synthesis, enzyme catalysis, either *in vivo* (metabolic engineering, biotransformation) or *in vitro* (chemoenzymatic methods), is a promising approach [18, 21–23]. Catalyst-controlled, regioselective glycosylation of DTG was described by Beale and Taylor [18] using 2-aminoethyldiphenylborinate and silver (I) oxide as the catalysts responsible for the regioselectivity of the reaction [18] (▶ **Fig. 1**). Stereoselectivity of the reaction was explained by a selective activation of the equatorial OH group of *cis*-1,2-diols motifs of an appropriate pyranoside substrate emerged by using borinic ester and silver (I) oxide as a catalyst [18]. We adapted their protocol, described for the synthesis of purpureaglycoside A and its analogs, to produce GEV using EV obtained by DTG degradation as the educt (see above). The reaction yielded three products, two of them were identified by UPLC-ESI-MS as 2,3,4,6-tetra-*O*-acetyl GEV (**5**) and 2,3,4,6-tetra-*O*-acetyl-N-GEV (**6**) derivatives (Rt = 6.57 min and Rt = 6.86; *m/z* 834, respectively) (▶ **Fig. 2**). The third compound (**7**) was tentatively identified as an orthoester regioisomer of GEV, the formation of which has been previously described [18]. The 2,3,4,6-tetra-*O*-acetyl GEV derivative **5** was obtained as the main product. The ratio of compound **5** to compound **6** was around 80:20, as indicated by UPLC-ESI-MS analyses and NMR data (▶ **Fig. 2** and **Table 1**).

The method was further optimized to meet our demands. We found that the molar ratio of acetobromoglucose (ABG) was not



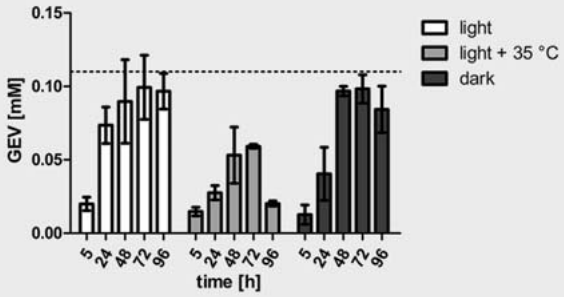
▶ **Fig. 3** Biotransformation reaction of EV (**2**) by *Dl*K1OHD suspension cells. **A** GEV (**3**) production is depicted in relation to the EV concentration added in mM in cells. **B** The EV (**2**) level was followed over time in media. Error bars represent the SD of three biological replicates each consisting of three technical replicates.

the limiting factor of the reaction. The reaction was conducted in the dark to stabilize the ABG, and/or in smaller volumes to optimize the surface/volume ratio. Under optimized conditions, 93% of the educt was consumed after 20 h, yielding mainly compound **5** (▶ **Table 1**). The reaction carried out without the catalyst 2-aminoethyl-diphenylborinate yielded only small amounts of **6**. Different reactional conditions were tested and are summarized in ▶ **Table 1**, along with the ratios of the products obtained, according to UPLC-ESI-MS analyses. An 80:20 ratio of compounds **5** and **6** in the reaction favored glycosylation at the 4′-*O* position. However, selectivity was lower compared to that obtained by Beale and Taylor [18], who reported a 95:5 ratio in favor of the 4′′′-*O*-glycosylation of digitoxin. Beale and Taylor [18] described that the 4′′′-OH group of digitoxin experienced efficient organoboron-catalyzed glycosylation, as it is the most sterically accessible position. Using EV as the educt, we observed a shift towards glycosylation at the 3′-*O* position, though glycosylation at the 4′-*O* position still is the favored reaction. This let us assume that EV provides a 3′-hydroxyl group more easily sterically accessible than the 3′′′-OH of digitoxin for chemical glycosylation, as it is only bound to the steroid aglycone. Using 2-aminoethyldiphenylborinate and silver (I) oxide as catalysts clearly force regioselective glycosylation on the 4′-*O* position compared to non-catalyst controlled glycosylation reaction. The non-catalyst controlled reaction resulted in only less conversion of EV and a low amount of product glycosylated in the 3′-*O* position.

Extending the reaction time to about 30 h promoted the cleavage of the acetyl groups, resulting in a mixture of GEV (**3**) and N-GEV (**4**) that could not be separated easily due to their similar polarities. For this reason, the acetylated derivatives of the two GEV isomers were first isolated and purified. The recovery rates of compounds **5** and **6** after purification were 45 and 90%, respectively (**Fig. 3S**, Supporting Information). Compounds **5** and **6** were then deacetylated [19] (▶ **Fig. 1**) to yield GEV (**3**), naturally glycosylated in the 1–4 linkage (77% yield), and N-GEV (**4**), non-naturally glycosylated in 1–3 (70% yield), respectively. The identity and purity of the obtained compounds were accessed by UPLC-ESI-MS and NMR analyses (**Fig. 4S**, Supporting Information). Glycosylation at the 4′-OH position of GEV (**3**) was confirmed by the significant downfield shift of the C-4′ resonance signal as compared to a ring carbon with the free OH-group (▶ **Fig. 1** and **Fig. 5S**, Supporting Information). Moreover, the downfield ^{13}C signal of C-4′ exhibits a ^{3}J-coupling with the methyl protons of C-6′ (via HMBC), thus confirming the 4′-OH glycosylation. In summary, the catalyst-controlled, regioselective glycosylation of EV to GEV resulted in a 30% overall yield on a molar basis. All individual chemical reaction starting from the degradation of DTG leading to EV and the further glycosylation as well as hydrolysis to obtain GEV yielded conversion rates higher than 70%. The chemical semisynthesis of GEV therefore offers a potential tool to provide required amounts to supply further *in vitro* and *in vivo* studies.

Digitalis suspension culture cells are able to transform exogenous cardenolides and other steroids [24–26]. Depending on their chemical structure, exogenous cardenolides may undergo glycosylation/deglycosylation and acetylation/deacetylation reactions in the side chain, along with hydroxylation of the steroid scaffold [27–29]. Although several glycosylation reactions such as glucosylation, fucosylation and digitalosylation were reported to take place in *D. lanata* tissue cultures [30], digitoxosylation of digitoxigenin has not yet been reported to occur *in vivo* in plant tissue cultures. Therefore, we did not aim at the production of evatromonoside using a biotransformation process. Since *Dl*K1OHD cell suspension cultures were described as possessing high glycosyltransferase activity [25] and to be able to convert EV into GEV [27, 29], we used this particular cell line to establish a biotransformation process for the production of GEV.

EV substrate concentrations between 0.3–1.2 mM were added to cell suspension cultures 3 days after transfer into fresh culture medium followed with GEV accumulation in the cells. The highest product levels were obtained after administration of 1.2 mM EV and the highest substrate : product ratio (1 : 1) was obtained when 0.3 mM EV was given (▶ **Fig. 3**). The highest glycosylation rates were recorded at 3 to 7 days after substrate administration. This was also reported for digitoxin glycosylation using the same cell culture [25]. GEV concentration decreased after 7 days, probably as a result of degradation [27, 30]. A series of attempts were carried out to increase the biotransformation efficacy. For example, 10 mM ascorbate or 50 µM MeJA were added to the suspension



▶ **Fig. 4** Glycosylation of EV (**2**) by biotransformation reaction under different conditions. Influence on GEV (**3**) formation, extracted from the suspension cells, in heat, and darkness versus control reactions performed in light. Dashed line marks 50% conversion rate. Error bars represent the SD of at least three biological replicates each consisting of three technical replicates.

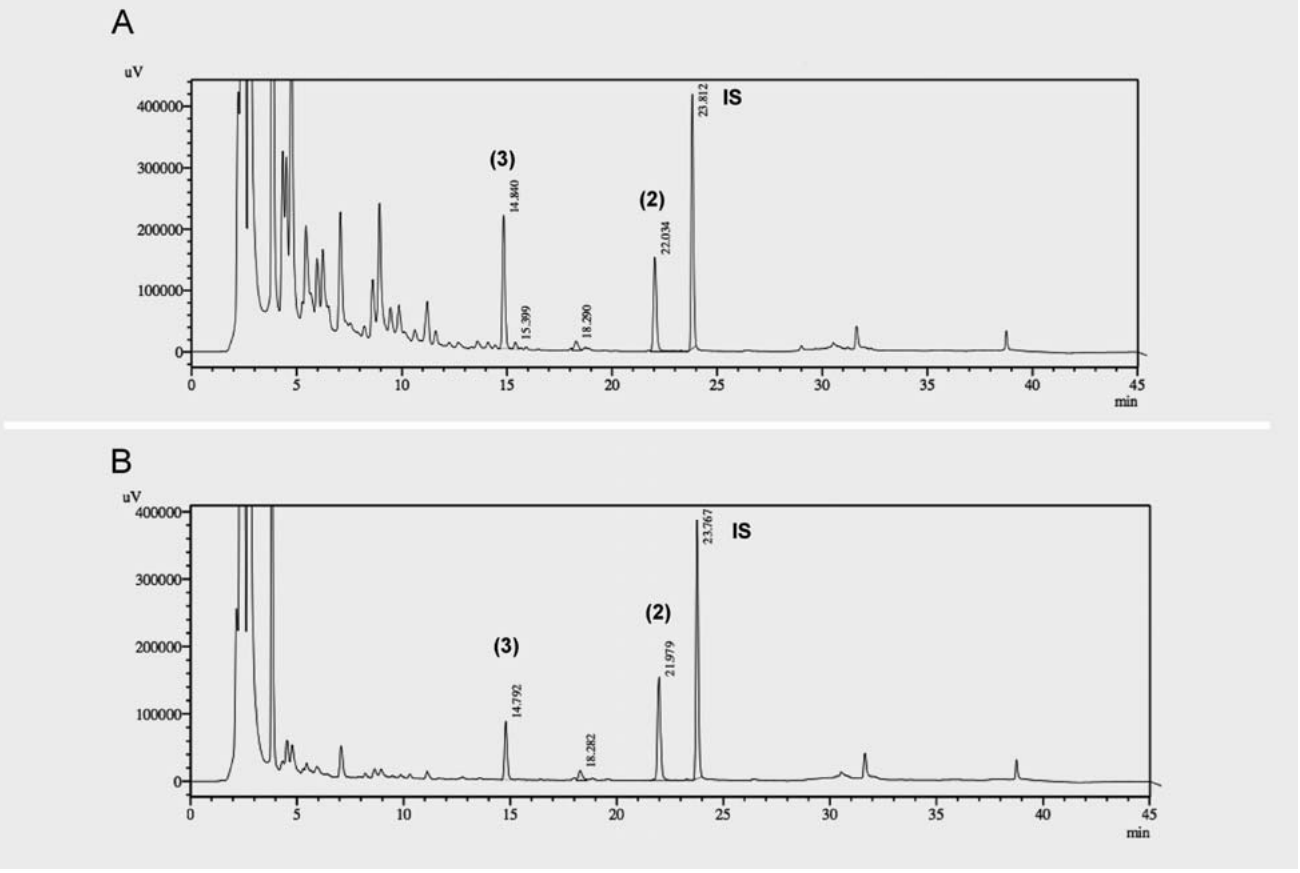
cultures. MeJA is known to induce the biosynthesis of various natural products *in planta* and can also affect cardenolide biosynthesis [31]. However, it had no influence on the desired biotransformation reaction. Biotransformation experiments were also performed in dark and light conditions, but the production rates were similar (▶ **Fig. 4**). Cultivation in the dark is the preferred option since no chlorophyll is formed, which may impair purification, and energy costs are reduced (▶ **Fig. 5**).

In order to obtain GEV and to investigate and compare the efficacy of the biotransformation reaction, EV was added to batch cultivation of *Dl*K1OHD suspension cells at a concentration of 0.3 mM, a similar concentration used in the chemical reaction. The two-step purification afforded 98% pure GEV. Using the added substrate as an indicator, GEV resulted in an 18% overall yield on a molar basis.

At this stage, chemical synthesis starting from EV seems to be the more promising and straightforward approach to produce GEV in bulk amounts than glycosylation by plant cell cultures. The biotransformation process can be further optimized and scaled up to larger vessels and higher cell density to improve the production rates. In addition, cell cultures with a higher glycosylation capacity might well be selected by cell aggregate cloning as described earlier [25]. Another approach would be the identification of glucosyltransferase genes encoding enzymes capable of converting EV or similar cardenolides. These enzymes can be recombinantly expressed in *Escherichia coli* or yeast, and these strains can be used for biotransformation *in vivo* or *in vitro* as previously described for flavonoids [32–34]. Work in this area is in progress. Although it is feasible to carry out enzymatic conversions *in vitro*, the use of glucosyltransferases is a less promising alternative since they require activated sugars as co-substrates [34].

The effect of the synthesized EV (**2**), GEV (**3**), and N-GEV (**4**) on the viability of renal cell carcinoma (Caki-1, 786-O) and prostate cancer (DU-145 and PC3) cell lines was investigated and compared to DTG (**1**) determined by [35, 36]. All cardiac glycosides exhibited cytotoxic effects against renal and prostate cancer cell lines (▶ **Table 2**), however, they differed in their impact on the investigated cell lines. EV and GEV showed higher cytotoxicity compared to DTG towards all four tested cancer cell lines. The DU145 prostate cancer cell line and 786-O renal cell carcinoma cell line expressed similar sensitivity for EV and GEV. However, especially for the Caki-1 renal cell carcinoma cell line and the PC3 prostate cancer cell line, GEV (**4**) was the most bioactive and cytotoxic compound. It was already shown that cardiac glycosides might have a different impact on various cancer cell lines [36]. In case of, non-small cell lung cancer A549 cells, EV seems a very promising candidate [12], and a well-established synthesis for this compound is an additional advantage of this study. However, in the cases of renal and prostate cancer cell lines mentioned above as well as in antiviral activity, GEV is far more active [5]. The IC_{50} values obtained for the synthetic non-natural isomer N-GEV were significantly higher than those of GEV (▶ **Table 2**). A similar pattern of cytotoxicity was shown for the acetylated compounds **5** and **6** against PC3 cell lines. The IC_{50} value of **5** was determined to be 37.23 nM and **6** was 390 nM. Along with the IC_{50} values of GEV and N-GEV, this indicates the importance of a free 3′-OH group within the sugar moiety of the CGs. This coincides with the values for the inhibition of the α1, 2, 3 subunit of the porcine Na^+/K^+-ATPase (▶ **Table 2**). The inhibition rate in that case was not measured for a specific Na^+/K^+-ATPase isoform, but offers a clue on the overall inhibition activity. A free hydroxyl group at position 3′ seems to be important for the bioactivity. The reduced activity of N-GEV might be associated with the lower binding affinity towards Na^+/K^+-ATPase rather than enzyme inhibition. This can be explained as the sugar moiety is not necessary for the inhibition of the Na^+/K^+-ATPase [37]. However, the glycosylation pattern and hydrophilic surface have an influence on the binding affinity. Steric hindrance also has a great impact on the proper orientation of the molecule in the enzyme complex [38]. In addition, the sugar moiety is described as the key feature towards specificity of CG on Na^+/K^+-ATPase binding [38, 39]. Amino acids within the core regions of the protein are highly conserved, however, amino acids around the entrance of the CG binding cavity show isoform specific differences [38, 40]. The non-natural 1–3 linkage found in N-GEV is influencing the binding affinity. The sugar residues have been demonstrated to stabilize the enzyme-CG complex, especially by the interactions of the 3′-α-hydroxyls with both proton-donating and proton-accepting groups on the enzyme [41]. Possible amino acids for the interaction with the 3′-α-hydroxyl group are Glu-319 and Arg-887 (aa numbering for the shark Na^+/K^+-ATPase) [42]. For N-GEV, the position of 3′-α-hydroxyls is blocked, and therefore the stability of the CG-enzyme complex is decreased, resulting in a lower bioactivity.

In summary, we described two different approaches to obtain the antiviral and cytotoxic cardiac glycoside GEV. The synthesis of EV from DTG was optimized, so EV could be used as a precursor for both approaches and also for further pharmaceutical studies, as EV also shows promising anticancer activity [12]. The molar product yield of GEV obtained by the biotransformation reaction with plant suspension cultures was only half of that achieved by chemical semisynthesis. The straightforward chemical synthesis approach produces bulk amounts of the non-commercially available GEV, which are required for further pharmacological and pharmaceutical studies, including *in vivo* studies. In addition, the reaction side product N-GEV showed lower cytotoxic activity



▶ **Fig. 5** *Dl*K1OHD suspension cell extract of biotransformation reaction with EV (**2**) as the substrate. **A** Chromatogram of extracted cells, 5 h after feeding in light conditions. **B** Chromatogram of extracted cells, previously adapted to the dark, 5 h after feeding in dark conditions. GEV (**3**): Rt = 14.8 min; EV (**2**): Rt = 22.0 min; internal standard digitoxigenin (IS): Rt = 23.8 min. For chromatographic conditions, see Material and Methods.

▶ **Table 2** Cytotoxic effects of GEV and N-GEV on renal cell carcinoma (Caki-1,786-O) and prostate cancer (DU-145 and PC3) cell lines and inhibition of Na^+/K^+-ATPase. IC_{50} values for cytotoxicity are in nM (48 h) and IC_{50} values for the α1, 2, 3 subunit of the porcine Na^+/K^+-ATPase inhibition are in µM. The IC_{50} values represent the mean from three experiments each consisting of three technical replicates; values for DTG were evalutated by * [35] and † [36].

Compound	Caki-1	786-O	DU-145	PC3	Inhibition Na^+/K^+-ATPase
GEV (3)	25.66 nM	25.54 nM	27.82 nM	38.01 nM	5.50 µM
N-GEV (4)	n. d.	1513 nM	1998 nM	911.9 nM	14.0 µM
EV (2)	54.69 nM	21.27 nM	27.30 nM	69.53 nM	2.57 µM
DTG (1)	33.31 nM†	57.13 nM†	43.80 nM*	121.8 nM*	12.1 µM†

against tumor cell lines than GEV. This may be explained by a lower affinity of N-GEV to the targeted Na^+/K^+-ATPase or a reduced stability of the inhibitor-enzyme complex.

Material And Methods

Isolation of digitoxin from *Digitalis lanata* extract

Digitoxin was isolated and purified from a *D. lanata* methanolic extract enriched in A-series cardenolide by flash chromatography. Detailed isolation and purification protocol is provided in Supporting Information.

General procedure for preparation of evatromonoside

The synthesis of EV was based on the US Patent 3,856,944 (1974) [20], according to the examples 1, 18, and 7 thereof. Some modifications were introduced regarding solvents, the molar ratio of reactants, and the hydrolysis conditions to optimize the preparation procedure. The reaction was conducted in two main steps. The first one includes the obtaining of digitxogenin-bisdigitoxo-

side and the second the preparation of evatromonoside. Detailed synthesis protocol is available in Supporting Information.

Chemical synthesis of glucoevatromonoside

The chemical synthesis of GEV consisted of two individual reaction steps containing an intermediate purification step of the interim reaction compounds. First, a glycosylation reaction was performed after Beale and Taylor [18], whereas removal of the acetyl groups was based on Zhou and O'Doherty [19].

Literature reaction (reaction 1). The reaction was performed according to Beale and Taylor [18], with EV as the substrate. To EV (50 mg, 0.1 mmol) and Ag_2O (47 mg, 0.2 mmol), 2-aminoethyldipheynlborinate (5.6 mg, 0.025 mmol) in 2 mL dried CH_2Cl_2 and ABG (82.5 mg, 0.2 mmol) in 3 mL CH_2Cl_2 were added. The reaction was stirred for 20 h at room temperature. The reactional medium was diluted with dichloromethane, filtrated, and the organic solvent was evaporated under reduced pressure for purification.

Reaction with double proportion of reagents (reaction 2). The reaction was performed as described for reaction 1, but with changes in the amounts of reagents: EV (100 mg, 0.2 mmol) and Ag_2O (95 mg, 0.4 mmol) were added to 2-aminoethyldipheynlborinate (11.25 mg, 0.05 mmol) in 2 mL dried CH_2Cl_2 and ABG (165.2 mg, 0.4 mmol) in 3 mL CH_2Cl_2. The reactional medium was diluted with dichloromethane, filtrated, and the organic solvent was evaporated under reduced pressure for purification.

Reaction in the dark (reaction 3). The reaction was performed as described for reaction 1. However, the suspension was stirred for 20 h at room temperature in the dark. Afterwards, the reaction medium was diluted with dichloromethane, filtrated, dried, and the reaction mixture was analyzed on TLC and UPLC-ESI-MS.

Optimized reaction conditions (reaction 4). EV (100 mg, 0.2 mmol) and Ag_2O (95 mg, 0.4 mmol) were added to a glass flask containing a stir bar. The substrate was dissolved by adding the solutions of 2-aminoethyldiphenylborinate (11.25 mg, 22.5 mM, 0.05 mmol) in dried CH_2Cl_2 (2 mL) and acetyl protected glycosyl bromide (2,3,4,6-tetra-*O*-acetyl-*α*-D-glucopyranosyl bromide, ABG, 165.2 mg, 133 mM, 0.4 mmol) in dried CH_2Cl_2 (3 mL). The resulting suspension was stirred at room temperature in total for 20 h in the dark. After 15 h, the reaction was monitored by TLC. A mobile phase of ethyl acetate : dichloromethane (1 : 1) was used for elution, and detection was made by spraying anisaldehyde/sulphuric acid and Kedde reagent. In case there was still more than around 30 % of substrate remaining, quantified by ImageJ software (https://imagej.nih.gov/ij/, 1.48 v), the volume of the reaction mixture was reduced by evaporation and the reaction was stopped, at the latest, after 20 h by diluting the suspension with CH_2Cl_2 and filtrating the reaction mixture twice. The organic solvent was evaporated and the reaction mixture was purified by silica gel column chromatography.

Non-catalyzed reaction. EV (50 mg, 0.1 mmol) and silver oxide (47 mg, 0.2 mmol) were dissolved in a solution of 82.5 mg ABG in 5 mL of dichloromethane. The reaction was stirred for 20 h at room temperature. Afterwards, the reactional medium was diluted with dichloromethane, filtrated, and the reaction mixture was analyzed on TLC.

For each reactional condition assayed, the resulting materials were purified by flash column chromatography (0 = > 100 % ethyl acetate in dichloromethane, 10 mL fractions, 0, 20, 40, 50, 60, 80, 100 %).

Hydrolysis of acetyl groups. $LiOH^*H_2O$ (30 mg, 715 µmol, 1 : 3) was added to a solution of the acetylated product (200 mg, 240 µmol) in a mixture of MeOH/H_2O (4 : 1, 50 mL), and the reaction was stirred for a maximum of 1 h at room temperature. The reaction was then neutralized with 0.05 M HCl (17 mL). The organic solvent was reduced and the remaining residue was diluted with water (17 mL). The mixture was extracted with 3 × 50 mL EtOAc and washed with 3 × 50 mL water. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The reaction was analyzed by UPLC-ESI-MS and TLC. For the latter, a mobile phase of methanol : acetone (5 : 95) was used.

Biotransformation experiments and purification of glucoevatromonoside

Suspension cell cultures K1OHD of *D. lanata* that were described having a high glycosyltransferase activity [25] were subcultured weekly to fresh MS media. Fifteen g of cells were transferred to an Erlenmeyer flask containing 100 mL of growth medium. Erlenmeyer flasks were kept on rotary shakers (110 rpm) at 21 ± 2 °C under 16 h of light and 8 h of dark.

Erlenmeyer flasks containing 100 mL of growth medium and 15 g of cell suspensions culture of *D. lanata*, 3 days old, were feed with 1 mL of the substrate solution in DMSO (fc: 0.3–1.2 mM) under aseptic conditions. Each experiment included a control with 1 mL of DMSO and a media control with the respective substrate. After feeding, the Erlenmeyer flasks were kept on a rotary shaker (110 rpm) at 21 ± 2 °C under 16 h of light and 8 h of dark or completely in the dark. To enhance the glycosylation reaction, 50 µM methyl jasmonate and 10 mM ascorbate were added to the suspension cell culture. In addition, the biotransformation experiment was conducted at 35 °C. Cell material (1 g) and 1 mL of growth medium aliquots were removed for analysis after intervals of 1, 24, 48, 96, 168 and 240 h of feeding.

The frozen cells (300 mg) were extracted with 600 µL of HPLC grade methanol in a 2-mL Eppendorf flask by vortexing and keeping the mixture in an ultrasonic bath for 10 min at 40 °C. Ten µL of methanol solution of digitoxigenin (stock solution: 10 mg/mL) were added as the internal standard. Afterwards, the extract was centrifuged for 10 min at 10 000 *g* and the supernatant was collected and analyzed by HPLC-ESI-MS or TLC.

The growth medium (0.5 mL) was extracted with 250 µL of chloroform : isopropanol (3 : 2, v/v) in a 2-mL Eppendorf flask. Ten µL a methanol solution of digitoxigenin (stock solution 10 mg/mL) were added as the internal standard. The mixture was kept in an ultrasounic bath at room temperature for 5 min. The extract was centrifuged for 5 min at 10 000 *g* and the organic phase was separated and evaporated in a heating block (34 °C) to dryness. The samples were kept at − 18 °C until analysis. The residues were dissolved in 100 µL HPLC grade methanol and analyzed by HPLC or TLC.

Suspension cells were harvested by vacuum filtration and grounded to a fine powder by liquid nitrogen. The cells (15 g) were extracted by MeOH (30 mL) under sonication (30 min). After

filtration of the grounded cell material, the methanol was removed by evaporation under reduced pressure at 50 °C and the resulting residue was purified by flash chromatography over a silica gel column (0 = > 100 % acetone in dichloromethane; 0, 60, 70, 100 %; 20 > 100 % MeOH in acetone, 20 %, 100 %). Fractions eluted with methanol : acetone (20 : 80) were shown to concentrate GEV, according to TLC analysis. They were combined, the solvent removed, and the obtained residue (55 mg) submitted to purification over a Sephadex LH20 column. Isocratic elution with methanol afforded 130 fractions, 1 mL each, which were analyzed by TLC or HPLC after solvent removal.

Chromatography analysis

Detailed information for UPLC-ESI-MS, HPLC, and TLC analysis are provided in Supporting Information.

NMR analysis

NMR spectra were recorded on a JEOL Alpha500 spectrometer (500 MHz, 11.7 T) by using a tunable 5 mm probe head and an inverse field gradient 5 mm probe head, respectively. Detailed information on NMR analysis is available in Supporting Information.

Cell lines

The renal cell carcinoma cell lines (Caki-1, 786-O) and the prostate cancer cell lines (DU145, PC-3) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured in RPMI-1640 medium or DMEM (Caki-1, 786-O, and PC-3; Sigma) supplemented with 10 % heat-inactivated fetal bovine serum, 1 nmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2.

MTT assays

The colorimetric MTT assay was performed using 3-(4,5-dimethyl-1,3-thiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT). Briefly, prostate (DU-145 and PC3) and renal (Caki-1, 786-O) cells were seeded in 96-well culture plates (6×10^3 and 4×10^3 cells/well, respectively) and, after 24 h, were treated with different concentrations of the compounds. Then, 24, 48, and 72 h later, the medium was replaced by 50 µL of MTT reagent (1 mg/mL) and the cells were incubated for a further 4 h. DMSO was added in order to dissolve the formazan crystals, and the absorbance was measured at 570 nm using a plate spectrophotometer (VersaMax ELISA Microplate Reader, Molecular Devices). Cells treated with 0.5 % DMSO served as a negative control to define 100 % of viability. The percentage of viable cells was plotted against the compound concentration and the IC_{50} values were determined by nonlinear regression and calculated using log (compound) compared with a normalized response (variable slope) using GraphPad Prism 5 Software (GraphPad).

Na^+/K^+-ATPase assay

Enzymatic activities of the Na^+/K^+-ATPase α1, 2, 3 subunit of porcine cortex (Sigma) were assayed as described by [36, 43]. Detailed information of the assay conditions is provided within the Supporting Information.

Statistical analysis

All data are expressed as the mean ± standard deviation of the mean. Means between the various groups were compared by one-way analysis of variance (ANOVA followed by Tukey's post hoc test). In case of multiple comparisons, a post hoc Bonferroni correction was applied. P values < 0.001 were considered statistically significant. Data were analyzed using GraphPad Prism 5 Software (GraphPad).

Supporting information

UPLC/MS data and HPLC-DAD chromatograms obtained for compounds **1**–**6**, isolation and purification protocol for compound **1**, a plot of the HMQC-NMR spectrum of compound **2** as well as the dose-response curves of MTT assays for compounds **2**–**4** and the conditions of the Na^+/K^+-ATPase assay along with the bar graphs for compounds **1**–**4** are available as Supporting Information. Detailed descriptions of EV (**2**) synthesis and experimental conditions of UPLC-ESI-MS, HPLC, TLC, and NMR analysis are also provided as Supporting Information.

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Conflict of Interest

All authors declare to have no conflicts of interest.

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