HPLC-Based Activity Profiling: Discovery of Piperine as a Positive $GABA_A$ Receptor Modulator Targeting a Benzodiazepine-Independent Binding Site

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A plant extract library was screened for $GABA_A$ receptor activity making use of a two-microelectrode voltage clamp assay on *Xenopus laevis* oocytes. An ethyl acetate extract of black pepper fruits [*Piper nigrum* L. (Piperaceae) 100 μg/mL] potentiated GABA-induced chloride currents through $GABA_A$ receptors (composed of α_1, β_2, and γ_{2S} subunits) by 169.1 ± 2.4%. With the aid of an HPLC-based activity profiling approach, piperine (**5**) was identified as the main active compound, together with 12 structurally related less active or inactive piperamides (**1**−**4**, **6**−**13**). Identification was achieved by on-line high-resolution mass spectrometry and off-line microprobe 1D and 2D NMR spectroscopy, using only milligram amounts of extract. Compound **5** induced a maximum potentiation of the chloride currents by 301.9 ± 26.5% with an EC_{50} of 52.4 ± 9.4 μM. A comparison of the modulatory activity of **5** and other naturally occurring piperamides enabled insights into structural features critical for $GABA_A$ receptor modulation. The stimulation of chloride currents through $GABA_A$ receptors by compound **5** was not antagonized by flumazenil (10 μM). These data show that piperine (**5**) represents a new scaffold of positive allosteric $GABA_A$ receptor modulators targeting a benzodiazepine-independent binding site.

Gamma-aminobutyric acid type A ($GABA_A$) receptors are the major inhibitory neurotransmitter receptors in the brain. The assembly of five subunits forms a central pore that is permeable for chloride ions upon activation by the endogenous ligand γ-aminobutyric acid (GABA). A total of 19 different subunit isoforms have been identified in the human genome, which form $GABA_A$ receptors in numerous combinations.[1] The most abundant $GABA_A$ receptor subtype consists of 2 α_1, 2 β_2, and 1 γ_2 subunits, and more than 10 subtypes composed of other subunit combinations have been identified.[2] $GABA_A$ receptor subtypes differ in tissue localization, functional characteristics, and their pharmacological properties.[3,4]

The therapeutic action of the benzodiazepines and other pharmacological compounds used to treat anxiety, panic, insomnia, and epilepsy is mediated by an enhancement of GABAergic neuronal inhibition through $GABA_A$ receptors.[5,6] Various natural products modulating $GABA_A$ receptors (e.g., flavonoids, monoterpenes, diterpenes, neolignans, and β-carbolines) have been identified.[7,8] Little is known in most cases, however, about their subunit selectivity, and presently no natural product derived compound is in clinical development.

We recently embarked on a project aimed at the discovery of $GABA_A$ receptor modulating compounds with scaffolds new for the target. In a screening of 880 plant and fungal extracts with an automated functional assay using *Xenopus* oocytes expressing $GABA_A$ ($\alpha_1\beta_2\gamma_{2S}$) receptors, an ethyl acetate extract of *Piper nigrum* showed promising activity. This observation was intriguing insofar as it somehow seemed to corroborate traditional use of pepper in Asian folk medicine as antiepileptic, antianxiety, sedative, and sleep-inducing herbal preparations.[9−11] Therefore, we deemed this extract sufficiently interesting to identify the constituent(s) responsible for the $GABA_A$ receptor modulating activity with the aid of HPLC-based activity profiling. HPLC-based activity profiling is a rapid and miniaturized approach for localization, dereplication, and characterization of bioactive natural products in extracts.[12] We have successfully used it with various cell-based and biochemical assays[13−15] and recently developed and validated a profiling protocol for the discovery of new $GABA_A$ receptor ligands.[16] Here, we describe the identification of piperine (**5**) as a new scaffold of positive allosteric modulators of the $GABA_A$ receptor targeting a benzodiazepine-independent binding site.

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 ($\alpha_1\beta_2\gamma_{2S}$).[17] When tested at 100 μg/mL, the *P. nigrum* ethyl acetate extract enhanced GABA-induced chloride ion current (I_{GABA}) by 169.1 ± 2.4%. The extract was submitted to HPLC-based activity profiling using a validated protocol.[16] The chromatogram of a semipreparative separation of extract (5 mg) and the corresponding activity profile of the time-based fractionation (22 microfractions of 90 s each) are shown in Figure 1.

A prominent peak of activity was found in fractions 7 and 8 (potentiation of I_{GABA} by 316.1 ± 7.0% and 248.1 ± 10.6%, respectively), which contained the major compound of the extract. Fraction 9 showed moderate activity (35.5 ± 1.1%), while fractions 6, 10, and 13 were only marginally active. However, nonresolved peaks occurred in the chromatogram, in particular in the time window of the activity peak. Therefore, separation conditions were optimized for full resolution of the critical HPLC peaks. These conditions were then used to measure high-resolution LC-MS data and for peak-based microfractionation by semipreparative HPLC for subsequent off-line microprobe NMR.

The semipreparative HPLC chromatogram obtained with 10 mg of extract is shown in Figure 2A. A total of 30 peaks were collected and submitted to parallel evaporation. For each peak a ^{1}H NMR spectrum (128 scans) was recorded with a 1 mm TXI probe. For 13 peaks, the spectra were of sufficient quality for reliable structure identification. Molecular formulas were calculated for compounds **1**−**13** using accurate mass data obtained by HPLC-PDA-ESI-TOFMS analysis of the extract. Since *P. nigrum* is phytochemically well studied (95 compound entries in the Chapman and Hall Dictionary of Natural Products[18]), 1−4 entries were found for each

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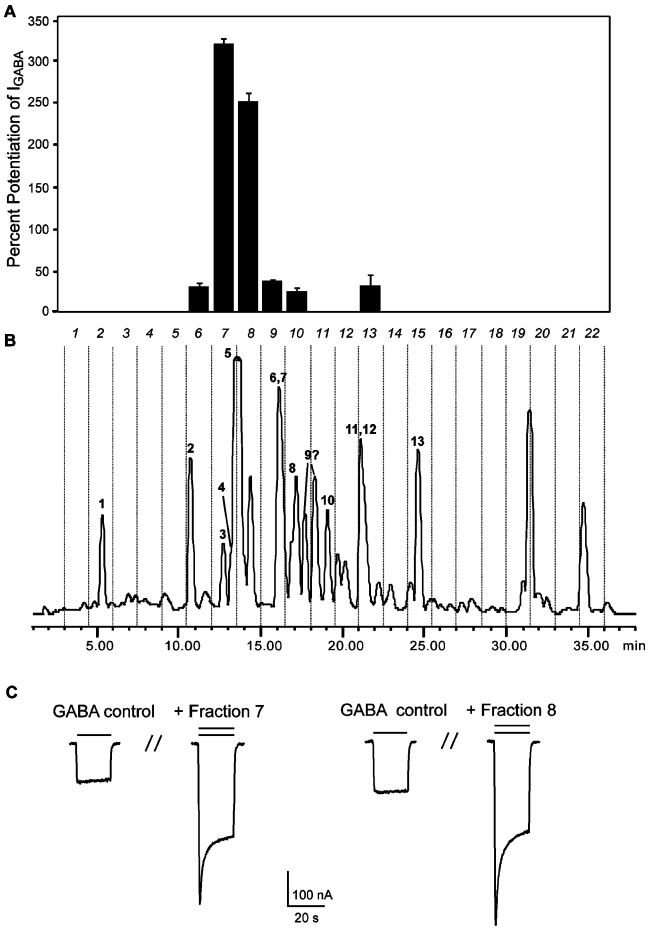


Figure 1. HPLC-based activity profiling of the black pepper extract for $GABA_A$ receptor modulating properties. The HPLC chromatogram (254 nm) of a semipreparative separation of 5 mg of extract is shown in B. Peak numbering corresponds to compounds **1**−**13**. The 22 collected time-based fractions, 90 s each, are indicated with dashed lines. The potentiation of the GABA-induced chloride current in *Xenopus* oocytes (I_{GABA}) by each fraction is shown in A. Part C shows typical traces for the modulation of GABA-induced chloride currents through $GABA_A$ ($\alpha_1\beta_2\gamma_{2S}$) receptors by fractions 7 and 8 of the *P. nigrum* EtOAc extract.

of the 13 molecular formulas. Results of the HPLC-PDA-ESI-TOFMS analysis and database search are summarized in Table 1.

Compounds **1**−**13** were unambiguously identified with the aid of ^{1}H NMR data and comparison with published reference data.[19−29] The major peak was piperine (**5**), the main pungent piperamide in *P. nigrum*,[30] and the remaining compounds were all structurally related amides (Chart 1). Data for **1**−**13** are provided as Supporting Information. Figure 2B shows ^{1}H NMR spectra of minor amides **3** and **4**, and **5** collected from the peak-based microfractionation. Representative HSQC and HMBC spectra of compounds **4** and **5**, respectively, are shown in Figure 2C to provide an impression of the quality of spectra that can be obtained with this off-line HPLC-microprobe NMR approach.

For a quantitative determination of $GABA_A$ receptor activity of compounds in the active time window, piperlonguminine (**3**), piperanine (**4**), and piperine (**5**) were purified at preparative scale, along with structurally related trichostachine (**2**) and piperettine (**7**). These compounds were tested at a concentration of 100 μM in the oocyte assay. Piperine (**5**) was most efficient, as it potentiated I_{GABA} by 226 ± 26%, while piperanine (**4**) at the same concentration was less efficient (potentiation of I_{GABA} by 138 ± 20%). Weak enhancement of I_{GABA} (32 ± 24%) was observed for **7**, and compounds **2** and **3** slightly inhibited I_{GABA} (−29 ± 13% and −10 ± 3%, respectively) (Chart 1). Given the lack of I_{GABA} potentiation in other fractions of the activity profile (Figure 1A), the other amides must be considered inactive.

As shown in Figure 3, both piperanine (**4**) and piperine (**5**) enhanced I_{GABA} at a GABA EC_{5-10} in a dose-dependent manner. The currents were stimulated at concentrations ≥ 1 μM. Maximum I_{GABA} enhancement by **4** and **5** (187 ± 10%, n = 3, and 302 ± 26%, n = 3, respectively) occurred at ∼300 μM with EC_{50} values of 56 ± 19 and 52 ± 9 μM, respectively. The application of **5** prior to GABA showed no activity, indicating an allosteric modulation of the receptor (response to application of 100 μM **5** in the absence of GABA is shown in Figure 3C). Furthermore, the application of 100 μM trichostachine (**2**), piperlonguminine (**3**), piperanine (**4**), and piperettine (**7**) in the absence of GABA displayed as well no activity on $GABA_A$ receptors composed of $\alpha_1\beta_2\gamma_{2S}$ subunits (see Figure 3C).

To investigate a possible interaction of **5** with the benzodiazepine binding site, we analyzed its effect on I_{GABA} in the presence of the benzodiazepine receptor antagonist flumazenil (10 μM). Potentiation of I_{GABA} by 300 μM piperine (**5**) was not significantly affected by flumazenil (304 ± 40%, n = 3 control vs 334 ± 108% in the presence of flumazenil, n = 3) (Figure 4A and B). Figure 4C illustrates the additive effects of 100 μM **5** (180 ± 69%, n = 3) and diazepam (1 μM) (204 ± 48%, n = 3) on I_{GABA} when coapplied (391 ± 104%, n = 3) (Figure 4C and D).

The example of piperamides highlights the advantages of an HPLC-based approach and, in particular, the possibility of obtaining valuable preliminary structure−activity information via the characterization of focused compound subsets without a need for preparative purification. Activity can be easily localized in the extract, and all peaks in the critical time window rapidly separated by semipreparative HPLC under optimized conditions (Figures 1 and 2). A series of compounds structurally related to **5** could be identified by a combination of on-line (HPLC-PDA-HRMS) and off-line (microprobe NMR) requiring only milligram amounts of extract. Off-line NMR with disposable 1 mm tubes has several attractive features for profiling. Collected HPLC peaks can be processed in parallel (evaporation, sample preparation for NMR). An NMR autosampler permits unattended measurement of 1D ^{1}H NMR spectra, on the basis of which the need for more advanced NMR experiments can be checked. The microtubes can be stored for a certain time similar to classical NMR tubes, and time-consuming experiments can be performed at a later moment. By extending the profiling beyond the active compounds toward inactive but structurally related molecules, small focused "virtual" libraries are generated that provide valuable information for preliminary structure−activity considerations. In the present case it was clear that the nature of the amide moiety and the chain length between the aromatic ring and the amide were critical for the observed allosteric modulation of $GABA_A$ receptors. Rigidity of the chain might also be important for the efficiency, as the 4,5-dihydro derivative **4** was significantly less efficient in stimulating I_{GABA} compared to **5** (Figure 3).

Very recently, Pedersen et al. reported **5** as a $GABA_A$ receptor ligand presumed to bind to the benzodiazepine binding site.[31] However, only low affinity (IC_{50} of 1.2 mM in a [^{3}H]-flumazenil binding assay) was reported. We assume that the activity observed by Pedersen et al.[31] was due to low-affinity binding to the benzodiazepine binding site at very high compound concentrations. Besides the fact that the affinity was extremely low, a binding assay provides neither information on the intrinsic activity of a compound nor insights into the molecular mechanism of its action.

In contrast, our functional assay clearly demonstrated a positive allosteric modulation of the $GABA_A$ receptor by **5** and **4** with comparable potencies and revealed a higher efficiency of **5** in stimulating I_{GABA} (Figure 3). Studies with the benzodiazepine receptor antagonist flumazenil (Figure 4A and B) and the additive

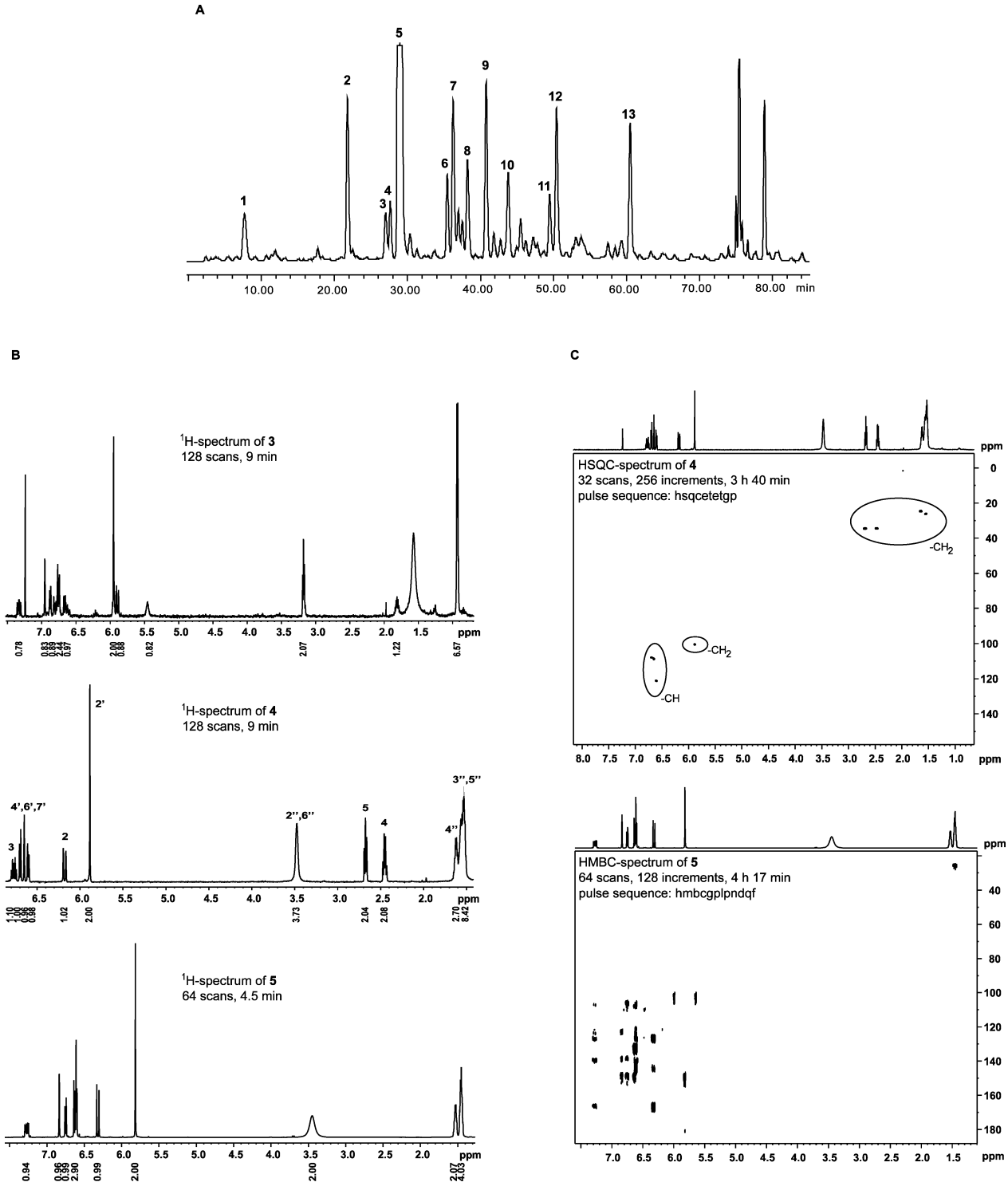


Figure 2. Part A shows the chromatogram (254 nm) of the optimized, semipreparative HPLC separation of the active *P. nigrum* extract (10 mg in 100 μL of DMSO). A total of 30 peaks were collected for off-line microprobe NMR. Peak labeling corresponds to compounds **1**−**13**. Part B shows ^{1}H NMR spectra of selected compounds obtained by the separation mentioned above, whereas part C shows the spectra of two representative 2D NMR experiments.

effects of **5** and diazepam (Figure 4C and D) clearly demonstrate that **5** does not interact with the benzodiazepine binding site.

Benzodiazepines may cause undesirable effects including reduced coordination, cognitive impairment, increased accident proneness, physiological dependence, and withdrawal symptoms.[5,32] $GABA_A$ receptor ligands with fewer side effects are, therefore, an unmet medical need.[5,33]

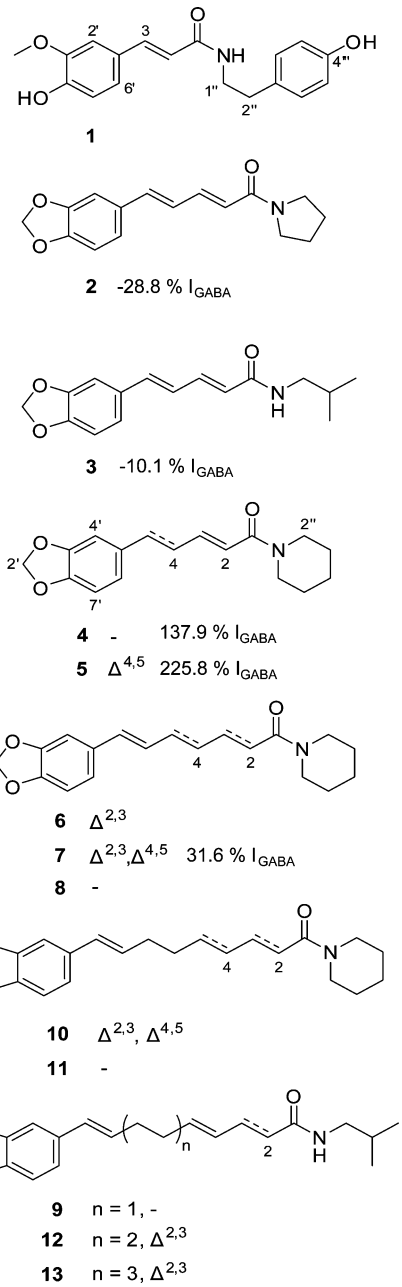
Our data show that **5** represents a new scaffold for positive allosteric $GABA_A$ receptor modulators interaction with a benzodiazepine-independent binding site. Our study provides a molecular basis for earlier reports on the antidepressant[34,35] and anticonvulsant activity of piperine (**5**)[9,36,37] in rodents and corroborates traditional uses of *Piper nigrum* and other *Piper* species.[9−11,38] Even though a number of other pharmacological properties have been reported,[39−42] **5** may represent an interesting lead structure. It complies in all respects with Lipinski's "rule of five" (MW: 285 g/mol, ClogP: 3.31, H-bond-donor/acceptor sites: 0/4.),[43] is orally bioavailable, and is readily accessible. Piperine (**5**) is, however, known

Table 1. Data of HPLC-PDA-TOFMS Analysis and Associated Database Findings of Compounds **1**−**13**, Which Were Purified from the Active Ethyl Acetate Extract by Semipreparative HPLC

cpd	t_{R1} (min)[a]	t_{R2} (min)[b]	λ_{max} (nm)	acc. mass found	acc. mass calcd	calcd formula	DNP hits[c]
1	6.1	6.6	221, 294, 316	313.1295	313.1308	$C_{18}H_{19}NO_3$	2
2	21.5	20.6	241, 309, 342	271.1195	271.1202	$C_{16}H_{17}NO_3$	1
3	26.8	25.4	243, 309, 338	273.1346	273.1359	$C_{16}H_{19}NO_3$	2
4	27.9	26.2	232, 285	287.1513	287.1515	$C_{17}H_{21}NO_3$	2
5	29.0	27.1	255, 310, 338	285.1361	285.1359	$C_{17}H_{19}NO_3$	4
6	36.3	33.7	213, 263, 305	313.1673	313.1672	$C_{19}H_{23}NO_3$	2
7	36.9	34.5	348	311.1512	311.1515	$C_{19}H_{21}NO_3$	1
8	39.2	36.4	210, 263, 310, 358	315.1826	315.1828	$C_{19}H_{25}NO_3$	2
9	41.7	38.7	261	327.1816	327.1828	$C_{20}H_{25}NO_3$	2
10	44.9	41.6	268, 305	339.1837	339.1828	$C_{21}H_{25}NO_3$	2
11	50.4	47.3	261, 307	343.2145	343.2141	$C_{21}H_{29}NO_3$	2
12	51.4	48.0	213, 263, 303	355.2141	355.2141	$C_{22}H_{29}NO_3$	3
13	61.1	58.5	261, 303	383.2460	383.2454	$C_{24}H_{33}NO_3$	3

[a] Retention time in the HPLC-PDA-ESITOFMS analysis. [b] Retention time in the semipreparative HPLC separation (Figure 2A). [c] Hits in the natural products database (Chapman and Hall Dictionary of Natural Products); search query limited by the term "*piper*".

Chart 1. Structures of Piperamides **1**−**13** and Potentiation of GABA-Induced Chloride Current (I_{GABA}) in *Xenopus* Oocytes by 100 μM **2**−**5** and **7**



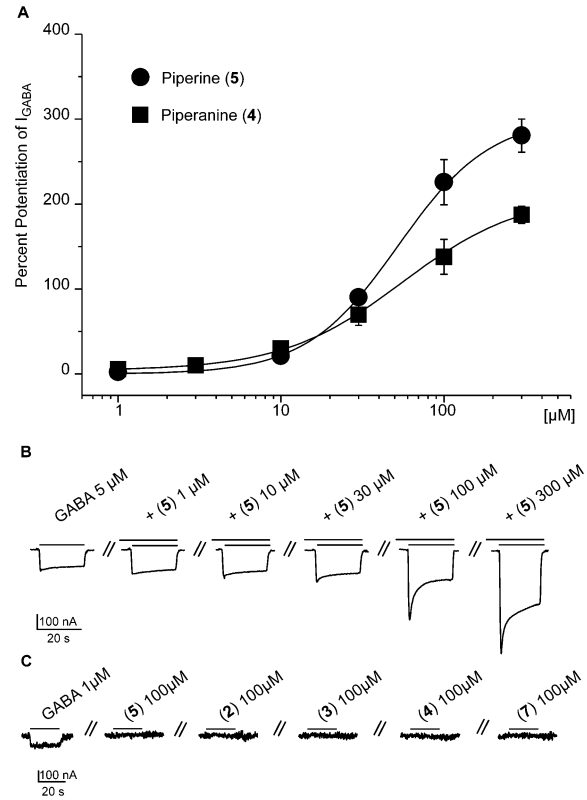


Figure 3. Part A shows the concentration−response curves for compounds **4** and **5** on $GABA_A$ receptors composed of α_1, β_2, and γ_{2S} subunits using a GABA EC_{5-10}. Part B displays typical traces for modulation of chloride currents through $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors by piperine (**5**). In part C representative currents illustrate the absence of direct activation of $GABA_A$ receptors ($\alpha_1\beta_2\gamma_{2S}$) by piperine (**5**), trichostachine (**2**), piperlonguminine (**3**), piperanine (**4**), and piperettine (**7**) at 100 μM in comparison to a GABA-induced current at 1 μM.

to activate TRPV1 receptors[40] and is a general inhibitor of both phase I and phase II metabolism,[44] which might cause side effects and drug interactions. It should be explored to what extent pharmacological promiscuity of piperamides can be reduced through structural modifications.

Experimental Section

General Experimental Procedures. NMR spectra were recorded at room temperature with a Bruker Avance III spectrometer operating at 500.13 MHz. Proton NMR experiments and 1D and 2D homonuclear and heteronuclear NMR spectra were measured with a 1 mm TXI probe.

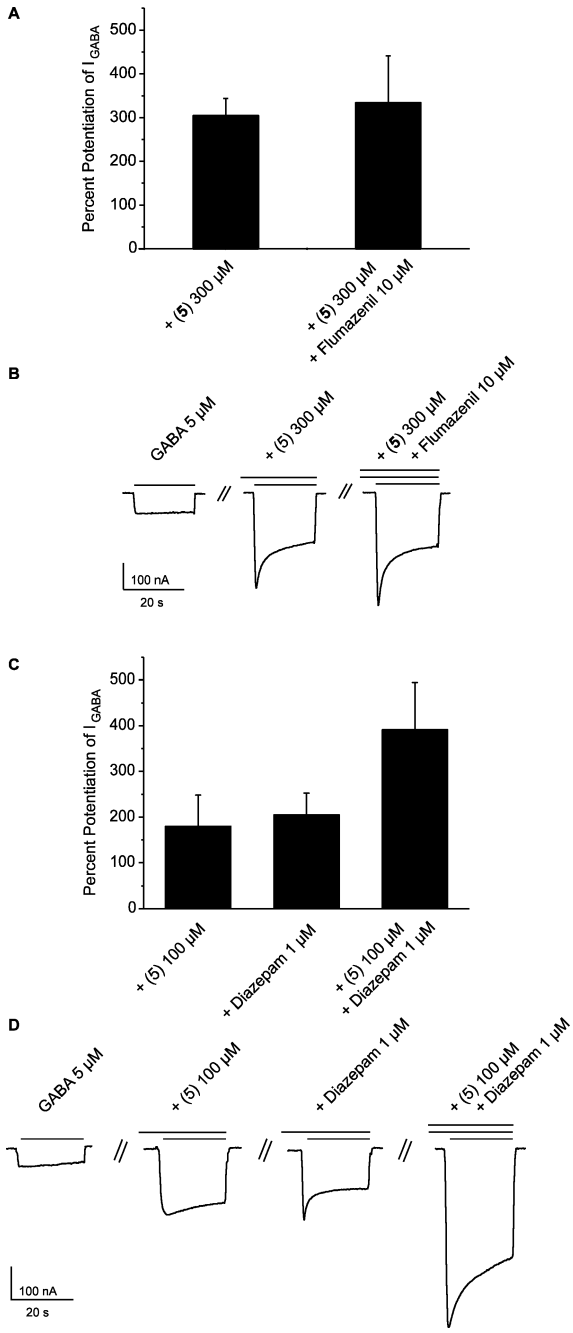


Figure 4. Effect of (**5**) on I_{GABA} in the presence of flumazenil and diazepam. (A) Stimulation of I_{GABA} by **5** in the presence of flumazenil (10 μM). The left bar shows the positive allosteric modulation of the GABA (EC_{5-10})-induced chloride current by 300 μM piperine (**5**). The right bar illustrates that flumazenil does not antagonize the **5**-induced enhancement of I_{GABA}. (B) Typical GABA-induced chloride currents in the absence and presence of the indicated concentrations of **5**, or **5** and flumazenil, respectively. (C) Additive effects of **5** and diazepam on I_{GABA}. The left bar illustrates the enhancement of I_{GABA} by 100 μM (**5**); the bar in the middle, by 1 μM diazepam, and the right bar illustrates enhancement of I_{GABA} when both compounds were coapplied. (D) Representative chloride currents induced by 5 μM GABA (corresponding to EC_{5-10}), current enhancement by **5** (100 μM) and diazepam (1 μM), and I_{GABA} during coapplication of both compounds.

Spectra were analyzed using Bruker TopSpin 2.1 software. High-resolution mass spectra (HPLC-PDA-ESITOFMS) were obtained on a micrOTOF ESI-MS system (Bruker Daltonics) connected via a 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). Semipreparative HPLC separations for activity profiling and off-line 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 semipreparative HPLC fractions was performed with a Genevac EZ-2 plus vacuum centrifuge (Avantec). SunFire C18 (3.5 μm, 3.0 × 150 mm) and SunFire Prep C18 (5 μm, 10 × 150 mm) columns (Waters) were used for HPLC-PDA-ESITOFMS and semipreparative HPLC, respectively. HPLC-grade acetonitrile (Scharlau Chemie S.A.) and water were used for HPLC separations. Solvents used for extraction and column chromatography were of analytical grade. Petroleum ether of technical grade was purified by distillation for extraction and column chromatography. Silica gel (63−200 μm, Merck) was used for column chromatography.

Plant Material. Dried fruits of *P. nigrum* L. were purchased from the Juhuayuan Herbal Market in Kunming (Yunnan Province, China). A voucher specimen (00 286) 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 the temperature was set at 70 °C. For isolation of the piperamides, 343 g of ground fruits was extracted by maceration at room temperature with petroleum ether (4 × 2.5 L, 2 h each), followed by ethyl acetate (4 × 2.5 L, 2 h each). The solvents were evaporated at reduced pressure to yield 14.22 and 18.84 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,[16] with minor modifications: separation was carried out on a semipreparative HPLC column with acetonitrile (solvent A) and water (solvent B) using the following gradient: 30% A to 100% A for 30 min, hold for 10 min. The flow rate was 4 mL/min, and 50 μL of extract (100 mg/mL in DMSO) was injected. A total of 22 time-based microfractions of 90 s each were collected. Microfractions were evaporated in parallel and submitted to activity testing.

HPLC-PDA-ESITOFMS. The ethyl acetate extract of *P. nigrum* was analyzed with acetonitrile (solvent A) and water containing 0.1% formic acid (solvent B) using an optimized gradient profile: 30% A isocratic for 5 min, 30% to 80% A in 65 min, 80% to 100% A in 1 min, hold for 9 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 200 μL. Conditions for ESITOFMS were as follows: spectra were recorded in the range m/z 100−600 in positive 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 2-propanol/water (1:1) containing 5 mM NaOH.

Semipreparative HPLC and Off-Line Microprobe NMR. Separation of *P. nigrum* 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 4 mL/min, and the injected volume of extract was 100 μL at a concentration of 100 mg/mL in DMSO. A total of 30 peak-based fractions were collected manually, evaporated in parallel, and redissolved in d_4-methanol, d_1-chloroform, or d_6-DMSO. For NMR experiments of the collected fractions, the following settings were used: 64 or 128 scans to record ^{1}H spectra; 8 scans for ^{1}H^{1}H-COSY spectra using the *cosygpqf* pulse program; 32 scans and 256 increments to record HSQC experiments using the *hsqcedetgp* or *hsqcetgpsi2* pulse program, and for HMBC-NMR, 64 scans, 128 increments, and the *hmbcgplpndqf* pulse program.

Isolation of Piperamides. A portion (16.6 g) of the ethyl acetate extract was separated by chromatography on a silica gel column (70 × 6.5 cm i.d.) using a step gradient of petroleum ether (solvent A) and ethyl acetate (solvent B) in ratios of 10:0, 8:2, 6:4, 4:6, 2:8, and 10:0 (2 L each), respectively, to yield 20 fractions (1−20). Fractions 10 and 11 (2.02 and 9.02 g, respectively) were used for crystallization of **5** (7.16 g). The residue of the mother liquor of fraction 10 (830 mg) was separated into 18 fractions (10A−10R) by medium-pressure liquid chromatography on a silica gel cartridge (40−63 μm, 150 × 40 mm i.d.). A gradient of 10% B to 50% B in 95 min and 50% B to 100% B

in 60 min was applied at a flow rate of 20 mL/min. From fraction 10L (140 mg), a total of 65.3 mg was separated by injecting different volumes of a concentration of 10 mg/mL DMSO onto the semipreparative HPLC column in order to isolate **4** (1.5 mg) and **7** (13.4 mg). The gradient profile using acetonitrile (solvent C) and water containing 0.1% formic acid (solvent D) was 40% C to 55% C in 25 min. The flow rate was set at 4 mL/min. Further on, a total of 68.3 mg of fraction 19 (1.2 g) and 56.2 mg of fraction 9 (60 mg) were separated using the semipreparative HPLC system by repeated injection of different volumes of 10 mg/mL DMSO dilutions. Separation of fraction 19 yielded compound **2** (10.5 mg) using the following gradient system: 40% isocratic C for 2 min, followed by 40% C to 45% C in 18 min. Fraction 9 was separated isocratically at 50% C to yield compound **3** (6.5 mg). The flow rate for both separations was 4 mL/min. Compounds **2**−**4** and **7** were identified by comparison of physicochemical data (^{1}H NMR and UV−vis) with published values[20,23,24,27] and recorded data for the peaks in the off-line HPLC microprobe NMR approach. The purity of the compounds was >95% (^{1}H NMR).

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. (2006).[45] Female *Xenopus laevis* (NASCO, Fort Atkinson, WI) were anesthetized by exposing them for 15 min to a 0.2% MS-222 (methanesulfonate salt of 3-aminobenzoic acid ethyl, Sigma) solution before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/mL collagenase (Type 1A, Sigma). Synthesis of capped runoff 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. The amount of injected cRNA mixture was determined by means of a NanoDrop ND-1000 (Kisker Biotech). To ensure expression of the gamma subunit in $\alpha_1\beta_2\gamma_{2S}$ receptors, rat cRNAs were mixed in a 1:1:10 ratio. Oocytes were then stored at 18 °C in ND96 solution.[46] 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) 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. Oocytes with maximal current amplitudes > 3 μA were discarded to exclude voltage clamp errors.

Fast Solution Exchange during I_{GABA} Recordings. Test solutions (100 μL) were applied to the oocytes at a speed of 300 μL/s by means of an automated fast perfusion system.[17] In order to determine GABA EC_{5-10} (typically between 3 and 8 μM), a dose−response experiment with GABA concentrations ranging from 0.1 μM to 1 mM was performed. Stock solution of *P. nigrum* (10 mg/mL in DMSO) was diluted to a concentration of 100 μg/mL with bath solution and then mixed with GABA EC_{5-10}. As previously described, microfractions collected from the semipreparative HPLC separations were dissolved in 30 μL of DMSO and subsequently mixed with 2.97 mL of bath solution containing GABA EC_{5-10}.[16] Stock solutions of compounds **2**−**5** and **7** (10 mM in DMSO) were diluted to a concentration of 100 μM with bath solution and then mixed with GABA EC_{5-10} or applied alone. For dose−response experiments, bath solution containing compound **4** or **5** in concentrations ranging from 1 to 300 μM was applied to the oocyte 90 s prior to application of the corresponding compound solution containing GABA EC_{5-10}. Diazepam and flumazenil (Sigma) were dissolved in DMSO (10 mM) and subsequently diluted in bath solution or bath solution containing GABA EC_{5-10}. Oocytes were preincubated for 90 s with flumazenil or diazepam before the corresponding GABA EC_{5-10} containing test solution was applied.[45]

Data Analysis. Enhancement of the chloride current (I_{GABA}) was defined as $I_{(GABA+Comp)}/I_{GABA} - 1$, where $I_{(GABA+Comp)}$ is the current response in the presence of a given compound, and I_{GABA} is the control GABA-induced chloride current. Data are given as mean ± SE of at least two oocytes and ≥2 oocyte batches.

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Supporting Information Available: Spectral characterization data of compounds **1**−**13**, including ^{1}H and ^{13}C chemical shifts from 1D-^{1}H NMR- and 2D-heteronuclear NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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