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Phytochemical profiling of *Curcuma kwangsiensis* rhizome extract, and identification of labdane diterpenoids as positive $GABA_A$ receptor modulators

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ABSTRACT

An ethyl acetate extract of *Curcuma kwangsiensis* S.G. Lee & C.F. Liang (Zingiberaceae) rhizomes (100 μg/ml) enhanced the GABA-induced chloride current (I_{GABA}) through $GABA_A$ receptors of the $\alpha_1\beta_2\gamma_{2S}$ subtype by 79.0 ± 7.0%. Potentiation of I_{GABA} was measured using the two-microelectrode voltage-clamp technique and *Xenopus laevis* oocytes. HPLC-based activity profiling of the crude extract led to the identification of 11 structurally related labdane diterpenoids, including four new compounds. Structure elucidation was achieved by comprehensive analysis of on-line (LC-PDA-ESI-TOF-MS) and off-line (microprobe 1D and 2D NMR) spectroscopic data. The absolute configuration of the compounds was established by comparison of experimental and calculated ECD spectra. Labdane diterpenes represent a new class of plant secondary metabolites eliciting positive $GABA_A$ receptor modulation. The highest efficiency was observed for zerumin A (maximum potentiation of I_{GABA} by 309.4 ± 35.6%, and EC_{50} of 24.9 ± 8.8 μM).

1. Introduction

Worldwide, anxiety and sleep difficulties, especially insomnia, are common and highly prevalent healthcare problems (Ringdahl et al., 2004; Uhde et al., 2009). A crucial target for anxiolytics, sedatives, hypnotics, anticonvulsants, and muscle relaxants is the gamma-aminobutyric acid type A ($GABA_A$) receptor, a ligand-gated ion channel that mediates inhibitory neurotransmission in the central nervous system (CNS). The $GABA_A$ receptor has a heteropentameric structure and can be assembled from 19 different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, ρ_{1-3}, and θ). The most abundant $GABA_A$ receptor in the mammalian brain consists of two α_1, two β_2, and one γ_{2S} subunits (Olsen and Sieghart, 2008). Despite the broad range of drugs that are clinically in use to treat anxiety and sleep disorders, there is an increasing demand for herbal preparations with such properties. Herbal products have become increasingly important during the last decades, owing to positive consumer acceptance (Biesalski, 2002). In addition to synthetic drug candidates, a wide range of plant-derived natural products have been shown to modulate the function of $GABA_A$ receptors (Johnston et al., 2006). Given the continued importance of natural products in drug discovery and development (Newman and Cragg, 2012), plant-derived compounds may provide inspiration for new scaffolds of $GABA_A$ receptor modulators.

In the search for positive $GABA_A$ receptor modulators of natural origin, we screened an in-house plant extract library, comprising major officinal herbal drugs of the European and Chinese Pharmacopoeias, for the ability to potentiate GABA-induced chloride currents. Extracts were tested with an automated two-microelectrode voltage clamp assay in *Xenopus laevis* oocytes expressing recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors, at a concentration of 100 μg/ml. A previously validated HPLC profiling protocol for the discovery of new $GABA_A$ receptor modulating natural products was applied to identify the active constituents (Kim et al., 2008). Using this approach, we successfully identified various plant secondary metabolites including alkaloids (Zaugg et al., 2010), lignans (Zaugg et al., 2011a), terpenes (Zaugg et al., 2011b,d), coumarins (Zaugg

Abbreviations: GABA, gamma-aminobutyric acid; I_{GABA}, GABA-induced chloride current; TCM, traditional Chinese medicine; ECD, electronic circular dichroism; TDDFT, time-dependent density function theory; CE, Cotton effect; OPLS, optimized potential for liquid simulations; CPCM, conductor-like polarizable continuum model; SCRF, self-consistent reaction field.

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et al., 2011c), sanggenons (Kim et al., 2012), and flavonoids (Yang et al., 2011) as positive $GABA_A$ receptor modulators.

In the course of our *in vitro* screening, an ethyl acetate extract from rhizomes of *Curcuma kwangsiensis* S.G. Lee & C.F. Liang (Zingiberaceae) displayed positive $GABA_A$ receptor modulation. While the activity was only moderate, the extract was selected for further investigation based on chemotaxonomic considerations and the fact that none of the typical metabolites of the genus *Curcuma* was reported to exhibit $GABA_A$ receptor modulating activity. Curcumae rhizoma (Ezhu) is the dried rhizome of *C. kwangsiensis* S.G. Lee & C.F. Liang, *C. wenyujin* Y.H. Chen & C. Ling, or *C. phaeocaulis* Val., and belongs to the best known herbs in traditional Chinese medicine (TCM). Ezhu is widely used as a digestive and analgesic agent, and also for the treatment of menstrual disorders (Chinese Pharmacopoeia Commission, 2010; Tang and Eisenbrand, 2011). The genus *Curcuma* counts approximately 100 species, among which only about one fifth have been studied extensively from a phytochemical viewpoint. Known compounds from *Curcuma* species belong to three major classes of plant secondary metabolites, including diphenylalkanoids, phenylpropanoids, and terpenoids (Nahar and Sarker, 2007). The phytochemistry of *C. kwangsiensis* is poorly studied compared to other *Curcuma* species. The rhizome is known to contain a number of structurally related diarylheptanoids (Li et al., 2011, 2010), and various mono- and sesquiterpenes which are the main components of the essential oil (Zeng et al., 2009).

We here describe the identification of $GABA_A$ receptor modulating labdane diterpenes via an HPLC-based discovery platform, along with the structure elucidation and *in vitro* pharmacological evaluation of the isolated compounds. The absolute configuration of the diterpenoids was established by comparing experimental and TDDFT simulated electronic circular dichroism (ECD) spectra.

2. Results and discussion

2.1. Isolation and structure elucidation

In a screening for new $GABA_A$ receptor modulators, an ethyl acetate extract from *C. kwangsiensis* rhizomes enhanced I_{GABA} by 79.0 ± 7.0% when tested at 100 μg/ml. To track the active principles responsible for positive $GABA_A$ receptor modulation, the extract was submitted to a process referred to as HPLC-based activity profiling. This approach combines physicochemical data recorded on-line with biological information obtained in parallel from time-based microfractionation (Potterat and Hamburger, 2006). An aliquot of the extract (5 mg) was separated by semi-preparative RP-HPLC, and collected peak-based microfractions were retested in the oocyte assay. Fig. 1 shows the active time window of the HPLC chromatogram (254 nm) and the activity of peak-based microfractions a–r. The activity was dispersed over a broad time window, suggesting that the activity of the extract was due to several related compounds. The highest activity was found in microfraction k (potentiation of I_{GABA} by 164.7 ± 47.6%), while other microfractions were less or only marginally active. By means of LC-PDA-ESI-TOF-MS analysis of the extract, in combination with off-line NMR data recorded after peak-based collection, the compound eluting at 42.5 min was readily identified as coronarin D (**6**), a labdane diterpene previously reported from *Hedychium coronarium* (Itokawa et al., 1988). Several HPLC peaks in the active time window exhibited UV and MS spectra similar to those of **6**, indicating structurally related compounds. Targeted preparative purification by a combination of flash chromatography on silica gel and semi-preparative RP-HPLC afforded a series of 11 labdane diterpenes (**1**–**11**) (Fig. 2), including four new natural products (**2**, **4**, **9**, and **11**). Structure elucidation was achieved by comprehensive analysis

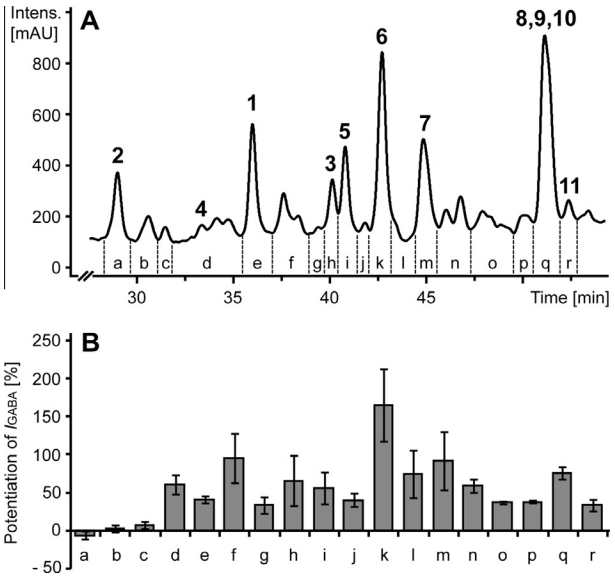

Fig. 1. Activity profiling of *C. kwangsiensis* rhizome extract for $GABA_A$ receptor modulating activity. (A) HPLC chromatogram (254 nm) of a semi-preparative separation of 5 mg extract. (B) Activity profile of collected peak-based microfractions a–r tested for I_{GABA} modulation. Peak labeling in the HPLC chromatogram refers to the isolated compounds **1**–**11**.

of on-line (LC-PDA-ESI-TOF-MS) and off-line (microprobe 1D and 2D NMR) spectroscopic data, and comparison with literature data. The relative configuration of compounds was established by NOESY and NOE difference experiments, while the absolute configuration was determined by comparison of experimental and calculated ECD spectra.

The ^1H and ^{13}C NMR data of compound **1** (Tables 1 and 2) were in agreement with the data published for curcuminol D, a diterpene isolated from *Curcuma wenyujin* (Zhang et al., 2008) and *Curcuma zedoaria* (Park et al., 2012). However, the relative configuration of curcuminol D was established only for the decalin ring system, whereas the configuration at C-15 remained undefined. We here report the unequivocal determination of the relative and absolute configuration of compound **1** based on NMR spectral assignments and ECD spectroscopy. Assignment of the relative configuration of the *trans*-decalin system was supported by NOESY correlation between H-5 and H-9, and between CH_3-20 and H-2β. Hence, H-5, H-9, and CH_3-20 were in an axial position, indicative for two possible absolute configurations of the decalin ring system (5*R*,9*R*,10*R* or 5*S*,9*S*,10*S*). In addition, 1D NOE difference experiments were performed to assign the relative configuration at C-15 (Fig. 3). Presaturation of H-15 resulted in the enhancement of H-14a, H-16a, and H-16b. Irradiation of H-14b enhanced H-14a, H-9, and H-16a, while no enhancement of H-15 was observed. A selective 1D TOCSY experiment was used to unambiguously determine the multiplicities of H-14a, H-14b, H-16a, and H-16b (Table 1). Excitation of H-15 unraveled H-14b as a doublet of doublet with coupling constants J = 13.7, and 12.6 Hz, indicative for the *trans*-orientation of both protons. Consequently, the hydroxyl group at C-15 had to be in α-orientation. This was further corroborated by the vicinal coupling constants between H-14a/H-15 ($^3J_{H-H}$ = 4.1 Hz), H-16a/H-15 ($^3J_{H-H}$ = <4.0 Hz), and H-16b/H-15 ($^3J_{H-H}$ = 3.1 Hz) which corresponded to dihedral angles of approx. 50°, 50–60°, and 60°, respectively.

In order to establish the absolute configuration of **1**, ECD spectra were calculated for two stereoisomers (5*S*,9*S*,10*S*,15*S* and 5*S*,9*S*,10*S*,15*R*). Conformational analysis using OPLS 2005 molecular mechanic force field in H_2O revealed nine and eight conformers,