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Identification of GABA A receptor modulators in *Kadsura longipedunculata* and assignment of absolute configurations by quantum-chemical ECD calculations

Janine Zaugg^a, Samad Nejad Ebrahimi^{a,b}, Martin Smiesko^c, Igor Baburin^d, Steffen Hering^d, and Matthias Hamburger^{a,*}

^aDivision of Pharmaceutical Biology, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland ^bDepartement of Phytochemistry, Medicinal Plant and Drugs Research Institute, Shahid Beheshti University, G.C., Tehran, Iran ^cDivision of Molecular Modeling, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland ^dInstitute of Pharmacology and Toxicology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

Abstract

A petroleum ether extract of *Kadsura longipedunculata* enhanced the GABA-induced chloride current (I_{GABA}) by $122.5 \pm 0.3\%$ ($n = 2$) when tested at $100 \mu\text{g/ml}$ in *Xenopus laevis* oocytes expressing GABA A receptors ($\alpha_1\beta_2\gamma_{2S}$ subtype) in two-microelectrode voltage clamp measurements. Thirteen compounds were subsequently identified by HPLC-based activity profiling as responsible for GABA A receptor activity and purified in preparative scale. 6-Cinnamoyl-6,7-dihydro-7-myrceneol and 5,6-dihydrocuparenic acid were thereby isolated for the first time. The determination of the absolute stereochemistry of these compounds was achieved by comparison of experimental and calculated ECD spectra. All but one of the 13 isolated compounds from *K. longipedunculata* potentiated I_{GABA} through GABA A receptors composed of $\alpha_1\beta_2\gamma_{2S}$ subunits in a concentration-dependent manner. Potencies ranged from 12.8 ± 3.1 to $135.6 \pm 85.7 \mu\text{M}$, and efficiencies ranged from $129.7 \pm 36.8\%$ to $885.8 \pm 291.2\%$. The phytochemical profiles of petroleum ether extracts of *Kadsura japonica* fruits ($114.1 \pm 2.6\%$ potentiation of I_{GABA} at $100 \mu\text{g/ml}$, $n = 2$), and *Schisandra chinensis* fruits (inactive at $100 \mu\text{g/ml}$) were compared by HPLC-PDA-ESIMS with that of *K. longipedunculata*.

Keywords

Kadsura longipedunculata; Schisandraceae; HPLC-based activity profiling; GABA A receptor; Lignans; Terpenoids; Circular dichroism; Quantum-chemical calculations

1. Introduction

Gamma-aminobutyric acid type A (GABA A) receptors are hetero-pentameric chloride ion channels and are targets for the major inhibitory neurotransmitter of the central nervous system, gamma-aminobutyric acid (GABA). Chloride channel opening of the GABA A receptor leads to a hyperpolarization of the neuronal membrane and inhibition of further

action potentials. To date, 11 GABA A receptor subtypes known to exist in the human brain differ in function and tissue localization, the most prominent being the $\alpha_1\beta_2\gamma_2\delta$ subtype (Olsen and Sieghart, 2008). Numerous sedative-anxiolytics and sedative-hypnotics, such as benzodiazepines and *z*-compounds (zolpidem, zaleplon etc.), and some general anesthetics and antiepileptics, bind to GABA A receptors. However, side-effects of these drugs due to lack of GABA A receptor subtype selectivity limit their clinical use. Thus, there is a continued medical need for GABA A receptor modulators with new structural scaffolds and potential subtype selectivity (Mohler, 2011).

We recently screened a library of fungal and plant extracts in an automated functional two-microelectrode voltage clamp assay on *Xenopus* oocytes (Baburin et al., 2006) which transiently expressed GABA A receptors of the $\alpha_1\beta_2\gamma_2\delta$ subtype. Among the 982 extracts of the library were nine extracts originating from three different species of the Schisandraceae family (*Kadsura longipedunculata*, *Kadsura japonica* and *Schisandra chinensis*). Among these, petroleum ether extracts of *K. longipedunculata* and *K. japonica* showed promising activity.

Kadsura and Schisandra species are scandent, woody vines growing throughout Eastern and Southeastern Asia, with the highest diversity lying in Southern China (Saunders, 1998, 2000). Both genera share highly similar morphologic and anatomic characteristics (Saunders, 1998). In Russia, *S. chinensis* fruits have been used as an adaptogen and stimulant (limonnika kitajskogo nastojka) (Panossian and Wikman, 2008), whereas in traditional Chinese medicine dried fruits of *S. chinensis* (Bei-Wuweizi) and *S. sphenanthera* (Nan-Wuweizi) are officinal drugs to treat respiratory malfunction, spermatorrhoea, enuresis, diarrhea, night sweating, insomnia and hepatitis (Haensel et al., 1993; Hou and Youyu, 2005; Lu and Chen, 2009; Stoeger, 2009). *K. japonica* fruits have more or less the same indications as Schisandra fruits and are used as an unofficial commercial grade of these (Haensel et al., 1993). *K. longipedunculata* is one of the most common species of Kadsura in China. Traditionally the stems and roots are used for medicinal purposes, while the fruits are eaten locally or serve as a source of fragrant oils (Saunders, 1998). Up to now, only roots and stems of *K. longipedunculata* have been investigated phytochemically, but not the fruits. Due to morphological similarity, *Schisandra* fruits may easily be confused with the dried fruits from *Kadsura* species (Fujita, 1929; Haensel et al., 1993; Xiao et al., 2010).

In this study, 13 natural products from a petroleum ether extract of *K. longipedunculata* fruits were identified as positive GABA A receptor modulators with the aid of HPLC-based activity profiling. This miniaturized approach enables rapid identification of bioactive constituents in extracts (Potterat and Hamburger, 2006) and has been successfully used in combination with various cell-based and biochemical assays (Adams et al., 2009; Danz et al., 2001; Dittmann et al., 2004; Potterat et al., 2004), including the discovery of new GABA A receptor modulators (Kim et al., 2008; Li et al., 2010; Pei et al., 1980; Yang et al., 2011; Zaugg et al., 2010, 2011a, 2011b, submitted for publication).

2. Results and discussion

2.1. Isolation and structure elucidation

A preformatted library of 982 extracts was tested in an automated, fast perfusion system during two-microelectrode voltage clamp measurements with *Xenopus* oocytes which transiently expressed GABA A receptors of the subunit combination $\alpha_1\beta_2\gamma_2\delta$ (Baburin et al., 2006). At a concentration of 100 $\mu\text{g/ml}$ petroleum ether extracts of *K. longipedunculata* and *K. japonica* fruits enhanced the GABA induced chloride current (I_{GABA}) by $122.5 \pm 0.3\%$ ($n = 2$) and $114.1 \pm 2.6\%$ ($n = 2$), respectively, while ethyl acetate and MeOH extracts

of both drugs were not active (data not shown). Interestingly, the petroleum ether, ethyl acetate and MeOH extracts from the taxonomically related *S. chinensis* fruits showed no activity. Next, we performed HPLC-based activity profiling with the active extract of *K. longipedunculata* using a validated protocol (Kim et al., 2008) to identify the constituents responsible for the activity. The chromatogram (266 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 Fig. 1B and A, respectively. The highest activity was found in fraction 12 which potentiated I_{GABA} by $498.0 \pm 150.4\%$ ($n = 3$). Fractions 4 and 13 also were active, albeit to a lesser degree (potentiation of I_{GABA} by $103.2 \pm 8.2\%$ ($n = 2$) and $160.5 \pm 19.9\%$ ($n = 3$), respectively). Fractions 8 and 14 showed minor activity (potentiation of I_{GABA} by $80.4 \pm 7.2\%$ ($n = 3$) and $70.3 \pm 11.1\%$ ($n = 3$), respectively). Preparative isolation of active compounds was achieved by liquid–liquid partitioning, normal phase open column chromatography and preparative RP-HPLC. Some structurally related but inactive compounds were also purified in view of preliminary structure–activity considerations. Nine known lignans, arisantetralones A–D (**1**–**4**), dihydroguaiaretic acid (**5**), mono-methyl dihydroguaiaretic acid (**8**), deoxyschizandrin (**9**), zuihonin A (**10**), anwulignan (**11**), and saururenin (**13**), and a known sesquiterpene, (+)- γ -cuparene (**6**), were identified by ESI-MS, 1D and 2D NMR spectroscopy, optical rotation, CD, and by comparison with published data (Cheng et al., 2009; da Silva and Lopes, 2004; Ikeya et al., 1980, 1979; Lee, 1981; Miyazawa et al., 1997; Nakatani et al., 1988; Sadhu et al., 2003; Schrecker, 1957; Urzua et al., 1987; Wang et al., 2000, 2006) (Chart 1). Analytical data of the known compounds are given as Supplementary data. In addition, a sesquiterpene structurally closely related to **6** [(–)-5,6-dihydrocuparene (**12**)] and a monoterpene cinnamic acid ester [(+)-6-cinnamoyl-6,7-dihydro-7-myrceneol (**7**)] (Chart 1) were identified as previously unreported natural products. Their structures, including absolute configurations, were established with the aid of HR-ESI-MS, 1 and 2 D NMR spectroscopic experiments, optical rotation, and ECD. Multiplicity-edited HSQC NMR and HMBC NMR unambiguously revealed the covalent structures of compounds **7** and **12** (Chart 1, Tables 1 and 2, for complete NMR spectral datasets see Figures S8–S14 of the Supplementary data). In both cases, NOESY NMR allowed the determination of the conformations of the predominant conformers in DMSO. A transoid diene conformation of the myrcenyl moiety of **7** was confirmed by correlations between H-7 and C6–CH₂ and between H-8a and H-5. Furthermore, the presence of an *E*-cinnamoyl residue was indicated by the NOESY correlation between H-2' and H-5'/H-9'. In the case of **12**, NMR shifts of the cyclopentane moiety closely matched with the data of **6** (Table 1). The mutual orientation of the cyclopentane and the cyclohexadiene units was established by a NOESY correlation between H-5'a (α -orientated) and H-3. The absolute configurations of **6**, **7** and **12** were determined and that of **6** confirmed by comparison of their CD spectra with ECD spectra calculated in the gas phase and in MeOH using time dependent density functional theory (DFT). For this purpose, 3D structures of *R*-**6** (**e6**), *S*-**7** (**e7**), and *R*-**12** (**e12**) were submitted to a systematic conformational search in H₂O (OPLS 2005), within an energy window of 5 kcal/mol. A total of 275 conformational species were found for **e7**, and 24 each were found for **e6** and **e12**. Conformers which were within an energy range of 1 kcal/mol (**e7**: 20) and 2 kcal/mol (**e6**: 16 and **e12**: 7) from the global minima were subject to geometrical optimization (DFT/B3LYP/6-31G**) in the gas-phase combined with calculation of vibrational modes to confirm these minima. No imaginary frequencies were found. For **e6**, **e7**, and **e12**, a total of 12, 7, and 17 species, respectively, could be confirmed (Figs. 2A, Fig. 3A, and Fig. 4A). For these conformations, ECD spectra were calculated (TDDFT/B3LYP/6-31G**) in the gas phase and in MeOH (SCRF/CPCM). Each calculated ECD spectrum was assigned a Boltzmann weight according to the energy of the minimized conformers at 298.15 K and overlaid prior to comparison with the particular experimental ECD spectrum (Figs. 2B, Figs. 3B, and Figs. 4B). The positive and negative CE of *S*-**e7** at

around 300 nm and 250 nm, respectively, appeared inverted in comparison with the experimental ECD spectrum of **7**. Mirror-images of the computed ECD spectra in the gas-phase and MeOH were in agreement with the experimental data. The mirror-image of the ECD calculation in MeOH closely matched the experimental spectrum, especially with respect to Cotton effects (CE) at lower wavelengths. In **7**, differences between calculated and experimental spectra presumably resulted from an overestimation of the UV absorbance in the calculations (Tayone et al., 2011), or may be due to minor differences between calculated and solution conformers (Kamel et al., 2009) of this flexible molecule. The predominant conformers of **e7** (7 species, 83%) had a cisoid enone conformation, while the remaining ones (10 species, 17%) showed a transoid enone conformation. Among these two major conformational groups, individual conformers further varied by bond rotations around C5-C6 and C2-C3 (Fig. 3). Boltzmann-weighted calculated ECD spectra of each conformer in the gas phase and in MeOH are given as Supplementary data (Fig. S3 and S4). Thus, compound **7** was identified as *R*-(+)-6-cinnamoyl-6,7-dihydro-7-myrceneol.

In a conformational search, compound **e6** occurred in two major core conformations A (66% contribution) and B (34% contribution) differing in the orientation of the substituted cyclopentane ring. Further variations arose by bond rotation around C1-C7 and rotation of the hydroxy group itself (Fig. 2A). The calculations of the **e6**-ECD spectra closely matched the experimental spectrum of **6**. The positive CE at around 220 nm was likely due to a $\pi \rightarrow \pi^*$ transition of the aromatic ring system. In agreement with the calculated ECD spectra it indicated an *R*-configuration of the stereocenter at C-1' (Fig. 2B), and compound **6** was thus identified as *R*-(+)- γ -cuparenol (Ito et al., 1965; Nayek et al., 2003). Conformational analysis and geometrical optimization of **e12** provided two major cores with positive helicity (P) (2 species; 42% contribution) and negative helicity (M) of the cyclic diene (5 species; 58% contribution), respectively. The latter further differed in the orientation of the cyclopentane residue (Fig. 4A; dihedral angles of the cyclic diene are listed in Table S2 of the Supplementary data). Inspection of the calculated ECD spectra of each conformer revealed that the change in helicity of the cyclic diene inverted the sign of the CE. P-helicity and M-helicity corresponded to a negative and a positive CE at 302 nm, respectively. Other conformational differences had no pronounced influence on the spectra (Fig. S5). Despite the apparent biosynthetic relation to **6**, calculation of ECD with *R*-**e12** did not match the negative CE at 302 nm observed in the experimental spectrum. The close match with the mirror-inverted calculated curve pointed towards an *S*-configuration of **12**. However, small conformational changes in **e12** strongly affected the sign of the calculated ECD spectra. Hence we feel that independent evidence is needed to unambiguously identify the absolute configuration of **12**.

2.2. Modulation of GABA A receptors

Modulation of GABA A receptors by compounds **1–13** (0.1–500 μ M) was assessed in an automated two-microelectrode voltage clamp assay on *Xenopus* oocytes transiently expressing $\alpha_1\beta_2\gamma_{2S}$ receptors. All compounds except the dibenzocyclooctadiene lignan (**9**) enhanced I_{GABA} at a GABA EC_{5-10} in a concentration-dependent manner (see Fig. 5 and Table 3 for details). The potencies (EC_{50} values) of the four arisantetralone lignans (**1–4**) did not significantly differ from each other ($p > 0.05$). In contrast, the structural differences between **2**, and **3** and **4** significantly affected efficiencies in stimulation of I_{GABA} at a GABA EC_{5-10} ($p < 0.05$). Compound **2** had the highest efficiency of I_{GABA} modulation ($885.8 \pm 291.2\%$, $n = 4$, Table 3). Interchange of substituents at C-7 and C-6, as in **1**, did not significantly alter the efficiencies of I_{GABA} modulation (**2** $\approx$ **1**, $p > 0.05$). Replacing the hydroxy group at C-4' of the benzylic moiety by a methoxy group, as in **3**, significantly lowered the activity ($p < 0.05$). Stereochemistry at C-2 had no influence on the efficiencies (**2** $>$ **3** $\approx$ **4**, $p < 0.05$). However, the inversion of absolute configuration at C-2 apparently

affected agonistic and antagonistic potential at $\alpha_1\beta_2\gamma_2\delta$ GABA A receptors: similar to **5**, **7**, **8**, and **12**, compounds **1–3** produced small chloride currents in the absence of GABA, as observed in preincubation experiments (Fig. 5 D–F). This partial agonistic activity did not exceed 10% of the maximal current induced by a saturating GABA concentration (1 mM).

The conformationally more flexible acyclic lignans **5**, **8**, **11**, and **13** showed equal to slightly higher potencies than the arisantetralones **1–4** at GABA A receptors of $\alpha_1\beta_2\gamma_2\delta$ subunit composition. Whereas the EC_{50} values of the investigated lignans were not much affected by replacement of the free hydroxy moiety by a methoxy group at C-4' (EC_{50} : **1** $\approx$ **3**; **5** $\approx$ **8**; **11** $\approx$ **13**, $p > 0.05$), this structural change decreased efficiency of acyclic lignans (**8** $<$ **5**; **13** $<$ **11**, $p < 0.05$; **3** $\approx$ **1**, $p > 0.05$, see Table 3). A dioxomethylene moiety at C-3 and C-4 significantly decreased potency and efficiency in the cases of **5** and **11**, but not of **8** and **13** (EC_{50} : **5** $>$ **11**, $p = 0.06$; efficiency **5** $>$ **11**, $p < 0.05$; EC_{50} , efficiency: **8** $\approx$ **13**) (Table 3). However, it visibly influenced the mode of action at the GABA A receptor. Interestingly, compounds **5** and **8** exerted partial agonistic activity, but not the dioxomethylene derivatives **11** and **13** (evident from the absence of chloride currents during the preincubation period in absence of GABA). Different kinetics of the I_{GABA} may be related structural differences among the studied compounds. Furthermore, near plateau-like currents induced by **11** and **13** are in contrast to the fast decaying I_{GABA} induced by **5** and **8** (similar to **4**, **6**, and **7**), reflecting either pronounced desensitization of GABA A receptors or an open channel block. Zuihonin A (**10**) and deoxyschizandrin (**9**) are structurally too different from the other lignans for structure–activity considerations. Compound **10**, however, can be grouped with anwulignan (**11**) and saururenin (**13**) considering potency, efficiency, and also with respect to the slowly decaying I_{GABA} induced by this compound. The cuparene sesquiterpenes **6** and **12** showed little to no agonistic activity at GABA A receptors of $\alpha_1\beta_2\gamma_2\delta$ subunit composition, but reasonably potent modulation of I_{GABA} with comparable potency and efficiency. The monoterpene cinnamic acid ester (**7**) showed partial agonistic activity as lignans **1–3**, and potent maximal I_{GABA} stimulation ($834.6 \pm 77.5\%$) (Fig. 5, Table 3; Chart 1).

2.3. Phytochemical differences between the genera *Kadsura* and *Schisandra*

Kadsura and *Schisandra* are closely related genera which share a similar phytochemical profile (Haensel et al., 1993; Saunders, 1998). Therefore, *Kadsura* fruits were often used as a substitute for *Schisandra* (Haensel et al., 1993; Ookawa et al., 1981; Saunders, 1998). In the initial extract screening only the *Kadsura* species exerted GABA A receptor modulatory activity. We compared the phytochemical profiles of the petroleum ether extracts of *S. chinensis* and both *Kadsura* species by HPLC-PDA-ESIMS. The HPLC-based activity profiling of *K. longipedunculata* led to the identification of constituents (**1–8** and **10–13**) which were responsible, to varying degrees, for the GABA A receptor modulatory activity of the crude extract. Most of these compounds could also be detected in the *K. japonica* extract, which had previously shown comparable activity in the oocyte assay as *K. longipedunculata* (Fig. 6). In contrast, the HPLC profile of the inactive petroleum ether extract of *S. chinensis* strongly differed from those of the *Kadsura* extracts. Only traces of some of the active compounds could be detected by comparison of UV and mass spectra (Fig. 6).

The lignan content of *Schisandra sp.* fruits varies depending on geographical origin and time of harvest (Lu and Chen, 2009; Tang and Eisenbrand, 2011; Zhu et al., 2007), and constituents such as (+)-anwulignan (**11**) or meso-dihydroguaiaretic acid (**5**), which we identified as GABA A receptor active, have already been isolated from this source, however not as major compounds (Lu and Chen, 2009; Wei et al., 2010). Nonetheless, our results suggest that, despite a rather similar phytochemical profile of *Kadsura* and *Schisandra*

species, their specific lignan patterns are decisive for the GABA A receptor activities of the extracts. More samples should be tested to draw a more general conclusion on this issue. Interestingly, deoxyschisandrin (**9**), one of the extensively studied pharmacologically-active dibenzocyclooctadiene lignans (Hancke et al., 1999; Opletal et al., 2004) and a major compound of *S. sphenanthera* (Lu and Chen, 2009), does not affect GABA A receptors of the $\alpha_1\beta_2\gamma_{2S}$ composition. It is possible that a sedative action of *S. sphenanthera* in rodents is not due to GABA A receptor activity, but due to other mechanisms as Huang et al. conjectured (Huang et al., 2007). In contrast, the GABA A receptor modulating lignans of *Kadsura* species indeed reach a concentration sufficient for GABA A receptor activity of the crude extract. Animal behavioral experiments and pharmacokinetic studies with extracts and pure compounds are needed to assess the GABA A receptor related activity *in vivo*.

2.4. Conclusions

Compared to other natural products previously isolated and tested for GABA A receptor activity (Li et al., 2010; Yang et al., 2011; Zaugg et al., 2010, 2011a), the lignans of *K. longipedunculata* showed medium to high potencies and moderate efficiencies (except for **2**, **4**, and **5**). Apart from neolignans honokiol and magnolol (Ai et al., 2001), this is the first time that lignans are described as GABA A receptor modulators. With exception of **11** and **13**¹, all lignans possessed physico-chemical properties favorable for oral bioavailability and blood–brain-barrier penetration (H-acceptors 3, H-donors 3, MW 450, cLogP: 5, number of rotatable bonds 8, polar surface area (PSA) 60–70 (–90) Å²) (Pajouhesh and Lenz, 2005). Arisantetralones A–C (**1**–**3**) showed an interesting mode of action, combining partial agonistic activity, positive modulation, and possibly an open channel block at high concentrations, which warrants a more detailed investigation of their binding site(s), and potential GABA A receptor subtype selectivity of this compound class.

Interestingly, two different compound classes, lignans and isoprenoids, contribute to the GABA A receptor modulating activity of the *K. longipedunculata* extract. Among the 3 active terpenoids **6**, **7** and **12**, two were newly-described natural products. Their absolute configurations were determined by comparison of experimental and calculated ECD. ECD calculations have been increasingly used for the interpretation of CD spectra (Bringmann et al., 2009). However, the example of **12** shows the current limitations of this approach and highlights the sensitivity of circular dichroism to conformational changes. Therefore, proper conformational analysis and geometrical optimizations of the conformers prior to calculations of energy transitions is essential.

3. Experimental

3.1. General experimental procedures

Optical rotation was measured on a Perkin Elmer polarimeter (model 341) equipped with a 10 cm microcell. The optical rotation for the sodium D line (589 nm) was extrapolated from the lines of a mercury lamp using the Drude equation (Fluegge, 1970). CD spectra of compounds **1**–**4** were recorded in MeOH (50 µg/ml) on a Chirascan CD spectrometer and analyzed with the Pro-Data V2.4 software. CD spectra of other compounds were measured on an AVIV CD Spectrometer Model 62ADS and analyzed with the AVIV 60DS V4.1 software. NMR spectra were recorded at target temperature of 18 °C on a Bruker Avance III 500 MHz spectrometer operating at 500.13 MHz for ¹H and 125.77 MHz for ¹³C. A 1 mm TXI-microprobe with a z-gradient was used for ¹H-detected experiments; ¹³C-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-ESI-TOF-MS) in

¹Compounds **11** and **13** have a cLogP of 5.59. The other criteria are fulfilled.

positive mode were obtained on a Bruker micrOTOF ESI-MS system connected via T-splitter (1:10) to an Agilent HP 1100 series system consisting of a binary pump, autosampler, column oven and diode array detector (G1315B) with settings as previously reported (Zaugg et al., 2010). Data acquisition and processing was performed using Bruker HyStar 3.0 software. Semi-preparative HPLC separation for activity profiling and compound isolation was performed on an Alliance 2690 Separation module connected to a 996 photodiode array detector. Data acquisition and processing was performed using Waters Empower Pro Software. Waters SunFire C18 (3.5 μm , 3.0×150 mm) and SunFire Prep C18 (5 μm , 10×150 mm) columns were used for analytical HPLC and semipreparative RP-HPLC, respectively. Semipreparative NP-HPLC was performed on a Merck LiChroSorb 100 Diol (10 μm , 10×250 mm) column. Medium pressure liquid chromatography (MPLC) was done on a pre-packed normal phase cartridge (40–63 μm , 40×150 mm) using a Buchi Sepacore system consisting of a control unit C-620, two pump modules C-605, and a fraction collector C-660. The MPLC unit was controlled with the SepacoreControl software (version 1.0.3000.1). Preparative HPLC separation was performed with a Waters SunFire Prep C18 OBD (5 μm , 30×150 mm) column on a Shimadzu LC-8A preparative separation chromatograph equipped with a SPD-M10A VP diode array detector. HPLC-grade MeOH (Scharlau Chemie S.A.) and H₂O were used for HPLC separations. HPLC solvents contained 0.1% HCO₂H for analytical separations. CDCl₃ (100 Atom% D, stab. with Ag) and DMSO-*d*₆ (100 Atom%) for NMR was purchased from Armar Chemicals. 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.

3.2. Plant material

Dried fruits of *K. longipedunculata* Finet & Gagnep., *K. japonica* (L.) Dunal, and *S. chinensis* (Turczaninow) Baillon were purchased by Mr. Jinfu Wan, Yunnan Baiyao group Co. Ltd., Kunming, China, at the Juhuayan herbal market in Kunming, Yunnan, China, and identified at the labs of Yunnan Baiyao group. Voucher specimens (00 450, 00 440, and 00 451, respectively) are deposited at the Institute of Pharmaceutical Biology, University of Basel. The organoleptic, macroscopic, and microscopic characteristics of apocarps and seeds were confirmed by Janine Zaugg by comparison with lit. (Fujita, 1929; Saunders, 1998, 2000). A detailed description of the plant material is provided as Supplementary data.

3.3. Extraction

The plant material was frozen with liquid N₂ and ground with a Retsch ZM1 ultracentrifugal mill. The extract for the screening and HPLC-based activity profiling was prepared with a Dionex ASE 200 extraction system with solvent module by extraction with pet-ether, b.p. 40–60°, redistilled. In total, 3 extraction cycles were performed at an extraction pressure of 120 bar and a temperature of 70 °C. For compound isolation 1.08 kg of ground fruits were macerated at room temperature with *n*-hexane (4 $\times$ 3.2 l, 1 h each). The solvent was evaporated at reduced pressure to yield 109.7 g of extract. The extracts were stored at –20 °C until use.

3.4. HPLC microfractionation

Microfractionation for GABA A receptor activity profiling was performed as previously described (Kim et al., 2008), with minor modifications: separation was carried out on a semipreparative RP-HPLC column with MeOH (solvent A) and H₂O (solvent B) using the following gradient: 65% A–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) were injected. A total of 28 time-based microfractions of each 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 N₂ gas, and submitted to bioassay.

3.5. Isolation

A portion of the extract (51.4 g) was partitioned by liquid–liquid extraction with *n*-hexane and MeOH:H₂O [9:1] to yield a lipophilic fraction (36.0 g) and a polar fraction (14.8 g). The latter was separated by open column chromatography (9 × 42 cm, 1.0 kg silica gel) using a step gradient of *n*-hexane–EtOAc–MeOH (100:0:0, 2 l; 95:5:0, 2 l; 90:10:0, 2 l; 80:20:0, 2 l; 70:30:0, 2 l; 60:40:0, 2 l; 50:50:0, 1 l; 40:60:0, 1 l; 30:70:0, 1 l; 20:80:0, 1 l; 10:90:0, 1 l; 0:100:0, 2 l; 0:50:50, 1 l; 0:0:100, 1 l). The flow rate was ca. 50 ml/min. The effluent was combined to 23 fractions (1–23) according to TLC patterns (detection at 254 nm, 366 nm and at daylight after staining with anisaldehyde–sulphuric acid reagent). Crystallization of fraction 9 (1.68 g) yielded 692 mg of the major compound anwulignan (**11**). The mother liquor of fraction 9 as well as other fractions used for compound isolation were diluted in MeOH (125–500 mg/ml) and separated by preparative HPLC with different gradients of MeOH and H₂O and a flow rate of 20 ml/min as follows: 50 mg of fraction 7 (102 mg), gradient of 70:30 to 85:15 in 14 min, yielded (+)-Zuihonin A (**10**) (10 mg); 200 mg of the mother liquor of fraction 9, gradient of 80:20 to 100:0 in 17 min yielded more anwulignan (**11**) (111 mg) and saurenin (**13**) (19.5 mg), 100 mg of fraction 11 (474 mg), gradient of 73:27–90:10 in 22 min, yielded 8 mg of a cinnamic acid ester of 6,7-dihydro-6,7-myrcenediol (Bohlmann et al., 1983), 6-cinnamoyl-6,7-dihydro-7-myrceneol (**7**) and the dibenzocyclooctadiene lignan deoxyschizandrin (**9**) (1.3 mg); 60 mg of fraction 12 (322 mg), gradient of 73:27–90:10 in 22 min, yielded *meso*-dihydroguaiaretic acid (**5**) (17 mg) and *meso*-mono-methyldihydroguaiaretic acid (**8**) (1 mg); 100 mg of fraction 14 (770 mg), gradient of 65:35–70:30 in 5 min, yielded arisantetralone A(**1**) (2 mg), B (**2**) (1 mg), C (**3**) (29 mg), and D (**4**) (1 mg) (Cheng et al., 2009; da Silva and Lopes, 2004; Liu et al., 1988).

An aliquot (980 mg) of fraction 10 (5.95 g) was separated by MPLC using a CH₂Cl₂:THF mixture [97:3] and *n*-hexane in a gradient ranging from 10:90 to 90:10 in 2 h, followed by 90:10–100:0 in 10 min, hold for 30 min. The flow rate was set at 15 ml/min and 12 fractions (A–L) were collected based on TLC analysis. Fractions 10B (76 mg), 10C (28 mg), and 10G (61 mg) were dissolved in *n*-hexane containing 4% *iso*-PrOH (100 mg/ml) and separated by semipreparative NP-HPLC using *n*-hexane and *iso*-PrOH (0.01% HCO₂H) as mobile phase. Isocratic conditions (10B, 10C: [98:2]; 10G: [99.8:0.2]) were used; the flow rate was 5 ml/min. Manual peak collection yielded anwulignan (**12**) (21 mg of fraction 10B and 10 mg of fraction 10C) and γ -cuparenol (**7**) (4.5 mg) of fraction 10G. A part (960 mg) of fraction 15 (1.52 g) was separated by MPLC using a gradient of dichloromethane and MeOH (100:0–90:10 in 2 h, followed by 90:10–0:100 in 30 min, flow rate 15 ml/min). Five fractions (A–E) were collected based on TLC pattern. Repeated injections of fraction 15B (55 mg) onto semipreparative NP-HPLC using *n*-hexane and *iso*-PrOH (0.01% HCO₂H) in an isocratic mode [99.5:0.5] and a flow rate of 5 ml/min yielded the 5,6-dihydro derivative of cuparenic acid (Enzell and Erdtman, 1958)(**13**) (4 mg).

3.6. Spectroscopic data of new compounds

3.6.1. R-(+)- γ -cuparenol (Ito et al., 1965; Nayek et al., 2003)(6)—White amorphous substance; $[\alpha]_D^{20}$: +20.7° (MeOH; *c* 0.38); UV: $\lambda_{\max}$ (nm) 222, 263 sh; CD (MeOH, *c* = 1.8 × 10^{−3} M, 0.1 cm path-length): $[\theta]_{200}$ + 2 667, $[\theta]_{209}$ + 1 009, $[\theta]_{220}$ + 2 127; ESIMS *m/z* 219 [M + H]⁺. For ¹H and ¹³C NMR data see Table 1.

3.6.2. R-(+)-6-cinnamoyl-6,7-dihydro-7-myrceneol (7)—Colorless oil; $[\alpha]_D^{20}$: +15.2° (CHCl₃; *c* 0.66); UV: $\lambda_{\max}$ (nm) 218, 222, 230 sh; 278.5, 230 sh; CD (MeOH, *c* = 6.6 ×

10^{-4} M, 0.1 cm path-length): $[\theta]_{205} + 13\,380$, $[\theta]_{216} + 2\,207$, $[\theta]_{229} + 6\,677$, $[\theta]_{239} 0$, $[\theta]_{253} - 3\,025$, $[\theta]_{282} 0$, $[\theta]_{294} + 1\,059$; HRESIMS m/z 323.1649 (calcd. for $C_{19}H_{24}NaO_3$: 323.1618). For ^1H and ^{13}C NMR data see Table 2.

3.6.3. S-(–)-5,6-Dihydrocuparenic acid (12)—White amorphous substance; $[\alpha]_D^{20}$: -31.3° ($CHCl_3$; c 0.47); UV: λ_{max} (nm) 215, 302; (MeOH, $c = 1.7 \times 10^{-3}$ M, 0.1 cm path-length): $[\theta]_{205} + 1\,938$, $[\theta]_{233} 0$, $[\theta]_{302} - 1\,905$; HRESIMS m/z 257.1519 (calcd. for $C_{15}H_{22}NaO_2$: 257.1512). For ^1H and ^{13}C NMR data see Table 1.

3.7. Conformational analysis, geometrical optimization, and CD calculation

Conformational analyses of compounds **e6**, **e7** and **e12** were performed with Schrödinger MacroModel 9.1 software using the OPLS 2005 (Optimized Potential for Liquid Simulations) force field in H_2O . Conformers of **e7** and **e6/e12** occurring within a 1 and 2 kcal/mol energy window from the particular global minimum, respectively, were chosen for geometrical optimization and energy calculation using density functional theory (DFT) with the B3LYP functional and the 6-31G** basis-set in the gas-phase with the Gaussian 03 program package (Frisch et al., 2004). Vibrational analysis was done at the same level to confirm minima (Bringmann et al., 2009). TD-DFT/B3LYP/6-31G** (in the gas phase and in MeOH using the “self-consistent reaction field” method (SCRF) with the CPCM polarizable conductor calculation model) was employed to calculate excitation energy (denoted by wavelength in nm) and rotatory strength R in dipole velocity (R_{vel}) and dipole length (R_{len}) forms. ECD curves were calculated based on rotatory strengths using half bandwidth of 0.2 eV with conformers of **e6**, **e7** and **e12**. The spectra were combined after Boltzmann-weighting according to their population contribution.

3.8. Expression of GABA A receptors

Stage V–VI oocytes from *Xenopus laevis* were prepared and cRNA was injected as previously described (Khom et al., 2006). Female *X. laevis* (Nasco) were anesthetized by exposing them for 15 min to a 0.2% MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate, Sigma–Aldrich) 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 run-off 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_2O (Sigma–Aldrich) containing different cRNAs at a concentration of ca. 300–3000 pg/nL per subunit. The amount of injected cRNA mixture was determined by means of a NanoDrop ND-1000 (Kisker Biotech). Rat cRNAs were mixed in a 1:1:10 ratio to ensure expression of the γ -subunit in $\alpha_1\beta_2\gamma_{2S}$ receptors. Oocytes were then stored at $18^\circ C$ 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) (Methfessel et al., 1986). Voltage clamp measurements were performed between days one and five after cRNA injection.

3.9. Two-microelectrode voltage clamp studies

Electrophysiological experiments were performed by the two-microelectrode voltage clamp method making use of a TURBO TEC 03X amplifier (npi electronic) at a holding potential of -70 mV and pCLAMP 10 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.

3.10. 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 $\mu\text{L/s}$ by means of the ScreeningTool automated fast perfusion system (npi electronics) (Baburin et al., 2006). In order to determine GABA EC_{5-10} (typically between 3 and 10 μM for receptors of the subunit combination $\alpha_1\beta_2\gamma_2\text{S}$), a concentration-response experiment with GABA concentrations ranging from 0.1 μM to 1 mM was performed. Stock solution of plant extracts (10 mg/ml in DMSO) were diluted to a concentration of 100 $\mu\text{g/ml}$ with bath solution containing GABA EC_{5-10} according to a validated protocol (Kim et al., 2008). As previously described, microfractions of the *K. longipedunculata* extract collected from the semi-preparative HPLC separation were dissolved in 30 μl DMSO and subsequently mixed with 2.97 ml of bath solution containing GABA EC_{5-10} (Kim et al., 2008). For concentration-response experiments, stock solutions of compounds 1–13 (100 mM in DMSO) were diluted to concentrations ranging from 0.1 to 500 μM with bath solution and applied to the oocyte for measuring agonistic activity. After a preincubation period of 20 s, a second application immediately followed containing the corresponding compound solution combined with GABA EC_{5-10} to measure GABA A receptor modulation.

3.11. Analyzing concentration-response curves

Enhancement of the GABA-induced chloride current (I_{GABA}) was defined as $I_{(\text{GABA} + \text{Comp})}/I_{\text{GABA}} - 1$, where $I_{(\text{GABA} + \text{Comp})}$ 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,

USA). Data were fitted to the equation
$$\frac{1}{1 + \left(\frac{\text{EC}_{50}}{[\text{Comp}]} \right)^{n_H}}$$
 , 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 $\pm$ S.E. of at least 2 oocytes and 2 oocyte batches. Statistical significance was calculated using *t*-test by ANOVA with confidence intervals of $p < 0.05$ and $p < 0.01$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

HPLC-based activity profiling of a petroleum ether extract of *K. longipedunculata* fruits. The chromatogram (266 nm) of a semipreparative separation of 10 mg extract is displayed in part (B). The numbers correspond to the isolated compounds. The 28 collected microfractions (90 s each) are indicated with dashed lines. The corresponding GABA A receptor modulatory activity of each microfraction is shown in part (A) (in percent potentiation of the GABA-induced chloride current, error bars correspond to S.E.).

Fig. 2.

(A) Minimized conformers of *R-e6* in the gas phase using DFT at the B3LYP/6-31G** level. Two major core conformations occurred within a 2 kcal/mol range from the global minimum. According to Boltzmann weights, core conformation A (8 species) accounted for 66%, and core conformation B (4 species) accounted for 34% of the total population. (B) Experimental ECD spectrum of (+)- γ -cuparenol (**6**) in MeOH and calculated ECD spectra of *R-e6* in gas-phase and MeOH using density function theory at the B3LYP/6-31G** level.

Fig. 3.

(A) Minimized conformers of *S-e7* in the gas-phase using DFT at the B3LYP/6-31G** level. Two major core conformations occurred within a 1 kcal/mol range from the global minimum. According to Boltzmann weights, a transoid enone core (10 species) accounted for 17%, and a cisoid enone core (7 species) accounted for 83% of the total population. (B) Experimental ECD spectrum of (+)-6-cinnamoyl-6,7-dihydro-7-myrceneol (**7**) in MeOH and calculated ECD spectra of *S-e7* in gas-phase and MeOH using density function theory at the B3LYP/6-31G** level. Mirror images reflecting *R*-configuration are displayed with dashed lines.

Fig. 4.

(A) Minimized conformers of *R*-**e12** in the gas-phase using DFT at the B3LYP/6-31G** level. Two major core conformations occurred within a 2 kcal/mol range from the global minimum. According to Boltzmann weights, two species accounting for nearly half of the total population (42%) showed P-helicity of the cyclic diene. They only differed in the orientation of the carboxylic group. Conformers with an M-helicity (58% in total) showed further conformational variations in the orientation of the cyclopentane residue. (B) Experimental ECD spectrum of (–)-5,6-dihydrocuparenic acid (**12**) in MeOH and calculated ECD spectra of *R*-**e12** in gas-phase and MeOH using density function theory at the B3LYP/6-31G** level. Mirror images reflecting *S*-configuration are displayed with dashed lines.

Fig. 5.

(A–C) Concentration-dependent potentiation of the GABA-induced chloride current (I_{GABA}) through $\alpha_1\beta_2\gamma_2\delta$ GABA A receptors by compounds 1–8 and 10–13. (D–F) Typical currents elicited by GABA control samples (GABA EC_{5-10}), and different concentrations of compounds 1–8 and 10–13 in absence and presence of GABA EC_{5-10} (preincubation experiments). Note that compounds 1–3, 5, 7, 8, and 12 induce small chloride currents in the absence of GABA suggesting partial agonist activity.

Fig. 6. HPLC-PDA-ESIMS analysis of *K. longipedunculata*, *Kadsura japonica* and *Schisandra chinensis* fruits (gradient 65% to 100% MeOH + HCO₂H 0.1% in 30 min). Numbers correspond to isolated compounds from *K. longipedunculata* with (black) and without (grey) GABA A receptor modulatory activity. The potentiation of the GABA induced chloride current by the crude extract (100 µg/ml) is indicated on the upper right side of each chromatogram. UV traces recorded at 266 nm are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Chart 1.
Isolated compounds from *K. longipedunculata* fruits.

Table 1

¹H NMR and ¹³C NMR spectroscopic data of compounds **6** and **12**.^a

C	6 ^b	12	H	6 ^b	12
C1	138.0	124.4	1	–	–
C2	126.6	137.3	2	7.26 <i>d</i> (8.7)	7.14 <i>d</i> (6.1)
C3	127.4	118.7	3	7.35 <i>d</i> (8.7)	5.93 <i>d</i> (6.1)
C4	147.4	156.1	4	–	–
C5	127.4	26.0	5	7.35 <i>d</i> (8.7)	2.27 <i>m</i>
C6	126.6	24.7	6	7.26 <i>d</i> (8.7)	2.39 <i>m</i>
C7	65.4	173.3	7	4.63 <i>s</i>	–
C1'	50.7	52.5	1'	–	–
C2'	44.5	44.1	2'	–	–
C3'	39.9	40.5	3'a	1.70 <i>m</i> β	1.61–1.69 <i>m</i> β
			3'b	1.57 <i>m</i> α	1.50–1.56 <i>m</i> α
C4'	20.0	19.6	4'	1.81 <i>m</i>	1.66 <i>m</i>
C5'	37.0	36.5	5'a	2.51 <i>m</i> α	2.20 <i>m</i> α
			5'b	1.72 <i>m</i> β	1.44–1.50 <i>m</i> β
C1'-CH ₃	24.6	22.1	1'-CH ₃	1.28 <i>s</i>	1.06 <i>s</i>
C2'-CH ₃	24.5 β	25.1 β	2'-CH _{3a}	1.09 <i>s</i> β	1.03 <i>s</i> β
C2'-CH ₃	26.6 α	26.9 α	2'-CH _{3b}	0.57 <i>s</i> α	0.82 <i>s</i> α

^aRecorded in CDCl₃ at 500 MHz (¹H) and 125 MHz (¹³C), with chemical shift (ppm) of solvent used as internal standard.^bShift differences between **6** and **12** are due to the anisotropic effect of the aromatic ring.

Table 2¹H NMR and ¹³C NMR spectroscopic data of compound **7**^a.

C^b		H	
C1	24.5	1	1.21 <i>s</i>
C2	71.3	2	-
C2-CH ₃	24.5	2-CH ₃	1.21 <i>s</i>
C3	79.6	3	4.98 <i>dd</i> (2.3, 10.3)
C4	27.9	4a	1.98 <i>dddd</i> (14.4, 9.7, 6.4, 2.3)
		4b	1.81 <i>dddd</i> (14.4, 10.3, 9.7, 5.4)
C5	28.2	5a	2.29 <i>ddd</i> (14.6, 5.4, 9.7)
		5b	2.21 <i>ddd</i> (14.6, 9.7, 6.4)
C6	146.0	6	-
C6-CH ₂	115.1	6-CH ₂	5.01 <i>s</i>
C7	138.9	7	6.36 <i>dd</i> (17.6, 12.0)
C8	112.3	8a	5.22 <i>d</i> (17.6)
		8b	5.04 <i>d</i> (12.0)
C1'	167.5	1'	-
C2'	117.7	2'	6.57 <i>d</i> (16.0)
C3'	145.0	3'	7.75 <i>d</i> (16.0)
C4'	134.4	4'	-
C5'	127.7	5'	7.59 <i>m</i>
C6'	128.7	6'	7.39 <i>m</i>
C7'	130.2	7'	7.39 <i>m</i>
C8'	128.7	8'	7.39 <i>m</i>
C9'	127.7	9'	7.59 <i>m</i>

^aRecorded in MeOD at 500 MHz (1H) with chemical shift (ppm) of solvent used as internal standard.^b¹³C shifts from HSQC and HMBC NMR experiment.

Table 3

Potencies and Efficiencies of Compounds 1–13 at the GABA A receptor ($\alpha_1\beta_2\gamma_{2S}$).

Compounds	EC ₅₀ μ M	Max. stimulation of I _{GABA} at EC ₅₋₁₀ %	Hill-Coeff. n_H	Nr of exp. (n)
<i>Arisantetralone lignans (Fig. 5A and D)</i>				
1	52.2 $\pm$ 24.8	245.0 $\pm$ 59.6	1.4 $\pm$ 0.5	6
2	135.6 $\pm$ 85.7	885.8 $\pm$ 291.2 ^{a,b}	1.1 $\pm$ 0.3	4
3	36.6 $\pm$ 16.4	168.7 $\pm$ 41.5 ^a	1.7 $\pm$ 0.8	5
4	118.7 $\pm$ 54.4	129.7 $\pm$ 36.8 ^b	1.3 $\pm$ 0.5	4
<i>Acyclic lignans (Fig. 5B and E)</i>				
5	79.2 $\pm$ 19.4 ^{cd}	793.4 $\pm$ 107.4 ^{egh}	1.5 $\pm$ 0.2	4
8	54.6 $\pm$ 28.8	362.5 $\pm$ 87.1 ^e	1.4 $\pm$ 0.6	4
11	31.5 $\pm$ 7.1 ^d	395.6 $\pm$ 27.2 ^{fg}	1.9 $\pm$ 0.7	4
13	12.8 $\pm$ 3.1 ^c	288.8 $\pm$ 23.7 ^{fh}	1.2 $\pm$ 0.2	4
<i>Other lignans (Fig. 5B and E)</i>				
9	n.d.	n.d.	n.d.	0
10	21.8 $\pm$ 7.5	218.1 $\pm$ 20.8	1.2 $\pm$ 0.5	4
<i>Sesquiterpenes (Fig. 5C and F)</i>				
6	57.3 $\pm$ 19.7	383.5 $\pm$ 89.3	2.6 $\pm$ 1.0	4
12	118.4 $\pm$ 29.9	413.4 $\pm$ 66.3	1.7 $\pm$ 0.3	4
<i>Monoterpene derivatives (Fig. 5C and G)</i>				
7	70.6 $\pm$ 12.2	834.6 $\pm$ 77.5	1.9 $\pm$ 0.3	4

^{a-h} Statistically significant differences between the measured values in the columns are indicated by letters.^d $p = 0.06$; $p < 0.05$.^g $p < 0.01$.