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Nitrogenated honokiol derivatives allosterically modulate $GABA_A$ receptors and act as strong partial agonists

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ABSTRACT

In traditional Asian medicinal systems, preparations of the root and stem bark of *Magnolia* species are widely used to treat anxiety and other nervous disturbances. The biphenyl-type neolignan honokiol together with its isomer magnolol are the main constituents of *Magnolia* bark extracts. We have previously identified a nitrogen-containing honokiol derivative (3-acetylamino-4′-*O*-methylhonokiol, **AMH**) as a high efficient modulator of $GABA_A$ receptors. Here we further elucidate the structure-activity relation of a series of nitrogenated biphenyl-neolignan derivatives by analysing allosteric modulation and agonistic effects on $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. The strongest I_{GABA} enhancement was induced by compound **5** (3-acetamido-4′-ethoxy-3′,5-dipropylbiphenyl-2-ol, E_{max}: 123.4 ± 9.4% of $I_{GABA\text{-}max}$) and **6** (5′-amino-2-ethoxy-3′,5-dipropylbiphenyl-4′-ol, E_{max}: 117.7 ± 13.5% of $I_{GABA\text{-}max}$). Compound **5** displayed, however, a significantly higher potency (EC_{50} = 1.8 ± 1.1 μM) than compound **6** (EC_{50} = 20.4 ± 4.3 μM).

Honokiol, **AMH** and four of the derivatives induced significant inward currents in the absence of GABA. Strong partial agonists were honokiol (inducing 78 ± 6% of $I_{GABA\text{-}max}$), **AMH** (63 ± 6%), 5′-amino-2-*O*-methylhonokiol (**1**) (59 ± 1%) and 2-methoxy-5′-nitro-3′,5-dipropylbiphenyl-4′-ol (**3**) (52 ± 1%). 3-*N*-Acetylamino-4′-ethoxy-3′,5-dipropyl-biphenyl-4′-ol (**5**) and 3-amino-4′-ethoxy-3′,5-dipropyl-biphenyl-4′-ol (**7**) were less efficacious but even more potent (**5**: EC_{50} = 6.9 ± 1.0 μM; **7**: EC_{50} = 33.2 ± 5.1 μM) than the full agonist GABA.

1. Introduction

γ-Aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the mammalian central nervous system (CNS). The action of GABA is primarily exerted through ligand-gated ion channels, the $GABA_A$ receptors. The $GABA_A$ receptor is a co-assembly of five subunits, which together form a central pore in the cell membrane for selective chloride ion transport.[12] $GABA_A$ receptors exist in different subtypes, which are characterized by the type of subunit and the respective assemblage and they depend on the tissue in which they occur. The different $GABA_A$ subtypes exert different physiological effects[15,17] and react differently to $GABA_A$ receptor modulatory compounds making the search for subtype-selective chemical entities interesting.[18] The $GABA_A$ receptor plays a crucial role in several disorders of the CNS such as depression, anxiety, epilepsy. Among many other classes of $GABA_A$ receptor modulators, two classes that are clearly identifiable upon their mode of action are benzodiazepines that exert their action upon the presence of a γ_2 subunit within the presence of either α_1, α_2, α_3 or α_5 subunits[21] and barbiturates, etomidate, propofol, valerenic acid, which do not require the presence of a γ subunit.[7,19,10]

The study of Asian medicinal preparations with anxiolytic and CNS relaxing effects such as Saiboku-to from Japan led to the identification of the biphenyl neolignans honokiol and magnolol as the major active constituents of the Asian *Magnolia* bark preparations that contain, for example, *Magnolia officinalis* Rehd. et Wils.[13]. Besides the great multitude of pharmacological activities that are ascribed to especially honokiol (**H**),[14] the CNS activity of honokiol and magnolol could be linked to their interaction with $GABA_A$ receptors.[1]

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The modulatory effect of honokiol on chloride currents through a set of $GABA_A$ receptor subtypes expressed in *Xenopus* oocytes was previously investigated in our group using a series of 31 analogs of honokiol. It led to the discovery of the very potent 3-acetylamino-4′-*O*-methylhonokiol (**AMH**) that enhanced I_{GABA} trough $\alpha_1\beta_2$ receptors by more than 2600%.[20] In that communication, it was also shown that for **H**, the potentiation was about equal for $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_2$ receptor subtypes, that is, the potentiation did not require the presence of a γ_{2S} subunit, which hints to a binding site of **H** different from the benzodiazepine binding site. Accordingly, Baur et al.[4] could demonstrate through an indepth study on subunit-specificity of 4′-*O*-methylhonokiol (**MH**) that the current potentiation by **MH** was also not depending on the presence of a γ_{2S} subunit. The binding of benzodiazepine requires the presence of a γ_2 receptor subunit, however, benzodiazepine effects are usually accompanied by undesired side effects[23] rendering a drug candidate interacting with a novel (non benzodiazepine) binding site especially interesting. Recent data of Alexeev et al.[2] who analysed the effects of several point mutations on **H** action suggest that its binding site may be separate from the binding site of neurosteroids, anesthetics, ethanol and picrotoxin.

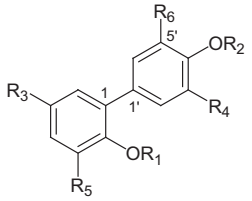
The structural similarity of **AMH** to **H** and **MH** prompted us to further explore this lead as a candidate with potentially lacking of benzodiazepine side-effects through the study of structure activity-relationships of nitrogenated honokiol derivatives by analysing allosteric modulation of $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors with particular focus on partial agonistic effects.

2. Results and discussion

2.1. Syntheses

Seven honokiol derivatives with nitrogen-containing moieties (**1**–**7**; Scheme 1, Table 1) were synthesized and the enhancement of GABA-induced chloride currents (I_{GABA}) was studied. Aside, a potential induction of chloride currents through $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_{2S}$ subunits was analysed subsequently.

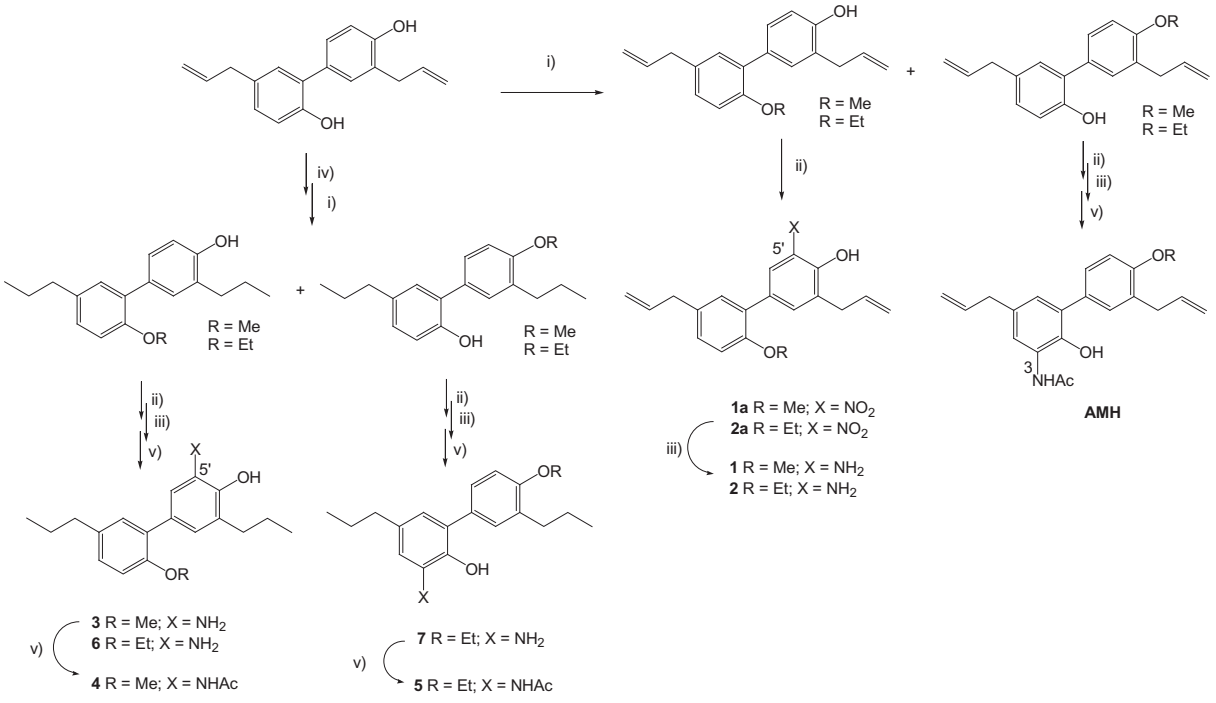
Table 1
Structures of compounds based on nitrogenated honokiol for the evaluation of $GABA_A$ receptor modulatory activity including the previously identified highly efficient 3-acetylamino-4′-*O*-methylhonokiol (**AMH**, i.e., compd **31** in Ref. 20) and honokiol (**H**)



Compd	R_1	R_2	R_3	R_4	R_5	R_6
H	–H	–H	–2-Propenyl	–2-Propenyl	–H	–H
AMH	–H	$-CH_3$	–2-Propenyl	–2-Propenyl	$-NHCOCH_3$	–H
1	$-CH_3$	–H	–2-Propenyl	–2-Propenyl	–H	$-NH_2$
2	$-C_2H_5$	–H	–2-Propenyl	–2-Propenyl	–H	$-NH_2$
3	$-CH_3$	–H	Propyl	Propyl	–H	$-NH_2$
4	$-CH_3$	–H	Propyl	Propyl	–H	$-NHCOCH_3$
5	–H	$-C_2H_5$	Propyl	Propyl	$-NHCOCH_3$	–H
6	$-C_2H_5$	–H	Propyl	Propyl	–H	$-NH_2$
7	–H	$-C_2H_5$	Propyl	Propyl	$-NH_2$	–H

The syntheses aimed at combining pharmacophore features that turned out to be most promising from previous $GABA_A$ receptor modulatory studies,[20] that is, nitrogenation of the aromatic ring using either an amino function or an acetylated amino function as well as the substitution of the free hydroxy groups with either methyl or ethyl moieties. The hydrogenation of the initial 2-propenyl chain into a propyl chain was in most cases undertaken to enhance overall chemical stability.

The 2-*O*-alkylated honokiols 2-*O*-methylhonokiol and 2-*O*-ethylhonokiol resp. were nitrated in ortho position to the free



i) MW irradiation 1. KOH, 2. Me_2SO_4 or Et_2SO_4; ii) HNO_3 (65%), EtOAc; iii) $SnCl_2$ x $2H_2O$, EtOH; iv) H_2, [Pd/C]; v) Ac_2O, H_2O

Scheme 1. Synthesis of a series of nitrogenated honokiol analogs.

hydroxy group according to Johnson and Corey[8] resulting in 2-*O*-methyl-5′-nitrohonokiol (**1a**) and 2-*O*-ethyl-5′-nitrohonokiol (**2a**), resp., which were reduced to the corresponding amines (**1**) and (**2**) according to literature.[22] The synthesis of the five hydrogenated honokiol derivatives **3**–**7** is described in Bernaskova et al.,[5] the general route to alkylated honokiols is described in Schuehly et al.[16]

2.2. Pharmacological evaluation

2.2.1. Concentration-dependent enhancement of I_{GABA} by honokiol derivatives

I_{GABA} (EC_{3-7}) modulation by derivatives **1**–**7** was determined (Fig. 1, Table 2).

2.2.2. Honokiol derivatives as partial agonist on $GABA_A$ receptors

Honokiol and its nitrogenated derivatives **AMH**, **1**, **3**, **5** and **7** induced chloride currents through $GABA_A$ receptors in the absence of GABA (see Fig. 1D for representative currents evoked by 100 μM of the indicated compound). Figure 1C illustrates the partial agonistic effects. Inward currents are expressed as fractions of $I_{GABA\text{-}max}$ induced by 1 mM GABA.

H, **AMH** and **1** were identified as the strongest partial agonists on $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors with maximal inward currents ranging between 59 ± 1% (**1**, $n = 3$) and 78 ± 6% (**H**, $n = 4$) of $E_{max\text{-}dir}$, followed by the slightly less efficient compound **3** (52 ± 1%, $n = 3$). The weakest partial agonists were compounds **5** and **7**, however still inducing approximately 30% of $E_{max\text{-}dir}$ (Table 3, Fig. 1C). Compounds **2**, **4** and **6** did not induce chloride currents in the absence of GABA (Table 3).

3. Conclusion

In a previous study on I_{GABA} modulation by honokiol and derivatives, it has been found that derivatives comprising nitrogen-containing moieties potentiate I_{GABA} more efficiently

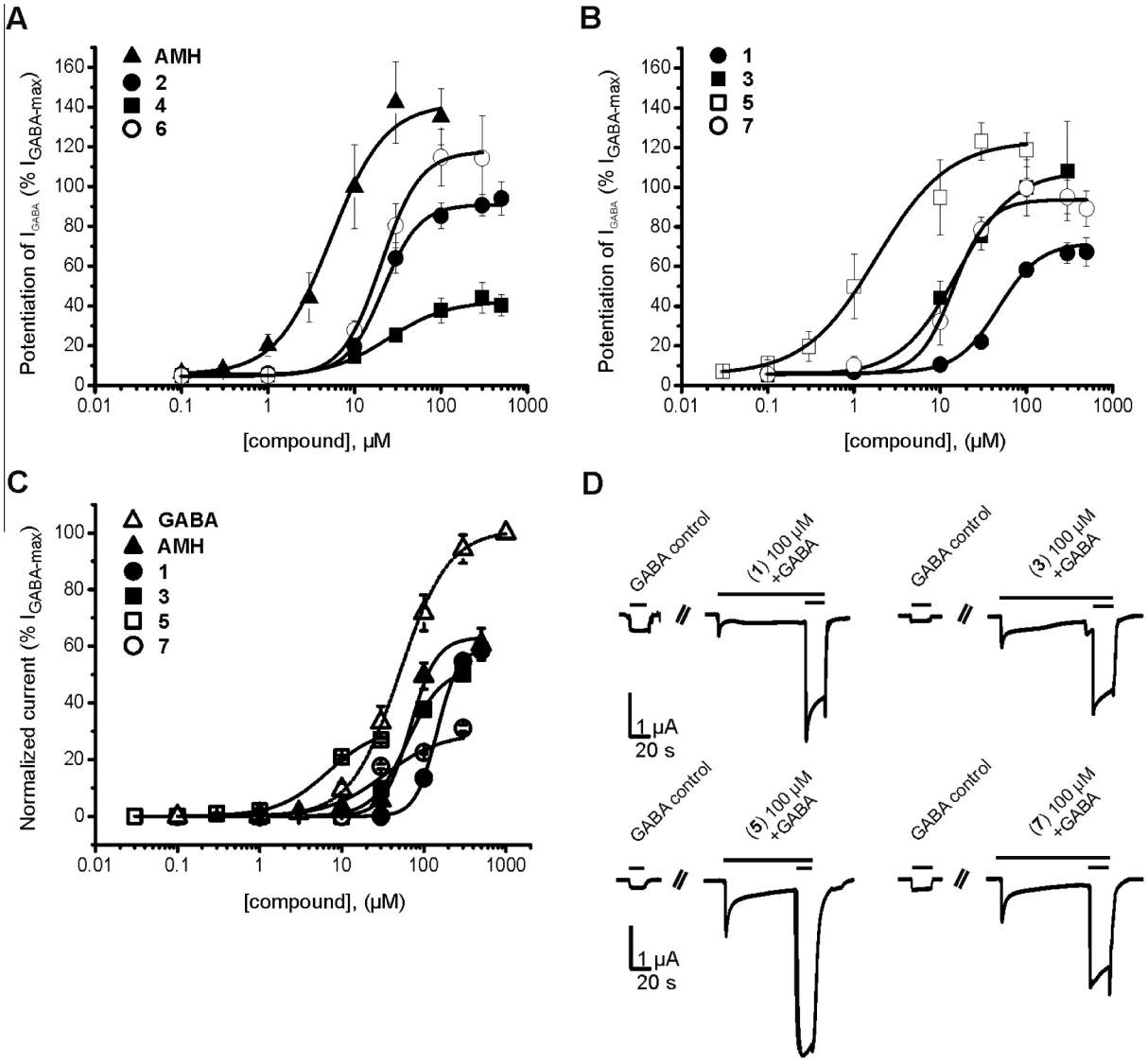


Figure 1. Concentration–effect curves for I_{GABA} potentiation ($\alpha_1\beta_2\gamma_{2S}$) by (A) **AMH** (▲), **2** (●), **4** (■) and **6** (○) and (B) **1** (●), **3** (■), **5** (□) and **7** (○). (C) Partial agonistic effect induced by **AMH** (▲), **1** (●), **3** (■), **5** (□) and **7** (○) on $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors compared to I_{GABA} induced by the full agonist GABA (△, from[3]). Each data point represents the mean ± SE from at least three oocytes and two different frogs. (D) Typical inward currents illustrating direct activation of $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors (single horizontal bar) and I_{GABA} modulation (double horizontal bar) by 100 μM of compounds **1**, **3**, **5** and **7**.

Table 2
Efficiency and potency of I_{GABA} modulation ($\alpha_1\beta_2\gamma_{2S}$) by **AMH** and derivatives **1**–**7**. E_{max} indicates maximum enhancement of chloride current through $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors induced by the indicated compound in % of the maximal I_{GABA} induced by 1 mM GABA. Hill-coefficient (n_H) and number of experiments are given

Compound	E_{max} (%)	EC_{50} (μM)	n_H	n
AMH	141.6 ± 14.1	5.3 ± 1.9	1.4 ± 0.3	5
1	72.0 ± 4.8	47.6 ± 6.8	1.8 ± 0.2	6
2	91.0 ± 4.2	21.5 ± 3.3	2.1 ± 0.4	4
3	108.0 ± 8.0	15.8 ± 4.4	1.3 ± 0.1	4
4	42.5 ± 4.9	24.6 ± 7.4	1.3 ± 0.3	4
5	123.4 ± 9.4	1.8 ± 1.1	1.0 ± 0.3	7
6	117.7 ± 13.5	20.4 ± 4.3	1.9 ± 0.3	5
7	93.7 ± 5.8	14.4 ± 3.2	2.2 ± 0.8	4

Table 3
Direct activation of $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors by honokiol derivatives. $E_{max\text{-}dir}$ indicates maximum chloride current through $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors induced by a saturating concentration of the indicated compound in % of the maximal I_{GABA} induced by 1 mM GABA (see Fig. 1C). EC_{50} value and n_H of the GABA concentration–response curves for comparison were taken from[3]

Compound	$E_{max\text{-}dir}$ (%)	EC_{50} (μM)	n_H	n
GABA	100	51.0 ± 3.0*	1.4 ± 0.1	27
H	78 ± 6	76.2 ± 10.3	2.6 ± 0.4	4
AMH	63 ± 6	68.1 ± 9.7	2.7 ± 0.4	3
1	59 ± 1	144.3 ± 5.7**	3.3 ± 0.3	3
2	No agonist activity			
3	52 ± 1	62.2 ± 2.4	2.1 ± 0.1	3
4	No agonist activity			
5	32 ± 2	6.9 ± 1.0**	1.4 ± 0.1	3
6	No agonist activity			
7	29 ± 2	33.2 ± 5.1**	1.3 ± 0.2	3

Asterisks indicate statistically significant differences to $I_{Honokiol}$ as follows.
* $p < 0.05$.
** $p < 0.01$.

and also display higher potencies compared to the parent molecule honokiol.[20] Based on these findings, 7 nitrogen-containing honokiol derivatives have been synthesized combining molecular features that were recognized to be important functional groups and subsequently studied for I_{GABA} enhancement and direct activation of $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_{2S}$ subunits. Altogether two compounds of the tested series and two precursors are new chemical entities.

Besides their modulatory activity, **H**, **AMH** and four of the newly synthesized derivatives activated $GABA_A$ receptors in the absence of GABA (Fig. 1C and D, Table 3). Partial agonism was most pronounced for **H**, **AMH** and **1** followed by **3**. Compounds **5** and **7** are only weak partial agonists but apparently more potent on $\alpha_1\beta_2\gamma_{2S}$ receptors than the full agonist GABA. Partial agonist activity was previously reported for **H** and magnolol (at concentrations >10 μM) by Alexeev et al.,[2] though in a different cell system. Our data confirm and extend this finding to nitrogenated derivatives such as **AMH**, **1**, **3**, **5** and **7** (Table 3). Remarkably, small structural changes completely diminish partial agonism while preserving positive allosteric modulation of $GABA_A$ receptors (**2**, **4**, **6** in Tables 2 and 3). First studies with **H** on mutated $GABA_A$ receptors (including $\alpha1_{(Q240W)}$, essential for the action of neurosteroids; $\beta3_{(M286W)}$, preventing the action of general anesthetics; $\beta3_{(T256F)}$ or $\alpha1_{(T260F)}$ essential for the interaction with picrotoxine) did not affect allosteric modulation of $GABA_A$ receptors by either **H** or magnolol suggesting that these molecules interact with an yet unidentified binding site.[2] We show here that the agonistic activity of **H** and the studied nitrogenated derivatives does not correlate with allosteric modulation. Future studies will show whether agonistic and modulatory effects of these compounds are mediated via separate binding sites.

4. Experimental

4.1. General

Infrared spectra were recorded on a Bruker Alpha Platinum ATR spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian 400 MHz spectrometer (400 and 100 MHz) using chloroform-d as solvent and were referenced using TMS as internal standard. It is worth to note that the carbons of the B-ring of the amines **1** and **2** give very broad signals in the ^{13}C NMR-spectra. Therefore their resonances are often only visible in the HMBC spectra. C-5 of **2** is not even definitely found in HMBC.

EI-MS were recorded on an Agilent Technologies HP 7890A instrument fitted with detector HP 5975C VL MSD (70 eV, ion source 250 °C, quadrupole temperature 150 °C). Column: Agilent HP-5MS 30 m, ID 0.25 mm, film 5% phenyl95%methylpolysiloxane 9.25 μm. Oven temperature was kept at 45 °C for 2 min and programmed to 300 °C at a rate of 3 °C/min, then kept constant at 300 °C for 20 min.

ESI-MS were recorded in ESI positive and negative mode on a Thermo Finnigan LCQ Deca XP Plus mass spectrometer with autosampler. Column: Zorbax SB-C18 (3.5 μm; 150 × 2.1 mm; Agilent Technologies) with guard column at a flowrate of 300 μL/min.

The purity of synthesized compounds was verified using HPLC on an Agilent 1260 series equipped with diode array detector and by NMR spectroscopy. For analytical HPLC-DAD, an SB-C18 Zorbax column (3.5 μm; 150 × 2.1 mm; Agilent Technologies) equipped with guard column at a flow rate of 300 μL/min was used. The gradient elution program was as follows: CH_3CN in water (0 → 25 min/10 → 90%, 25 → 30 min/90 → 100%, 30 → 38 min/100%).

For TLC analysis, precoated Si60 F_{254} plates (Merck, Darmstadt) were used. Detection was done by UV/254 nm and spraying with molybdato-phosphoric acid and subsequent heating.

Compound mixtures were separated by PTLC (Merck; PLC silica gel 60 F_{254}, 1 mm), using cyclohexane/ethyl acetate mixtures. Honokiol was purchased from APIChem Technology Co., Hangzhou, China (purity >98%).

4.2. Synthesis

4.2.1. Synthesis of 2-*O*-methyl-5′-nitro-honokiol (1a)

Nitric acid (65%, 3.6 mmol, 0.25 mL) was added under intense stirring within ca. 5 s to a solution of 2-*O*-methyl-honokiol (101 mg, 0.360 mmol; synthesis see[16]) in ethyl acetate (10 mL) at room temperature. The reaction mixture was stirred for 10 min and neutralized with NaOH (2 N). The organic phase was separated and the water phase was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with brine (15 mL), dried over Na_2SO_4 and concentrated under reduced pressure yielding 115 mg (98%) of methyl-5′-nitro-honokiol (**1a**) as orange oil.

1a: IR (ATR, ν_{max}, cm^{-1}): 3209, 3079, 2909, 2835, 1638, 1621, 1536, 1498, 1464, 1431, 1323, 1239, 1179, 1129, 1027, 912, 810, 768, 676, 606; ^1H NMR ($CDCl_3$) δ 10.97 (s, 1H, O*H*), 8.15 (d, J = 1.8 Hz, 1H, H-6′), 7.65 (d, J = 1.8 Hz, 1H, H-2′), 7.16 (dd, J = 8.4, 2.2 Hz, 1H, H-4), 7.10 (d, J = 2.2 Hz, 1H, H-6), 6.92 (d, J = 8.4 Hz, 1H, H-3), 6.01 (ddt, J ~17, 10.3, 6.8 Hz, 1H, H-2‴), 5.97 (ddt, J ~17, 10.2, 6.6 Hz, 1H, H-2″), 5.13 (m, 2H, H-3‴), 5.09 (m, 2H, H-3″), 3.80 (s, 3H, OCH_3), 3.53 (d, J = 6.6 Hz, 2H, H-1‴), 3.38 (d, J = 6.6 Hz, 2H, H-1″); ^{13}C NMR ($CDCl_3$) δ 154.8 (C-2), 152.3 (C-4′), 139.1 (C-2′), 137.5 (C-2″), 135.3 (C-2‴), 133.3 (C-5′), 132.6 (C-5),

130.6 (C-3′), 130.5 (C-6), 130.2 (C-1′), 129.2 (C-4), 127.8 (C-1), 123.4 (C-6′), 116.7 (C-3‴), 115.8 (C-3″), 111.4 (C-3), 55.7 (OCH3), 39.3 (C-1″), 33.8 (C-1‴); MS (ESI$^-$) *m*/*z* (%): 324.22 ([M−H]$^-$, 100).

4.2.2. Synthesis of 5′-amino-2-*O*-methylhonokiol (1)

$SnCl_2 \times 2H_2O$ (70 mg, 0.310 mmol) was added to a solution of 2-*O*-methyl-5′-nitro-honokiol (**1a**) (98 mg, 0.301 mmol) in MeOH (10 mL) and was stirred for 72 h at room temperature, an additional amount of $SnCl_2 \times 2H_2O$ (100 mg, 0.443 mmol) was added and stirring was continued for 24 h. The foamy precipitate resulting from the addition of $NaHCO_3$ (1 N, 20 mL) was filtered off with Celite® and rinsed with EtOH (30 mL). After evaporation of the alcohols the resulting mixture was extracted with dichloromethane (3×10 mL). The combined extracts were dried over Na_2SO_4, concentrated under reduced pressure and purified by PTLC (silica, cyclohexane/ethyl acetate 5:3) to yield **1** (25 mg, 39%) as a brown oil. **1**: IR spectra (ATR, ν_{max}, cm^{-1}): 3373, 3313, 3074, 3000, 2974, 2903, 2832, 1637, 1606, 1488, 1240, 1141, 907, 809; ^{1}H NMR ($CDCl_3$) δ 7.12 (s, 1H, H-6), 7.10 (d, *J* = 8.8 Hz, 1H, H-4), 6.90 (d, *J* ~8 Hz, 1H, H-3), 6.89 (s, 1H, H-6′), 6.75 (s, 1H, H-2′), 6.00 (ddt, *J* = 16.9, 10.2, 6.4 Hz, 1H, H-2‴), 6.04 (ddt, *J* = 16.9, 10.9, 6.6 Hz, 1H, H-2″), 5.28 (d, *J* = 17.6 Hz, 1H, H-3‴), 5.21 (d, *J* = 9.9 Hz, 1H, H-3‴), 5.11 (dq, *J* = 16.9, 1.2 Hz, 1H, H-3″), 5.07 (d, *J* ~8 Hz, 1H, H-3″), 3.79 (s, 3H, OC*H*$_3$), 3.45 (d, *J* = 6.0 Hz, 2H, H-1‴), 3.37 (d, *J* = 6.5 Hz, 2H, H-1″); ^{13}C NMR ($CDCl_3$) δ 154.8 (C-2), 142.3 (C-4), 137.8 (C-2″), 136.7 (C-2‴), 134.4 (C-5′), 132.1 (C-5), 131.3 (C-1′), 131.0 (C-6), 130.5 (C-1), 127.8 (C-4), 124.7 (C-3′),122.1 (C-2′), 117.0 (C-6′), 116.7 (C-3‴), 115.5 (C-3″), 111.2 (C-3), 55.7 (OCH_3), 39.4 (C-1″), 36.0 (C-1‴); MS (ESI) *m*/*z* (%): 296.17 [M+H]$^+$ (100).

4.2.3. Synthesis of 2-*O*-ethyl-5′-nitro-honokiol (2a)

Nitric acid (65%, 0.182 mL, 2.62 mmol) was added under intense stirring within ca. 5 s to a solution of 2-*O*-ethyl-honokiol (77 mg, 0.262 mmol; synthesis see[16]) in ethyl acetate (10 mL) at room temperature. The reaction mixture was stirred for 60 s and carefully neutralized with NaOH (2 N). The organic phase was separated, and the aqueous phase was extracted with ethyl acetate (3×15 mL). The combined organic phases were washed with brine (3×15 mL), dried over Na_2SO_4, and concentrated under reduced pressure. Because of incomplete reaction the residue was solved again in ethyl acetate (10 mL) nitration and workup were repeated but with a reaction time of 10 min resulting in 88 mg of 2-*O*-ethyl-5′-nitro-honokiol (**2a**) as an orange oil, yield 98%.

IR (ATR, ν_{max}, cm^{-1}): 3204, 3079, 2978, 1638, 1621, 1536, 1499, 1466, 1323, 1238, 1129, 1042, 912, 674, 551; ^{1}H NMR ($CDCl_3$) δ 10.97 (s, 1H, O*H*), 8.20 (d, *J* = 2.2 Hz, 1H, H-6′), 7.73 (d, *J* = 1.5 Hz, 1H, H-2′), 7.13 (d, *J* ~7, 1H, H-4), 7.12 (s, 1H, H-6), 6.90 (d, *J* = 8.8 Hz, 1H, H-3), 6.02 (ddt, *J* ~17, 10.3, 6.7 Hz, 1H, H-2‴), 5.98 (ddt, *J* ~17, 9.9, 6.6 Hz, 1H, H-2″), 5.15 (m, 2H, H-3‴), 5.09 (m, 2H, H-3″), 4.03 (q, *J* = 6.9 Hz, 2H, OC*H*$_2$CH$_3$), 3.53 (d, *J* = 6.6 Hz, 2H, H-1‴), 3.38 (d, *J* = 6.6 Hz, 2H, H-1″), 1.35 (t, *J* = 6.9 Hz, 3H, OCH$_2$C*H*$_3$); ^{13}C NMR ($CDCl_3$) δ 154.1 (C-2), 152.2 (C-4′), 139.3 (C-2′), 137.5 (C-2″), 135.3 (C-2‴), 133.3 (C-5′), 132.5 (C-5), 130.4, 2 x 130.3 (C-6, C-1′, C-3′), 129.2 (C-4), 127.7 (C-1), 123.3 (C-6′), 116.8 (C-3‴), 115.8 (C-3″), 112.5 (C-3), 64.1 (OCH_2CH$_3$), 39.3 (C-1″), 33.7 (C-1‴), 14.8 (OCH$_2$C*H*$_3$); MS (ESI) *m*/*z* (%): 340.24 ([M+H]$^+$, 100).

4.2.4. Synthesis of 5′-amino-2-*O*-ethylhonokiol (2)

$SnCl_2 \times 2H_2O$ (426 mg, 1.89 mmol) was added to a solution of 2-*O*-ethyl-5′-nitro-honokiol (**2a**) (71 mg, 0.21 mmol) in EtOH (10 mL). After stirring for 72 h at room temperature $NaHCO_3$ (1 N, 30 mL) was added. The foamy precipitate was filtered off with Celite® and rinsed with EtOH (5×10 mL). The solutions were concentrated under reduced pressure and the resulting aqueous solution was extracted with dichloromethane (3×10 mL). The organic layer was concentrated to a final volume of 15 mL, washed with brine, dried over Na_2SO_4, concentrated under reduced pressure and purified by PTLC (silica, cyclohexane/ethyl acetate 5:3) to yield **2** (22 mg, 34%) as a brown oil yield **2**: IR (ATR, ν_{max}, cm^{-1}): 3374, 3313, 3075, 2976, 2922, 1638, 1607, 1489, 1437, 1472; 1410, 1392, 1236, 1142, 993, 909, 805, 732; ^{1}H NMR ($CDCl_3$) δ 7.13 (s, 1H, H-6), 7.06 (d, *J* = 8.3 Hz, 1H, H-4), 6.91 (s, 1H, H-6′), 6.88 (d, *J* = 8.3 Hz, 1H, H-3), 6.82 (s, 1H, H-2′), 6.06 (ddt, *J* = 16.9, 10.2, 5.9 Hz, 1H, H-2‴), 5.99 (ddt, *J* = 16.8, 9.9, 6.6 Hz, 1H, H-2″), 5.26 (d, *J* = 17.2 Hz, 1H, H-3‴), 5.20 (d, *J* = 10.1 Hz, 1H, H-3‴), 5.09 (d, *J* = 17.0 Hz, 1H, H-3″), 5.08 (d, *J* = 10.4 Hz, 1H, H-3″), 4.00 (q, *J* = 6.8 Hz, 2H, OC*H*$_2$), 3.44 (d, *J* = 5.9 Hz, 2H, H-1‴), 3.36 (d, *J* = 6.6 Hz, 2H, H-1″), 1.34 (t, *J* = 6.8 Hz, 3H, OCH$_3$C*H*$_3$); ^{13}C NMR ($CDCl_3$) δ 154.2 (C-2), 142.3 (C-4), 137.8 (C-2″), 136.7 (C-2‴), 132.3 (C-5), 131.4 (C-1′), 130.9 (C-6), 130.8 (C-1), 127.8 (C-4), 125.2 (C-3′), 122.1 (C-2′), 117.0 (C-6′), 116.4 (C-3‴), 115.4 (C-3″), 111.3 (C-3), 55.6 (OCH_3), 39.3 (C-1″), 35.6 (C-1‴); MS (ESI) *m*/*z* (%): 310.14 [M+H]$^+$ (100).

4.3. Pharmacological experiments

4.3.1. Expression of $GABA_A$ receptors in *Xenopus laevis* oocytes and two-microelectrode voltage-clamp experiments

Preparation of stage V–VI oocytes from *Xenopus laevis* and synthesis of capped runoff poly(A) cRNA transcripts from linearized cDNA templates (pCMV vector) was performed as previously described.[9] Female *Xenopus laevis* frogs (NASCO, USA) were anesthetized by 15 min incubation in a 0.2% MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester; Sigma Aldrich, Vienna, Austria) solution before removal of parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/mL collagenase (Type 1A, Sigma–Aldrich, Vienna, Austria).

Selected oocytes were injected with 10–50 nL of DEPC-treated water (diethyl pyrocarbonate, Sigma, Vienna, Austria) containing the different $GABA_A$ cRNAs at a concentration of approximately 300–3000 pg/nL/subunit. To ensure expression of the γ_{2S} subunit in the case of $\alpha_1\beta_2\gamma_{2S}$ receptors, cRNAs were mixed in a ratio of 1:1:10. The amount of cRNAs was determined by means of a NanoDrop ND-1000 (Kisker-Biotech, Steinfurt, Germany).

Oocytes were stored at +18 °C in modified ND96 solution (90 mM NaCl, 1 mM $CaCl_2$, 1 mM KCl, 1 mM $MgCl_2 \times 6H_2O$, and 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid); pH 7.4, all from Sigma–Aldrich, Vienna, Austria).

Chloride currents through $GABA_A$ receptors (I_{GABA}) were measured at room temperature (+21 ± 1 °C) by means of the two-microelectrode voltage clamp technique making use of a TURBO TEC-05X amplifier (npi electronic, Tamm, Germany). I_{GABA} were elicited at a holding potential of −70 mV. Data acquisition was carried out by means of an Axon Digidata 1322A interface using pCLAMP v.10 (Molecular Devices, Sunnyvale, CA, USA). The modified ND96 solution was used as bath solution. Microelectrodes were filled with 2 M KCl and had resistances between 1 and 3 MΩ.

4.3.2. Perfusion system

GABA and the studied derivatives were applied by means of the ScreeningTool (npi electronic, Tamm, Germany) perfusion system as described previously.[6,9] To elicit I_{GABA}, the chamber was perfused with 120 µL of GABA- or compound-containing solutions, respectively, at a volume rate of 300 µL/s.[11] Care was taken to account for possible slow recovery from increasing levels of desensitization in the presence of high drug concentrations. The duration of washout periods was therefore extended from 1.5 min (<10 µM compounds) to 30 min (⩾10 µM compounds), respectively.

Oocytes with maximal current amplitudes >3 μA were discarded to exclude voltage clamp errors.

4.3.3. Data analysis

Stimulation of chloride currents by modulators of the $GABA_A$ receptor was measured at a GABA concentration eliciting between 3% and 7% of the maximal current amplitude (EC_{3-7}). The GABA EC_{3-7} was determined for each oocyte individually. Enhancement of the chloride current was defined as $I_{(GABA+compound)}/I_{GABA\text{-}max} * 100\%$, where $I_{(GABA+compound)}$ is the current response in the presence of a given compound and $I_{GABA\text{-}max}$ is the current response induced by 1 mM GABA. Concentration–response curves were generated and the data were fitted by nonlinear regression analysis using Origin Software (OriginLab Corporation, USA). Data were fitted to the equation $1/(1 + (EC_{50}/[compound])^{nH})$, where n_H is the Hill coefficient. Each data point represents the mean ± SE from at least 3 oocytes and $\geqslant$2 oocyte batches. Statistical significance was calculated using paired Student *t*-test with a confidence interval of <0.05.

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Supplementary data

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