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Eudismic analysis of tricyclic sesquiterpenoid alcohols: Lead structures for the design of potent inhibitors of the human UDP-glucuronosyltransferase 2B7

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Abstract

The epimeric tricyclic sesquiterpenoid alcohols globulol, epiglobulol, cedrol, epicedrol, longifolol, and isolongifolol were investigated in their ability to inhibit the recombinant human UDP-glucuronosyltransferase (UGT) 2B7. The stereoisomers displayed rapidly reversible competitive inhibition, which was substrate-independent. Longifolol and its stereoisomer isolongifolol displayed the lowest competitive inhibition constants (K_{ic}) of 23 and 26 nM, respectively. The K_{ic} values of cedrol and its epimer epicedrol were 0.15 and 0.21 μM, those of globulol and epiglobulol were 5.4 and 4.0 μM, respectively. The diastereomeric alcohols exhibited nearly identical affinities toward UGT2B7 indicating that the spatial arrangement of the hydroxy group had no influence on the dissociation of the enzyme–terpenoid complex. The high affinities stemmed presumably from mere hydrophobic interactions between the hydrocarbon scaffold of the terpenoid alcohol and the binding site of the enzyme. Glucuronidation assays revealed that there were large differences in the rates at which the epimeric alcohols were conjugated. Therefore, the spatial arrangement of the hydroxy group controlled the rate of the UGT2B7-catalyzed reaction. The introduction of a methyl group into the side chain of isolongifolol and longifolol increased the steric hindrance. As a result, the rate of the UGT2B7-catalyzed reaction was decreased by more than 88%. The findings indicated that the rate

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of the UGT2B7-catalyzed glucuronidation is significantly controlled by stereochemical and steric factors. Considering the high inhibition levels exerted by the tricyclic sesquiterpenoid alcohols, these compounds might serve as valuable lead structures for the design of potent inhibitors for UGT2B7.

Keywords: Metabolism; Enzyme; Inhibition; Stereochemistry; UGT; Longifolene

1. Introduction

The UDP-glucuronosyltransferases (UGTs) are membrane-bound enzymes of the endoplasmic reticulum that catalyze the transfer of glucuronic acid (GlcA) from UDP-α-D-glucuronic acid (UDPGlcA) to many lipophilic endogenous and xenobiotic compounds [1–4]. This conjugation to GlcA increases the water solubility of the aglycone and aids in its urinary and biliary excretion. The human genome contains many UGT-encoding genes, which have been divided into two subfamilies, UGT1 and UGT2. The UGT1A isoforms are encoded by a single large gene cluster on chromosome 2 and the expression of individual UGTs of this subfamily is governed by exon sharing. In contrast, UGT2B isoforms are encoded by separate genes clustered on human chromosome 4 [5–7].

The UGTs play important roles in the metabolism of prominent endobiotics and xenobiotics such as morphine, codeine, androgens, oestrogens, cholic acid derivatives, bilirubin, nonsteroidal anti-inflammatory drugs such as ibuprofen, and terpenoid alcohols [8–11]. One of the major challenges in the study of UGTs is understanding their complex substrate selectivity. Most, if not all human UGTs exhibit promiscuity[1] with respect to their substrates, meaning that each isoform can glucuronidate more than one aglycone of rather variable chemical structure. This leads to partial overlap in the substrate selectivities of different UGTs. On the other hand, even isoforms that can glucuronidate the same compound often do it at different efficiencies. Furthermore, there are some aglycones that are highly selective for a single UGT, like morphine that is glucuronidated at the 6-hydroxy position by UGT2B7, although other isoforms can catalyze the glucuronidation of this opioid at the 3-hydroxy position [12]. Little is currently known, however, about the structural elements both in the aglycone and the UGTs that are directly involved in the determination of this complex substrate selectivity, also because the X-ray crystal structure of any of the UGT isoforms has not been resolved to date. However, studies employing computational chemistry tools led to predictive models for some UGT1A isoforms [13,14].

Despite their promiscuous behavior, we have recently shown that the UGT isoforms 2B7 and 2B17 displayed high selectivities toward stereoisomers that are related as mirror images, or enantiomers [15,16]. The enantiomers used in these studies displayed nearly identical affinities toward the enzymes, but they were glucuronidated at significantly different rates, meaning that chiral distinction[2] was limited to the actual UGT-catalyzed

[1] The term catalytic promiscuity has been originally used to describe the ability of an enzyme to catalyze an adventitious secondary activity at the active site responsible for the primary activity (see Copley SD. Enzymes with extra talents: moonlighting functions and catalytic promiscuity. Curr. Opin. Chem. Biol. 2003, 7, 265–272). However, in the case of metabolic enzymes like UGTs the term has been used to depict their overlapping substrate selectivities.

[2] Chiral discrimination is used synonymously for chiral distinction. It describes the stereoselective recognition of chiral guests (e.g., chiral drugs) by chiral molecular hosts (e.g., enzymes, receptors, transporters, channel proteins, etc.).

substitution reaction. It was concluded that the spatial arrangement of the hydroxy group had no influence on the affinity but merely on the glucuronidation reaction *per se*.

This study investigates stereochemical events during the formation of the enzyme–terpenoid complex and the UGT2B7-catalyzed GlcA-transfer reaction. The UGT isoform 2B7 was chosen for this study because this enzyme was identified as the key enzyme responsible for drug glucuronidation. In this respect, UGT2B7 accounts for the transformation of 40% of drugs that are metabolized by UGT isoforms [17]. Since many endobiotics and xenobiotics occur as chiral entities [18–20], and UGT2B7 has displayed stereoselective glucuronidation toward enantiomeric probes (*vide supra*) we decided to study the behavior of this enzyme towards diastereomeric compounds. For this purpose, the results of the eudismic analysis[3] of the epimeric sesquiterpenoid alcohols globulol, epiglobulol, cedrol, epicedrol, longifolol, and isolongifolol are reported (Scheme 1) [21]. These sesquiterpenoid alcohols are related as diastereomers and display distinct physicochemical properties, in contrast to enantiomers. However, the only configurational difference between the epimers is the spatial orientation of the hydroxy group in the case of globulol, epiglobulol, cedrol, and epicedrol, and the hydroxymethyl group in the case of longifolol and isolongifolol, the very site of the UGT-catalyzed glucuronidation reaction. This structural feature enables the investigation of chiral distinction during the formation of the enzyme–substrate complex as well as during the GlcA-transfer reaction. Furthermore, this study demonstrates that stereochemical analysis could provide the key to the design of potent inhibitors of UGT2B7.

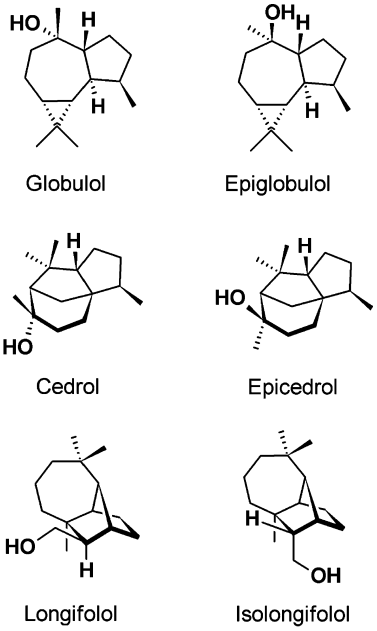
The affinity of the different stereoisomers toward UGT2B7 was determined by measuring their IC_{50} values and dissociation constants. Estriol was chosen as the reference substrate because this steroid was conveniently detected by fluorescence spectroscopy. In addition, estriol showed simple Michaelis–Menten kinetics without substrate inhibition, and the enzyme assays displayed good reproducibility. Moreover, estriol displayed high affinity toward UGT2B7 (K_m 4.6 μM), hence the total concentration of substrate and inhibitor in the enzyme assays was low. This became important when compounds that display lower solubilities such as the terpenoid alcohols in this study were assayed.

2. Materials and methods

2.1. Materials

(+)-Longifolol and (−)-isolongifolol were synthesized from (+)-longifolene following published procedures [28]. (+)-Longifolene was a generous gift from Camphor & Allied Products Ltd. (Mumbai, India) and was purified before use as previously described [29]. (−)-Globulol (CAS 489-48-1), (−)-epiglobulol (CAS 88728-58-9), (+)-cedrol (CAS 77-53-2), and (−)-epicedrol (CAS 19903-73-2) were obtained from Fluka (Buchs, Switzerland). All the terpenoid alcohols were purified by flash chromatography and, in addition,

[3] Eudismic analysis investigates the interaction between chiral compounds and their biological targets. This analytical method is used in drug design and development to increase the selectivity and potency of a chiral lead compound toward the pharmacological target [19,20]. The term eudismic analysis was derived from the expressions eutomer and distomer. The more biologically active stereoisomer is termed eutomer, the less active one is called distomer.



Scheme 1. The structures of the epimeric tricyclic alcohols.

solid terpenoid alcohols were recrystallized prior to use. The identity of the terpenoid alcohols was confirmed by ^{1}H NMR, ^{13}C NMR, and GC–MS. The purity of the compounds was determined by measuring the optical rotation and by combustion analysis. The longifolol and isolongifolol analogues **1** and **2** were synthesized from longifolyl aldehyde and isolongifolyl aldehyde[4] according to published procedures [30]. All terpenoid alcohols were identical in their ^{1}H NMR, ^{13}C NMR, GC–MS and optical rotation. The combustion analysis showed that all the applied terpenoid alcohols were of high purity (deviation from theoretical values <0.3%). UDPGlcA (trisodium salt, CAS 63700-19-6), saccharic acid-1,4-lactone (CAS 61278-30-6), estriol (CAS 50-27-1), estriol-17β-(β-D-glucuronide) (CAS 7219-89-8), scopoletin (CAS 92-61-5), and epitestosterone (CAS 481-30-1) were obtained from Sigma (St. Louis, MO, USA). 4-Nitrophenol (CAS 100-02-7) was from Aldrich (Schnelldorf, Germany). Radiolabeled [^{14}C]UDPGlcA was acquired from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). HPLC grade solvents

[4] Longifolyl and isolongifolyl aldehyde were prepared as follows: to a solution of (+)-longifolol or (−)-isolongifolol (0.23 g, 1.0 mmol) in anhydrous DMSO (15 mL) was added IBX (0.70 g, 2.5 mmol) and the resulting suspension was stirred under an atmosphere of dry argon at room temperature (23 °C) for 4 h. The reaction was slightly exothermic and the initial slurry became a colorless solution, which finally turned into a finely dispersed suspension. After completion of the reaction, water (5.0 mL) was added and the suspension was extracted with Et_2O (3×75 mL). The combined organic layers were washed with saturated $NaHCO_3$ (1×100 mL), water (3×100 mL), and brine (100 mL), dried over anhydrous Na_2SO_4, and filtered through a small pad of silica. The silica pad was washed with Et_2O (2×30 mL). The organic solvent was removed under reduced pressure and the resulting colorless oil was dried *in vacuo* affording the aldehydes in quantitative yields and high purities (>99%, GC–MS).

were used throughout the study. The recombinant human UGT2B7 was expressed as a His-tagged protein in baculovirus-infected insect cells as previously described [31].

2.2. Analytics

The compounds were analyzed by NMR on a Varian Mercury 300 MHz spectrometer (Varian, Palo Alto, CA, USA) and by GC–MS. The GC–MS system consisted of a 5890A gas chromatograph and a 5970 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). NMR spectra were processed by use of Varian VNMR software (Varian, Palo Alto, CA, USA) on a UNIX Solaris workstation. GC–MS spectra were analyzed with the HP ChemStation program on a Windows 3.1 workstation. Optical rotation was determined by use of a Polartronic E polarimeter with a micro tube for small sample volumes (100 mm, 1.10 mL; Schmidt + Haensch, Berlin, Germany). The elemental analyses were conducted by Robertson-Microlit Laboratories (Madison, NJ, USA).

2.3. IC_{50} values

The IC_{50} values of the terpenoid alcohols were determined at five concentrations bracketing their apparent IC_{50} (0.10, 0.25, 1.0, 4.0, and $10 \times IC_{50}$). Estriol was employed at 25 μM. The K_m value of estriol for UGT2B7 (4.6 μM) was determined frequently in our laboratory and the value represents the average value of experiments conducted during the last 7 months using the same enzyme batch. The reaction mixture consisted of phosphate buffer (50 mM, pH 7.4), 10 mM $MgCl_2$, and 5.0 mM saccharic acid-1,4-lactone. The enzyme was employed at 0.1 mg protein mL^{-1}. Control assays in the absence of inhibitor and blank runs in the absence of cosubstrate and estriol were performed for each inhibition assay. The enzyme reaction was initiated after a 5 min pre-incubation time at 37 °C by the addition of a solution of UDPGlcA to a final concentration of 5.0 mM. The enzyme reactions were terminated after an incubation time of 15 min at 37 °C by the addition of ice-cold perchloric acid (4.0 M) and transfer to ice. The mixtures were centrifuged (16,000*g*, 10 min) and aliquots of the supernatants were subjected to HPLC analysis. The data were analyzed by nonlinear regression applying the 2-parameter Hill equation. The results reflect a minimum of three replicate determinations.

2.4. Testing for reversibility

UGT2B7 at a concentration of 5.0 mg protein mL^{-1} was pre-incubated in buffered solution (phosphate buffer, 50 mM, pH 7.4) at 37 °C with a concentration of terpenoid alcohol equivalent to 10-fold its IC_{50}. After an equilibration time of 45 min, this mixture was diluted 50-fold into reaction buffer (37 °C) containing the reference substrate estriol (25 μM), phosphate buffer (50 mM, pH 7.4), UDPGlcA (5.0 mM), $MgCl_2$ (10 mM), and saccharic acid-1,4-lactone (5.0 mM) to initiate reaction. The formation of glucuronide was monitored every 2.5 min over 60 min by pipetting 100 μL of the reaction buffer to a vial containing 10 μL perchloric acid (4.0 M). The acidified mixtures were transferred to ice, centrifuged (16,000*g*, 10 min), and aliquots of the supernatants were subjected to HPLC analysis. Control assays in the absence of terpenoid alcohol were included. The results reflect a minimum of two replicate determinations.

2.5. Mode of inhibition

The mode of inhibition was determined by measuring the IC_{50} values of the terpenoid alcohols at six $[S]/K_m$ ratios of estriol (0.5, 1.0, 2.0, 5.0, 10, and 15). The terpenoid alcohol was employed at five concentrations bracketing its estimated IC_{50} (0.10, 0.25, 1.0, 4.0, and $10 \times IC_{50}$). One control assay to determine the % inhibition at each $[S]/K_m$ ratio of estriol employing longifolol at a fixed concentration of 0.10 μM was included. The assays were carried out as described above. The results reflect a minimum of two replicate determinations.

2.6. Inhibition constants

The K_{ic} values were determined at five estriol concentrations (2.5, 5.0, 10, 25, and 50 μM). The terpenoid alcohols were employed at three concentrations bracketing their estimated K_{ic} values (0.25, 1.0, and $4.0 \times K_{ic}$), which were calculated from the respective IC_{50} values by the Cheng–Prusoff equation [11]. The assays were carried out as described above. The results reflect a minimum of three replicate determinations.

2.7. Testing for substrate-independent inhibition

The IC_{50} values of the terpenoid alcohols longifolol and isolongifolol for UGT2B7 were determined by the use of four structurally different reference substrates. The assay conditions were balanced using the substrates at concentrations resembling their K_m values, which have been previously determined in our laboratory: 4-nitrophenol (500 μM, 0.10 mg protein mL^{-1}, incubation time 10 min), scopoletin (320 μM, 0.10 mg protein mL^{-1}, incubation time 10 min), morphine (200 μM, 0.20 mg protein mL^{-1}, incubation time 35 min), and epitestosterone (15 μM, 0.25 mg protein mL^{-1}, incubation time 15 min). Longifolol and isolongifolol were employed at five concentrations bracketing their IC_{50} values (0.10, 0.25, 1.0, 4.0, and $10 \times IC_{50}$). The assays were carried out as described above. The results reflect a minimum of three replicate determinations.

2.8. Glucuronidation assays

The formation of terpenoid glucuronide was measured by the use of radiolabeled [^{14}C]UDPGlcA [32]. A solution of [^{14}C]UDPGlcA (50 μL, 196 mCi $mmol^{-1}$, 0.02 mCi mL^{-1} in EtOH/water 7:1 v/v) was transferred to 1-mL vials and the solvent was evaporated *in vacuo*. The residue was dissolved in reaction buffer (90 μL), which consisted of UGT2B7 (2.0 mg protein mL^{-1}), cold UDPGlcA (450 μM), $MgCl_2$ (10 mM), phosphate buffer (50 mM, pH 7.4) and saccharic acid-1,4-lactone (5.0 mM). The reaction was initiated by the addition of a solution of terpenoid alcohol (10 μL, 1.0 mM in 50-vol % DMSO/water) to a final saturating concentration of 100 μM. After incubating for 14 h at 37 °C, the reactions were terminated by the addition of perchloric acid (10 μL, 4.0 M) and transfer to ice. The mixtures were centrifuged (16,000*g*, 10 min) and aliquots of the supernatants were subjected to HPLC analysis. Control assays in the presence of estriol (25 μM) and blank runs were included. The detection limit was determined by subjecting dilutions of reaction buffer containing estriol [^{14}C]β-D-glucuronide to HPLC analysis. The results reflect a minimum of six replicate determinations.

2.9. HPLC methods

The HPLC system consisted of the Agilent 1100 series degasser, binary pump, autosampler, thermostated column compartment, multiple wavelength detector, and fluorescence detector (Agilent Technologies, Palo Alto, CA, USA). The resulting spectra were analyzed with Agilent ChemStation software (Rev B.01.01). Glucuronidation reaction products were separated and detected as follows. Estriol β-D-glucuronide: Hypersil BDS-C18 (150 × 4.6 mm; Agilent Technologies, Palo Alto, CA, USA); 35% MeOH in phosphate buffer (50 mM, pH 3.0); flow rate 1.0 mL min^{-1}; detection by fluorescence spectroscopy (excitation at λ 335 nm, emission at λ 455 nm); retention time 4.2 min. 4-Nitrophenol β-D-glucuronide: Chromolith SpeedROD (50 × 4.6 mm; Merck, Darmstadt, Germany); 15% acetonitrile in phosphate buffer (50 mM, pH 3.0); flow rate 1.0 mL min^{-1}; detection by UV spectroscopy (λ 300 nm); retention time 1.3 min. Scopoletin β-D-glucuronide: Chromolith SpeedROD (50 × 4.6 mm); 10% MeOH in phosphate buffer (50 mM, pH 3.0); flow rate (gradient run) 0.8 mL min^{-1} (0.0–4.5 min), 0.8 mL → 2.5 mL min^{-1} (4.5–5.0 min), 2.5 mL min^{-1} (5.0–11.0 min), 2.5 → 0.8 mL min^{-1} (11.0–12 min); detection by fluorescence spectroscopy (excitation at λ 335 nm, emission at λ 455 nm); retention time 4.2 min. Epitestosterone β-D-glucuronide: Chromolith SpeedROD (50 × 4.6 mm); 43% MeOH in phosphate buffer (50 mM, pH 3.0); flow rate 2.0 mL min^{-1}; detection by UV spectroscopy (λ 246 nm); retention time 5.9 min. Morphine 6-β-D-glucuronide: Hypersil BDS-C18 (250 × 4.0 mm); 1% acetonitrile in phosphate buffer (50 mM, pH 3.0); flow rate 0.80 mL min^{-1}; detection by fluorescence spectroscopy (excitation at λ 285 nm, emission at λ 335 nm); retention time 14.7 min. Terpenoid [^{14}C]β-D-glucuronides: Chromolith SpeedROD (50 × 4.6 mm); gradient run 5% MeOH in phosphate buffer (50 mM, pH 3.0; 0.0–3.5 min), 5% → 80% MeOH (3.5–10 min), 80% MeOH (10–15 min), 80% → 5% MeOH (15–20 min), 5% MeOH (20–25 min); flow rate 1.0 mL min^{-1}; retention times 9.0–9.4 min for terpenoid [^{14}C]β-D-glucuronides; retention times 1.5–1.7 min for [^{14}C]UDPGlcA; detection by use of a 9701 HPLC Radioactivity Monitor (Reeve Analytical, Glasgow, Scotland).

3. Results

The IC_{50} values of the sesquiterpenoid alcohols was measured using the reference substrate estriol at a concentration of 25 μM. This concentration, which is higher than the K_m of estriol for UGT2B7 (4.6 μM), was chosen to allow for a reliable integration of the estriol glucuronide signal also when high-affinity substrates such as longifolol and isolongifolol were assayed at higher concentrations. It should be mentioned that estriol is selectively glucuronidated by UGT2B7 at the hydroxy group in position 17β. The IC_{50} values along with the eudismic ratios are displayed in Table 1. The eudismic ratio was calculated by dividing the IC_{50} values of the epimeric alcohols. The eudismic ratio therefore quantifies the preferential dissociation of one enzyme–epimer complex over the other. As can be seen from Table 1, the IC_{50} values of the terpenoid alcohols ranged from 74 nM to 29 μM, representing an approximately 400-fold difference. Longifolol and isolongifolol displayed the highest affinity toward UGT2B7 with IC_{50} values of 74 and 97 nM, respectively. The epimeric alcohols displayed nearly identical affinities as expressed by eudismic ratios that ranged merely between 1.1 and 1.3. However, these eudismic ratios were meaningful only when the stereoisomeric compounds displayed identical modes of

Table 1
IC_{50} and K_{ic} values of the epimeric alcohols

Compound	IC_{50}[a] (μM)	$CI_{95\%}$[b]	Eudismic ratio[d]	K_{ic}[c] (μM)	$CI_{95\%}$[b]	Eudismic ratio[e]
Globulol	29.2 ± 1.5	26.0–32.3	1.2	5.4 ± 0.79	3.9–7.0	1.4
Epiglobulol	24.5 ± 1.6	21.0–28.0		4.0 ± 0.32	3.4–4.7	
Cedrol	0.63 ± 0.042	0.54–0.72	1.1	0.15 ± 0.012	0.13–0.18	1.4
Epicedrol	0.60 ± 0.045	0.50–0.69		0.21 ± 0.016	0.18–0.25	
Longifolol	0.074 ± 0.0055	0.063–0.086	1.3	0.023 ± 0.0011	0.021–0.025	1.1
Isolongifolol	0.097 ± 0.0070	0.082–0.11		0.026 ± 0.0020	0.022–0.030	

[a] Mean value ± standard deviation ($n = 15$).
[b] 95% Confidence interval.
[c] Mean value ± standard deviation ($n = 60$).
[d] Calculated by dividing the higher IC_{50} value of one epimer by the lower value of its respective stereoisomer.
[e] Calculated from the respective K_{ic} values.

inhibition. These findings could also have been a result of unspecific, noncompetitive and uncompetitive binding to the enzyme, blurring the chiral distinction of the active-site directed, competitive interaction. In this respect, the compounds were tested for reversibility and their mode of inhibition. The reversibility of inhibition was assessed according to standard procedures by measuring the recovery of enzymatic activity after a rapid and large dilution. It was found that the sesquiterpenoid alcohols were identical in their behavior and they were rapidly reversible inhibitors (Fig. 1).

The mode of inhibition—either competitive, uncompetitive, noncompetitive, or mixed-type—was determined by measuring the effects of the $[S]/K_m$ ratio on the apparent IC_{50} values of the terpenoid alcohols [22]. As can be seen from Fig. 2, the stereoisomeric alcohols displayed competitive inhibition toward UGT2B7 because the corresponding IC_{50} values increased linearly with increasing concentration of the reference substrate estriol. Taken together, the six tricyclic sesquiterpenoid alcohols displayed an identical mode of inhibition and the calculated eudismic ratios were therefore indeed meaningful. The eudismic ratios close to unity expressed that both epimers had similar affinities toward the binding site of UGT2B7. Therefore, there was no chiral distinction during the formation

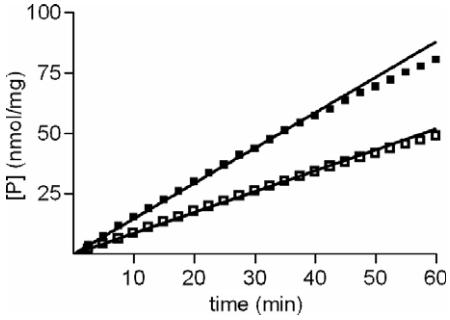


Fig. 1. The reversibility of inhibition was determined by measuring the recovery of enzyme activity after a rapid and large dilution. The data for longifolol are displayed (□, $n = 2$). One control assay in the absence of inhibitor was included (■, $n = 2$). The curve represents the expected behavior for a rapidly reversible inhibitor. The other epimeric alcohols displayed similar curves. The ordinate displays the amount of formed estriol β-D-glucuronide per mg protein.

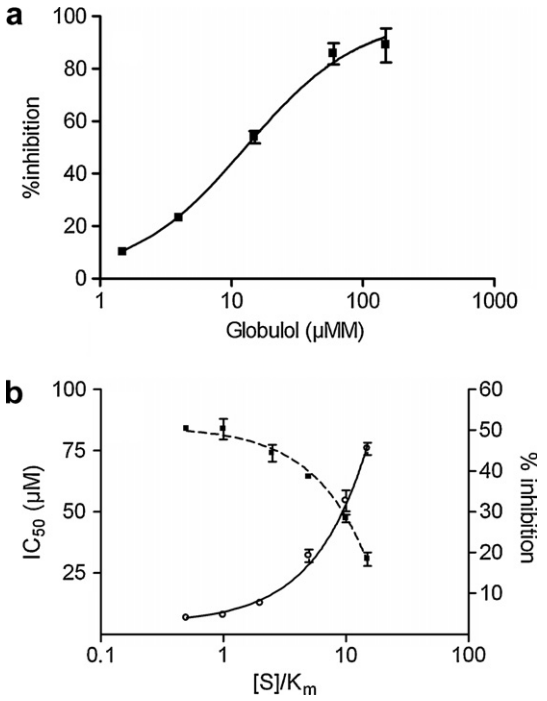


Fig. 2. (a) Typical semilogarithmic concentration-response plot for the inhibition assays. Globulol was used as the inhibitor. Estriol was used as the reference substrate at a concentration of 10 μM. The IC_{50} for globulol was 12.7 μM ± 0.88 ($CI_{95\%}$ 10.8–14.7). (b) IC_{50} values for globulol (○) at different $[S]/K_m$ ratios of estriol. The abscissa is plotted on a logarithmic scale for clarity. The mean values and standard deviations are displayed ($n = 10$). Control assays to determine the % inhibition exerted by longifolol at a fixed concentration of 0.10 μM were included at each $[S]/K_m$ ratio (■, $n = 2$). The curves represent the expected behavior for a competitive inhibitor. The other terpenoid alcohols displayed similar curves.

of the encounter complex, even though the epimers possessed different physicochemical properties.

The competitive inhibition constants (K_{ic}) of the sesquiterpenoid alcohols were determined to derive a kinetic parameter that was independent of the substrate concentration. Furthermore, the K_{ic} values were measured to confirm the high affinities, low eudismic ratios and the mechanism of inhibition displayed by the tricyclic alcohols as indicated by the studies described above (Table 1). The data of this analysis were fitted to the competitive, noncompetitive, mixed-type, and uncompetitive inhibition models. The different models were ranked according to the corrected Akaike's information criterion (results not shown). Based on these results, the active-site directed, competitive inhibition model was chosen and the resulting K_{ic} values were in good agreement with those calculated from the respective IC_{50} values by the Cheng–Prusoff equation (not shown) [23]. Accordingly, longifolol and isolongifolol displayed very low K_{ic} values of 23 and 26 nM, respectively. The K_{ic} values of cedrol and its diastereomer epicedrol were approximately 10-fold higher (0.15 and 0.21 μM, respectively), and those of globulol and epiglobulol were 5.4 and 4.0 μM, respectively (Table 1). The K_{ic} values of the epimers were nearly identical, in agreement with the corresponding IC_{50} values (*vide supra*). This study substantiated the

finding that there was no significant level of chiral distinction during the formation of the enzyme–epimer complex, meaning that the configuration at the chiral center bearing the hydroxy or hydroxymethyl group had no significant influence on the affinity toward UGT2B7.

Furthermore, it was determined that the compounds displayed similar affinity levels independently of a second substrate. The substrate-independency was assayed by measuring the IC_{50} values of the sesquiterpenoid alcohols longifolol and isolongifolol by the use of four structurally different substrates of UGT2B7. In this respect, the IC_{50} values of the two epimeric alcohols were determined using the reference substrates 4-nitrophenol, morphine, scopoletin, and epitestosterone. It should be mentioned here that morphine was included as a substrate because morphine glucuronidation at the 6-hydroxy position is selectively catalyzed by UGT2B7 [24]. The measured IC_{50} values that were determined with the four reference substrates ranged from 45 to 69 nM (standard deviation <9.6%; $n = 10$) and from 42 to 78 nM (standard deviation <8.7%; $n = 10$) for longifolol and isolongifolol, respectively. The corresponding eudismic ratios were between 1.1 and 1.4. These results confirmed that the inhibition exerted by longifolol and isolongifolol was independent of the second substrate applied. Also the eudismic ratio as a measure of the chiral distinction during the formation of the encounter complex was independent of the employed second substrate.

In the preceding sections, the influence of the spatial orientation of the hydroxy group on the affinity was investigated. Furthermore, the mode of inhibition and substrate-independency were assessed. The subsequent sections deal with the influence of structural features, i.e., stereochemistry and steric hindrance, on the rate of the UGT2B7-catalyzed glucuronidation reaction.

The sesquiterpenoid alcohols and their corresponding β-D-glucuronides could not be detected either by UV or fluorescence spectroscopy due to the lack of appropriate chromophores in the saturated tricyclic hydrocarbon scaffold. Therefore, the formation of the terpenoid β-D-glucuronides was assayed by the use of radiolabeled [^{14}C]UDPGlcA after incubating the reaction buffer for 14 h at a high UGT2B7 concentration of 2.0 mg protein mL^{-1}. The detection limit was 8.0 pmol terpenoid β-D-glucuronide at a signal-to-noise ratio of 10. The high concentration of enzyme and the long incubation time were necessary to unambiguously identify and detect the formed terpenoid [^{14}C]β-D-glucuronides. The concentration of radiolabeled cosubstrate was limited due to the high costs and to minimize the radioactive waste output. However, the employed cosubstrate concentration of 500 μM was sufficient for the purpose of this study. The tricyclic alcohols were employed at a saturating concentration of 100 μM to ensure that depletion of terpenoid alcohol did not occur. The best conversion was 8.1% (longifolol), therefore, no depletion of terpenoid alcohol due to glucuronide formation took place and the results of this study were consistent and comparable. Hence, the amount of formed β-D-glucuronide could be applied as a measure of the glucuronidation rate. The radiochromatograms of this analysis are displayed in Fig. 3 and the quantification results are summarized in Table 2. It was found that UGT2B7 glucuronidated globulol at a much higher rate than its epimer epiglobulol. The amount of globulol glucuronide was 789 pmol, that of its stereoisomer epiglobulol was below the detection limit of 8.0 pmol. A similar pattern was observed for the UGT2B7-catalyzed glucuronidation of longifolol and its epimer isolongifolol. A large amount of longifolol glucuronide was formed (810 pmol), in contrast to 65 pmol of isolongifolol glucuronide. Cedrol and epicedrol were significantly poorer substrates of

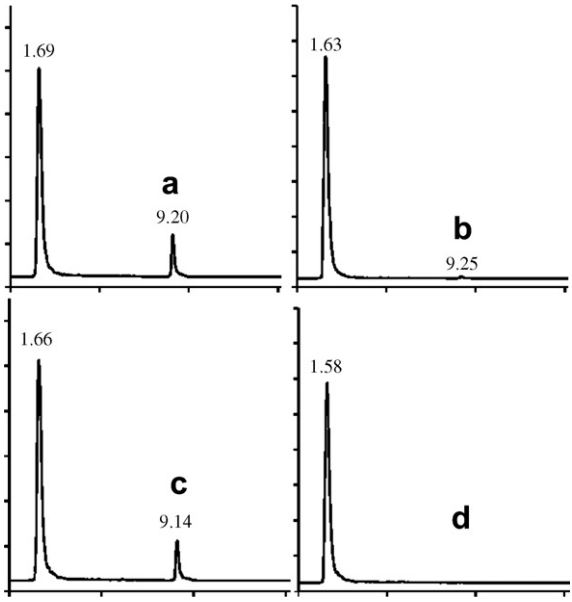


Fig. 3. Radiochromatograms of the formed terpenoid [^{14}C]β-D-glucuronides of longifolol (a), isolongifolol (b), globulol (c), and epiglobulol (d). The curves depict clear differences in the amount of formed glucuronide (retention time approx. 9.2 min). Longifolol and globulol were much better substrates of UGT2B7 compared to their epimers isolongifolol and epiglobulol. The glucuronide of epiglobulol was not detected. The peak at approx. 1.6 min is the not consumed radiolabeled cosubstrate.

Table 2
Glucuronidation of the sesquiterpenoid alcohols

Compound	Glucuronide[a] (pmol)	% Conversion	Eudismic ratio
Globulol	789 ± 55	7.9	>98
Epiglobulol	<8[b]	<0.08	
Cedrol	31 ± 1.6	0.31	>3.9
Epicedrol	<8[b]	<0.08	
Longifolol	810 ± 70	<8.1	12
Isolongifolol	65 ± 4.2	0.65	

[a] Mean value ± standard deviation ($n = 6$).
[b] Signal below detection limit.

UGT2B7 compared to the other sesquiterpenoid alcohols. The response of epicedrol glucuronide was below the detection limit and the amount of cedrol glucuronide was 31 pmol. This analysis showed that the eudismic ratios were significantly higher than those of the IC_{50} and K_{ic} values, indicating significant levels of chiral distinction during the actual glucuronidation reaction. Especially the UGT2B7-catalyzed glucuronidation of globulol was highly favored over the enzymatic conjugation of its stereoisomer epiglobulol (eudismic ratio > 98). Furthermore, the glucuronidation of longifolol and cedrol were preferred over the conjugation of their respective stereoisomers by factors of 12 and more than 3.9, respectively.

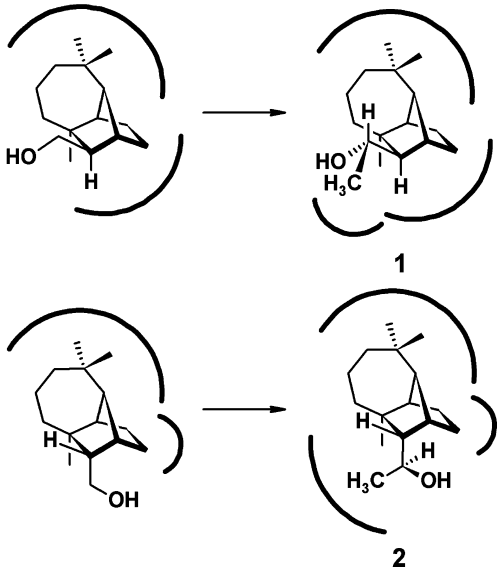


Fig. 4. Graphical representation of steric hindrance. The introduction of one additional methyl group to the side chain in longifolol (compound **1**, top) and isolongifolol (compound **2**, bottom) increased steric hindrance (depicted by the solid arcs). This resulted in decreased accessibility to the hydroxy group, which resulted in lower glucuronidation rates.

Two derivatives of longifolol and its epimer isolongifolol were synthesized by the formal replacement of one H-atom by a methyl group to investigate the influence of steric repulsion on the glucuronidation rate (Fig. 4). The accessibility of the hydroxy group for nucleophilic substitution in the derivatives **1** and **2** was decreased due to the introduction of an additional alkyl substituent in the vicinity of the reaction center. Accordingly, a decrease in the glucuronidation rate was anticipated. As was expected, the amount of formed glucuronide of the derivatives **1** and **2** (31 and < 8 pmol, respectively) decreased significantly compared to those of longifolol and isolongifolol. The concentration of formed glucuronide was reduced by 96% (compound **1**) and by more than 88% (compound **2**) compared to longifolol and isolongifolol, respectively. On the other hand, both compounds displayed high affinity toward the enzyme. The IC_{50} of compound **1** was 77 nM (standard deviation $= 5.3$, $n = 15$) and that of compound **2** was 0.40 μM (standard deviation $= 0.039$, $n = 15$). This result showed that the steric hindrance exerted by the additional methyl group had a significant influence on the glucuronidation rate, whereas the high affinity toward UGT2B7 was retained.

4. Discussion

The IC_{50} and K_{ic} values of the epimeric alcohols were nearly identical (Table 1). This finding indicated that the spatial orientation of the hydroxy and hydroxymethyl group had no effect on the affinity toward the binding site of UGT2B7. Since the IC_{50} and K_{ic} relate to the dissociation (formation) of the enzyme–epimer complex [22], it was concluded that the formation of the encounter complex was not influenced by the configuration at the

asymmetric carbon atom bearing the hydroxy or hydroxymethyl group. Therefore, the enzyme exhibited no significant chiral distinction during this recognition process towards the epimeric terpenoid alcohols, and UGT2B7 accepted the different stereoisomers at nearly identical affinities. This might suggest that there was no significant interaction between the hydroxy group of the epimeric alcohols and the binding site of UGT2B7 that could contribute to the affinity level, and therefore did not result in different IC_{50} and K_{ic} values between the two stereoisomers. Taking this into account, it might be concluded that the high affinities of especially longifolol and isolongifolol stemmed merely from hydrophobic interactions between the tricyclic hydrocarbon scaffold of the terpenoid alcohols and the binding site of UGT2B7. This indicated that the enzyme did not recognize the hydroxy group within the tricyclic substrates during the formation of the enzyme–terpenoid complex.

In contrast to the formation of the enzyme–terpenoid complex, the actual UGT2B7-catalyzed transfer of GlcA was stereoselective, because globulol, longifolol, and cedrol were conjugated at significantly higher rates than their respective epimers (Table 2). This finding indicated chiral distinction during the GlcA-transfer reaction. Especially the conjugation of globulol was highly stereoselective as indicated by its eudismic ratio of >98 and the glucuronidation of longifolol was favored over the conjugation of its epimer by a factor of 12. The smaller eudismic ratio of the latter compound might be explained by the higher flexibility of the hydroxymethyl group, rendering also isolongifolol a better substrate. In the cases of globulol and cedrol the hydroxy group is directly attached to the rigid tricyclic hydrocarbon scaffold, which results in decreased flexibility of the hydroxy group (Scheme 1). The eudismic ratio of cedrol and epicedrol glucuronidation could not be assessed sufficiently due to very low conjugation rates. However, the eudismic ratio was at least 3.9 indicating chiral distinction that favors the glucuronidation of cedrol over its epimer.

Cedrol and epicedrol were poor substrates of UGT2B7 despite their high affinities toward this enzyme (Tables 1 and 2). The common characteristic of these two epimers was the high steric hindrance in the vicinity of the nucleophilic hydroxy group, resulting in decreased rates of the UGT2B7-catalyzed conjugation reaction. Furthermore, also epiglobulol was found to be a very poor substrate since its glucuronide could not be detected. The common feature between cedrol, epicedrol, and epiglobulol was the methyl group at the asymmetric carbon atom that bears the nucleophilic hydroxy group. It was therefore concluded that the steric hindrance exerted by this methyl group could be partly responsible for the low conjugation rates. Since the methyl group is also encountered in globulol, which was a very good substrate of UGT2B7, we concluded that the combination of spatial orientation and steric hindrance resulted in low glucuronidation rates. To test this hypothesis, two derivatives of longifolol and isolongifolol were synthesized (Fig. 4). It was anticipated that the additional methyl group would decrease the accessibility to the hydroxy group during the UGT2B7-catalyzed reaction, resulting in a decreased glucuronidation rate. The glucuronidation assays showed that the introduction of the methyl group into the longifolol and isolongifolol scaffold indeed decreased the glucuronidation by at least 88%. Therefore, it was concluded that the glucuronidation rate was controlled by the orientation of the hydroxy group as well as by steric hindrance.

In this study, it was demonstrated that the spatial arrangement of the hydroxy and hydroxymethyl group and the steric demand in the vicinity of this nucleophilic group governed the glucuronidation of epimeric terpenoid alcohols. However, the affinity level was not affected by the configuration at the asymmetric carbon atom bearing the hydroxy

group. The latter finding might indicate that there was no significant interaction between the hydroxy group and the binding site of UGT2B7.

5. Conclusions

The enzymatic glucuronidation of high-affinity substrates such as longifolol and isolongifolol might be prevented by addressing steric factors and stereochemical features. We conclude that the glucuronidation of substrates can be prevented by introducing bulky substituents in the vicinity of the reaction center to turn high-affinity substrates into potent true inhibitors. This is especially important for the design of inhibitors of UGT isoforms, since functional groups that usually promote water solubility such as hydroxy, thiol, amino, and carboxy groups are commonly glucuronidated by these enzymes. This study suggests that it might be possible to overcome this major obstacle by considering stereochemical and steric properties to prevent glucuronidation of these functional groups. Especially longifolol and its epimer isolongifolol represent suitable lead compounds for the design of inhibitors, since both epimers displayed very low K_{ic} values—a feature that is rarely seen with UGT enzymes [17]—and they bear a primary hydroxy group that is readily accessible to synthetic modifications. This could be exploited to synthesize derivatives of these epimers to obtain detailed structure–activity relationships that provide more insight in the features that control affinity and glucuronidation rate and that could finally lead to potent true inhibitors of this UGT isoform. In this context it should be mentioned that to date no sufficiently UGT isoform-selective and potent inhibitor has been discovered [25]. Such inhibitors are important in the study of drug glucuronidation because they could be used to identify UGT isoforms that mediate a particular glucuronidation in human tissues and employed to verify drug–drug interactions [26]. Furthermore, selective probes can be used to measure the phenotype of genetical polymorphism [24,27]. One advantage of inhibitors based on a hydrocarbon scaffold such as the terpenoid alcohols investigated in this study would be that they are not detectable by UV or fluorescence spectroscopy and therefore would not interfere with the detection and analysis of UV and fluorescence active substrates. Furthermore, detailed eudismic analysis could provide novel insights in the UGT-catalyzed glucuronidation reaction at the molecular level. Presently, we are conducting advanced studies to investigate the intriguing behavior of UGT isoforms toward stereoisomers and to demonstrate that the principles outlined in this study can be used for the design of potent true inhibitors of UGT2B7.

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