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Note

# Simultaneous preparation of α/β-zearalenol glucosides and glucuronides

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ABSTRACT

An improved and reproducible procedure for the preparation of four different glycosides of the mycotoxins α- and β-zearalenol (α,β-ZEL), both metabolites of the *Fusarium* toxin zearalenone (ZEN), is reported. These conjugated or masked mycotoxins are formed during phase II metabolism in plants (glucosides) or animals and humans (glucuronides). Improved regioselective Königs–Knorr glucuronidation was applied to ZEN followed by reduction of the keto group of the mycotoxin, leading to α- and β-configuration of ZEL and also to a partial reduction of the glucuronic acid methyl ester to obtain the corresponding glucosides. After deprotection of the sugar moiety, α- and β-zearalenol-14-β,D-glucuronide as well as the corresponding glucosides were isolated at once using preparative HPLC. The reduction step was studied under different reaction conditions to finally develop an optimized and also tunable procedure for the first simultaneous preparation of both, glucosides and glucuronides of a xenobiotic substance in reasonable amounts to be used as reference materials for bioanalytical and toxicological investigations.



Zearalenone (ZEN, **1**, Fig. 1A) is a mycotoxin produced by several plant pathogenic *Fusarium* species, including *F. graminearum*, *F. culmorum* and *F. cerealis*. This mycotoxin is common in maize, but barley, oats, wheat and rice are also susceptible to contamination with ZEN.[1] *Fusarium* species are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found in cereals grown not only in America, Europe and Asia, but also in Africa.[2,3] ZEN and its phase I metabolites possess estrogenic activity in mammals, including pigs, cattle and sheep.[4] Problems of the reproductive tract as well as impaired fertility and abnormal fetal development in farm animals can be caused by ZEN.[5] Biotransformation of ZEN involves the formation of α- and β-zearalenol (α,β-ZEL, Fig. 1A), the major mammalian phase I metabolites,[6] which are subsequently conjugated with glucuronic acid to form the corresponding glucuronides.[7] Both α-ZEL (**2α**) and β-ZEL (**2β**) are also produced by *Fusarium* spp. in lower concentrations than ZEN and frequently contaminate cereals and other plant products.[8,9] Rat erythrocytes and the intestinal mucosa of swine have also been shown to form α- and β-ZEL by the reduction of ZEN.[10,11] Furthermore, these metabolites can interfere with various enzymes involved in steroid metabolism, which was recently investigated.[12–14] The estrogenic activity of α-ZEL is even higher than that of ZEN.[15] Therefore, ZEN and its metabolites α- and β-ZEL are important from an agricultural, economic and health perspective.[16]

Additionally conjugated mycotoxins, especially glucosides (Fig. 1B), can emerge after metabolization by living plants. The occurrence of ZEN-14-β,D-glucoside (**3**) in wheat was shown by Schneweis et al.[17] The formation of α,β-ZEL-14-β,D-glucosides (**4α**/**4β**) by plant enzymes was investigated in maize cell suspension cultures[18] and in *Arabidopsis thaliana*.[19] Awareness of such altered forms, often called masked mycotoxins, is increasing, but reliable analytical methods, standards as well as occurrence and toxicity data are still scarce.[20,21]

Conjugation with glucuronic acid is one of the major metabolic pathways of mycotoxins in animals as well as humans and is important for inactivation and excretion,[4,22–24] especially in the case of α- and β-ZEL.[25] These glucuronides are excreted in urine and could therefore serve as possible biomarkers for daily uptake measurement.[26] Furthermore, detailed metabolic studies have been hindered by the lack of authentic specimens of mycotoxin glucuronides. Basically there are two sites for glycosylation present in ZEN and three in ZEL, but conjugation in position 14 is strongly favored compared to positions 7 (ZEL) and 16 (ZEN, ZEL)[22,27] (ring system numbering following the scheme proposed by Metzler[28]).

Synthesis of ZEN-14-β,D-glucoside (**3**) via the Königs–Knorr procedure under phase transfer conditions has been reported[29] but the analogous reaction to produce ZEN-14-β,D-glucuronide

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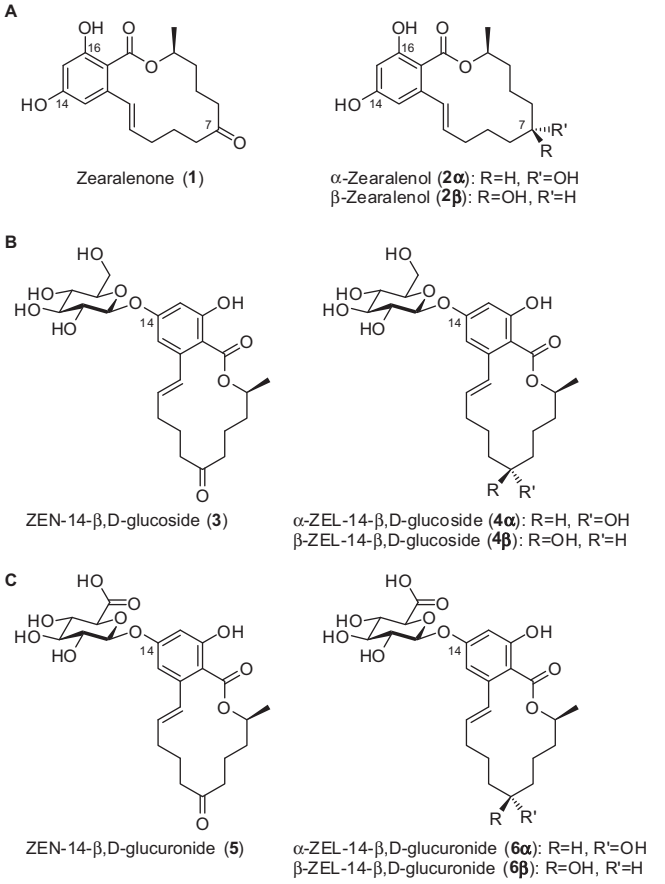
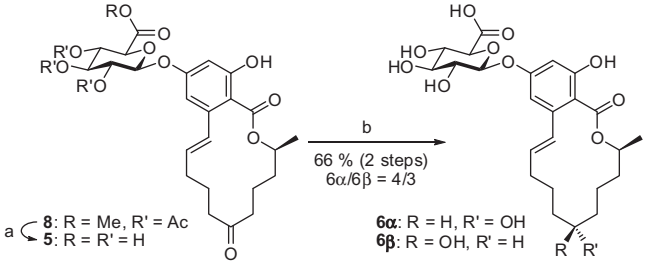



**Figure 1.** Structures of zearalenone (**1**), α/β-zearalenol (**2α**/**2β**) and corresponding 14-glycosides.

(**5**) was described to be unsuccessful under a variety of coupling conditions.[27] Therefore, Stevenson et al. used preparative enzymatic synthesis to obtain small amounts of glucuronides of ZEN and its metabolites for identification and characterization.[27] Nevertheless, we were able to develop a fast and reproducible chemical synthesis of ZEN-14-β,D-glucuronide (**5**) using standard equipment and common reagents, which was published very recently.[30] In the course of ongoing research in the emerging field of masked and conjugated mycotoxins we also focused on the synthesis of ZEL glucuronides (**6α**/**6β**, Fig. 1C).

We herein report an efficient and tunable method for the simultaneous preparation of α/β-zearalenol glucosides (Glc) and glucuronides (GlcA), applying optimized $NaBH_4$ reduction as key step after glucuronidation of ZEN. Since all chemicals and reagents are readily and commercially available, reproduction of this approach should be possible in other labs without much difficulty to obtain all glycosides in a short time. Additionally, this is the first described method for simultaneous preparation of glucosides and glucuronides of xenobiotic substances in reasonable amounts to be used as reference materials for bioanalytical and toxicological investigations.




**Scheme 2.** Preparation of ZEL-14-β,D-glucuronides by reduction of (crude) **5**. Reagents and conditions: (a) KOH, THF/$H_2O$; (b) $NaBH_4$, MeOH.

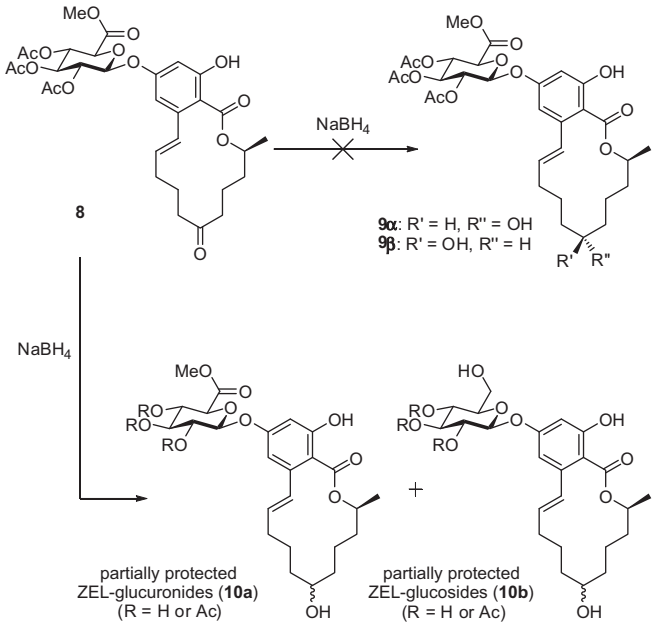
Starting from ZEN (**1**), an improved Königs–Knorr procedure was used for glucuronidation to obtain the protected glucuronide **8**. Compared to previously described results,[30] the yield of this step was significantly increased from 50% to 74%, using bromo sugar **7** as glucuronyl donor, activated by silver(I) oxide in dry acetonitrile (Scheme 1). High purity of $Ag_2O$ and highest available anhydrous quality of acetonitrile as well as the use of new and freshly activated molecular sieves were crucial for the outcome of this reaction.

As already mentioned above, the protected glucuronide **8** was previously used as intermediate for the synthesis of ZEN-14-β,D-GlcA (**5**). An optimized protocol applying potassium hydroxide in THF/water (4:1) gave the desired product in 78% yield (without undesired basic hydrolysis of the lactone moiety).[30] After reproducing this step we were able to prepare both zearalenol-14-β,D-glucuronides (**6α**, **6β**) by $NaBH_4$ reduction (Scheme 2) without purification of the intermediate **5** in an overall yield of 66% (α-ZEL-14-GlcA/β-ZEL-14-GlcA = 4/3) after separation using preparative high performance liquid chromatography (PHPLC).

By reaction of the protected glucuronide **8** with $NaBH_4$, we observed partial reduction of the glucuronic acid methyl ester as well as cleavage of acetyl groups to yield the corresponding partially deprotected glucuronides (**10a**) as well as glucosides (**10b**) even after 10 min of reaction time at −20 °C (Scheme 3).

Since this reduction step seemed to be applicable for fast and cost-effective simultaneous preparation of both, glucosides and glucuronides of zearalenol, the reduction of **8** was studied under different reaction conditions in terms of time and temperature and analyzed using LC–MS/MS (Table 1, Fig. 2A). Approximate quantification was done applying NLS (neutral loss scan) MS/MS mode, since breaking of the glycosidic bond forming oxocarbenium ions after neutral loss of ZEL (−319 amu) was identified as the predominant fragmentation of all considered products (as indicated in Table 1). Masses (*m*/*z*) of molecule ions of all detected compounds


**Scheme 1.** Improved Königs–Knorr procedure for glucuronidation of zearalenone (**1**).




**Scheme 3.** Reaction of protected ZEN-14-β,D-glucuronide (**8**) with $NaBH_4$ leading to partially protected ZEL-glucuronides (**10a**) and ZEL-glucosides (**10b**).

were used to identify (partially de-acetylated) glucosides and glucuronides.

During this analysis ZEL configured glycosides were not distinguished. Partial reduction of the glucuronic acid methyl ester was obtained already after 10 min of reaction time at −20 °C and as expected, this reaction proceeds faster at elevated temperatures. At 0 and 20 °C also undesired partial reduction of the macrocyclic lactone of ZEL was observed after 24 h or even 30 min, respectively, indicated by appropriate *m*/*z* values of molecule ions and a neutral loss of 323 amu (Fig. 2B).

Therefore, we decided to carry out this reduction at preparative scale at −20 °C and for a reaction time of 60 min to obtain ZEL-14-β,D-glucuronides as well as ZEL-14-β,D-glucosides in comparable amounts, avoiding degradation of the conjugated mycotoxin. After reaction of **8** with $NaBH_4$ and subsequent deprotection applying KOH in THF/water, the crude product mixture was separated by PHPLC yielding all desired ZEL-glycosides (**4α**, **4β**, **6α**, and **6β**) in reasonable yields (Scheme 4) and molecular ratios, which were in good agreement with data obtained by LC–MS/MS analysis (Table 1).

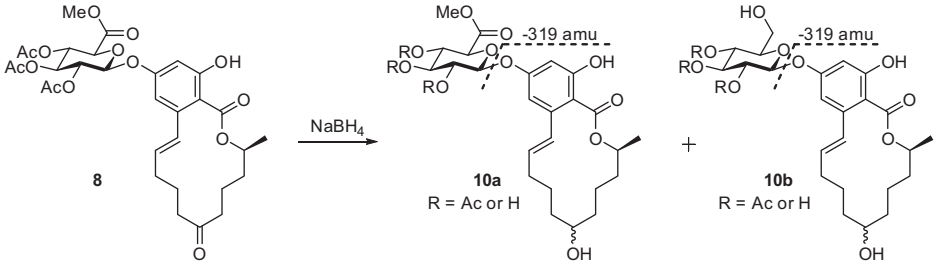
In summary, efficient and reproducible procedures for the synthesis of ZEL-14-β,D-glucuronides and ZEL-14-β,D-glucosides were developed. Protected ZEN-glucuronide **8** was prepared applying optimized Königs–Knorr glucuronidation and used as key intermediate for selective synthesis of ZEL-14-β,D-glucuronides (**6α, 6β**) by reduction of ZEN-14-β,D-glucuronide (**5**) as well as for simultaneous preparation of ZEL-14-β,D-glucosides (**4α, 4β**) and ZEL-14-β,D-glucuronides (**6α, 6β**) applying a tunable procedure for $NaBH_4$ reduction of **8** and subsequent basic hydrolysis of remaining acetyl protective groups. Since all chemicals and reagents are commercially available, these methods can be reproduced in other labs to obtain the desired mycotoxin conjugates on milligram scale for further investigations. Furthermore optimized $NaBH_4$ reduction of protected glucuronic acid methyl esters can be applied for fast and simultaneous preparation of glucosides and glucuronides of other xenobiotic compounds yielding reasonable amounts of potential phase II conjugates for bioanalytical and toxicological studies.

## 1. Experimental

### 1.1. General methods

All reactions were performed under an argon atmosphere. The progress of reactions was monitored by thin-layer chromatography (TLC) over silica gel 60 F254 (Merck). The chromatograms were visualized by irradiation with ultraviolet light or by heat staining with ceric ammonium molybdate in ethanol/sulfuric acid. LC-ESI-MS/MS was performed on an HCT ion trap mass spectrometer (Bruker, Germany) in full scan and NLS (neutral loss scan) mode. Chromatographic separation was done on a 1200 series HPLC system (Agilent Technologies, Germany) using a Luna RP-C18 column

**Table 1**
Yields of $NaBH_4$ reduction[a] of **8** at different reaction temperatures, yielding (partially) protected ZEL-glucuronides (**10a**) and ZEL-glucosides (**10b**),[b] quantified by LC–MS/MS




| Reaction time (min) | Conversion to partially protected ZEL-14-β,D-glucosides (**10b**) at different reaction temperatures ($b/(a+b)$ mol %)[b,c] | | |
|---|---|---|---|
| | −20 °C | 0 °C | 20 °C |
| 10 | 11 | 32 | 47 |
| 30 | 34 | 50 | 53[c] |
| 60 | 42 | 54 | [d] |
| 120 | 44 | 55 | [d] |
| 300 | 51 | 57 | [d] |
| 1200 | 61 | [d] | [d] |

[a] 10 M equiv of $NaBH_4$ added at once to a solution of **8** in MeOH.
[b] **10a** + **10b** = 100 mol %.
[c] *a* = mol % **10a**; *b* = mol % **10b**.
[d] Neutral loss of 323 amu indicated (partial) reduction of the macrocyclic lactone.

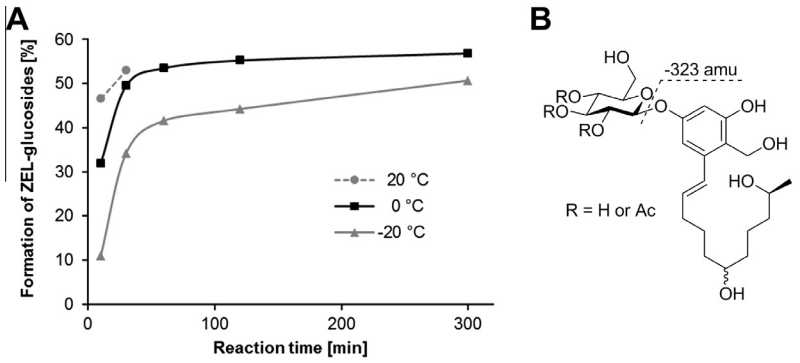
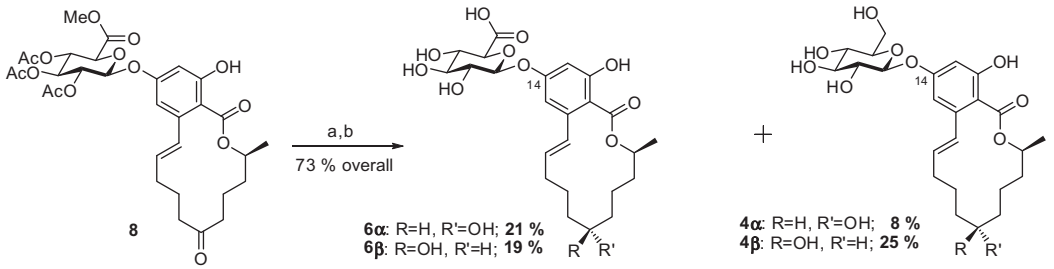



**Figure 2.** (A) Formation of (partially) protected ZEL-14-β,D-glucosides (**10b**) by $NaBH_4$ reduction of **8** depending on reaction time ($t$ = 10–300 min) and temperature (see also Table 1 for further details); (B) undesired reduction of the macrocyclic lactone indicated by a neutral loss of 323 amu.




**Scheme 4.** Simultaneous preparation of ZEL-14-β,D-glucuronides (**6α**, **6β**) and ZEL-14-β,D-glucosides (**4α**, **4β**). Reagents and conditions: (a) $NaBH_4$, MeOH, −20 °C, 1 h; (b) KOH, THF/$H_2O$, 20 °C, 4 h, PHPLC.

(3.0 × 150 mm, 3 μm particle size, Phenomenex, Germany) in combination with a linear acetonitrile/water gradient (10–90% acetonitrile) containing 0.1% acetic acid and a flow rate of 1 mL/min. Application of pure substances was achieved using a TLC-MS interface (Camag, Germany). Preparative HPLC separation was done on an 1100 Series preparative HPLC system (Agilent Technologies, Germany) using a SunFire Prep C18 OBD, 5 μm, 19 × 100 mm column (Waters, Germany) to separate the early eluting **4β** and the late eluting **6α**. Eluents were composed of water, MeOH and glacial acetic acid (A: 79.5:20:0.5, v:v:v; B: 0:99.5:0.5, v:v:v) and the flow rate was 16 mL/min. After an initial hold time at 30% B for 0.1 min, the proportion of B was linearly increased to 70% within the next 12.9 min, and to 100% B within another 0.1 min. Thereafter, the column was flushed with 100% B for 1.8 min followed by column re-equilibration at 30% B for 2 min. The retention times were 5.0 for **4β**, 7.25 and 7.65 for the not fully separated mix of **4α** and **6β** and 10.0 for **6α** (for chromatograms see Supplementary data). A second run using a Zorbax Eclipse XDB C8 Prep HT, 5 μm, 21.2 × 150 mm column (Agilent Technologies, Germany) was necessary to separate **6β** and **4α**. The same eluents and flow rate as for the first preparative HPLC step were used and the gradient was modified to the following conditions: The starting conditions of 20% B were held for 0.1 min. Then a linear gradient to 60% B in 12.9 min was applied, followed by an increase to 100% B within the next 0.1 min. As before, the column was flushed with 100% B for 1.8 min followed by column re-equilibration at 20% B for 2 min. The retention times of 8.4 min for **6β** and 11.4 min for **4α** allowed good separation (for chromatograms see Supplementary data). LC–HR–MS(/MS) spectra were acquired on an 6550 iFunnel Q-TOF instrument after electrospray ionization coupled to a 1290 Infinity UHPLC system (both Agilent Technologies, Germany). A Zorbax SB C18 Rapid Resolution High Definition column (150 × 2.1 mm, 1.8 μm particle size, Agilent Technologies, Germany) was used in combination with a MeOH–water gradient containing 0.1% formic acid. Preparative column chromatography was performed on silica gel 60 (40–63 μm, Merck, Germany) using a Sepacore™ Flash System (Büchi, Switzerland). $^1H$ and $^{13}C$ NMR spectra were recorded on an Avance DRX-400 MHz spectrometer (Bruker, Germany). Data were recorded and evaluated using TOPSPIN 1.3 (Bruker Biospin). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals. Zearalenone was obtained from Fermentek (Israel) and all other chemicals were purchased from ABCR (Germany) or Sigma–Aldrich (Austria/Germany).

### 1.2. Preparation of zearalenone-14-(2,3,4-tri-*O*-acetyl-β,D-glucuronic acid methyl ester) (8)[30]

To a solution of 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-β,D-glucuronic acid methyl ester (**7**) (99.3 mg, 0.25 mmol) and ZEN (31.8 mg, 0.1 mmol) in dry acetonitrile (2 mL) powdered molecular sieves (3 Å, 100 mg) were added. The reaction mixture was stirred for 3 h at room temperature. $Ag_2O$ (34.8 mg, 0.15 mmol) was added and stirring was continued in the dark for 24 h. After further addition of **7** (99.3 mg, 0.25 mmol) and $Ag_2O$ (34.8 mg, 0.15 mmol), reaction control via TLC indicated full consumption of ZEN after 24–36 h. The reaction mixture was diluted with dichloromethane, filtered through celite, concentrated and subjected to column chromatography (hexanes/EtOAc, gradient elution from 8/1 to 1/1) to yield **8** (47.0 mg, 74%) as a white solid: $^1H$ NMR (acetone-$d_6$): $\delta$ 11.85 (s, 1H, OH), 7.05 (d, 1H, $J_{11,12}$ 15.4 Hz, H-12), 6.65 (d, 1H, $J_{13,15}$ 2.4 Hz, H-13), 6.57 (d, 1H, H-15), 5.82 (ddd, 1H, $J_{10a,11}$ 10.3, $J_{10b,11}$ 4.5 Hz, H-11), 5.75 (d, 1H, $J_{1',2'}$ 7.9 Hz, H-1'), 5.48 (t, 1H, $J_{2',3'}$ 9.6, $J_{3',4'}$ 9.6 Hz, H-3'), 5.26 (t, 1H, $J_{4',5'}$ 9.7 Hz, H-4′), 5.25 (dd, 1H, H-2′), 5.05 (sext, 1H, $J_{3,4}$ 5.9, $J_{3,Me}$ 5.9 Hz, H-3), 4.70 (d, 1H, H-5′), 3.72 (s, 3H, $OCH_3$), 2.87–2.76 (m, 1H, H-8a), 2.66–2.58 (m, 1H, H-6a), 2.37–2.04 (m, 5H, H-6b, H-8b, H-9a, H-10a, H-10b), 2.04 (s, 3H, $COCH_3$), 2.02 (s, 3H, $COCH_3$), 2.01 (s, 3H, $COCH_3$),

1.85–1.75 (m, 2H, 2 H-5), 1.70–1.65 (m, 2H, 2 H-4), 1.62–1.50 (m, 1H, H-9b), 1.41 (d, 3H, $CH_3$); $^{13}C$ NMR (acetone-$d_6$): $\delta$ 209.4 (s, 1C, C-7), 171.0 (s, 1C, C-1), 169.3 (s, 1C, $COCH_3$), 169.0 (s, 1C, $COCH_3$), 168.8 (s, 1C, $COCH_3$), 166.8 (s, 1C, C-6′), 164.7 (s, 1C, C-16), 160.7 (s, 1C, C-14), 143.5 (s, 1C, C-12a), 133.3 (d, 1C, C-11), 132.3 (d, 1C, C-12), 108.6 (d, 1C, C-13), 106.0 (s, 1C, C-16a), 102.6 (d, 1C, C-15), 97.0 (d, 1C, C-1′), 73.9 (d, 1C, C-3), 72.0 (d, 1C, C-5′), 71.6 (d, 1C, C-3′), 70.7 (d, 1C, C-2′), 69.2 (d, 1C, C-4′), 52.1 (q, 1C, $OCH_3$), 42.5 (t, 1C, C-6), 36.0 (t, 1C, C-8), 34.5 (t, 1C, C-4), 30.9 (t, 1C, C-10), 21.9 (t, 1C, C-5), 20.8 (t, 1C, C-9), 19.8 (q, 1C, $CH_3$), 19.6 (q, 1C, $COCH_3$), 19.6 (q, 1C, $COCH_3$), 19.5 (q, 1C, $COCH_3$); ESI-MS: *m*/*z* 657 $[M+Na]^+$; HRMS: *m*/*z* calcd for $C_{31}H_{38}O_{14}Na$ $[M+Na]^+$: 657.2154. Found: 657.2171.

### 1.3. General procedure for deprotection of glycosides

Starting material (**8** or crude **10a**/**10b**) was dissolved in THF/water (4:1, 5 mL/100 μmol), KOH (10 equiv) was added and the reaction mixture was stirred for 4 h at room temperature. After addition of 0.1 N HCl (pH 3), the solution was diluted with water and extracted with EtOAc. The combined organic layer was dried over $Na_2SO_4$ and concentrated to yield the crude product(s).

### 1.4. General procedure for $NaBH_4$ reduction

The starting material (crude **5** or **8**) was dissolved in dry MeOH (20 mL/0.1 mmol) and reacted with $NaBH_4$ (10 equiv) for 1 h at 20 °C (reduction of **5**) or −20 °C (reduction of **8**), respectively. The reaction mixture was quenched with diluted acetic acid (3% in $H_2O$, 50 mL/0.1 mmol) and immediately extracted with EtOAc. The organic layer was washed with satd. $NaHCO_3$ solution, dried over $Na_2SO_4$ and concentrated.

### 1.5. General procedure for LC–MS/MS analysis of $NaBH_4$ reduction of 8

Protected glucuronide **8** (5 mg, 7.9 μmol) was dissolved in dry MeOH (2 mL). $NaBH_4$ (3 mg, 79 μmol) was added at the appropriate temperature and the reaction mixture was stirred for 20 h. Samples (100 μL) after 10, 30, 60, 120, 300 and 1200 min were diluted with EtOAc (1 mL) and washed with 0.5 N HCl. The organic layer was separated, dried over $Na_2SO_4$ and diluted with MeOH (1:1). A volume of 0.5 mL was filtrated and used for LC–MS/MS analysis.

### 1.6. Preparation of α,β-ZEL-14-β,D-glucuronides (6α, 6β)[27] and α,β-ZEL-14-β,D-glucosides (4α, 4β)[31]

Starting from protected ZEN-14-β,D-glucuronide **8** (5 mg, 7.9 μmol), deprotection and subsequent $NaBH_4$ reduction of the crude intermediate **5** at 20 °C, following the general procedures described above, yielded glucuronides **6α** (1.5 mg, 38%) and **6β** (1.1 mg, 28%).

$NaBH_4$ reduction of **8** (15.9 mg, 25 μmol) at −20 °C, followed by deprotection of the resulting mixture of partially protected glucuronides (**10a**) and glucosides (**10b**), simultaneously led to **6α** (2.6 mg, 21%), **6β** (2.4 mg, 19%), **4α** (1.0 mg, 8%) and **4β** (3.0 mg, 25%) after preparative HPLC, yielding the desired products as white solids: $^1H$ and $^{13}C$ NMR data matched those reported;[27,31] **6α**: ESI-MS: *m*/*z* 495 $[M-H]^-$; HRMS: *m*/*z* calcd for $C_{24}H_{31}O_{11}$ $[M-H]^-$: 495.1872. Found: 495.1881; **6β**: ESI-MS: *m*/*z* 495 $[M-H]^-$; HRMS: *m*/*z* calcd for $C_{24}H_{31}O_{11}$ $[M-H]^-$: 495.1880. Found: 495.1877; **4α**: ESI-MS: *m*/*z* 505 $[M+Na]^+$; HRMS: *m*/*z* calcd for $C_{24}H_{33}O_{10}$ $[M-H]^-$: 481.2079. Found: 481.2079; **4β**: ESI-MS: *m*/*z* 505 $[M+Na]^+$; HRMS: *m*/*z* calcd for $C_{24}H_{33}O_{10}$ $[M-H]^-$: 481.2079. Found: 481.2077.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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2013.03.002.

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