

Published in final edited form as:

Mol Divers. 2011 May ; 15(2): 361–372. doi:10.1007/s11030-010-9297-7.

HPLC-based activity profiling for GABA_A receptor modulators from the traditional Chinese herbal drug Kushen (*Sophora flavescens* root)

Xinzhou Yang,

Institute of Pharmaceutical Biology, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland

Igor Baburin,

Institute of Pharmacology and Toxicology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

Inken Plitzko,

Institute of Pharmaceutical Biology, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland

Steffen Hering, and

Institute of Pharmacology and Toxicology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

Matthias Hamburger

Institute of Pharmaceutical Biology, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland, e-mail: Matthias.Hamburger@unibas.ch

Abstract

An EtOAc extract from the roots of *Sophora flavescens* (Kushen) potentiated γ -aminobutyric acid (GABA)-induced chloride influx in *Xenopus* oocytes transiently expressing GABA_A receptors with subunit composition, $\alpha_1\beta_2\gamma_2\delta$. HPLC-based activity profiling of the extract led to the identification of 8-lavandulyl flavonoids, kushenol I, sophoraflavanone G, (–)-kurarinone, and kuraridine as GABA_A receptor modulators. In addition, a series of inactive structurally related flavonoids were characterized. Among these, kushenol Y (**4**) was identified as a new natural product. The 8-lavandulyl flavonoids are first representatives of a novel scaffold for the target.

Keywords

GABA_A receptor modulators; HPLC-based activity profiling; 8-Lavandulyl flavonoids; *Sophora flavescens*; Fabaceae

Introduction

Gamma-aminobutyric acid type A (GABA_A) receptors mediate inhibitory neurotransmission in the brain. The receptors are composed of five subunits forming a central pore that is permeable for chloride ions upon activation by the endogenous ligand γ -aminobutyric acid

(GABA). Up to now 19 different subunit isoforms have been identified in the human genome and form GABA_A receptors in numerous combinations [1]. The most abundant GABA_A receptor subtype is composed of 2 α ₁, 2 β ₂, and 1 γ ₂ subunits. More than 10 subtypes composed of other subunit combinations have been identified [2], which differ in tissue localization, functional characteristics, and pharmacological properties [3,4]. GABA_A receptors possess several binding sites for small molecules, and are target for numerous drugs used to treat anxiety, panic, insomnia, and epilepsy [5,6]. However, use of the most frequently prescribed benzodiazepines is associated with undesirable side effects including reduced coordination, cognitive impairment, increased accident proneness, physiological dependence, and withdrawal symptoms [5,7]. Rational lead discovery approaches are presently not possible due to the lack of a high-resolution structure of the GABA_A receptor [3,8].

Various natural products modulating GABA_A receptors (e.g., flavonoids, monoterpenes, diterpenes, neolignans, β -carbolines, and polyacetylenes) have been identified [9, 10]. Given the diversity of these natural product ligands which structurally differ from synthetic GABA_A receptor modulators, we recently initiated a project aiming at the discovery of GABA_A receptor agonists with natural product scaffolds new for the target. In a screening of 880 plant and fungal extracts with an automated functional assay using *Xenopus* oocytes expressing human GABA_A receptors of the most abundant subtype composition (α ₁ β ₂ γ _{2S}), an EtOAc extract of the traditional Chinese herbal drug Kushen (roots of *Sophora flavescens* Aiton, Fabaceae) displayed promising activity.

HPLC-based activity profiling is a miniaturized and highly effective approach for localization, dereplication, and characterization of bioactive natural products in extracts [11]. This approach has been successfully established and used in our labs with various cell-based and biochemical assays [12–14]. We recently developed and validated a profiling approach for the discovery of new GABA_A receptor ligands [15], which we subsequently employed to identify scaffolds new for the target [16,17]. We here describe HPLC-based activity profiling of the active *S. flavescens* extract, identification of the active constituents, and preliminary SAR (structure activity relationship) considerations based on a focused compound library generated from the extract.

Experimental section

General experimental procedures

Optical rotations were measured on a M341 polarimeter (Perkin-Elmer). CD spectra were recorded on a JASCO J-720W spectrophotometer. UV spectra were measured on Hewlett-Packard 8452A diode array spectrophotometer (λ_{max} in nm). IR spectra were measured on a Nicolet Magna-FT-IR-750 spectrometer (ν_{max} in cm^{-1}). NMR spectra were recorded at 291 K with a Bruker Avance IIITM spectrometer operating at 500.13 MHz for ¹H, and 125.77 MHz for ¹³C, respectively. ¹H-NMR experiments and 2D homonuclear and heteronuclear NMR spectra were measured with a 1 mm TXI probe, and ¹³C-NMR spectra were obtained in 3 mm tubes (Armar Chemicals) with a 5 mm BBO probe. Spectra were analyzed using Bruker TopSpin 2.1 software. High resolution mass spectra (HPLC–PDA–ESITOFMS) were obtained on a micro TOF ESI–MS system (Bruker Daltonics) connected via T-splitter (1:10) to an HP 1100 series system (Agilent) consisting of a binary pump, autosampler, column oven, and diode array detector (G1315B). Data acquisition and processing was performed using HyStar 3.0 software (Bruker Daltonics). Semi-preparative HPLC separations for activity profiling and offline microprobe NMR was performed with an HP 1100 series system (Agilent) consisting of a quaternary pump, autosampler, column oven, and diode array detector (G1315B). Parallel evaporation of microfractions and semi-preparative HPLC fractions was performed with an EZ-2 plus vacuum centrifuge (Genevac).

SunFire C18 (3.5 μm , 3.0 \times 150 mm i.d.) and a SunFire Prep C18 (5 μm , 10 \times 150 mm i.d.) columns (Waters) were used for HPLC–PDA–ESITOFMS and semi-preparative HPLC, respectively. HPLC-grade acetonitrile (Scharlau Chemie) and water were used for HPLC separations. Solvents used for extraction and column chromatography were of analytical grade. Sephadex LH-20 gel was obtained from Amersham Biosciences (Uppsala, Sweden). Silica gel 60 F254 pre-coated Al sheets (0.25 mm) and silica gel 60 GF254 glass plates (1.00 mm) (both Merck) were used for TLC and preparative TLC, respectively.

Plant material

Kushen (dried roots of *S. flavescens*) was purchased from Yong Quan GmbH (Ennepetal, Germany). A voucher specimen (P00414) is deposited at the Institute of Pharmaceutical Biology, University of Basel.

Extraction

The plant material was frozen with liquid nitrogen and ground with a ZM1 ultracentrifugal mill (Retsch). The extract for the screening and HPLC-based activity profiling was prepared with an ASE 200 extraction system with solvent module (Dionex) by extraction with ethyl acetate. Extraction pressure was 120 bar and temperature was set at 70 °C. Extract was obtained by two extraction cycles of 5 min each. For isolation of larger amounts of pure compounds for pharmacological testing, 50 g of ground roots were extracted by maceration at room temperature with petroleum ether (4 \times 0.5 l, 4 h each), followed by ethyl acetate (4 \times 0.5 l, 4 h each). The solvents were evaporated at reduced pressure to yield 0.9 and 2.3 g of petroleum ether and ethyl acetate extract, respectively. The extracts were stored at –20 °C until use.

Microfractionation for activity profiling

Microfractionation for GABA_A receptor activity profiling was performed as previously described [15], with minor modifications: separation was carried out on a semi-preparative HPLC column with acetonitrile (solvent A) and water containing 0.1% formic acid (solvent B) using the following gradient: 20% A to 100% A for 30 min, hold at 100% A for 5 min. The flow rate was 5 mL/min, and 50 μL of extract (100 mg/ml in DMSO) were injected. A total of 22 time-based microfractions of 90 s each were collected. Solvent removal of microfractions was carried out with a centrifugal evaporator (Genevac AZ-2), and films reconstituted in DMSO prior to activity testing.

HPLC–PDA–ESITOFMS

The ethyl acetate extract of *S. flavescens* was analyzed with acetonitrile (solvent A) and water containing 0.1% formic acid (solvent B) using an optimized gradient profile: 20–100% A in 30 min, 100% A, hold at 100% A for 5 min. The flow rate was 0.5 mL/min. The sample was dissolved in DMSO at a concentration of 10 mg/mL, and the injection volume was 10 μL . ESITOFMS spectra were recorded in the range of m/z 100–1500 in positive ion mode. Nitrogen was used as a nebulizing gas at a pressure of 2.0 bar and as a drying gas at a flow rate of 9.0 L/min (dry gas temperature 240 °C). Capillary voltage was set at 4500 V, hexapole at 230.0 Vpp. Instrument calibration was performed using a reference solution of sodium formate 0.1% in isopropanol/water (1:1) containing 5 mM sodium hydroxide.

Preparative isolation of compounds for GABA_A receptor activity test

A portion (0.8 g) of the ethyl acetate extract was dissolved in 2 mL of DMSO and the solution was filtered. Separation of the ethyl acetate extract was carried out with the same solvent system and gradient elution as for HPLC–PDA–ESITOFMS. The flow rate was set at 5 mL/min and the injected volume of extract was 200 μL at a concentration of 400 mg/

mL in DMSO. A total of 13 peak-based fractions were collected manually. A total of 10 injections were carried out, and corresponding fractions were combined. Final purification were as follows: peaks 2–5, 7, 8, and 10–13 were filtered through a Sephadex LH-20 column (300 × 10 mm, MeOH containing 0.1% formic acid) to yield pure compounds **3** (5.6 mg), **4** (1.8 mg), **5** (2.3 mg), **6** (3.8 mg), **9** (67 mg), **10** (2.9 mg), **13** (4.3 mg), **14** (3.1 mg), **15** (2.1 mg), and **16** (1.2 mg). Peak 1 was separated by preparative TLC (CHCl₃:MeOH:formic acid—100:10:0.5) to afford compounds **1** (2.9 mg) and **2** (3.3 mg). Peak 6 was separated by preparative TLC (CHCl₃:MeOH:formic acid—150:10:0.5) to give compounds **7** (3.4 mg) and **8** (2.2 mg). Peak 9 was also purified by preparative TLC (CHCl₃:MeOH:formic acid—150:10:0.5) to give compounds **11** (2.4 mg) and **12** (3.6 mg).

Microprobe NMR analysis

1 mm NMR tubes (Bruker) were used for ¹H detected 1D and 2D NMR experiments, and 0.1 mg of the above 16 pure compounds was dissolved in 10 μl CD₃OD. The following settings were used: 128 scans to record ¹H-spectra; 8 scans for ¹H, ¹H-COSY spectra using *cosygpqf* pulse program; 32 scans and 256 increments to record HSQC experiments using the *hsqcedetgp* or *hsqcetgps2* pulse program. HMBC spectra were recorded with 64 scans and 128 increments using the *hmbcgpplndqf* pulse program.

Kushenol Y (4)

Light yellow amorphous powder; $[\alpha]_D^{20} +22.0$ ($c=0.17$, MeOH). CD (MeOH) $[\theta]_{315} + 5897$, $[\theta]_{293} - 17061$. UV (MeOH) λ_{\max} (log ϵ): 287 (4.13), 326 (3.78) nm. IR (film) ν_{\max} : 3401 (br), 2968, 2943, 1651, 1610, and 1268 cm⁻¹. ¹H-NMR (500.13 MHz, CD₃OD) δ : 1.11 (s, 6H, H-6'', 7''), 1.23 (m, 2H, H-3''), 1.35 (m, 2H, H-4''), 1.59 (s, 3H, H-10''), 2.38 (m, 1H, H-2''), 2.42 (m, 2H, H-1''), 4.52 (s, 1H, H-9''), 4.59 (s, 1H, H-9''), 4.74 (d, 1H, $J=11.6$ Hz, H-3), 5.39 (d, 1H, $J=11.6$ Hz, H-2), 5.96 (s, 1H, H-6), 6.35 (d, 1H, $J=2.1$ Hz, H-3'), 6.39 (dd, 1H, $J=8.0, 2.1$ Hz, H-5'), 7.28 (d, 1H, $J=8.0$ Hz, H-6'); ¹³C-NMR (125.77 MHz, CD₃OD) δ : 196.5 (C-4), 165.7 (C-9), 160.7 (C-7), 158.0 (C-5), 157.9 (C-2'), 155.9 (C-4'), 148.3 (C-8''), 128.8 (C-6'), 115.2 (C-1'), 110.8 (C-9''), 108.8 (8), 107.8 (C-5'), 103.6 (C-10), 100.4 (C-3'), 96.1 (C-6), 78.1 (C-2), 72.7 (C-3), 71.3 (C-5''), 46.9 (C-2''), 41.4 (C-4''), 26.4 (C-3''), 28.6 (C-6''), 27.3 (C-1''), 28.8 (C-7''), 18.7 (C-10''). HREIMS m/z : 481.1846 (calcd. for C₂₅H₃₀O₈Na, 481.1838).

Expression of GABA_A receptors

Stage V–VI oocytes from *Xenopus laevis* were prepared and cRNA was injected as previously described by Khom et al. [18]. Female *X. laevis* (NASCO, Fort Atkinson, WI, USA) were anesthetized by exposing them for 15 min to a 0.2% MS-222 (methanesulfonate salt of 3-aminobenzoic acid ethyl, Sigma, Vienna, Austria) solution before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/mL collagenase (Type 1A, Sigma, Vienna, Austria). 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 water (Sigma) containing different cRNAs at a concentration of approximately 300–3000 pg/nL per subunit. To ensure expression of $\alpha_1\beta_2\gamma_2S$ receptors, rat cRNA of α_1, β_2 , and γ_2S subunits were mixed in a 1:1:10 ratio. The amount of injected cRNA mixture was determined by means of a NanoDrop ND-1000 (Kisker Biotech, Steinfurt, Germany). Oocytes were then stored at 18 °C in ND96 solution (90mM NaCl, 1mM KCl, 1mM MgCl₂, 1 mM CaCl₂, and 5 mM HEPES; pH 7.4) [19] Voltage clamp measurements were performed between days 1 and 5 after cRNA injection.

Two-microelectrode voltage clamp studies

Electrophysiological experiments were performed by the two-microelectrode voltage clamp method making use of a TURBO TEC-03X amplifier (npi electronic GmbH, Tamm, Germany) at a holding potential of -70 mV and pCLAMP 10 data acquisition software (Molecular Devices, Sunnyvale, CA, USA). Currents were low-pass-filtered at 1 kHz and sampled at 3 kHz. ND 96 solution was used as bath solution. The electrode filling solution consisted of 2 M KCl. Oocytes with maximal current amplitudes >3 μ A were discarded to exclude voltage clamp errors. Before application of test solutions, a dose–response experiment with GABA concentrations ranging from 0.1 to 1 mM was performed to determine GABA EC_{3–10} (typically between 3 and 10 μ M).

Test solution application

Test solutions were applied by means of a ScreeningTool (npi electronic GmbH, Tamm, Germany) automated fast perfusion system [20]. As previously described, microfractions collected from the semi-preparative HPLC separations were dissolved in 30 μ L DMSO and mixed with 2.97 mL of bath solution [15]. After addition of GABA EC_{3–10}, 100 μ L of this solution was applied to the oocytes at a perfusion speed of 300 μ L/s. Stock solutions of compounds **6–16** (10 mM in DMSO) were diluted to a concentration of 100 μ M with bath solution and then mixed with GABA EC_{3–10}. Of each solution, 100 μ L was applied to the oocytes at a speed of 300 μ L/s.

Data analysis

Enhancement of the chloride current (I_{GABA}) was defined as $I_{\text{GABA+Comp}}/I_{\text{GABA}}-1$, where $I_{\text{GABA+Comp}}$ is the current response in the presence of a given compound, and I_{GABA} is the control GABA induced 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 $1 + \frac{1}{\left(\frac{EC_{50}}{[Comp]}\right)^{n_H}}$, where EC₅₀ is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50%, and n_H is the Hill coefficient. Data are given as mean \pm S.E. of at least two oocytes and 2 oocyte batches.

Results and discussion

Extracts were screened by means of an automated, fast perfusion system during two-microelectrode voltage clamp measurements in *Xenopus* oocytes expressing functional GABA_A receptors with defined subunit composition ($\alpha_1\beta_2\gamma_{2S}$) [20]. When tested at 100 μ g/mL, the *S. flavescens* EtOAc extract enhanced GABA induced chloride ion current (I_{GABA}) by $55.0 \pm 7.4\%$ ($n = 3$, Fig. 1).

The extract was then submitted to HPLC-based activity profiling using a previously validated protocol [15]. The chromatogram of a semi-preparative separation of 5 mg extract, the corresponding potentiation of I_{GABA} by the time-based fractionations (27 microfractions of 90 s each), and representative currents of the most active fractions are shown in Fig. 2. Fractions 11 and 12 displayed the highest potentiation of I_{GABA} ($155.6 \pm 22.2\%$, $n = 2$ and $135.1 \pm 6.6\%$, $n = 2$, respectively). These fractions contained the major compounds of the extract. Fractions 14 and 15 potentiated I_{GABA} to a lesser extent ($45.0 \pm 5.0\%$, $n = 2$ and $62.5 \pm 12.5\%$, $n = 2$, respectively), while the effects of fractions 9, 18, 19, and 21 were not significantly different from zero ($P > 0.05$, see Fig. 2).

Considering that some HPLC peaks showed partial overlap, separation conditions were optimized for full resolution of the critical HPLC peaks. These conditions were then used to measure high-resolution LC–MS data (Fig. 3a; Table 1). Results of the HPLC–PDA–TOFMS analysis and a database search are summarized in Table 1. The same optimized conditions were also used in preparative isolation of target compounds. A typical semi-preparative HPLC chromatogram obtained with an injection of 80 mg of extract is shown in Fig. 3b. A total of 13 peaks were collected and submitted to further purification with Sephadex LH-20 column and preparative TLC to afford 16 compounds for which 1D and 2D NMR spectra were recorded with a 1 mm TXI probe. The purity was checked by analytical HPLC under conditions as in Fig. 3a.

The major peak was attributed to (–)-kurarinone (**9**), the main lavandulyl flavonoid in *S. flavescens*. The remaining compounds were all structurally related 8-lavandulyl flavonoids (Fig. 4) which were identified as kushenol H (**1**), kushenol K (**2**), kurarinol (**3**), kushenol P (**5**), norkurarinol (**6**), kushenol I (**7**), kushenol N (**8**), (–)-kurarinone (**9**), kushenol X (**10**), neokurarinol (**11**), kushenol C (**12**), sophoraflavanone G (**13**), leachianone A (**14**), kuraridine (**15**), and kushenol A (**16**) by comparison of their MS, UV, and NMR spectra with published reference data [21–27].

A new but pharmacologically inactive flavonoid **4** was obtained as a light yellow amorphous powder. Its molecular formula $C_{25}H_{30}O_8$ was deduced from HR-ESIMS ($M+Na^+$ found 481.1846, calcd. 481.1838). The UV spectrum showed absorption maxima at 287 and 326 nm. The 1H -NMR spectrum revealed the presence of a 1,2,4-trisubstituted aromatic ring at δ_H 7.28 (d, 1H, $J=8.0$ Hz, H-6'), 6.39 (dd, 1H, $J=8.0, 2.1$ Hz, H-5') and 6.35 (d, 1H, $J=2.1$ Hz, H-3'), an isolated aromatic proton at δ_H 5.96 (s, 1H, H-6), and two protons at δ_H 4.74 (d, 1H, $J=11.6$ Hz, H-3) and 5.39 (d, 1H, $J=11.6$ Hz, H-2) characteristic of 2,3-*trans*-flavanonol derivatives [24]. Additional signals at δ_H 1.11 (s, 6H, H-6'', 7''), 1.23 (m, 2H, H-3''), 1.35 (m, 2H, H-4''), 1.59 (s, 3H, H-10''), 2.38 (m, 1H, H-2''), 2.42 (m, 2H, H-1''), 4.52 (s, 1H, H-9''), and 4.59 (s, 1H, H-9'') were attributable to a 5-hydroxy-2-isopropenyl-5-methylhexyl moiety and were virtually superimposable with data reported for lavandulyl moieties of related flavonoids such as kushenol H (**1**) [21]. Compared to **1** the only difference in the spectrum of **4** observed was a hydroxy group at C-5 instead of a methoxy residue in **1**. The ^{13}C -NMR spectrum of **4** contained signals of four oxygenated aromatic carbons and one carbonyl carbon. Chemical shifts and signal patterns in the 1H - and ^{13}C -NMR spectra indicated that the hydroxyl groups were at C-5, C-7, C-2', and C-4'. These assignments and the position of the side chain were further confirmed by HSQC and HMBC experiments. Representative spectra obtained with the 1 mm TXI probe after HPLC peak collection are shown in Fig. 5. As in other lavandulyl flavonoids reported from *S. flavescens*, the absolute configuration at C-2'' has not been determined [24]. The CD spectrum of **4** showed a positive absorption at $[\theta]_{315} + 5897$ and a negative absorption at $[\theta]_{293} - 17061$, from which the absolute configuration at C-2 and C-3 were determined as 2*R* and 3*R*, respectively [28]. Thus, **4** was (2*R*,3*R*)-8-(5-hydroxy-2-isopropenyl-5-methylhexyl)-5,7,2',4'-tetrahydroxyflavanonol. We named the compound kushenol Y, in accord with trivial names of related lavandulyl flavonoids from *S. flavescens*.

For further evaluation of compounds in the active time windows, **6**, **7**, and **9–16** (10 μ M) were co-applied with a GABA (EC_{3-10}) to oocytes expressing $\alpha_1\beta_2\gamma_2S$ receptors. Kushenol I (**7**), (–)-kurarinone (**9**), sophoraflavanone G (**13**), and kuraridine (**15**) induced the strongest I_{GABA} enhancement (Fig. 6). For these compounds the concentration–response relationships for I_{GABA} enhancement were determined (Fig. 7). Given the lack of activity in other fractions of the activity profile (Fig. 1), the other flavonoids were considered as inactive. Interestingly, application of saturating concentrations of kushenol I (**7**), (–)-kurarinone (**9**), sophoraflavanone G (**13**), and kuraridine (**15**) induced chloride inward currents in the

absence of GABA. These currents did not exceed 10% of the maximal I_{GABA} induced by a saturating GABA concentration (1 mM). This partial agonist activity was most pronounced for kuraridine (**15**) (Fig. 8).

(-)-Kurarinone (**9**) and kuraridine (**15**) are the first representatives of a new scaffold of GABA_A receptor modulators with a flavonoid moiety. Interaction with GABA_A receptors have been investigated since the 1980s, when first evidence was produced from displacement studies with radiolabelled benzodiazepines [29]. Subsequent binding studies demonstrated that a range of naturally occurring and synthetic flavonoids including the monomeric flavonoids apigenin [30], 6-hydroxyflavone [31], baicalin [32], hesperidin, and 6-methylapigenin [33,34], and biflavonoids such as agathisflavone and amentoflavone [35–37] bind to GABA_A receptors with nanomolar affinity. Quercetin, apigenin, morine, and chrysin have been shown to inhibit I_{GABA} through $\alpha_1\beta_1\gamma_2\delta$ GABA_A receptors [38]. Simple flavones appear to interact with the benzodiazepine binding site [34,39], whereas amentoflavone is a negative modulator of GABA_A receptors whose action appears independent of benzodiazepine modulatory sites on the receptor [36]. Evidence for in vivo activity mediated through GABA_A receptor modulation has been reported for selected flavonoids. Simple flavones and flavonols such as chrysin, apigenin, wogonin, 6-methylapigenin, hispidulin, and luteolin-7-*O*-(2-rhamnosylglucoside) appear to induce predominantly anxiolytic effects and seem less efficient as sedatives, anticonvulsants, and myorelaxants [34,39–42].

The pharmacology of *Sophora flavescens* flavonoids has been intensively studied. Neuroprotective [43] and anti-inflammatory [44] properties have been reported, but no pharmacological effects that could be related to modulation of GABA_A receptors. Given the close structural relationship of **1–16**, some preliminary structure activity considerations can be derived from this focused library via our activity profiling approach (Fig. 9): The compounds differ in some specific attributes, namely in the substitution at C-5'' of the side chain, the substituents at C-5, C-2', and C-4'' of the flavonoid moiety, and in the nature of the basic flavonoid structure, viz. flavanone, flavanonol or chalcone. Absence of a hydroxy group at C-5'' of the side chain appears essential, as compounds **1–6** bearing a hydroxyl group were all inactive. The methoxy substituent at C-5 contributes to activity, since all compounds with a hydroxy group either showed less (eg. **13**) or no activity. Comparison of (-)-kurarinone (**9**) and kushenol A (**16**) indicates that a hydroxy group at C-4'' also contributes to activity. Active compounds bear a hydroxy substituent at C-2'' instead of a methoxy group as, for example, in neokurarinol (**11**). Flavanone (e.g., **9**) and chalcone derivatives (e.g., **15**) show higher activity than corresponding flavanonol derivatives (**7** and **8**). In summary, it appears that several structural features contribute to some degree to the GABA_A receptor modulating properties of 8-lavandulyl flavonoids and that absence of a hydroxyl group at C-5'' is essential (Fig. 5).

The discovery of a new scaffold for potent GABA_A modulators (EC_{50} s between 5.0 and 15.0 μM) enlarges the spectrum of natural products acting on this target. The example of 8-lavandulyl flavonoids highlights the advantages of an HPLC-based approach, in particular, the possibility of obtaining valuable preliminary structure–activity information via the rapid characterization of structurally related compound series. (-)-Kurarinone (**9**) fulfils most criteria of Lipinski's rule of five [45] and has a polar surface area that may enable CNS penetration [46]. We currently investigate the binding site of the most active compounds **9** and **15**, their subunit specificity, and in vivo activity.

Acknowledgments

Financial support from the Swiss National Science Foundation (Projects 31600-113109 and 205321-116157/1), the Steinegg-Stiftung, Herisau, the Fonds zur Förderung von Lehre und Forschung, Basel (M.H.), the FWF Project P19614-B11 (S.H.) is gratefully acknowledged. We thank Jian Gao (Department of Chemistry, University of Basel) for measuring optical rotation, circular dichroism, UV and IR spectra. We thank Dr. Sophia Khom for valuable comments.

Abbreviations

TOFMS	Time of flight mass spectrometry
PDA	Photodiode array
HMBC	Heteronuclear multiple-bond correlation
HSQC	Heteronuclear single quantum coherence
CD	Circular dichroism

References

- Simon J, Wakimoto H, Fujita N, Lalande M, Barnard EA. Analysis of the set of GABA_A receptor genes in the human genome. *J Biol Chem.* 2004; 279:41422–41435. doi:10.1074/jbc.M401354200. [PubMed: 15258161]
- Olsen RW, Sieghart W. International union of pharmacology. LXX. Subtypes of γ -aminobutyric acid_A receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev.* 2008; 60:243–260. doi:10.1124/pr.108.00505. [PubMed: 18790874]
- Barrera NP, Edwardson JM. The subunit arrangement and assembly of ionotropic receptors. *Trends Neurosci.* 2008; 31:569–576. doi:10.1016/j.tins.2008.08.001. [PubMed: 18774187]
- Sieghart W, Sperk G. Subunit composition, distribution and function of GABA_A receptor subtypes. *Curr Top Med Chem.* 2002; 2:795–816. [PubMed: 12171572]
- Whiting PJ. GABA-A receptors: a viable target for novel anxiolytics? *Curr Opin Pharmacol.* 2006; 6:24–29. doi:10.1016/j.coph.2005.08.005. [PubMed: 16359919]
- Riss J, Cloyd J, Gates J, Collins S. Benzodiazepines in epilepsy: pharmacology and pharmacokinetics. *Acta Neurol Scand.* 2008; 118:69–86. doi:10.1111/j.1600-0404.2008.01004.x. [PubMed: 18384456]
- Kaplan EM, DuPont RL. Benzodiazepines and anxiety disorders: a review for the practicing physician. *Curr Med Res Opin.* 2005; 21:941–950. doi:10.1185/030079905X48401. [PubMed: 15969894]
- Rupprecht R, Eser D, Zwanzger P, Moeller HJ. GABA_A receptors as targets for novel anxiolytic drug. *World J Biol Psychiatry.* 2006; 7:231–237. doi:10.1080/15622970600868525. [PubMed: 17071543]
- Johnston GAR, Hanrahan JR, Chebib M, Duke RK, Mewett KN. Modulation of ionotropic GABA receptors by natural products of plant origin. *Adv Pharmacol.* 2006; 54:285–316. doi:10.1016/S1054-3589(06)54012-8. [PubMed: 17175819]
- Tsang SY, Xue H. Development of effective therapeutics targeting the GABA_A receptor: naturally occurring alternatives. *Curr Pharm Design.* 2004; 10:1035–1044. doi:10.2174/1381612043452767.
- Potterat O, Hamburger M. Natural products in drug discovery—concepts and approaches for tracking bioactivity. *Curr Org Chem.* 2006; 10:899–920. doi:10.2174/138527206776894401.
- Potterat O, Wagner K, Gemmecker G, Mack J, Puder C, Vettermann R, Streicher R. BI-32169, a bicyclic 19-peptide with strong glucagon receptor antagonist activity from *Streptomyces* sp. *J Nat Prod.* 2004; 67:1528–1531. doi:10.1021/np040093o. [PubMed: 15387654]
- Danz H, Stoyanova S, Wippich P, Brattstroem A, Hamburger M. Identification and isolation of the cyclooxygenase-2 inhibitory principle in *Isatis tinctoria*. *Planta Med.* 2001; 67:411–416. doi:10.1055/s-2001-15805. [PubMed: 11488453]

14. Dittmann K, Gerhaeuser C, Klimo K, Hamburger M. HPLC-based activity profiling of *Salvia miltiorrhiza* for MAO-A and iNOS inhibitory activities. *Planta Med.* 2004; 70:909–913. doi: 10.1055/s-2004-8326156. [PubMed: 15490317]
15. Kim HJ, Baburin I, Khom S, Hering S, Hamburger M. HPLC-based activity profiling approach for the discovery of GABA_A receptor ligands using an automated two microelectrode voltage clamp assay on *Xenopus* oocytes. *Planta Med.* 2008; 74:521–526. doi:10.1055/s-2008-1074491. [PubMed: 18543148]
16. Zaugg J, Baburin I, Strommer B, Kim HJ, Hering S, Hamburger M. HPLC-based activity profiling: discovery of piperine as a positive GABA_A receptor modulator targeting a benzodiazepine-independent binding site. *J Nat Prod.* 2010; 73:185–191. doi:10.1021/np900656g. [PubMed: 20085307]
17. Li Y, Plitzko I, Zaugg J, Hering S, Hamburger M. HPLC-based activity profiling for GABA_A receptor modulators: a new dihydroisocoumarin from *Haloxylon scoparium*. *J Nat Prod.* 2010; 73:768–770. doi:10.1021/np900803w. [PubMed: 20210326]
18. Khom S, Baburin I, Timin EN, Hohaus A, Sieghart W, Hering S. Pharmacological properties of GABA_A receptors containing γ 1 subunits. *Mol Pharmacol.* 2006; 69:640–649. doi:10.1124/mol.105.017236. [PubMed: 16272224]
19. Methfessel C, Witzemann V, Takahashi T, Mishina M, Numa S, Sakmann B. Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels. *Pflug Arch Eur J Phy.* 1986; 407:577–588.
20. Baburin I, Beyl S, Hering S. Automated fast perfusion of *Xenopus* oocytes for drug screening. *Pflug Arch Eur J Phys.* 2006; 453:117–123. doi:10.1007/s00424-006-0125-y.
21. Wu LJ, Miyase T, Ueno A, Kuroyanagi M, Noko T, Fukushima S. Studies on the constituents of *Sophora flavescens* Ait. III. *Yakugaku Zasshi.* 1985; 105:736–741.
22. Sato S, Takeo J, Aoyama C, Kawahara H. Na⁺-glucose co-transporter (SGLT) inhibitory flavonoids from the roots of *Sophora flavescens*. *Bioorg Med Chem.* 2007; 15:3445–3449. doi: 10.1016/j.bmc.2007.03.011. [PubMed: 17374486]
23. Kim JH, Ryu YB, Kang NS, Lee BW, Heo JS, Jeong IY, Park KH. Glycosidase inhibitory flavonoids from *Sophora flavescens*. *Biol Pharm Bull.* 2006; 29:302–305. doi:10.1248/bpb.29.302. [PubMed: 16462036]
24. Kuroyanagi M, Arakawa T, Hirayama Y, Hayashi T. Anti-bacterial and antiandrogen flavonoids from *Sophora flavescens*. *J Nat Prod.* 1999; 62:1595–1599. doi:10.1021/np99000051d. [PubMed: 10654410]
25. Hatayama K, Komatsu M. Studies on the constituents of *Sophora* species. V. Constituents of the root of *Sophora angustifolia* SIEB. et. ZUCC. *Chem Pharm Bull.* 1971; 19:2126–2131.
26. Hillerns PI, Wink M. Binding of flavonoids from *Sophora flavescens* to the rat uterine estrogen receptor. *Planta Med.* 2005; 71:1065–1068. doi:10.1055/s-2005-871302. [PubMed: 16320211]
27. Kyogoku K, Hatayama K, Komatsu M. Constituents of Chinese drug, Kushen (the root of *Sophora flavescens* Arr.). Isolation of five new flavonoids and formononetin. *Chem Pharm Bull.* 1973; 21:2733–2738. [PubMed: 4783169]
28. Gaffield W. Circular dichroism, optical rotatory dispersion and absolute configuration of flavanones, 3-hydroxyflavanones and their glycosides: determination of aglycone chirality in flavanone glycosides. *Tetrahedron.* 1970; 26:4093–4108. doi:10.1016/S0040-4020(01)93050-9.
29. Luk KC, Stern L, Weigele M, O'Brien RA, Spirt N. Isolation and identification of “diazepam-like” compounds from bovine urine. *J Nat Prod.* 1983; 46:852–861. doi:10.1021/np50030a005. [PubMed: 6330305]
30. Avallone R, Zanoli P, Puia G, Kleinschnitz M, Schreier P, Baraldi M. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla*. *Biochem Pharmacol.* 2000; 59:1387–1394. doi:10.1016/S0006-2952(00)00264-1. [PubMed: 10751547]
31. Ren L, Wang F, Xu Z, Chan WM, Zhao C, Xue H. GABA_A receptor subtype selectivity underlying anxiolytic effect of 6-hydroxyflavone. *Biochem Pharmacol.* 2010; 79:1337–1344. doi: 10.1016/j.bcp.2009.12.024. [PubMed: 20067772]

32. Wang F, Xu Z, Ren L, Tsang SY, Xue H. GABA_A receptor subtype selectivity underlying selective anxiolytic effect of baicalin. *Neuropharmacology*. 2008; 55:1231–1237. doi:10.1016/j.neuropharm.2008.07.040. [PubMed: 18723037]
33. Wasowski C, Marder M, Viola H, Medina JH, Paladini AC. Isolation and identification of 6-methylapigenin, a competitive ligand for the brain GABA_A receptors, from *Valeriana wallichii* D. C. *Planta Med*. 2002; 68:934–936. [PubMed: 12391561]
34. Marder M, Viola H, Wasowski C, Fernandez S, Medina JH, Paladini AC. 6-Methylapigenin and hesperidin: new valeriana flavonoids with activity on the CNS. *Pharmacol Biochem Behav*. 2003; 75:537–545. doi:10.1016/S0091-3057(03)00121-7. [PubMed: 12895671]
35. Svenningsen AB, Madsen KD, Liljefors T, Stafford GI, van Staden J, Jäger AK. Biflavones from *Rhus* species with affinity for the GABA_A/benzodiazepine receptor. *J Ethnopharmacol*. 2006; 103:276–280. doi:10.1016/j.jep.2005.08.012. [PubMed: 16168585]
36. Hanrahan JR, Chebib M, Davucheron NLM, Hall BJ, Johnston GAR. Semisynthetic preparation of amentoflavone: a negative modulator at GABA_A receptors. *Bioorg Med Chem Lett*. 2003; 13:2281–2284. doi:10.1016/S0960-894X(03)00434-7. [PubMed: 12824018]
37. Nielsen M, Frøkjær S, Braestrup C. High affinity of the naturally-occurring biflavonoid, amentoflavon, to brain benzodiazepine receptors in vitro. *Biochem Pharmacol*. 1988; 37:3285–3287. doi:10.1016/0006-2952(88)90640-5. [PubMed: 2840912]
38. Goutman JD, Waxemberg MD, Doñate-Oliver F, Pomata PE, Calvo DJ. Flavonoid modulation of ionic currents mediated by GABA_A and GABA_C receptors. *Eur J Pharmacol*. 2003; 461:79–87. doi:10.1016/S0014-2999(03)01309-8. [PubMed: 12586201]
39. Hui KM, Huen MSY, Wang HY, Zheng H, Sigel E, Baur R, Ren H, Li ZW, Wong JTF, Xue H. Anxiolytic effect of wogonin, a benzodiazepine receptor ligand isolated from *Scutellaria baicalensis* Georgi. *Biochem Pharmacol*. 2002; 64:1415–1424. doi:10.1016/S0006-2952(02)01347-3. [PubMed: 12392823]
40. Coleta M, Batista MT, Campos MG, Carvalho R, Cotrim MD, Lima TC, Cunha AP. Neuropharmacological evaluation of the putative anxiolytic effects of *Passiflora edulis* Sims, its subfractions and flavonoid constituents. *Phytother Res*. 2006; 20:1067–1073. doi:10.1002/ptr.1997. [PubMed: 17009209]
41. Medina JH, Viola H, Wolfman C, Marder M, Wasowski C, Calvo D, Paladini AC. Neuroactive flavonoids: new ligands for the benzodiazepine receptors. *Phytomedicine*. 1998; 5:235–243. [PubMed: 23195847]
42. Kavvadias D, Sand P, Youdim KA, Qaiser MZ, Rice-Evans C, Baur R, Sigel E, Rausch WD, Riederer P, Schreier P. The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood-brain barrier and exhibits anticonvulsive effects. *Br J Pharmacol*. 2004; 142:811–820. doi:10.1038/sj.bjp.0705828. [PubMed: 15231642]
43. Park SJ, Nam KW, Lee HJ, Cho EY, Koo U, Mar W. Neuroprotective effects of an alkaloid-free ethyl acetate extract from the root of *Sophora flavescens* Ait. against focal cerebral ischemia in rats. *Phytomedicine*. 2010; 16:1042–1051. doi:10.1016/j.phymed.2009.03.017. [PubMed: 19427179]
44. Jin JH, Kim JS, Kang SS, Son KH, Chang HW, Kim HP. Anti-inflammatory and anti-arthritic activity of total flavonoids of the roots of *Sophora flavescens*. *J Ethnopharmacol*. 2009; 127:589–595. doi:10.1016/j.jep.2009.12.020. [PubMed: 20034551]
45. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*. 1997; 23:3–25. doi:10.1016/S0169-409X(96)00423-1.
46. Pajouhesh H, Lenz GR. Medicinal chemical properties of successful central nervous system drugs. *NeuroRX*. 2005; 2:541–553. doi:10.1602/neurorx.2.4.541. [PubMed: 16489364]

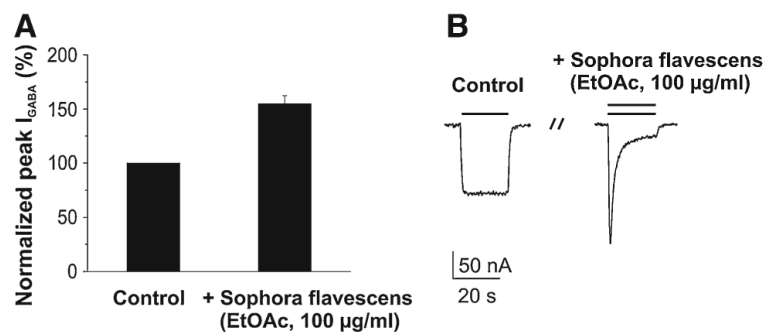


Fig. 1. Potentiation of I_{GABA} by *Sophora flavescens* extract (EtOAc, 100 μ g/mL). **a** The mean I_{GABA} potentiation of $55.0 \pm 7.4\%$ ($n = 3$; compared to 100% control) was observed. **b** Chloride currents through $\alpha_1\beta_2\gamma_2S$ GABA_A receptors induced by GABA EC₃₋₁₀ (control; *left column*) and currents during co-application of GABA (EC₃₋₁₀) and 100 μ g/mL of the *Sophora flavescens* extract (*right column*)

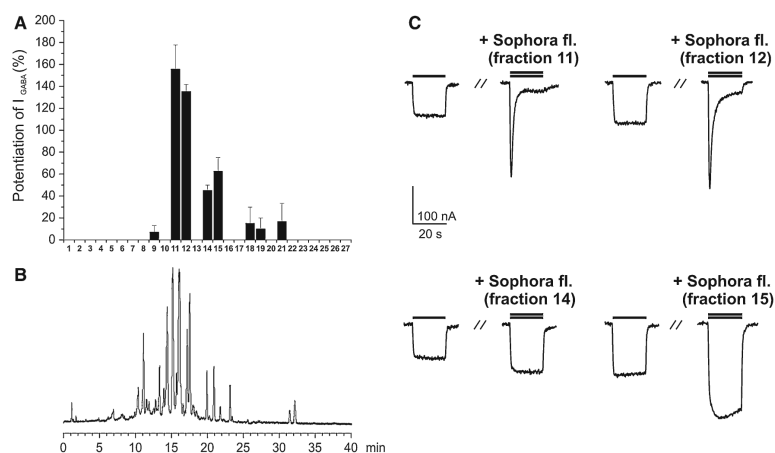


Fig. 2. HPLC-based activity profiling of *Sophora flavescens* extract (EtOAc) for GABA_A receptor modulating properties. **b** The HPLC chromatogram (254 nm) of a semi-preparative separation of 5 mg extract is shown. **a** Potentiation (in %) of I_{GABA} through $\alpha_1\beta_2\gamma_2S$ GABA_A receptors by 27 fractions of 90 s each fraction is shown above. **c** Representative currents through $\alpha_1\beta_2\gamma_2S$ GABA_A receptors in the presence of GABA (EC₃₋₁₀, *single bar*, control) and currents recorded during co-application of GABA (EC₃₋₁₀) and the indicated fraction (*double bar*). Current recordings for four most active fractions (11, 12, 14, and 15) are shown

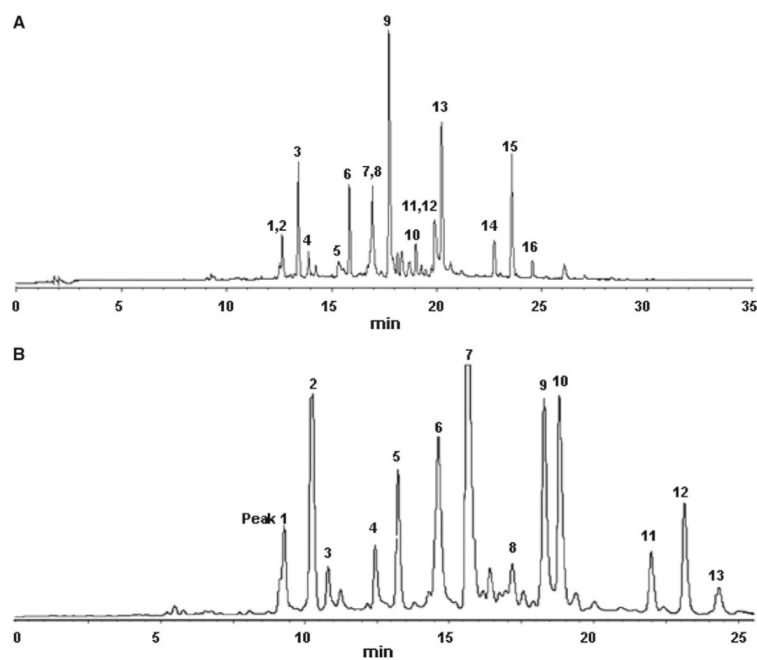


Fig. 3.

a The optimized HPLC separation for LC–PDA–TOFMS analysis of the EtOAc extract is shown. Peak labeling corresponds to compounds **1–16**. 200 μg extract in 20 μL DMSO were injected. The HPLC trace was recorded at 254 nm. **b** The chromatogram (254 nm) of the optimized, semi-preparative HPLC separation of the EtOAc extract (80 mg in 200 μL DMSO) is shown. Peaks 1–13 were collected for microprobe NMR and further purified if required

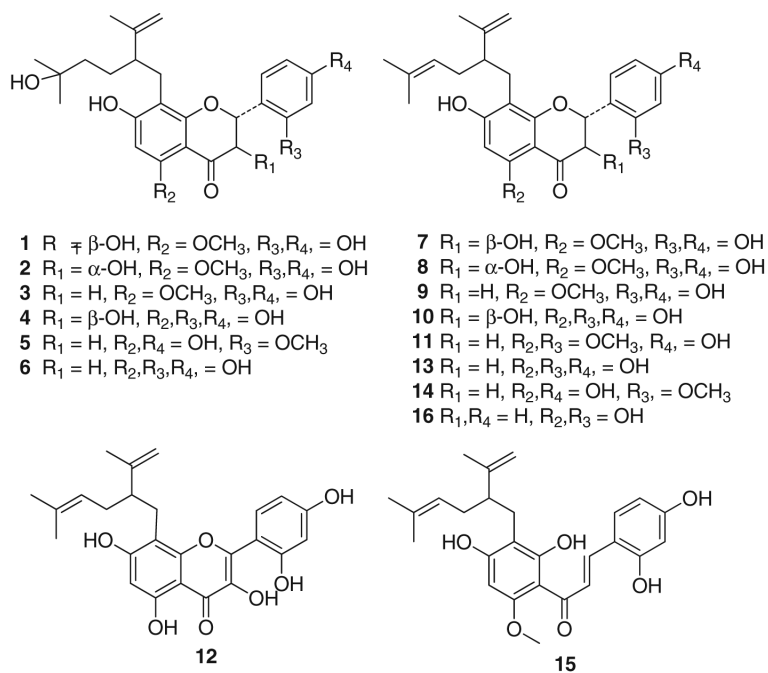


Fig. 4.
Structures of flavonoids **1–16**

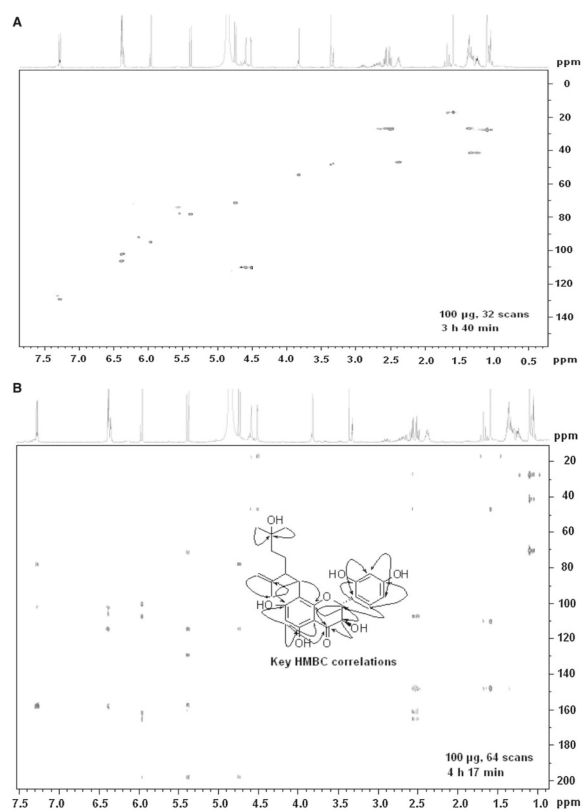


Fig. 5.
a HSQC spectrum of kushenol Y (**4**) recorded with the TXI probe after HPLC peak collection and **b** HMBC spectrum and key correlations

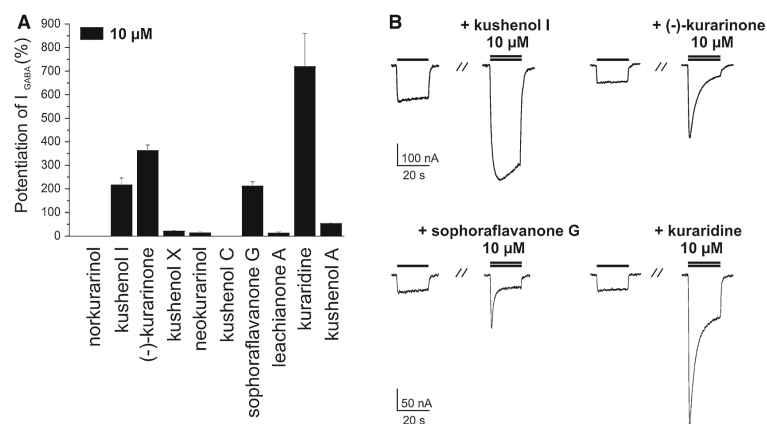


Fig. 6. **a** Potentiation of I_{GABA} through $\alpha_1\beta_2\gamma_2S$ GABA_A receptors by 10 μ M of norkurarinol ($0 \pm 0\%$, $n = 2$), kushenol I ($216.5 \pm 29.3\%$, $n = 5$), (-)-kuraridine ($362.3 \pm 23.8\%$, $n = 5$), kushenol X ($21.6 \pm 3.4\%$, $n = 2$), neokurarinol ($13.7 \pm 4.6\%$, $n = 2$), kushenol C ($0 \pm 0\%$, $n = 2$), sophoraflavanone G ($211.6 \pm 18.6\%$, $n = 3$), leachianone A ($13.1 \pm 5.1\%$, $n = 2$), kuraridine ($719.7 \pm 140.3\%$, $n = 3$), and kushenol A ($53.1 \pm 3.1\%$, $n = 2$). **b** Representative currents through $\alpha_1\beta_2\gamma_2S$ GABA_A receptors induced by GABA (EC_{3-10} , single bar, control) and traces recorded during co-application of GABA (EC_{3-10}) and the indicated compound (double bar). Current potentiation induced by the four most active compounds, kushenol I (7), (-)-kuraridine (9), sophoraflavanone G (13), and kuraridine (15) is shown

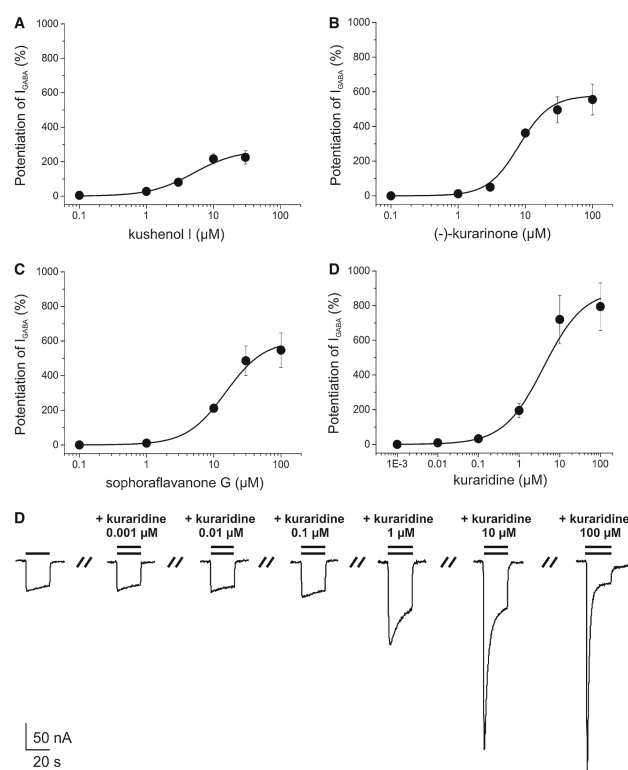


Fig. 7.

Concentration–response curves for I_{GABA} enhancement by **a** kushenol I ($EC_{50} = 5.0 \pm 2.3 \mu\text{M}$, $n_H = 1.3 \pm 0.3$, $n = 5$); **b** (–)-kurarinone ($EC_{50} = 8.1 \pm 1.4 \mu\text{M}$, $n_H = 1.9 \pm 0.1$, $n = 4$); **c** sophoraflavanone G ($EC_{50} = 15.0 \pm 3.6 \mu\text{M}$, $n_H = 1.5 \pm 0.2$, $n = 3$); and **d** kuraridine ($EC_{50} = 4.0 \pm 2.4 \mu\text{M}$, $n_H = 0.9 \pm 0.1$) in *Xenopus* oocytes expressing $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_2S$ subunits. A maximum potentiation of I_{GABA} by kushenol I (**a**, $267.6 \pm 56.6\%$, $n = 5$), (–)-kurarinone (**b**, $578.5 \pm 68.8\%$, $n = 4$), sophoraflavanone G (**c**, $604.9 \pm 108.2\%$, $n = 3$), kuraridine (**d**, $891.5 \pm 163.0\%$, $n = 3$) was observed. **e** Representative currents through $\alpha_1\beta_2\gamma_2S$ $GABA_A$ receptors in the presence of GABA (EC_{3-10} , *single bar*, control) and currents recorded during co-application of GABA (EC_{3-10}) and 0.001, 0.01, 0.1, 1, 10, and 100 μM of kuraridine (*double bar*)

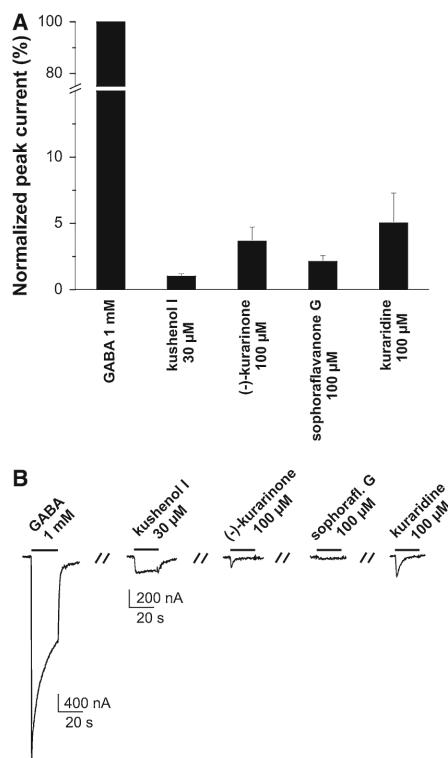
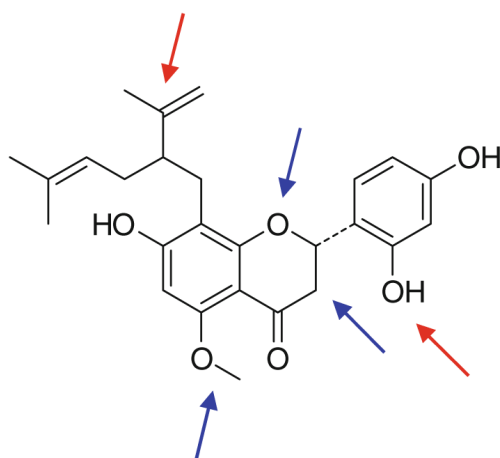


Fig. 8. Activation of GABA_A receptors by kushenol I, (-)-kurarinone, sophoraflavanone G, and kuraridine. **a** Bar graphs illustrate the amplitudes of chloride currents induced in the absence of GABA by saturating concentrations of kushenol I ($1.0 \pm 0.2\%$, $n = 3$), (-)-kurarinone ($3.7 \pm 1.1\%$, $n = 3$), sophoraflavanone G ($2.1 \pm 0.4\%$, $n = 3$) and kuraridine ($5.0 \pm 2.2\%$, $n = 3$). 100% correspond to the maximal I_{GABA} induced by a saturating concentration (1 mM) of GABA. **b** Representative currents illustrating direct activation of GABA_A receptors ($\alpha_1\beta_2\gamma_2\delta$) by kushenol I, (-)-kurarinone, sophoraflavanone G, and kuraridine in comparison to I_{GABA} induced by 1 μ M GABA



xlogP	4.4
MW	438
H-Bond Donors	3
H-Bond Acceptors	6
Polar Surface Area	96.2

Fig. 9. Summary of preliminary structure activity information on lavandulyl flavonoids and calculated physicochemical data for (-)-kurarinone (**9**) (from Pubchem)

Table 1

Key data from HPLC–PDA–ESITOFMS analysis, semi-preparative HPLC, and database search for compounds 1–16

Cpd	R _{t1} (min) ^a	R _{t2} (min) ^b	λ _{max} (nm)	Accurate mass [M + Na] ⁺ found	Accurate mass [M + Na] ⁺ calc.	Molecular formula	DNP hits ^c
1	12.6	9.4	207, 288, 328	495.1987	495.1995	C ₂₆ H ₃₂ O ₈	2
2	12.6	9.4	207, 288, 328	495.1987	495.1995	C ₂₆ H ₃₂ O ₈	2
3	13.4	10.3	210, 292, 338	479.2055	479.2046	C ₂₆ H ₃₂ O ₇	3
4	13.9	11.7	209, 287, 326	481.1846	481.1838	C ₂₅ H ₃₀ O ₈	1
5	15.3	12.5	212, 293, 340	479.2035	479.2046	C ₂₆ H ₃₂ O ₇	3
6	15.8	13.3	210, 292, 338	465.1901	465.1889	C ₂₅ H ₃₀ O ₇	2
7	16.9	14.2	212, 290, 333	477.1896	477.1889	C ₂₆ H ₃₀ O ₇	5
8	16.9	14.2	212, 290, 333	477.1896	477.1889	C ₂₆ H ₃₀ O ₇	5
9	17.8	15.6	213, 289, 321	461.1952	461.1940	C ₂₆ H ₃₀ O ₆	16
10	19.1	17.2	209, 296, 341	463.1741	463.1733	C ₂₅ H ₂₈ O ₇	8
11	19.9	18.3	nd	475.2089	475.2097	C ₂₇ H ₃₂ O ₆	2
12	19.9	18.3	273, 304, 363	461.1585	461.1576	C ₂₅ H ₂₆ O ₇	1
13	20.3	19.3	208, 292, 336	447.1788	447.1784	C ₂₃ H ₂₈ O ₆	10
14	22.8	22.0	213, 294, 318, 340	461.1949	461.1940	C ₂₆ H ₃₀ O ₆	16
15	23.7	23.2	246,	394 461.1937	461.1940	C ₂₆ H ₃₀ O ₆	16
16	24.6	24.4	242, 295, 321	431.1839	431.1834	C ₂₅ H ₂₈ O ₅	10

^a Retention time in the HPLC–PDA–ESITOFMS analysis.

^b retention time in the semi-preparative HPLC separation.

^c hits in the natural products database (Chapman and Hall Dictionary of Natural Products); search query limited by the term “*Sophora*” and the corresponding molecular formula

nd_N Not determined