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Enzyme-assisted synthesis and structure characterization of glucuronic acid conjugates of losartan, candesartan, and zolarsartan

Anna Alonen [a], Johanna Jansson [a], Sirkku Kallonen [a], Alexandros Kiriazis [a], Olli Aitio [b], Moshe Finel [c], Risto Kostiainen [a,*]

[a] *Faculty of Pharmacy, Division of Pharmaceutical Chemistry, University of Helsinki, P.O. Box 56 (Viikinkaari 5 E), FIN 00014 University of Helsinki, Finland*
[b] *Program in Structural Biology and Biophysics, Institute of Biotechnology, NMR Laboratory, University of Helsinki, P.O. Box 56 (Viikinkaari 9), FIN 00014 University of Helsinki, Finland*
[c] *Faculty of Pharmacy, Drug Discovery and Development Technology Center, University of Helsinki, P.O. Box 56 (Viikinkaari 5 E), FIN 00014 University of Helsinki, Finland*

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Abstract

Three angiotensin II receptor antagonists—losartan, candesartan, and zolarsartan—were investigated. All the compounds, which are structural analogues, are metabolized via conjugation to glucuronic acid. Interestingly, both *O*- and *N*-glucuronidation take place, so that regioisomers are formed. One ether *O*-glucuronide, two acyl *O*-glucuronides, and five tetrazole-*N*-glucuronides were biosynthesized, in milligram scale, from the three sartan aglycones. Liver microsomes from bovine, moose, rat, and pig and recombinant human UDP-glucuronosyltransferases were used as catalysts. The synthesized compounds were identified as sartan glucuronides by mass spectrometry, while the sites of glucuronidation were determined by nuclear magnetic resonance spectroscopy. Drug metabolites are needed as standards for pharmaceutical research and, as the present study shows, they can easily be produced with enzymes as catalyst.

Keywords: Losartan; Candesartan; Zolarsartan; GR117289; Glucuronide; Biosynthesis; Regioisomer

1. Introduction

Glucuronidation is a phase II metabolism reaction in which glucuronic acid is conjugated to a small lipophilic compound [1–3]. The reaction is catalyzed by UDP-glucuronosyltransferases (UGTs), and the substrates are a wide range of endogenous and exogenous compounds, including hormones, dietary compounds, and drugs. Glucuronidation is an S_N2-reaction where the configuration of the glucuronic acid changes from α- to β-anomer. The most typical glucuronides are *O*- and *N*-glucuronides, while the *C*- and *S*-glucuronides are rare [2]. Glucuronidation increases the hydrophility of the aglycone, and the conjugate is more easily excreted from the body. Human UGTs are classified into families UGT1 and UGT2, and UGT2 is further divided into subfamilies UGT2A and UGT2B [4].

This work deals with three sartans: losartan, candesartan, and zolarsartan (GR117289). All three are receptor AT_1 antagonists that block the hypertensive effects of angiotensin II. Losartan and candesartan are presently in clinical use for the treatment of hypertension, and zolarsartan is known to be an active compound [5,6]. Losartan is partly metabolized to even more active carboxylic acid. Candesartan is released during the absorption process from its prodrug candesartan cilexetil via ester hydrolysis. Losartan, candesartan, and zolarsartan are structural analogues: they all have a tetrazole ring and there is a carboxylic acid group in candesartan and zolarsartan (Fig. 1). Losartan, candesartan, and zolarsartan can be metabolized through glucuronidation [7–12]. Interestingly, two or three different glucuronide conjugates are produced from each

* Corresponding author. Fax: +358 9 19159556.
E-mail address: risto.kostiainen@helsinki.fi (R. Kostiainen).

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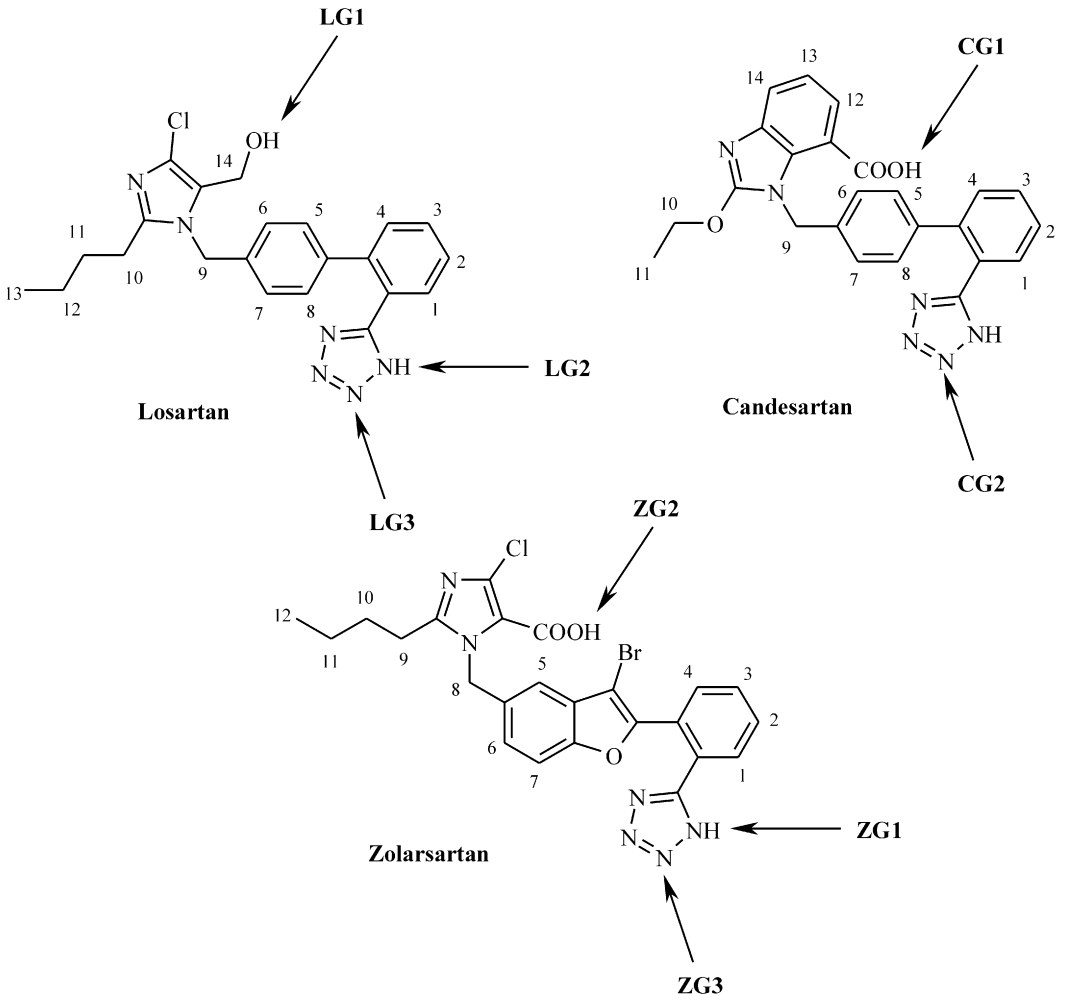

Fig. 1. Structures of losartan, candesartan, and zolarsartan. Arrows indicate the glucuronidation sites and the abbreviation for the regioisomer.

sartan. In other words, glucuronide regioisomers are formed. *O*-Glucuronides of sartans are produced from reaction at the hydroxyl or carboxylic acid groups and *N*-glucuronides from reaction at the tetrazole ring.

Glucuronidation at the carboxylic acid group produces acyl glucuronide carrying an ester linkage between the aglycone and glucuronic acid [13–16]. Owing to the electrophilicity of the ester group carbon, acyl glucuronides tend to be unstable. In addition to enzymatic hydrolysis catalyzed by esterases and β-glucuronidase in vivo, acyl glucuronides are prone to hydrolysis in vitro, especially at basic pH. In addition, intramolecular rearrangement often takes place in acyl glucuronides. In this migration reaction the acyl group rearranges to an adjacent hydroxyl group of the glucuronic acid, forming C-2 isomer and the reaction can further continue to C-3 and C-4 isomers. Acyl migration is more likely to take place at neutral to high pH (7–9) and elevated temperature. The acyl migration products, C-2, C-3, and C-4 isomers, are hemiacetals that can interconvert between β- and α-anomer. Hence, the lability of acyl glucuronides is strongly dependent on environmental factors, mainly pH and temperature, as well as on the particular aglycone.

Although glucuronidation is usually a detoxification reaction, the reactivity of acyl glucuronides due to the electrophilic carbon may lead to adverse drug reactions [13–16]. Either the acyl glucuronide itself or the migration isomers may cause unwanted effects by binding covalently to macromolecules, such as proteins or DNA. Thus, there is a clear need for activity and toxicity assays, and for synthetic glucuronides that can serve as analytical standards in pharmacological, toxicological, pharmacokinetic, and metabolism studies.

At present only a few glucuronide conjugates are commercially available. Both chemical [17,18] and enzymatic [19–26] methods have been exploited for the preparation of glucuronide conjugates. Biosynthesis of glucuronide metabolites is preferred when milligram scale yields are sufficient. Unlike traditional chemical syntheses, enzyme-catalyzed glucuronide reactions do not require multiple steps. Chemical syntheses usually produce the α-anomer and other byproducts in addition to the desired β-anomer, which leads to more complicated purification. In the case of aglycones that contain several possible glucuronidation sites, chemical syntheses will yield a mixture of mono- and polyglucuronides unless the unwanted glucuronidation

sites are protected. Enzymes produce mainly monoglucuronides, and they can be regio- and even stereoselective. Therefore enzymatic synthesis of glucuronides has gained increased interest in production of glucuronides in small-scale [19–26]. Although most of the enzymatic synthesis methods are focused on the production of *O*-glucuronides, the development of regioselective synthesis of *N*-glucuronide standards is important as well. The characterization of the synthesis products and especially the site of glucuronidation is a challenge. It seems that nuclear magnetic resonance spectroscopy (NMR) and especially two-dimensional NMR is required, since mass spectrometry (MS) and other techniques only seldom can offer specific information on the site of glucuronidation [8,24,27].

The aim of this study was to develop and optimize methods for enzyme-assisted synthesis of *O*- and *N*-glucuronides of losartan, candesartan, and zolarsartan. Depending on the substrate and the desired product, liver microsomes from various species or human recombinant UGT-enzymes were used as catalyst for the biosyntheses. Synthesis products were identified and characterized by tandem mass spectrometry (MS/MS) and NMR. The enzyme-assisted synthesis proved to be an easy and effective method to produce glucuronide metabolites from sartans. The metabolites that were obtained can be used as standard compounds, and the milligram amounts are sufficient for numerous analyses.

2. Materials and methods

2.1. Chemicals

Losartan (potassium salt), candesartan, and zolarsartan (GR117289) were donated by Merck (Rahway, NJ), AstraZeneca (Mölndal, Sweden), and GlaxoSmithKline (Hertfordshire, United Kingdom), respectively. Saccharic acid 1,4-lactone and uridine-5′-diphospho-α-D-glucuronic acid (trisodium salt) were purchased from Sigma (St. Louis, MO). All reagents and solvents were of analytical or high-performance liquid chromatography (HPLC) grade. Rat liver microsomes were prepared from Aroclor 1254 induced male Sprague–Dawley rats, and pig, moose, and bovine liver microsomes were from untreated animals, as previously described [28]. The recombinant human UGTs 1A3, 1A8, and 1A10 were expressed in baculovirus-infected insect cells [29,30]. Protein concentrations of microsomes and UGTs were measured with the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

2.2. Biosyntheses of sartan glucuronides

The reactions contained 5 mM saccharic acid 1,4-lactone, 5 mM UDP-glucuronic acid, 5 mM $MgCl_2$, and 50 mM phosphate buffer (pH 7.4). Recombinant human UGTs and liver microsomes from various animals were tested as the biocatalysts for the syntheses. Expect for the *ZG1* and *ZG3*, aglycone concentrations between 250 and 2000 μM were tested to find a suitable concentration for a particular glucuronide product. A lower range of 25–2000 μM was used for *ZG1* and *ZG3*, because of substrate inhibition. Protein concentrations (0.5–3.5 mg/mL) and incubation times (up to 50 h) were optimized as well. Optimization reactions were performed in small-scale (250 μL). On the basis of the small-scale incubations, the reaction volumes were then scaled up to produce milligram amounts of the target glucuronide. The details of the larger-scale incubations are presented in Table 1. Following the incubation, the pH of the losartan glucuronide reactions was adjusted to about 3 with H_3PO_4. The reactions of candesartan and zolarsartan glucuronides were not acidified since the substances would have precipitated under acidic conditions. Proteins were removed from the reaction mixture by centrifugation (26,700*g*, 10 min, 6 °C), and the supernatants were filtered and solid-phase extracted (Bakerbond C18 PolarPlus, 3 g; J.T.Baker, Phillipsburg, NJ).

The cartridges for the solid-phase extraction (SPE) were preconditioned with methanol (losartan and zolarsartan glucuronides) or acetonitrile (candesartan glucuronides) and 50 mM phosphate buffer, with pH 3 used for the extraction of losartan glucuronides and pH 7.4 for candesartan and zolarsartan glucuronides. After loading of the samples, the cartridges were washed with water. Since small proportions of the candesartan and zolarsartan glucuronides were eluted at the sample loading or washing step, the part of sample that came through the cartridge was extracted once again, with use of 50 mM phosphate buffer at pH 3 (candesartan glucuronides) and pH 7.4 (zolarsartan glucuronides) for washing. Again, part of the *ZG2* and *ZG3* came through the cartridge during the sample loading or washing step and the extraction was repeated once again with use of 50 mM phosphate buffer at pH 3 in both the conditioning and washing steps. Finally, the glucuronides were eluted with methanol (losartan and zolarsartan glucuronides) or acetonitrile (candesartan glucuronides). SPE elution solvents were evaporated and the residue was dissolved in the HPLC eluent (see below). (For the candesartan and zolarsartan glucuronides, water replaced acetic acid and formic acid, respectively.)

Sartan glucuronides were fractionated using an Agilent 1100 HPLC equipped with an autosampler, a UV multiple wavelength detector, and a fraction collector (Agilent Technologies, Waldbronn, Germany). The HPLC separation was performed by reversed phase chromatography using an HP Hypersil BDS C18 (5 μm 150 × 4.6 mm) column for the losartan glucuronides and an HP Hypersil BDS C18 (5 μm 250 × 4.0 mm) column for candesartan and the *ZG1*. For the fractionation of *ZG1*, also a semipreparative Merck LiChrocart® 250-10 LiChrospher® WP 300 RP-18 (12 μm) column was used. *ZG2* and *ZG3* were separated with an Agilent Zorbax Eclipse Plus C18 (5 μm 250 × 4.6 mm) column. The HPLC method for separation of the losartan glucuronides was isocratic with flow rate of 1 mL/min and the eluent was 20 mM ammonium acetate buffer (pH 4.5)—acetonitrile 73:27. Gradient elutions were

Table 1
Reaction conditions for the synthesis of losartan, candesartan, and zolarsartan glucuronides[a]

Glucuronide	Substrate (mg)	Protein (mg)[b]	Reaction volume (mL)	Incubation time (h)	Yield (mg)
LG1	34.6	50 (BLM)	50	24	0.5 (1.1%)
LG2	33.6[c]	n.d.[d] (UGT1A10)	50	48	0.9 (2.2%)
LG3	20.8	60 (MLM)	30	5	2.0 (7.4%)
CG1	22.1	77 (UGT1A8)	50	48	0.9 (2.8%)
CG2	26.4	99 (UGT1A3)	40	48	1.5 (4.0%)
ZG1	7.9	77 (RLM)	70	7	3.6 (34.6%)
ZG2	28.0	126 (PLM)	50	7	1.6 (4.3%)
ZG3	15.2[c]	120 (UGT1A3)	80	26	6.8 (33.9%)

Incubations were carried out at +37 °C with stirring.

[a] *LG1*, losartan-*O*-glucuronide; *LG2*, losartan-*N*1-glucuronide; *LG3*, losartan-*N*2-glucuronide; *CG1*, candesartan-*O*-glucuronide; *CG2*, candesartan-*N*2-glucuronide; *ZG1*, zolarsartan-*N*1-glucuronide; *ZG2*, zolarsartan-*O*-glucuronide; *ZG3*, zolarsartan-*N*2-glucuronide.

[b] Protein source given in parenthesis: BLM, bovine liver microsomes; MLM, moose liver microsomes; RLM, rat liver microsomes; PLM, pig liver microsomes.

[c] Substrate was added in two portions, at the beginning (24.1 mg of losartan; 8.9 mg of zolarsartan) and after 24-h (the reaction of losartan) or 7-h (the reaction of zolarsartan) incubation (6.5 mg of losartan; 6.3 mg of zolarsartan).

[d] Not determined in milligram units.

used for candesartan and zolarsartan glucuronides. The gradient elution of candesartan glucuronides was performed at a flow rate of 1 mL/min of aqueous 1% acetic acid and acetonitrile, with the following acetonitrile composition: 0–7 min 28%, 7–25 min 28–45%, 25–30 min 45–55%, 30–31 min 55–28%, 31–46 min 28%. The eluent for the zolarsartan glucuronides was aqueous 0.1% formic acid and acetonitrile at a flow rate of 1 mL/min (5 mL/min for the semipreparative column). The acetonitrile gradient was 0–9 min (0–14.4 min for the semipreparative column) 36%, 9–14 min (14.4–22.4 min) 36–40%, 14–21 min (22.4–25 min) 40%, 21–23 min (25–28.2 min) 40–80%, 23–26 min (28.2–33 min) 80%, 26–27 min (33–34.6 min) 80–36%, and 27–42 min (34.6–58.6 min) 36% (time frames of 80% elution and equilibration were prolonged to 7 and 25 min, respectively, for the Agilent Zorbax Eclipse Plus C18 column). The oven temperatures of the HPLC methods were set to 40 °C for losartan and zolarsartan glucuronides and to 25 °C for candesartan glucuronides. The collection of glucuronide fractions was based on UV detection with wavelengths of 256 nm, 210 nm, and 306 nm for losartan, candesartan, and zolarsartan glucuronides, respectively.

The collected glucuronide fractions were evaporated, and salts were removed from losartan and candesartan glucuronides by SPE (Bakerbond C18 PolarPlus, 100 mg). Finally all glucuronides were lyophilized until constant mass was reached. The purities of the synthesized sartan glucuronides were determined by HPLC with the methods described above and an UV diode array detector.

2.3. Structure characterization

The mass spectrometric analyses of the isolated *LG1*, *LG3*, and candesartan glucuronides were carried out on an API 3000 triple quadrupole mass spectrometer (MDS Sciex, Toronto, Ont., Canada) coupled to an Agilent HPLC and an ion spray source. Similar experiments were performed for *LG2* and the zolarsartan glucuronides with a quadrupole time-of-flight mass spectrometer (Q-TOF Micro Waters/Micromass, Manchester, UK) coupled to an Acquity ultra performance LC (Waters, Milford, MA) and an electrospray source. The eluent for the chromatography in the MS studies of *LG1* and *LG3* was aqueous 0.1% formic acid and acetonitrile (0–5 min 30–80%, 5–8 min, 80% of acetonitrile) with flow rate of 0.7 mL/min (the flow was split 1/100 before MS). Candesartan glucuronides were analyzed with 5 mM ammonium acetate (pH 4.5) and acetonitrile (0–5 min 10–90%, 5–7 min 90%, 7–8 min 90–10% of acetonitrile) with a flow rate of 1 mL/min (the flow was split 1/100 before MS). Aqueous 0.1% formic acid and acetonitrile were used as eluent for *LG2* and the zolarsartan glucuronides. The gradient curve of acetonitrile for *LG2* was 0–4 min 5–60%, 4–5 min 60–90%, and 5–5.5 min 90–5% and for the zolarsartan glucuronides it was 0–4 min 10–90%, 4–5 min 90%, and 5–5.5 min 90–10% with flow rate of 1 mL/min. A Waters XTerra MS C18 (2.5 μm 4.6 × 30 mm) was used as LC column for *LG1*, *LG3*, and candesartan glucuronides, and a Waters Acquity UPLC™ BEH C18 (1.7 μm 2.1 × 50 mm) column for *LG2* and zolarsartan glucuronides. Air was used as a nebulizing gas and nitrogen as a collision and a curtain gas.

In the triple quadrupole mass spectrometer experiments, ion spray needle voltage and orifice voltage were 5500 V and 30 V in positive ion mode and −4500 V and −60 V in negative ion mode, respectively. With the quadrupole time-of-flight mass spectrometer, capillary voltages were 2200 V (*LG2*) and 3000 V (zolarsartan glucuronides), and sample cones were 32 V (*LG2*) and 20 V (zolarsartan glucuronides). Collision offset voltages in positive and negative ion modes were 25 eV and −25 eV (*LG1* and *LG3*), 15 eV and −15 eV (*LG2*), 20 eV and −40 eV (candesartan glucuronides), and 15 eV and −25 eV (zolarsartan glucuronides), respectively.

The NMR studies of the synthesized glucuronides and their aglycones were performed at room temperature on a

Varian 300 MHz mercury plus spectrometer (Varian, Inc., Palo Alto, CA). The heteronuclear multiple bond correlation (HMBC) spectrum of candesartan glucuronide *CG1* was measured using a Varian Unity Inova 600 spectrometer equipped with a cold probe. Deuteriated methanol containing tetramethylsilane as an internal reference was used as solvent. NMR experiments were performed as previously described [24].

3. Results and discussion

Eight sartan glucuronides were synthesized from three sartan aglycones; namely, losartan, candesartan, and zolarsartan. The enzyme sources for these biosyntheses were recombinant human UGT-enzymes or liver microsomes from different animals. First, small-scale reactions were carried out to find suitable conditions for each synthesis, including the best enzyme source. (The results of the screening assays with recombinant human UGTs and various animal liver microsomes will be published elsewhere.) The production of glucuronides was then scaled up, with the conditions described in Table 1 used in the biosyntheses. Recombinant human UGTs are active for a much longer time than liver microsomes, and the optimal incubation time varied with the enzyme source. The yields of the glucuronides were 0.5–6.8 mg (1.1–34.6%) (Table 1). These amounts are great enough for the synthesized products to serve as standards in analytical studies.

HPLC combined with a UV diode array detector was used to test the purity of the synthesized sartan glucuronides. The amounts of impurities in the losartan glucuronides were 2.5% in *LG1*, 0.7% in *LG2*, and 2.6% in *LG3*. *CG1* and *CG2* contained 13.3% and 9.9% impurities, and *ZG1*, *ZG2*, and *ZG3* 16.0%, 37.1%, and 0.2%, respectively.

The MS spectra showed protonated and deprotonated molecules of synthesized sartan glucuronides in positive and negative ion modes, respectively. The compounds were identified as glucuronides of sartans on the basis of *m*/*z* values, which were 176 Da higher than for the corresponding sartan aglycone. These glucuronide ions were selected as precursors for the MS/MS analysis. Table 2 presents the MS/MS data for the losartan, candesartan, and zolarsartan glucuronides. The MS/MS data confirmed that the compounds were glucuronides, but NMR measurements were required to determine the sites of glucuronidation.

Comparison of the one-dimensional ^{1}H NMR spectra of losartan glucuronides with previously published spectra [8] led to the identification of losartan-*O*-glucuronide (*LG1*), losartan-*N*1-glucuronide (*LG2*), and losartan-*N*2-glucuronide (*LG3*). Assignments of the chemical shifts of losartan and its glucuronides are presented in Table 3.

One-dimensional ^{1}H NMR spectra were also measured for *CG1* and *CG2* and for the candesartan aglycone (Table 4). In the case of *CG2* we used correlated spectroscopy to assist with the chemical shift assignments. Since the signals of protons H9 were singlet in the spectra of candesartan and *CG2*, but the AB spin system was found in the spectrum of *CG1*, *CG1* was assigned as candesartan-*O*-glucuronide. To strengthen the structural characterization of *CG1* and to determine the glucuronidation site of *CG2*, we measured HMBC and nuclear overhauser enhancement (NOE) spectra. Also a one-dimensional ^{13}C NMR spectrum of

Table 2
Tandem mass spectrometric data for losartan, candesartan, and zolarsartan glucuronides[a]

Glucuronide	Precursor	Products [interpretation]
	m/*z* (relative abundance)	
Positive ion mode		
LG1	599 (15)	405 (100) $[M+H-glu-H_2O]^+$; 207 (20) $[M+H-glu-H_2O-N_2-2\text{-butyl-4-Cl-5-}CH_2\text{-imidazole}]^+$; 377 (17) $[M+H-glu-H_2O-N_2]^+$
LG2	599 (8)	405 (100) $[M+H-glu-H_2O]^+$; 581 (65) $[M+H-H_2O]^+$; 377 (51) $[M+H-glu-H_2O-N_2]^+$
LG3	599 (67)	553 (100) $[M+H-H_2O-CO]^+$; 383 (55) $[M+H-H_2O-N_2-2\text{-butyl-4-Cl-5-}CH_2\text{-imidazole}]^+$; 380 (40) $[M+H-glu-CH_3CH_2CH_2]^+$
CG1	617 (30)	423 (100) $[M+H-glu-H_2O]^+$; 263 (95) $[M+H-glu-N_2-CH_3CH_2-Ar\text{-}COOH]^+$; 441 (72) $[M+H-glu]^+$
CG2	617 (78)	441 (100) $[M+H-glu]^+$; 423 (96) $[M+H-glu-H_2O]^+$; 263 (47) $[M+H-glu-N_2-CH_3CH_2-Ar\text{-}COOH]^+$
ZG1	733 (19)	557 (100) $[M+H-glu]^+$; 355 (20) $[M+H-glu-COOH-2\text{-butyl-4-Cl-5-imidazole}]^+$
ZG2	733 (33)	557 (100) $[M+H-glu]^+$; 355 (31) $[M+H-glu-COOH-2\text{-butyl-4-Cl-5-imidazole}]^+$
ZG3	733 (100)	557 (89) $[M+H-glu]^+$; 355 (54) $[M+H-glu-COOH-2\text{-butyl-4-Cl-5-imidazole}]^+$
Negative ion mode		
LG1	597 (100)	421 (9) $[M-H-glu]^-$; 554 (5) $[M-H-CH_3CH_2CH_2]^-$
LG2	597 (100)	421 (7) $[M-H-glu]^-$
LG3	597 (100)	421 (54) $[M-H-glu]^-$; 569 (18) $[M-H-N_2]^-$
CG1	615 (−)	439 (100) $[M-H-glu]^-$; 309 (68) unidentified; 292 (23) unidentified
CG2	615 (−)	161 (100) [2-etoxy-benzimidazole]$^-$; 439 (71) $[M-H-glu]^-$; 133 (45) [2-hydroxy-benzimidazole]$^-$
ZG1	731 (18)	509 (100) $[M-H-glu-COOH]^-$; 555 (41) $[M-H-glu]^-$; 175 (29) $[glu-H]^-$
ZG2	731 (5)	555 (100) $[M-H-glu]^-$; 113 (55) $[glu-H-H_2O-CO_2]^-$; 175 (47) $[glu-H]^-$
ZG3	731 (9)	157 (100) $[glu-H-H_2O]^-$; 509 (95) $[M-H-glu-COOH]^-$; 555 (69) $[M-H-glu]^-$

[a] *LG1*, losartan-*O*-glucuronide; *LG2*, losartan-*N*1-glucuronide; *LG3*, losartan-*N*2-glucuronide; *CG1*, candesartan-*O*-glucuronide; *CG2*, candesartan-*N*2-glucuronide; *ZG1*, zolarsartan-*N*1-glucuronide; *ZG2*, zolarsartan-*O*-glucuronide; *ZG3*, zolarsartan-*N*2-glucuronide.

Table 3
^{1}H chemical shifts (ppm) of losartan and its glucuronides[a]

^{1}H	Losartan	*LG1*	*LG2*	*LG3*
H1 ($^3J_{1,2}/^4J_{1,3}$)	7.38–7.55 (m)	7.63 (d, 8.1)	7.64 (m)	7.85 (dd, 7.5/1.5)
H2 ($^3J_{1/3,2}/^4J_{2,4}$)	7.38–7.55 (m)	7.53 (m)	7.58–7.61 (m)	7.51 (dt, 7.2/1.5)
H3 ($^3J_{2/4,3}/^4J_{1,3}$)	7.38–7.55 (m)	7.53 (m)	7.71–7.77 (m)	7.59 (dt, 7.5/1.5)
H4 ($^3J_{3,4}/^4J_{2,4}$)	7.38–7.55 (m)	7.60 (d, 6.6)	7.58–7.61 (m)	7.47 (dd, 7.2/1.8)
H5/H8 ($^3J_{5,6/7,8}$)	7.11 (d, 8.4)	7.10 (d, 8.4)	7.16 (d, 8.4)	7.15 (d, 7.8)
H6/H7 ($^3J_{5,6/7,8}$)	6.91 (d, 8.4)	7.00 (d, 8.1)	6.96 (d, 8.4)	7.00 (d, 8.1)
H9	5.25 (s)	5.32 (s)	5.25 (s)	5.32 (s)
H10 ($^3J_{10,11}$)	2.57 (t, 7.8)	2.60 (t, 8.1)	2.55 (t, 7.2)	2.60 (t, 7.2)
H11 ($^3J_{10/12,11}$)	1.53 (quint, 7.2)	1.55 (m)	1.50 (quint, 7.2)	1.54 (quint, 7.2)
H12 ($^3J_{11/13,12}$)	1.32 (sex, 7.2)	1.30 (m)	1.30 (sex, 7.2)	1.29 (sex, 7.2)
H13 ($^3J_{12,13}$)	0.86 (t, 7.2)	0.87 (t, 7.2)	0.85 (t, 7.2)	0.86 (t, 7.2)
H14 ($^2J_{14,14'}$)	4.43 (s)	4.56 (d, 12.9)	4.45 (s)	4.49 (s)
H14′ ($^2J_{14,14'}$)	4.43 (s)	4.70 (d, 12.9)	4.45 (s)	4.49 (s)
GH1 ($^3J_{G1,G2}$)		4.28 (d, 7.8)	5.11 (d, 9.0)	5.84 (d, 9.0)
GH2 ($^3J_{G1/G3,G2}$)		3.18 (t, 9)	4.10 (t, 9.3)	4.03–4.09 (m)
GH3 ($^3J_{G2/G4,G3}$)		3.38 (o)	3.40 (t, 9.3)	3.58–3.70 (m)
GH4 ($^3J_{G3/G5,G4}$)		3.48 (t, 9.3)	3.58 (t, 9.3)	3.58–3.70 (m)
GH5 ($^3J_{G4,G5}$)		3.71 (d, 9.9)	3.70 (d, 9.9)	4.03–4.09 (m)

[a] *LG1*, losartan-*O*-glucuronide; *LG2*, losartan-*N*1-glucuronide; *LG3*, losartan-*N*2-glucuronide; *J*, coupling constant; s, singlet; d, doublet; dd, doublet of doublet; dt, doublet of triplet; t, triplet; quint, quintet; sex, sextet; m, multiplet; o, overlapping signals.

Table 4
^{1}H chemical shifts (ppm) of candesartan and its glucuronides[a]

^{1}H	Candesartan	*CG1*	*CG2*
H1 ($^3J_{1,2}/^4J_{1,3}$)	7.60–7.66[b] (m)	7.58–7.61 (m)	7.80 (dd, 7.8/1.2)
H2 ($^3J_{1/3,2}/^4J_{2,4}$)	7.48–7.55[b] (m)	7.47–7.52 (m)	7.43 (dt, 7.5/1.2)
H3 ($^3J_{2/4,3}/^4J_{1,3}$)	7.48–7.55[b] (m)	7.58–7.61 (m)	7.55 (dt, 7.5/1.5)
H4 ($^3J_{3,4}/^4J_{2,4}$)	7.60–7.66[b] (m)	7.47–7.52 (m)	7.49 (dd, 7.5/1.5)
H5/H8 ($^3J_{5,6/7,8}$)	6.96–7.02 (m)	6.99 (d, 8.4)	7.03 (d, 8.4)
H6/H7 ($^3J_{5,6/7,8}$)	6.96–7.02 (m)	6.91 (d, 8.1)	6.95 (d, 8.4)
H9	5.69 (s)	5.62 (AB system)	5.68 (s)
H9′	5.69 (s)	5.61 (AB system)	5.68 (s)
H10 ($^3J_{10,11}$)	4.60 (q, 6.9)	4.61 (q, 6.9)	4.61 (q, 6.9)
H11 ($^3J_{10,11}$)	1.46 (t, 6.9)	1.47 (t, 6.9)	1.48 (t, 6.9)
H12 ($^3J_{12,13}/^4J_{12,14}$)	7.60–7.66[b] (m)	7.77 (dd, 8.1/1.2)	7.63 (dd, 5.7/1.2)
H13 ($^3J_{12/14,13}$)	7.20 (t, 7.5)	7.22 (t, 8.1)	7.19 (t, 7.5)
H14 ($^3J_{13,14}/^4J_{12,14}$)	7.60–7.66[b] (m)	7.68 (dd, 8.1/1.2)	7.60 (dd, 5.7/1.2)
GH1 ($^3J_{G1,G2}$)		5.68 (o)	5.84 (d, 9.6)
GH2 ($^3J_{G1/G3,G2}$)		3.54 (o)	4.13 (t, 9.0)
GH3		3.54 (o)	3.65 (o)
GH4		3.64 (o)	3.65 (o)
GH5 ($^3J_{G4,G5}$)		3.96 (d)	4.07 (d, 9.3)

[a] *CG1*, candesartan-*O*-glucuronide; *CG2*, candesartan-*N*2-glucuronide; *J*, coupling constant; s, singlet; d, doublet; dd, doublet of doublet; dt, doublet of triplet; t, triplet; q, quartet; m, multiplet; o, overlapping signals.
[b] May have to be interchanged.

candesartan aglycone was measured. The shifted ^{13}C signal of the acyl carbon (candesartan 170 ppm, *CG1* 166 ppm, *CG2* 170 ppm) supported the characterization of *CG1*. In the HMBC spectrum of *CG1* the correlation between GH1 (5.68 ppm) and acyl carbon (166 ppm) confirmed the compound as the acyl glucuronide of candesartan. The signal of the tetrazole ring carbon was shifted in the spectrum of *CG2* (candesartan 157 ppm, *CG1* 157 ppm, *CG2* 166 ppm) indicating that the compound was the *N*-glucuronide. *CG2* was concluded to be *N*2-glucuronide rather than *N*1-glucuronide because there was no correlation between the glucuronide moiety and the benzene rings in the NOE experiments. In addition, previously *O*-glucuronide and *N*2-glucuronide but not *N*1-glucuronide of candesartan was found to form via biotransformation [11].

The ^{1}H chemical shifts of zolarsartan aglycone, *ZG1*, *ZG2*, and *ZG3* are presented in Table 5. *ZG2* was identified as the *O*-glucuronide since the multiplicity of the methylene protons H8 of zolarsartan had changed from singlet to the AB spin system, similarly to what Bowers et al. [10] previously discovered. To differentiate between glucuronides *ZG1* and *ZG3*, which were assumed to be *N*1-glucuronide and *N*2-glucuronide, we measured rotating frame overhauser effect (ROESY) spectra. (Owing to the size of the mol-

Table 5
^{1}H chemical shifts (ppm) of zolarsartan and its glucuronides[a]

^{1}H	Zolarsartan	*ZG1*	*ZG2*	*ZG3*
H1 ($^3J_{1,2}/^4J_{1,3}$)	7.87 (dd, 7.2/2.1)	7.91 (d, 8.1)	7.80 (m)	7.85 (m)
H2 ($^3J_{1/3,2}/^4J_{2,4}$)	7.72 (dt, 7.2/1.5)	7.72 (t, 7.8)	7.58 (m)	7.69 (t, 7.8)
H3 ($^3J_{2/4,3}/^4J_{1,3}$)	7.77 (dt, 7.2/1.8)	7.81 (t, 7.8)	7.58 (m)	7.70 (t, 7.8)
H4 ($^3J_{3,4}/^4J_{2,4}$)	7.95 (dd, 9.0/1.8)	8.09 (d, 7.8)	7.80 (m)	8.11 (m)
H5	7.19 (s)	7.21 (s)	7.17 (s)	7.18 (s)
H6 ($^3J_{6,7}/^4J_{5,6}$)	7.13 (dd, 8.4/1.8)	7.11 (d, 10.2)	7.10 (d, 8.4)	7.09 (d, 8.4)
H7 ($^3J_{6,7}$)	7.38 (d, 8.4)	7.28 (d, 8.4)	7.33 (d, 8.4)	7.36 (d, 8.4)
H8 ($^2J_{8,8'}$)	5.77 (s)	5.80 (s)	5.65 (d, 16.8)	5.78 (s)
H8′ ($^2J_{8,8'}$)	5.77 (s)	5.80 (s)	5.87 (d, 16.2)	5.78 (s)
H9 ($^3J_{9,10}$)	2.71 (t, 7.5)	2.59 (t, 7.2)	2.69 (t, 7.5)	2.72 (t, 7.8)
H10 ($^3J_{9/11,10}$)	1.56 (quint, 7.5)	1.47 (m)	1.57 (m)	1.57 (quint, 7.8)
H11 ($^3J_{10/12,11}$)	1.32 (sex, 7.5)	1.29 (m)	1.29 (m)	1.33 (sex, 7.8)
H12 ($^3J_{11,12}$)	0.85 (t, 7.5)	0.82 (t, 7.2)	0.86 (t, 7.5)	0.86 (t, 7.8)
GH1 ($^3J_{G1,G2}$)		5.32 (d, 9.3)	5.74 (d, 11.1)	5.81 (d, 9.6)
GH2 ($^3J_{G1/G3,G2}$)		4.36 (t, 9,6)	3.47[b] (o)	4.00 (o)
GH3		3.48–3.73 (o)	3.47[b] (o)	3.55[b] (o)
GH4 ($^3J_{G3/G5,G4}$)		3.48–3.73 (o)	3.55[b] (o)	3.55[b] (o)
GH5 ($^3J_{G4,G5}$)		3.48–3.73 (o)	3.67[b] (o)	4.00[b] (o)

The assignments for zolarsartan are adapted from Cannell et al. [36].
[a] *ZG1*, zolarsartan-*N*1-glucuronide; *ZG2*, zolarsartan-*O*-glucuronide; *ZG3*, zolarsartan-*N*2-glucuronide; *J*, coupling constant; s, singlet; d, doublet; dd, doublet of doublet; dt, doublet of triplet; t, triplet; m, multiplet; o, overlapping signals.
[b] May have to be interchanged.

ecule a NOE experiment on *ZG1* using the 300 MHz magnetic field did not result in any enhancement.) *ZG1* was identified as *N*1-glucuronide of zolarsartan, on the basis of the correlation between the protons GH1 (5.32 ppm) and H1 (7.91 ppm), in addition to a weaker correlation between protons GH2 (4.36 ppm) and H1. No correlation between the sugar moiety and the aglycone was detected in the ROESY spectrum of *ZG3*, suggesting that conjugation was at the N2 atom of the tetrazole ring.

Availability of drug metabolites is critical for pharmaceutical investigations. Acyl glucuronide metabolites require special attention in drug development owing to their possible toxicity. Enzyme-catalyzed production of glucuronides is an important tool alongside traditional chemical synthesis. Although the yield of a minor metabolite will probably be better in a traditional synthesis than in biosynthesis multiple steps are not required in biosynthesis. In the planning of an enzyme-assisted synthesis, some preliminary experiments need to be performed to determine roughly the kinetics of the enzyme. Although there are many different parameters that could be optimized in the case of enzyme-catalyzed synthesis, as we show here, optimization of the most important parameters is sufficient for easy and efficient synthesis of glucuronide metabolites.

4. Conclusions

Glucuronide metabolites of losartan, candesartan, and zolarsartan were synthesized enzymatically in milligram scale. Three glucuronides were obtained from losartan, three from zolarsartan, and two from candesartan. Identification and structure characterization were performed by mass spectrometry and NMR. The *O*-glucuronides of candesartan and zolarsartan were acyl glucuronides, which are usually considered to be relatively labile. However, neither hydrolysis nor acyl migration were detected in the syntheses of *O*-glucuronides. Earlier studies of glucuronidation at the tetrazole ring have suggested that enzyme-catalyzed reactions dominantly yield tetrazole-*N*2-glucuronide and rarely tetrazole-*N*1-glucuronides [7,8,31–35]. Nevertheless, *N*1-glucuronides of both losartan and zolarsartan were synthesized enzymatically in this study, along with the *N*2-glucuronide.

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References

[1] A. Radominska-Pandya, P.J. Czernik, J.M. Little, E. Battaglia, P.I. Mackenzie, Drug Metab. Rev. 31 (1999) 817–899.
[2] C.D. King, G.R. Rios, M.D. Green, T.R. Tephly, Curr. Drug Metab. 1 (2000) 143–161.
[3] R.H. Tukey, C.P. Strassburg, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 581–616.
[4] P.I. Mackenzie, K. Walter Bock, B. Burchell, C. Guillemette, S. Ikushiro, T. Iyanagi, J.O. Miners, I.S. Owens, D.W. Nebert, Pharmacogenet. Genomics 15 (2005) 677–685.
[5] M.J. Robertson, J.C. Barnes, G.M. Drew, K.L. Clark, F.H. Marshall, A. Michel, D. Middlemiss, B.C. Ross, D. Scopes, M.D. Dowle, Br. J. Pharmacol. 107 (1992) 1173–1180.
[6] A. Hilditch, A.A. Hunt, C.J. Gardner, D.J. Twissell, J. Polley, A. Travers, G.M. Drew, D. Middlemiss, B.C. Ross, M.J. Robertson, Br. J. Pharmacol. 111 (1994) 137–144.
[7] S.W. Huskey, R.R. Miller, S.H.L. Chiu, Drug Metab. Dispos. 21 (1993) 792–799.

[8] R.A. Stearns, G.A. Doss, R.R. Miller, S.H. Chiu, Drug Metab. Dispos. 19 (1991) 1160–1162.
[9] R.A. Stearns, R.R. Miller, G.A. Doss, P.K. Chakravarty, A. Rosegay, G.J. Gatto, S.H. Chiu, Drug Metab. Dispos. 20 (1992) 281–287.
[10] G.D. Bowers, P.J. Eddershaw, S.Y. Hughes, G.R. Manchee, J. Oxford, Rapid Commun. Mass Spectrom. 8 (1994) 217–221.
[11] T. Kondo, K. Yoshida, Y. Yoshimura, M. Motohashi, S. Tanayama, J. Mass Spectrom. 31 (1996) 873–878.
[12] T. Kondo, K. Yoshida, Y. Yoshimura, M. Motohashi, S. Tanayama, Arzneimittelforschung 46 (1996) 594–600.
[13] B.C. Sallustio, L. Sabordo, A.M. Evans, R.L. Nation, Curr. Drug Metab. 1 (2000) 163–180.
[14] M. Shipkova, V.W. Armstrong, M. Oellerich, E. Wieland, Ther. Drug Monit. 25 (2003) 1–16.
[15] M.J. Bailey, R.G. Dickinson, Chem. Biol. Interact 145 (2003) 117–137.
[16] A.V. Stachulski, J.R. Harding, J.C. Lindon, J.L. Maggs, B.K. Park, I.D. Wilson, J. Med. Chem. 49 (2006) 6931–6945.
[17] F.M. Kaspersen, C.A. Van Boeckel, Xenobiotica 17 (1987) 1451–1471.
[18] A.V. Stachulski, G.N. Jenkins, Nat. Prod. Rep. 15 (1998) 173–186.
[19] P. Reiss, D.A. Burnett, A. Zaks, Bioorg. Med. Chem. 7 (1999) 2199–2202.
[20] L. Luukkanen, I. Kilpelainen, H. Kangas, P. Ottoila, E. Elovaara, J. Taskinen, Bioconjug. Chem. 10 (1999) 150–154.
[21] D.E. Stevenson, U. Hubl, Enzyme Microb. Technol. 24 (1999) 388–396.
[22] M.G. Soars, E.L. Mattiuz, D.A. Jackson, P. Kulanthaivel, W.J. Ehlhardt, S.A. Wrighton, J. Pharmacol. Toxicol. Methods 47 (2002) 161–168.
[23] T. Kuuranne, O. Aitio, M. Vahermo, E. Elovaara, R. Kostiainen, Bioconjug. Chem. 13 (2002) 194–199.
[24] A. Alonen, O. Aitio, K. Hakala, L. Luukkanen, M. Finel, R. Kostiainen, Drug Metab. Dispos. 33 (2005) 657–663.
[25] S.E. Jantti, A. Kiriazis, R.R. Reinila, R.K. Kostiainen, R.A. Ketola, Steroids 72 (2007) 287–296.
[26] L. Hintikka, T. Kuuranne, O. Aitio, M. Thevis, W. Schanzer, R. Kostiainen, Steroids 73 (2008) 257–265.
[27] B. Zhu, D. Bush, G.A. Doss, S. Vincent, R.B. Franklin, S. Xu, Drug Metab. Dispos. 36 (2008) 331–338.
[28] L. Luukkanen, E. Elovaara, P. Lautala, J. Taskinen, H. Vainio, Pharmacol. Toxicol. 80 (1997) 152–158.
[29] M. Kurkela, J.A. Garcia-Horsman, L. Luukkanen, S. Morsky, J. Taskinen, M. Baumann, R. Kostiainen, J. Hirvonen, M. Finel, J. Biol. Chem. 278 (2003) 3536–3544.
[30] T. Kuuranne, M. Kurkela, M. Thevis, W. Schanzer, M. Finel, R. Kostiainen, Drug Metab. Dispos. 31 (2003) 1117–1124.
[31] T.J. Chando, D.W. Everett, A.D. Kahle, A.M. Starrett, N. Vachharajani, W.C. Shyu, K.J. Kripalani, R.H. Barbhaiya, Drug Metab. Dispos. 26 (1998) 408–417.
[32] L. Perrier, M. Bourrie, E. Marti, C. Tronquet, D. Masse, Y. Berger, J. Magdalou, G. Fabre, J. Pharmacol. Exp. Ther. 271 (1994) 91–99.
[33] S.W. Huskey, G.A. Doss, R.R. Miller, W.R. Schoen, S.H. Chiu, Drug Metab. Dispos. 22 (1994) 651–658.
[34] S.W. Huskey, J. Magdalou, M. Ouzzine, G. Siest, S.H. Chiu, Drug Metab. Dispos. 22 (1994) 659–662.
[35] J.C. Stevens, J.L. Fayer, K.C. Cassidy, Drug Metab. Dispos. 29 (2001) 289–295.
[36] R.J. Cannell, A.R. Knaggs, M.J. Dawson, G.R. Manchee, P.J. Eddershaw, I. Waterhouse, D.R. Sutherland, G.D. Bowers, P.J. Sidebottom, Drug Metab. Dispos. 23 (1995) 724–729.