Bioactivity-Guided Isolation of GABA$_A$ Receptor Modulating Constituents from the Rhizomes of *Actaea racemosa*

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Black cohosh (*Actaea racemosa*) is a frequently used herbal remedy for the treatment of mild climacteric symptoms. In the present study, the modulation of γ-aminobutyric acid (GABA)-induced chloride currents (I_{GABA}) through GABA type A (GABA$_A$) receptors by black cohosh extracts and isolated compounds was investigated. GABA$_A$ receptors, consisting of α$_1$, β$_2$, and γ$_{2S}$ subunits, were expressed in *Xenopus laevis* oocytes, and potentiation of I_{GABA} was measured using the two-microelectrode voltage clamp technique. In a bioactivity-guided isolation procedure the positive modulation of I_{GABA} could be restricted to the plant terpenoid fractions, resulting in the isolation of 11 cycloartane glycosides, of which four significantly ($p < 0.05$) enhanced I_{GABA}. The most efficient effect was observed for 23-*O*-acetylshengmanol 3-*O*-β-D-xylopyranoside (**4**, 100 μM), enhancing I_{GABA} by 1692 ± 201%, while actein (**1**), cimigenol 3-*O*-β-D-xylopyranoside (**6**), and 25-*O*-acetylcimigenol 3-*O*-α-L-arabinopyranoside (**8**) were significantly less active. In the absence of GABA, only **4** induced small (not exceeding 1% of $I_{GABA-max}$) chloride inward currents through GABA$_A$ receptors. It is hypothesized that the established positive allosteric modulation of GABA$_A$ receptors may contribute to beneficial effects of black cohosh extracts in the treatment of climacteric symptoms.

Actaea racemosa L. [syn.: *Cimicifuga racemosa* (L.) Nutt.], Ranunculaceae, commonly known as black cohosh, is a perennial herb growing in temperate and cool temperate regions of the northern hemisphere.[1] The center of its distribution is in the southeastern United States, where native North American groups used the rhizomes of black cohosh for the treatment of rheumatism and menstrual disorders.[1,2] After being included in the U.S. Pharmacopoeia for more than 100 years, *A. racemosa* became popular in Europe in the middle of the last century, where it underwent several pharmacological and clinical investigations.[3] Although the plant's active principle was extensively studied, its mechanism of action remained unclear.[4] Initial animal experiments of black cohosh extracts suggested a hormone-like activity of the lipophilic components (e.g., triterpene glycosides), whereupon an estrogenic effect was postulated,[5−8] but later studies disproved this theory.[9−17] However, there is evidence for antiproliferative and proapoptotic gene expression induced by black cohosh extracts and isolated cycloartane-type triterpenoids.[18] An enhancement of apoptosis and repressed cell cycle genes further suggests that *A. racemosa* might be useful in the prevention and therapy of breast cancer.[19,20] In addition, there is evidence for an interaction of *A. racemosa* and its main constituents with the central nervous system (CNS).[21,22] Such interactions have been suggested, including the central endogenous opioid system,[23] the μ-opioid receptor,[24] 5-HT$_{1A}$, 5-HT$_{1D}$, and 5-HT$_7$ receptors,[25] or D$_2$ receptors.[26] Furthermore, black cohosh preparations reduced moderate vasomotor symptoms associated with menopause more effectively than placebo in clinical trials.[15−17] In particular, improvements in sleep and mood disturbances were observed.[17]

In the present study, the interaction of black cohosh extracts with γ-aminobutyric acid (GABA) type A (GABA$_A$) receptors was analyzed. GABA$_A$ receptors mediate the inhibitory neurotransmission in the mammalian CNS.[27−30] They represent ligand-gated chloride channels that are assembled from different subunits, forming a pentameric structure. Nineteen subunits of mammalian GABA$_A$ receptors have been cloned so far: α$_{1−6}$, β$_{1−3}$, γ$_{1−3}$, δ, ε,

π, ρ$_{1−3}$, and θ.[30,32] Consequently, a large variety of different receptor subtypes can be formed.[30,31] GABA$_A$ receptors are the molecular target of multiple clinically important drugs, such as benzodiazepines, barbiturates, and general anesthetics,[28−34] but also natural compounds of plant origin including flavonoids such as methylapigenin[35] and wogonin (from *Scutellaria baicalensis*),[36,37] the monoterpenes borneol[38] and thymol,[39] the polyacetylenes MS-1, MS-2, and MS-4 from *Cussonia zimmermannii*,[40] and valerenic acid from *Valeriana officinalis*.[41−43]

In order to evaluate the effect of black cohosh extracts on GABA-induced chloride currents (I_{GABA}) through GABA$_A$ receptors, receptors were expressend in *Xenopus* oocytes. The active principles were then identified in a bioactivity-guided approach (Figure S1, Supporting Information), leading to the isolation of 11 cycloartane glycosides (**1−11**).

Results and Discussion

Dried and cut subaerial parts of *A. racemosa* were ground and extracted with MeOH. The resulting crude extract (A0) was partitioned subsequently between water and organic solvents of increasing polarity, giving Et$_2$O (A1), EtOAc (A2), and *n*-butanol (A3) fractions. The extracts obtained and the remaining aqueous layer (A4) were tested for their ability to modulate I_{GABA} at a concentration of 100 μg/mL (Figure S2 and Table S1, Supporting Information). To analyze the modulation of I_{GABA} by black cohosh extracts and isolated compounds, GABA$_A$ receptors composed of α$_1$, β$_2$, and γ$_{2S}$ subunits were expressed in *Xenopus* oocytes. Stimulation of I_{GABA} was analyzed by means of the two-micro-electrode voltage-clamp technique. These and all subsequent experiments were carried out at a GABA EC$_{3−10}$ concentration (see Experimental Section).

The crude MeOH extract (A0) significantly enhanced I_{GABA} (162 ± 19%, $n = 5$). Partitioning the MeOH extract with Et$_2$O resulted in a significantly greater potentiation of I_{GABA} (270 ± 53%, $n = 4$, $p < 0.05$), indicating that activity to be attributable to the more nonpolar constituents of the plant. HPLC-UV and HPLC-ELSD analysis of extract A1 showed a high content of weak UV-absorbing compounds, which were assumed to be cycloartane glycosides. HPLC chromatograms of extract A2 were similar to those of A1, apart from a higher ratio of UV-absorbing substances. Stimulation
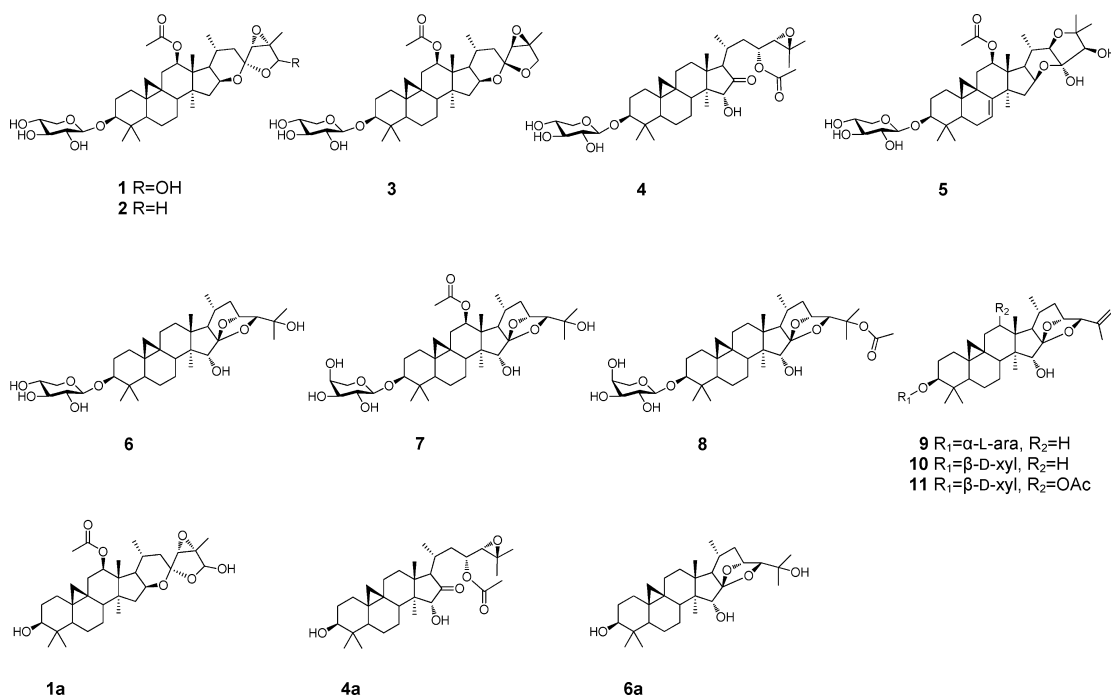
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Chart 1

1 R=OH
2 R=H

3

4

5

6

7

8

9 R₁=α-L-ara, R₂=H
10 R₁=β-D-xyl, R₂=H
11 R₁=β-D-xyl, R₂=OAc

1a

4a

6a

of I_{GABA} by the EtOAc extract A2 was less pronounced compared to extract A1 (113 ± 14%, $n = 4$). No significant stimulation of I_{GABA} was induced by extracts A3 (*n*-butanol) and A4 (aqueous). In these two extracts no triterpenoids were detected by HPLC-ELSD and TLC analysis, suggesting that the cycloartane glycosides may be responsible for the potentiation of I_{GABA}.

Subsequently, extract A1 was separated by silica gel column chromatography. A nonpolar solvent mixture was chosen to initially elute oily and resinous constituents from the column, while keeping the glycosides adsorbed onto the material, resulting in eight fractions (A1A−A1H). Thereupon, MeOH was added to the solvent mixture and triterpenoids were eluted, yielding another six fractions (A1I−A1N). Finally, the column was flushed with MeOH (A1O). The effects of these fractions on I_{GABA} (EC$_{3−10}$) were analyzed on *Xenopus* oocytes at a concentration of 100 µg/mL (Figure S3 and Table S2, Supporting Information).

While fractions A1A−A1H, consisting of dark brown, viscous material, displayed significant but relatively weak (A1A−A1C) potentiation of I_{GABA} or no effect (A1D−A1E), fractions A1I−A1N showed strong I_{GABA} enhancement (Table S2, Supporting Information), suggesting that the active components could be enriched. These latter fractions appeared as white to pale yellow powders except for fraction A1I, which was ochre. Fraction A1O was a brown powder and found to contain less apolar substances. HPLC-UV/ELSD analysis of active fractions A1I−A1N showed that the content of UV-absorbing substances in these fractions was reduced to a minimum and triterpene glycosides were highly enriched. Thus, further work concentrated on the isolation of the main compounds from fractions A1I−A1N and on the subsequent characterization of GABA_A receptor modulation by these compounds.

Fraction A1J showed four major compounds, which were isolated by silica gel column chromatography and identified as actein (**1**),[44] 26-deoxyactein (**2**),[45] 23-*epi*-26-deoxyactein (**3**),[46] and 23-*O*-acetylshengmanol 3-*O*-β-D-xylopyranoside (**4**),[46] with **1** and **3** as the most abundant constituents. Fractions A1K−A1N did not differ in their constituent pattern, as compounds **4−7** were apparent in all fractions, with decreasing contents of **4** and **7** and increasing amounts of compounds **5** and **6**. These four fractions were combined and repeatedly chromatographed to obtain cimiracemoside F (**5**), cimigenol 3-*O*-β-D-xylopyranoside (**6**), and cimiracemoside D (**7**).[47] Fraction A1I showed three major components (**8−10**), which were

isolated (along with one minor constituent **11**) using different chromatographic methods. Compounds identified were 25-*O*-acetylcimigenol 3-*O*-α-L-arabinopyranoside (**8**),[48] 25-*O*-anhydro-cimigenol 3-*O*-α-L-arabinopyranoside (**9**), 25-*O*-anhydrocimigenol 3-*O*-β-D-xylopyranoside (**10**), and cimiracemoside K (**11**).[49] All isolated substances were analyzed for GABA_A receptor modulation, whereas significant enhancement of I_{GABA} could be determined for compounds **1**, **4**, **6**, and **8**.

As illustrated in Figure 1, 100 µM of compounds **1**, **4**, **6**, and **8** significantly enhanced ($p < 0.05$) I_{GABA} by more than 150%, when coapplied with GABA (EC$_{3−10}$). Compound **4** induced an about 10-times more potent I_{GABA} enhancement than the other compounds. Compared to established GABA_A receptor modulators like the benzodiazepine diazepam (see Figure 1 for comparison) the effect of **4** was about 8 times more pronounced. Except compound **4**, none of these compounds induced chloride currents when applied in the absence of GABA. The chloride currents induced by
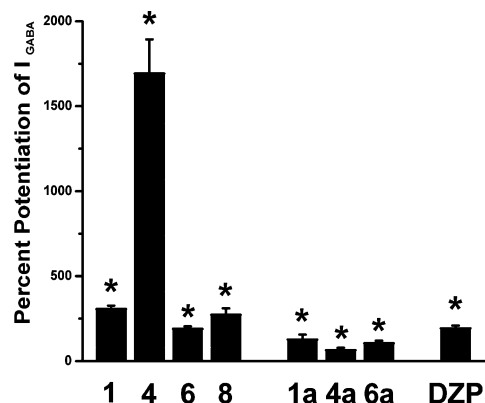

Figure 1. Modulation of I_{GABA} in % by the indicated pure compounds isolated from black cohosh. Bars illustrate the enhancement of I_{GABA} upon coapplication of GABA (EC$_{3−10}$) and 100 µM of the indicated compound. Diazepam (DZP) was used as a control and was applied at a concentration of 0.3 µM. Each bar represents the mean ± SEM from at least three oocytes and ≥ two oocyte batches. [(*) indicates significantly different from zero ($p < 0.05$, *t*-test by ANOVA)].

Table 1. Stimulation of I_{GABA} (GABA EC$_{3-10}$ concentrations) at 100 μM of the Indicated Compound

Compound	Stimulation of I_{GABA} (%)	Number of Experiments (n)
1	305 ± 20	7
4	1692 ± 201	6
6	189 ± 14	6
8	271 ± 39	5
1a	126 ± 29	4
4a	64 ± 14	4
6a	104 ± 15	4

Table 2. Summary of Efficiencies and Potencies of the Isolated Compounds **1**, **4**, **6**, and **8**

Compound	EC$_{50}$ (μM)	Maximal Stimulation of I_{GABA}	Number of Experiments (n)
1	36 ± 14	378 ± 64	7
4	27 ± 8	1947 ± 185	6
6	28 ± 17	256 ± 40	6
8	26 ± 7	289 ± 45	5

compound **4** were, however, relatively small and did not exceed 1% of $I_{GABA-max}$ (induced by application of 1 mM GABA; data not shown).

To elucidate whether hydrolysis of the pentose moiety affects I_{GABA} modulation, the active compounds **1**, **4**, and **6** were hydrolyzed to 12-*O*-acetylacteol (**1a**),[50] 23-*O*-acetylshengmanol (**4a**),[51] and cimigenol (**6a**),[50] respectively. I_{GABA} modulation by the resulting aglycons was significantly less pronounced. This loss of activity was most evident for **4** (from 1692 ± 201% to 64 ± 14%) and less pronounced for **1**, **6**, and **8** (Figure 1, Table 1).

Compounds **1**, **4**, **6**, and **8** dose dependently enhanced I_{GABA} with comparable potencies (EC$_{50}$ ranged from 26 ± 7 (**8**) to 36 ± 14 (**1**), $p > 0.05$). Compound **4** induced the greatest I_{GABA} potentiation at 300 μM (1947 ± 185%), while compounds **1**, **6**, and **8** were considerably less efficient (I_{GABA} enhancement <300%, Figure 2, Table 2).

The present work describes the bioassay-guided isolation and pharmacological evaluation of four active principles from the rhizomes of *A. racemosa*. Besides actein (**1**) and cimigenol derivatives **6** and **8**, which showed a significant enhancement of I_{GABA}, most notably 23-*O*-acetylshengmanol 3-*O*-β-D-xylopyranoside (**4**) exhibited a strong effect and therefore may be considered as a lead structure for the development of new GABA$_A$ receptor modulators.

The established allosteric modulation of GABA$_A$ receptors by the identified compounds may cause anxiolytic, sedative, and hypnotic effects, for example, which potentially contribute to beneficial effects described for black cohosh extracts in the treatment of postmenopausal symptoms. A detailed characterization of their subunit specific effects (analysis of the interaction with different GABA$_A$ receptor subtypes) and in vivo studies will help to understand the CNS effects of these novel GABA$_A$ receptor modulators. So far, little is known about the pharmacokinetics and metabolites of black cohosh extracts. A recent study, however, provided the first evidence for the bioavailability of actein after peroral application to rats.[52] Even now, it remains to be clarified whether these compounds penetrate the blood brain barrier for efficient GABA$_A$ receptor modulation in vivo.

Experimental Section

General Experimental Procedures. All reagents were of analytical quality, with organic solvents for isolation distilled before use and Nanopure water utilized. Column chromatography was performed with silica gel (40−63 μm particle size) (Merck, Germany) or with Sephadex LH-20 (Sigma Aldrich, St. Louis, MO). TLC was performed on silica gel 60 F$_{254}$ plates (VWR, Germany) using chloroform−acetone−MeOH (70:15:15) as mobile phase and vanillin−sulfuric acid (reagent A: 1% vanillin in MeOH; reagent B: 5% sulfuric acid in MeOH) for detection. Preparative LPLC was accomplished using a Merck Hitachi L-7100 pump and Lichroprep RP-18 silica gel (40−63 μm particle size) (Merck, Germany). Semipreparative HPLC was carried out on a Dionex system (Dionex, Sunnyvale, CA) with a P580 pump, ASI-100 autosampler, UVD 170U detector, Gilson 206 fraction collector, and Phenomenex Synergi Max-RP column (250 × 10 mm, 10 μm particle size). Extracts, fractions, and pure compounds were analyzed by HP 1090, connected to an autosampler, column heater, DAD, and Alltech ELSD 2000 instrument, using a Phenomenex Synergi Max-RP column (150 × 4.6 mm, 4 μm particle size). MS analysis was carried out by direct injection using a Bruker Daltonics Esquire 3000^{plus} instrument with atmospheric pressure chemical ionization (APCI). NMR spectra were recorded on a Bruker DRX-300 instrument (Bruker, Germany).

Plant Material. Dried and cut subaerial parts of *Actaea racemosa* were obtained from Mag. Kottas Heilkräuter, Austria (Rd. Cimicifugae Cs. EB 6, lot number: KLA50226). A voucer specimen (ARR-070201-CSS) is deposited at the Institute of Pharmacy/Pharmacognosy, University of Innsbruck.

Extraction and Isolation. Dried rhizomes (700 g) were ground, and extracted with 2 L of MeOH under reflux for 3 days, and the solvent was evaporated under reduced pressure to afford 108 g of a crude extract. The extract was then partitioned between Et$_2$O and water 10 times, and the Et$_2$O layer was rewashed with water and concentrated to dryness, yielding a residue of 27 g. The Et$_2$O extract (26 g) was subjected to silica gel column chromatography (25 × 10 cm) and eluted with petroleum ether−acetone−EtOAc (1:1:1), yielding eight yellow fractions (A1I−A1H). After the eluting solvent turned colorless, 10% MeOH was added to the solvent mixture to collect fractions A1I−A1N.
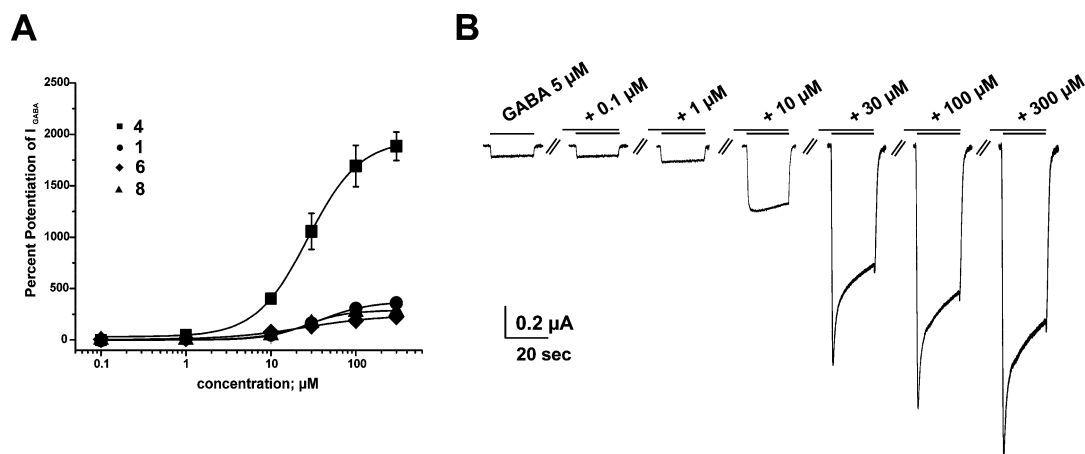

Figure 2. (A) Enhancement of I_{GABA} by compound **4** (■), compound **1** (●), compound **6** (◆), and compound **8** (▲) at a GABA EC$_{3-10}$ concentration through $\alpha_1\beta_2\gamma_{2S}$ receptors. Each data point represents the mean ± SEM from at least three oocytes and ≥ two oocyte batches. (B) Representative currents for the I_{GABA} modulation by compound **4**.

A1O was obtained by flushing the column with MeOH. Fraction A1J (2.40 g) was chromatographed over a silica gel column (100 × 3.5 cm) using *n*-heptane−EtOAc−MeOH (10:10:0.5 to 10:10:3) in a gradient manner, yielding 12 subfractions (A1J1−A1J12). Fraction A1J12 gave 74 mg of compound **1**, and fraction A1J10 gave 121 mg of compound **3**. Fractions A1J8 and A1J6 were recrystallized from MeOH to afford 23 mg of compound **2** and 57 mg of compound **4**. Fractions A1K−A1N were combined (4.45 g) and subjected to silica gel column chromatography (45 × 5.5 cm), using a solvent system of CH$_2$Cl$_2$−MeOH (in a step gradient from 9:1 to 7:3), obtaining six subfractions (A1KN1−A1KN6). Fraction A1KN2 (1.65 g) was chromatographed with a silica gel column (100 × 3.5 cm) using *n*-heptane−EtOAc−MeOH (10:10:0.5 to 10:10:3) in a gradient manner, yielding seven subfractions (A1KN21−A1KN27). Subfraction A1KN23 afforded 26 mg of compound **7**. Also fraction A1KN4 (0.83 g) was subjected to silica gel column chromatography using *n*-heptane−EtOAc−MeOH (10:10:1 to 10:10:3), giving eight subfractions (A1KN41−A1KN48). Subfractions A1KN42 and A1KN43 were combined (204 mg) and subjected to LPLC using H$_2$O−MeCN (in a gradient from 7:3 to 4:6 over 4 h) to obtain 10 mg of compound **5** and 54 mg of compound **6**. Fraction A1I (0.98 g) was subjected to Sephadex LH-20 column chromatography (80 × 2 cm) using CH$_2$Cl$_2$−acetone (85:15) as solvent mixture, yielding nine subfractions (A1I1−A1I9). Subfraction A1I3 (180 mg) was separated with semipreparative HPLC using H$_2$O−MeCN (50:50), yielding 23 mg of compound **8** and 9 mg of compound **9**. Subfraction A1I4 (96 mg) was also subjected to semipreparative HPLC using H$_2$O−MeCN (45:55) to give 5 mg of compound **11**. Semipreparative HPLC of subfraction A1I5 (78 mg) using H$_2$O−MeCN (40:60) afforded 8 mg of compound **10**.

Enzymatic Hydrolysis. For hydrolysis of β-D-xylose from position C-3, compounds **1** (35 mg), **4** (26 mg), and **6** (24 mg) were each suspended in 3.5 mL of sodium acetate buffer (pH 4.3) containing 140 mg of naringinase and incubated at 20 °C for 72 h under constant stirring. The reaction process was controlled every 24 h by TLC. The aglycons were extracted from the reaction mixtures using *n*-hexane (10 mL, 5 times). The *n*-hexane layers were combined, rewashed with Nanopure water, and evaporated to dryness, giving yields of 23 mg of 12-*O*-acetylacteol (**1a**), 14 mg of 23-*O*-acetylshengmanol (**4a**), and 12 mg of cimigenol (**6a**). Identity of the aglycons was confirmed by NMR spectroscopy.

Expression and Functional Characterization of GABA$_A$ Receptors. The preparation of stage V−VI oocytes from *Xenopus laevis* and the synthesis of capped off runoff poly(A$^+$) cRNA transcripts from linearized cDNA templates (pCMV vector) were performed as described previously.[41,43] Briefly, female *Xenopus laevis* (Nasco, Fort Atkinson, WI) were anesthetized by exposing them for 15 min to a 0.2% solution of MS-222 (the methane sulfonate salt of 3-aminobenzoic acid ethyl ester; Sandoz) before surgically removing parts of the ovaries. Follicle membranes from the isolated oocytes were enzymatically digested with 2 mg/mL collagenase (Type 1A, Sigma, Germany). One day after isolation, the oocytes were injected with about 10−50 nL of DEPC-treated water (diethyl pyrocarbonate; Sigma, Germany) containing the different cRNAs at a concentration of approximately 150−3000 ng/μL/subunit. The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-biotech, Germany).

To ensure expression of the gamma-subunit, cRNAs were mixed in a ratio of 1:1:10.[53] Incorporation of gamma subunits into GABA$_A$ receptors was determined by coapplication of a GABA EC$_{3−10}$ concentration and 0.3 μM diazepam. Oocytes were stored at 18 °C in ND96 solution.[54] Electrophysiological experiments were performed using the two-microelectrode voltage-clamp method at a holding potential of −70 mV making use of a TURBO TEC 01C amplifier (npi electronic, Germany) and an Axon Digidata 1322A interface (Molecular Devices, Sunnyvale, CA). Data acquisition using pCLAMP v.9.2 was carried out. The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM MgCl$_2$·6H$_2$O, 1 mM CaCl$_2$, and 5 mM HEPES (pH 7.4). Microelectrodes were filled with 2 M KCl and had resistances between 1 and 3 MΩ.[41]

Perfusion System. GABA and the test material (extract, fraction, or pure compound) were applied by means of fast perfusion system.[55] Test material or control solutions were applied by means of a TECAN Miniprep 60 permitting automation of the experiments. Oocytes with maximal current amplitudes > 3 μA were discarded to exclude voltage-clamp errors.[55]

Analyses of Concentration−Response Curves. Stimulation of chloride currents by modulators of the GABA$_A$ receptor was measured at a GABA concentration eliciting between 3% and 10% of the maximal current amplitude (EC$_{3−10}$). The EC$_{3−10}$ was determined at the beginning of each experiment. Enhancement of the chloride current was defined as $(I_{(GABA+Comp)}/I_{GABA}) − 1$, where $I_{(GABA+Comp)}$ is the current response in the presence of a given test material (extract, fraction, or pure compound) and I_{GABA} is the control GABA current. To measure the sensitivity of the GABA$_A$ receptor for a given compound, it was applied for an equilibration period of 1 min before applying GABA (EC$_{3−10}$). 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 n_H is the Hill coefficient. Each data point represents the mean ± SE from at least four oocytes and ≥two oocyte batches. Statistical significance was calculated using the paired Student *t*-test with a confidence interval of $p < 0.05$.

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