A Cycloartane Glycoside Derived from *Actaea racemosa* L. Modulates GABA<sub>A</sub> Receptors and Induces Pronounced Sedation in Mice

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**ABSTRACT**

23-O-Acetylshengmanol 3-O-β-D-xylopyranoside (Ac-SM) isolated from *Actaea racemosa* L.—an herbal remedy for the treatment of mild menopausal disorders—has been recently identified as a novel efficacious modulator of GABA<sub>A</sub> receptors composed of α<sub>1</sub>, β<sub>2</sub>, and γ<sub>2S</sub>-subunits. In the present study, we analyzed a potential subunit-selective modulation of GABA-induced chloride currents (I<sub>GABA</sub>) at GABA concentrations eliciting 3-8% of the maximal GABA response (EC<sub>50</sub>-a) through nine GABA<sub>A</sub> receptor isoforms expressed in Xenopus laevis oocytes by Ac-SM with two-microelectrode voltage clamp and behavioral effects 30 minutes after intraperitoneal application in a mouse model. Efficacy of I<sub>GABA</sub> enhancement by Ac-SM displayed a mild α-subunit dependence with α<sub>2</sub>β<sub>2</sub>γ<sub>2S</sub> (maximal I<sub>GABA</sub> potentiation [E<sub>max</sub> = 1545 ± 97%] and α<sub>2</sub>β<sub>2</sub>γ<sub>2S</sub> (E<sub>max</sub> = 1408 ± 87%) receptors being most efficaciously modulated, followed by slightly weaker I<sub>GABA</sub> enhancement through α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub> (E<sub>max</sub> = 1187 ± 1666), α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub> (E<sub>max</sub> = 1174 ± 218%), and α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub> (E<sub>max</sub> = 1171 ± 274%) receptors and less pronounced effects on receptors composed of α<sub>4</sub>β<sub>2</sub>γ<sub>2S</sub> (E<sub>max</sub> = 752 ± 53%) subunits, whereas potency was not affected by the subunit composition (EC<sub>50</sub> values ranging from α<sub>1</sub>β<sub>2</sub>γ<sub>2S</sub> = 35.4 ± 12.3 μM to α<sub>4</sub>β<sub>2</sub>γ<sub>2S</sub> = 50.9 ± 11.8 μM). Replacing β<sub>2</sub>- with β<sub>1</sub>- or β<sub>3</sub>-subunits as well as omitting the γ<sub>2S</sub>-subunit affected neither efficacy nor potency of I<sub>GABA</sub> enhancement by Ac-SM. Ac-SM shifted the GABA concentration-response curve toward higher GABA sensitivity (about 3-fold) and significantly increased the maximal GABA response by 44 ± 13%, indicating a pharmacological profile distinct from a pure allosteric GABA<sub>A</sub> receptor modulator. In mice, Ac-SM significantly reduced anxiety-related behavior in the elevated plus maze test at a dose of 0.6 mg/kg, total ambulation in the open field test at doses ≥6 mg/kg, stress-induced hyperthermia at doses ≥0.6 mg/kg, and significantly elevated seizure threshold at doses ≥20 mg/kg body weight. High efficacy and long biologic half-life of Ac-SM suggest that potential cumulative sedative side effects upon repetitive intake of *A. racemosa* L. preparations might not be negligible.

**Introduction**

GABA mediates fast inhibitory neurotransmission in the mammalian brain by activating synaptic and extrasynaptic GABA<sub>A</sub> receptors. GABA<sub>A</sub> receptors are composed of five subunits that form a chloride-conducting ion pore (Macdonald and Olsen, 1994; Sieghart, 1995; Sieghart and Sperk, 2002; Sigel and Steinmann, 2012). Nineteen different GABA<sub>A</sub> receptor subunits have been identified in the human genome (Simon et al., 2004), theoretically allowing the formation of approximately 800 subunit combinations. However, so far the existence of only 11 native GABA<sub>A</sub> receptor subtypes has been confirmed (Olsen and Sieghart, 2008). There is consensus that the mainly expressed GABA<sub>A</sub> receptor isofrom consists of α<sub>1</sub>, β<sub>2</sub>, and γ<sub>2</sub> subunits (Olsen and Sieghart, 2008) with a commonly accepted 2α:3β:1γ subunit stoichiometry (Tretter et al., 1997; Baumann et al., 2001, 2002). The subunit combination determines the pharmacologic properties of GABA<sub>A</sub> receptors (Olsen and Sieghart, 2009). GABA<sub>A</sub> receptors play a major role in controlling the excitability of the mammalian brain and are thus involved in regulating, among others, sleep, vigilance, mood, and emotions (Möhler et al., 2005; Möhler, 2006). GABA<sub>A</sub> receptors are modulated by structurally diverse compounds including clinically applied drugs, such as benzodiazepines, barbiturates, neurosteroids, and anesthetics, as well as a large number of natural products, including flavonoids, terpenoids, or polyacetlyenes (for review, see Johnston et al., 2006; Olsen and Sieghart, 2009; Hanrahan et al., 2011; Nilsson and Sterner, 2011). Recently, we have identified four cycloartane glycosides

**ABBREVIATIONS:** Ac-SM, 23-O-acetylshengmanol 3-O-β-D-xylopyranoside; ANOVA, analysis of variance; EPM, elevated plus maze; DMSO, dimethylsulfoxide; EC<sub>50</sub>-a, concentration eliciting between 3 and 8% of the maximal current amplitude; E<sub>max</sub>, maximal potentiation of I<sub>GABA</sub>; GABA<sub>A</sub>, HC, home cage; I<sub>GABA</sub>, GABA-induced chloride currents; MS-222, methane sulfonate salt of 3-aminobenzoic acid ethyl ester; OA, open arm; OF, open field test; PTZ, pentylenetetrazole; SIH, stress-induced hyperthermia; T, body temperature elevation.
(actein, cimigenol 3-O-β-D-xylopyranoside, 25-O-acetyl-cimigenol 3-O-α-l-arabinopyranoside, and 23-O-acetylshengmanol 3-O-β-D-xylopyranoside) as novel efficacious GABA_A modulators isolated from Actaea racemosa L. Whereas enhancement of GABA-induced chloride currents (\(I_{GABA}\)) by actein, cimigenol 3-O-β-D-xylopyranoside, and 25-O-acetyl-cimigenol 3-O-α-l-arabinopyranoside averaged at about 300%, a more than 5-fold stronger \(I_{GABA}\) potentiation was observed for cycloartane glycoside 23-O-acetylshengmanol 3-O-β-D-xylopyranoside (Ac-SM) (Ciecko et al., 2010).

The aim of the present study was to obtain deeper insights into the molecular mechanism of Ac-SM action by analyzing a potential subunit-dependent modulation of nine different GABA_A receptor subtypes expressed in Xenopus laevis oocytes. In addition, potential anxiolytic, stress-reducing, anticonvulsant effects as well as changes in locomotor activity upon application of Ac-SM were studied in mice to investigate the potential effects of this compound on mood, sedation, and seizure threshold.

### Materials and Methods

All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European Convention (Directive 2010/63/EU) for the protection of vertebrate animals used for experimental and other scientific purposes (European Treaty Series no. 123). Every effort was taken to minimize the number of animals used.

**Chemicals.** Ac-SM (Fig. 1) was isolated from *Actaea racemosa* L. as previously described (Ciecko et al., 2010). Stock solutions (100 mM for in vitro experiments and 1 mg/10 μl for in vivo experiments, respectively) were prepared in dimethylsulfoxide (DMSO). All chemicals were obtained from Sigma-Aldrich (Vienna, Austria).

Due to low solubility in the bath solution, Ac-SM was used only up to a concentration of 300 μM in in vitro experiments. Equal amounts of DMSO were present in control and compound-containing solutions. The maximum DMSO concentration in the bath (0.3%) did not affect \(I_{GABA}\) (Khom et al., 2006).

For in vivo experiments, working concentrations were adjusted by dilution with 0.9% sodium chloride; the final concentration of DMSO was fixed at 10% including control solutions. To enhance solubility of the compound, Tween 80 (3% final concentration) was added to all dilutions with 0.9% sodium chloride; the final concentration of DMSO remained at 0.3%.

**Preparation of stage V–VI oocytes.** To minimize the number of animals used, females were anesthetized by exposing them for 15 minutes to a 0.2% solution of MS-222 (methylene sulfonate salt of 3-aminobenzoic acid ethyl ester) before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (type IA). Oocytes were stored at 18°C in a medium containing 90 mM NaCl, 1 mM KCl, 1 mM MgCl_2·6H_2O, 1 mM CaCl_2, and 5 mM HEPES (Methfessel et al., 1986). Each oocyte was further incubated for 1 hour in the presence of high GABA or compound concentrations, the duration of which was at least 15 minutes.

**Expression and Functional Characterization of GABA_A Receptors.** Preparations of stage V–VI oocytes from *Xenopus laevis* (Nasco, Fort Atkinson, WI) and synthesis of capped off run-off poly(A+)
 rRNA transcripts from linearized cDNA templates (pCMV vector) were performed as previously described (Khom et al., 2006). Briefly, female *X. laevis* were anesthetized by exposing them for 15 minutes to a 0.2% solution of MS-222 (methylene sulfonate salt of 3-aminobenzoic acid ethyl ester) before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (type IA). Oocytes were stored at 18°C in a medium containing 90 mM NaCl, 1 mM KCl, 1 mM MgCl_2·6H_2O, 1 mM CaCl_2, and 5 mM HEPES (Methfessel et al., 1986). Each oocyte was further incubated for 1 hour in the presence of high GABA or compound concentrations, the duration of which was at least 15 minutes.

**Perfusion System.** GABA and Ac-SM were applied by means of fast perfusion system (for details, see Baburin et al., 2006). Drug or control solutions were applied by means of a TECAN Miniprep 60 (NIPI Electronic, Sunnyvale, CA). Data acquisition was carried out using pCLAMP version 9.2 (Molecular Devices). The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM MgCl_2·6H_2O, 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 (Khom et al., 2006).

**Enhancement of the chloride current was defined as**

\[
\frac{I_{GABA + Comp}}{I_{GABA}} - 1
\]

where \(I_{GABA + Comp}\) is the current response in the presence of Ac-SM, 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 minute before applying GABA (EC_50). 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:

\[
\frac{1}{1 + \left(\frac{EC_{50}}{X_{Comp}}\right)^{n_H}}
\]

where \(n_H\) is the Hill coefficient. Each data point represents the mean ± S.E.M. from at least 3 oocyte batches.
Behavioral Studies. Male mice (C57BL/6N) were obtained from Charles River Laboratories (Sulzfeld, Germany). For maintenance, mice were group-housed (maximum five mice per type III cage) with free access to food and water. At least 24 hours before the commencement of experiments, mice were transferred to the testing facility, with continued ad libitum access to food and water. The temperature in the holding and testing facilities was 22°C, the humidity was 40–60%, and a 12-hour light/dark cycle was in operation (lights on from 7:00 AM to 7:00 PM). Male mice aged 3–6 months were tested.

Intraperitoneal injection of control or Ac-SM-containing solutions was usually done 30 minutes before the test, except for home cage (HC) activity analysis (60 minutes before test) and measurement of stress-induced hyperthermia (3 hours before test), to reduce the impact of stress on the analysis. Application of the solvent alone did not influence animal behavior. All doses are indicated as milligrams per kilogram body weight of the animal.

Open Field Test. Exploration of a novel environment was tested over 10 minutes in a 50 × 50–cm box built from gray PVC equipped with infrared beams. Illumination intensity was set to 150 lux in the center, center. Animals with infrared beams. Illumination intensity was set to 150 lux in the over 10 minutes in a 50

TABLE 1

<table>
<thead>
<tr>
<th>Subunit Combination</th>
<th>EC50</th>
<th>Maximal Potentiation of IGABA</th>
<th>nH</th>
<th>Number of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>αβγ2</td>
<td>47.1 ± 20.9</td>
<td>1187 ± 166</td>
<td>1.0 ± 0.1</td>
<td>6</td>
</tr>
<tr>
<td>αβγ2</td>
<td>39.1 ± 8.4</td>
<td>1454 ± 97</td>
<td>1.7 ± 0.3</td>
<td>7</td>
</tr>
<tr>
<td>αβγ2</td>
<td>47.5 ± 11.7</td>
<td>1174 ± 218</td>
<td>2.0 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>αβγ2</td>
<td>46.3 ± 4.7</td>
<td>752 ± 53</td>
<td>1.4 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>αβγ2</td>
<td>50.9 ± 11.8</td>
<td>1408 ± 67</td>
<td>1.7 ± 0.4</td>
<td>3</td>
</tr>
<tr>
<td>αβγ2</td>
<td>46.0 ± 22.9</td>
<td>1171 ± 274</td>
<td>1.0 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>αβγ2</td>
<td>45.0 ± 11.4</td>
<td>1277 ± 243</td>
<td>1.5 ± 0.3</td>
<td>5</td>
</tr>
<tr>
<td>αβγ2</td>
<td>43.4 ± 15.9</td>
<td>1069 ± 214</td>
<td>1.6 ± 0.4</td>
<td>6</td>
</tr>
<tr>
<td>αβγ2</td>
<td>35.4 ± 12.3</td>
<td>1247 ± 222</td>
<td>1.7 ± 0.4</td>
<td>4</td>
</tr>
</tbody>
</table>
short, a temperature probe, lubricated with glycerol, was inserted into the rectum of the mouse to a depth of up to 2 cm to measure basal temperature (T1). The temperature probe remained in the animal until a stable temperature was reached (maximum 10 seconds). Measurement of T1 served also as the handling stressor. After 15 minutes, the measurement was repeated (T2), and the rise in temperature was considered as stress-induced hyperthermia ΔT (Olivier et al., 2003).

**Seizure Threshold.** Seizure threshold was determined by pentylepentylenetetrazole (PTZ) tail-vein infusion on freely moving animals at a rate of 100 ml/min (10 mg/ml PTZ in saline, pH = 7.4). Infusion was stopped when animals displayed generalized clonic seizures. Animals were immediately killed by cervical displacement after onset of seizures. The seizure threshold dose was calculated from the infused volume in relation to body weight.

**Statistical Analysis.** Statistical significance was calculated by one-way analysis of variance (ANOVA) and, where appropriate, by two-way ANOVA (followed by a post-hoc mean comparison with Bonferroni; GraphPad Software, La Jolla, CA). *P* values, 0.05 were accepted as statistically significant. All data are given as the mean ± S.E.M.

**Results**

In the present study, we demonstrate a comparable potency and efficacy of IGABA modulation by Ac-SM (for structural formula, see Fig. 1) on nine different GABA<sub>A</sub> receptor

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**Fig. 3.** Ac-SM shifts the GABA concentration-response curve toward higher GABA sensitivity. GABA concentration-response curves for α<sub>1</sub>β<sub>3</sub> GABA<sub>A</sub> receptors in the absence (control, ♦) and in the presence of 300 μM Ac-SM (○) are compared. Each data point represents the mean ± S.E.M. from at least three oocytes from two different oocyte batches.

**Fig. 4.** Ac-SM dose dependently reduces locomotor activity in the OF test. Bars indicate the total distance traveled (A), the time spent in the center (B), the number of entries to the center (C), and the distance traveled in the center (D) (percentage of the total distance) of mice treated with the indicated dose of Ac-SM [mg/kg body weight (gray bars) compared with vehicle-treated control littermates (white bars)]. Bars always represent means ± S.E.M.; the number of animals per group is given in the respective bar. Statistically significant differences with *P < 0.05 and **P < 0.01 compared with control (ANOVA with post-hoc Bonferroni analysis).
subtypes. $I_{\text{GABA}}$ modulation was independent of the presence of a $\gamma_2$-subunit. Ac-SM induces anxiolytic, stress-reducing, sedative, and anticonvulsant effects.

**$I_{\text{GABA}}$ Enhancement by Ac-SM Does Not Require the Presence of a $\gamma_2$-Subunit.** Ac-SM efficaciously potentiates $I_{\text{GABA}}$ through GABA$_A$ receptors composed of $\alpha_1\beta_2\gamma_2S$ subunits (Cicek et al., 2010). As illustrated in Fig. 2A, Ac-SM, however, also modulated GABA$_A$ receptors lacking a $\gamma_2$-subunit with similar efficacy and potency (maximal potentiation of $I_{\text{GABA}}$, $E_{\text{max}}(\alpha_1\beta_2\gamma_2S) = 1187 \pm 166\%$ and $EC_{50}(\alpha_1\beta_2\gamma_2S) = 47.1 \pm 20.9 \mu M$ versus $E_{\text{max}}(\alpha_1\beta_2) = 1277 \pm 243\%$ and $EC_{50}(\alpha_1\beta_2) = 45.0 \pm 11.4 \mu M$; see also Table 1).

**Ac-SM Enhances $I_{\text{GABA}}$ Irrespective of the GABA$_A$ Receptor Subunit Composition.** To investigate a possible $\alpha$-subunit–dependent $I_{\text{GABA}}$ enhancement, the $\alpha_1$-subunit was substituted with $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, and $\alpha_6$-subunits, respectively, and expressed in combination with $\beta_2$- and $\gamma_2S$-subunits (Fig. 2B; Table 1). The highest efficacy was observed for receptors containing either $\alpha_2$- or $\alpha_5$-subunits, with a maximal $I_{\text{GABA}}$ potentiation of $E_{\text{max}}(\alpha_2\beta_2\gamma_2S) = 1454 \pm 97\%$ (n = 7) and $E_{\text{max}}(\alpha_5\beta_2\gamma_2S) = 1408 \pm 87\%$ (n = 3), respectively (Table 1), followed by receptors comprising either $\alpha_7$– $E_{\text{max}}(\alpha_7\beta_2\gamma_2S) = 1174 \pm 218\%$ (n = 4), or $\alpha_6$-subunits ($E_{\text{max}}(\alpha_6\beta_2\gamma_2S) = 1171 \pm 274\%$; n = 3). Ac-SM, however, displayed a lower efficacy on GABA$_A$ receptors comprising $\alpha_2$-subunits compared with $\alpha_1\beta_2\gamma_2S$, $\alpha_2\beta_2\gamma_2S$, $\alpha_6\beta_2\gamma_2S$ ($P < 0.05$), $\alpha_3\beta_2\gamma_2S$, and $\alpha_5\beta_2\gamma_2S$ ($P < 0.001$) receptors ($E_{\text{max}}(\alpha_2\beta_2\gamma_2S) = 752 \pm 53\%$; n = 3; $F(5,20) = 9.185$; Table 1).

In contrast to this moderate $\alpha$-subunit dependence, replacing the $\beta_2$-subunit in $\alpha_1\beta_2\gamma_2S$ receptors by either $\beta_1$- or $\beta_3$-subunits (Fig. 2C) did not affect the strength of $I_{\text{GABA}}$ enhancement by Ac-SM ($E_{\text{max}}(\alpha_1\beta_1\gamma_2S) = 1069 \pm 214\%$, n = 6 versus $E_{\text{max}}(\alpha_1\beta_2\gamma_2S) = 1187 \pm 166\%$, n = 6 versus $E_{\text{max}}(\alpha_1\beta_3\gamma_2S) = 1247 \pm 222\%$, n = 4).

Comparison of the potencies of the respective GABA$_A$ receptor isofoms revealed no significant differences, with $EC_{50}$ values ranging from $35.4 \pm 12.3 \mu M$ ($\alpha_1\beta_2\gamma_2S$) to $50.9 \pm 11.8 \mu M$ ($\alpha_2\beta_2\gamma_2S$) (see Table 1). Typical current traces for concentration-dependent $I_{\text{GABA}}$ enhancement by Ac-SM are illustrated in Fig. 2D.

**Ac-SM Shifts the GABA Concentration-Response Curves and also Enhances Saturating GABA Concentrations.** To gain further insight into the mechanism of $I_{\text{GABA}}$ modulation by Ac-SM, we compared GABA concentration-response curves in the presence and absence of Ac-SM on $\alpha_1\beta_3$ channels. Ac-SM was applied at a saturating concentration of 300 $\mu M$. At this concentration, Ac-SM shifted the concentration-effect curve toward significantly lower GABA concentrations ($EC_{50}(\text{GABA}) = 3.7 \pm 0.4 \mu M$ versus $EC_{50}(\text{GABA}) = 1.2 \pm 0.2 \mu M$, $P < 0.01$) and increased the maximal GABA response by 44% (n = 3; see Fig. 3). Ac-SM at 300 $\mu M$ induced only small currents in the absence of GABA (not exceeding 1% of maximal $I_{\text{GABA}}$ induced by 1 mM GABA), indicating modulatory effects (see also Cicek et al., 2010).

**Ac-SM Dose-Dependently Reduces Locomotor Activity.** To get first insight into the in vivo effects of Ac-SM, the explorative behavior of male C57BL/6N mice was studied 30 minutes after intraperitoneal application in the open field test (OF). As illustrated in Fig. 4A, no changes in total ambulation were observed upon application of Ac-SM at doses $\leq 2$ mg/kg. Reduced ambulation compared with solvent-treated controls started at doses $\geq 6$ mg/kg reaching a maximum at a dose of 60 mg/kg by reducing the total distance by more than 60% (control: 38.0 $\pm 1.2$ m versus Ac-SM 60 mg/kg: 14.7 $\pm 3.1$ m; $F(6,34) = 19.597$, $P < 0.01$). Time spent in the center of the OF did not significantly differ between the compound-treated (doses $\geq 20$ mg/kg) and control groups. Only mice treated with Ac-SM at a dose of 60 mg/kg spent significantly more time in the center area of the OF (control: 57.7 $\pm 6.1$ seconds versus Ac-SM 60 mg/kg: 212.5 $\pm 101.6$ seconds; $F(6,34) = 2.815$, $P < 0.05$; see Fig. 4B), reflecting primarily the strong suppression of motor activity. The number of entries into the center area mostly paralleled the time spent in this compartment. However, mice treated with 0.2 mg/kg Ac-SM tended to visit the center area more frequently compared with the control group (control: 28.2 $\pm 2.1$ versus Ac-SM 0.2 mg/kg: 33.1 $\pm 4.1$). In addition, in line with reduced ambulation, the number of center entries significantly dropped at a dose of 60 mg/kg (control: 28.2 $\pm 2.1$ versus Ac-SM 60 mg/kg: 5.9 $\pm 1.6$; $F(6,34) = 8.828$, $P < 0.01$; see Fig. 4C). Control mice and Ac-SM–treated mice covered a comparable percentage of total

Fig. 5. Ac-SM reduces locomotor activity for 12 hours without rebound effect. Activity (given as arbitrary activity counts) in the home cage of control (black circles) and Ac-SM–treated (20 mg/kg; red circles) mice is compared. The experiment was started at 5:00 PM (time point 0), lasting for 72 hours. Light-on phases of the light-dark cycle are highlighted as yellow bars on the x-axis. Each data point represents a mean $\pm$ S.E.M. from eight mice.
distances in the center area (see Fig. 4D). Doses higher than 60 mg/kg were not tested due to limited solubility of the compound.

To investigate the duration of in vivo Ac-SM effects, we investigated locomotor activity of mice treated with a dose inducing marked reduction of ambulation. Home cage activity analysis (Fig. 5) revealed a long-lasting reduction of motor activity at a dose of 20 mg/kg for at least 6–12 hours. No rebound effect on the subsequent time periods was measured [two-way ANOVA, drug effect: $F_{1,670} = 16.81, P < 0.0001$; time effect: $F_{5,670} = 5.77, P < 0.0001$].

**Ac-SM Paradigm Dependently Reduces Anxiety-Related Behavior and Stress-Induced Hyperthermia.** Ac-SM slightly reduced motor activity at doses >2 mg/kg in the OF compared with vehicle (see Fig. 4A). Thus, to assess changes in anxiety-related behavior, the effect of lower doses was analyzed in the elevated plus maze test: as illustrated in Fig. 6A, Ac-SM–treated mice (0.6 mg/kg) visited the OAs of the EPM more frequently [control: 15.0 ± 1.6% of total entries versus Ac-SM 0.6 mg/kg: 28.3 ± 4.5% of total entries; $F_{2,37} = 4.667, P < 0.05$]. In addition, a tendency toward more time spent (Fig. 6B) and longer distances covered on the OA compared with control animals was observed (Fig. 6C); these effects were, however, not statistically significant. Total motor activity was not affected at these doses (data not shown).

Studying potential anxiolytic effects at higher doses was, however, hampered by the concomitantly occurring reduction of motor activity that might easily mask anxiolytic effects, as the behavioral tests (OF and EPM) rely on exploratory behavior. Therefore, we included a locomotion-independent test of a physiologic stress response. Activation of the autonomic nervous system in response to stress, such as exposure to noise, heat, or pain, in animals can be measured by changes in body temperature—a process referred to as stress-induced hyperthermia (SIH). SIH is a short-lasting body temperature elevation ($\Delta T$) that can be reduced or even ablated by anxiolytic drugs (Vinkers et al., 2008). Basal body temperature measured before compound application did not differ between control and compound-treated mice (data not shown).

As shown in Fig. 7, body temperature upon stress exposure (i.e., insertion of the temperature probe) was raised in salinetreated control mice by 1.2 ± 0.1°C. Three hours after application, Ac-SM at doses ≥0.6 mg/kg significantly reduced $\Delta T$ in a dose-dependent manner [0.6 mg/kg: $\Delta T = 0.6 ± 0.1°C; F_{4,41} = 15.530, P < 0.01$]. The maximal effect was reached at a dose of 6 mg/kg [$\Delta T = 0.1 ± 0.1°C; F_{4,41} = 15.350, P < 0.01$], where SIH was not traceable any more.

**Ac-SM Increases the Seizure Threshold upon Pentylentetrazole Infusion.** A common feature of most positive allosteric modulators of GABA$_A$ receptors is an increased threshold against pentylentetrazole-induced seizures (De Deyn and Macdonald, 1989; Rudolph et al., 1999; Gerak and France, 2011; Löscher et al., 2013). Indeed, Ac-SM significantly increased the seizure threshold at doses ≥20 mg/kg [control: 40.4 ± 3.5 mg/kg PTZ versus Ac-SM 20 mg/kg: 58.5 ± 1.7 mg/kg PTZ; $F_{6,23} = 19.903, P < 0.001$; see Fig. 8].

**Discussion**

Black cohosh (A. racemosa L.) preparations are widely used, mainly to relieve menopausal symptoms, although their efficacy is still controversially discussed (Borrelli and Ernst, 2008; Rees, 2009; Sassarini and Lumsden, 2010; Bedell et al., 2014; Wuttke et al., 2014). Despite extensive studies on the plant’s potential active principles, its mechanism of action is still not entirely understood (Palacio et al., 2009; Bedell et al., 2014; Wuttke et al., 2014). Understanding its molecular mechanism(s) of action is, however, a conditio sine qua non for considering potential beneficial and unwanted side effects. The initially suggested estrogenic activity of lipophilic components, such as triterpenoids, was disproved by later studies (Gaube et al., 2007; Mercado-Feliciano et al., 2012; Hajirahimkhan et al., 2013). There, however, is evidence that A. racemosa L.
preparations interact with distinct neurotransmitter systems in the central nervous system, including \( \mu \)-opioid receptors (Rhyu et al., 2006; Reame et al., 2008), serotonin (5-HT) receptors (subtypes 5-HT1A, 5-HT1D, and 5-HT7) (Burdette et al., 2003), and dopamine subtype 2 receptors (Jarry et al., 2003). We have previously shown that \textit{A. racemosa} \textit{L.} extracts efficaciously modulate GABA\textsubscript{A} receptors and identified four structurally related cycloartane glycosides in \textit{A. racemosa} \textit{L.} extracts as a novel class of GABA\textsubscript{A} receptor ligands (Cicek et al., 2010). Although the potency of these compounds was apparently rather low, we observed a remarkably high efficacy of \( I_{\text{GABA}} \) enhancement compared with established GABA\textsubscript{A} receptor modulators, such as benzodiazepines (e.g., maximal \( I_{\text{GABA}} \) enhancement by the highly potent benzodiazepine site ligand triazolam \( E_{\text{max}(1/2g2S)} = 253 \pm 12\% \) (data taken from Khom et al., 2006) versus \( I_{\text{GABA}} \) enhancement by Ac-SM \( E_{\text{max}(1/2g2S)} = 1187 \pm 166\% \) in this study (Sigel and Baur, 1988)). Here, we analyzed potential subunit-selective effects of the most efficacious compound, Ac-SM. Ac-SM positively allosterically modulated \( I_{\text{GABA}} \) through nine major GABA\textsubscript{A} receptor subtypes with equal potency, but slightly different efficacy (Fig. 2, A–C). Enhancement of \( I_{\text{GABA}} \) by Ac-SM was only slightly influenced by the \( \alpha \)-subunit isoform incorporated into the channel, but not by the \( \beta \)-subunit isoform, and did not depend on the presence of a \( \gamma_{2S} \)-subunit. Based on these observations, we exclude an interaction of Ac-SM with the benzodiazepine binding site (Sigel and Baur, 1997; Rudolph et al., 1999; Olsen and Sieghart, 2009; Richter et al., 2012; Sigel and Steinmann, 2012). In addition, as Ac-SM efficaciously modulates a broad variety of GABA\textsubscript{A} receptor subtypes/does not discriminate between various GABA\textsubscript{A} receptor subtypes, its profile is apparently similar to GABA\textsubscript{A} receptor modulators such as barbiturates or neurosteroids. However, whether Ac-SM also shares a common binding site with these compounds, or whether Ac-SM interacts with a novel, yet undefined binding site on GABA\textsubscript{A} receptors remains to be exploited in future studies.

Our in vivo experiments revealed a wide spectrum of effects induced by low doses of Ac-SM, which fit the profile of nonsubtype-selective GABA\textsubscript{A} agonists or positive allosteric modulators (Rudolph et al., 1999; Sieghart, 2000; Olsen and Sieghart, 2009; Möhler, 2011; Sigel and Steinmann, 2012). These effects include reduction of motor activity paralleled by reduced stress-induced hyperthermia. Due to the strong motoric inhibition at higher doses (Fig. 4A), suggesting pronounced sedative effects, potential anxiolytic effects of Ac-SM (Fig. 6) are difficult to assess; however, increased exploration of the open arms of the elevated plus maze was observed at low doses not affecting motor activity. The motor activity–independent SIH test revealed a marked dose-dependent reduction of stress response (Fig. 7), supporting the assumption of anxiolytic effects induced by Ac-SM (Olivier et al., 2003). Furthermore, we observed a dose-dependent increase in PTZ-induced seizure threshold (Fig. 8). The long-lasting reduction of activity in the home cage suggests a long biologic half-life of Ac-SM (Fig. 5).

Taken together, our data demonstrate that the recently identified novel GABA\textsubscript{A} receptor modulator Ac-SM enhances \( I_{\text{GABA}} \) through nine receptor subtypes with similar potency but slightly different efficacies (\( \alpha_{1/2g5d} \beta_{1/2g2S} \sim \alpha_{2g} \beta_{2g} \)). Due to the positive GABA\textsubscript{A} allosteric modulatory effects of Ac-SM (and potentially other cycloartane glycosides; Cicek et al., 2010), it is tempting to speculate that this GABA\textsubscript{A} receptor modulation leading to sedation and muscle relaxation contributes to the alleviation of mild to moderate symptoms in menopause reported in clinical studies with preparations from \textit{A. racemosa} \textit{L.}, especially when hot flushes are associated with sleep and mood disturbances (Wuttke et al., 2003; Frei-Kleiner et al., 2005; Osners et al., 2005; Verhoeven et al., 2005; Newton et al., 2006; Pockaj et al., 2006; Uebelhack et al., 2006; Cheema et al., 2007).

\textit{A. racemosa} \textit{L.} preparations are currently standardized for their 23-epi-26-deoxyacteine content (Pepping, 1999), one of the five main Actaea triterpenoids (actein, 23-epi-26-deoxyacteine, cimigenol \( 3-O-\beta-D\)-xylopyranoside, cimiracemoside C, and cimiracemoside F). Significant, although varying, amounts of
Ac-SM were recently identified in A. racemosa L. plant material (ranging from 0.0137 ± 1.65% to 0.0092 ± 1.97%) as well as in four Actaea preparations (ranging from 0.004 ± 6.42% to 0.0184 ± 1.92%) that are commercially available in Austria (Ciecke et al., 2011). Whether these estimated amounts of pure Ac-SM are also sufficient to induce anxiolytic, stress-reducing, anticonvulsant, and sedative effects in humans remains, however, to be clarified. Due to the efficacy of Ac-SM and its long biologic half-life (Fig. 5), cumulative sedative side effects of repetitive intake of A. racemosa L. preparations might not be negligible. In addition, Actaea preparations are consumed in most cases without prescription. As Ac-SM as well as other hitherto unknown components/metabolites of A. racemosa L. preparations interact among others with various targets in the central nervous system, potential harmful interactions with other psychoactive drugs—in particular, central nervous system–depressant drugs—should be also carefully considered.

Authorship Contributions

Participated in research design: Khom, Schwarzer, Hering.
Conducted experiments: Strommer, Khom, Kastenberger, Schwarzer.
Contributed new reagents or analytic tools: Ciecke, Stuppner.
Performed data analysis: Strommer, Khom, Kastenberger, Schwarzer.
Wrote or contributed to the writing of the manuscript: Khom, Schwarzer, Hering.

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