Positive GABA_A Receptor Modulators from *Acorus calamus* and Structural Analysis of (+)-Dioxosarcoguaiacol by 1D and 2D NMR and Molecular Modeling

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Abstract
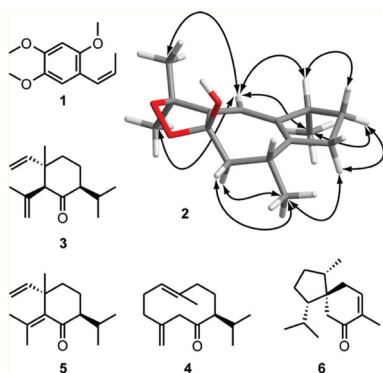
In a two-microelectrode voltage clamp with *Xenopus laevis* oocytes, a petroleum ether extract of *Acorus calamus* rhizomes enhanced the GABA-induced chloride current through GABA_A receptors of the $\alpha_1\beta_2\gamma_{2S}$ subtype by 277% ± 9.7% (100 μg/mL). β-Asarone (**1**), (+)-dioxosarcoguaiacol (**2**), (+)-shyobunone (**3**), and (+)-preisocalamenediol (**4**) were subsequently identified as main active principles through HPLC-based activity profiling and targeted isolation. The compounds induced maximum potentiation of the chloride current ranging from 588% ± 126% (EC$_{50}$: 65.3 ± 21.6 μM) (**2**) to 1200% ± 163% (EC$_{50}$: 171.5 ± 34.6 μM) (**1**), whereas (−)-isoshyobunone (**5**) and (−)-acorenone (**6**) exhibited weak GABA_A modulating properties (**5**: 164% ± 42.9%; EC$_{50}$: 109.4 ± 46.6 μM and **6**: 241% ± 23.1%; EC$_{50}$: 34.0 ± 6.7 μM). The relative configuration of 2 was established as 4*R**8*S**10*R** by NOESY experiments and conformational analysis.

Acorus calamus L. (Acoraceae), commonly known as "sweet flag", is a polyploidic marsh plant indigenous to Asia and is now distributed along trade routes all over the northern hemisphere.[1] The aromatic rhizome has been widely used as an herbal remedy. It contains 1.7–9.3% of a volatile oil composed of monoterpenes, sesquiterpenes, and phenylpropanoids. The most characteristic constituent of the oil is β-asarone, even though

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its concentration may vary considerably (96% in the tetraploidic form found in eastern and tropical southern Asia, 5% in the triploidic form found in Europe, Himalayan, and temperate Indian regions, and 0% in the diploidic variety growing from North America to Siberia).[1–4] In European folk medicine, *A. calamus* rhizomes have been mainly used as "*Amarum aromaticum*" to alleviate gastrointestinal ailments such as acute and chronic dyspepsia, gastritis and gastric ulcer, intestinal colic, and anorexia.[5,6] Ayurvedic medicine and traditional Chinese medicine (TCM) use the drug preferably to treat central nervous system (CNS) related diseases such as epilepsy, insanity, mental weakness, or insomnia.[7–9] Several in vivo studies support a sedative and tranquillizing action of the essential oil and of ethanolic and aqueous extracts of *A. calamus*.[10–12] Up to now, the underlying mechanism of action has remained elusive despite various investigations, even though the pharmacological effect could be mainly attributed to α-asarone and β-asarone.[11,13–15]

$GABA_A$ receptors are pentameric ligand-gated chloride ion channels that are activated by GABA, the major inhibitory neurotransmitter in the CNS. Most likely more than 11 different receptor subtypes, assembled from five varying subunits, exist in the human brain. These are involved in distinct neuronal circuits and are targeted by numerous classes of drugs such as benzodiazepines, barbiturates, and some general anesthetics. The most abundant $GABA_A$ receptor in the human brain consists of two α_1, two β_2, and one γ_{2S} subunit.[16–18]

We recently screened a library of 982 extracts using an automated functional two-microelectrode voltage clamp assay with *Xenopus* oocytes[19] that transiently expressed $GABA_A$ receptors of the $\alpha_1\beta_2\gamma_{2S}$ subtype. A petroleum ether extract of *A. calamus* rhizomes showed promising activity. As previously shown, HPLC-based activity profiling is a miniaturized, effective approach to discover new bioactive natural products,[20–24] and we have successfully applied it to the discovery of new $GABA_A$ receptor modulators of natural origin.[25–28] Herein, we describe the identification of $GABA_A$ receptor modulating compounds in *A. calamus* (**1**–**6**) and provide information suggesting the complete relative configuration of (+)-dioxosarcoguaiacol (**2**), a sesquiterpene previously unknown in this plant.

RESULTS AND DISCUSSION

Plant extracts were screened in an automated, fast perfusion system during two-microelectrode voltage clamp measurements with *Xenopus* oocytes that transiently expressed $GABA_A$ receptors of the subunit combination $\alpha_1\beta_2\gamma_{2S}$.[19] At a concentration of 100 μg/mL, the *A. calamus* petroleum ether extract potentiated the GABA-induced chloride current (I_{GABA}) by 277% ± 9.7% (Figure 1C). To localize the activity within the extract, it was submitted to HPLC-based activity profiling using a previously validated protocol.[29] The chromatogram (254 nm) of a semipreparative HPLC separation (10 mg of extract) and the corresponding activity profile of the time-based microfractionation (28 microfractions of 90 s each) are shown in Figure 1B and A, respectively. A major peak of activity was found in fraction 14 (potentiation of I_{GABA} by 237% ± 5.4%), which contained an unresolved complex of peaks containing compounds **3**–**6**. Minor activity was found in fractions 1, 6–8, and 10. Fractions 6 and 7 contained a major compound of the extract and enhanced I_{GABA} by 54.7% ± 9.3% and 73.9% ± 17.7%, respectively. Fraction 8, consisting of a minor compound, showed a potentiation of I_{GABA} by 50.6% ± 17.4%, whereas fraction 10 enhanced I_{GABA} by 33.8% ± 10.3%. Fraction 1 showed marginal activity (38.3% ± 2.9%) but was not further pursued.

Preparative isolation was focused on the purification of compounds in the active time-based fractions and was started by an open column separation of the extract. An HPLC-ESIMS analysis of the resulting 21 fractions revealed that the extract was significantly more

complex than suggested by the semipreparative HPLC chromatogram at 254 nm (Figure 1B). Peaks with retention times fitting to active microfractions were detected in several fractions. Fractions A–E and L–U were submitted to bioassay at a test concentration of 10 μg/mL (Figure 1C). The most active fractions, A, C, L, and M (potentiation of I_{GABA} by 132% ± 41.5%, 157% ± 20.7%, 235% ± 59.0%, and 158% ± 72.9%, respectively), contained peaks that corresponded to the active time windows in the HPLC-based activity profiling (Figure 1D) and were further purified. Fraction D was not further pursued since it was very similar to fraction C. Finally, β-asarone (**1**), (+)-dioxosarcoguaiacol (**2**), (+)-shyobunone (**3**), (+)-preisocalamenediol (**4**), (−)-isoshyobunone (**5**), and (−)-acorenone (**6**) were isolated and unambiguously identified by ESI-TOF-MS, 1D and 2D microprobe NMR, optical rotation, and comparison with published data.[30–37] Compounds **1** and **3–6** have been previously isolated from *A. calamus*.[38–41] Their spectroscopic data are given as Supporting Information.

(+)-Dioxosarcoguaiacol (**2**), a sesquiterpene new for *A. calamus*, had been described from a Red Sea soft coral (*Sarcophyton glaucum*). However, assignment of the relative configuration was limited to stereocenters 4S* and 10R*, while the configuration at the bridgehead C-8 was not established.[35] Optical rotation and NMR data of **2** were identical to published data. To confirm the relative configuration of the stereocenters at C-4 and C-10, we submitted the four possible stereoisomers of **2** (4R*8S*10R*, 4S*8S*10R*, 4R*8S*10S*, 4S*8S*10S*) to conformational analysis and compared $^3J_{HH}$ coupling constants from ^{1}H NMR and selective TOCSY experiments, and NOESY correlations with the structures of the calculated conformers (relevant NMR data are summarized in Table 1; NMR spectra of **2** are given as Supporting Information). For each stereoisomer, 1–3 conformers were obtained within a 1 kcal/mol range from the corresponding global energy minimum (Figure S1, Supporting Information). However, only one conformer of each stereoisomer reasonably fit to the observed NMR data. These conformers were then submitted to geometrical optimization using density function theory (B3LYP/6-31G*), prior to comparison of optimized dihedral angles and interatomic distances with the experimental data.
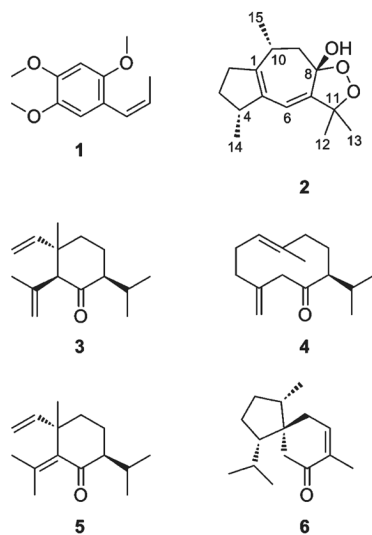
Unambiguous assignment of the methylene protons at C-2 and C-3 was established on the basis of $^3J_{HH}$ H-2b/H-3b of 0 Hz, indicative of their perpendicular orientation. This was corroborated by a NOESY correlation between H-3b and H-2a. $^3J_{HH}$ coupling constants H-9b/H-10 (5.2 Hz) and H-9a/H-10 (13.4 Hz) corresponded to dihedral angles of

approximately 60° and 180°, respectively. Geometrically optimized conformers matched with the above-mentioned NMR data. However, two stereoisomers ($4R^*8S^*10S^*$, $4S^*8S^*10S^*$) could be discarded since the interatomic distances between H-2a and CH_3-15, and between H-6 and both methyl groups at C-11, were not in accord with the observed NOESY correlations. Assignment of the relative configuration at C-4 was supported by NOESY correlations between H-3a and H-4, and H-3b and CH_3-14. Hence, only the $4R^*8S^*10R^*$ stereoisomer fully matched with the NMR data of (+)-dioxosarcoguaiacol (**2**) (Figure 2). A synoptical table (Table S6) of the stereoisomers is given as Supporting Information.

(+)-Dioxosarcoguaiacol[35] was previously published with a $4S^*10R^*$ configuration, and with NMR shifts and optical rotation identical to compound **2**. $4R10R$-Calamusenone, the 8-oxo, $\Delta^{7,11}$ analogue of **2**, was previously reported from *A. calamus* essential oil, and its absolute configuration determined by X-ray crystallographic analysis.[42] The formation of **2** can be plausibly explained via photo-oxygenation of calamusenone by "ene-type" addition of O_2 at C-11 and subsequent ring closure to the endoperoxide.[43] ^1H NMR integrals of **2** showed an enantiomeric excess of the $8S^*$ epimer [8-OH: δ 3.08, $8S^*$ and δ 2.94, $8R^*$ (95:5); H-6: δ 5.55, $8S^*$ and δ 5.51, $8R^*$ (95:5)]. The comparison of computationally optimized conformers of **2** with structural information obtained by 1D and 2D NMR experiments allowed us to unambiguously identify the relative configuration of (+)-dioxosarcoguaiacol (**2**) as $4R^*8S^*10R^*$, which contradicted the previously reported structure.[35] Our findings were supported by the existence of a putative precursor, $4R10R$-calamusenone, with known absolute configuration.

Compounds **1**–**6** were tested in the oocyte assay at concentrations ranging from 0.1 to 1000 μM. To varying degrees, all compounds enhanced I_{GABA} at a GABA EC_{5-10} in a concentration-dependent manner (Figure 3). Compared to other natural products,[25,27,44] compounds **1**–**4** showed high efficiencies on $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. Potentiation of I_{GABA} ranged from 588% ± 126% (**2**) to 1197% ± 163% (**1**) (Table 2). Compounds with higher efficiency usually displayed lower potencies (higher concentrations for half-maximal stimulation of I_{GABA}; EC_{50}) and vice versa. Compound **6** showed the highest potency (34.0 ± 6.7 μM) but a maximum stimulation of I_{GABA} of only 241% ± 23.1%.

Even though the set of compounds tested was too small for a study of structure–activity relationships, some interesting observations were made: (+)-shyobunone (**3**) and (+)-preisocalamenediol (**4**), which both derive from acoragermacrone,[40,45] showed comparably high efficiencies. The potency of **4** was lower, which could be due to the high conformational flexibility of the germacrane-type scaffold. (−)-Isoshyobunone (**5**) was much less efficient than its presumed precursor **3**[46] (Figure 3; Table 2), which can only be explained by the varying C-2 substitution and the consequential conformational difference. Interestingly, **5** showed a weak (less than GABA EC_{5-10}) direct activation of the $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor, whereas **3** did not evoke any agonistic effect. Only weak $GABA_A$ receptor modulation was found for (—)-acorenone (**6**). Compounds **2**–**6** broaden the spectrum of sesquiterpenes acting at the $GABA_A$ receptors. Up to now only a few sequiterpenes have been reported as $GABA_A$ receptor modulators. The most prominent is picrotoxin, a strong $GABA_A$ receptor inhibitor[47] that is widely used as an experimental compound for animal convulsion models. Valerenic acid from *Valeriana officinalis* roots is a $\beta_{2/3}$-subunit-specific positive allosteric modulator[44,48] with anxiolytic effects in vivo.[48,49] This compound has served as a new scaffold for $GABA_A$ receptor ligands.[49,50]

Among the compounds identified by the profiling of *Acorus* extract, the simple phenylpropanoid β-asarone (**1**) induced the highest potentiation of I_{GABA} (1200% ± 163% with an EC_{50} of 171.5 ± 34.6 μM). Its efficiency at a GABA EC_{5-10} was significantly

higher than that of known GABA$_A$ receptor modulators such as benzodiazepines (triazolam: 253% ± 12%, midazolam: 342% ± 64%, clotiazepam: 260% ± 27%)[51] or natural products such as valerenic acid (400% ± 78%).[44] The structure of **1** is somewhat reminiscent of propofol, a general anesthetic acting at the GABA$_A$ receptor. However, the pharmacological potential of **1** is certainly limited due to its known toxicity.[52–54] There are many reports of sedative and tranquillizing properties of *Acorus* extracts and essential oil in animal models, but the mechanism of action was not established up to now.[10–15,55] Given that β-asarone (**1**) is a major compound of the essential oil, its sedative and tranquillizing activities may be due to the GABA$_A$ receptor modulating properties of **1**. The quantity of β-asarone in *Acorus* rhizome and essential oil, however, depends on the chemotype and thus varies considerably.[3,4] Medicinal preparations made of the diploidic or triplodic form are free of the toxic β-asarone and are, therefore, recommended for therapeutic use.[8] It is interesting to note that there seems to be a correlation between β-asarone content in the three chemotypes of *A. calamus* and their traditional uses. The tetraploidic, β-asarone-rich types growing in Asia have been traditionally used in Ayurvedic medicine and TCM as sedatives, whereas such uses have not been reported from the β-asarone-poor European and North American chemotypes. The reason for "Eastern" traditional usage as a sedative and "Western" traditional usage as an aromatic bitter could possibly be explained by limited access in the past to the geographically separated chemotypes of *A. calamus*.

The essential oil and extracts of *A. calamus* roots have multiple pharmacological and biological effects,[56] but there is not much published data on bioactivity of sesquiterpenes **3**–**6** and (+)-dioxosarcoguaiacol (**2**). The compounds represent interesting drug-like structures, since they all fulfill Lipinski's "rule of five".[57] From a pharmacological perspective, however, further investigations may be limited to the highly efficient sesquiterpenes **3** and **4** and to the minor compound **2**. Further pharmacological and toxicological studies are needed to substantiate the sedative and tranquillizing properties of pure compounds and of the essential oil.

EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotations of compounds **2**–**6** were measured on a Perkin-Elmer polarimeter (model 341) equipped with a 10 cm microcell. The optical rotation for the Na-D-line (589 nm) was extrapolated from the lines of a mercury lamp using the Drude equation.[58] NMR spectra were recorded at room temperature with a Bruker Biospin Avance III spectrometer operating at 500.13 MHz. ^{1}H NMR, COSY, DEPT-edited HSQC, HMBC, 1D-TOCSY, and 2D-NOESY spectra were measured with a 1 mm TXI probe. Spectra were analyzed using Bruker TopSpin 2.1 software. ESI-TOF-MS spectra of compounds **1**–**6** were recorded in positive mode on a Bruker Daltonics microTOF ESIMS system. 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 performed using a reference solution of sodium formate 0.1% in 2-propanol/water (1:1) containing 5 mM NaOH.

HPLC-PDA-ESIMS spectra were obtained in positive mode on a Bruker Daltonics Esquire 3000 Plus ion trap MS system connected via T-splitter (1:5) to an Agilent HP 1100 series system consisting of a binary pump, autosampler, column oven, and diode array detector (G1315B). Data acquisition and processing of all mass spectra was performed using Bruker HyStar 3.0 software.

Semipreparative HPLC separations were performed on a Waters Alliance 2690 separation module connected to a 996 photodiode array detector. Data acquisition and processing was

performed using Waters Empower Pro software. Preparative HPLC separation was performed using a Shimadzu LC-8A preparative separation chromatograph equipped with a SPD-M10A VP diode array detector. Medium-pressure liquid chromatography (MPLC) was done on prepacked normal-phase cartridges (40–63 μm, 40 × 150 mm) using a Buchi Sepacore system consisting of a control unit C-620, a fraction collector C-660, and two pump modules C-605. The MPLC unit was controlled with the Buchi SepacoreControl software (version 1.0.3000.1). Deuterated chloroform (100 atom % D, stabilized with Ag) for NMR experiments was purchased from Armar Chemicals. HPLC-grade MeOH (Scharlau Chemie S.A.) and water were used for HPLC separations. Solvents used for extraction, open column chromatography, and MPLC were of technical grade and purified by distillation. Silica gel (63–200 μm, Merck) was used for open column chromatography.

Plant Material

Dried and cut rhizomes of *A. calamus* Ph. Helv. were purchased from Haenseler AG (Herisau, Switzerland) (batch 2009.12.0224). The identity of the plant material was confirmed at the Division of Pharmaceutical Biology, University of Basel, where a voucher specimen (00 380) is deposited.

Microfractionation for Activity Profiling

An aliquot (approximately 20 g) of ground roots was macerated at room temperature with petroleum ether (3 × 0.5 L, 1 h each). Microfractionation for GABA$_A$ receptor activity profiling was performed as previously described,[25,27–29] with minor modifications; separation was carried out on a Waters SunFire Prep C18 (5 μm, 10 × 150 mm) column with MeOH (solvent A) and H$_2$O (solvent B) using the following gradient: 60% A to 100% A in 30 min, hold for 10 min. The flow rate was 4 mL/min, and 100 μL of the extract (100 mg/ mL in DMSO) was injected. A total of 28 time-based microfractions of 90 s each were collected. Microfractions were evaporated in parallel with a Genevac EZ-2 Plus vacuum centrifuge. The dry films were redissolved in 1 mL of MeOH; aliquots of 0.5 mL were dispensed in two vials, dried under N$_2$ gas, and submitted to bioassay.

Preparative Extraction and Isolation

The plant material was ground with a Retsch ZM1 ultracentrifugal mill. Ground roots (630 g) were extracted by maceration at room temperature with petroleum ether (4 × 2.5 L, 1 h each). The solvent was evaporated at reduced pressure to yield 20.7 g of petroleum ether extract. A portion of the extract (18.8 g) was separated by open column chromatography (9 × 80 cm, 1.2 kg silica gel) using a step gradient of petroleum ether and ethyl acetate (95:5, 10 L; 90:10, 4 L; 80:20, 2 L; 70:30, 2 L; 60:40, 2 L; 50:50, 4 L; 25:75, 1.8 L; 100:0, 2 L). The flow rate was approximately 15 mL/min. The effluents were combined to 21 fractions (A–U) based on TLC patterns (detection at 254 nm and at daylight after staining with anisaldehyde–sulfuric acid reagent). Fractions A–U were analyzed by HPLC-PDA-ESIMS on a Waters SunFire C18 (3.5 μm, 3.0 × 150 mm) column with MeOH (solvent A) and H$_2$O (solvent B), both containing 0.1% formic acid. A gradient of 60% A to 100% A in 30 min, hold for 10 min, and a flow rate of 0.4 mL/min were used. The sample concentration was 10mg/mL in DMSO, and the injection volume was 5 μL. The fractions were then submitted to bioassay. A portion (700 mg) of fraction A (1.32 g) was separated into 21 fractions (A1– A21) by MPLC using toluene, hexane, and CH$_2$Cl$_2$ in the following gradient: 10:90:0 to 100:0:0 in 3 h, 0:0:100 for 1 h at a flow rate of 30 mL/min. Fractions A10 (60.9 mg), A14 (140.8 mg), and A18 (19.2 mg) consisted of (+)-shyobunone (**3**), (+)-preisocalamenediol (**4**), and (−)-isoshyobunone (**5**), respectively. A portion (750 mg) of fraction C (920 mg) was separated into five fractions (C1–C5) by MPLC using a gradient of hexane and ethyl acetate (100:0 to 95:5 in 1.5 h, flow rate 30 mL/min) to yield 376 mg (fraction C3) of (−)-acorenone

(**6**). Fraction L (385 mg) was separated by MPLC using a gradient of a CHCl$_3$/EtOAc [9:1] mixture and hexane (20:80 to 50:50 in 2 h, flow rate 30 mL/min), which yielded 15 fractions (L1–L15). Fraction L13 (29.5 mg) was dissolved in 400 μL of DMSO and separated by preparative HPLC on a Waters SunFire Prep C18 OBD (5 μm, 30 × 150 mm) column using a gradient of MeOH/H$_2$O (50:50 to 100:0 in 30 min, flow rate 20 mL/min) to obtain 3.8 mg of (+)-dioxosarcoguaiacol (**2**). A portion (790 mg) of fraction M (952 mg) was separated by MPLC using a gradient of a CH$_2$Cl$_2$/EtOAc [9:1] mixture and hexane (20:80 to 50:50 in 2 h, flow rate 30 mL/min), which yielded 12 fractions (M1–M12). Fraction M2 (290.9 mg) was identified as β-asarone (**1**). A portion (30 mg) of fraction M5 (83.4 mg) was dissolved in hexane (300 μL) and submitted repeatedly to semipreparative HPLC on a Merck LiChroSorb 100 Diol (10 μm, 10 × 250 mm) column (hexane/2-propanol (97:3) isocratic, flow rate 5 mL/min) to isolate another 18.5 mg of (+)-dioxosarcoguaiacol (**2**).

(+)-Dioxosarcoguaiacol (2): yellow oil; [α]$^{22}_D$ +36 (*c* 0.16, CHCl$_3$); UV (MeOH) λ_{max} 260 (sh), 267 nm; NMR data see Table 1 and Supporting Information; ESI-TOF-MS *m*/*z* 273.1447 [M + Na]$^+$ (calcd for C$_{15}$H$_{22}$O$_3$Na, 273.1463).

Compounds **1** and **3**–**6** were unambiguously identified by means of 1D and 2D NMR experiments, ESI-TOF-MS, and optical rotation in the case of chiral compounds. The data were compared with published values[30–37] and are available as Supporting Information.

Conformational Analysis and Geometrical Optimization

Conformational analysis of the stereoisomers of **2** was performed with Schrödinger 9.1 software at the OPLS_2005 level in CHCl$_3$. Selection of the conformers was done within a 1 kcal/mol energy window. Conformers not fitting to NMR data were discarded (exclusion criteria: (i) dihedral angles clearly mismatching the $^3J_{HH}$ coupling constants between H-10/H-9a and H-10/H-9b or (ii) dihedral angles mismatching $J_{2b,3b}$ 0 Hz and interatomic distances between H-6/H-12 and H-6/H-13 mismatching the corresponding NOESY correlations). The Gaussian 03 package[59] was used for optimizing the remaining starting geometries by means of the density function theory with the B3LYP functional and the 6-31G* basis set in the gas phase.[60] Dihedral angles and interatomic distances were taken from minimized conformers and compared with NMR data.

Expression of GABA$_A$ Receptors

Stage V–VI oocytes from *Xenopus laevis* were prepared and cRNA was injected as previously described by Khom et al. (2006).[51] Female *X. laevis* (NASCO, Fort Atkinson, WI) were anesthetized by exposing them for 15 min to a 0.2% MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate, 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 from *Clostridium histolyticum* (Type 1A, Sigma-Aldrich). Synthesis of capped runoff poly(A+) cRNA transcripts was obtained from linearized cDNA templates (pCMV vector). One day after enzymatic isolation, the oocytes were injected with 50 nL of DEPC-treated H$_2$O (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). Rat cRNAs were mixed in a 1:1:10 ratio to ensure expression of the gamma subunit in α$_1$β$_2$γ$_{2S}$ receptors. 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% of penicillin–streptomycin solution (Sigma-Aldrich).[61] Voltage clamp measurements were performed between days 1 and 5 after cRNA injection.

Two-Microelectrode Voltage Clamp Studies

Electrophysiological experiments were performed by the two-microelectrode voltage clamp method at a holding potential of −70 mV making use of a TURBO TEC 03X amplifier (npi Electronic GmbH, Tamm, Germany) and an Axon Digidata 1322A interface (Molecular Devices, Sunnyvale, CA). Data were recorded by using pCLAMP v10.2 data acquisition software (Molecular Devices). 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). Electrode filling solution contained 2 M KCl.

Fast Solution Exchange during I_{GABA} Recordings

Test solutions (100 μL) of extracts, fractions, and pure compounds were applied to the oocytes at a speed of 300 μL/s by means of the Screening Tool automated fast perfusion system (npi Electronic GmbH).[19] In order to determine GABA EC_{5-10} (typically between 3 and 10 μM for receptors of the subunit combination $\alpha_1\beta_2\gamma_{2S}$), a concentration–response experiment with GABA concentrations ranging from 0.1 μM to 1 mM was performed. Stock solutions (10 mg/mL in DMSO) of *A. calamus* extract and open column fractions (1–21) were diluted to concentrations of 100 and 10 μg/mL, respectively, with bath solution containing GABA EC_{5-10}. As previously described in a validated protocol, microfractions collected from the semipreparative HPLC separations were dissolved in 30 μL of DMSO and subsequently mixed with 2.97 mL of bath solution containing GABA EC_{5-10}.[29] For concentration–response experiments, bath solution containing compounds **1**–**6** in concentrations ranging from 0.1 to 1000 μM was applied to the oocyte. After a 20 s incubation period, a second application immediately followed containing the corresponding compound solution combined with GABA EC_{5-10}.

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. Data were analyzed using the ORIGIN 7.0 SR0 software (OriginLab Corporation) and are given as mean ± SE of at least 2 oocytes and 2 oocyte batches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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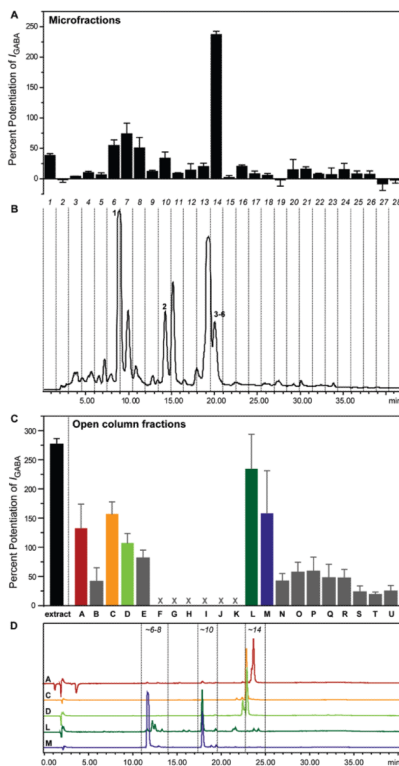
Figure 1.
HPLC-based activity profiling of a petroleum ether extract of *Acorus calamus* L. for
GABA$_A$ modulating properties. The HPLC chromatogram (254 nm) of a semipreparative
separation of 10 mg of extract is shown in B. Peak numbering corresponds to compounds **1**–
6. The 28 collected time-based fractions, 90 s each, are indicated with dashed lines.
Potentiation of the GABA-induced chloride current in *Xenopus* oocytes (I_{GABA}) of
microfractions is shown in A. Potentiation of I_{GABA} by the extract (100 μg/mL) and by open
column fractions A–E and L–U (10 μg/mL) are shown in C. Fractions F–K were not tested
due to limited amounts (<100 μg). Part D shows analytical HPLC traces (254 nm) of open
column fractions A, C, D, L, and M. The active time-windows from the HPLC-based
activity profiling (approximately corresponding to microfractions 6–8, 10, and 14) are
indicated with dashed lines.
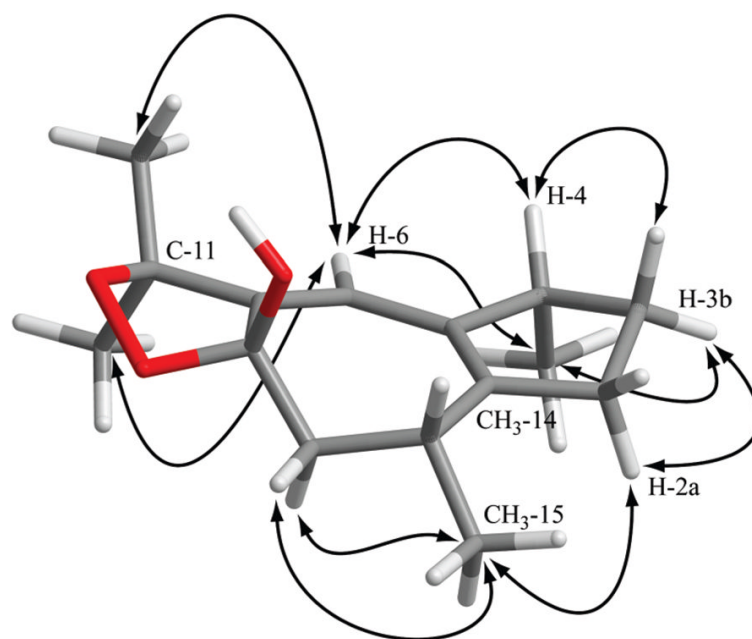
Figure 2.
Geometrically optimized structure of 4R*8S*10R*-(+)-dioxosarcoguaiacol (**2**). Critical NOESY correlations for assigning the relative configuration are indicated with arrows. The geometrical optimization was performed using density function theory at the B3LYP/6-31G* level.
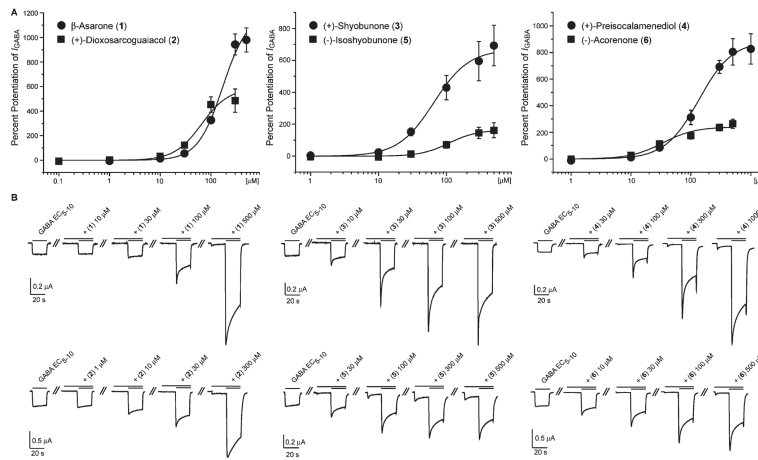
Figure 3.
Part A shows the concentration–response curves for compounds **1**–**6** on GABA$_A$ receptors (α_1, β_2, and γ_{2S} subunit composition) using a GABA EC$_{5-10}$. Typical traces for modulation of chloride currents through $\alpha_1 \beta_2 \gamma_{2S}$ GABA$_A$ receptors for compounds **1**–**6** are given in B.

Table 1

NMR Spectroscopic Data (500 MHz, CDCl$_3$) for (+)-Dioxosarcoguaiacol (2)

position	$\delta_C{}^a$	mult.	δ_H (J in Hz)	HMBC^b	NOESY^c
1	145.8	C			
2	35.6	CH$_2$	H-2a: 2.58, ddd (16.7, 8.5, 7.2) H-2b: 2.21, ddd (16.7, 10.9, 0)		H-2a ↔ H-3b, 15
3	29.9	CH$_2$	H-3a: 1.95, m H-3b: 1.40, dddd (16.6, 8.5, 3.5, 0)		H-3a ↔ 4 H-3b ↔ H-2a, 14
4	44.3	CH	2.63, m		
5	132.5	C			
6	114.6	CH	5.55, s	C-11, 4, 8, 7, 5	H-13, 12, 14, 4
7	153.2	C			
8	103.4	C			
8-OH			3.08, br s		
9	38.0	CH$_2$	H-9a: 1.63, dd (13.4, 13.4) H-9b: 2.03, dd (13.4, 5.2)	C-14, 10, 8, 7, 1	H-9a ↔ H-15 H-9b ↔ H-15
10	31.5	CH	2.71, m		
11	85.3	C			
12	28.0	CH$_3$	1.37, s	C-15, 11, 7	H-6
13	24.8	CH$_3$	1.43, s	C-12, 11, 7	H-6
14	20.1	CH$_3$	1.04, d (7.1)	C-3, 4, 5	
15	19.6	CH$_3$	1.12, d (7.1)	C-10, 9, 8, 1	H-9a, 9b, 2a

[a]13C shifts are deduced from HSQC and HMBC experiments.

[b]HMBC correlations from *hmbcgp* experiment (NS: 128, DS: 16), optimized for 10 Hz, are from proton(s) stated to the indicated carbon.

[c]Critical NOESY correlations from *noesygpphpp* experiment (mixing time 0.75 s, NS: 32, DS: 16)

Table 2

Potencies (EC$_{50}$) and Efficiencies (Maximum Stimulation of the GABA-Induced Chloride Current) of Compounds 1–6 for GABA$_A$ Receptors of the Subunit Composition α$_1$β$_2$γ$_{2S}$

compd	EC$_{50}$ [μM] ± SE	maximum stimulation of I_{GABA} (EC$_{5-10}$) ± SE [%]	Hill coefficient[a] (n_H) ± SE	no. of experiments (n)
1	171.5 ± 34.6	1200 ± 163	1.8 ± 0.2	6
2	65.3 ± 21.6	588 ± 126	1.6 ± 0.3	5
3	64.8 ± 19.8	669 ± 112	1.7 ± 0.3	6
4	135.1 ± 34.4	886 ± 105	1.6 ± 0.2	5
5	109.4 ± 46.6	164 ± 42.9	2.1 ± 0.8	6
6	34.0 ± 6.7	241 ± 23.1	1.6 ± 0.3	5

[a]Indicates the slope of the concentration–response curve at the EC50. Hill coefficients > 1 indicate positive cooperativity during receptor binding.[62]