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Moderate concentrations of 4-*O*-methylhonokiol potentiate $GABA_A$ receptor currents stronger than honokiol

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ABSTRACT

Background: Magnolia bark preparations from *Magnolia officinalis* of Asian medicinal systems are known for their muscle relaxant effect and anticonvulsant activity. These CNS related effects are ascribed to the presence of the biphenyl-type neolignans honokiol and magnolol that exert a potentiating effect on $GABA_A$ receptors. 4-*O*-methylhonokiol isolated from seeds of the North-American *M. grandiflora* was compared to honokiol for its activity to potentiate $GABA_A$ receptors and its $GABA_A$ receptor subtype-specificity was established.
Methods: Different recombinant $GABA_A$ receptors were functionally expressed in *Xenopus* oocytes and electrophysiological techniques were used determine to their modulation by 4-*O*-methylhonokiol.
Results: 3 μM 4-*O*-methylhonokiol is shown here to potentiate responses of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor about 20-fold stronger than the same concentration of honokiol. In the present study potentiation by 4-*O*-methylhonokiol is also detailed for 12 $GABA_A$ receptor subtypes to assess $GABA_A$ receptor subunits that are responsible for the potentiating effect.
Conclusion: The much higher potentiation of $GABA_A$ receptors at identical concentrations of 4-*O*-methylhonokiol as compared to honokiol parallels previous observations made in other systems of potentiated pharmacological activity of 4-*O*-methylhonokiol over honokiol.
General significance: The results point to the use of 4-*O*-methylhonokiol as a lead for $GABA_A$ receptor potentiation and corroborate the use of *M. grandiflora* seeds against convulsions in Mexican folk medicine.

1. Introduction

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. It acts at two types of receptors, the G-protein coupled $GABA_B$ receptor and the $GABA_A$ receptor, that constitute an ion channel. Numerous subunits have been cloned [for review see 1–5], indicating that many receptor isoforms exist [4]. These subunits show homology to subunits of the nicotinic acetylcholine receptors, the glycine receptor and the $5HT_3$ receptor. They show a differential distribution in brain [6]. The $GABA_A$ receptors are heteromeric protein complexes consisting of five subunits, which are arranged around a central Cl^--selective channel [1]. The major receptor isoform of the $GABA_A$ receptor in the brain presumably consists of α_1, β_2 and γ_2 subunits [1–5]. Different approaches have indicated a 2α:2β:1γ subunit stoichiometry for this receptor [7–10] with a subunit arrangement γβαβα anti-clockwise as seen from the synaptic cleft [10–12]. The pharmacological properties depend both on subunit composition [13] and arrangement [14]. $GABA_A$ receptors do not exclusively locate to synapses. Some receptor subtypes, among them the ones containing the δ subunit, have been found localized to extra-synaptic regions where they are involved in tonic inhibition [15]. The $GABA_A$ receptor is the target of many clinically used and experimental drugs [for review see 16,17].

The biphenyl-neolignans magnolol and honokiol are the major secondary metabolites in the botanical drug Magnoliae cortex, i.e., the bark of *Magnolia officinalis* [18]. Reports on curare-like activity of infusions or decoctions of *Magnolia* bark in scientific literature date back to 1921 and Watanabe et al. [19] demonstrated CNS related effects including muscle relaxation and anticonvulsant activity of extracts of *M. officinalis* bark. Biphenyls are considered privileged structures in pharmaceutical drug design [20] and to date, various pharmacological activities are ascribed to the presence of the biphenyls magnolol and honokiol [21] including their anxiolytic action [22] as well as a specific $GABA_A$ modulatory activity [22–25]. Interestingly, in several pharmacological assays, the less polar derivative 4-*O*-methylhonokiol turned out to exhibit higher potency than honokiol, e.g., in its inhibitory potency

Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$ receptor, γ-aminobutyric acid type A receptor; 2-AG, 2-arachidonylglycerol; THDOC, tetrahydrodeoxycorticosterone

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against COX-1/2 activity and 5-LOX-mediated LTB_4 formation [26,27] and in its action as a cannabinoid-2 receptor inverse agonist [28], a fact that prompted us to investigate its $GABA_A$ receptor activity in more detail. The structures of honokiol and 4-*O*-methylhonokiol are shown in Fig. 1. 4-*O*-Methylhonokiol is not a major constituent of Asian *Magnolia* bark; however, it is contained to c. 10% in the oil-containing seed coat of the North American *M. grandiflora*. The ethnomedicinal use of *M. grandiflora* seeds against epilepsy was reported and pharmacologically evaluated [29], but such an indication is not listed in Asian medicinal systems, which may have to do with the fact that the Asian species of *Magnolia* have much lower contents in 4-*O*-methylhonokiol. In this context, it may be noteworthy to mention that ethnomedicinal records for the genus *Magnolia* in North America and Asia are generally very similar and reflect the disjunct geographic distribution of the family Magnoliaceae [30].

A strong preference for 4-*O*-methylhonokiol over honokiol is documented here for the potentiation of $GABA_A$ receptor currents. We also performed an in-depth study of the potency of 4-*O*-methylhonokiol toward $GABA_A$ receptor composed of distinct subunits.

2. Materials and methods

2.1. Expression of $GABA_A$ receptors in Xenopus oocytes

Capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized plasmids with a cytomegalovirus promotor (pCMVvectors) containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, and the dried pellet was dissolved in water and stored at −80 °C. cRNA mixtures were prepared from these stock solutions and stored at −80 °C. *Xenopus laevis* oocytes were prepared, injected and defolliculated as described previously [31,32]. They were injected with 50 nL of the cRNA solution containing, in the case of αβ receptors α_1 and β_2 subunits at a concentration of 75 nM:75 nM, in the case of αβγ receptors α_1 or α_2, α_3, α_5, α_6 and β_2 and γ_2 subunits at a concentration of 10 nM:10 nM:50 nM [33] and in the case of αβδ receptors α_1, β_2 and δ subunits at a concentration of 10 nM:10 nM:50 nM. Subsequently, the oocytes were incubated in modified Barth's solution at +18 °C for at least 24 h before the measurements for αβ and αβγ receptors and 48 h for αβδ receptors.

2.2. Functional characterization of the $GABA_A$ receptors

Currents were measured using a modified two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments) in combination with an XY-recorder (90% response time 0.1 s) or digitized at 100 Hz using a PowerLab 2/20 (AD Instruments) using the computer programs Chart (ADInstruments GmbH, Spechbach, Germany). Tests with a model oocyte were performed to ensure linearity in the larger current range. The response was linear up to 15 μA.

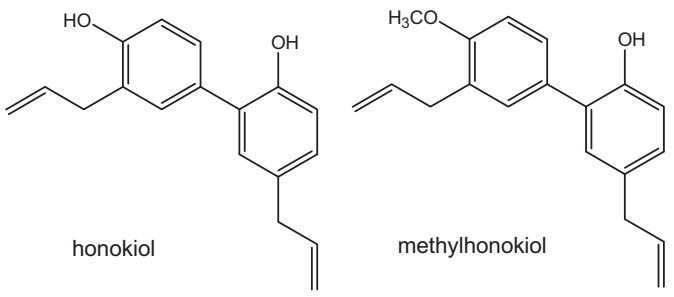

Fig. 1. Structure of honokiol and 4-*O*-methylhonokiol.

Electrophysiological experiments were performed by using the two-electrode voltage clamp method at a holding potential of −80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Na-HEPES (pH 7.4) and was applied by gravity flow 6 mL/min. The perfusion medium was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about 0.4 mm from the surface of the oocyte.

Concentration–response curves for 4-*O*-methylhonokiol were fitted with the equation $I(c) = I_{max} / (1 + (EC_{50} / c)^n)$, where c is the concentration of 4-*O*-methylhonokiol, EC_{50} is the concentration of 4-*O*-methylhonokiol eliciting half maximal current amplitude, I_{max} is the maximal current amplitude, I is the current amplitude and *n* is the Hill coefficient. The individual curves were standardized to I_{max} and subsequently averaged. Allosteric modulation was measured at a GABA concentration eliciting 0.5–1.0% of the maximal GABA current amplitude in the corresponding receptor. GABA was applied for 20 s alone or in combination with allosteric compound. Potentiation of GABA currents was expressed as $(I_{(modulator + GABA)} / I_{GABA} - 1) \times 100\%$. The perfusion system was cleaned between drug applications by washing with DMSO to avoid contamination. In each case DMSO was washed out before placing the next oocyte.

The data were analyzed by one-way ANOVA with Dunnett's post test for significance of differences.

2.3. Extraction of 4-*O*-methylhonokiol

4-*O*-Methylhonokiol was obtained from the dichloromethane extract of *M. grandiflora* seeds collected in northern Mississippi. The crude methylhonokiol was obtained as an oil after chromatography of the extract on silica gel (40–63 μm) using *n*-hexane/ethyl acetate mixtures (100% -- >70% *n*-hexane in ethyl acetate). After evaporation of the solvent, 4-*O*-methylhonokiol was obtained as light colored oil. Its spectroscopic data (NMR, MS) were in full agreement with data published in literature [37]. The purity of >99% was verified by HPLC and NMR.

Honokiol (and also magnolol) was obtained as a minor product from the more polar fraction of the seed oil of *M. grandiflora* during the isolation of 3-*O*-methylhonokiol. Honokiol was purified by column chromatography using hexane–ethyl acetate gradients (70% -- >40% *n*-hexane in ethyl acetate) and subsequently identified using NMR and MS data published in literature [34]. Its purity was >99% as seen in the HPLC chromatogram and using NMR.

3. Results and discussion

In initial experiments recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in *Xenopus* oocytes. 3 μM 4-*O*-methylhonokiol potentiated currents elicited by 0.5 μM GABA by 3883 ± 609% ($n = 6$). In the absence of GABA, 3 μM 4-*O*-methylhonokiol elicited small currents amounting to 0.21 ± 0.16% ($n = 4$) of the maximal current amplitude elicited by GABA in the same oocyte. Potentiation by 3 μM 4-*O*-methylhonokiol should be compared with that by 3 μM honokiol that amounted to 192 ± 63% ($n = 4$) (Fig. 2b). This indicates that the potentiation by 3 μM 4-*O*-methylhonokiol is about 20-fold more than that by 3 μM honokiol. A low concentration of GABA was chosen to prevent truncation of the observed strong potentiation.

A concentration–response curve of 4-*O*-methylhonokiol in recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors is shown in Fig. 2a. After two applications of 0.5 μM GABA, the same concentration of GABA together with increasing concentrations of 4-*O*-methylhonokiol was applied. It should be noted that in these experiments a maximum level of potentiation was not obtained. At concentrations higher than 10 μM, a bell shaped behavior was observed, that is the observed potentiation decreased again. The reason for this phenomenon is not clear. If the observed agonistic effects

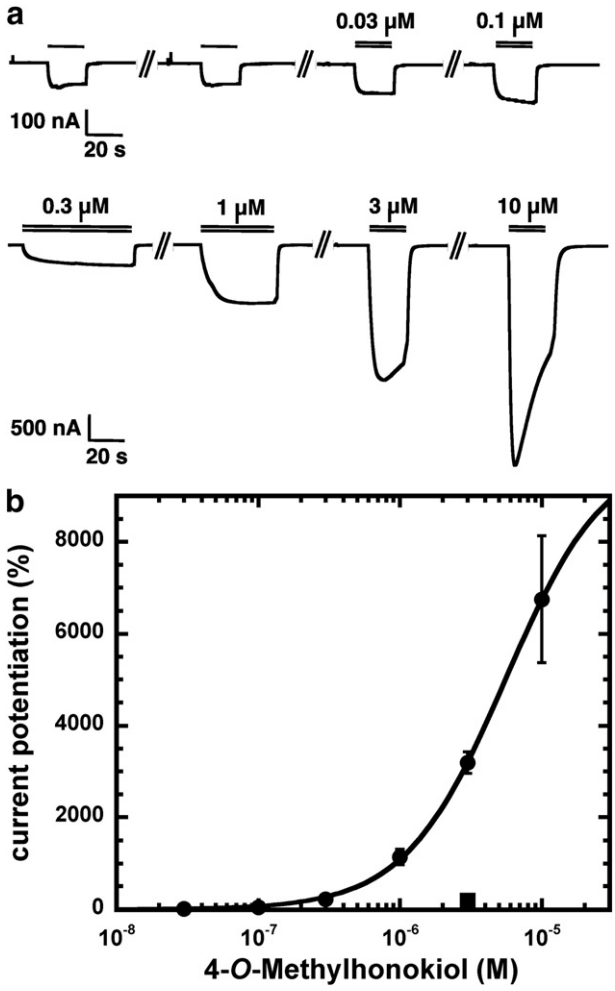

Fig. 2. 4-*O*-Methylhonokiol stimulates GABA currents mediated by $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors in a concentration-dependent way. $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in *Xenopus* oocytes. a) Current traces recorded after exposure of a single oocyte to 0.5 μM GABA or 0.5 μM GABA in combination with increasing concentrations of 4-*O*-methylhonokiol. The concentration of 4-*O*-methylhonokiol is indicated in μM above the bar. Please note that the current scale for the first four traces is different from the one for the second four traces. Drugs were applied during 20 s, except for the co-application of GABA with 0.3 μM and 1 μM where application was made until a plateau was reached. b) Concentration-dependent potentiation by 4-*O*-methylhonokiol (circles). Four experiments were fitted individually to the equation given in the methods section, standardized to the fitted maximal current amplitude and then averaged. Shown is mean ± SD. Potentiation by 3 μM honokiol is shown for comparison (square).

cause this phenomenon, they would have to override the effects of potentiation. Parameters of the concentration-response curve were estimated after averaging of four such experiments. The EC_{50} was estimated 5.4 ± 1.8 μM, maximal potentiation 10'100 ± 3'100% and the Hill coefficient 1.26 ± 0.07. The threshold of simulation was observed at 0.1 μM 4-*O*-methylhonokiol, where the potentiation amounted to 43 ± 23%. Please note that in cumulative concentration–response curves potentiation was somewhat smaller than the one observed in single applications of 4-*O*-methylhonokiol. Previous estimates of concentration response parameters for honokiol were an EC_{50} of about 36 μM and a maximal potentiation of about 1300% [27].

To identify subunits required for the potentiation, we expressed $GABA_A$ receptors of different subunit composition. For the determination of the subunit specificity entire concentration–response curves would be desired. This would give information on the effect the potency and efficacy at each receptor subtype. As indicated above entire concentration–response curves could not be made. Therefore, the potentiation was tested at a fixed concentration of 3 μM 4-*O*-methylhonokiol, using a GABA concentration of $EC_{0.5–1.0}$. First, we replaced α_1 in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors by different α subunit isoforms. As shown in Fig. 3, $\alpha_2\beta_2\gamma_2$ and $\alpha_3\beta_2\gamma_2$ receptors displayed a potentiation comparable to $\alpha_1\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$ showed a non-significantly reduced potentiation and $\alpha_5\beta_2\gamma_2$ showed a significantly reduced potentiation. These results indicate that the type of α subunit has some effect on the potentiation. Next, we investigated the role of the β subunit. $\alpha_1\beta_3\gamma_2$ showed a similar potentiation as $\alpha_1\beta_2\gamma_2$, while significantly smaller potentiation was observed in $\alpha_1\beta_1\gamma_2$. These results indicate that the type of β subunit is important for the potentiation. Further, we investigated the role of the γ subunit. Omission of γ_2 from $\alpha_1\beta_2\gamma_2$ to obtain $\alpha_1\beta_2$, or change to $\alpha_1\beta_3\delta$ did not significantly alter potentiation. Potentiation at the presumably extrasynaptic $\alpha_4\beta_3\delta$ receptors was less than at $\alpha_1\beta_3\delta$, but amounted still to about 2000%. The presence of a γ or a δ subunit is not important.

The point mutated receptors $\alpha_1\beta_2$N265Sγ_2 and $\alpha_1\beta_2$V436Tγ_2 are, unlike the wild type receptors, insensitive to modulation by loreclezole [35,36] and 2-AG [37,38], respectively. As documented in Fig. 3, in $\alpha_1\beta_2$V436Tγ_2 solid potentiation was maintained. These findings make it unlikely that 4-*O*-methylhonokiol acts at the binding site for 2-AG. Potentiation in $\alpha_1\beta_2$N265Sγ_2 was significantly reduced to about 40%, indicating that this mutation at least influences the action of 4-*O*-methylhonokiol.

In order to investigate whether the benzodiazepine binding site is implied in the potentiation by 4-*O*-methylhonokiol, we tried to inhibit this potentiation by the benzodiazepine antagonist Ro15-1788 (Fig. 4). In the absence of the antagonist the response at 0.5 μM GABA was potentiated by 0.5 μM 4-*O*-methylhonokiol by 443 ± 28% (SEM, $n = 4$) and in the presence of 1 μM Ro15-1788 by 381 ± 30% (SEM, $n = 4$). These values did not differ significantly (unpaired *t*-test), indicating that 4-*O*-methylhonokiol does not act at the benzodiazepine binding site.

Further experiments addressed the question whether 4-*O*-methylhonokiol acts at the sites for the neurosteroid THDOC or

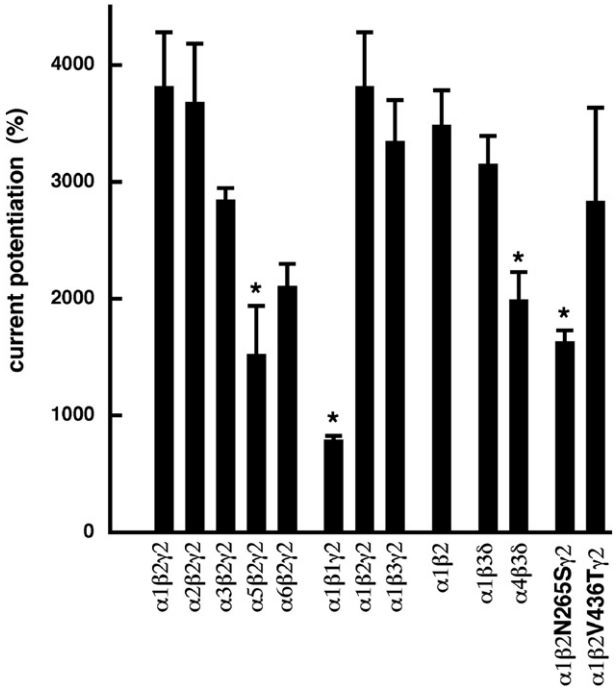

Fig. 3. Subunit specificity of the current potentiation. Different subunit combinations were expressed in *Xenopus* oocytes. Potentiation of GABA currents at a GABA concentration eliciting 0.5–1.0% of the maximal current amplitude was determined using 3 μM 4-*O*-methylhonokiol. Mean ± SEM from 4 to 6 determinations in 1–3 batches of oocytes is shown. An asterisk above the bar indicates a significantly different potentiation as compared to the value obtained from $\alpha_1\beta_2\gamma_2$ receptors.

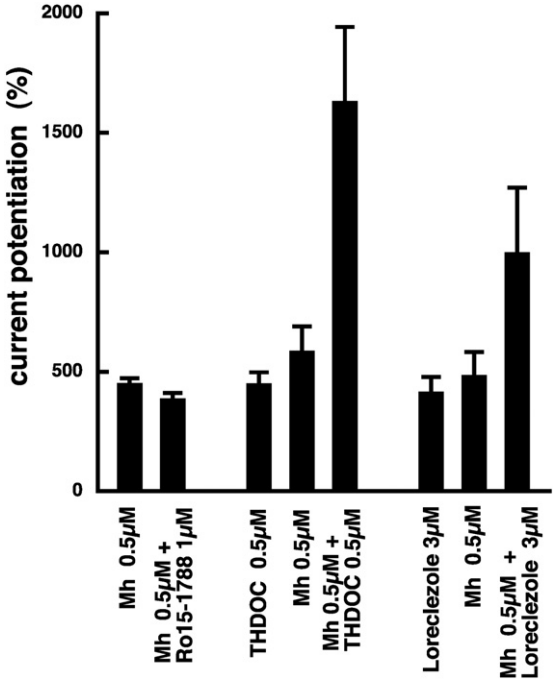

Fig. 4. Effect of co-application of 4-*O*-methylhonokiol with Ro15-1788, THDOC and loreclezole. In order to evaluate whether 4-*O*-methylhonokiol acts at the site for benzodiazepines, neurosteroids or loreclezole, these compounds were applied together with 4-*O*-methylhonokiol. Mean ± SEM from 4 determinations is shown.

loreclezole. 0.5 μM THDOC in combination with 0.5 μM 4-*O*-methylhonokiol acts in an additive or even super-additive way, indicating that the two substances act at different sites. 3 μM loreclezole in combination with 0.5 μM 4-*O*-methylhonokiol acts in an additive way. The EC_{50} for potentiation by loreclezole has been reported to be 1.2 μM [35], indicating again that the two substances act at different sites. The observation that a point mutation abolishing the potentiation by loreclezole reduces potentiation by 4-*O*-methylhonokiol may be due to an allosteric effect by the mutation.

The observation that addition of a methyl group to one phenolic –OH group of honokiol makes the molecule much more active at 3 μM reflects the interaction of the ligand with the receptor. Either removal of the ability to form a hydrogen bridge or introduction of a hydrophobic group in the corresponding position is beneficial for the functional effect.

The present data should also be compared with earlier studies of the effect of honokiol on neuronal $GABA_A$ receptors [25] and recombinant $GABA_A$ receptors expressed in HEK-cells [25] or *Xenopus* oocytes [24]. This comparison is justified if it is assumed that honokiol and 4-*O*-methylhonokiol both act at the same site. In hippocampal dentate granule neurones, extra-synaptic currents were strongly potentiated and decay times of inhibitory post-synaptic currents (IPSC) slowed down by honokiol, while the amplitudes of the IPSCs were unaffected [25]. For recombinant $\alpha_1\beta_3\gamma_2$ expressed in HEK-cells an EC_{50} of about 1 μM and a maximal potentiation of about 500% were reported. No dependence of the potentiation on the isoform of α subunits, β subunits or γ subunits was found [25]. Extensive work with point mutations led to the conclusion that honokiol must act via a new allosteric site. In the oocyte expression system, a similar lower extent of potentiation at β_1 containing receptors was observed as in the present study. Honokiol and derivatives were compared here by their action on $\alpha_1\beta_2$ $GABA_A$ receptors at an exceedingly high concentration of 30 μM. Under these conditions, 4-*O*-methylhonokiol was only twice as active as honokiol. The most active compound there was 3-*N*-acetylamino-4′-*O*-methylhonokiol, with an EC_{50} of about 4 μM and a maximal potentiation of about 2600%.

The seed coat (sarcotesta) of the North American *M. grandiflora* contains 4-*O*-methylhonokiol at a level of about 10%. Ingestion of 20 g seeds would lead to an uptake of about 1000 mg 4-*O*-methylhonokiol (the seed coat represents about 50% of the dry weight of the seed). The molecular weight of 4-*O*-methylhonokiol is 280 g/Mol. If it is assumed that there is little degradation during the time needed for distribution in the body and if it is assumed that distribution occurs throughout the body fluids in an adult with a body weight of 75 kg and a fluid portion of 60%, it may be estimated that *in vivo* concentrations of 78 μM are reached. Based on a threshold of action at the $GABA_A$ receptor of 0.1 μM reported here, 4-*O*-methylhonokiol may – even assuming a substantial degradation and an incomplete distribution – show significant effects in human after consumption of North American *M. grandiflora* seeds. Indeed, it has been shown that seed extracts exert an anticonvulsant effect on rats [29]. The CNS related effects observed after ingestion of extracts of *M. officinalis* bark [19] may also at least partially be due to 4-*O*-methylhonokiol, despite the fact that it is only a minor compound in the bark as compared to honokiol.

4. Conclusion

As the same concentration of 4-*O*-methylhonokiol potentiates $GABA_A$ receptors much stronger than honokiol, it may be used as an easily accessible lead compound in the search of new modulators of $GABA_A$ receptors and it may be at least partially be responsible for biological effects observed after ingestion of *Magnolia* extract. 4-*O*-Methylhonokiol shows an overall high potency against the most common $\alpha_1\beta_2\gamma_2$ receptor subtype [39], therefore indicating a potential to develop 4-*O*-methylhonokiol into a sedative, amnestic and also anticonvulsant agent. The fact that the absence or presence of a δ subunit, which is often regarded as crucially involved in e.g. seizures and epilepsies [40], has not a significant effect on the potentiation of $GABA_A$ receptors by 3-*O*-methylhonokiol does not necessarily thwart this goal. Taken all results together, the situation appears far more complex because 3-*O*-methylhonokiol shows overall high activity against various combinations of α, β and γ subunit containing receptors indicating a broad potential that could be developed through e.g. derivatizations. However, such derivatives of 3-*O*-methylhonokiol may turn out showing a completely different specificity toward the $GABA_A$ receptor subunit composition and therefore a clear prediction of the potential would be shady.

Last, but not least 3-*O*-methylhonokiol also illustrates the phenomenon that a change in the polarity of a molecule due to methylation, as found here in comparison to honokiol, has a remarkable effect on its pharmacological potency.

Acknowledgments

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