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Identification and Characterization of GABA$_A$ Receptor Modulatory Diterpenes from *Biota orientalis* That Decrease Locomotor Activity in Mice

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Ⓢ *Supporting Information*

ABSTRACT: An ethyl acetate extract of *Biota orientalis* leaves potentiated GABA-induced control current by 92.6% ± 22.5% when tested at 100 μg/mL in *Xenopus laevis* oocytes expressing GABA$_A$ receptors ($\alpha_1\beta_2\gamma_{2S}$ subtype) in two-microelectrode voltage clamp measurements. HPLC-based activity profiling was used to identify isopimaric acid (**4**) and sandaracopimaric acid (**5**) as the compounds largely responsible for the activity. Sandaracopimaradienolal (**3**) was characterized as a new natural product. Compounds **4** and **5** were investigated for GABA$_A$ receptor subtype selectivity at the subtypes $\alpha_1\beta_1\gamma_{2S}$, $\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_3\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, and $\alpha_5\beta_2\gamma_{2S}$. Sandaracopimaric acid (**5**) was significantly more potent than isopimaric acid (**4**) at the GABA$_A$ receptor subtypes $\alpha_1\beta_1\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, and $\alpha_5\beta_2\gamma_{2S}$ (EC$_{50}$ **4**: 289.5 ± 82.0, 364.8 ± 85.0, and 317.0 ± 83.7 μM vs EC$_{50}$ **5**: 48.1 ± 13.4, 31.2 ± 4.8, and 40.7 ± 14.7 μM). The highest efficiency was reached by **4** and **5** on α_2- and α_3-containing receptor subtypes. In the open field test, ip administration of **5** induced a dose-dependent decrease of locomotor activity in a range of 3 to 30 mg/kg body weight in mice. No significant anxiolytic-like activity was observed in doses between 1 and 30 mg/kg body weight in mice.
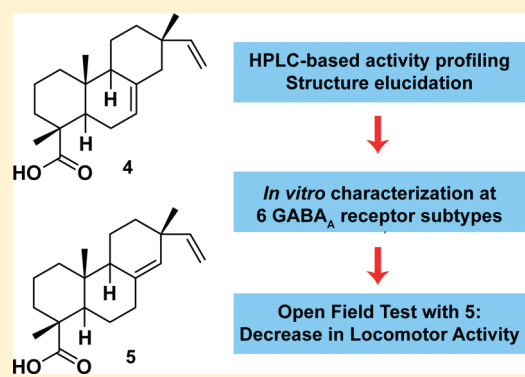
Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS). GABA binding to the GABA$_A$ receptor leads to opening of the intrinsic chloride ion channel. This causes a hyperpolarization of the neuronal membrane and thus to an inhibition of further action potential triggering.[1,2] The quaternary structure of the GABA$_A$ receptor is an assembly of five varying subunits. The human genome encodes for 19 subunits, namely, α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-3}, δ, ε, π, and θ, which may combine into numerous heteropentamers.[3] So far, 11 GABA$_A$ receptor subtypes with distinct pharmacological properties are known to be expressed in human neurons.[4,5] CNS-related diseases such as insomnia, anxiety, and epilepsy are often treated with GABA$_A$ receptor modulating drugs, such as benzodiazepines or barbiturates, which, however, suffer from various side-effects.[6]

We screened a library consisting of 982 plant and fungal extracts for GABA$_A$ receptor modulatory activity with the objective of identifying new scaffolds for the target. For this purpose, we used an automated, functional, two-microelectrode voltage clamp assay with *Xenopus* oocytes[7] that transiently expressed rat GABA$_A$ receptors of the subunit composition $\alpha_1\beta_2\gamma_{2S}$. An ethyl acetate extract of *Biota orientalis* (L.) Endl. (Cupressaceae) leaves and twigs showed promising activity.

B. orientalis originates from Eastern Asia,[8] but nowadays is also widely cultivated in Europe. In addition to various flavonoids, phenylpropanoids, and some lignans, over 100 different terpenoids

have been identified from *B. orientalis* (a synoptical table with secondary metabolites identified in *B. orientalis* is provided as Supporting Information).[8−28] The crude drug consisting of leaves and twigs is known as Cebaye in China and is one of the 50 fundamental herbs of traditional Chinese medicine (TCM). It is used to treat disorders such as diarrhea, respiratory malfunction,[29−31] gout, rheumatism, leukotrichia, and alopecia.[32] The TCM drug Baiziren consists of seeds of *B. orientalis* and is prescribed, among other indications, to treat anxiety.[31]

HPLC-based activity profiling is a miniaturized and highly effective approach for rapid dereplication and characterization of bioactive natural products in extracts[33] and can be combined with various cell-based and biochemical assays.[34−40] We recently developed and validated a profiling protocol for the discovery of new GABA$_A$ receptor ligands,[41] which was successfully applied in the investigation of several active plant extracts.[42−46] Here we report the structural elucidation of a new diterpene, sandaracopimaradienolal (**3**), and the identification of isopimaric acid (**4**) and sandaracopimaric acid (**5**) as GABA$_A$ receptor modulatory constituents via HPLC-based activity profiling of the active *B. orientalis* extract. The GABA$_A$ receptor subtype specificity of **4** and **5** was characterized at receptors of the composition $\alpha_1\beta_1\gamma_{2S}$,
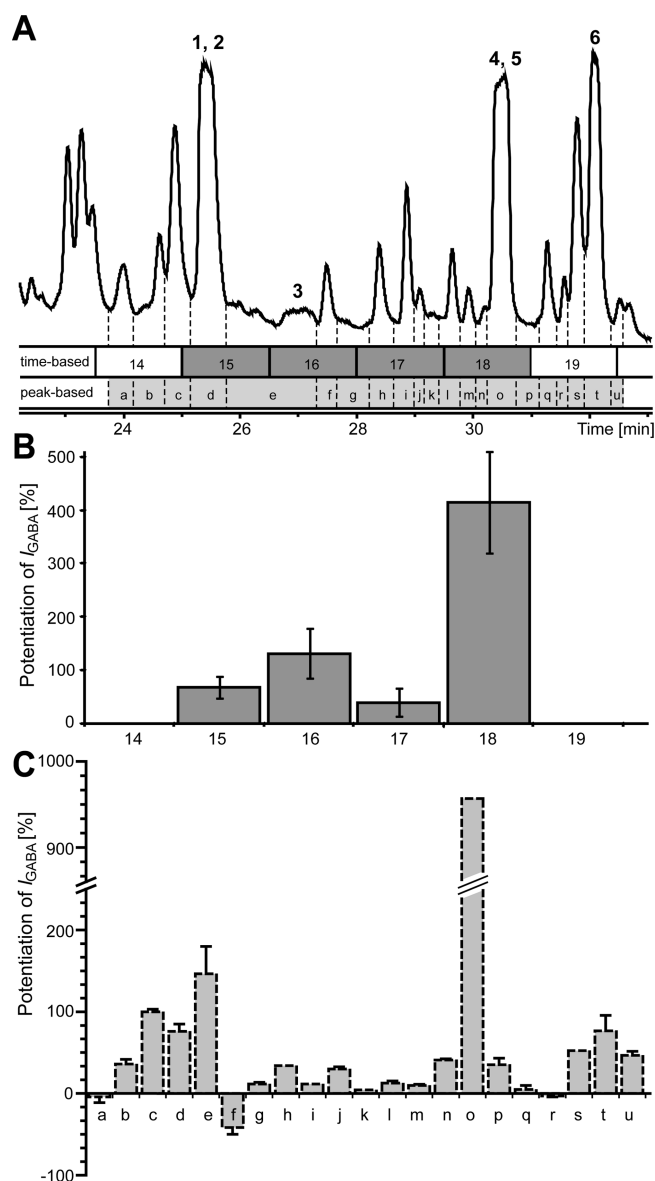
Figure 1. HPLC-based activity profiling of *Biota orientalis* ethyl acetate extract. Part A shows critical time window of a semipreparative HPLC separation (10 mg of extract, detection at 210 nm). Time windows of the time-based microfractionation (90 s each) are highlighted in dark gray, and corresponding potentiation of GABA-induced control current by fractions 14—19 is shown in B. Subsequent peak-based fractionation is shown in light gray bars in A, and potentiation of GABA-induced control current by peak-based microfractions *a*—*u* is displayed in C.

$\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_3\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, and $\alpha_5\beta_2\gamma_{2S}$, and the most potent compound, sandaracopimaric acid (**5**), was examined in a mouse model by means of the open field test.

■ RESULTS AND DISCUSSION

Extracts were screened by means of an automated, fast perfusion system used for two-microelectrode voltage clamp measurements in *Xenopus* oocytes that expressed functional GABA$_A$ receptors with the subunit composition $\alpha_1\beta_2\gamma_{2S}$.[7] When tested at 100 μg/mL, an ethyl acetate extract of *B. orientalis* leaves and twigs enhanced the GABA-induced chloride ion current (I_{GABA}) by 92.6% ± 22.5%. To localize the activity within the

extract, we performed an HPLC-based activity profiling after a validated protocol.[41] The relevant time window of the chromatogram (210 nm) from a semipreparative HPLC separation (10 mg of extract, 28 microfractions of 90 s each) is shown in Figure 1A. GABA$_A$ receptor modulatory activity was concentrated in fractions 15 to 18 (Figure 1A, dark gray bar). Fraction 18 induced the strongest potentiation of I_{GABA} (414.5% ± 95.3%), and fractions 15, 16, and 17 enhanced GABA-induced chloride currents by 67.6% ± 21.0%, 131.5% ± 47.3%, and 39.2% ± 25.8%, respectively (Figure 1B). The active region of the chromatogram was rather complex, and the time-based fractionation did not provide the necessary resolution to track the active peaks. This was achieved by a peak-based fractionation. Peak-based fractions *a*—*u* are shown in Figure 1A by a light gray bar, and the corresponding activity profile is given in Figure 1C. The high activity of time-based fraction 18 could thus be assigned to peak *o* (potentiation of I_{GABA} by 956.9% ± 0.0%). Fraction 16 was resolved into peaks *e*, *f*, and *g*. However, only *e* showed an appreciable potentiation of I_{GABA} (146.45% ± 33.3%). Fraction 15 corresponded to part of peak *e*, to *d*, and to part of *c*. Moderate activity was found in *c* and *d* (potentiation of I_{GABA} by 100.0% ± 3.0% and 76.1% ± 8.9%, respectively). Although time-based fractions 14 and 19 did not show any activity, the corresponding peak-based microfractions were collected and tested. Moderate activity was found in peak-based fraction *t* (enhancement of I_{GABA} by 76.6% ± 18.8%).

For structure elucidation and for further pharmacological testing, compounds corresponding to the active peaks were purified at preparative scale. Liquid—liquid extraction of the EtOAc extract, separation on a silica gel MPLC column, and semipreparative RP-HPLC afforded four pure compounds (**1**—**3**, **6**) and an inseparable mixture of **4** and **5**. Structures were established by 1D NMR and 2D NMR experiments as pinusolide (**1**), sandaracopimaradienediol (**2**), totarol (**6**), and a mixture of isopimaric acid (**4**) and sandaracopimaric acid (**5**) in a ratio of 77:23 (^{1}H NMR). Analytical data of **1**, **2**, **4**, **5**, and **6** are provided as Supporting Information. Structural analysis of minor compound **3** revealed a pimaradiene-type diterpene that differed from **2** only by the oxidation state of C-18. Due to its close relationship to **2** and the aldehyde function at C-18, compound **3** was named sandaracopimaradienolal.
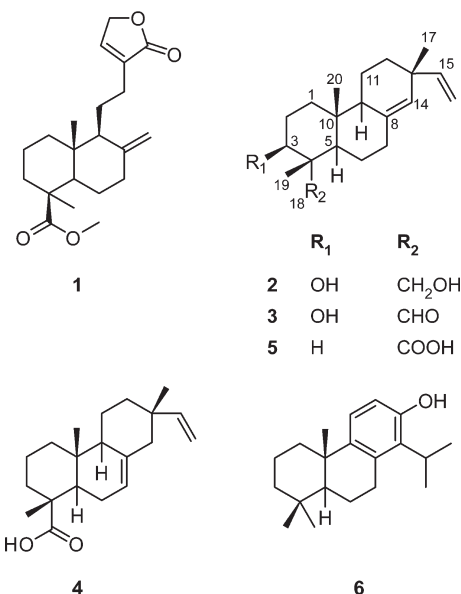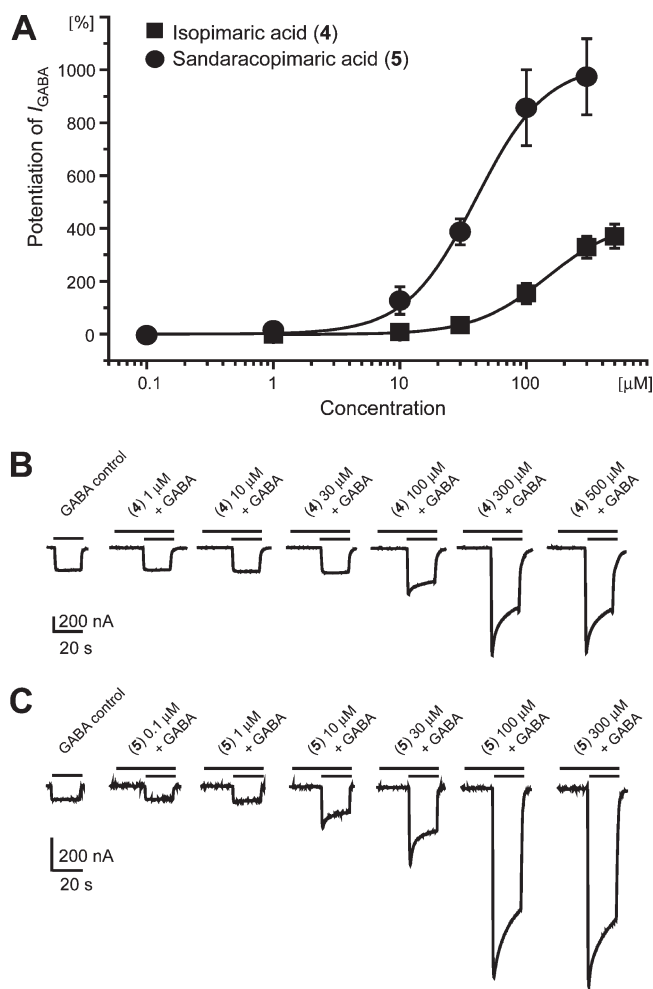
Figure 2. Part A shows concentration−response curves of isopimaric acid (4) and sandaracopimaric acid (5) at $GABA_A$ receptors of the subunit composition $\alpha_1\beta_2\gamma_{2S}$, using a GABA EC_{5-10}. Typical traces for modulation of chloride currents through $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors without direct activation by 4 and 5 are given in B and C, respectively.

Pure compounds and the isomer mixture were tested at concentrations of 10 and 100 μM in the oocyte assay for a preliminary activity profile at $\alpha_1\beta_2\gamma_{2S}$ receptors. The isomer mixture 4/5 potentiated I_{GABA} by 43.9% ± 1.1% and 318.9% ± 101.1% at 10 and 100 μM, respectively (n = 2). Rather unexpectedly, none of the other compounds induced a significant potentiation of I_{GABA} at the tested concentrations (10 μM: −13.4% ± 2.0% (1), 13.8% ± 1.3% (2), 3.8% ± 3.8% (3), −10.6% ± 4.8% (6); 100 μM: −13.1% ± 2.6% (1), 7.7% ± 7.7% (2), 3.0% ± 3.0% (3), −15.1% ± 15.1% (6) (n = 2)). Therefore, further concentration−response experiments were performed only with 4 and 5, which were commercially obtained as pure substances. Modulation of I_{GABA} through $\alpha_1\beta_2\gamma_{2S}$ receptors was studied with concentrations ranging from 0.1 to 500 μM. Maximum I_{GABA} potentiation by 4 (425.2% ± 96.5%, n = 5) was observed at ∼500 μM, with an EC_{50} of 141.6 ± 68.0 μM. Sandaracopimaric acid (5) was more potent (EC_{50}: 33.3 ± 8.7 μM) and more efficient than 4 (max. potentiation of I_{GABA}: 855.7% ± 114.9%, n = 4) (Figure 2; Table 1).

Next, the concentration-dependent I_{GABA} modulation of compounds 4 and 5 was tested on distinct $GABA_A$ receptor subtypes ($\alpha_1\beta_1\gamma_{2S}$, $\alpha_1\beta_3\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, and $\alpha_5\beta_2\gamma_{2S}$) to elucidate potential subunit specificity. As displayed in Figure 3 and summarized in Table 1, sandaracopimaric acid (5) showed higher potencies (EC_{50}) than isopimaric acid (4) on all receptor subtypes, reflected by potency ratios $[EC_{50}(4)/EC_{50}(5)]$ of 4.25 ($\alpha_1\beta_2\gamma_{2S}$ receptors) and upward (Table 2). However, only on receptor subtypes $\alpha_1\beta_1\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, and $\alpha_5\beta_2\gamma_{2S}$ was this difference statistically significant. Moreover, on $\alpha_1\beta_2\gamma_{2S}$ receptors, 5 was twice as efficient in stimulating I_{GABA} compared to 4, as reflected by the efficiency ratio $[I_{max}(4)/I_{max}(5)]$ (Table 2). Overall, 5 seemed slightly more efficient, except at subtype $\alpha_1\beta_1\gamma_{2S}$ (differences not statistically significant). The huge discrepancies between potency and efficiency of 4 and 5 are of particular interest in light of the small structural differences between these compounds. The two diterpenes differ only in the position of a double bond ($\Delta^{7,8}$ in 4 vs $\Delta^{8,14}$ in 5).

However, neither of the compounds exerted significant particular subtype specificity, as reflected by their comparable EC_{50} values at the subtypes of investigation (p > 0.05) (Table 1). The order of efficiency of isopimaric acid (4) on $GABA_A$ receptors comprising different α-subunits was $\alpha_1\beta_2\gamma_{2S} \approx \alpha_5\beta_2\gamma_{2S} < \alpha_2\beta_2\gamma_{2S}$ (p < 0.05). The apparently higher efficiency of 4 on $\alpha_3\beta_2\gamma_{2S}$ receptors compared to α_1/α_5-containing subtypes, however, was not statistically significant. A different order of efficiency was observed for 5 with $\alpha_5\beta_2\gamma_{2S} < \alpha_1\beta_2\gamma_{2S} \approx \alpha_2\beta_2\gamma_{2S} \approx \alpha_3\beta_2\gamma_{2S}$ (p-values see Table 1). On $GABA_A$ receptors comprising varying β-subunits, only 5 showed a significant difference in efficiency ($\alpha_1\beta_1\gamma_{2S} < \alpha_1\beta_2\gamma_{2S}$; p < 0.05) (Table 1). When tested at the $\alpha_1\beta_2\gamma_{2S}$ subtype, benzodiazepines (triazolam, midazolam, and clotiazepam) were clearly more potent than 4 and 5 (Table 1). Efficiencies, however, ranged from 253% ± 12% (triazolam) to 342 ± 64% (midazolam) potentiation of I_{GABA}[47] and are thus comparable with efficiencies of 4 and 5 (Table 1). To determine a possible dependency on the γ-subunit, which is involved in the benzodiazepine binding site, 4 and 5 were tested on $GABA_A$ receptors comprising α_1 and β_2 subunits only. No significant difference in activity was found between $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ subtypes at 30 and 100 μM, which suggested a γ-subunit-independent binding site (Figure 4). Pre-incubation experiments revealed that neither 4 nor 5 showed direct activation on any of the expressed $GABA_A$ receptor subtypes (Figure 3, parts B, D, F, and H; Figure 4, parts B and D). Therefore, isopimaric acid (4) and sandaracopimaric acid (5) can be described as positive allosteric $GABA_A$ receptor modulators devoid of direct agonistic activity and particular subtype specificity.

Modulation of $GABA_A$ receptors comprising α_1, α_2, and α_3 subunits by 4 and 5 (Figure 3, Table 1) suggests sedative-like and anxiolytic-like action in vivo (see Mohler et al. 2010 for a recent review).[48] We, therefore, studied the in vivo effects of the more potent compound (5) in mice in the open field (OF) test. The OF test is based on the natural behavior of rodents such as an innate fear of open spaces and represents a standard behavioral paradigm for evaluation of anxiolytic-like properties of drugs and for the measurement of locomotor activity.[49] We analyzed the explorative behavior of male c57Bl/6N mice 30 min after intraperitoneal injection of either vehicle (= control) or 5 in the OF test. As illustrated in Figure 5A, ambulation of control mice and mice treated with 1 mg/kg BW 5 did not significantly differ (control: 31.8 ± 1.7 m, n = 10 vs 1 mg/kg BW: 31.8 ± 2.3 m, n = 10; p > 0.05). Administration of 5 at a dose of 3 mg/kg BW, however, resulted in significantly reduced ambulation (25.1 ± 2.0 m, n = 19; p < 0.05), which reached its

Table 1. Potencies and Efficiencies of Compounds 4 and 5 for GABA$_A$ Receptors of Different Subtype Compositions and of Reference Compounds (Benzodiazepines) at the $\alpha_1\beta_2\gamma_{2S}$ Subtype

	Isopimaric Acid (4)		
subtype	EC$_{50}$ [μM]	max. potentiation of I_{GABA} (EC$_{5-10}$) [%](I_{max})	Hill coeff (n_H)[a]	n[b]
$\alpha_1\beta_1\gamma_{2S}$	289.5 ± 82.0	715.9 ± 143.3	1.6 ± 0.2	6
$\alpha_1\beta_2\gamma_{2S}$	141.6 ± 68.0	425.2 ± 96.5[c-h]c	1.6 ± 0.4	5
$\alpha_1\beta_3\gamma_{2S}$	257.0 ± 121.2	475.7 ± 150.9	1.5 ± 0.3	4
$\alpha_2\beta_2\gamma_{2S}$	364.8 ± 85.0	1031.5 ± 173.9[c-h]c,d	1.9 ± 0.3	5
$\alpha_3\beta_2\gamma_{2S}$	724.1 ± 340.7	1074.0 ± 370.5	1.2 ± 0.1	6
$\alpha_5\beta_2\gamma_{2S}$	317.0 ± 83.7	472.2 ± 93.7[c-h]d	2.0 ± 0.3	5

	Sandaracopimaric Acid (5)		
subtype	EC$_{50}$ [μM]	max. potentiation of I_{GABA} (EC$_{5-10}$) [%]	Hill coeff (n_H)[a]	n[b]
$\alpha_1\beta_1\gamma_{2S}$	48.1 ± 13.4	501.6 ± 55.7[c-h]e	1.8 ± 0.3	4
$\alpha_1\beta_2\gamma_{2S}$	33.3 ± 8.7	855.7 ± 114.9[c-h]e,f	1.6 ± 0.4	4
$\alpha_1\beta_3\gamma_{2S}$	24.9 ± 6.3	519.7 ± 83.8	2.1 ± 0.5	4
$\alpha_2\beta_2\gamma_{2S}$	31.2 ± 4.8	1093.7 ± 60.1[c-h]g	2.1 ± 0.3	4
$\alpha_3\beta_2\gamma_{2S}$	56.6 ± 10.6	1101.1 ± 97.8[c-h]h	1.7 ± 0.3	5
$\alpha_5\beta_2\gamma_{2S}$	40.7 ± 14.7	512.7 ± 98.3[c-h]f−h	1.6 ± 0.3	6

	Benzodiazepines at $\alpha_1\beta_2\gamma_{2S}$ (Data by Khom et al., 2006)[47]	
compound	EC$_{50}$ [nM]	max. potentiation of I_{GABA} (EC$_{5-10}$) [%]
triazolam	22 ± 3	253 ± 12
midazolam	143 ± 88	342 ± 64
clotiazepam	184 ± 88	260 ± 27

[a] Indicates the slope of the concentration−response curve at the EC$_{50}$. Hill coefficients > 1 indicate positive cooperativity during ligand binding.[69] [b] Number of experiments. [c−h] Each letter separately shows significantly different efficiencies ([c−e]$p < 0.05$, [f]$p = 0.06$, [g,h]$p < 0.01$).

maximum at a dose of 30 mg/kg BW (22.3 ± 1.2 m; $n = 17$; $p < 0.05$). Control mice and mice treated with 1−30 mg/kg BW of **5** spent the same amount of time in the center of the OF (no statistically significant difference observed). Hence no anxiolytic-like effect was observed under influence of **5** (Figure 5B).

With the example of *B. orientalis*, HPLC-based activity profiling led to the identification of two positive GABA$_A$ receptor modulators with a diterpene scaffold that is new for the target. In addition, a new natural product, sandaracopimaradienolol (**3**), was identified. The GABA$_A$ receptor modulation observed with the time-based fractions 15−18 could be located at higher resolution in the peak-based fractions. However, only the compounds responsible for the activity of 18/*o* were finally identified. Further work is required to identify the active compounds located in peak-based microfractions *c*−*e*, *o*, and *t*. A thorough characterization of isopimaric acid (**4**) and sandaracopimaric acid (**5**) at several GABA$_A$ receptor subtypes revealed two novel positive GABA$_A$ receptor modulators with virtually the same efficiencies but varying potencies at the investigated subtypes. The two diterpenes differ only in the position of a double bond ($\Delta^{7,8}$ in **4** vs $\Delta^{8,14}$ in **5**), which seems to affect potency rather than efficiency. Future investigation of structurally related diterpenes could, therefore, provide deeper insights on how potency and efficiency relate to structural features of pimarane diterpenoids. Interestingly, activity of **4** and **5** was independent of the γ-subunit, which clearly indicated that both compounds interact with a non-benzodiazepine binding site (Figure 4).

The reduced locomotor activity observed with intraperitoneal administration of **5** may result from an enhancement of the GABAergic system—in particular a positive allosteric modulation of GABA-induced currents and thus an enhanced inhibitory neurotransmission—but might be also due to interaction with completely different molecular targets. Analysis of the EEG or measurement of body temperature upon administration of the compound would substantiate whether the observed decrease of locomotor activity was due to sedation.[50,51] Interestingly, the observed effects of **5** occurred at doses comparable to those of known GABA$_A$ receptor modulators and sedatives such as midazolam or zolpidem.[52,53]

The absence of an anxiolytic-like effect was inconsistent with the in vitro data (high efficiency at α_2- and α_3-containing subtypes) (Figure 3, Table 1). Other behavioral paradigms for anxiolysis might reveal whether the observed decrease in locomotor activity negatively interfered with an anxiolytic-like effect in the OF. Sandaracopimaric acid (**5**) is a molecule with suitable physicochemical properties for oral bioavailability (H-acceptors/donors 2:1, MW 302, cLogP 4.16, rotatable bonds 2).[54] In addition, a relatively small polar surface area of 37.3 Å^2 is favorable for blood-brain-barrier (BBB) penetration.[54,55] However, further studies on metabolism and BBB penetration of **5** are needed. This first preliminary in vivo evaluation was performed with intraperitoneal administration, which circumvents the liver first-pass. Oral administration and comparison with clinically used GABA$_A$ receptor ligands will be needed to determine the therapeutic potential of **5**.

■ EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded at target temperature 18 °C on a Bruker Avance III 500 MHz
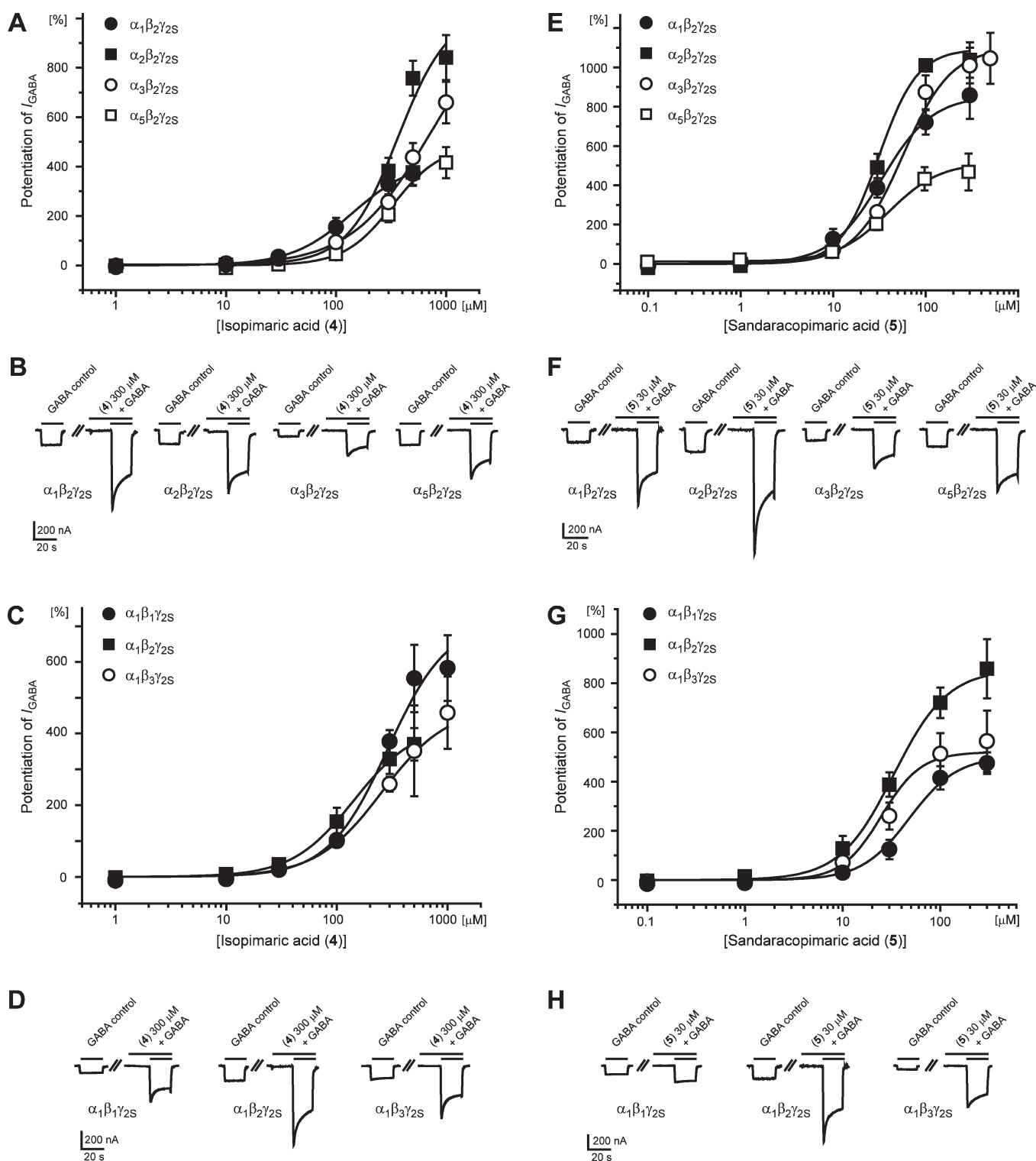
Figure 3. Parts A and E display the α-subunit dependency of isopimaric acid (**4**) and sandaracopimaric acid (**5**), respectively, reflected by concentration−response curves with $GABA_A$ receptors of subunit compositions $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, and $\alpha_5\beta_2\gamma_{2S}$. Parts C and G show the β-subunit dependency of **4** and **5**, respectively, reflected by concentration−response curves at $GABA_A$ receptors of the subunit compositions $\alpha_1\beta_1\gamma_{2S}$, $\alpha_1\beta_2\gamma_{2S}$, and $\alpha_1\beta_3\gamma_{2S}$. Typical traces reflecting modulation of chloride currents without direct activation were recorded with compounds **4** and **5** at all expressed $GABA_A$ receptor subtypes (typical currents of **4** and **5** are displayed in B and D, and F and H, respectively). All measurements were performed with a GABA EC_{5-10}.

spectrometer (Bruker BioSpin, Faellanden, Switzerland) operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C. A 1 mm TXI-microprobe with a z-gradient was used for ^1H-detected experiments; ^{13}C NMR spectra were recorded with a 5 mm BBO-probe head with z-gradient. Spectra were analyzed using Bruker TopSpin 2.1 software. High-resolution mass spectra (HPLC-PDA-ESITOFMS) in positive mode

Table 2. Potency Ratio and Efficiency Ratio for Isopimaric Acid (4) and Sandaracopimaric Acid (5)

subtype	$\alpha_1\beta_1\gamma_{2S}$	$\alpha_1\beta_2\gamma_{2S}$	$\alpha_1\beta_3\gamma_{2S}$	$\alpha_2\beta_2\gamma_{2S}$	$\alpha_3\beta_2\gamma_{2S}$	$\alpha_5\beta_2\gamma_{2S}$
potency ratio $[EC_{50}(4)/EC_{50}(5)]$	6.02^a	4.25	10.32	11.69^b	12.79	7.79^a
efficiency ratio $[I_{max}(4)/I_{max}(5)]$	1.43	0.50^a	0.92	0.94	0.98	0.92

[a] Statistically significant ($p < 0.05$). [b] Statistically significant ($p < 0.01$).
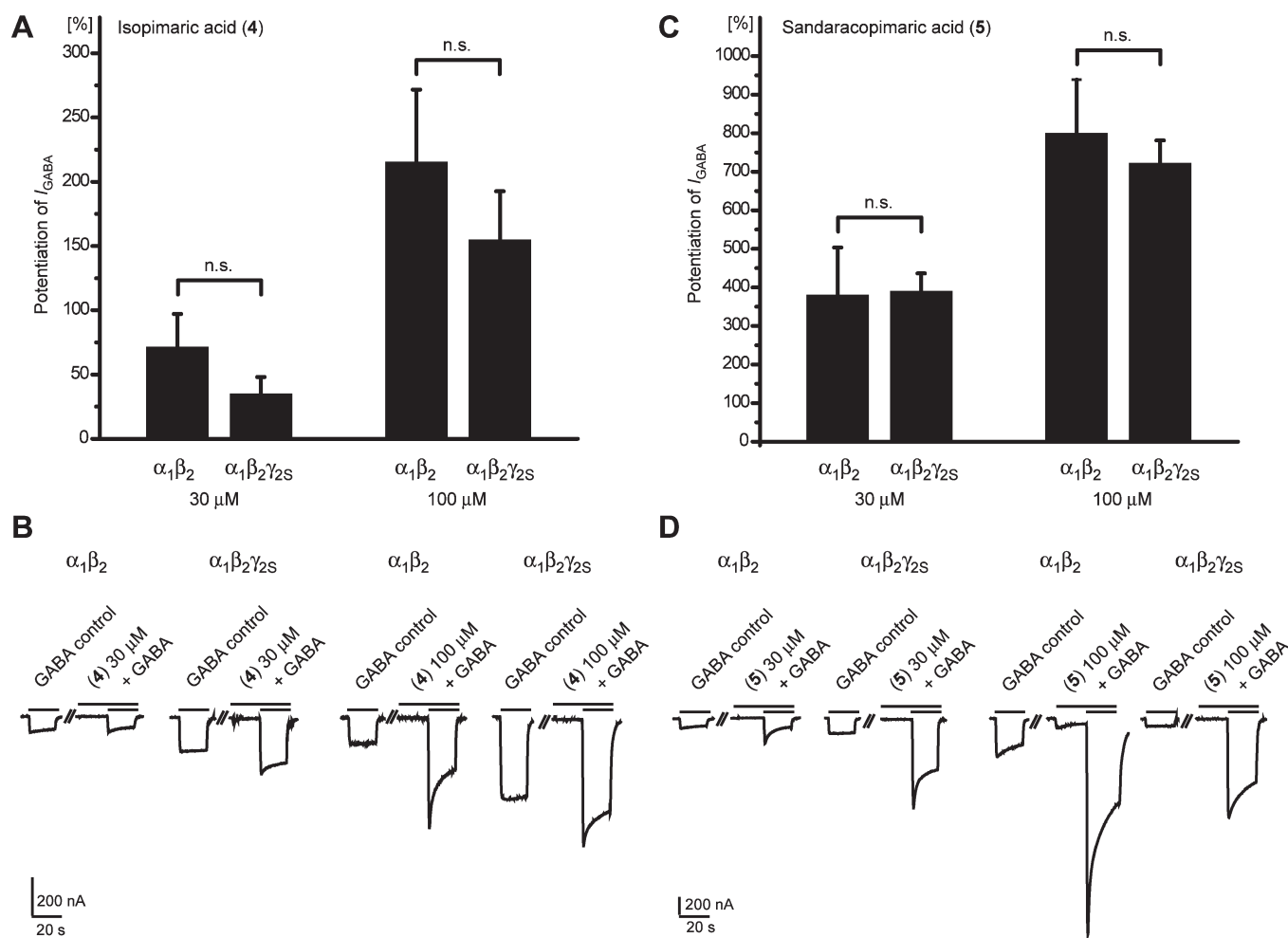
Figure 4. Parts A and C display the γ-subunit dependency of isopimaric acid (4) and sandaracopimaric acid (5), respectively. The bar graphs indicate the potentiation of GABA-induced control currents by 30 and 100 μM of compound, at GABA$_A$ receptors of the subunit compositions $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$. Traces show typical modulation of chloride currents in GABA$_A$ receptor subtypes by compounds 4 and 5 (parts B and D, respectively).

were obtained on a Bruker micrOTOF ESIMS system (Bruker Daltonics, Bremen, Germany) connected via a T-splitter (1:10) to an Agilent HP 1100 Series system consisting of a binary pump, autosampler, column oven, and diode array detector (G1315B) (Agilent Technologies, Waldbronn, Germany). Nitrogen was used as a nebulizing gas at a pressure of 2.0 bar and as a drying gas at a flow rate of 9.0 L/min (dry gas temperature 240 °C). Capillary voltage was set at 4500 V; hexapole at 230.0 Vpp. Instrument calibration was done with a reference solution of sodium formate 0.1% in 2-propanol/water (1:1) containing 5 mM NaOH. Data acquisition and processing were performed using Bruker HyStar 3.0 software. Semipreparative HPLC separations for activity profiling were carried out with an Agilent HP 1100 Series system consisting of a quaternary pump, autosampler, column oven, and diode array detector (G1315B). HPLC fractions were evaporated with a Genevac EZ-2 Plus vacuum centrifuge (Genevac Ltd., Ipswich, United Kingdom). Waters SunFire C18 (3.5 μm, 3.0 × 150 mm) and SunFire

Prep C18 (5 μm, 10 × 150 mm) columns (Waters, Wexford, Ireland) were used for HPLC-PDA-ESITOFMS and semipreparative HPLC, respectively. Medium-pressure liquid chromatography (MPLC) was performed with a glass column (49 × 460 mm) packed with silica gel (0.015–0.040 μm; Merck) on a Buchi Sepacore system consisting of two C605 pumps, a C635 detector, a C620 control unit, and a C660 fraction collector (Buchi Labortechnik AG, Flawil, Switzerland). Sample introduction was carried out with a precolumn packed with the sample absorbed onto silica gel. The separation was monitored with Buchi Sepacore Control 1.0 software.

Plant Material. Cebaye (dried twigs and leaves of *B. orientalis*) was purchased from a local market in Shanxi Province, China. Identity of the sample was confirmed with the aid of the corresponding monograph of the Chinese Pharmacopoeia IX and other literature[56] at the Division of Pharmaceutical Biology, University of Basel, where a voucher specimen (00 305) is deposited.

A

B
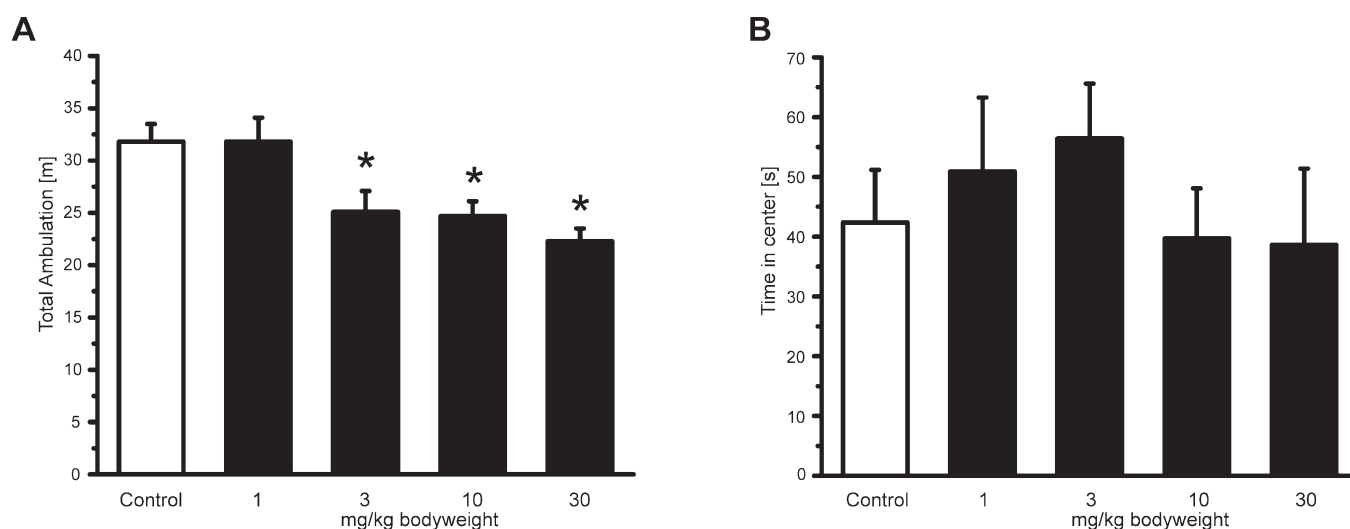
Figure 5. Ambulation and explorative behavior in the open field test assessed over 10 min for control and sandaracopimaric acid (**5**)-treated mice at the indicated doses (doses represent mg/kg BW). Bars indicate the total distance traveled (A) and the time spent in the center (B). Bars represent mean $\pm$ SEM from ≥ 10 mice. The asterisks indicate statistically significant differences ($p < 0.05$).

Extraction. The plant material was frozen with liquid nitrogen and ground with a ZM1 ultracentrifugal mill (Retsch). The EtOAc extract for screening and HPLC-based activity profiling was prepared with an ASE 200 extraction system with solvent module (Dionex, Sunnyvale, CA). Extraction pressure was 120 bar, temperature was set at 70 °C, and three extraction cycles of 5 min each were performed. For preparative isolation, 430 g of ground plant material was macerated overnight with 1.25 L of EtOAc, followed by percolation with EtOAc (2×1 L). The solvent was evaporated at reduced pressure to yield 22.8 g of extract. The extracts were stored at -20 °C until use.

Microfractionation for Activity Profiling. Time-based microfractionation for $GABA_A$ receptor activity profiling was performed as previously described,[41] with minor modifications. Separation for both time-based and peak-based microfractionation was carried out on a semipreparative HPLC column with acetonitrile containing 0.1% formic acid (solvent A) and water containing 0.1% formic acid (solvent B) using the following gradient: 10% A to 100% A for 30 min, hold for 10 min. In the time-based fractionation, 28 microfractions of 90 s each were collected from an injection of 10 mg of extract (in 500 μL of DMSO). The flow rate was 7 mL/min. In peak-based fractionation (10 mg of extract in 100 μL of DMSO), 21 microfractions were collected. The flow rate was 4 mL/min. All microfractions were dried, evenly distributed to two vials, and submitted to activity testing.

Isolation. The EtOAc extract (22.8 g) was dissolved in MeOH and extracted with n-hexane to remove essential oils. The methanolic portion was then redissolved in $CHCl_3$ and extracted with H_2O to remove polar constituents. The residue (11.1 g) was coated onto silica gel (44.9 g) and packed into a precolumn prior to elution onto the MPLC column. Elution was done with an n-hexane (solvent A) and EtOAc (solvent B) gradient: 0% B to 30% B in 4 h, followed by 30% A to 100% B in 4 h. The flow rate was set at 15 mL/min. Fractions of 15 mL were collected and were later combined to 20 fractions (1–20) on the basis of a TLC analysis. Selected fractions were submitted to semipreparative gradient HPLC with acetonitrile (solvent C) and H_2O containing 0.1% formic acid (solvent D) as eluents. The flow rate was 4 mL/min. Methanolic stock solutions (100 mg/mL) were prepared and repeatedly injected in portions of 30 to 100 μL. A portion (140 mg) of fraction 2 (322.4 mg) afforded compound **6** (12.0 mg). The gradient profile was 30% C to 100% C in 20 min. An aliquot (56 mg) of fraction 7 (265.7 mg) gave a mixture of **4** and **5** (30.5 mg). A gradient of 70% C to 100% C over

20 min was used. The same gradient was used for separation of an aliquot (120 mg) of fraction 13 (281.6 mg). Compounds **1** (24.4 mg) and **3** (2.6 mg) were obtained. Compound **2** (5.2 mg) was isolated from 80 mg of fraction 16 (2.035 g). A gradient of 65% C to 73% C over 16 min was used. Several attempts to separate the mixture of **4** and **5** failed.

Compounds **1**–**6** were identified by comparison of physicochemical data (NMR, ESI-TOF-MS, and UV–vis spectroscopy) with published values.[11,57−66] The purity (except for the mixture of **4** and **5**) was >95% (purity check by ^{1}H NMR).

Sandaracopimaradienolal (**3**): ^{1}H NMR ($CDCl_3$, 500.13 MHz) δ 9.38 (1H, s, H-18), 5.73 (1H, dd, $J = 17.7, 10.7$ Hz, H-15), 5.24 (1H, br s, H-14), 4.87 (1H, dd, $J = 17.7, 2.0$ Hz, H-16a), 4.85 (1H, dd, $J = 10.7, 2.0$ Hz, H-16b), 3.77 (1H, dd, $J = 11.6, 4.5$ Hz, H-3), 2.21 (1H, ddd, 14.2, 4.3, 2.3 Hz, H-7a), 2.04 (1H, ddm, $J = 14.2, 12.9$ Hz, H-7b), 1.80 (1H, ddd, $J = 13.7, 3.0, 3.0$ Hz, H-1a), 1.74 (1H, m, H-9), 1.74 (1H, m, H-2a), 1.63 (1H, m, H-2b), 1.60 (1H, m, H-11a), 1.53 (1H, dd, $J = 12.7, 1.7$ Hz, H-5), 1.52 (1H, m, H-11b), 1.47 (1H, m, H-6a), 1.45 (1H, m, H-12a), 1.36 (1H, m, H-12b), 1.22 (1H, ddd, $J = 13.7, 13.7, 4.5$ Hz, H-1b), 1.08 (3H, s, H-19), 1.07 (1H, m, H-6b), 1.02 (3H, s, H-17), 0.84 (3H, s, H-20); ^{13}C shifts ($CDCl_3$, 125.77 MHz) δ 206.86 (CH, C-18), 148.57 (CH, C-15), 135.66 (C, C-8), 129.80 (CH, C-14), 110.41 (CH_2, C-16), 72.12 (CH, C-3), 55.30 (C, C-4), 50.06 (CH, C-9), 46.87 (CH, C-5), 37.45 (C, C-13), 37.12 (C, C-10), 36.75(CH_2, C-1), 35.21 (CH_2, C-7), 34.25 (CH_2, C-12), 26.56 (CH_2, C-2), 26.02 (CH_3, C-17), 24.08 (CH_2, C-6), 18.74 (CH_2, C-11), 15.36 (CH_3, C-20), 9.22 (CH_3, C-19); relevant NOESY correlations, H-3 $\leftrightarrow$ H-17, H-3 $\leftrightarrow$ H-5, H-5 $\leftrightarrow$ H-17, H-5 $\leftrightarrow$ H-9, H-20 $\leftrightarrow$ H-19, H-20 $\leftrightarrow$ H-17; HR-ESIMS m/z 325.2153 $[M + Na]^+$ (calcd for $C_{20}H_{30}O_2Na$, 325.2143). NMR spectra of **3** are available as Supporting Information.

Expression and Functional Characterization of $GABA_A$ Receptors. The preparation of stage V–VI oocytes from *Xenopus laevis*, the synthesis of capped-off runoff poly(A+) cRNA transcripts from linearized cDNA templates (pCMV vector), and cRNA injection into oocytes were performed as previously described.[47] In summary, female *X. laevis* (Nasco, Fort Atkinson, WI) were anesthetized by a 15 min exposure to a 0.2% MS-222 (methanesulfonate salt of 3-aminobenzoic acid ethyl, Sigma-Aldrich, Munich, Germany) solution before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/mL collagenase (Type 1 A, Sigma-Aldrich). One day after enzymatic isolation, the

oocytes were injected with approximately 10–50 nL of DEPC-treated water (diethyl pyrocarbonate, Sigma-Aldrich) containing different cRNAs at a concentration of approximately 300–3000 pg/nL per subunit. The amount of injected cRNA mixture was determined by means of a NanoDrop ND-1000 (Kisker Biotech, Steinfurt, Germany). To ensure expression of the γ_{2S} subunits in $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_2\gamma_{2S}$, $\alpha_3\beta_2\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$, and $\alpha_1\beta_3\gamma_{2S}$ receptors, cRNAs were mixed in a 1:1:10 ratio, except $\alpha_1\beta_1\gamma_{2S}$ (ratio 3:1:10). For $\alpha_1\beta_2$ receptors, the cRNAs were mixed in a 1:1 ratio. Oocytes were then stored at 18 °C in an aqueous solution of 90 mM NaCl, 1 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 5 mM HEPES (pH 7.4), containing 1% penicillin–streptomycin solution (Sigma-Aldrich).[67] Voltage clamp measurements were performed between days 1 and 5 after cRNA injection. Electrophysiological experiments on a two-microelectrode voltage clamp setup were performed at a holding potential of −70 mV making use of a TURBO TEC 01C amplifier (npi electronic, Tamm, Germany) and an Axon Digidata 1322A interface (Molecular Devices, Sunnyvale, CA). Data were recorded by using pCLAMP v10.2. Currents were low-pass-filtered at 1 kHz and sampled at 3 kHz. The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 5 mM HEPES (pH 7.4). Electrodes with resistances between 1 and 3 MΩ were used and filled with 2 M KCl.

Sample Application during Current Recordings. Of each sample, 100 μL was applied to the oocytes at a perfusion speed of 300 μL/s by the ScreeningTool automated fast perfusion system (npi electronic).[7] Before application of test solutions, concentration–response experiments with GABA concentrations ranging from 0.01 μM to 1 mM were performed to determine the GABA concentration eliciting 5–10% of the maximal current amplitude at 1 mM (GABA EC$_{5-10}$), which typically ranged from 3 to 10 μM for receptors comprising a γ_{2S} subunit and 0.3 to 1 μM for $\alpha_1\beta_2$ receptors. The stock solution (10 mg/mL in DMSO) of *B. orientalis* extract was diluted to a concentration of 100 μg/mL with bath solution containing GABA EC$_{5-10}$. As previously described in a validated protocol, time-based and peak-based microfractions collected from the semipreparative HPLC separations were dissolved in 30 μL of DMSO and mixed with 2.97 mL of bath solution containing GABA EC$_{5-10}$.[41] For concentration–response experiments, bath solution containing compounds **4** and **5** in concentrations ranging from 0.1 to 1000 μM was applied to the oocyte. After a preincubation period of 20 s, a second application immediately followed containing the corresponding compound solution combined with GABA EC$_{5-10}$. Pure isopimaric acid (**4**) (≥98%) and GABA were purchased from Sigma-Aldrich, and sandaracopimaric acid (≥95%) (**5**) was purchased from Orchid Cellmark (Princeton, NJ).

Data Analysis. Enhancement of the GABA-induced chloride current (I_{GABA}) was defined as $I_{(GABA+Comp)}/I_{GABA} - 1$, where $I_{(GABA+Comp)}$ is the current response in the presence of a given compound, and I_{GABA} is the control GABA-induced chloride current. Concentration–response curves were generated, and the data were fitted by nonlinear regression analysis using ORIGIN software (OriginLab Corporation, Northampton, MA). Data were fitted to the equation $1/[1 + (EC_{50}/[Comp])^{n_H}]$, where EC$_{50}$ is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50%, and n_H is the Hill coefficient. The maximum potentiation of I_{GABA} by a given compound was derived from the fit. Data are given as mean ± SE of at least 2 oocytes and ≥2 oocyte batches. Statistical significance was calculated using the paired Student t-test with confidence intervals of $p < 0.05$ unless otherwise stated.

In Vivo Experiments. Male mice (C57Bl/6N) were obtained from Charles River Laboratories (Wilmington, MA). For breeding and maintenance mice were group housed with free access to food and water. Temperature was fixed to 23 ± 1 °C and 60% humidity with a 12 h light–dark cycle (lights on 0700–1900 h). Male mice at 3–6 months of age were tested in all experiments. All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention for the protection of vertebrate

animals used for experimental and other scientific purposes ETS no.: 123. Every effort was taken to minimize the number of animals used.

Chemicals. A stock solution of sandaracopimaric acid was prepared in 100% DMSO (50 mg/mL). Working concentrations were adjusted by dilution with 0.9% NaCl. NaOH was used to adjust the pH to 7.2–7.4. For ip administration the compound was solubilized with 3% Polysorbate 80 and with DMSO, whereby the final DMSO concentrations did not exceed 10% (see Broadwell et al. 1982 for DMSO effects on the permeability of the blood brain barrier).[68] All solutions were freshly prepared every day prior to experiments.

Open Field Test. Ambulation 30 min after ip injection of either control (vehicle without compound) or test solution (vehicle containing compound at the indicated doses) was tested over 10 min in 50 × 50 cm Flexfield boxes equipped with infrared rearing detection. Animals were video monitored, and their explorative behavior was analyzed using ActiMot 2 equipment and software (TSE Systems, Bad Homburg, Germany). Arenas were subdivided into border (up to 8 cm from wall), center (20 × 20 cm, i.e. 16% of total area), and intermediate area according to the recommendations of EMPRESS (European Mouse Phenotyping Resource of Standardised Screens; http://empress.har.mrc.ac.uk).

For comparison of control groups and compound-treated groups the unpaired Student's t test was used. Comparison of more than two groups was done by one-way ANOVA. p-values of <0.05 were accepted as statistically significant.

■ ASSOCIATED CONTENT

ⓢ Supporting Information. ^{1}H and ^{13}C NMR spectra of compound **3**, spectral data of compounds **1**, **2**, and **4**–**6**, and a synoptical table with currently known secondary metabolites from *Biota orientalis*. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

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