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Identification of dihydrostilbenes in *Pholidota chinensis* as a new scaffold for $GABA_A$ receptor modulators

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ABSTRACT

A dichloromethane extract of stems and roots of *Pholidota chinensis* (Orchidaceae) enhanced GABA-induced chloride currents (I_{GABA}) by 132.75 ± 36.69% when tested at 100 μg/mL in a two-microelectrode voltage clamp assay, on *Xenopus laevis* oocytes expressing recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. By means of an HPLC-based activity profiling approach, the three structurally related stilbenoids coelonin (**1**), batatasin III (**2**), and pholidotol D (**3**) were identified in the active fractions of the extract. Dihydrostilbene **2** enhanced I_{GABA} by 1512.19 ± 176.47% at 300 μM, with an EC_{50} of 52.51 ± 16.96 μM, while compounds **1** and **3** showed much lower activity. The relevance of conformational flexibility for receptor modulation by stilbenoids was confirmed with a series of 13 commercially available stilbenes and their corresponding semisynthetic dihydro derivatives. Dihydrostilbenes showed higher activity in the oocyte assay than their corresponding stilbenes. The dihydro derivatives of tetramethoxy-piceatannol (**12**) and pterostilbene (**20**) were the most active among these derivatives, but they showed lower efficiencies than compound **2**. Batatasin III (**2**) showed high efficiency but no significant subunit specificity when tested on the receptor subtypes $\alpha_1\beta_2\gamma_{2s}$, $\alpha_2\beta_2\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$, $\alpha_4\beta_2\gamma_{2s}$, $\alpha_5\beta_2\gamma_{2s}$, $\alpha_1\beta_1\gamma_{2s}$, and $\alpha_1\beta_3\gamma_{2s}$. Dihydrostilbenes represent a new scaffold for $GABA_A$ receptor modulators.

1. Introduction

$GABA_A$ receptors are ligand-gated chloride channels physiologically activated by GABA, the major inhibitory neurotransmitter in the brain. Structurally, they are heteropentameric assemblies forming a central chloride-selective channel. Up to now 19 different subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3) have been identified. Depending on the nature, stoichiometry, and arrangement of these subunits, the receptor subtypes exhibit distinct pharmacological profiles providing the potential for rational drug therapy in several disorders related with impaired GABAergic function, such as epilepsy, insomnia, anxiety, and mood disorders.[1–3] $GABA_A$ receptors are the target for many clinically important drugs such as benzodiazepines (BDZs), barbiturates, neuroactive steroids, anesthetics, and certain other CNS depressants. Due to their lack of subunit specificity, these drugs show a number of adverse side effects. Hence, there is a need for subtype-selective drugs devoid of the side effects of the classical BDZs. Despite the availability of experimental subunit-specific GABAergic drugs for more than a decade, no subtype-selective $GABA_A$ receptor modulators have been introduced into clinical practice.[3,4]

In a search for new natural product-derived $GABA_A$ receptor modulators, we screened a library of 880 fungal and plant extracts in an automated functional two-microelectrode voltage clamp assay on *Xenopus* oocytes[5] transiently expressing $GABA_A$ receptors of the $\alpha_1\beta_2\gamma_{2S}$ subtype, the most abundant subunit combination in the human brain.[2] In this screening the dichloromethane extract of stems and roots of *Pholidota chinensis* LINDL (Orchidaceae) showed promising activity.

Orchidaceae is the largest family of flowering plants, with around 25,000 species in over 800 genera. The family shows worldwide distribution, with greatest diversity in tropical and subtropical climate zones. Apart from their ornamental value, many orchids have been used as medicinal plants. In traditional Chinese medicine we find the earliest written records for medicinal uses of orchids.[6–8] In Chinese folk medicine, the whole plant of *P. chinensis* (*shi xian tao*) has long been used in the treatment of diverse conditions, such as hypertension, headache, gastroenteritis, and bronchitis. Previous pharmacological studies on *P. chinensis* reported sedative and anticonvulsant activities.[9–12] The genus *Pholidota* comprises approximately 30 species distributed from tropical Asia to tropical

Abbreviations: BDZs, benzodiazepines; GABA, gamma-aminobutyric acid; $GABA_A$Rs, gamma-aminobutyric acid type A receptors.

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Australia. Phytochemical studies on the genus showed the presence of triterpenes, steroids, lignans, benzoxepines, and stilbenoids.[10] Stilbenoids exhibit a limited but heterogeneous distribution in the plant kingdom, and have been most widely reported from the Orchidaceae family. Dihydrostilbenes and 9,10-dihydrophenanthrenes have been previously identified in the genus *Pholidota*.[13–17]

In this study, batatasin III (**2**) isolated from a DCM extract of *P. chinensis* was identified as a positive $GABA_A$ receptor modulator by means of HPLC-based activity profiling,[18] a miniaturized approach for the rapid identification of new bioactive natural compounds,[19–22] that we have been successfully applying in the discovery of $GABA_A$ receptor ligands of natural origin.[23–27] The subunit selectivity of **2** was assessed at $GABA_A$ receptor subtypes $\alpha_2\beta_2\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$, $\alpha_4\beta_2\gamma_{2s}$, $\alpha_5\beta_2\gamma_{2s}$, $\alpha_1\beta_1\gamma_{2s}$, and $\alpha_1\beta_3\gamma_{2s}$. Furthermore, dihydrostilbenes were established as a new scaffold for $GABA_A$ receptor modulators, by comparison of the performance of a series of commercially available stilbenes and their semisynthetic dihydro derivatives on the oocyte assay.

2. Experimental

2.1. General procedures

1D and 2D NMR spectra were measured at room temperature on a Bruker Avance III 500 MHz spectrometer (Bruker BioSpin, Fällanden, Switzerland) operating at 500.13 MHz. Spectra were recorded at 291.2 K with a 1 mm TXI probe with z-gradient. The following settings were used: 128 scans for ^1H spectra; 8 scans for $^1H^1H$-COSY spectra (*cosygpqf* pulse program); 32 scans and 256 increments for HSQC experiments (*hsqcedetgp* pulse program); 64 scans and 128 increments for HMBC spectra (*hmbcgp* pulse program). Spectra were analyzed by TopSpin 3.0 software (Bruker). High resolution mass spectra (HPLC–ESITOFMS) were recorded in positive mode, *m*/*z* range 100–800, on a Bruker microTOF ESIMS system (Bruker Daltonics, Bremen, Germany) connected via a T-splitter (1:10) to an Agilent HP 1100 system consisting of a degasser, a binary mixing pump, autosampler, column oven, and a diode array detector (G1315B) (Agilent Technologies, Waldbronn, Germany). Nitrogen was used as a nebulizing gas at a pressure of 2.0 bar, and as drying gas at a flow rate of 9.0 L/min (dry gas temperature 240 °C). Capillary voltage was set at 45,000 V; hexapole at 230.0 Vpp. Instrument calibration was done with a reference solution of sodium formate 0.1% in 2-propanol/water (1:1) containing 5 mM NaOH. Data acquisition and processing was performed with Bruker Daltonics Hystar 3.0 software. Semi-preparative HPLC separations for activity profiling and purification were performed with an Agilent HP 1100 series system consisting of a quaternary pump, autosampler, column oven, and diode array detector (G1315B). Waters SunFire™ C18 (3.5 μm, 3.0 × 150 mm) and SunFire™ Prep C18 (5 μm, 10 × 150 mm) columns were used for analytical and semi-preparative HPLC analysis, respectively (Waters, Wexford, Ireland). Parallel evaporation of semi-preparative HPLC fractions was performed with a Genevac EZ-2 plus vacuum centrifuge (Genevac Ltd, Ipswich, United Kingdom). Flash chromatography was performed with pre-packed Buchi Sepacore® silica flash cartridges (40–63 μm, 40 × 150 mm) on a Buchi Sepacore® system consisting of two C-605 pumps, a C-620 control unit, and a C-660 fraction collector (Buchi, Flawil, Switzerland). Sample introduction was carried out with a Buchi Prep Elut adapter filled with the sample adsorbed onto silica gel. The separation was monitored by TLC. Preparative HPLC separations were performed with a Waters SunFire Prep C18 OBD column (5 μM, 30 × 150 mm) connected to a Shimadzu LC-8A preparative HPLC with SPD-M10A VP diode array detector. HPLC-grade MeOH (Scharlau Chemie S.A.), acetonitrile (Scharlau Chemie S.A.) and water were used for HPLC separations. For analytical separations, HPLC solvents contained 0.1% of HCO_2H. NMR spectra were recorded in methanol-d_4 (Armar Chemicals). For extraction and flash chromatography, distilled technical grade solvents were used. Silica gel (63–200 μm, Merck) was used for open column chromatography.

2.2. Plant material

Shi Xian Tao (dried stems/roots of *P. chinensis* Lindl.) was purchased in 2008 from a local herbal market in Kunming and authenticated by Dr. X. Yang (previously Pharmaceutical Biology, University of Basel, now Kunming Institute of Botany, Chinese Academy of Science, Kunming, PR China). A voucher specimen (463) is deposited at the Division of Pharmaceutical Biology, University of Basel.

2.3. Extraction

The plant material was frozen with liquid nitrogen and ground with a ZM1 ultracentrifugal mill (Retsch). The DCM extract for screening and HPLC-based activity profiling was prepared with an ASE 200 extraction system with solvent module (Dionex, Sunnyvale CA). Three extraction cycles (5 min each) were performed, at an extraction pressure of 120 bar and a temperature of 70 °C. For preparative isolation, 293 g of ground plant material was macerated with DCM (4 × 1 L, 3 h each, permanent magnetic stirring). The solvent was evaporated at reduced pressure to yield 10.3 g of extract. The extracts were stored at 2–8 °C until use.

2.4. Microfractionation for activity profiling

Time-based microfractionation for $GABA_A$ receptor activity profiling was performed as previously described,[23,27,28] with minor modifications: separation was done on a semi-preparative HPLC column with MeOH (solvent A) and water (solvent B), using a gradient from 50% A to 80% A in 30 min, followed by 80% A to 100% A in 2 min. The flow rate was 4 mL/min, and 10 mg of extract (in 200 μL of DMSO) were injected. A total of 24 microfractions of 90 s each were collected. After parallel evaporation of solvents, the dry films were redissolved in 1 mL of methanol, and aliquots of 0.5 mL were dispensed in two vials, dried under N_2 gas, and submitted to bioassay.

2.5. Isolation

An aliquot of the DCM extract (450 mg) was dissolved in $CHCl_3$ and adsorbed onto silica gel (3 g), prior to packing into a Buchi Prep Elut adapter. Separation was performed on a Sepacore® silica gel cartridge eluted with an *n*-hexane (solvent A) and EtOAc (solvent B) gradient: 0% B to 30% B in 90 min, followed by 30% B to 50% B in 30 min, and 50% B to 80% B in 30 min. The flow rate was set at 15 mL/min. Fractions of 15 mL were collected and later combined into 18 fractions (1–18) on the basis of a TLC analysis (detection at 254, 366, and at daylight after staining with anisaldehyde–sulfuric acid reagent). Fractions 1–18 were submitted to analytical HPLC–PDA–ESIMS with MeOH (solvent C) and water (solvent D), using a gradient from 50% C to 100% C in 30 min, hold for 15 min. The flow rate was 0.4 mL/min, and 10 μg of each fraction (in 10 μL of DMSO) were injected. Fractions 13 and 14 were found to contain the compounds of interest and were submitted to semi-preparative HPLC using solvents C and D as eluents. A gradient of 50% C to 60% C in 30 min was used for fraction 13, and isocratic conditions (50% C, 30 min) for fraction 14. The flow rate was 4 mL/min. Stock solutions in DMSO (50 mg/mL) were prepared and repeatedly injected in portions of 50–100 μL. A portion

(17 mg) of fraction 13 (25 mg) afforded compounds **1** (2.3 mg) and **2** (6.5 mg). Compound **3** (2 mg) was isolated from 10 mg of fraction 14 (16 mg).

Compounds **1**–**3** were identified by comparison of their physiochemical data (NMR, ESI-TOFMS, and UV–vis) with published values.[14,29–31] The purity was >95% (purity check by ^{1}H NMR).

2.5.1. Coelonin (1)

^{1}H NMR (methanol-d_4, 500.13 MHz) δ_H (ppm): 8.13 (1H, d, J = 8.4 Hz, H-5), 6.62 (1H, dd J = 8.3 and 2.7 Hz, H-6), 6.61 (1H, d, J = 2.6 Hz, H-8), 6.30 (1H, d, J = 2.5 Hz, H-3), 6.26 (1H, d, J = 2.5 Hz, H-1), 3.67 (3H, s, 4-OCH_3), 2.59 (4H, s, H-9 and H-10); ^{13}C shifts (derived from multiplicity-edited HSQC and HMBC spectra), δ_C (ppm): 158.3 (C, C-4), 155.4 (C, C-2), 154.8 (C, C-7), 139.8 (C, C-8a), 138.7 (C, C-10a), 128.6 (CH, C-5), 125.2 (C, C-4b), 114.8 (C, C-4a), 113.8 (CH, C-8), 112.2 (CH, C-6), 104.8 (CH, C-1), 100.1 (CH, C-3), 54.2 (4-OCH_3), 30.8 (CH_2, C-10) 30.1 (CH_2, C-9). HR-ESIMS m/z 243.1016 $[M+H]^+$ (calcd for $C_{15}H_{15}O_3$, 243.1016).

2.5.2. Batatasin III (2)

^{1}H NMR (methanol-d_4, 500.13 MHz) δ_H (ppm): 7.03 (1H, dd, J = 7.9 and 7.5 Hz, H-5′), 6.64 (3H, m, H-2′, H-4′, and H-6′), 6.29 (1H, br s, H-2), 6.23 (2H, m, H-4 and H-6), 3.63 (3H, s, 5-OCH_3), 2.75 (4H, m, H-α and H-β); ^{13}C shifts (derived from multiplicity-edited HSQC and HMBC spectra), δ_C (ppm): 160.9 (C, C-5), 157.6 (C, C-3), 156.7 (C, C-3′), 144.9 (C, C-1), 143.3 (C, C-1′), 129.0 (CH, C-5′), 119.8 (CH, C-6′), 115.3 (CH, C-2′), 112.4 (CH, C-4′), 108.1 (CH, C-2), 105.5 (CH, C-6), 98.7 (CH, C-4), 54.3 (CH_3, 5-OCH_3), 37.6 (CH_2, C-β), 37.0 (CH_2, C-α). HR-ESIMS m/z 245.1176 $[M+H]^+$ (calcd for $C_{15}H_{17}O_3$, 245.1172).

2.5.3. Pholidotol D (3)

^{1}H NMR (methanol-d_4, 500.13 MHz) δ_H (ppm): 7.17 (1H, dd, J = 7.9 and 7.8 Hz, H-5′), 7.00–6.95 (4H, m, H-2′, H-6′, H-α and H-β), 6.69 (1H, dd, J = 8.2 and 2.2 Hz, H-4′), 6.58 (2H, m, H-2 and H-6), 6.31 (1H, t, J = 2 Hz, H-4), 3.76 (3H, s, 5-OCH_3); ^{13}C shifts (derived from multiplicity-edited HSQC and HMBC spectra), δ_C (ppm): 160.8 (C, C-5), 157.7 (C, C-3), 156.0 (C, C-3′), 139.7 (C, C-1), 138.5 (C, C-1′), 129.4 (CH, C-5′), 128.6 (CH, C-β), 128.4 (CH, C-α), 117.8 (CH, C-6′), 114.4 (CH, C-4′), 112.4 (CH, C-2′), 105.8 (CH, C-2), 103.4 (CH, C-6), 100.3 (CH, C-4), 54.4 (CH_3, 5-OCH_3). HR-ESIMS m/z 243.1017 $[M+H]^+$ (calcd for $C_{15}H_{15}O_3$, 243.1016).

Further purification of compound **2** for subunit specificity tests was achieved by separating a portion of the extract (7.3 g) by open column chromatography (6 × 69 cm, 700 g of silica gel), using a step gradient of *n*-hexane–EtOAc (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100, 1 L each) and washing in the end with MeOH 100% (1.5 L). The flow rate was ca. 50 mL/min. The effluent was combined to 15 fractions (1–15) based on TLC patterns. After HPLC–PDA–MS analysis, fractions 7 and 8 were selected for isolation of compound **2** by preparative HPLC, with acetonitrile (solvent A) and water (solvent B), using a gradient from 40% A to 50% A in 30 min, followed by 50% A to 100% A in 5 min, hold for 10 min. The flow rate was 20 mL/min. Stock solutions in THF (100 mg/mL) were prepared and repeatedly injected in portions of 300–400 μL. The separation of fractions 7 (129 mg) and 8 (132 mg), yielded compound 10.8 mg of **2**.

2.6. Synthesis of dihydrostilbenes

2.6.1. Stilbenes

Compounds **4**–**7**, **9**, **11**, **13**, **15**, **17**, and **19** were purchased from TCI Europe N.V. Compounds **21** and **25** were purchased from Sigma–Aldrich Co. Compound **23** was purchased from Santa Cruz Biotechnology, Inc.

2.6.2. General procedure

Dihydro derivatives of compounds **7**, **9**, **11**, **13**, **15**, **17**, **19**, **21**, and **23** were prepared by hydrogenation of corresponding stilbenes. A standard protocol was followed,[32] with minor modifications. Solutions of each stilbene (10 mg) in absolute EtOH (5 ml) were stirred under H_2 for 3 h in the presence of 10% Pd/C. The reaction mixtures were filtered over Celite to remove the catalyst, and evaporated to dryness. The resulting residues were purified by flash column chromatography, using a hexane/EtOAc gradient, to afford target compounds **8**, **10**, **12**, **14**, **16**, **18**, **20**, **22**, and **24**, respectively, in yields of 85–95%. The spectroscopic data of compounds were in agreement with the literature, except for compound **24**, for which no report was found (^{1}H NMR spectrum is provided as Supporting information).[32–41]

2.6.3. *trans*-2-Fluoro-4′-methoxy-dihydrostilbene (24)

^{1}H NMR (chloroform-d_4, 500.13 MHz) δ_H (ppm): 7.26–7.08 (4H, m), 7.08–7.98 (2H, m), 6.90–6.80 (2H, m), 3.81 (3H, s), 3.05–2.84 (4H, m). HRESI-MS m/z 253.1589 $[M+Na]^+$ (calcd formula weight for $C_{15}H_{15}FO$, 230.2774).

2.7. Expression of $GABA_A$ receptors

Stage V–VI oocytes from *Xenopus laevis* were prepared, and cRNA injected as previously described.[23] 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 from *Clostridium histolyticum* (Type 1A, Sigma). Synthesis of capped runoff poly(A+) cRNA transcripts was obtained from linearized cDNA templates (pCMV vector). Directly 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 containing 1% of penicillin–streptomycin solution (Sigma–Aldrich). Voltage clamp measurements were performed between days 1 and 5 after cRNA injection.

2.8. Positive control

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, Sigma, purity not less than 98%) was used as positive control. At 1 μM diazepam enhanced I_{GABA} up to 231.3 ± 22.6% (n = 3). See also Figure S1, Supporting information.

2.9. Two-microelectrode voltage clamp studies

Electrophysiological experiments were performed by the two-microelectrode voltage clamp method making use of a TURBO TEC 03X amplifier (npi electronic 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.

2.10. 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 the ScreeningTool (npi electronic, Tamm,

Germany) automated fast perfusion system.[5] In order to determine GABA EC_{3-10} (typically between 3 and 10 μM for receptors of subunit composition $\alpha_1\beta_2\gamma_{2s}$), a dose–response experiment with GABA concentrations ranging from 0.1 μM to 1 mM was performed. Stock solution of the DCM extract (10 mg/mL in DMSO) was diluted to a concentration of 100 μg/mL with bath solution containing GABA EC_{3-10} according to a validated protocol.[23] As previously described, microfractions collected from the semi-preparative HPLC separations were dissolved in 30 μL of DMSO and subsequently mixed with 2.97 mL of bath solution containing GABA EC_{3-10}.[23] A stock solution of each pure compound tested (100 mM in DMSO) was diluted to concentrations of 0.1, 1.0, 3.0, 10, 30, 100, 300, and 500 μM with bath solution for measuring direct activation, or with bath solution containing GABA EC_{3-10} for measuring modulation of $GABA_A$ receptors. The final DMSO concentration in all the samples including the GABA control samples was adjusted to 1% to avoid solvent effect at the $GABA_A$ receptor.

2.11. Data analysis

Enhancement of the 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 were analyzed using the ORIGIN 7.0 SR0 software (OriginLab Corporation) and are given as mean ± SE of at least two oocytes and ⩾2 oocyte batches.

3. Results and discussion

3.1. Isolation and structure elucidation of active compounds

Screening for $GABA_A$ modulating activity was performed with *Xenopus laevis* oocytes transiently expressing $GABA_A$ receptors of the subtype $\alpha_1\beta_2\gamma_{2s}$. In an automated fast-perfusion system used for two-microelectrode voltage clamp measurements,[5] a dichloromethane extract (100 μg/mL) of *P. chinensis* roots enhanced the GABA-induced chloride ion current (I_{GABA}) by 132.8 ± 36.7%. To track the activity in the extract, we used HPLC-based activity profiling with a validated protocol.[23] The chromatogram (210–700 nm) of a semipreparative HPLC separation (10 mg of extract) and the corresponding activity profile of the time-based fractionation (24 microfractions of 90 s each) are shown in Figure 1B and A, respectively. The major peak of activity was found in fraction 9, which potentiated I_{GABA} by 119.1 ± 19.1%. Fraction 8 showed marginal activity (enhancement of I_{GABA} by 26.5 ± 4.7%). All the remaining fractions showed minimal activity and were not considered further.

Isolation of the active compounds was achieved by flash chromatography and subsequent purification by semi-preparative HPLC. Compounds were tracked with the aid of TLC and HPLC–ESIMS. The three structurally related stilbenoids coelonin (**1**), batatasin III (**2**), and pholidotol D (**3**) (Fig. 2) were identified by ESI-TOF-MS, 1D and 2D microprobe NMR, and comparison with published data.[14,29–31] The *Z* configuration in compound **3** was corroborated by proton NMR, using the chemical shifts and coupling constant of the two olefinic protons at δ_H 6.95 (2H, d, J = 6.0 Hz, H-β and α), which discards the presence of the *trans*-stereoisomer thunalbene. Detailed spectroscopic data of compounds **1**–**3** are available as Supporting information.

Stilbenoids are the major secondary metabolites in the genus *Pholidota*,[10] and the identification of compounds **1**–**3** in the active fractions of *P. chinensis* DCM extract was not surprising. The three compounds have been previously isolated from the species,[10,12,14] but they have not been reported as $GABA_A$ receptor modulators.

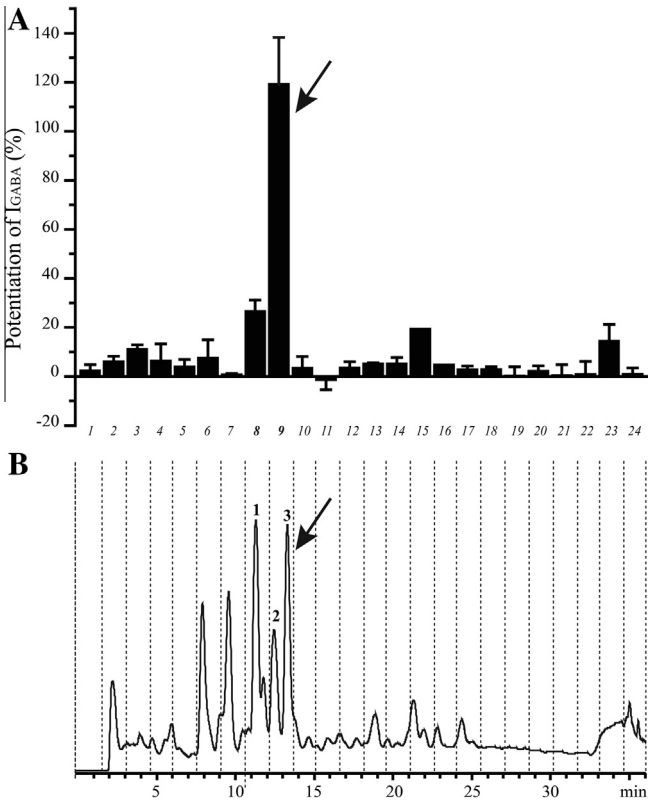

Figure 1. HPLC-based activity profiling of a DCM extract of stems and roots of *P. chinensis*, for $GABA_A$ receptor modulatory activity. (B) HPLC chromatogram (210–700 nm) of a semipreparative separation of 10 mg of extract. The numbers above peaks designate compounds **1**–**3**. The 24 time-based fractions of 90 s each are indicated with dashed lines. (A) Potentiation of the I_{GABA} by each microfraction (error bars correspond to SE).

3.2. Modulation of $GABA_A$ receptors

For a preliminary activity profile at $GABA_A$Rs of the subtype $\alpha_1\beta_2\gamma_{2s}$, **1**–**3** were tested at a concentration of 100 μM in the *Xenopus* oocyte assay. Batatasin III (**2**) was the most efficient among the three compounds. It potentiated I_{GABA} by 628.3 ± 87.1%, while compounds **1** and **3** exhibited weaker enhancements (139.5 ± 14.4% and 192.0 ± 64.1%, respectively) (Fig. 3A). Further concentration–response experiments on $\alpha_1\beta_2\gamma_{2s}$ receptors were performed with compounds **1**–**3**, at concentrations ranging from 1 to 300 μM (500 μM for compound **3**). As shown in Figure 3B, all stilbenoids enhanced I_{GABA} at a GABA EC_{3-10} in a concentration-dependent manner. The bibenzyl batatasin III (**2**) displayed strong $GABA_A$ receptor modulatory activity, with an efficiency (maximal stimulation of I_{GABA}) of 1512.9 ± 176.5% and a potency (higher concentration for half-maximal stimulation of I_{GABA}, or EC_{50}) of 52.5 ± 17.0 μM. The structurally related stilbene pholitodol D (**3**) showed much lower activity, with an efficiency of 786.8 ± 72.1% and potency of 175.5 ± 25.5 μM. The dihydrophenanthrene coelonin (**1**) showed activity similar to compound **2**, but no saturation of the receptors was reached at the highest concentration tested (300 μM). None of the compounds induced direct activation of the receptors when applied prior to GABA, at concentrations lower than 100 μM. This was indicative of an allosteric modulation of the receptor with the subunit composition $\alpha_1\beta_2\gamma_{2s}$, rather than direct agonistic activity (Fig. 3C).

Compared to other natural products tested in the same in vitro model and $GABA_A$ receptor subtype,[24,27,28,33] batatasin III (**2**) exhibited much higher efficiency. The efficiency of **2** in $GABA_A$Rs of the

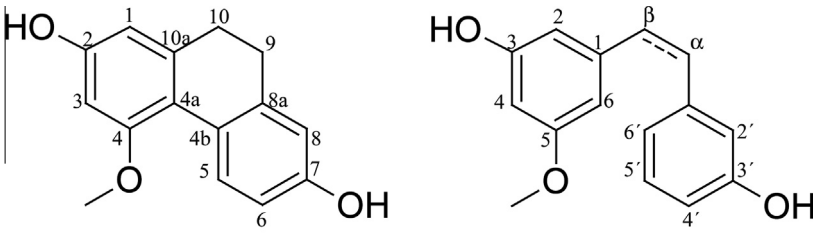

Figure 2. Chemical structures of compounds **1**–**3**.

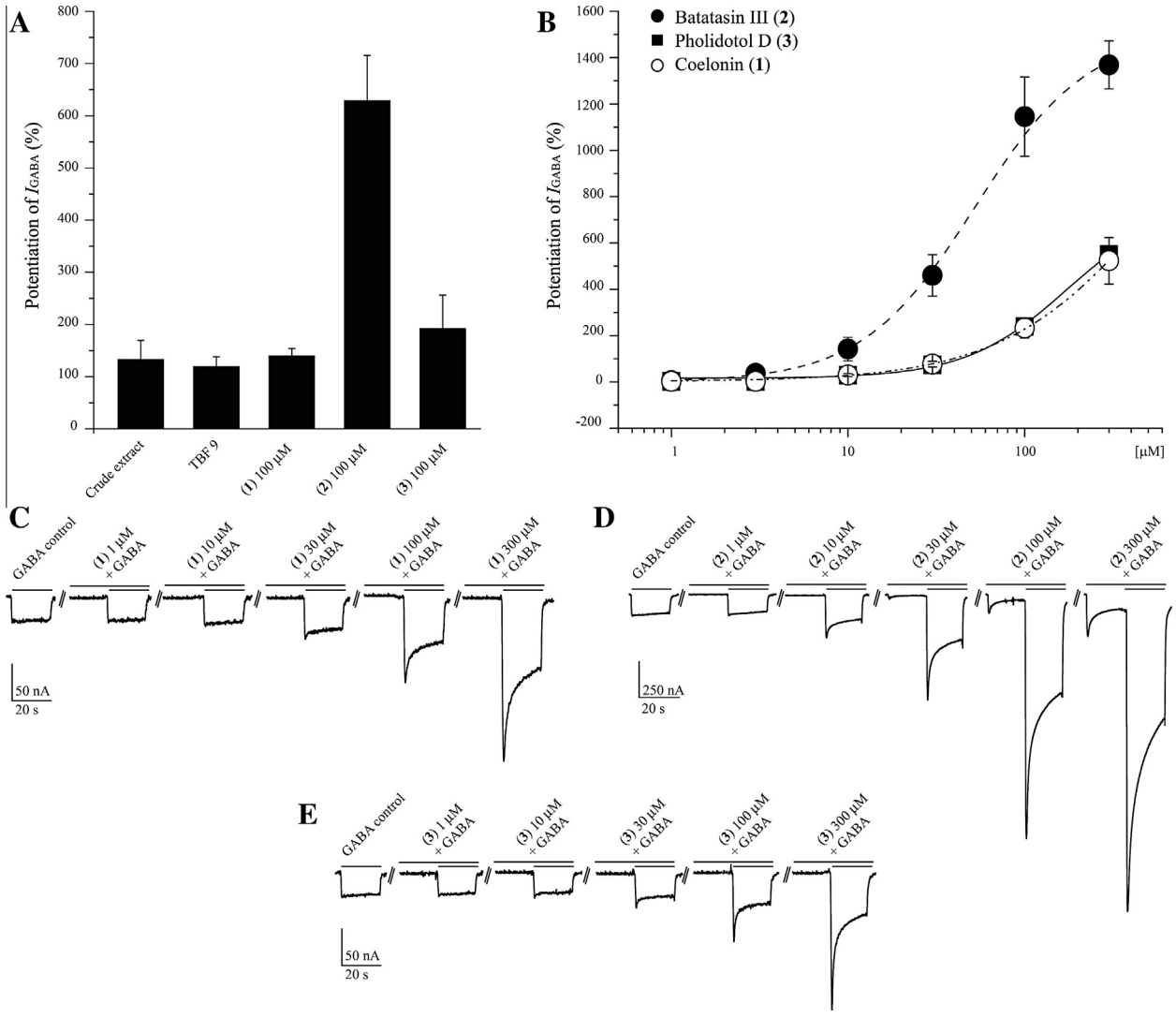

Figure 3. (A) Potentiation of I_{GABA} by the DCM extract of *P. chinensis* stems and roots (100 μg/mL), by microfraction 9, and compounds **1**–**3** (100 μM). (B) Concentration–response curve for compounds **1**–**3** on $GABA_A$ receptors of the subunit composition $\alpha_1\beta_2\gamma_{2S}$. (C–E) Typical traces for modulation of I_{GABA} by compounds **1**–**3**, respectively. The flat segments in the currents indicate the absence of direct activation of the receptors. All experiments in A–E were carried out using a GABA EC_{3-10}.

subtype $\alpha_1\beta_2\gamma_{2s}$ was also significantly higher than that of classical BDZs, with a potentiation of I_{GABA} at least fourfold that of triazolam, clotiazepam, and midazolam.[34] However, its EC_{50} value was significantly higher than that of BDZs and indicated a much lower binding affinity.

Despite the small number of compounds, preliminary structure–activity considerations could be derived. Conformational flexibility as in batatasin III (**2**) appeared to be critical for the modulatory activity of stilbenoids, since introduction of a double bond $\Delta^{\beta,\alpha}$ in pholidotol D (**3**) drastically decreased potency and efficiency. The importance of flexibility was confirmed by the weak activity of coelonin (**1**) in which the dihydrophenanthrene ring conferred additional rigidity to the structure. Although stilbenoids such as resveratrol have been described as neuroprotective

agents,[17,35–38] none of them has been reported as $GABA_A$ receptor ligand so far. Batatasin III (**2**) is thus the first representative of a new scaffold for $GABA_A$ receptor modulators. It is noteworthy that compounds with biosynthetically related scaffolds such as flavonoids,[25,39] coumarins,[24] and lignans[40] have been previously shown to possess $GABA_A$ receptor modulatory properties.

3.3. $GABA_A$ receptor subtype selectivity

Batatasin III (**2**) was tested for potential α subunit specificity, by replacing the α_1 subunit in the receptor subtype $\alpha_1\beta_2\gamma_{2s}$ with α_2, α_3, α_4, and α_5. Likewise, β subunit specificity was evaluated by replacing β_2 with β_1 and β_3. Concentration-dependent I_{GABA} modulation of compound **2** was evaluated on receptor subtypes $\alpha_2\beta_2\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$, $\alpha_4\beta_2\gamma_{2s}$, $\alpha_5\beta_2\gamma_{2s}$, $\alpha_1\beta_1\gamma_{2s}$, and $\alpha_1\beta_3\gamma_{2s}$ (Table 1).

As shown in Figure 4 and summarized in Table 1, compound **2** did not exhibit subtype specificity, as reflected by comparable EC_{50} values with all receptor subtypes studied ($p > 0.05$). The order of potency of batatasin III (**2**) in receptor composed by different α subunits was $\alpha_4\beta_2\gamma_{2s} > \alpha_5\beta_2\gamma_{2s} > \alpha_1\beta_2\gamma_{2s} > \alpha_3\beta_2\gamma_{2s} > \alpha_2\beta_2\gamma_{2s}$. The lower potency on $\alpha_2\beta_2\gamma_{2s}$ receptors compared to $\alpha_4\beta_2\gamma_{2s}$ was statistically significant, while there were no significant differences in efficiency among the other α-containing receptor subtypes. On $GABA_A$ receptors comprising different β subunits, almost no differences in potency and efficiency were observed. Thus, batatasin III (**2**) was a positive allosteric modulator of $GABA_A$Rs, devoid of significant subtype specificity.

Table 1
Potencies and efficiencies of batatasin III (**2**) for $GABA_A$ receptors of different subunit compositions

Subtype	EC_{50} (μM)	Max. potentiation of I_{GABA} (EC_{3-10}) (I_{max}) (%)	Hill coeff. (n_H)	n[a]
$\alpha_1\beta_2\gamma_{2s}$	52.5 ± 17.0	1512.9 ± 176.5	1.4 ± 0.3	5
$\alpha_2\beta_2\gamma_{2s}$	80.8 ± 22.1	1026.5 ± 139.2	1.2 ± 0.1	6
$\alpha_3\beta_2\gamma_{2s}$	67.3 ± 18.6	1694.2 ± 229.0	1.2 ± 0.1	5
$\alpha_4\beta_2\gamma_{2s}$	26.2 ± 3.6	1588.2 ± 97.5	1.5 ± 0.1	6
$\alpha_5\beta_2\gamma_{2s}$	46.7 ± 9.0	1375.7 ± 76.5	1.3 ± 0.1	5
$\alpha_1\beta_1\gamma_{2s}$	66.7 ± 21.0	1251.3 ± 157.0	1.8 ± 0.4	5
$\alpha_1\beta_3\gamma_{2s}$	67.2 ± 10.5	1252.9 ± 79.9	1.4 ± 0.1	5

[a] Number of experiments.

3.4. $GABA_AR$ modulatory activity of dihydrostilbenes

Flexibility appeared to be a critical factor for the $GABA_AR$ modulatory activity of stilbenoids. To confirm the influence of the double bond $\Delta^{\beta,\alpha}$, 13 commercially available stilbenoids and their corresponding dihydro derivatives (compounds **4**–**25**; Fig. 5) were tested in the *Xenopus* oocyte assay. Compounds were initially tested at a concentration of 100 μM on $GABA_A$Rs of the subtype $\alpha_1\beta_2\gamma_{2s}$. As expected, dihydrostilbenes showed higher activity than the corresponding stilbenes (Table 2, Fig. 6A). These differences in the activity of stilbenes and their dihydro derivatives were statistically significant in almost every case, with the exception of the pairs **4** and **5**/**6**, **9**/**10**, **13**/**14**, and **23**/**24** ($p > 0.05$).

Among the stilbenes, tetramethoxy-piceatannol (**11**), resveratrol (**13**), pterostilbene (**19**), and resveratrol triacetate (**21**), displayed the highest activity, potentiating I_{GABA} between 100% and 200%. Their corresponding dihydro derivatives showed the highest activity among dihydrostilbenes, but only compounds **12** and **20** showed efficiencies comparable to that of batatasin III (**2**) (544.5 ± 104.4% and 660.6 ± 100.2% respectively). A comparison of the activity of the dihydrostilbenes at 100 μM revealed that the bibenzyl scaffold alone (**6**) does not possess any $GABA_AR$ modulatory activity. In general, substituents at C-3 and C-5 (**12**, **14**, **16**, **18**, **20**, and **22**) resulted in an enhancement of the activity. Increasing the lipophilicity by replacing the hydroxy groups at C-3 and C-5 with bulkier oxygenated functions (**12**, **20**, and **22**) enhanced the

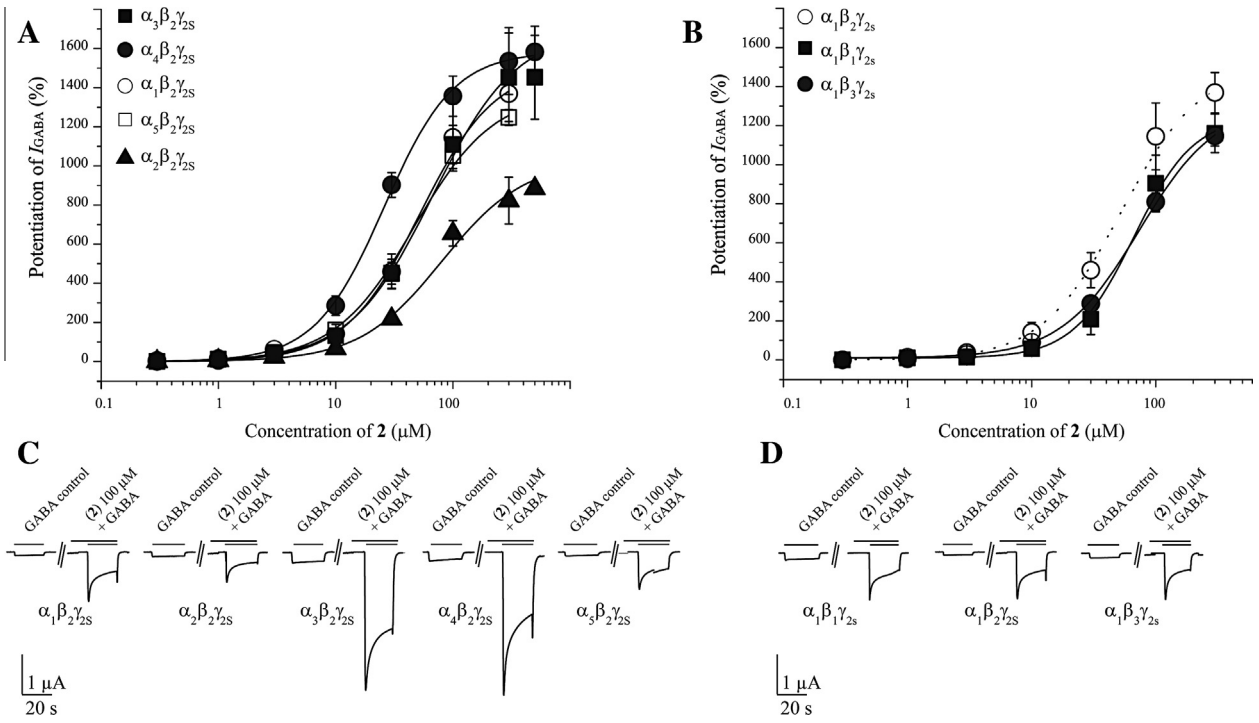

Figure 4. (A) α-Subunit dependency of batatasin III (**2**), depicted as concentration–response curves, with $GABA_A$ receptors of the subunit compositions $\alpha_1\beta_2\gamma_{2s}$, $\alpha_2\beta_2\gamma_{2s}$, $\alpha_3\beta_2\gamma_{2s}$, $\alpha_4\beta_2\gamma_{2s}$, and $\alpha_5\beta_2\gamma_{2s}$. (B) β-Subunit dependency of batatasin III (**2**), depicted as concentration–response curves with $GABA_A$ receptors of the subunit compositions $\alpha_1\beta_1\gamma_{2s}$, $\alpha_1\beta_2\gamma_{2s}$, and $\alpha_1\beta_3\gamma_{2s}$. (C and D) Typical traces for modulation of I_{GABA} by compound **2**, in receptors with different α and β subunit composition, respectively. All experiments were performed using a GABA EC_{3-10}.

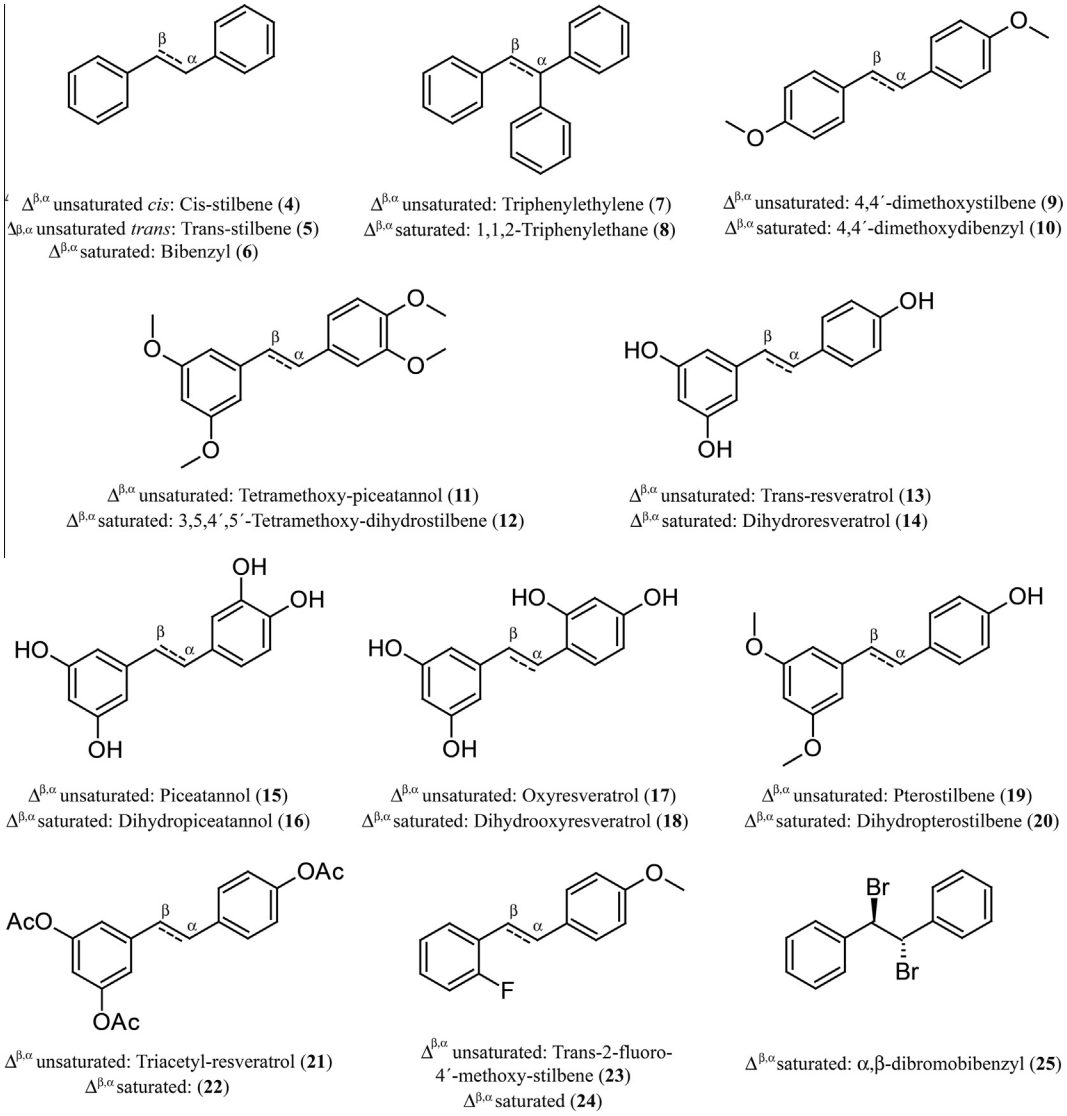

Figure 5. Chemical structures of compounds **4**–**25**.

Table 2
Potentiation of I_{GABA} in $\alpha_1\beta_2\gamma_{2s}$ receptors by compounds **4**–**25**, at a test concentration of 100 μM

Stilbenes			Dihydrostilbenes		
Compound	Max. potentiation of I_{GABA}	n[a]	Compound	Max. potentiation of I_{GABA}	n[a]
4	12.6 ± 9.2	3	**6**	8.3 ± 22.9	3
5	−11.3 ± 12.3	3			
7	−7.3 ± 0.1	3	**8**	51.6 ± 1.1	3
9	−24.8 ± 4.8	3	**10**	−20.9 ± 3.7	3
11	101.3 ± 0.9	3	**12**	544.5 ± 140.4	3
13	121.9 ± 21.8	3	**14**	162.2 ± 17.5	3
15	−35.4 ± 9.8	3	**16**	86.3 ± 9.9	3
17	−19.7 ± 3.9	3	**18**	44.1 ± 19.7	3
19	212.4 ± 10.9	3	**20**	660.6 ± 100.2	3
21	122.8 ± 18.6	3	**22**	227.7 ± 1.3	2
23	−22.9 ± 7.5	3	**24**	−16.8 ± 7.9	3
Diazepam[b] (1 μM)	231.3 ± 22.6	3	**25**	−12.9 ± 0.4	3

[a] Number of experiments.
[b] Positive control.

activity of dihydrostilbenes. The role of substituents in ring B was less clear within this compounds series. In the case of compounds **12** and **20**, different substitution patterns in ring B did not influence the activity. In contrast, when comparing compounds **14**, **16**, and **18**, addition of a hydroxy group in C-4′ or C-6′ led to a significant decrease of activity. Introduction of a halogen atom as in **25** induced slight negative receptor modulation, and substitution at C-4 (compound **10**) decreased activity. Since we had only one pair of *cis* and *trans* isomers (**4** and **5**, both inactive at 100 μM), the role of geometric isomerism could not be assessed in more detail.

The dihydro derivatives of tetramethoxy-piceatannol and pterostilbene (compounds **12** and **20**, respectively) were submitted to further concentration–response experiments on $\alpha_1\beta_2\gamma_{2s}$ receptors. Both compounds enhanced I_{GABA} at a GABA EC_{3-10} in a concentration-dependent manner (Fig. 6B). Compounds **12** and **20** had lower efficiency than the natural dihydrostilbene **2** (Table 3), with maximal stimulations of I_{GABA} of 870.7 ± 106.8% and 694.2 ± 86.0%, respectively. In terms of potency, **20** was comparable to **2** (EC_{50} 54.5 ± 13.4 μM), whereas **12** was twice as potent (EC_{50} 20.2 ±

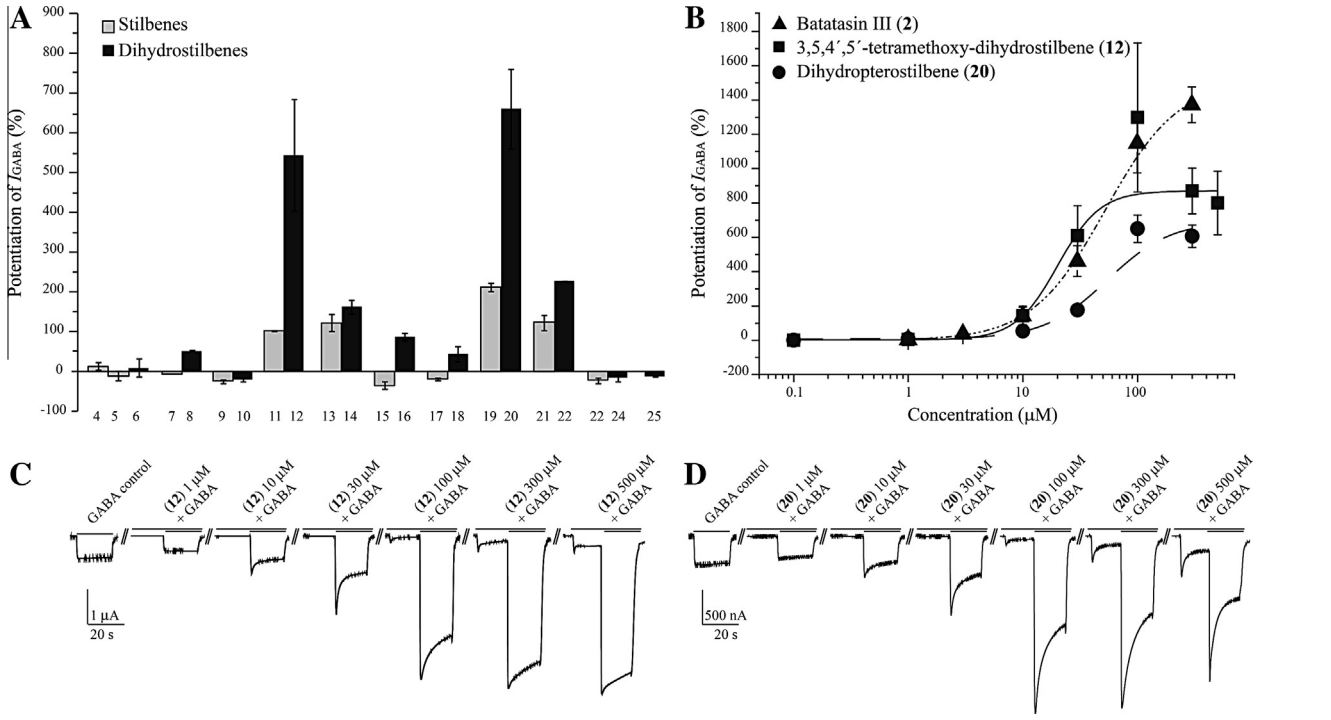

Figure 6. (A) Potentiation of I_{GABA} by compounds **4**–**25** (100 μM). (B) Potentiation of I_{GABA} by compounds **2**, **12** and **20**. Concentration–response curves are shown for $GABA_A$ receptors of the subunit composition $\alpha_1\beta_2\gamma_{2S}$. (C and D) Typical traces for modulation of I_{GABA} by compounds **12** and **20**, respectively. The inward currents induced in the absence of GABA (C and D) indicate direct activation of the receptors. All experiments shown in A–D were performed using a GABA EC_{3-10}.

Table 3
Potencies and efficiencies of compounds **2**, **3**, **12** and **20** for $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptors

Compound	EC_{50} (μM)	Max. potentiation of I_{GABA} (EC_{3-10}) (I_{max}) (%)	Hill coeff. (n_H)	n[a]
2	52.5 ± 17.0	1512.9 ± 176.5	1.2 ± 0.1	5
3	175.5 ± 25.5	786.8 ± 72.1	1.5 ± 0.2	5
12	20.2 ± 6.4	870.7 ± 106.8	2.3 ± 1.0	4
20	54.5 ± 13.4	694.2 ± 86.0	1.6 ± 0.2	4

[a] Number of experiments.

6.4 μM). This suggests that increased lipophilicity of ring B may have a positive effect on the potency of dihydrostilbenes. However, further studies with a larger series of compounds are needed for confirmation. None of the compounds induced direct activation of the receptors when applied prior to GABA, at concentrations lower than 100 μM (Fig. 6C).

Stilbenoids have attracted significant attention in recent years due to their wide range of useful properties, including applications in optics, biochemistry, and chemotherapy.[13,41] The stilbenoid scaffold can be considered as a priviledged structure.[42,43] However, there have been no reports on $GABA_A$ receptor modulatory activity of stilbenoids up to now, despite a significant number of publications on biological activities of natural stilbenoids, and in particular, on resveratrol. Dihydrostilbenes such as **2** may thus be an interesting starting point for the synthesis of new $GABA_A$ receptor modulators.

4. Conclusions

With the aid of an HPLC-based profiling approach, we identified batatasin III (**2**) as the major compound responsible for $GABA_AR$ modulatory activity of the dichloromethane extract of *P. chinensis*. This dihydrostilbene showed allosteric modulation in $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptors with a higher efficiency than any other natural products tested up to now, but its EC_{50} value was significantly higher than that of BDZs. Dihydrostilbenes represent a new scaffold for $GABA_A$ receptor modulators.

The conformational flexibility of dihydrostilbenoids appeared critical for $GABA_AR$ modulatory properties. For a further exploration of this scaffold, conformationally restricted derivatives should be synthesized in order to explore in more detail the optimal orientation of the aromatic rings and substituents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.01.008.

References and notes

1. Olsen, R. W.; Sieghart, W. *Pharmacol. Rev.* **2008**, *60*, 243.
2. Rudolph, U.; Knoflach, F. *Nat. Rev. Drug Disc.* **2011**, *10*, 685.
3. D'Hulst, C.; Atack, J. R.; Kooy, R. F. *Drug Discovery Today* **2009**, *14*, 866.
4. Tan, K. R.; Rudolph, U.; Lüscher, C. *Trends Neurosci.* **2011**, *34*, 188.
5. Baburin, I.; Beyl, S.; Hering, S. *Pflug. Arch. Eur. J. Physiol.* **2006**, *453*, 117.
6. Pérez Gutiérrez, R. M. *J. Med. Plants Res.* **2010**, *4*, 592.
7. Williams, R.; Martin, S.; Hu, J.-F.; Garo, E.; Rice, S.; Norman, V.; Lawrence, J.; Hough, G.; Goering, M.; O'Neil-Johnson, M.; Eldridge, G.; Starks, C. *Planta Med.* **2011**, *78*, 160.
8. Bulpitt, C. J.; Li, Y.; Bulpitt, P. F.; Wang, J. *J. R. Soc. Med.* **2007**, *100*, 558.
9. Wang, J.; Matsuzaki, K.; Kitanaka, S. *Chem. Pharm. Bull. (Tokyo)* **2006**, *54*, 1216.
10. Bandi, A. K. R.; Lee, D.-U. *Chem. Biodiversity* **2011**, *8*, 1400.
11. Wu, B.; Qu, H.; Cheng, Y. *Chem. Biodiversity* **1803**, *2008*, 5.

12. Yao, S.; Tang, C.-P.; Li, X.-Q.; Ye, Y. *Helv. Chim. Acta* **2008**, *91*, 2122.
13. Rivière, C.; Pawlus, A. D.; Mérillon, J.-M. *Nat. Prod. Rep.* **2012**, *29*, 1317.
14. Majumder, P. L.; Roychowdhury, M.; Chakraborty, S. *Phytochemistry* **1998**, *49*, 2375.
15. Kovacs, A.; Vasas, A.; Hohmann, J. *Phytochemistry* **2008**, *69*, 1084.
16. Shen, T.; Wang, X.-N.; Lou, H.-X. *Nat. Prod. Rep.* **2009**, *26*, 916.
17. Greger, H. *J. Nat. Prod.* **2012**, *75*, 2261.
18. Potterat, O.; Hamburger, M. *Curr. Org. Chem.* **2006**, *10*, 899.
19. Danz, H.; Stoyanova, S.; Wippich, P.; Brattstrom, A.; Hamburger, M. *Planta Med.* **2001**, *67*, 411.
20. Dittmann, K.; Gerhauser, C.; Klimo, K.; Hamburger, M. *Planta Med.* **2004**, *70*, 909.
21. Adams, M.; Christen, M.; Plitzko, I.; Zimmermann, S.; Brun, R.; Kaiser, M.; Hamburger, M. *J. Nat. Prod.* **2010**, *73*, 897.
22. Adams, M.; Zimmermann, S.; Kaiser, M.; Brun, R.; Hamburger, M. *Nat. Prod. Commun.* **2009**, *4*, 1377.
23. Kim, H. J.; Baburin, I.; Khom, S.; Hering, S.; Hamburger, M. *Planta Med.* **2008**, *74*, 521.
24. Li, Y.; Plitzko, I.; Zaugg, J.; Hering, S.; Hamburger, M. *J. Nat. Prod.* **2010**, *73*, 768.
25. Yang, X.; Baburin, I.; Plitzko, I.; Hering, S.; Hamburger, M. *Mol. Diversity* **2011**, *15*, 361.
26. Zaugg, J.; Khom, S.; Eigenmann, D.; Baburin, I.; Hamburger, M.; Hering, S. *J. Nat. Prod.* **2011**, *74*, 1764.
27. Zaugg, J.; Eickmeier, E.; Rueda, D. C.; Hering, S.; Hamburger, M. *Fitoterapia* **2011**, *82*, 434.
28. Zaugg, J.; Baburin, I.; Strommer, B.; Kim, H. J.; Hering, S.; Hamburger, M. *J. Nat. Prod.* **2010**, *73*, 185.
29. Wang, J.; Wang, L.; Kitanaka, S. *J. Nat. Med.* **2007**, *61*, 381.
30. Majumder, P.; Laha, S.; Datta, N. *Phytochemistry* **1982**, *21*, 478.
31. Hashimoto, T.; Hasegawa, K.; Yamaguchi, H.; Saito, M.; Ishimoto, S. *Phytochemistry* **1974**, *13*, 2849.
32. Oh, K. B.; Kim, S. H.; Lee, J.; Cho, W. J.; Lee, T.; Kim, S. *J. Med. Chem.* **2004**, *47*, 2418.
33. Zaugg, J.; Eickmeier, E.; Ebrahimi, S. N.; Baburin, I.; Hering, S.; Hamburger, M. *J. Nat. Prod.* **2011**, *74*, 1437.
34. Khom, S.; Baburin, I.; Timin, E. N.; Hohaus, A.; Sieghart, W.; Hering, S. *Mol. Pharmacol.* **2006**, *69*, 640.
35. Richard, T.; Pawlus, A. D.; Iglésias, M.-L.; Pedrot, E.; Waffo-Teguo, P.; Mérillon, J.-M.; Monti, J.-P. *Ann. N.Y. Acad. Sci.* **2011**, *1215*, 103.
36. Vingtdeux, V.; Dreses-Werringloer, U.; Zhao, H.; Davies, P.; Marambaud, P. *BMC Neurosci.* **2008**, *9*, S6.
37. Sun, A. Y.; Wang, Q.; Simonyi, A.; Sun, G. Y. *Mol. Neurobiol.* **2010**, *41*, 375.
38. Zhang, F.; Liu, J.; Shi, J. S. *Eur. J. Pharmacol.* **2010**, *636*, 1.
39. Hanrahan, J. R.; Chebib, M.; Johnston, G. A. *Br. J. Pharmacol.* **2011**, *163*, 234.
40. Zaugg, J.; Ebrahimi, S. N.; Smiesko, M.; Baburin, I.; Hering, S.; Hamburger, M. *Phytochemistry* **2011**, *72*, 2385.
41. Gray, E. E.; Rabenold, L. E.; Goess, B. C. *Tetrahedron Lett.* **2011**, *52*, 6177.
42. Welsch, M. E.; Snyder, S. A.; Stockwell, B. R. *Curr. Opin. Chem. Biol.* **2010**, *14*, 1.
43. Breinbauer, R.; Vetter, I. R.; Waldmann, H. *Angew. Chem., Int. Ed.* **2002**, *41*, 2878.