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FULL-LENGTH PAPER

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

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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, $\alpha_1\beta_2\gamma_{2S}$. 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

Abbreviations

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

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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 $2\alpha_1$, $2\beta_2$, and $1\gamma_2$ 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 ($\alpha_1\beta_2\gamma_{2S}$), 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 III™ spectrometer operating at 500.13 MHz for ^1H, and 125.77 MHz for ^{13}C, respectively. ^1H-NMR experiments and 2D homonuclear and heteronuclear NMR spectra were measured with a 1 mm TXI probe, and ^{13}C-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 × 150 mm i.d.) and a SunFire Prep C18 (5 µm, 10 × 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 precoated 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 (4×0.5 l, 4 h each), followed by ethyl acetate (4 × 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 formiate 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 ($CHCl_3$: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 ($CHCl_3$: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 ($CHCl_3$: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 ^{1}H detected 1D and 2D NMR experiments, and 0.1 mg of the above 16 pure compounds was dissolved in 10 µl CD_3OD. The following settings were used: 128 scans to record ^{1}H-spectra; 8 scans for ^{1}H,^{1} H-COSY spectra using *cosygpqf* pulse program; 32 scans and 256 increments to record HSQC experiments using the *hsqcedetgp* or *hsqcetgpsi2* pulse program. HMBC spectra were recorded with 64 scans and 128 increments using the *hmbcgplpndqf* 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} ($\log \varepsilon$): 287 (4.13), 326 (3.78) nm. IR (film) ν_{max}: 3401 (br), 2968, 2943, 1651, 1610, and 1268 cm^{-1}. ^{1}H-NMR (500.13 MHz, CD_3OD) 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′); ^{13}C-NMR (125.77 MHz, CD_3OD) 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 $C_{25}H_{30}O_8Na$, 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 (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 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 µA were discarded to exclude voltage clamp errors. Before application of test solutions, a dose–response experiment with GABA

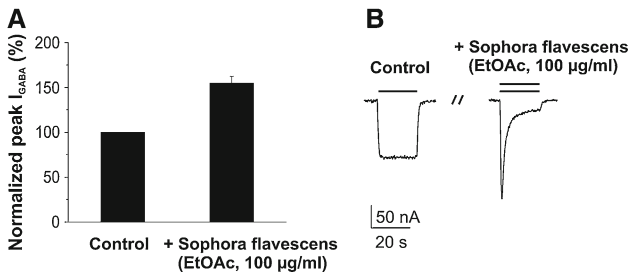


Fig. 1 Potentiation of I_{GABA} by *Sophora flavescens* extract (EtOAc, 100 μg/mL). **a** The mean I_{GABA} potentiation of $55.0 \pm 7.4\%$ ($n = 3$; compared to 100% control) was observed. **b** Chloride currents through $\alpha_1\beta_2\gamma_{2S}$ $\mathrm{GABA_A}$ receptors induced by GABA $\mathrm{EC_{3-10}}$ (control; *left column*) and currents during co-application of GABA ($\mathrm{EC_{3-10}}$) and 100 μg/mL of the *Sophora flavescens* extract (*right column*)

concentrations ranging from 0.1 to 1 mM was performed to determine GABA $\mathrm{EC_{3-10}}$ (typically between 3 and 10 μM).

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 μL DMSO and mixed with 2.97 mL of bath solution [15]. After addition of GABA $\mathrm{EC_{3-10}}$, 100 μL of this solution was applied to the oocytes at a perfusion speed of 300 μL/s. Stock solutions of compounds **6–16** (10 mM in DMSO) were diluted to a concentration of 100 μM with bath solution and then mixed with GABA $\mathrm{EC_{3-10}}$. Of each solution, 100 μL was applied to the oocytes at a speed of 300 μL/s.

Data analysis

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

Results and discussion

Extracts were screened by means of an automated, fast perfusion system during two-microelectrode voltage clamp

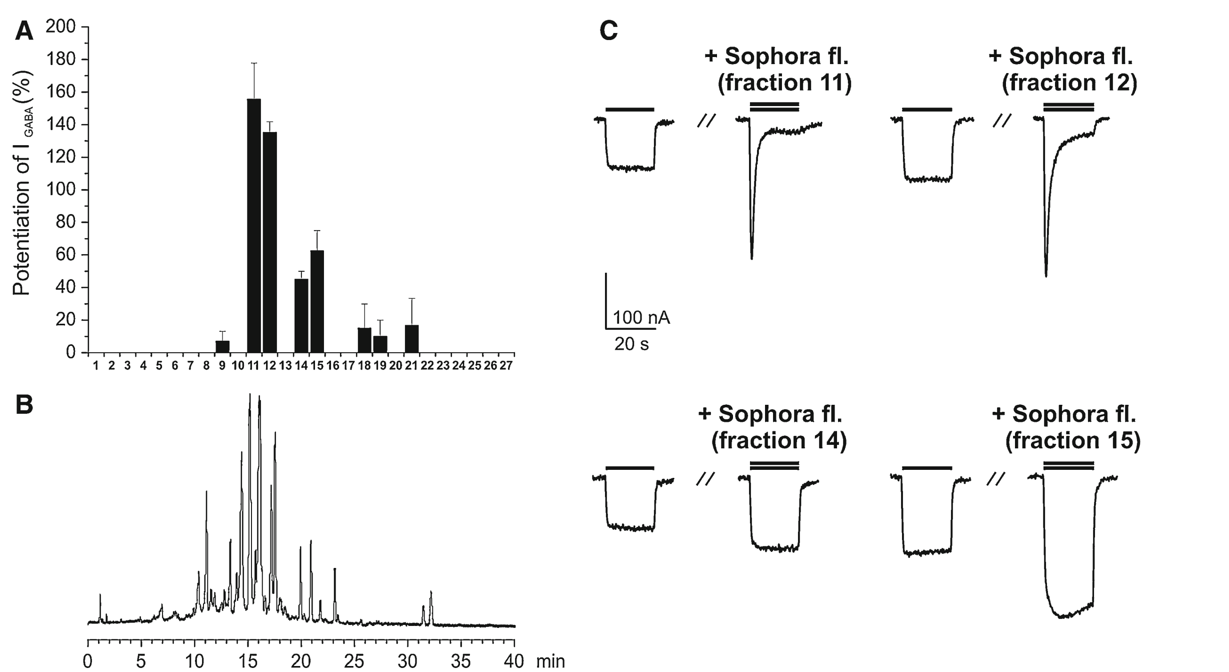


Fig. 2 HPLC-based activity profiling of *Sophora flavescens* extract (EtOAc) for $\mathrm{GABA_A}$ receptor modulating properties. **b** The HPLC chromatogram (254 nm) of a semi-preparative separation of 5 mg extract is shown. **a** Potentiation (in %) of I_{GABA} through $\alpha_1\beta_2\gamma_{2S}$ $\mathrm{GABA_A}$ receptors by 27 fractions of 90 s each fraction is shown above. **c** Representative currents through $\alpha_1\beta_2\gamma_{2S}$ $\mathrm{GABA_A}$ receptors in the presence of GABA ($\mathrm{EC_{3-10}}$, *single bar*, control) and currents recorded during co-application of GABA ($\mathrm{EC_{3-10}}$) and the indicated fraction (*double bar*). Current recordings for four most active fractions (11, 12, 14, and 15) are shown

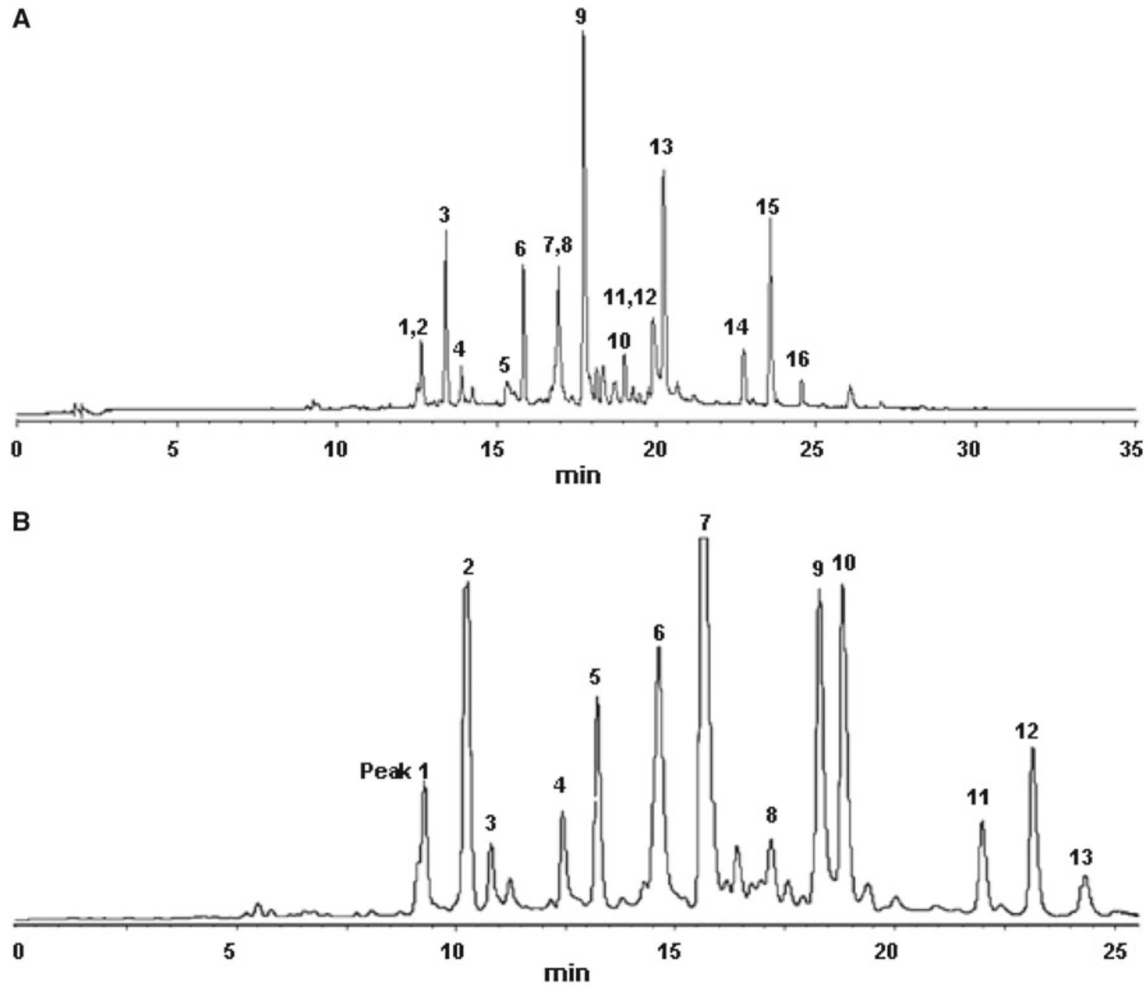


Fig. 3 **a** The optimized HPLC separation for LC–PDA–TOFMS analysis of the EtOAc extract is shown. Peak labeling corresponds to compounds **1**–**16**. 200 μg extract in 20 μL DMSO were injected. The HPLC trace was recorded at 254 nm. **b** The chromatogram (254 nm) of the optimized, semi-preparative 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

measurements in *Xenopus* oocytes expressing functional $GABA_A$ receptors with defined subunit composition ($\alpha_1\beta_2\gamma_{2S}$) [20]. When tested at 100 μg/mL, the *S. flavescens* EtOAc extract enhanced GABA induced chloride ion current (I_{GABA}) by 55.0 ± 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 ± 22.2%, $n = 2$ and 135.1 ± 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 ± 5.0%, $n = 2$ and 62.5 ± 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).

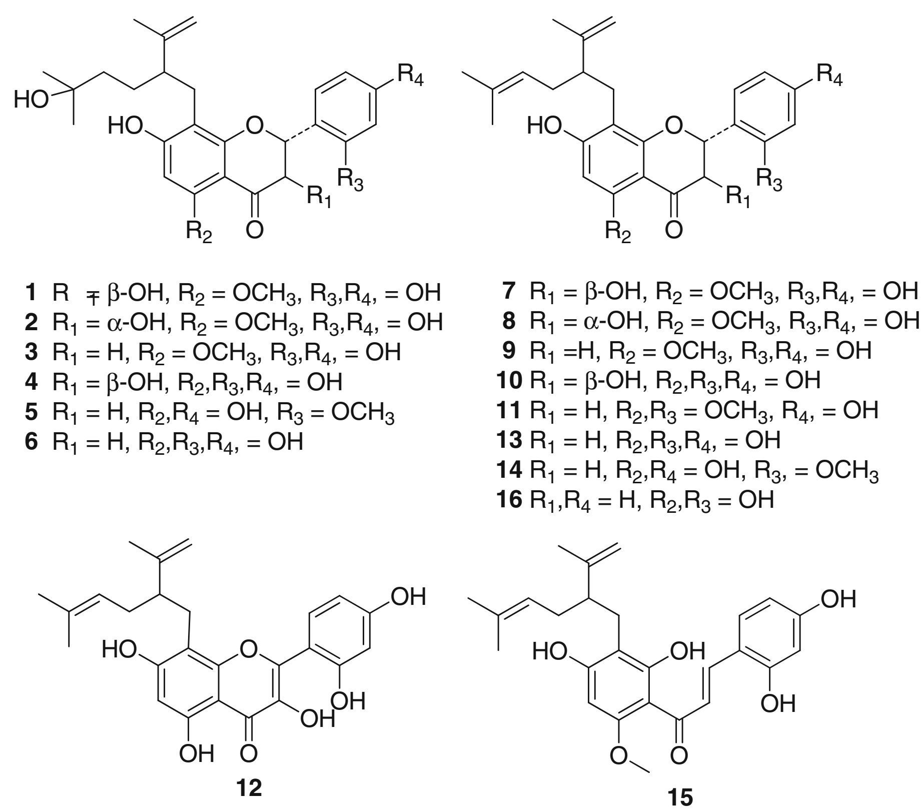
Considering that some HPLC peaks showed partial overlap, separation conditions were optimized for full resolution of the critical HPLC peaks. These conditions were then used to measure high-resolution LC–MS data (Fig. 3a; Table 1). Results of the HPLC–PDA–TOFMS analysis and a database search are summarized 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.

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

Cpd	Rt_1 (min)[a]	Rt_2 (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	$C_{25}H_{28}O_6$	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 Not determined

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



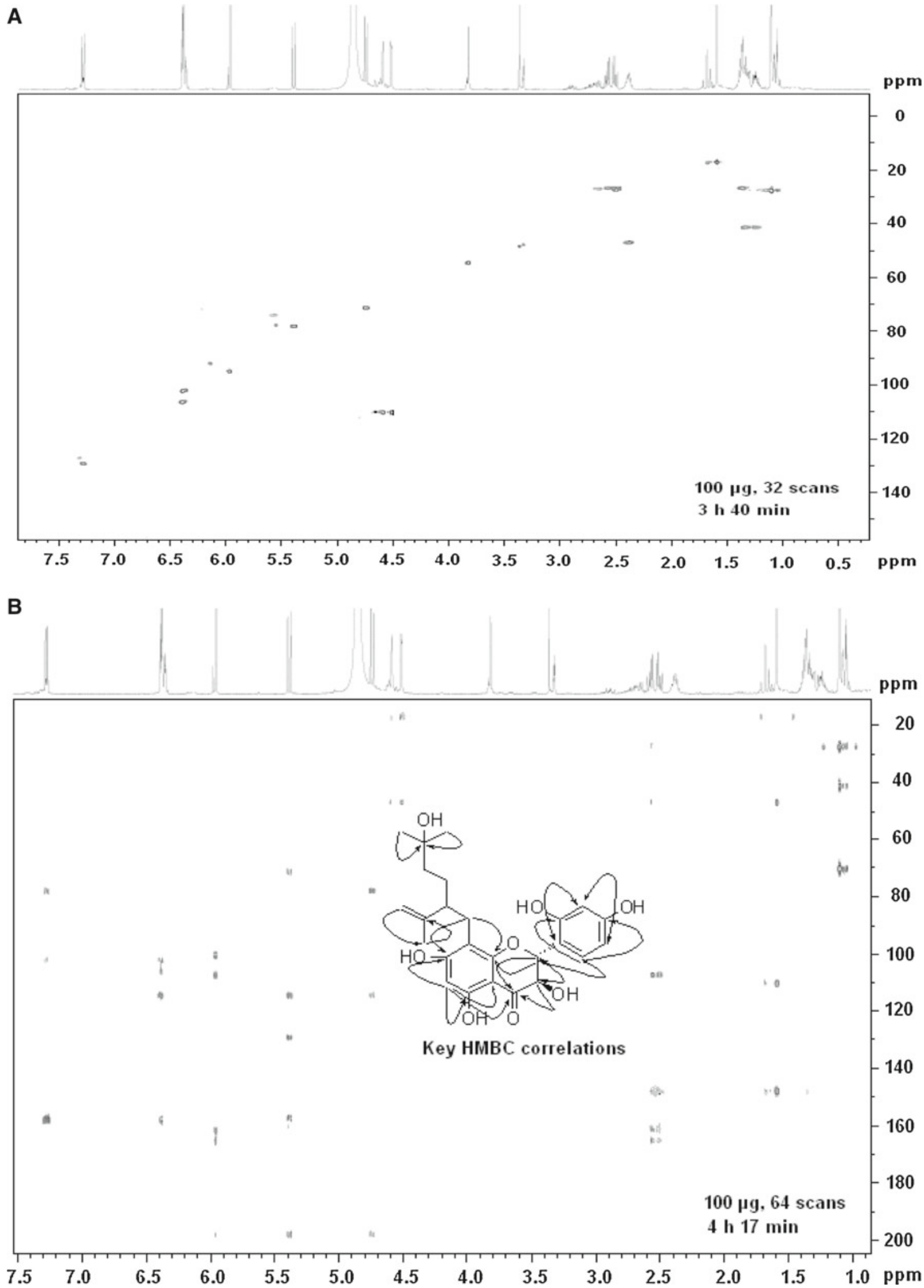


Fig. 5 **a** HSQC spectrum of kushenol Y (**4**) recorded with the TXI probe after HPLC peak collection and **b** HMBC spectrum and key correlations

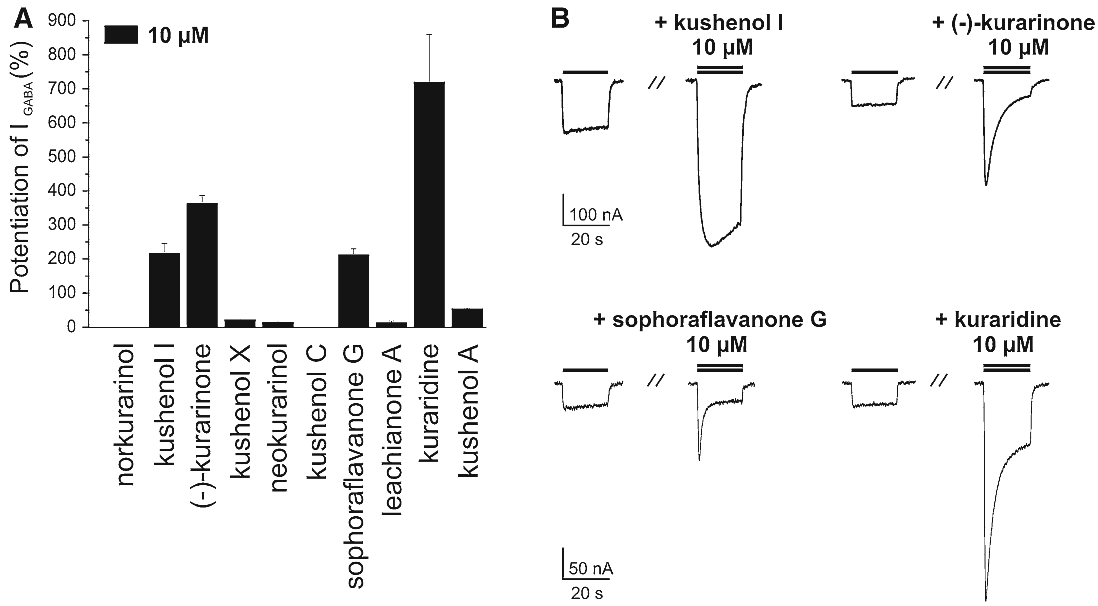


Fig. 6 **a** Potentiation of I_{GABA} through $\alpha_1\beta_2\gamma_{2S}$ $\mathrm{GABA_A}$ receptors by $10\,\mu$M of norkurarinol ($0 \pm 0\%, n = 2$), kushenol I ($216.5 \pm 29.3\%, n = 5$), (−)-kurarinone ($362.3 \pm 23.8\%, n = 5$), kushenol X ($21.6 \pm 3.4\%, n = 2$), neokurarinol ($13.7 \pm 4.6\%, n = 2$), 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 $\alpha_1\beta_2\gamma_{2S}$ $\mathrm{GABA_A}$ receptors induced by GABA ($\mathrm{EC_{3-10}}$, *single bar*, control) and traces recorded during co-application of GABA ($\mathrm{EC_{3-10}}$) and the indicated compound (*double bar*). Current potentiation induced by the four most active compounds, kushenol I (**7**), (−)-kurarinone (**9**), sophoraflavanone G (**13**), and kuraridine (**15**) is shown

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 ^{1}H-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 ^{1}H- 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** ($10\,\mu$M) were co-applied with a GABA ($\mathrm{EC_{3-10}}$) to oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors. Kushenol I (**7**), (−)-kurarinone (**9**), sophoraflavanone

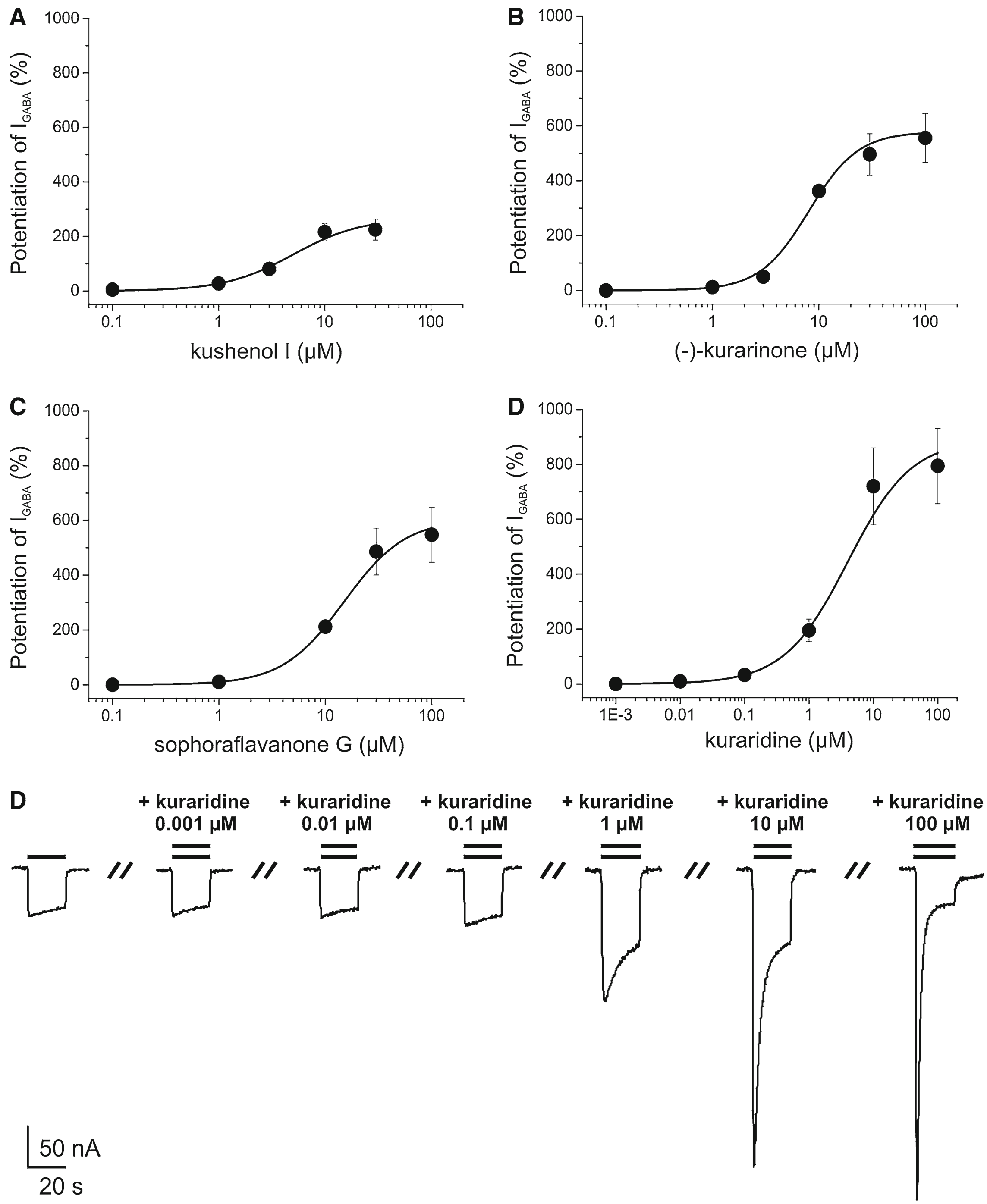


Fig. 7 Concentration–response curves for I_{GABA} enhancement by **a** kushenol I ($EC_{50} = 5.0 \pm 2.3\,\mu M$, $n_H = 1.3 \pm 0.3$, $n = 5$); **b** (−)-kurarinone ($EC_{50} = 8.1 \pm 1.4\,\mu M$, $n_H = 1.9 \pm 0.1$, $n = 4$); **c** sophoraflavanone G ($EC_{50} = 15.0 \pm 3.6\,\mu M$, $n_H = 1.5 \pm 0.2$, $n = 3$); and **d** kuraridine ($EC_{50} = 4.0 \pm 2.4\,\mu M$, $n_H = 0.9 \pm 0.1$, $n = 3$) in *Xenopus* oocytes expressing $GABA_A$ receptors composed of α_1, β_2, and γ_{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*)

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 $\alpha_1\beta_1\gamma_{2S}GABA_A$ 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

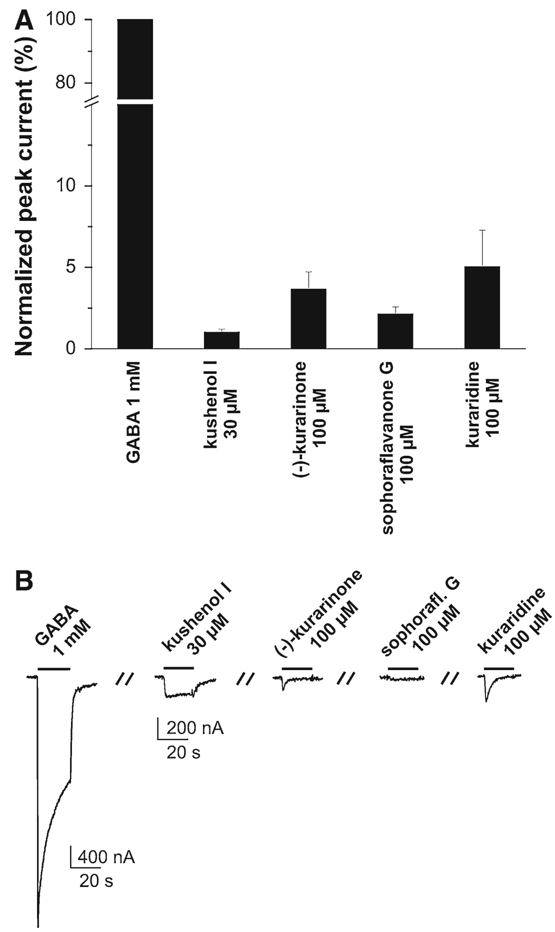
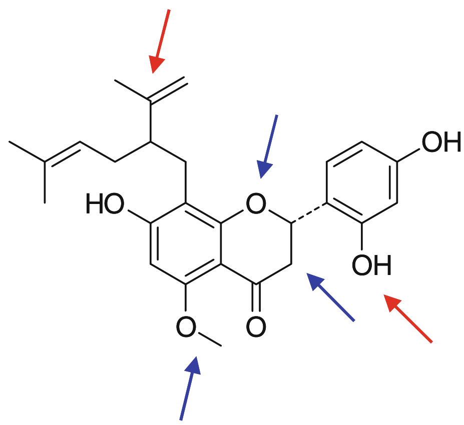


Fig. 8 Activation of $GABA_A$ receptors by kushenol I, (−)-kurarinone, sophoraflavanone G, and kuraridine. **a** Bar graphs illustrate the amplitudes of chloride currents induced in the absence of GABA by saturating concentrations of kushenol I (1.0 ± 0.2%, $n = 3$), (−)-kurarinone (3.7 ± 1.1%, $n = 3$), sophoraflavanone G (2.1 ± 0.4%, $n = 3$), and kuraridine (5.0 ± 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 ($\alpha_1\beta_2\gamma_{2S}$) by kushenol I, (−)-kurarinone, sophoraflavanone G, and kuraridine in comparison to I_{GABA} induced by 1 µM GABA

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



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)

investigate the binding site of the most active compounds **9** and **15**, their subunit specificity, and in vivo activity.

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