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# DISSERTATION

Titel der Dissertation

## „Isolation of positive, allosteric GABA$_A$ receptor modulators from Chinese herbal drugs traditionally used in the treatment of anxiety and insomnia"

Verfasserin

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*For Maximillian & Lennox*

| | |
|---|---|
| 5-HT$_3$ receptor | 5-hydroxytryptamine type 3 receptor |
| ACh | acetyl choline |
| ASR | anisaldehyde sulphuric acid reagent |
| ATF4/CREB | activating transcription factor 4/cAMP-response element binding protein 2 |
| BIG2 | brefeldin A-inhibited GDP/GTP exchange factor 2 |
| BZ | benzodiazepine |
| CAM | complementary and alternative medicine |
| CC | column chromatography |
| cF | cumulative fraction |
| CHM | Chinese Herbal Medicine |
| CHOP | CCAAT/enhancer binding protein (C/EBP), epsilon homologous protein |
| CM | Chinese Medicine |
| CNS | central nervous system |
| DAD | diode array detector |
| DEPC | diethylpyrocarbonate |
| DZP | diazepam |
| EBOB | ethynylbicycloorthobenzoate |
| ELSD | evaporative light scattering detector |
| EPM | elevated plus maze |
| ESI | electro spray ionization |
| EtOAc | ethyl acetate |
| FLZ | flumazenil |
| GABA | γ-aminobutyric acid |
| GABA$_A$ receptor | GABA type A receptor |
| GABA$_B$ receptor | GABA type B receptor |
| GABARAP | GABA$_A$ receptor adaptor protein |
| GABA-T | GABA transaminase |
| GAD | glutamate dehydrogenase |
| GAT | GABA transporter |
| GODZ | Golgi specific DHHC zinc finger |

| | |
|---|---|
| GPCR | G protein coupled receptor |
| GRIF 1 | $GABA_A$ receptor interacting factor-1 |
| GRIP | glutamate receptor interacting protein |
| HAc | acetic acid |
| HAP | huntingtin associated protein |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HPLC | high performance liquid chromatography |
| $I_{GABA}$ | GABA-induced chloride current |
| LGIC | ligand gated ion channel |
| MeCN | acetonitrilee |
| MeOH | methanol |
| MS | mass spectrometer/mass spectrometry |
| msec7-1 | mammalian homologue of yeast sec7p-1 |
| n ACh receptor | nicotinic acetyl choline receptor |
| NMR | nuclear magnet resonance |
| NSF | n-ethylmaleimide sensitive fusion protein (or receptor) |
| pA | pro Analysis |
| PE | petroleum ether |
| Plic-1 | protein linking IAP (CD74) to cytoskeleton-1 |
| PRIP | PLC-related catalytically inactive protein |
| PTX | picrotoxin(in) |
| PTZ | pentylenetetrazol |
| Rf | retention factor |
| S.E.M | standard error of the mean |
| SSADH | succinic semialdehyde dehydrogenase |
| TBPS | t-butylbicyclophosphorothionate |
| THPROG | tetrahydroprogesterone |
| TLC | thin layer chromatography |
| TM | transmembrane domain |
| VGAT | vesicular GABA transporter |
| VLC | vacuum liquid chromatography |

# 1 Introduction

## 1.1 Natural products in drug discovery

Plants have always been a good source for (small molecule) drugs, since they produce a wide range of bioactive primary and secondary metabolites. Conservatively estimated, natural products (NPs) and products derived thereof including semi-synthetic drugs and natural product-mimetics, still account for approximately 30 % of all newly approved drugs between 1981 and 2006 (Fig. 1; Harvey *et al.*, 2010; Newman, 2008; Potterat *et al.*, 2008). Furthermore, the impact of natural products on our knowledge about basic biological processes can be recognized in the great variety of drug targets that are named after compounds from natural origin like μ-(morphine)-opioid, nicotinic and muscarinic acetyl choline-, cannabinoid-, or kainate receptor.
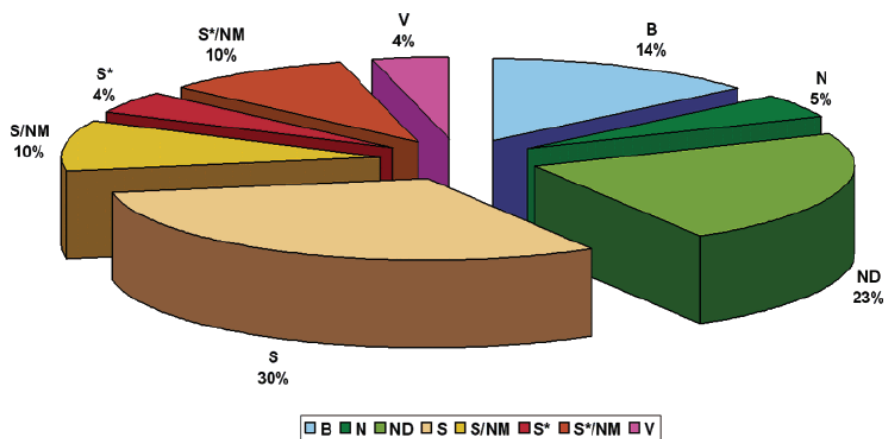


**Figure 1.** Natural products in drug discovery. The pie chart displays all new chemical entities approved as drugs between 1981 and 2006. "B" Biological; usually a large (>45 residues) peptide or protein either isolated from an organism/cell line or produced by biotechnological means in a surrogate host. "N" Natural product. "ND" Derived from a natural product and is usually a semisynthetic modification. "S" Totally synthetic drug, often found by random screening/modification of an existing agent. "S*" Made by total synthesis, but the pharmacophore is/was from a natural product. "V" Vaccine. "NM" Natural product mimic (Newman and Cragg, 2007).

The discovery of new drugs from natural sources mainly focuses on anti-cancer or anti-infective/-microbial activities and a comparably smaller number of studies deals with the effects of natural compounds on the central nervous system (CNS) (Butler, 2008). However, basic knowledge in neuroscience was primarily gained by studies of CNS-active natural products, and they still remain crucial research tools in the field of neuroscience as well as

viable therapeutic targets for disease of the CNS. Moreover, such disorders are not limited to a small group of patients but affect a great number of the worldwide population (Tab. 1.)

| Disorder | Population (%) | references |
|---|---|---|
| Alzheimer's Disease | 13.0 % older 65y | (Castellani *et al.*) |
| Anxiety | 16.6 % | (Somers *et al.*, 2006) |
| Depression/mood disorders | 5.3 % US | (Gelenberg, 2010) |
| Epilepsy | 0.5 - 1.0 % | (WHO, 2005) |
| Sleep disorders | 30,00% | (Roth, 2007) |
| Other mental disorders | 13.0 % | (WHO, 2004) |

**Table 1.** Worldwide prevalence of common mental health disorders

As can be seen in Table 1, sleep and anxiety disorders are among the most prevalent mental disorders. In western countries, several synthetic drug classes are applied in the treatment of these diseases, including histamine-1 receptor ($H_1$)-antagonist for sedation, antidepressants (e.g. serotonin/norepinephrine reuptake inhibitors) for anxiolysis or benzodiazepines for both. The use of CAM is equally common; many people even favor the use of alternative treatment strategies over the use of synthetic derivatives since the risk of unwanted side effects is considered a lower one (van der Watt *et al.*, 2008; Sarris and Byrne, [Online early access]). One molecular target both, anxiety and insomnia, share is the γ-amino butyric acid (GABA) type A ($GABA_A$) receptor. Thus, in the following chapter, GABAergic neurotransmission will be elucidated focusing on the $GABA_A$ receptor

## 1.2 γ-Aminobutyric acid (GABA)

From the many targets for CNS drugs, the GABAergic system is of crucial importance, since GABA is the major inhibitory neurotransmitter in the mammalian brain (Somogyi *et al.*, 1998). GABA is counteracted by glutamate, which is responsible for the majority of excitatory neurotransmission, and balance of GABAergic and glutamergic signals is vital for the functioning of the CNS. Thus, an imbalance of these two neurotransmitter systems is probably involved in many if not all CNS disorders to some extent, making the GABAergic system a viable target for CNS drugs (Olsen, 2002). This might also be due to the fact, that

most of the interneurons found in the CNS, which regulate the activity of other neurotransmitter systems, are GABAergic (Mody and Pearce, 2004).

Nonetheless, manipulation of the GABAergic neurotransmission is more commonly related to a distinct set of disorders like epilepsy (Galanopoulou, 2010; Macdonald *et al.*, 2010), anxiety disorders (Kalueff and Nutt, 2007; Durant *et al.*, 2010), and insomnia (Ebert *et al.*, 2006; Winsky-Sommerer, 2009). But there is evidence for involvement of this receptor in schizophrenia and other neuropsychiatric disorders (Lewis *et al.*, 2008; Möhler, 2009; Vinkers *et al.*, 2010) or in the development of tolerance and addiction (Enoch, 2008; Tan *et al.*, 2010). Furthermore, it may also proof useful to target $GABA_A$ receptors in the treatment of neuropathic pain (Enna and McCarson, 2006; Munro *et al.*, 2009; Zeilhofer *et al.*, 2009).
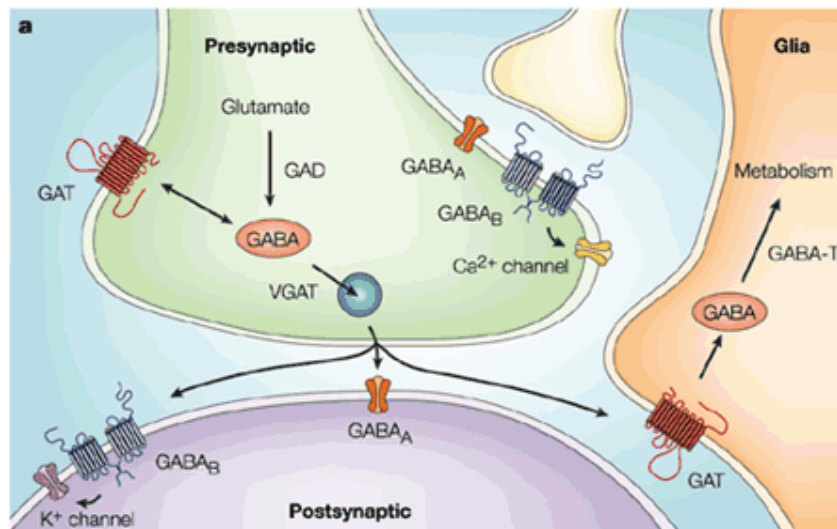


**Figure 2.** The GABAergic synapse. GABA is synthesized from glutamate by GAD, stored in vesicles and transported to the presynaptic membrane for release in the synapse, where it activates the GABA receptors type A and B. It is rapidly taken up through GATs, stored in vesicles again or degraded by GABA-T and SSADH. GAD: glutamate dehydrogenase VGAT: vesicular GABA transporter, GAT: GABA transporter, GABA-T: GABA transaminase, SSADH: succinic semi-aldehyde dehydrogenase (Owens and Kriegstein, 2002).

GABA itself is derived enzymatically from glutamate by glutamate decarboxylase (GAD), and 2 subtypes of this enzyme are known so far ($GAD_{65}$ and $GAD_{67}$). It is degraded into α-ketoglutarate or succinic acid through GABA-Transaminase (GABA-T) and succinic semi-aldehyde dehydrogenase (SSADH), respectively before it enters the Krebs cycle. This enzyme machinery is also referred to as the GABA shunt (Hassel *et al.*, 1998; Yogeeswari *et al.*, 2005). GABA is stored in small vesicles, in which it is transported through the vesicular GABA transporter (VGAT). GABA is released into the synaptic cleft by fusion of the small vesicles to the presynaptic membrane, where it can bind to its target receptors. Shortly

thereafter it is taken up again in the pre-synapse or in surrounding glia cells by GABA transporters (GAT 1-3 and the low affinity transporter BGT 1), where it is stored in vesicles or degraded through GABA-T and SSADH (Owens and Kriegstein, 2002) as depicted in figure 2, p. 3.

There are two types of GABA receptors: (1) $GABA_A$ and $GABA_{A-\rho}$ receptors, latter also referred to as $GABA_C$ receptors, are ligand gated ion channels and thus responsible for fast inhibition of neurotransmission (Mody and Pearce, 2004). (2) $GABA_B$ receptors are G-protein coupled receptors (GPCRs). These metabotropic receptors mainly influence $K^+$ and $Ca^{2+}$ channels, as well as second messenger systems. This again results in a decrease of neurotransmission, albeit slower as the inhibitory signals mediated by $GABA_A$ receptors (Bowery et al., 2002).

## 1.2.1 The $GABA_A$ receptor

The binding of GABA to $GABA_A$ receptors leads to conformational changes of the channel/receptor complex. These allow the channel to open and chloride as well as bicarbonate anions can influx the cell following their potential gradient. The increased amount of negative charge in the intracellular space hyperpolarizes the membrane. This results in an inhibitory postsynaptic potential that decreases the possibility of neurotransmission. This process is balanced by glutamate and finally the sum of excitatory and inhibitory signals determines if the postsynaptic neuron transmits a signal (D'Hulst et al., 2009). Although most of the $GABA_A$ receptors can be found in the synapse (post- and presynaptic), there are peri- and extrasynaptic receptors as well. While postsynaptically assembled receptors mediate phasic inhibition, extrasynaptic receptors seem to be responsible for tonic inhibition of neurotransmission (Mody and Pearce, 2004).

## 1.2.1.1 $GABA_A$ receptor structure

$GABA_A$ receptors are Cys-Loop pentameric ligand gated ion channels (LGIC) and closely related to nicotinic acetyl choline (nACh), 5-hydrosytryptamine type 3 ($5-HT_3$) and glycine receptors. Both, the C- and the N-terminus stretch out in the extracellular room, with a highly

conserved Cys-loop in the extracellular domain of the N-terminus. Generally, each of the 5 subunits consists of four transmembrane (TM) domains. They form a central pore in which the four TMs2 build the inner wall of this pore, which functions as the ion channel. The transmembrane domains TM3 and TM4 are linked through a large intracellular loop, which is responsible for most of the interactions between the receptor and the cytosol as depicted in figure 3A (Jacob *et al.*, 2008).

Although the crystal structure of the GABA$_A$ receptor could not be solved so far, there are computational homology models that derive its presumed structure using other proteins of the Cys-loop pentameric receptor superfamily based on the fact, that all these receptors share at least 30 % sequence similarity. This includes the nACh receptor of the marbled electric ray (*Torpedo marmorata*) which is depicted in figure 3B (Unwin, 2005).
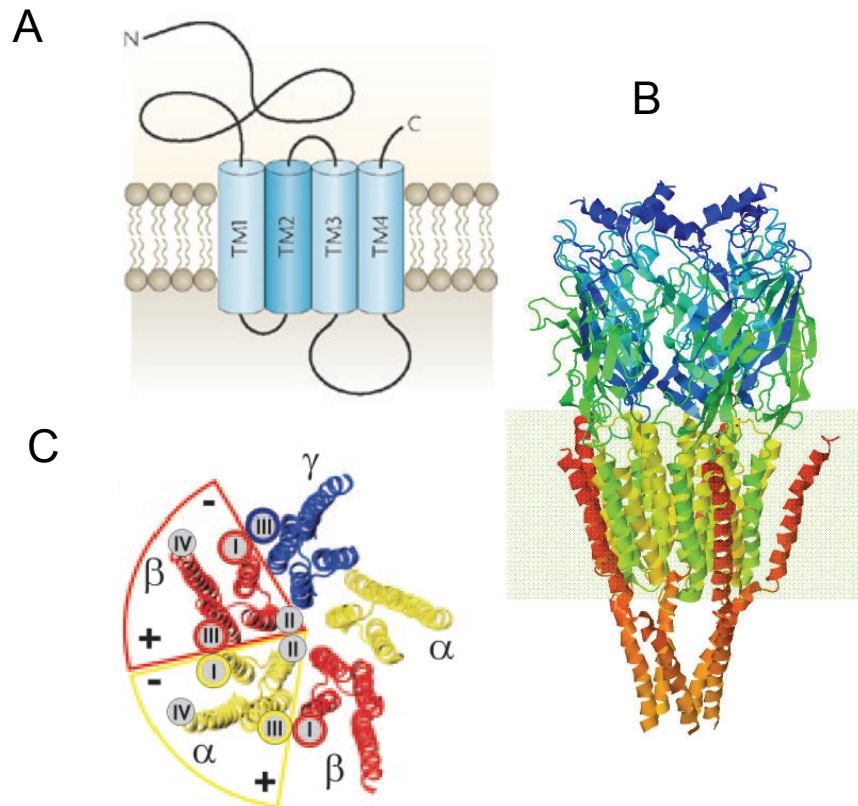


**Figure 3.** Structure of Cys-Loop pentameric ligand gated ion channels. A. Each of the five subunits consists of 4 transmembrane domains, whereby TM3 and TM4 are linked through a large intracellular loop. Both, the N- and C-terminus, are situated in the extracellular room. (Jacob *et al.*, 2008). B. Top view of a $\alpha_n\beta_n\gamma_n$ GABA$_A$ receptor channel. The 5 TM2s, here indicated as helix II build the inner wall of the pore (Ernst et al., 2005) C. 4 Å crystal structure of the nACh receptor isolated from *Torpedo marmorata* (Unwin, 2005) (left). The transmembrane domains are enclosed in the green box.

Furthermore, the soluble acetylcholine binding protein from the mollusk *Lymnaea stagnalis* (Brejc *et al.*, 2001) or the water soluble portion of the muscle nACh receptor (Dellisanti *et al.*,

2007; Chen, 2010) were used for homology models. There is good scientific evidence, that most functional $GABA_A$ receptors are build from 3 different types of subunits and that these 3 subunit-types are arranged alternately. In figure 3C, p. 5 (Ernst *et al.*, 2005), the top view of a representative αβγ-recpetor, is displayed in its typical α-β-α-β-γ arrangement (Tretter *et al.*, 1997; Baumann *et al.*, 2002; Minier and Sigel, 2004). Each interface between the different subunits is formed by contacts between the membrane spanning helices II (TM2s), as well as by contacts of helix I (TM1) of the minus side with helix III (TM3) of the plus side in two connected subunits (Ernst *et al.*, 2005).

### 1.2.1.2 Subunits and subtypes

$GABA_A$ receptors are widely distributed within the CNS. They show a considerable heterogeneity arising from the existence of 19 different subunits identified in the human genome: $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, δ, ε, $\rho_{1-3}$, θ and π (Simon *et al.*, 2004), whereby various splice variants add to this variety e.g. $\gamma_{2S}$ and $\gamma_{2L}$ (Whiting *et al.*, 1990). Since the $GABA_A$ receptor is formed from 5 subunits, this would theoretically allow the existence of some thousand different receptor subtypes, whereas only a few combinations have been conclusively identified *in situ* (Olsen and Sieghart, 2008). In general, $\alpha_{1-6}$, $\beta_{1-3}$, and $\gamma_2$ are considered the major $GABA_A$ receptor subunits in the mammalian brain, while the abundance of $\gamma_1$, $\gamma_3$, θ, ε, π and $\rho_{1-3}$ is rather low and/or scientific investigations on native receptors containing these subunits is still rare (Olsen and Sieghart, 2009). However, most of the native receptors are made up from two α, two β and one γ subunit. The most abundant receptor subtype is $\alpha_1\beta_2\gamma_2$, which represent around 60 % of all $GABA_A$ receptors in the mammalian brain, followed by $\alpha_2\beta_3\gamma_2$ (15-20 %) and $\alpha_3\beta_n\gamma_2$ (10 to 15 %). $\alpha_4\beta_n\delta$, $\alpha_5\beta_n\gamma_2$ as well as $\alpha_6\beta_n\delta$ receptors each occur to less than 5 % in the mammalian brain (Sieghart and Sperk, 2002; Moehler, 2006).

Different $GABA_A$ receptor subtypes exert different pharmacological profiles as extensively reviewed by Olsen and Sieghart (2008, 2009). In addition, these subtypes differ in their distribution pattern throughout the brain or even in their localization in a single neuron (Wisden *et al.*, 1992; Korpi *et al.*, 2002; Sieghart and Sperk, 2002). Thus, the different $GABA_A$ receptor subtypes are thought to be highly specialized for a specific neuronal circuit and thus for different CNS disorders.

*Receptor pharmacology based on subunits*

That the pharmacological profile of a receptor subtype depends on the subunits it is co-assembled from was convincingly shown for the first time through altered reaction of α-subunit knock-in mutated mice to diazepam. It was shown that the mutation of $\alpha_1$ led to the loss of diazepam mediated sedation (Rudolph *et al.*, 1999). In several subsequent studies it was found that different α-subunits are responsible for different pharmacological effects. Thus, while $\alpha_1$ subunits are mostly responsible for sedation (McKernan *et al.*, 2000), $\alpha_2$ mediates the anxiolytic effects of diazepam (Löw *et al.*, 2000), which seems to be valid for $\alpha_3$ as well (Atack *et al.*, 2005; Dias *et al.*, 2005). Furthermore, $\alpha_3$ and $\alpha_5$ are connected to psychiatric disorders (Yee *et al.*, 2004; Hauser *et al.*, 2005). Receptors containing an $\alpha_5$ subunit are also involved in learning and memory (Martin *et al.*, 2009), which is enhanced when this subunit is inactivated or $\alpha_5$ selective inverse agonist are applied (Crestani *et al.*, 2002; Collinson *et al.*, 2006; Dawson *et al.*, 2006; Prut *et al.*, 2010)

Since both, $\alpha_4$ and $\alpha_6$ subunits, are in-sensitive to diazepam, such experiments could not be carried out for these two α-subunits. However, they are often co-assembled with the δ-subunits in extra- or perisynaptic receptors (Nusser *et al.*, 1998; Peng *et al.*, 2002; Wei *et al.*, 2003). These receptors exhibit a strongly different pharmacological profile (higher affinity for GABA and other drugs than BZs, lower desensitization rate) and are thought to be responsible for most of the tonic inhibition in the CNS (Caraiscos *et al.*, 2004; Mody and Pearce, 2004; Farrant and Nusser, 2005).

Following the studies on the diazepam-sensitive α-subunits, other subunits were also investigated for distinct pharmacological profiles. In general, most β-subunit incorporating receptors contain $\beta_2$, followed by $\beta_3 > \beta_1$ (Sur *et al.*, 2001). β-subunits are not as well characterized concerning subunit-specific effects, although a variety of β-selective substances has been found e.g. the anticonvulsant loreclezole (Wingrove *et al.*, 1994). Additionally, the GABAergic effects of alcohol seem to be majorly mediated via the β-subunits as well (Wallner *et al.*, 2003). Mutations in $\beta_2$ and $\beta_3$ subunits showed, that the sedative actions of etomidate are mediated through $\beta_2$ (Reynolds *et al.*, 2003), the anesthetic/immobilizing actions through $\beta_3$ subunits (Jurd *et al.*, 2003). Moreover, there is evidence for involvement of $\beta_3$-containing receptor subtypes in alcohol tolerance and withdrawal (Sanchis-Segura *et al.*, 2007) as well as epilepsy (Homanics *et al.*, 1997).

From γ-subunits it is known, that they are crucial for the activity of benzodiazepines. BZs display a somewhat different pharmacology on subtypes incorporating $\gamma_2$ (most sensitive)

compared to $\gamma_1$ (low affinity) and $\gamma_3$, where classical BZs like diazepam show no binding compared to e.g. z-drugs like zaleplone or zopiclone (Sieghart, 1995; Hevers and Luddens, 1998; Khom *et al.*, 2006). The $\gamma_2$-subunit is present in 75-80 % of all GABA$_A$ receptors. It can be substituted through $\gamma_1$ and $\gamma_3$, as well as $\delta$ or $\varepsilon$, while the $\theta$ and $\pi$ are only barely characterized (Olsen and Sieghart, 2008).

### 1.2.1.3 GABA$_A$ receptor dynamics

The life cycle of the GABA$_A$ receptor is highly regulated and can be modified at various stages beginning with expression and assembly of the different subunits to functional receptors in the endoplasmatic reticulum. Although there is increasing evidence for a wide range of proteins interacting with the GABA$_A$ receptor (Fig. 4, p. 9), two of them are strongly connected to GABA$_A$ receptor dynamics, the GABA receptor associated protein (GABARAP) and gephyrin (Jacob *et al.*, 2008).

Gephyrin was the first protein directly associated with GABA$_A$ receptors and it is tightly co-localized with them in the post-synaptic membrane (Kneussel and Betz, 2000; Lüscher and Keller, 2004). It anchors the receptors in the membrane, thus being responsible for stabilization and clustering. Inhibition of gephyrin expression leads to a total loss of $\alpha_2$ and $\gamma_2$ subunits in hippocampal cultures (Kneussel, 2002) and a dramatic decrease in GABA$_A$ receptor clustering was observed in gephyrin knock-out mice (Essrich *et al.*, 1998). The clusters that could form in absence of gephyrin showed high mobility and receptors diffused all over the neuronal membrane instead of accumulating at the synapse (Jacob *et al.*, 2005).

GABARAP seems to be an adaptor protein which selectively interacts with the $\gamma_2$-subunits of assembled receptors and can bind to microtubules as well as the anchoring protein gephyrin or the membrane fusion factor NSF (N-ethylmaleimide-sensitive fusion protein). Thus, GABARAP plays a major role in trafficking and clustering of $\gamma$-subunit containing receptors (Chen and Olsen, 2007). Knock-out mice lacking GABARAP nonetheless display normal phenotypes, thus indicating that other proteins can substitute for GABARAP (O'Sullivan *et al.*, 2005).
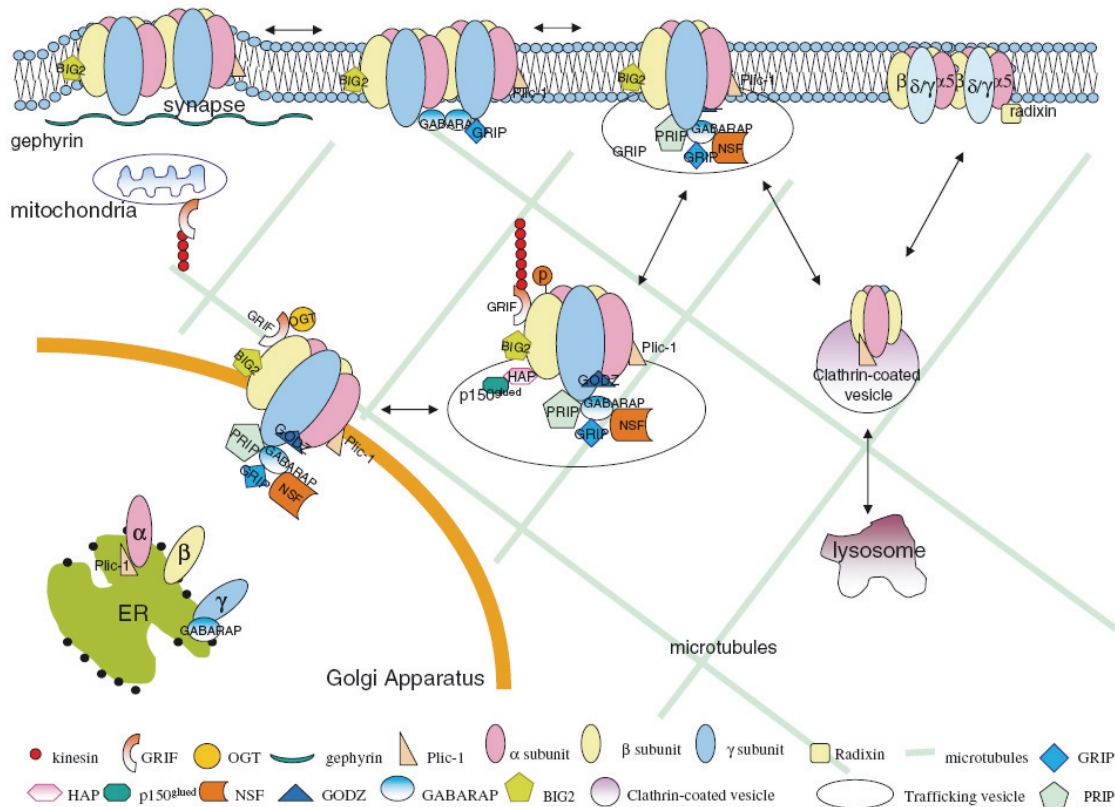
**Figure 4.** GABA$_A$ receptor dynamics. A range of proteins, as indicated in the figure, can influence successful expression and assembly of functional GABA$_A$ receptors at the cell surface and their internalization, often interacting with specific subunits (Chen and Olsen, 2007). GODZ: Golgi-specific DHHC zinc finger protein, BIG2: Brefeldin A-inhibited GDP/GTP exchange factor 2, Plic-1: proteins linking IAP (CD47) to cytoskeleton-1, PRIP: PLC-related catalytically inactive protein, HAP: huntingtin-associated protein, GRIP: glutamate receptor interacting protein, GRIF-1: GABA$_A$ receptor interacting factor-1.

Internalization of GABA$_A$ receptors into clathrin coated vesicles is mediated by adaptor protein 2 (AP-2) (Kittler *et al.*, 2002; Smith *et al.*, 2008) and such internalized GABA$_A$ receptors are transferred to the intracellular space. Here they await their recycling near the postsynaptic site, bound to gephyrin (Van Rijnsoever *et al.*, 2005) or are degraded in the endosomal system (Kittler and Moss, 2003). Still, many mechanisms of receptor dynamics remain to be elucidated.

### 1.2.1.4 GABA$_A$ receptor ligands

Actually, GABA$_A$ receptor pharmacology began in the 1970ies, when the convulsant bicuculline was found to inhibit certain inhibitory actions of GABA (Curtis *et al.*, 1971),

approximately 20 years after GABA was initially detected in the brain (Awapara, 1950). Subsequently the essential role of GABA in inhibitory neurotransmission and its target receptors were investigated and a wide range of $GABA_A$ receptor ligands was discovered (Fig. 5).

GABA itself interacts with a binding site situated on the interface between α and β subunits. This site is highly conserved in all subunits, albeit different subunits show variations in the potency of GABA binding (Ebert *et al.*, 1997). Next to GABA some other compounds mediate their effects through this binding site like the agonists muscimol, isoguvacine, the partial agonist gaboxadol (Krogsgaard-Larsen *et al.*, 1977) and the aforementioned bicuculline. Additionally, there are some non-competitive antagonist, which exert their activity on a binding site inside of the channel pore (Ffrench-Constant *et al.*, 1993), like picrotoxin(in) (PTX) and t-butylbicyclophosphorothionate (TBPS) as well as pentylentetrazol (PTZ). The clinical relevance of these compounds is low, but especially the competitive and non-competitive antagonists are often used to assess anticonvulsive activity of drugs in animal models.
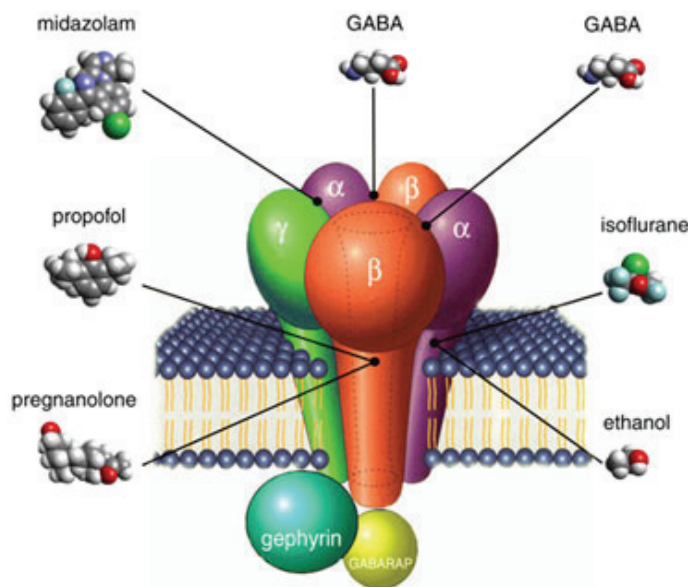


**Figure 5.** Endogenous ligands and drugs for the $GABA_A$ receptor. The PTX binding site, not depicted in this figure, is believed to be situated inside the channel pore (Harrison, 2007).

However, a range of clinically important drugs target the $GABA_A$ receptor and they are used in the treatment of anxiety, epilepsy and insomnia, as well as for induction and maintenance of anesthesia and to ease alcohol withdrawal (Sieghart, 1995; Korpi *et al.*, 2002). From all the

drugs targeting the $GABA_A$ receptor, the benzodiazepines (BZs) are probably the most commonly known ligands, since they are often prescribed in the treatment of anxiety and sleep disorders.

### Benzodiazepines

Generally, the binding site for BZs is distinct from the GABA binding site and situated between α and γ-subunits. Although the β-subunit is needed for functional receptors, it does not greatly influence the receptors sensitivity to BZs (Hadingham *et al.*, 1993). Moreover, differences in the pharmacological activities of BZs are mostly mediated via the α-subunit, as already mentioned before.

Benzodiazepines are used against insomnia since the 1960ies and for almost 10 years Valium® was the most prescribed drug worldwide (D'Hulst *et al.*, 2009). They superseded the barbiturates as hypnotic-sedative drugs due to their more favorable pharmacological profile. BZs are positive allosteric modulators and can not directly activate $GABA_A$ receptors, compared to barbiturates and thus are not as easily overdosed (Korpi *et al.*, 2002). Next to their sedative effects, BZs have a very broad range of activities and depending on their plasma-half life they are used for different disorders. Short- and intermediate-half life BZs are primarily used in the treatment of insomnia (e.g. lorazepam or temazepam). BZs with a longer plasma half-life are preferentially used to treat anxiety disorders especially generalized anxiety disorder (GAD) or panic disorders e.g. diazepam (WHO, 2009). Their anticonvulsant activity also makes BZs viable drugs in the therapy of epilepsy e.g. clobazam (Brodie, 2010).

Although the use of BZs is comparably save, they can generate a variety of side effects like fatigue, somnolence, drowsiness, ataxia, lethargy, sedation, involuntary muscle relaxation and worse, amnesia, tolerance, dependence and withdrawal symptoms (Lader, 2008). This of course limits their clinical use especially in the treatment of chronic disorders like anxiety or insomnia (WHO, 2009). Nowadays the BZs are often replaced by non-benzodiazepines, also referred to as the Z-drugs (zopiclone, zolpidem and zaleplone). These drugs, albeit structurally different, also interact with the BZ binding site. Still, their clinical advantage over BZs is not convincingly proven (Siriwardena *et al.*, 2008).

There is also evidence for naturally occurring BZs (Sangameswaran *et al.*, 1986), so called endozepines that are biosynthesized in humans probably through the intestinal flora (Baraldi *et al.*, 2009) or ingested with food, since they also have been found in various (edible) plants (Medina *et al.*, 1989).

*Barbiturates*

They have long been used as sedative and anxiolytic agents but due to their unfavorable pharmacological profile now have only limited use e.g. in the treatment of generalized seizures or to induce anesthesia. Among the barbiturates still on the market are thiopental, pento-, and phenobarbital. As already mentioned, barbiturates can not only modulate GABA activity but directly activate the $GABA_A$ receptor which makes them a more potent but also a more dangerous drug class compared to BZs.

*Non-volatile and volatile anesthetics*

Many anesthetic drugs exert their activity via the $GABA_A$ receptor; among them are the above mentioned barbiturates as well as etomidate, propofol and volatile anesthetics like enflurane or halothane (Korpi *et al.*, 2002). While propofol and etomidate are used to induce anesthesia (Li *et al.*, 2010a), the volatile anesthetics like en- or isoflurane are used for anesthesia maintenance. They exert their activity at least partly over $GABA_A$ receptors (Jones *et al.*, 1992), but the exact mechanism of their action or the putative binding sites need further characterization (Solt and Forman, 2007; Li *et al.*, 2010a).

*Neurosteroids*

Compared to the above mentioned drugs, neurosteroids are endogenous ligands of the $GABA_A$ receptor. It was already found in the 1940ies, that certain pregnane steroids have strong sedative and anesthetic effects (Selye, 1941). Neurosteroids (5α,3α and 5β,3α-THPROG, tetrahydroprogesterone) are biosynthesized from progesterone and can either act as positive allosteric modulators in low (nM) concentrations or as direct agonists in higher (more than 100 nM) concentrations (Lambert *et al.*, 1990). They not only act as sedative and anesthetic agents, but have anxiolytic and anticonvulsant activity (Mihalek *et al.*, 1999). There is now increasing evidence that these steroidal compounds are synthesized *in situ* in the CNS and act from the intracellular compartment on the receptors, exerting paracrine and autocrine effects (Lambert *et al.*, 2009).

Several binding domains could be identified so far, which seem to be located at the α and the β subunit, but are distinct from all other binding sites found at the $GABA_A$ receptor (Hosie *et al.*, 2007; Hosie *et al.*, 2009; Ator *et al.*, 2010). Neurosteroids preferentially bind to δ-subunit containing receptor subtypes or at least $δ^{-/-}$ mice are devoid of the normally observed neurosteroidal effects and the abundance of δ-subunit containing receptors is probably highly regulated by neurosteroid levels (Herd *et al.*, 2007). In their recent publication, Rupprecht and

co-workers (Rupprecht *et al.*, 2009) targeted the neurosteroids by applying the translocator protein (18 kD)-ligand XBD173, which promoted the synthesis of neurosteroids and thus exhibited anti-panic activity in humans. Thus neurosteroid-targeting drugs or neurosteroid-analogues will most likely add to the variety of $GABA_A$ receptor-interacting drugs for therapy of CNS disorders in the near future.

## 1.2.2 The $GABA_B$ receptor

The discovery of $GABA_B$ receptors by Bowery et al. (1980) was due to their insensitivity to bicuculline, and although activated by GABA, the receptors actions were not mediated by influx of $Cl^-$. Moreover, the muscle relaxant baclofen binds specifically to these receptors, and is the only $GABA_B$ receptor modulator used in clinics so far.

Successively, it was found that, in contrast to $GABA_A$ ion channels, $GABA_B$ receptors are metabotropic receptors and like all GPCRs, both of the two subunits consist of seven transmembrane domains (Millar and Newton, 2010). The $GABA_B$ receptor itself is an inhibitory $G_{i/o}$ receptor. The $G_{\alpha i}$ subunit blocks cAMP formation trough inhibition of adenylate cyclase and thus decreases the cell's conductance for $Ca^{2+}$ and influences many intracellular signaling pathways. The $G_{\beta\gamma}$ protein binds to voltage gated $K^+$ (Kir3) channel and finally hyperpolarizes the neuronal membrane.  Both pathways lead to a inhibition of neurotransmission and, more importantly, to adaptations and changes in receptor expression (Padgett and Slesinger, 2010). Remarkably, the $GABA_B$ receptor was also the first GPCR where it was convincingly shown that heterodimerization can be necessary for the building of a functional receptor (Jones *et al.*, 1998). So far, only two $GABA_B$ receptor subunits could be found $GABA_{B1}$ and $GABA_{B2}$, whereby $GABA_{B1}$ exists in two isoforms ($GABA_{B1a}$ and $GABAB_{B1b}$). The heterodimeric $GABA_B$ receptor is assembled from a $_{B1}$ and a $_{B2}$ subunit but it is possible that functional *homo*dimeric $GABA_{B1}$ receptors exists (Bettler *et al.*, 2004; Gassmann *et al.*, 2004; Villemure *et al.*, 2005).

Immunolabeling studies show that $GABA_B$ subunits are present in both, GABAergic and glutamergic pre- and postsynaptic sites, and can be found outside of the synapse as well. And it is believed that there is a strong interaction between $GABA_B$ and glutamergic signaling leading to changes in synaptic plasticity and to inhibition of action potential generation via $GABA_B$ (Chalifoux and Carter, 2010).

As recently reviewed, a range of proteins responsible for expression, trafficking, cell-surface expression and internalization of GABA$_B$ receptors have been found (Bettler and Tiao, 2006) e.g. Coat protein complex 1 (COP1), msec7-1, the two transcription factors ATF4/CREB (Vernon *et al.*, 2001) and CHOP (Sauter *et al.*, 2005), or Marlin-1, a RNA binding protein (Vidal *et al.*, 2007). Interestingly, GABA$_{B1}$ subunits can interact with the GABA$_A$ receptor $\gamma_2$-subunit and this interaction leads to an increase in agonist-induced internalization of the GABA$_B$ receptor (Bettler and Tiao, 2006).

GABA$_B$ receptor agonists and modulators could proof useful in the treatment of addiction, depression and gastro-esophageal reflux disease (Cryan and Slattery, 2010; Lehmann *et al.*, 2010; Tyacke *et al.*, 2010).

## 1.3 Natural compounds as GABA$_A$ receptor ligands

A vast variety of natural compounds, mostly but not solely derived from plants, have already been investigated for their activity on GABAergic targets (Johnston *et al.*, 2006b). Next to substances acting as GABA-T inhibitors e.g. gastrodin (An *et al.*, 2003) triterpene acids (Awad *et al.*, 2009b) or coumarins (Choi *et al.*, 2005); and GAT-inhibitoires (Zhao *et al.*, 2010), many natural products exert their effects via interaction with the GABA$_A$ receptor.

### *Alkaloids*

The alkaloid bicuculline (Fig. 6A, p. 15) was the first compound recognized as GABA$_A$ receptor-affecting natural compounds (see chapter 1.2.1.4, p. 9), which can be found in *Corydalis* or other plant species. Other prominent representatives are the bicuculline-related compound (+)hydrastine (Huang and Johnston, 1990) or the agonist muscimol (Fig. 6B, p. 15) isolated from *Amanita muscaria* (Johnston *et al.*, 1968). All three compounds directly interact with the GABA binding site. Securinine - an indolizidine alkaloid isolated from *Flueggea suffruticosa* (Pall.) Baill. - and related components were found to function as GABA$_A$ receptor antagonists, probably also due to direct interaction with the GABA binding site (Beutler *et al.*, 1985).
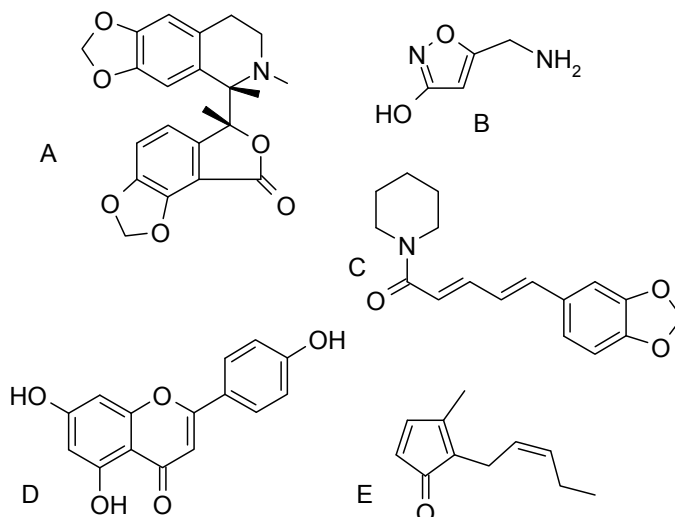
**Figure 6.** Examples for alkaloids, flavonoids and fragrant compounds affecting the GABA$_A$ receptor. **(A)** Bicucculine. **(B)** Muscimol. **(C)** Piperine. **(D)** Apigenin. **(E)** Cis-jasmone**.**

Furthermore, the diterpene-alkaloid songorine, found in *Aconitum* species, acts as a GABA$_A$ receptor antagonist (Zhao *et al.*, 2003), and the purine alkaloids theobromine and theophylline even displayed negative modulatory effects when tested in a functional assay (Hossain *et al.*, 2003). More recently, the pungent principle of *Piper* sp. - piperine (Fig. 6C) - was found to enhance I$_{GABA}$ to some extent (Zaugg et al., 2010).

*Fatty acids*

Linoleic acid and its hydro-peroxide displayed inhibitory activity on GABA$_A$ receptor channels in a functional assay using whole rat brain mRNA injected in *X. laevis* oocytes (Aoshima, 1996). Another fatty acid, docosahexaenoic acid, which can be found in high amounts in fish oil or is produced by certain algae, was also found to inhibit I$_{GABA}$ (Nabekura *et al.*, 1998). Both, linoleic and docosahexaenoic acid are considered essential fatty acids, but what the above mentioned observations mean in context to physiological processes in the CNS still needs further elucidation.

*Flavonoids and related compounds*

Flavonoids are known for an immense range of bioactivities (Middleton jr. *et al.*, 2000; Andersen, 2006; de Pascual-Teresa *et al.*) and many of them have already been investigated for their activity on GABA$_A$ receptors or their affinity to the BZ binding site, with miscellaneous results.

For example, the biflavonoid amentoflavone displays high affinity for the BZ binding site (Nielsen *et al.*, 1988; Baureithel *et al.*, 1997) and is a negative allosteric modulator of GABA$_A$

receptors in the functional assay (Hanrahan *et al.*, 2003). The actions of apigenin (Fig. 6D, p. 15) on the GABA$_A$ receptor are more complex since it binds with high affinity to the BZ binding site, is a negative allosteric modulator co-applied with GABA. Interestingly, it enhances the positive effects of diazepam on I$_{GABA}$ at least *in vitro*. Upon this finding the term of "second order modulator" was created (Campbell *et al.*, 2004).

Methylated and methoxylated flavonoids like 6-methylflavanone (Hall et al., 2005), 6-methylflavone (Ai *et al.*, 1997; Hall *et al.*, 2004b), 6-methyl-apigenin (Wasowski *et al.*, 2002) or hispidulin (Kavvadias *et al.*, 2004) exert highest activity on the GABA$_A$ receptor so far. Only recently, a range of highly active synthetically derived flavon-3-ol derivatives were investigated for their positive modulatory effects on I$_{GABA}$. They were found to preferentially bind to α$_2$ containing receptors compared to α$_1$, exerting the expected anxiolytic effects *in vivo* without inducing sedation (Fernandez et al., 2008). Furthermore it was possible to define distinct structural features for I$_{GABA}$ enhancement - trans-configuration of the residues in positions 2 and 3, acetylation of the hydroxyl group in position 3 and methoxy substituents at the B ring (Mewett *et al.*, 2009).

Moreover, baicalin (Wang *et al.*, 2008), 6-hydroxyflavone (Ren *et al.*, 2010) and 2'-OH-flavones (Huen *et al.*, 2003) target the GABA$_A$ receptor and partly display anxiolytic activity *in vivo*. Additionally, certain flavonoid-glycosides seem to exert effects on the GABA$_A$ receptor e.g. linarin and hesperidin (Fernández *et al.*, 2004). Controversially, in a study from Fernandez et al. (2006), it was found that other flavonoid glycosides, although expressing affinity for the BZ binding site, did not positively enhance I$_{GABA}$ in a functional assay *in vitro*. Nonetheless, they showed anxiolytic activity *in vivo* (Fernández *et al.*, 2006). A compound related to the flavonoids - epicatechingallate - also displayed second order modulatory activity, comparable to apigenin (Campbell *et al.*, 2004).

### *Fragrances in food stuff and alcoholic beverages*

A range of aliphatic alcohols and aldehydes have been investigated for their effects on the GABA$_A$ receptor, whereby most of these components are fragrant/volatile substances found in different alcoholic beverages or in food stuff e.g. octen-3-ol, sotolone, cis-jasmone (Fig. 6E, p.15), or leaf alcohol (Aoshima and Hamamoto, 1999; Aoshima *et al.*, 2001; Hossain *et al.*, 2002; Hossain *et al.*, 2003; Aoshima *et al.*, 2005; Mitou *et al.*, 2008).

Moreover, ethanol itself is a GABA$_A$ receptor modulator and could be considered a natural product, since it is biosynthesized from glucose. Many effects of ethanol are mediated via the GABA$_A$ receptor including impairment in motor function and cognition, sedation and

anxiolysis (Kumar *et al.*, 2009). Not surprising, alcohol dependence seems (at least partly) mediated by the GABA$_A$ receptor as well (Enoch, 2008).

### *Terpenes*

The class of terpenes is one of the largest natural compound classes and displays great structural variety since they can be build from two to several C5- (prenyl-) units. This leads to the generation of different terpenoid structures i.e. mono-, sesqui-, di-, tri-, and tetraterpenes. Although no tetra-terpenoid compounds are known to interact with the GABA$_A$ receptor, there are various representatives from each of the other groups exerting effects on this receptor.

The mostly volatile **monoterpenes** (C10) are common constituents of essential oils and one can assume that these compounds easily penetrate the blood-brain-barrier giving rise to a variety of CNS effects. So far, most of the investigated substances in this group are cyclic oxygenated derivatives like the positive allosteric modulators borneol (Fig. 7A, p. 18) which can be found in high quantities in *Valeriana* sp. and *Salvia* sp (Granger *et al.*, 2005), menthol and related compounds from *Mentha* sp. (Hall *et al.*, 2004a; Watt *et al.*, 2008; Corvalán *et al.*, 2009), thymol from *Thymus* sp. (Priestley *et al.*, 2003) - a phenolic monoterpene - or linalool, which is a compound found in numerous essential oils (Aoshima and Hamamoto, 1999). Additionally, the convulsant thujone from *Artemisia absinthium* L. was found to be a negative allosteric modulator of GABA$_A$ receptors (Hold *et al.*, 2000).

The C15 compounds, the **sesquiterpenes**, do not only exhibit convulsant activity, as it is long known for picrotoxinin (Fig. 7B, p. 18) from *Anamirta cocculus* (L.) Weight & Arn. (Ticku and Maksay, 1983) or for bilobalide (Fig. 7D, p. 18) from *Gingko biloba* L. (Huang *et al.*, 2003). *Valeriana officinalis*, used in European phytotherapy as anxiolytic and sedative agent, contains next to GABAergic borneol and linarin the positive modulator valerenic acid (Fig. 7C, p. 18). While valerenic acid even displays certain selectivity for $\beta_2$-subunit containing receptors, the related acetoxy-valerenic acid and hydroxyl-valerenic acid are not able to enhance I$_{GABA}$ (Khom *et al.*, 2007). In addition, valerenic acid and some synthetic derivatives show promising anxiolytic activity *in vivo* (Khom *et al.*, 2010).

In search of insecticidal components, antagonists for the GABA$_A$ receptor were isolated from *Senecio palmensis* (Nees) C. Sm. ex Link. These so called silphinenes are sesquiterpene tri-lactones and strongly resemble picrotoxinin in structure (González-Coloma *et al.*, 2002). They

proved to inhibit Cl⁻ influx into cell cultures and thus are assumed to have antagonistic activity on the GABA$_A$ receptor (Bloomquist, 2003).

A C20 ***diterpene*** - miltirone (Fig. 7E) - was isolated from *Salvia millitorrhiza*, a TCM herbal drug used in the treatment of stroke and, more generally, cardiovascular disease. The compound itself and several semi-synthetic derivatives were found to interact with the BZ binding site using an *in vitro* 3H diazepam replacement assay (He *et al.*, 1990; Chang *et al.*, 1991).
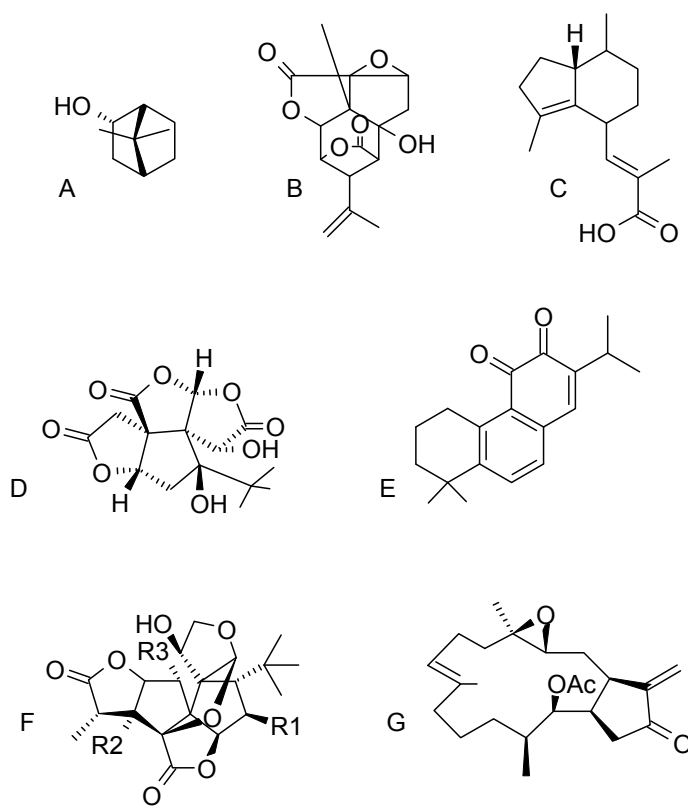


**Figure 7.** Terpenes influencing the GABA$_A$ receptor. **(A)** The monoterpene borneol. **(B)** The sesquiterpenes PTX, **(C)** valerenic acid and **(D)** bilobalide. The diterpenes **(E)** miltirone, **(F)** ginkgolide basic structure and **(G)** eupalmerin acetate.

About 15 years later, functional data on the I$_{GABA}$ effects were gained, at least for miltirone, but it neither exerted positive nor negative modulatory activity (Mostallino *et al.*, 2004). Nonetheless, miltirone was suggested to inhibit the up-regulation of α$_4$ subunit expression and thus probably has ameliorating effects on withdrawal symptoms caused by GABA$_A$ receptor modulators like alcohol.

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Related compounds from *Salvia officinalis*, namely carnosic acid, carnosol and galdosol were also found to interact with the GABA$_A$ receptor in a binding-study *in vivo*, although working on different binding sites (Rutherford *et al.*, 1992; Kavvadias *et al.*, 2003). Ginkgolides (Fig. 7F, p. 18), trilactone diterpenes from *Gingko biloba* L., displayed inhibitory activity on recombinant GABA$_A$ receptors (Huang *et al.*, 2003). A rather new class of GABA$_A$ receptor modulators is the cembranoids, macrocyclic diterpenes from mollusks or corals. In a recent study one such compound, eupalmerin acetate (Fig. 7G, p. 18), from *Eunicea* species (corals) displayed positive modulatory activity on the GABA$_A$ receptor *in vitro* probably via the neurosteroid binding site (Li *et al.*, 2008).

So far, several **triterpenes** were found to interact with the GABA$_A$ receptor: in a study from 2003 eight different ginsenosides were tested for their modulatory effects on GABA$_A$ receptors. It was found that ginsenosides are positive allosteric modulators and that ginsenoside C had the strongest ability to enhance I$_{GABA}$ (Choi *et al.*, 2003). The common triterpene betulin was investigated for its BZ binding site affinity and anticonvulsive activity *in vivo*. Related compounds betulinic acid and lupeol neither inhibited [$^3$H]flunitrazepam binding nor blocked bicuculline and picrotoxinin induced seizures (Muceniece *et al.*, 2008). Only recently, triterpenoid constituents from *Actaea racemosa* L. were found to strongly enhance I$_{GABA}$, with acetyl-shengmanole being the most active component (Cicek *et al.*, 2010).

**Neolignans** like honokiol (Fig. 8A, p. 20) and magnolol, dihydrohonokiol-B and some related components were investigated for their BZ binding affinity (Kuribara *et al.*, 1998; Squires *et al.*, 1999). They displayed anxiolytic-like effects *in vivo* in mice, using the elevated plus maze (EPM) and the Vogel Conflict test. They also seem to exert sedative effects *in vivo* by enhancing pentobarbital induced sleeping time

**Polyacetylenes** like cicutoxin (Fig. 8B, p. 20), virol A and cunaniol, isolated from water hemlock, *Cicuta virosa*, are antagonist (Uwai *et al.*, 2000) while the polyacetylenic compounds MS-1, -2, and -3 isolated from *Cussonia zimmermannii* display positive modulatory activity on the GABA$_A$ receptor with certain selectivity on β2-subunit containing receptor subtypes (Baur *et al.*, 2005).

*Various*

Only recently, a new compound class was discovered to enhance $I_{GABA}$, a dihydroisocoumarin isolated from *Haloxylon scoparium* displayed positive modulatory effects on GABA$_A$ receptors (Li *et al.*, 2010b). Another isocoumarin derivative isolated from the fungus *Neosartorya quadricincta* was shown to inhibit [(3)H]ethynylbicycloorthobenzoate (EBOB) binding, a compound interacting with the PTX binding site (Ozoe *et al.*, 2004). Thus it was concluded that this compound could be a new scaffold for insecticidal drugs. Caloporoside (Fig. 8C), a fungal metabolite isolated from *Caloporous dichrous*, was active in TBPS binding assay. Even disaccharides like lactose displayed a certain affinity for the GABA$_A$ receptor in a TBOB binding assay (Rezai *et al.*, 2003).
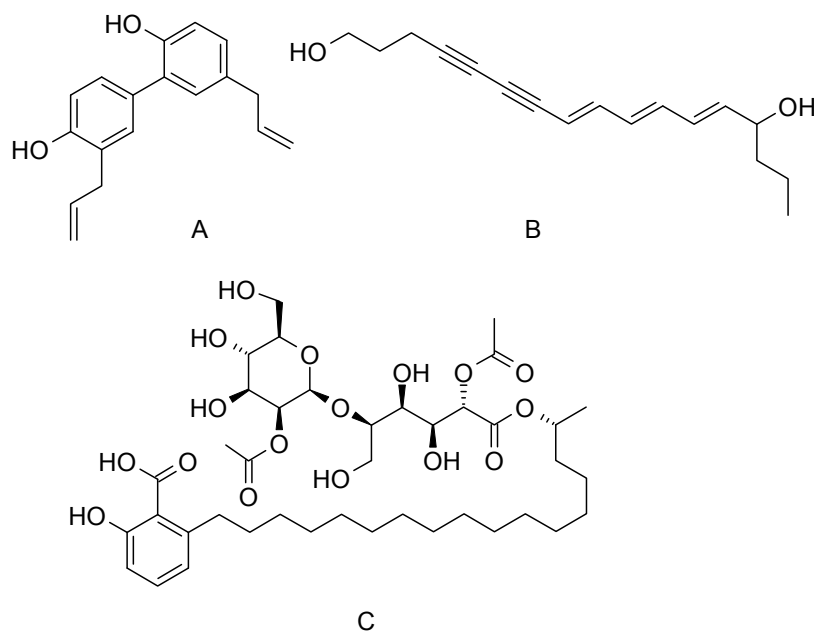


**Figure 8.** Examples for other GABA$_A$ receptor modulators. **(A)**The neolignan honokiol, **(B)** the polyacetylenic compound cicutoxin and **(C)** the benzoic acid derivative caloporoside.

In addition, some constituents already displayed promising activity *in vivo* without investigating the molecular mechanism of action. For example, dl-tetrahydropalmitate was found to exert anxiolytic effects *in vivo* and this effect could be blocked by flumazenil making interactions with the BZ binding site of the GABA$_A$ receptor likely (Leung et al., 2003). The same is valid for e.g. galphimines from *Galphimia glauca* (Herrera-Ruiz *et al.*, 2006) or polygalasaponins and other components from *Polygala* species (Duarte *et al.*, 2008; Yao *et al.*, 2010) etc. A range of different plants and natural compounds has been investigated for

their effects on the GABA$_A$ receptor but there is still a vast variety of plants traditionally applied as sedatives, anticonvulsants (Risa *et al.*, 2004; Moshi *et al.*, 2005; Jäger *et al.*, 2006; Awad *et al.*, 2009a; Pedersen *et al.*, 2009) or anxiolytic agents (Bourbonnais-Spear *et al.*, 2007; Quinlan, 2010) which lack functional data on their GABAergic activity or where not tested in this context so far.

**1.4 Aim of the study**

In the treatment of anxiety and insomnia people not only rely on synthetic drugs like the commonly prescribed benzodiazepines (BZs), but they often use herbal remedies since these are considered an effective alternative with lower risk of adverse effects. Some of the more popular traditional herbal drugs used in Western countries - like valerian (*Valeriana officinalis*) or kava kava (*Piper methysticum*) - have already been thoroughly investigated regarding their sedative/anxiolytic activity and their mechanism of action. Chinese Medicine (CM) also knows several anxiolytic and sedative remedies, but studies uncovering the molecular mechanisms underlying the proposed anxiolytic or sedative effects of these herbs are rare. Such ethnopharmacological studies are of great importance, since CM still plays a very important role in the Chinese health system and, furthermore, there is an ever-increasing interest in the Western countries for complementary and alternative medicine (CAM). Therefore, in this thesis 14 different Chinese Herbal medicines (CHMs) were selected mostly due to their use as sedative and/or anxiolytic agents and investigated for their activity on the $GABA_A$ receptor. This receptor is strongly involved in the regulation of sleep and arousal as well as pathological fear responses i.e. anxiety disorders and a viable target for sedative as well as anxiolytic drugs now used in clinics.

Hence, this work serves two aims. For one, these investigations aim to uncover at least part of the molecular mechanism of action using recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors, since they are a highly probable target for anxiolytic and sedative drugs. This would also provide scientific evidence for the ethnopharmacological use of the selected CHMs. Second, to isolate the compounds mainly responsible for the observed enhancement of $I_{GABA}$ in a bioactivity guided approach. This will lead to the discovery of new scaffolds for the development of anti-anxiety and anti-insomnia drugs.

## 2 Material and Methods

### 2.1 Plant material

All plant materials were purchased from Plantasia (Oberndorf, Austria). Voucher specimens are deposited in the Department of Pharmacognosy, University of Vienna.

| Plant name | drug | pinyin | lot No. | voucher No. |
|---|---|---|---|---|
| *Alibizia julibrissin* Durazz. (Fabaceae) | cortex | hé huān pí | 710805 | JS-11-07-AC |
| *Alibizia julibrissin* Durazz. (Fabaceae) | flos | hé huān huā | 410700 | JS-11-07-AF |
| *Arisaema erubescence* (Wall.) Schott.; *A. heterophyllum* BL.; *A. amurense* Maxim. (Araceae) | rhizoma | tiān nán xīng | 440141 | JS-11-07-AE |
| *Arnebia euchroma* (Royle) Johnst., *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) | rhizoma | zǐ cǎo | 002/7 | JS-11-07-AR |
| *Atractylodes macrocephala* Koidz. | rhizoma | bái zhú | 610043 | JS-11-07-AM |
| *Cnidium monnieri* (L.) Cuss. Ex Juss. (Apiaceae) | fructus | shé chuáng zǐ | 690341 | JS-11-07-CM |
| *Dimocarpus (Euphoria) longan* Lour. (Sapindaceae) | arillus | long yǎn ròu | 530395 | JS-11-07-DL |
| *Forsythia suspensa* (Thunb.) Val. (Oleaceae) | fructus | lián qiào | 440410 | JS-11-07-FS |
| *Juncus effusus* L. (Juncaceae) | medulla | dēng xīn cǎo | 660558 | JS-11-07-JE |
| *Lilium brownii* F.E. Brown *var. viridulum* Baker W; *L. lancifolium* Thunb.*, L. pumilum* DC. (Liliaceae) | bulbus | bǎi hé | 410169 | JS-11-07-LB |
| *Lophaterum gracile* Brong. (Gramineae) | herba | dàn zhú yè | 340528 | JS-11-07-LG |
| *Nelumbo nucifera* Gaertn. (Nymphaceaceae) | plumula | lián zǐ xīn | 520350 | JS-11-07-NN |
| *Polygonum multiflorum* Thunb. (Polygonaceae) | caulis | yè jiāo téng | 410853 | JS-11-07-PM |
| *Tribulus terrestris* L. (Zygophyllaceae) | fructus | cì jí lí | 410444 | JS-11-07-TT |

### 2.2 Chemicals and reagents

GABA, diazepam, flumazenil and reagents for ND96 buffer solution were purchased from Sigma (Vienna, Austria). All solvents used for extraction, chromatography and other phytochemical work were from highest purity grade available and purchased from VWR (Vienna, Austria).

### 2.3 Extraction

### 2.3.1 Extracts for initial screening

Extracts for the pre-liminary activity screening were produced as described in chapter 3.2./ manuscript chapter 2.3, p. 42.

### 2.3.2 n-Hexane extract from *Atractylodes macrocephala*

200 g of ground drug were exhaustively extracted with n-hexane on the water bath under reflux using 4 x 1 L n-hexane. After evaporation under reduce pressure, this yielded around 6 g of an oily, yellow residue.

### 2.3.3 Ethyl acetate (EtOAc) extract from *Juncus effusus*

2.2 kg of ground drug material were extracted on the water bath under reflux. This extraction was done portion-wise (between 30 and 50 g each) and exhaustively using 3 x 2 L of EtOAc for each portion. After evaporation under reduced pressure 13 g of a dark brown residue were gained.

### 2.4 Chromatography

### 2.4.1 Thin Layer Chromatography (TLC)

Stationary phase: aluminum plates coated with silica gel KG60 $F_{254}$ (Merck, Darmstadt, Germany) in varying dimensions (length to width: 10x10, 20x10, 20x12), which are given in the respective figure text.

The detection reagent anisaldehyde sulphuric acid reagent (ASR), which was used for the detection of compounds of *A. macrocephala* and *J. effusus*, was composed as following: MeOH:HAc:$H_2SO_4$conc:anisaldehyd in a ratio of 85:10:5:0.5. Briefly, 0.5 mL anisaldehyde were mixed with HAc and MeOH, then $H_2SO_4$conc was added drop-wise.

### 2.4.1.1 *Atractylodes macrocephala*

Mobile phase 1: n-hexane:EtOAc (95:5)
Mobile phase 2: n-hexane:EtOAc (80:20)
Detection: zones were detected in visible light and under $UV_{366}$ after spraying with ASR after heating for 5 to 10 min at 105 °C.

### 2.4.1.2 *Cnidium monnieri*

Mobile phase: n-hexane:EtOAc (90:10).
Detection: under $UV_{366}$ without further reagents.

### 2.4.1.3 *Juncus effusus*

Mobile phase: toluene:EtOAc:MeOH:HAc (35:10:1:2.5).
Detection: zones were detected in visible light and under $UV_{366}$ after spraying with ASR and heating for 5 to 10 min at 105 °C.

### 2.4.2 Column Chromatography (CC)

Stationary phase 1: silica gel KG60 (Merck, Darmstadt, Germany)
Stationary phase 2: Sephadex LH-20 (Sigma, Vienna, Austria).

### 2.4.2.1 *Atractylodes macrocephala*

Stationary phase 1.
Mobile phase: n-hexane:EtOAc 95:5.
Column dimensions for isolation of atractylenolide III: 60 x 0.5 cm i.d. (chapter 3.1, p. 32f.).
Column dimensions for isolation of atractylenolide II: 60 x 0.5 cm i.d. (chapter 3.1, p. 33f.).
Column dimensions for isolation of atractylenolide I: 90 x 4 cm i.d. (chapter 3.1, p. 35ff.).

**2.4.2.2 *Cnidium monnieri***

Stationary phase 1.

Mobile phase for fractionation of CM/HPLC/16 (chapter 3.3, p. 60): n-hexane:EtOAc (95:5).

Column dimensions for isolation of osthole and imperatorin: 60 x 0.5 cm i.d. (chapter 3.3, 60).

**2.4.2.3 *Juncus effusus***

Stationary phase 2.

Mobile phase for fractionation of JE/VLC/6 (chapter 3.5, p. 86): EtOAc.

Column dimensions for fractionation of JE/VLC/6 (chapter 3.5, p. 86): 65 x 1.5 cm i.d.

Mobile phase for fractionation of JE/VLC/5 (chapter 3.5, p. 88): EtOAc:MeOH (95:5).

Column dimensions for fractionation of JE/VLC/5 (chapter 3.5, p. 88): 70 x 1 cm i.d..

**2.4.3 Vacuum Liquid Chromatography (VLC)**

For all VLC columns following stationary phase was used: silica gel KG60 (Merck, Darmstadt, Germany).

**2.4.3.1 *Atractylodes macrocephala***

Mobile phases for VLC are given in chapter 3.1, Tab. 2, p. 35,

Column dimensions for VLC of the n-hexane extract: 90 x 4 cm i.d. (chapter 3.1, p. 35).

**2.4.3.2 *Juncus effusus***

Mobile phases for VLC are given in chapter 3.5, Tab. 3, p. 85.

Column for VLC: 65 x 3.5 cm i.d. (chapter 3.5, p. 85).

**2.4.5 High Performance Liquid Chromatography (HPLC)**

Instrument 1: consisting of a PE series 200 autosampler, a PE series 200 pump, a PE DAD 235C diode array detector and a PE nelson 600 series LINK interface.

Instrument 2: consisting of a DGU 20A5 degasser, a LC-20AD pump, a SIL 20ACHT autosampler, a SPD-M20A diode array detector, a CTO-20AC column oven and a CBM-20A communication bus module.

Instrument 3: consisting of a SIL-10AP autosampler, two LC-8A pumps, a SPD-M20A diode array detector, a FRC-10A fraction collector and a CBM-20A interface.

Column 1: Hypersil BDS-C18, 250 x 4.6 mm, 5 µm from Agilent Technologies.

Column 2: Hypersil C-18, 250 x 4.6 mm, 5 µm, from Thermo-Scientific Fisher.

Column 3: Nucleosil 100-7, C-18, 250 x 21, 5 µ from Machery-Nagel, Düren, Germany.

**2.4.5.1 *Atractylodes macrocephala***

For analysis and fractionation a method according to Wagner and Bauer (1997) was used, with a water (solvent A) and MeCN (solvent B) gradient as following: 62 to 70 % B in 10 min, 70 to 95 % in 2 min and held at 95 % for 8 min, with a flow rate of 1 mL/min for analysis and 27.6 mL/min for HPLC-aided fractionation.

**2.4.5.2 *Cnidium monnieri***

Analysis and fractionation were accomplished with a method according to Cheng et al. (2007) with a gradient consisting of water (solvent A) and MeCN (solvent B): 35 % B for 15 min, than increase to 80 % B in 5 min, steady concentration of B for 7 min followed by a decrease to 35 % B in 3 min. Analysis was ceased with a 5 min purge at 100 %. Flow rate was set at 1 mL/min for analysis and 27.6 mL/min for HPLC-aided fractionation.

**2.4.5.3** *Juncus effusus*

Analysis and fractionation were conducted with a water (solvent A)//MeOH (solvent B)-gradient: 60 to 80 %B in 15 min, with a flow rate of 1 mL/min for analysis and 27.6 mL/min for HPLC-aided fractionation. Analysis and fractionation were ceased with a 5 min purge phase at 100 % B.

**2.5 Mass Spectrometry (MS)**

MS was conducted as described in chapter 3.6, p. 100.

**2.6 Nuclear Magnetic Resonance Spectroscopy (NMR)**

**2.6.1 NMR of atractylenolide II and III**

NMR of atractylenolide II and III was conducted as described in chapter 3.2, p. 43 and spectra are shown in annex I (atractylenolide III) and annex II (atractylenolide II).

**2.6.1 NMR of atractylenolide I, osthole, imperatorin, effusol and dehydroeffusol**

NMR of the compounds was done as described in chapter 3.2, p. 43 and 3.6, p. 100. For the coumarins osthole (spectra see annex IV) and imperatorin (spectra see annex V) the same conditions as described for atractylenolide I (spectra see annex III) were used.

**2.7 Expression of GABA$_A$ Receptors in *Xenopus laevis* oocytes**

Expression of GABA$_A$ Receptors in *Xenopus laevis* oocytes was done as described in chapter 3.2, p. 44, 3.4, p. 67f. and 3.6, p. 101.

## 2.8 Two-microelectrode voltage clamp technique and automated fast perfusion system

The usage of the two-microelectrode voltage clamp technique and automated fast perfusion system was accomplished as described in chapter 3.2, p. 44f., 3.4, p. 67f.  and 3.6, p. 101f..

## 2.9 Data Analysis and statistics

Data analysis was conducted as described in chapter 3.2, p. 45, 3.4, p. 67 and 3.6, p. 102.

## 2.10. Molecular modeling

Binary classification tree and pharmacophore model were created as described in chapter 3.4, p. 69f..

## 3 Results

In this chapter the results will be displayed in form of three different manuscripts. While the first manuscript (chapter 3.2) deals with the GABA$_A$ receptor activity of 12 CHMs and focuses on *A. macrocephala*, the other two manuscripts describe the structural requirements for I$_{GABA}$ enhancement by coumarin derivatives (chapter 3.4) and the activity of *J. effusus* and two of its constituents (chapter 3.6). Further work and more detailed information on the phytochemical part of this work will be presented in separate chapters before the respective manuscript.

### 3.1 Isolation of the active principles from *Atractylodes macrocephala* Koidz.

*Atractylodes macrocephala* Koidz. (Asteraceae), also called largehead atractylodes, is a perennial plant growing in North-Eastern China. It is mainly collected in Zheijiang, Hubei and Hunnan province, whereby the plants from Yuqiang, Zheijing province are considered the best quality drug. The stem reaches heights of 0.4 to 0.6 m (Prajapati *et al.*, 2003), and is topped by big, purple inflorescences (Fig. 9).



**Figure 9.** Aerial parts of *A. macrocephala*, largehead atractylodes (downloaded from **http://www.botanyvn.com**, 14th of March 2011, 12:02 )

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The best time to collect the drug is end of October to beginning of winter. The part of the plant used as drug is the rhizome, which should be 2 to 3 years old to reach sufficient dimensions. The rhizomes are sliced into 2-3 cm thick slices and then either dried under the sun or bake/stir-fried with earth or bran.
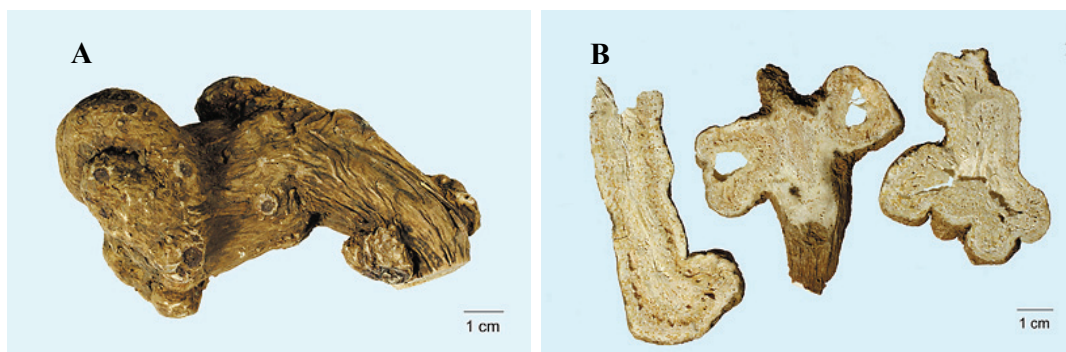


**Figure 10.** Whole **(A)** and sliced **(B)** rhizome of *A. macrocephala*, downloaded from http://www.tcmedicine.org, at the 28th of December 2010, 18:29

This drug is called bái zhú, white atractylodes. The name stems from the white crystals found on the surface of the dried and sliced drug (Fig. 10). The name white atractylodes also serves to distinguish this herbal drug from the related drug cāng zhú, which can be translated as black atractylodes (referring to the blackish-grey outer bark) and is derived from *A. lancea* (Thunb.) DC or *A. chinensis* (DC) Koidz.. Compared to the bái zhú, it contains high amounts of essential oil, which are freely visible as red dots sprinkling the surface. For this appearance the drug was formerly named chi zhú = red Atractylodes.

Bái zhú contains non-volatile sesquiterpene lactones and volatile sesquiterpene hydrocarbons, as well as polyacetylenes, fatty acids and polysaccharides. It is primarily used as a diuretic, digestive and spasmolytic agent; can stop spontaneous sweating and calm the fetus (Bensky *et al.*, 2006). Typically, 3-6 g of the drug are used, but the dosage can also go up to 20 g per decoction (Zhang, 2007).

So far, pharmacological investigations of this drug concentrated on its anti-inflammatory, anticancer or spasmolytic activity. Nonetheless, one of the most commonly used herbal remedies for insomnia is Jia-Wey-Shiau-Yau-San, which contains *A. macrocephala*, among other ingredients. It is also widely prescribed against symptoms related to menopause (Chen et al., 2009). Still, no investigations concerning its sedative effects were conducted.

After the initial extract screening, where the PE extract proofed to be the most active, semi-preparative HPLC (instrument 3, column 3) was engaged to fractionate the PE extract (3 x 50 mg in 4 mL MeCN each, injection volume: 3.5 mL). The following peak-based unification yielded 18 fractions in total, whereby fractions number 4 and 8 displayed highest activity. In some other fractions, e.g. fraction 18, residual activity could be observed (see chapter 3.2, p. 48, fig. 3A and p. 54, tab. S1).

First, fraction 4 and 8 were analyzed by TLC using n-hexane:EtOAc (80:20) as mobile phase. After spraying with ASR and heating at 105 °C zones could be detected under $UV_{366}$. Both fractions showed one prominent blue fluorescent zone with $R_f$ values of ~ 0.2 and ~ 0.4. After TLC screening, both fractions were subjected to HPLC analysis, displaying only one prominent peak each at Rt ~ 4.8 min and ~ 7.2 min (Fig. 11) at 215 nm. According to Wagner and Bauer (1997) these peaks are due to atractylenolide III and II, respectively.
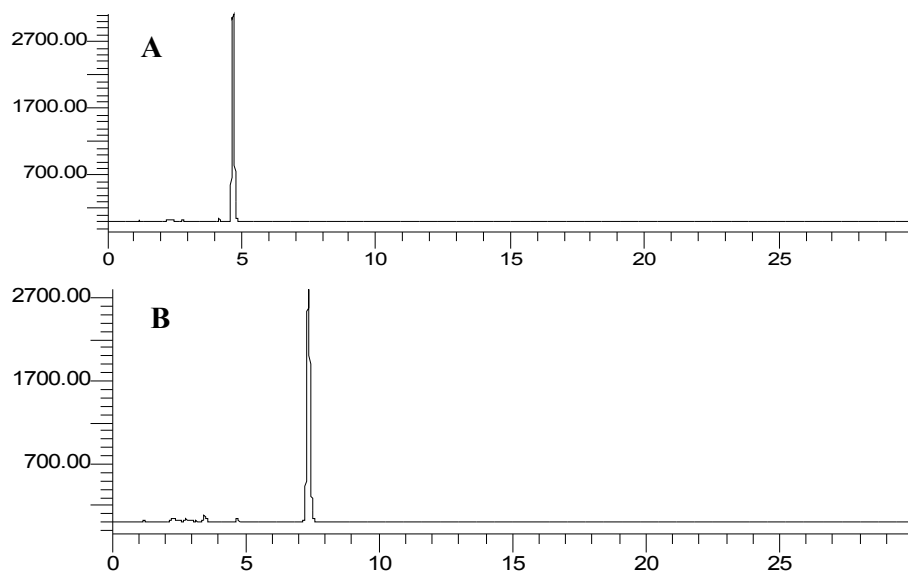


**Figure 11.** HPLC chromatograms of (A) fraction 4 and (B) fraction 8. Analysis method according to Wagner and Bauer (1997) with MeCN and water as mobile phase (chapter 2. 4.5.1, p. 27) on instrument 1 employing column 1. Injection volume was 20 µL and solutions of 1 mg/mL MeCN were used. Detection wavelength: 215 nm.

The two fractions were separately subjected to column chromatography (CC). Fraction AM/HPLC/4 (19 mg) was dissolved in MeOH and mixed with ~ 40 mg of silica gel. This mixture was evaporated to dryness under reduced pressure. For the column silica gel was

suspended in a sufficient amount of n-hexane:EtOAc (95:5) for 3 hours before it was filled in a glass column (60 x 0.5 cm i.d.). The fraction was then applied on top of the column and n-hexane:EtOAc (95:5) was used as mobile phase with a flow rate of 20 mL/h. 1 mL fractions were collected, resulting in a total of 60 fractions. After TLC screening the first 50 mL collected could be discarded (as pre-run), while in fractions 39 - 41 and 43 - 47 prominent blue fluorescent zones could be detected.

While fraction AM/HPLC/4/39-41 yielded 0.5 mg of an oily residue and was considered an impurity, fraction AM/HPLC/4/43-47 yielded 14 mg of white crystals. This fraction was further analyzed by HPLC (Fig. 12) and 1D- and 2D-$^1$H and $^{13}$C NMR identified this compound as atractylenolide III, one of the major sesquiterpene lactones of *A. macrocephala* (annex I).
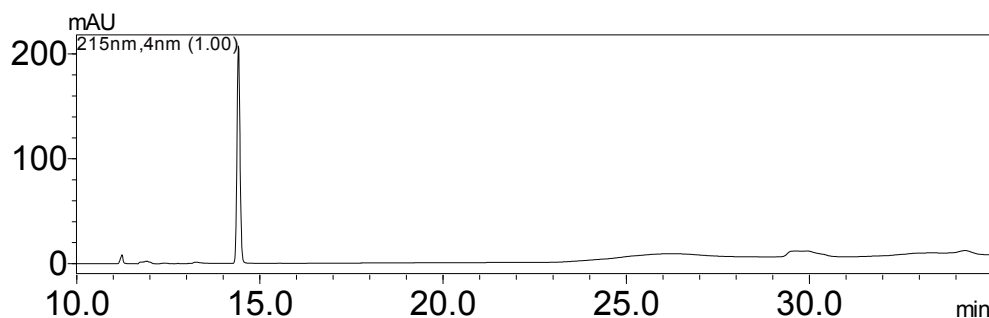


**Figure 12.** HPLC analysis of fraction AM/HPLC/4/43-47 on instrument 2, column 1. Injection volume: 5 µL of a solution of the fraction in a concentration of 0.5 mg/mL MeCN. Analysis method according to Wagner and Bauer (1997, chapter 2. 4.5.1, p. 27). Sample was injected at minute 10, after column equilibration, resulting in a final Rt of 4.9 min. of the major peak. Detection wavelength: 215 nm.

The purification of fraction AM/HPLC/8 (10 mg) was done likewise with n-hexane:EtOAc (95:5) as mobile phase and silica gel as stationary phase in a glass column of exactly the same dimensions. Fractions of 5 mL were collected, whereby the flow rate was set at 10 mL/h. Every 5th fraction was screened by TLC to reveal only one blue fluorescent zone in fraction AM/HPLC/4/25 (Fig.13, p. 34).
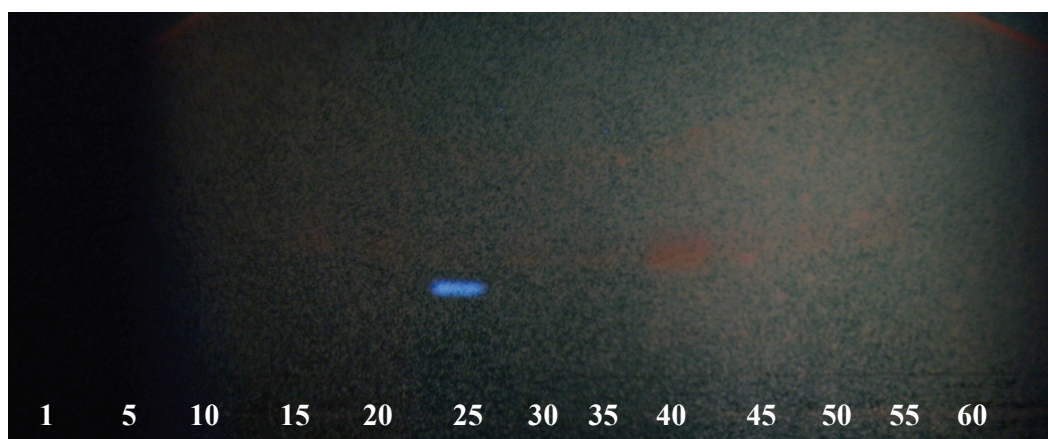
**Figure 13.** TLC screening of the fractionation of AM/HPLC/8 using silica gel plates (KG60 $F_{254}$, 20x10) revealed one blue fluorescent zone under $UV_{366}$ after spraying with ASR and heating at 105 °C for 5-10 min. The application volume of 20 µL was directly taken from the fractionation vials.

Thus, fractions 23 to 28 were analyzed (Fig.14) and fractions 23-27 could be unified to yield 5.6 mg (fraction AM/HPLC/8/23-27). This cumulative fraction was again analyzed by HPLC (Fig. 15, p. 35) and the structure was elucidated by $^{1}$H- and $^{13}$C-NMR as atractylenolide II (annex II).
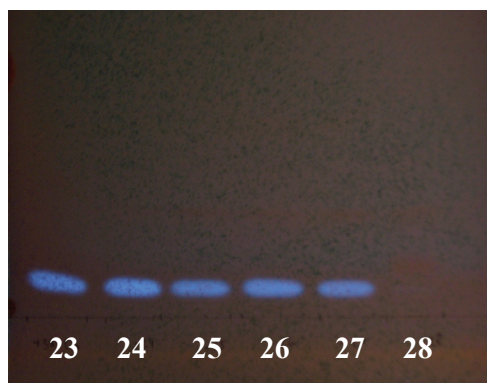


**Figure 14.** TLC screening of fractions AM/HPLC/8/23 to -/28 using silica gel plates (KG60 $F_{254}$, 10x10) revealed one blue fluorescent zone under $UV_{366}$ after spraying with ASR and heating at 105 °C for 5-10 min. The applied volume (20 µL) was taken directly from the fractionation vials.
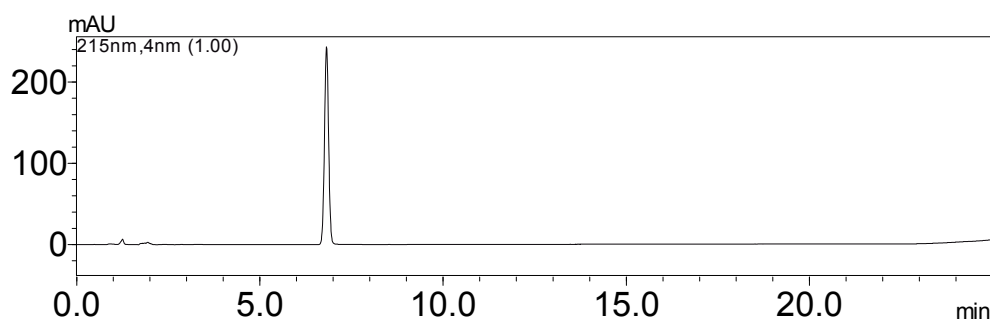
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**Figure 15.** HPLC chromatogram of the unified fraction AM/HPLC/8/23-28. HPLC instrument 2, column 1. Injection volume: 5 µL of a concentration of 0.5 mg/mL MeCN. Analysis method according to Wagner and Bauer (1997, chapter 2. 4.5.1, p. 27).

After determination of atractylenolide III and II as active principles in bái zhú, responsible for observed $I_{GABA}$ enhancement, a structurally similar compound, atractylenolide I, was isolated. For this, a freshly prepared n-hexane extract (chapter 2.3.2, p. 25) was first subjected to vacuum liquid chromatography (VLC) on silica gel (90 x 4 cm i.d.) for rough fractionation. Used solvents and solvent mixtures (1.5 L each) as well as the yields are given in table 2.

| fraction | solvent | ratio | yield (mg) | appearance |
|---|---|---|---|---|
| AM/VLC/1 | n-hexane | | 223 | clear |
| AM/VLC/2 | n-hexane:EtOAc | 8:2 | 2063 | yellow |
| AM/VLC/3 | n-hexane:EtOAc | 1:1 | 1385 | yellow |
| AM/VLC/4 | n-hexane:EtOAc | 2:8 | 281 | yellow |
| AM/VLC/5 | EtOAc | | 86 | light yellow |
| AM/VLC/6 | MeOH (wash out) | | 1841 | dark brown |
| **total yield** | | | **4889** | |

**Table 2.** VLC fractionation of an *A. macrocephala* n-hexane extract.

The VLC fractions were analyzed by HPLC to reveal that AM/VLC/2 contains atractylenolide I, II and III (Fig. 16, p. 36).
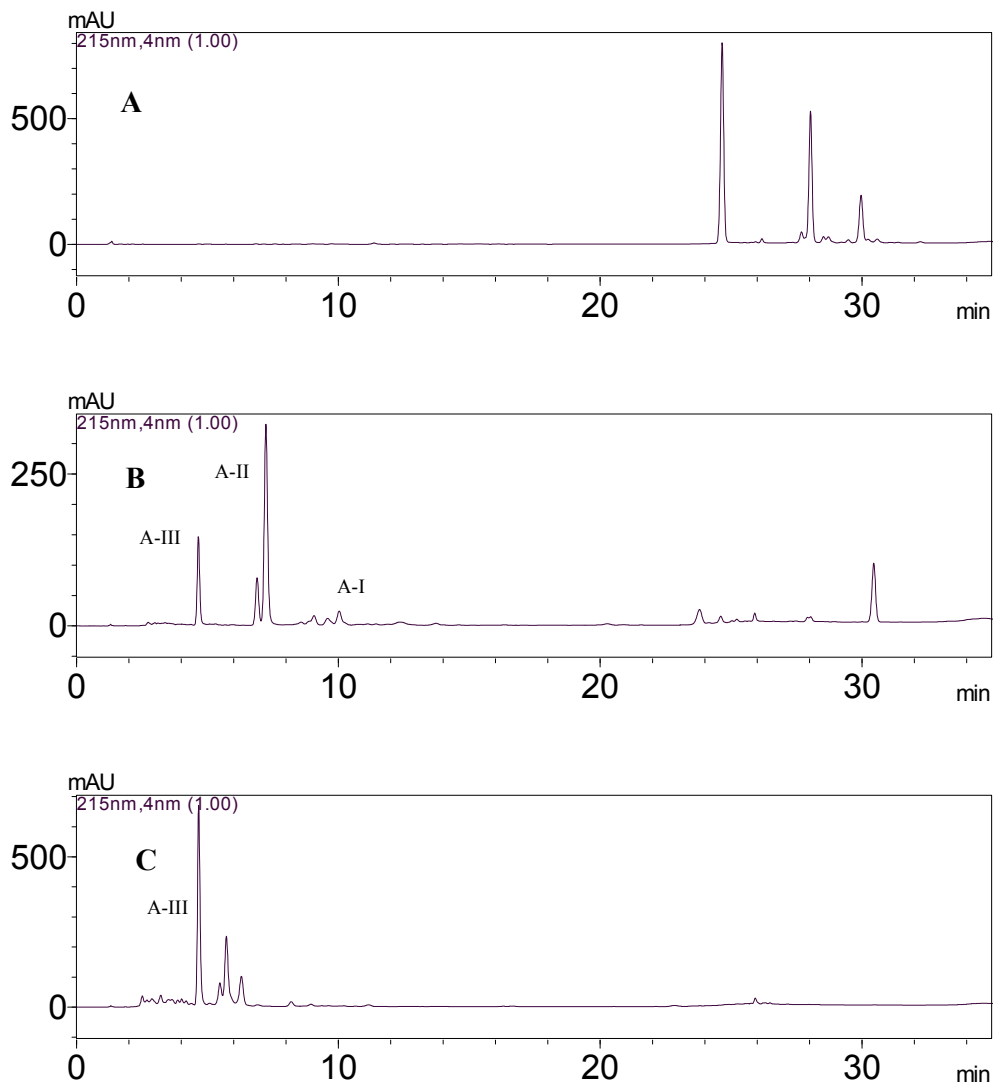
**Figure 16.** HPLC analysis of the VLC fractions (A) AM/VLC/1, (B) AM/VLC/2 and (C) AM/VLC/3 on instrument 2, column 1. Injection volume: 10 µL with a concentration of 1 mg/mL MeCN. Analysis method according to Wagner and Bauer (1997, chapter 2.5.4.1, p. 27). Detection wavelength: 215 nm. A-I: atractylenolide I, A-II: atractylenolide II, A-III: atractylenolide III.

The fraction was crystallized in n-hexane and could be divided in a crystalline (AM/VLC/2/CR, 77 mg) and an oily residue (AM/VLC/2/OR, 1.7 g). Subsequently, both residues were analyzed by HPLC (Fig. 17, p. 37) using not only 215 nm but also 255 nm as detection wavelength for the simultaneous detection of atractylenolide I, II and III. While in AM/VLC/2/CR atractylenolide II and III could be detected, AM/VLC/2/OR contained comparably higher amounts of atractylenolide I next to atractylenolide II and III.
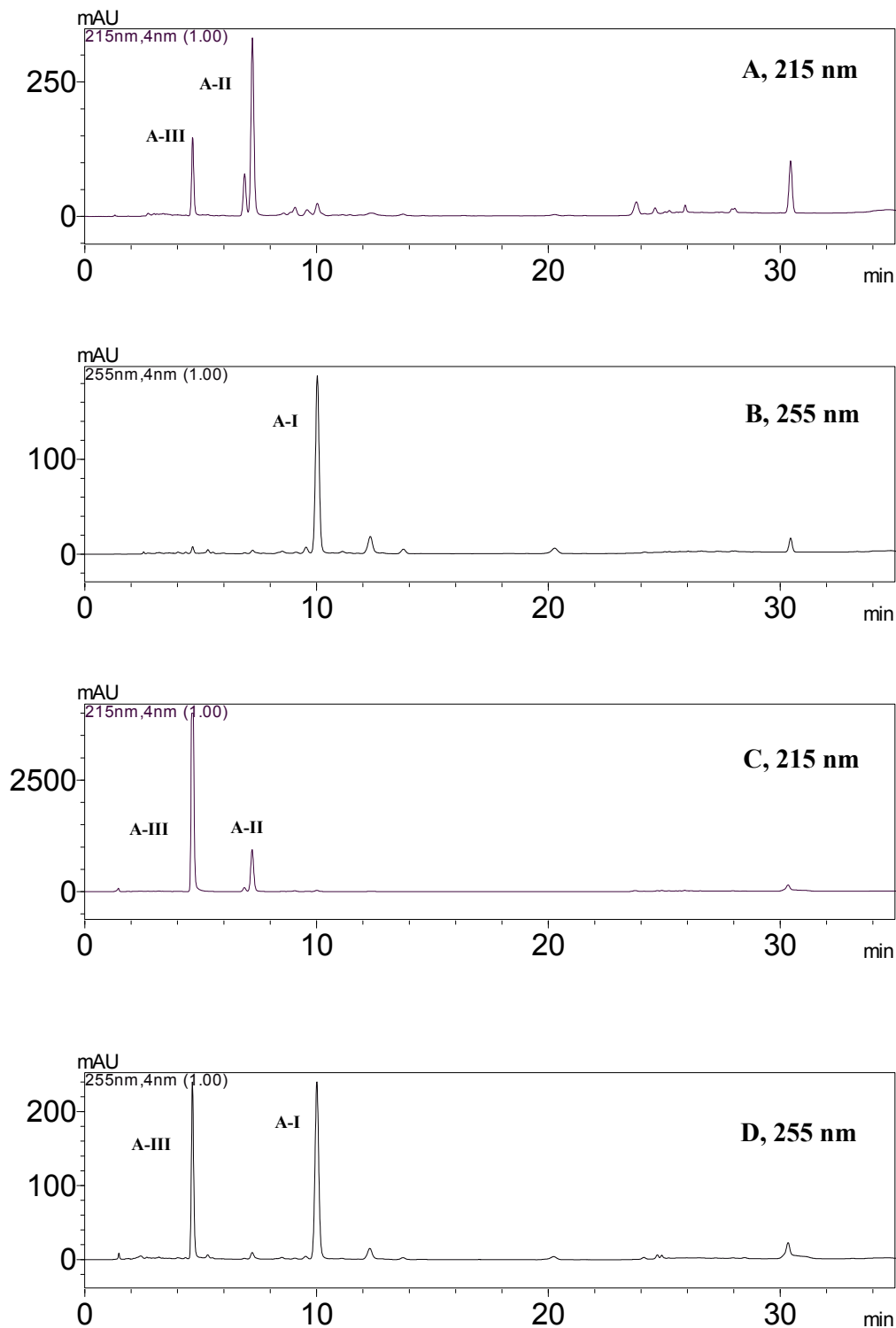
**Figure 17.**  HPLC analysis of AM/VLC/2/OR (A and B) and AM/VLC/2/CR (C and D) using instrument 2 and column 1 with the analysis method according to Wagner and Bauer (1997, chapter 2.5.4.1, p. 27). Injection volume: 10 µL with a concentration of 1 mg/mL MeCN. Detection wavelength was set at 215 nm (A and C) for better detection of impurities and at 255 nm (B and D) for simultaneous detection of atractylenolide I ($\lambda_{max}$ = 276 nm), which appears at Rt ~ 10.5 min, with atractylenolide II or III (no absorption at 276 nm).

Thus, AM/VLC/2/OR was subjected to CC on silica gel (90 x 4 cm i.d.) using n-hexane:EtOAc (95:5) as eluent. Flow rate was set at 6 mL/h, whereby 3 mL fractions were collected. For TLC screening n-hexane:EtOAc (8:2) was used as mobile phase. Fractions 426 to 461 contained one prominent blue and some weak to prominent orange fluorescent zones when spraying with ASR and subsequent detection under $UV_{366}$. Thus this fraction was unified to AM/VLC/2/OR/421-461 (31 mg), an oily residue with white crystals. It was assumed, that the blue fluorescent zone was due to atractylenolide I since the related atractylenolide II and III also give blue fluorescent zones under $UV_{366}$ after spraying with ASR. Subsequent HPLC analysis confirmed, that the fraction contained atractylenolide I, since Rt and UV spectrum of the major peak were in good accordance with the data published by Wagner and Bauer (1997). For purification of atractylenolide I, the fraction was dissolved in n-hexane:MeOH (3:1); crystals were washed with MeOH to yield 11 mg. HPLC analysis (Fig. 18) revealed one major peak with a $\lambda_{max}$ of 276 nm. This residue was finally identified as atractylenolide I by NMR (annex III).
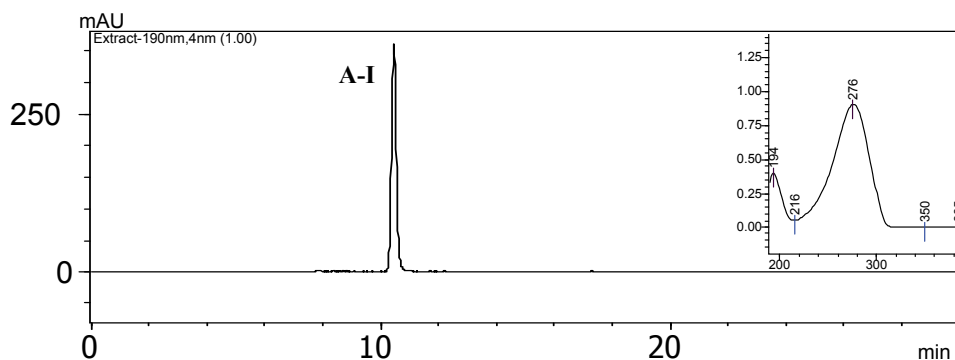


**Figure 18.** HPLC analysis of fraction AM/VLC/2/426-461 (1 mg/mL MeOH) using instrument 2 and column 1 with the analysis method published by Wagner and Bauer (1997, chapter 2.5.4.1, p. 27). Injection volume was 10 µL (0.5 mg/mL MeCN). Detection wavelength was set at 190 nm for better detection of impurities. Inlay: spectrum of the main peak at Rt 10.48 min.

For the following pharmacological studies i.e. concentration response curves and BZ-experiments higher amounts of atractylenolide II and III were needed. Thus they were isolated from fraction AM/VLC/2/CR (77 mg). Again silica gel as stationary phase (70 x 1 cm i.d.) and n-hexane:EtOAc (95:5) as mobile phase were used, yielding a total of 550 fractions (flow rate 6 mL/h, fraction size: 2 mL). Atractylenolide II (16 mg) could be isolated from fractions AM/VLC/CR/95-131, while atractylenolide III (31 mg) was gained from unification of fractions 381-421, whereby TLC and HPLC chromatograms were similar to already obtained data.

## 3.2 GABA$_A$ receptor modulators from Chinese Herbal Medicines traditionally applied against insomnia and anxiety (manuscript)

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This manuscript is in preparation for submission. All phytochemical work, except NMR, was done by the author of this thesis. NMR was conducted by Prof. Hanspeter Kählig (atractylenolide II and III) from the Department of Organic Chemistry and Prof. Ernst Urban (atractylenolide I), from the Department of Medicinal Chemistry, University of Vienna. From the pharmacological experiments, the dose response curves and the benzodiazepine experiments are to be contributed to the author as well, while the screening of extracts and HPLC fractions was accomplished by co-workers of the Department of Pharmacology and Toxicology, University of Vienna. The data of this manuscript is currently submitted as invention disclosure to the University of Vienna and thus was not yet submitted for publication to a Journal.

**Abstract**

*Ethnopharmacological relevance*: Several Chinese Herbal Medicines (CHMs) are used in the treatment of insomnia, restlessness, or anxiety. However, mechanisms underlying this effect and scientific proof for their traditional use are scarce.

*Aim of the Study:* In the present study CHMs were screened for their ability to modulate GABA-induced chloride currents ($I_{GABA}$) and isolate the active principles thus providing scientific evidence for their use as sedative and/or anxiolytic agents in CM.

*Material and methods:* Herbal drugs were extracted successively with petroleum ether, ethyl acetate, methanol and water and further fractionated according to their bioactivity. The obtained extracts, fractions and finally pure compounds were tested for their ability to potentiate $I_{GABA}$ using the two-microelectrode voltage clamp technique on recombinant $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors expressed in *Xenopus laevis* oocytes.

*Results:* From all tested extracts the petroleum ether extract of *Atractylodes macrocephala* Koidz. rhizomes showed the strongest $I_{GABA}$ potentiation and was studied in more detail.
This led to the isolation of the main components atractylenolide II and III, which seem to be responsible for the observed positive modulation of $I_{GABA}$ ($166 \pm 12$ %, n = 3 and $155 \pm 12$ %, n = 3, respectively) *in vitro*. They were more active than the analogous compound atractylenolide I ($96 \pm 3$ %, n = 3) which differs in an additional double binding in position 9 and 9a. Furthermore it could be shown that this effect is mediated independently of the benzodiazepine (BZ) binding site.

*Conclusion: A. macrocephala* exerts its *in vitro* activity on recombinant GABA$_A$ receptors mainly through the two sesquiterpene lactones atractylenolide II and III (Fig. 1). This positive allosteric modulation of $I_{GABA}$ can partially be responsible for the traditional ethnopharmacological use of this herbal drug as a sedative.

*Keywords*: *Atractylodes macrocephala,* GABA$_A$ receptors, sesquiterpene lactones, atractylenolide, voltage clamp, positive allosteric modulation.

## 1. Introduction

In Chinese Medicine (CM) several herbal drugs are applied in the treatment of insomnia or anxiety disorders, two highly prevalent mental disorders (Somers et al., 2006; Roth, 2007). Although such herbal remedies are frequently used and considered effective and well-tolerated, studies confirming their ethnopharmacological use are often lacking. From the 12 Chinese Herbal Medicines (CHMs) we selected for our study, only some have been investigated for anxiolytic, sedative or other GABA$_A$ receptor related effects *in vivo* or *in vitro*: sedative effects for flavonoids isolated from *Albizia julibrissin* flowers (Kang et al., 2000) and anxiolytic effects through *Albizia* bark extracts have been already published (Kim et al., 2004). Furthermore, the alkaloid harmane isolated from *Tribulus terrestris* is supposed to be mildly sedating and affect the locomotor activity in sheep (Bourke et al., 1992). *Nelumbo nucifera* seed embryos contain isoquinoline alkaloids like neferine and related constituents, which seem to have anxiolytic and sedative activity *in vivo* (Sugimoto et al., 2008). From *Dimocarpus longan*, adenosine was determined as the active anxiolytic principle in a bioactivity guided approach using the Vogel Conflict Test (Okuyama et al., 1999) and sedative-hypnotic activity was published for a decoction from the stem of *Polygonum multiflorum* (Yang et al., 1990). In search for the (other) molecular mechanism underlying these traditional sleep-remedies, they were investigated for their ability to modulate the γ-aminobutyric acid (GABA) type A (GABA$_A$) receptor. GABA is the major inhibitory neurotransmitter in the mammalian brain and hence the GABA$_A$ receptor represents a viable target when searching for anxiolytic or sedative components from natural origin (Johnston et al., 2006; Khom et al., 2007, Trauner et al. 2008; Zaugg et al., 2010). The GABA$_A$ receptor itself is a heteropentameric ligand gated ion channel responsible for fast inhibition of neurotransmission (Mody and Pearce, 2004), whereby from many possible receptor subtypes the α$_1$β$_2$γ$_{2S}$ receptor is the most abundant (Moehler, 2006; Olsen and Sieghart, 2008; 2009). In the present study effects of different CHM extracts, fractions and isolated compounds on GABA-induced chloride currents (I$_{GABA}$) were investigated using the two-microelectrode voltage clamp technique and an automated fast perfusion system on *Xenopus laevis* oocytes expressing α$_1$β$_2$γ$_{2S}$ GABA$_A$ receptors. Since the petroleum ether extract of bái zhú (rhizomes of *Atractylodes macrocephala* Koidz.) displayed highest activity it was further investigated using a bioactivity III (Fig. 1) guided approach. It was found that the sesquiterpene lactones atractylenolide II and are the active principles of bái zhú, which potentiated I$_{GABA}$ concentration dependently and independent of the benzodiazepine binding site.
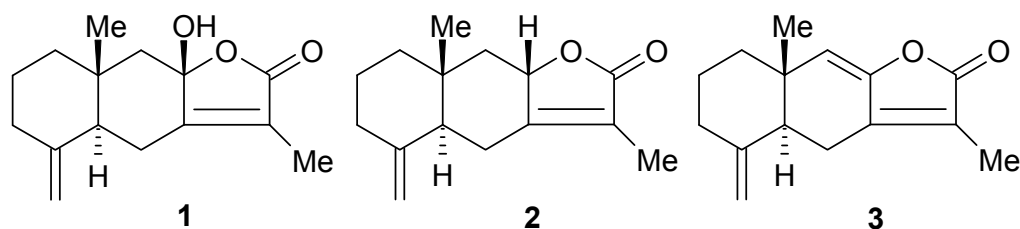
**Fig. 1.** Structures of the sesquiterpene lactones atractylenolide III (**1**), atractylenolide II (**2**) and atractylenolide I (**3**). While in atractylenolide III and II a hydroxyl or proton is positioned on C8, atractylenolide I bears an additional double bound in position 9 and 9a.

## 2. Materials

### 2.1. Chemicals

For HPLC and crystallization analytical grade solvents were used. For extraction and isolation on silica gel columns solvents of highest available purity were used (VWR, Vienna, Austria). Diazepam, flumazenil and ND96 reagents were purchased from Sigma (Vienna, Austria).

### 2.2. Plant Material

Herbal drugs were purchased from Plantasia (Oberndorf, Austria). Voucher specimens are deposited at the Department of Pharmacognosy, University of Vienna.

### 2.3. Extraction

The ground drugs were soaked in 500 mL petroleum ether for 10 min, and extracted on the water bath under reflux for 30 min. The obtained extracts were filtered and evaporated to dryness. The remaining drug material was air-dried overnight and extracted with each 500 mL of ethyl acetate (EtOAc), MeOH and distilled water likewise, whereby the water extract was lyophilized.

### 2.4. Fractionation by semi-preparative HPLC

HPLC activity profiling was carried out with a Shimadzu instrument consisting of two LC-8A pumps, a SIL-10AP autosampler, a SPD-M20A diode array detector, a FRC-10A fraction collector and a CBM-20A interface. A Nucleosil 100-7, C-18 (250 x 21, 5µm) column from Machery-Nagel (Düren, Germany) was employed. For fractionation following HPLC method was used (Wagner and Bauer, 1997): after equilibration (15min) at 62 % acetonitrilee (MeCN, solvent B) concentration of B was increased to 70 % B in 10 min, increased to 95 % B in 2

min and held on 95 % B for 8 min. A 1 min increase to 100 % B and 5 min purge phase with 100 % B ceased analysis. Flow rate was 27.8 mL/min and monitoring wavelengths were set at 190, 215 and 255 nm.

## 2.5. Isolation

HPLC fraction 4 (19 mg) was purified on silica gel (60 x 0.5 cm i.d) with n-hexane:EtOAc (95:5) as mobile phase and aliquots of 5 mL (flow rate: 20mL/h) were collected. Fractionation was monitored by TLC (silica gel KG60 $F_{254}$, Merck, Darmstadt, Germany) using n-hexane:EtOAc (8:2) as mobile phase and zones were visualized after spraying with anisaldehyde sulphuric acid reagent and heating at 105 °C for 5 to 10 min in visible light and under $UV_{366}$. Unification of fractions 4-386 to 4-421 yielded 14 mg of atractylenolide III (purity ≥ 99 %). HPLC fraction 8 (~ 7 mg) was purified by column chromatography on silica gel (60 x 0.5 cm i.d.) using n-hexane:EtOAc (95:5), collecting aliquots of 5 mL (flow rate: 10 mL/h). Fractionation was again monitored by TLC to yield 5.6 mg atractylenolide II (purity ≥ 99 %) from fractions 8-23 to 8-28. Atractylenolide I was isolated from a n-hexane extract of *A. macrocephala* (50 mg drug/mL n-hexane; 6 g extract in total). This extract was first subjected to VLC using the following solvents and solvent mixtures: n-hexane, n-hexane:EtOAc (8:2), n-hexane:EtOAc (1:1), n-hexane:EtOAc (2:8), EtOAc, EtOAc:CHCl$_3$ (1:1), CHCl$_3$, CHCl$_3$:MeOH (1:1), MeOH, MeOH:water (1:1) and water. The fraction (VLC2) gained from n-hexane:EtOAc (8:2) was further purified by column chromatography (90 x 4 cm i.d.) using n-hexane:EtOAc (95:5) as mobile phase (6 mL/h, 3 mL for each fraction). Fractionation was monitored by TLC. Fractions containing atractylenolide I (VLC2-426 to -461) were unified, crystallized in n-hexane:MeOH (3:1), and washed with MeOH to give 11 mg of pure compound (purity ≥ 98 %).

## 2.6. Spectroscopy

The isolated compounds were identified using NMR. Spectra for atractylenolide II and III were recorded on a Bruker Advance DRX 400 NMR with a resonance frequency at 600.13 MHz for $^1$H NMR and at 150.92 MHz for $^{13}$C NMR. Spectra for atractylenolide I were recorded in CD$_3$Cl or d6-DMSO on a Bruker Advance 500 spectrometer at 500 MHz ($^1$H NMR) and 125 MHz ($^{13}$C NMR).

## 2.7. Expression of GABA$_A$ Receptors

Preparation of stage V-VI oocytes from *Xenopus laevis* (NASCO, Fort Atkinson, WI, USA) and injection of cRNA were done as previously described (Khom et al., 2006). 15 min prior to surgery female frogs were anaesthetized using a 0.2 % solution of MS-222 (Ethyl 3-aminobenzoate methanesulfonic acid, Sigma, Vienna, Austria). Parts of the ovaries were removed and remaining follicle membranes were enzymatically digested with 2 mg/mL collagenase Type 1A (Sigma, Vienna, Austria). Synthesis of capped off run-off poly (A+) cRNA transcripts was performed from linearized cDNA templates (pCM vector). cRNAs were diluted with DEPC-treated water and stored at -80 °C. Injection of 10 − 50 nL of the different cRNA solutions was carried out on the day of isolation. To ensure the expression of the γ-subunit cRNAs of α$_1$, β$_2$ and γ$_{2S}$ were injected in a ratio of 1:1:10 (Baburin et al., 2008; Boileau et al., 2003). Injected oocytes were stored at 18 °C in penicillin and streptomycin supplemented ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$ x 6 H$_2$O, 1.8 mM CaCl$_2$ and 5 mM HEPES, pH 7.4).

## 2.8. Two-microelectrode voltage clamp technique and drug application

GABA$_A$ receptor expressing oocytes were screened for GABA-evoked currents 1 to 3 days after cRNA injection as previously described (Baburin et al., 2006). Electrophysiological experiments were performed by the two-microelectrode voltage clamp technique making use of a TURBO TEC-03X amplifier (npi electronic, Tamm, Germany) at a holding potential of -70 mV. Current measurements were digitized at 200 Hz and recorded with pCLAMP 10 data acquisition software (Molecular Devices, Sunnyvale, CA, USA). ND96 was used as bath solution. Glass electrodes (Harvard Apparatus, Kent, UK) were made using a micropipette puller (Narishige, Tokyo, Japan) and filled with 2 M KCl (1 − 3 MΩ). Oocytes with maximal current amplitudes ≥ 3 µA were discarded to exclude voltage clamp errors.

All experiments were performed at room temperature (23 to 25 °C). Stimulation of chloride currents by extracts and pure compounds was determined at a GABA concentration eliciting between 1 and 10 % of the maximal current amplitude (EC$_{1-10}$) and normally equaled a concentration between 1 and 10 µM GABA, depending on the oocyte. The GABA concentration needed for the measurements was established at the beginning of each experiment. Stock solutions of the extracts (10 mg/mL) and compounds (100 mM) were prepared in DMSO and stored at -20 °C, except for the 1mM GABA stock solution where ND 96 was used. Reservoirs for control and drug applications contained equivalent amounts of

DMSO, which did not exceed 1 %. At this concentration the measurements were not influenced. All stock solutions were dissolved in ND96 containing the appropriate amount of GABA to elicit currents of $EC_{1-10}$ immediately before the experiments, and the extracts were always diluted to a concentration of 100 µg/mL. Test solutions (100 $\mu$L) were applied to the oocytes at a speed of 300 $\mu$L/s by means of a ScreeningTool (npi electronics, Tamm, Germany) according to Baburin et al. (2006). A 5 to 10 min washout period was allowed between drug applications (concentration $\geq$ 30 µM) to avoid receptor desensitization.

## 2.9. Data Analysis

Potentiation of the GABA-induced chloride current ($I_{GABA}$) in percent was defined according to the formula $[I_{(GABA+Comp)}/I_{GABA}- 1]*100$, 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. Origin Software (OriginLab Corporation, Northhampton, MA, USA) was used to generate concentration-response curves. Data were fitted by nonlinear regression analysis to the equation $1/(1+(EC_{50}/[compound]n_H)$, where $EC_{50}$ is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50% of the compound-induced maximum response, and $n_H$ is the Hill coefficient. Responses were graphed as mean $\pm$ standard error (S.E.M.) from at least three oocytes out of $\geq$ two different batches. Statistical significance (*) was calculated using Student's $t$-test and one-way ANOVA with a confidence interval of $p < 0.05$.

# 3. Results

## 3.1. Screening of herbal extracts and fractions for modulation of $I_{GABA}$

In total 48, e.g. 4 extracts of different polarity from 12 TCM herbal drugs (Tab. 1), were investigated for their ability to enhance $I_{GABA}$. From these 48 extracts in total, the lipophilic extracts from *A. macrocephala* showed the most promising enhancement of $I_{GABA}$ (Fig. 2, Tab. 1) with the petroleum ether extract displaying the highest activity ($322 \pm 48$ %, n = 3).
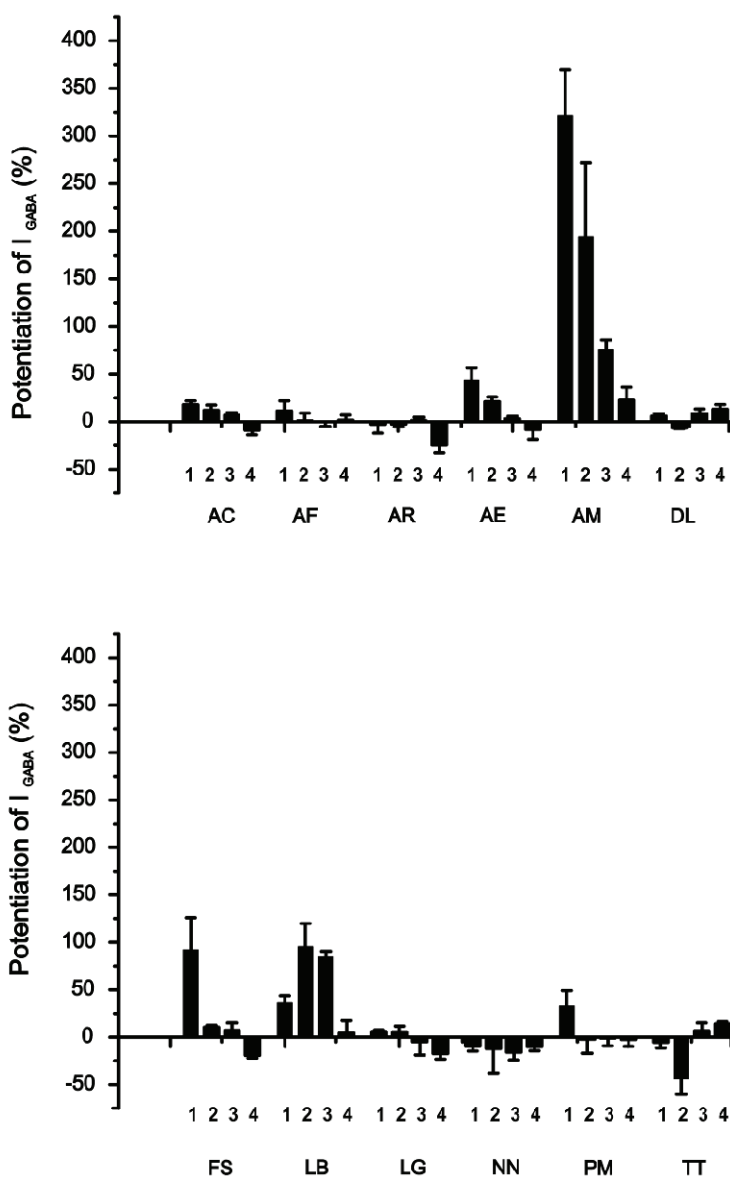


**Fig. 2.** Preliminary activity screening of the 48 different extracts (100 µg/mL). Potentiation of $I_{GABA}$ was measured at $EC_{1-10}$. CHM are abbreviated according to Table 1 with the plant name initials. 1 = PE/petroleum ether, 2 = ethyl acetate/EtOAc, 3 = MeOH, 4 = water/$H_2O$

| Abb. | Plant name | drug | pinyin | Potentiation of $I_{GABA}$ ± SEM (%) | | | |
|------|-----------|------|--------|------|------|------|------|
| | | | | PE | EtOAc | MeOH | water |
| AC | [1]*Alibizia julibrissin* Durazz. (Fabaceae) | cortex | hé huān pí | 18 ± 4 | 12 ± 6 | 7 ± 3 | -9 ± 5 |
| AF | [1]*Alibizia julibrissin* Durazz. (Fabaceae) | flos | hé huān huā | 11 ± 11 | 1 ± 8 | 0 ± 5 | 2 ± 6 |
| AR | [1]*Arisaema erubescence* (Wall.) Schott.; *A. heterophyllum* BL.; *A. amurense* Maxim. (Araceae) | rhizoma | tiān nán xīng | -3 ± 9 | -2 ± 3 | 1 ± 3 | -25 ± 8 |
| AE | [2]*Arnebia euchroma* (Royle) Johnst., *Lithospermum erythrorhizon* Sieb. et Zucc. | rhizoma | zǐ cǎo | 44 ± 13 | 21 ± 5 | 3 ± 3 | -8 ± 10 |
| AM | [1]*Atractylodes macrocephala* Koidz. | rhizoma | bái zhú | 322 ± 48 | 194 ±78 | 76 ± 10 | 22 ± 14 |
| DL | [3]*Dimocarpus (Euphoria) longan* Lour. (Sapindaceae) | arillus | long yǎn ròu | 6 ± 3 | -6 ± 1 | 8 ± 5 | 13 ± 5 |
| FS | [3]*Forsythia suspensa* (Thunb.) Val. (Oleaceae) | fructus | lián qiào | 92 ± 34 | 11 ± 2 | 7 ± 8 | -19 ± 3 |
| LB | [1]*Lilium brownii* F.E. Brown *var. viridulum* Baker W; *L. lancifolium* Thunb., *L. pumilum* DC. (Liliaceae) | bulbus | bǎi hé | 36 ± 8 | 95 ± 25 | 85 ± 6 | 4 ± 13 |
| LG | [3]*Lophaterum gracile* Brong. (Gramineae) | herba | dàn zhú yè | 6 ± 2 | 6 ± 6 | -5 ± 14 | -17 ± 6 |
| NN | [4]*Nelumbo nucifera* Gaertn. (Nymphaceaceae) | plumula | lián zǐ xīn | -9 ± 5 | -12 ± 26 | -16 ± 8 | -10 ± 4 |
| PM | [1]*Polygonum multiflorum* Thunb. (Polygonaceae) | caulis | yè jiāo téng | 33 ± 16 | -2 ± 15 | -1 ± 8 | -2 ± 7 |
| TT | [5]*Tribulus terrestris* L. (Zygophyllaceae) | fructus | cì jí lí | -44 ± 14 | -5 ± 6 | 7 ± 8 | 14 ± 2 |

[1](Bensky et al., 2006), in the case of Arisaematis rhizome prep. we refer to its use against convulsions since anti-epileptic agents can target the $GABA_A$ receptor as well

[2]referring to its heat-clearing effects which can possibly be also/ translated with "mind-cooling", meaning sedating or anxiolytic, http://alternativehealing.org/zi_cao.htm

[3](Hempen, 2007)

[4](Sugimoto et al., 2008)

[5](Huang, 1999)

**Tab. 1.** Selected 12 TCM herbal drugs for screening on recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor expressed in *X. laevis* oocytes. Footnotes indicate literature where the respective plant is mentioned as a sedative and/or anxiolytic agent. The extract-induced potentiation of $I_{GABA}$ is given as mean ± S.E.M from n = 3, from ≥ two different batches, whereby the extracts were always tested in concentrations of 100μg/mL.

Consequently, this extract was subjected to fractionation by semi-preparative HPLC fractions were first collected in a time-based manner and then combined according to the most prominent peaks. This lead to a total of 18 fractions from which HPLC fractions 4 (118 ± 7 %, n = 4), and 8 (109 ± 3 %, n = 4) showed the strongest $I_{GABA}$ enhancement (Fig. 3, Supp. Tab. 1).

**A**
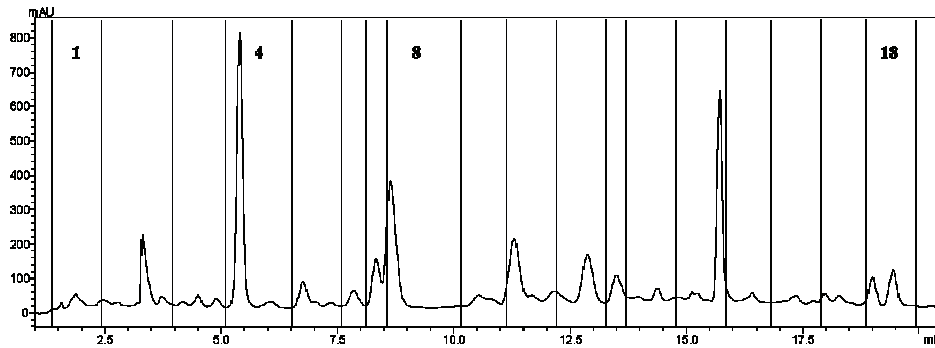


**B**
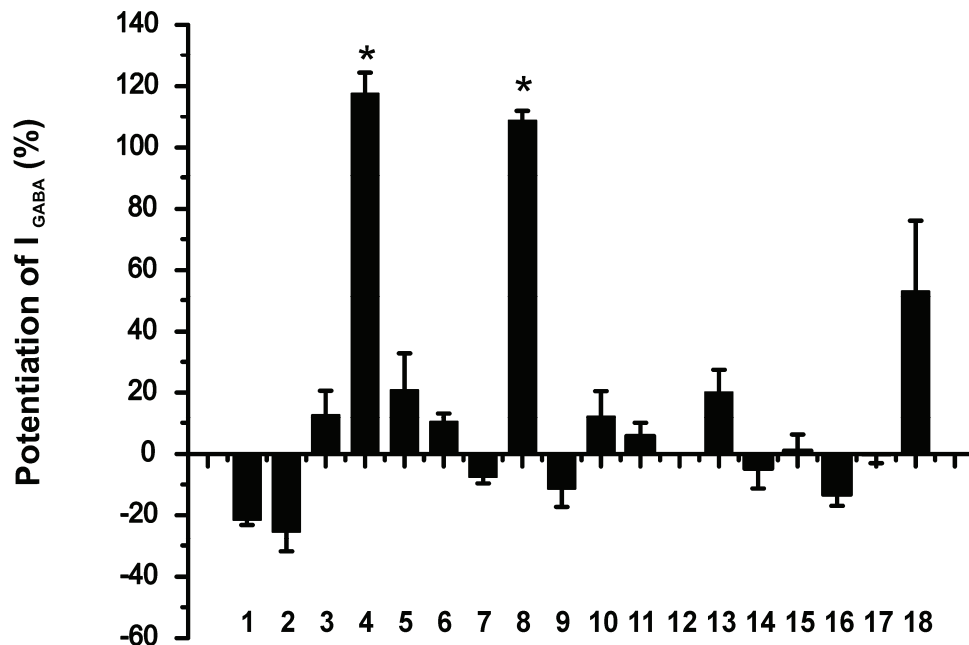


**Fig. 3. (A)** Fractionation pattern of *Atractylodes* petroleum ether extract. **(B)** Modulation of $I_{GABA}$ by the 18 fractions gained from semi-preparative HPLC. Bars represent mean ± S.E.M from at least three oocytes from ≥ two different batches. (*) indicates statistically significant (p < 0.05, Student's t-test). Fractions were tested in a concentration of 100 µg/mL.

Fractions 4 and 8 were each purified on silica gel (60 x 0.5 cm i.d.) with n-hexane:EtOAc (95:5) as eluent, leading to the isolation of atractylenolide III and II, respectively. Structures were confirmed by $^1$H and $^{13}$C-NMR and were in good accordance with published data (Hikino *et al.*, 1962). Another major component of *A. macrocephala* is the sesquiterpene lactone atractylenolide I (Fig. 1.3), which only differs from atractylenolide II and atractylenolide III by an additional double bound in position 9 and 9a. Interestingly, the HPLC fraction (fraction 11) which -according to Wagner and Bauer (1997) - contains atractylenolide I did not show promising activity in the screening. Thus atractylenolide I was isolated and investigated additionally to the above mentioned analogues, to gain first insights in the structure-activity relationship between these three sesquiterpene lactones. Spectral data of atractylenolide I was compared with literature (Endo et al., 1979) and in good accordance to the published data.

### 3.2. Concentration-dependent modulation of $I_{GABA}$ by atractylenolides I, II and II

Since the atractylenolides II and III (Fig. 1) were the most active constituents they were studied in more detail. For both compounds a concentration dependent potentiation of $I_{GABA}$ was observed (Fig. 3).
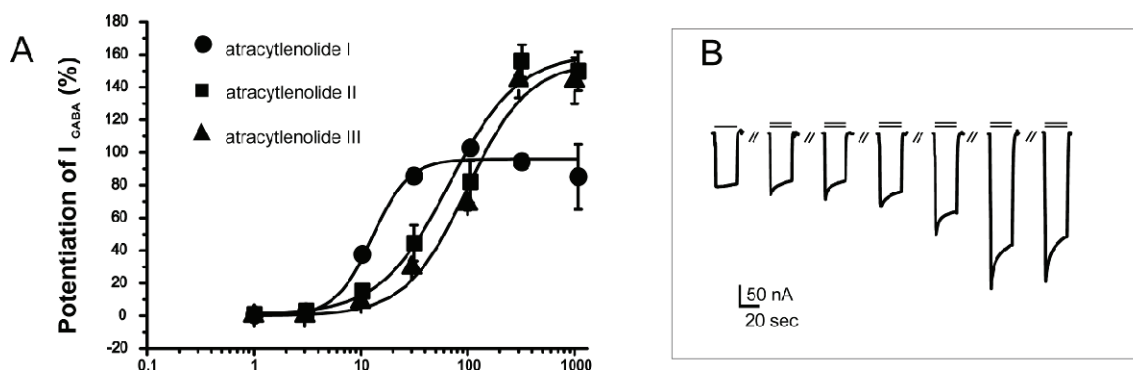


**Fig. 4.** Concentration dependent modulation of $I_{GABA}$ by (▲) atractylenolide III, (■) atractylenolide II and (●) atractylenolide I. **(A)** Concentration response curves of the three sesquiterepene lactones. **(B)** Typical GABA control current (single bar) and currents induced by co-application of GABA and A-II (double bar). Pure compounds were applied in concentrations ranging from 1 to 1000 µM. Each data point represents the mean ± S.E.M from 3 oocytes and at least two different batches.

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Atractylenolide II, co-applied in concentrations from 1 to 300 µM with GABA concentrations corresponding to $EC_{1-10}$ gave a maximum potentiation of $166 \pm 12$ %, with a half-maximum potentiation ($EC_{50}$) by $70 \pm 17$ µM and a Hill coefficient ($n$H) of $1.2 \pm 0.1$ (n = 3). The highest enhancement by atractylenolide III was $157 \pm 12$ % ($EC_{50}$: $99 \pm 20$ µM, $n$H: $1.5 \pm 0.4$, n = 3). The related atractylenolide I displayed a lower $I_{GABA}$ modulation by $96 \pm 3$ % ($EC_{50}$: $12 \pm 1$ µM, $n$H: $2.5 \pm 0.2$, n = 3).

### 3.3. Benzodiazepine binding site independent modulation by atractylenolide II

To determine if the sesquiterpene lactones modulate $GABA_A$ receptors by interaction with the benzodiazepine (BZ) binding site, atractylenolide II was used as a representative compound for this experiment.

Potentiation of $I_{GABA}$ by atractylenolide II (70 µM) was studied in the presence and absence of flumazenil (1 µM) or diazepam (300 nM).

Atractylenolide II-induced enhancement of $I_{GABA}$ was not inhibited by the co-application of flumazenil ($107 \pm 5$ % vs. $104 \pm 5$ %, n =3, Fig 4A). When atractylenolide II (70 µM) was co-applied with diazepam, an (additive) increase in modulation of $I_{GABA}$ from $97 \pm 4$ % (n =3) to $474 \pm 109$ % (n = 3) was observed compared to $I_{GABA}$ potentiation by diazepam alone ($347 \pm 52$ %, n = 3, Fig 4C).
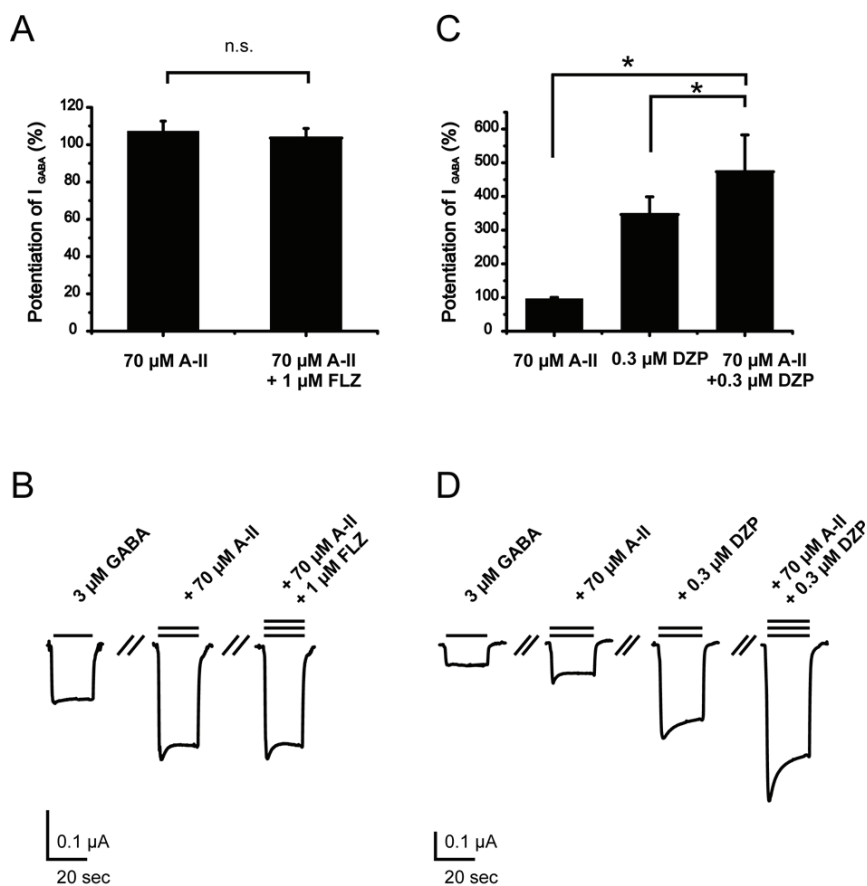
**Fig. 5.** Effects of atractylenolide II (A-II) on $I_{GABA}$ in the presence of flumazenil (FLZ) and diazepam (DZP) in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors. Bars represent the mean ± S.E.M from at least 3 oocytes, from ≥ two different batches. Statistical significance ($p < 0.05$, one-way ANOVA) is indicated with (*), n.s. = not significant ($p > 0.05$). (A) Potentiation of $I_{GABA}$ (EC$_{5-10}$) by A-II (70 µM) in the absence (left bar) and presence (right bar) of flumazenil (1 µM) is not significantly different ($p > 0.05$), indicating no involvement of the BZ binding site in the positive modulatory activity of A-II. (B) Typical GABA-induced chloride currents in the absence (GABA 3 µM, control, single bar) and presence of the indicated concentrations of A-II (double bar), or A-II and flumazenil (triple bar), respectively. (C) Additive effects of A-II and diazepam on $I_{GABA}$. The left bar shows the potentiation of $I_{GABA}$ by 70 µM A-II, the middle bar by 300 nM diazepam, and the right bar illustrates the stimulation of $I_{GABA}$ by co-application of A-II and diazepam. (D) Representative chloride currents induced by GABA (3 µM, control, single bar), by A-II (double bar), by diazepam (double bar) and A-II co-applied with diazepam (triple bar) at the indicated concentrations.

## 4. Discussion

When screening the extracts of 12 different CHMs regarding their ability to potentiate GABA-induced chloride currents, extracts from lián qiào (*Forsythia suspensa* fruits), băi hé

(*Lilium* sp. bulbs) and bái zhú (*Atractylodes macrocephala* rhizomes) displayed highest activity. Bái zhú are the sliced dried or fried rhizomes of *Atractylodes macrocephala* KOIDZ. (Asteraceae), which is a perennial herbaceous plant growing in North-Eastern China. This herbal drug contains essential oil (mostly sesquiterpene hydrocarbons), furano-sesquiterpenes, polyacetylenes and polysaccharides (Bensky et al., 2006; Chen, 1987; Ding et al., 2005; Endo et al., 1979). The use of bái zhú is indicated in a variety of disorders, but it is one of the most frequently prescribed ingredients in decoctions against insomnia (Chen et al., 2009) and traditionally applied in the treatment of anxiety accompanied with heart palpitations (Stahl-Biskup, 1998). However, to our knowledge this CHM was never investigated for its sedative or anxiolytic activities neither *in vitro* nor *in vivo*. Nonetheless, some other pharmacological effects have been examined according to its traditional use as tonic (Lee et al., 2007), anti-diabetic (Shan et al., 2003) or anti-abortive agent (Zhang et al., 2000; Zhang et al., 2008). Moreover, it displayed neuroprotective (Lin et al., 2009), anti-inflammatory (Dong et al., 2008; Li et al., 2007) and cytotoxic activities (Huang et al., 2005 ; Wang et al., 2006). From all constituents in bái zhú, the sesquiterpene lactones seem to be the active principles regarding positive modulatory activity on the $GABA_A$ receptor. Among the three compounds isolated, atractylenolide II and III showed similar $I_{GABA}$ enhancement, while the analogous atractylenolide I had lower efficacy (Fig. 4). This might be due to the additional double bond in position 8 and 9, which results in a more rigid structure (Fig. 1). Moreover, it was shown that an interaction with the BZ binding site is not likely, since the activity of atractylenolide II could not be inhibited by the BZ antagonist flumazenil (Fig. 5). This suggests that BZs typical adverse effects are avoided. Interestingly, $I_{GABA}$ enhancement through the petroleum ether extract was higher than the effect observed with the isolated compounds. This could be due to the loss of highly lipophilic and/or volatile compounds through RP-18 HPLC and following evaporation under reduced pressure. But it can also indicate additive and/or synergistic effects possibly through compounds that are not active when applied alone. This "second-order-modulation" of the $GABA_A$ receptor was already observed for certain natural compounds (Campbell et al. 2004) and would be worth further studies. Although the *in vivo* sedative/anxiolytic activity still needs to be elucidated, a study on the tissue distribution of atractylenolide III revealed that it can cross the blood-brain barrier and is enriched in the brain, specifically the cerebellum, when given orally to mice (Li et al., 2006). Based on the structural similarity this might be true for atractylenolide I and II as well. Thus all three components could display positive modulatory activities on the $GABA_A$ receptor *in vivo*

resulting in sedation or anxiolysis. This may explain the use of this herbal drug as a traditional sedative in CM.

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**Supplementary Tab. 1.** $I_{GABA}$ enhancement by the 18 fractions (100 µg/mL) gained from semi-preparative HPLC of the *A. macrocephala* PE extract. Potentiation of $I_{GABA}$ in percent is given as mean ± SEM from 3-4 oocytes from ≥ two different batches.

| Fraction | $I_{GABA}$ potentiation (%) | n |
|:--------:|:---------------------------:|:-:|
| 1 | -22 ± 2 | 3 |
| 2 | -25 ± 7 | 3 |
| 3 | 13 ± 8 | 3 |
| 4 | 118 ± 7 | 4 |
| 5 | 21 ± 12 | 3 |
| 6 | 10 ± 3 | 3 |
| 7 | -7 ± 2 | 3 |
| 8 | 109 ± 3 | 4 |
| 9 | -12 ± 6 | 3 |
| 10 | 12 ± 8 | 3 |
| 11 | 6 ± 4 | 3 |
| 12 | 0 ± 0 | 3 |
| 13 | 20 ± 7 | 3 |
| 14 | -5 ± 7 | 3 |
| 15 | 1 ± 5 | 3 |
| 16 | -13 ± 4 | 3 |
| 17 | 0 ± 3 | 3 |
| 18 | 53 ± 23 | 3 |

**3.3 Isolation of the active principles of *Cnidium monnieri* (L.) Cuss. ex Juss.**

*Cnidium monnieri* (L.) Cuss. ex Juss. (Apiaceae), Monnier's snowparsley, is an annual plant growing in many parts of China, but the drug is preferably collected in the East coastal provinces of North and North-Eastern China, namely Hebei, Shandong and Zheijiang province. The aerial parts reach heights between 0.1 and 0.8 m. The inflorescences form umbels, which are 2 to 5 cm in width and each of the finally branching umbellules carry 15 to 20 diminutive, white flowers (Fig. 19A). The ovoid fruits are very small with 1.5-3 x 1-2 mm i.d. (Fig. 19B), (eFloras, 2010).



**Figure 19. *Cnidium monnieri*. (A) *Cnidium monnieri*** aerial parts [http://www.horizonherbs.com, accessed 28th December, 19:09]. **(B)** Fruits of *Cnidium monnieri* photographed by I. Sprinzl (2007).

The ripe fruits are preferably collected in summer or autumn and should be yellowish green in color (Zhang, 2007). The drug is either used topically to treat skin diseases like eczema, pruritus and scabies or, when applied internally, as an aphrodisiac, analgesic or sedative agent. 3-9 g of drug are typically applied for internal use (Zhang, 2007). The main constituents in shé chuáng zǐ are fatty acids, essential oil components and coumarin derivatives (Bensky et al. 2006). From latter substance class, osthole is one of the major compounds found in *C. monnieri* fruits (Yan *et al.*, 2001) and more thoroughly studied than

other constituents. It was investigated for its anti-inflammatory (Liao *et al.*, 2010), cytotoxic (Yang *et al.*, 2003; Chou *et al.*, 2007), or even antidiabetic effects (Liang *et al.*, 2009).

From four extracts of different polarity investigated in the preliminary screening, the PE and the MeOH extracts displayed similar activity on the GABAA receptor (Fig. 20).
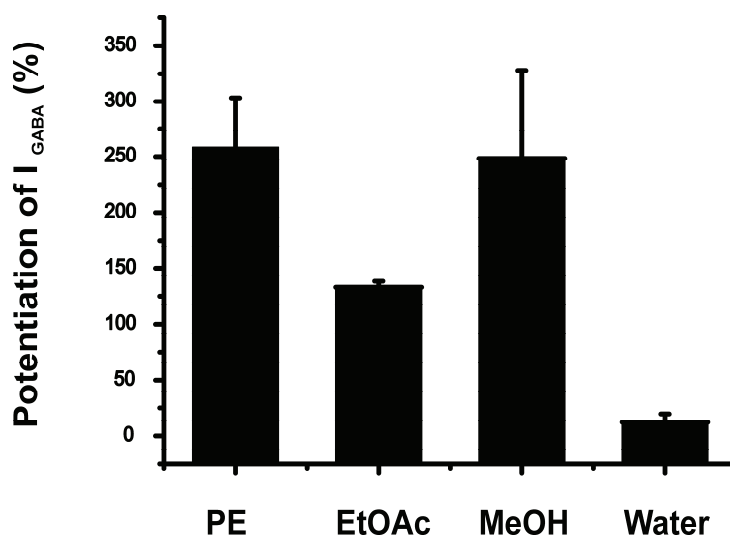


**Figure 20.** Activity screening of the four *C. monnieri* fruits extracts (100 µg/mL) from different polarity, whereby the PE extract displayed slightly higher activity. Bars represent mean potentiation of $I_{GABA} \pm SEM$ in percent. Data is derived from at least 3 oocytes from 2 different batches. This data was provided by co-workers of the Department of Pharmacology and Toxicology, University of Vienna.

By comparing the HPLC chromatograms of the PE, EtOAc and MeOH extracts, an almost identical peak pattern was observed, albeit higher amounts of the main constituents osthole and imperatorin can be found in the PE extract (Fig. 21, p. 58). In the water extract no peaks could be observed with the HPLC method used and thus it is not depicted in figure 21 (p. 58).
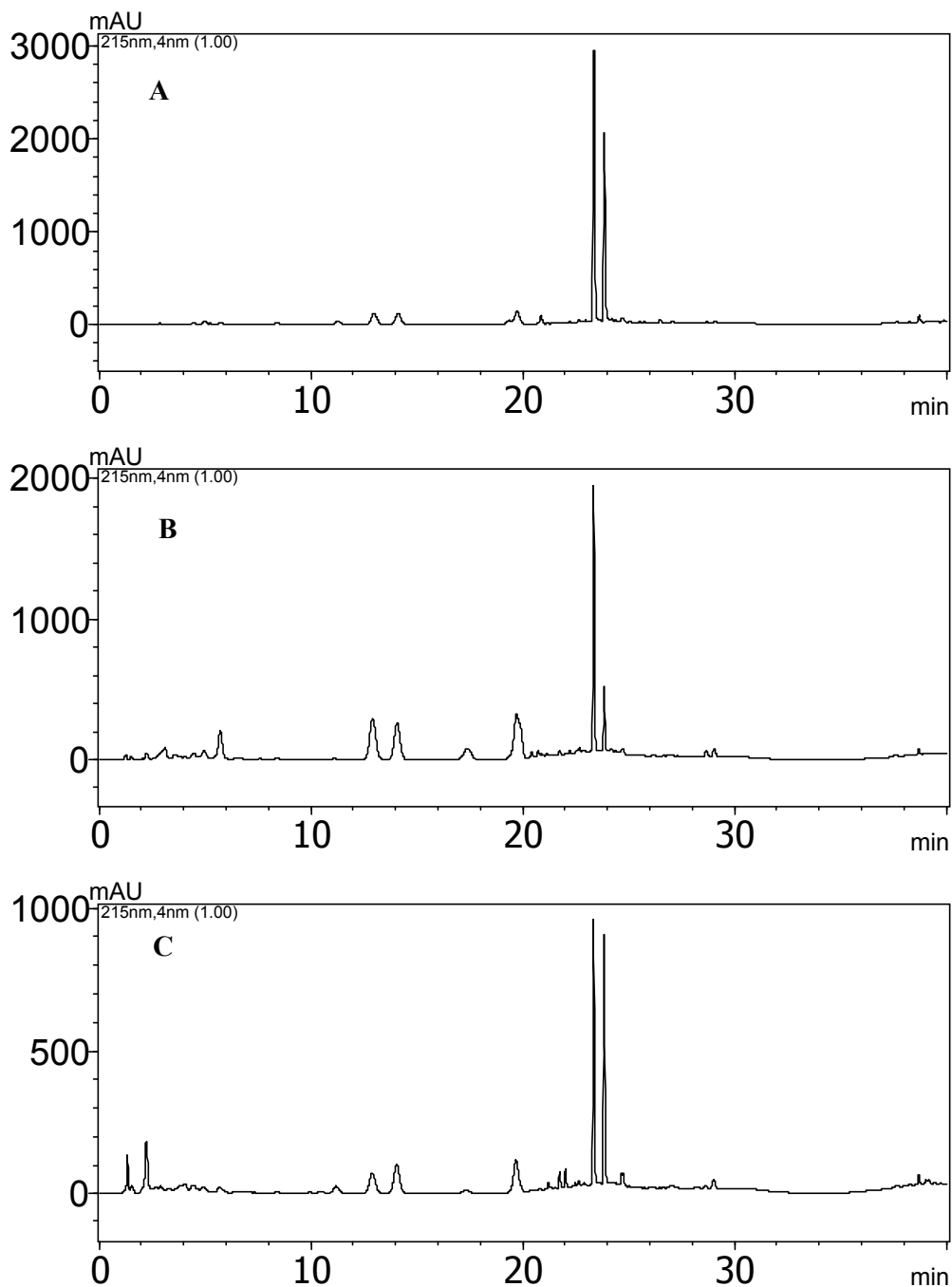
**Figure 21.** HPLC analysis of the different *C. monnieri* fruit extracts. Instrument 2, column 1 using a water/MeCN gradient (Chen *et al.*, 2007a, chapter 2.4.5.2, p. 27). Injection volume was 20 µL and solutions of 1 mg/mL MeOH were used. **(A)** Chromatogram of the PE extract. **(B)** Chromatogram of the EtOAc extract. **(C)** Chromatogram of the MeOH extract. Detection wavelength: 215 nm.

Since the PE extract exerted a slightly higher activity than the MeOH extract it was selected for fractionation on semi-preparative HPLC (instrument 3, column 3). For this, a total of 45mg (dissolved in 4 mL MeCN) of the PE extract were subjected to HPLC for fractionation,

whereby only 15 mg extract were used per run to ensure the separation of the two major peaks. Fractions were collected volume dependent (13 mL) and unified according to their peak pattern to yield 22 fractions in total (Fig. 22). From all fractions, only fraction CM/HPLC/16 displayed promising activity (Fig. 23).
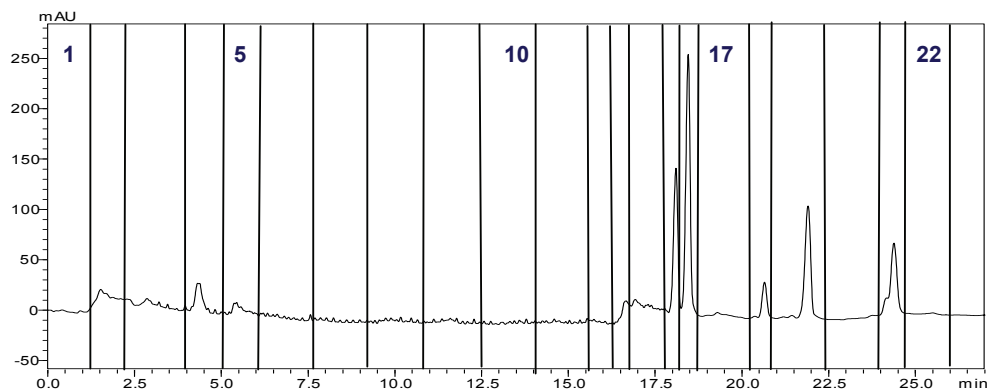


**Figure 22.** Fractionation scheme of the PE extract from *C. monnieri* fruits on instrument 3, employing column3 using the method from Chen et al (2007a) as described in chapter 2.4.5.2 (p. 27). First the fractions were collected volume-dependent, than unified according to their peak pattern. Detection wavelength is set at 280 nm.
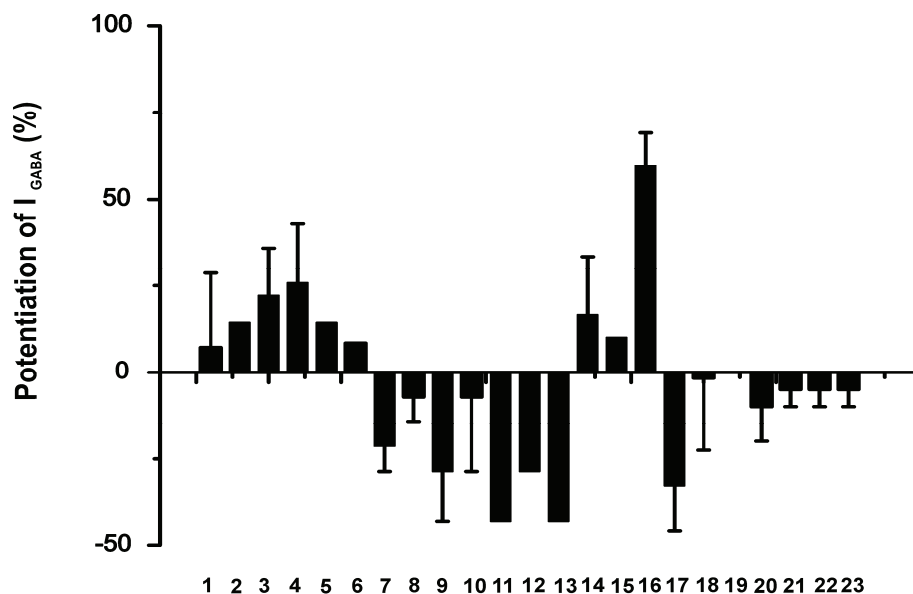


**Figure 23.** Activity screening of the HPLC fractions (100 µg/mL) from *C. monnieri* on $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors revealed that fraction 16 showed highest activity (n = 1-2). This data was provided by co-workers of the Department of Pharmacology and Toxicology, University of Vienna.

Fraction CM/HPLC/16 (~ 15 mg) was therefore subjected to normal phase CC on silica gel (60 x 0.5) using n-hexane:EtOAc (95:5) as mobile phase. Fractions of 5 mL were collected using a flow rate of 10 mL/h. As shown in figure 24, TLC screening (mobile phase: n-hexane:EtOAc (90:10), stationary phase: silica gel KG60 $F_{254}$) revealed to major spots in fractions CM/HPLC/16/30-35 (blue fluorescence) and CM/HPLC/16/50-60 (brown fluorescence). Consequently, fractions 25 to 36 and fractions 46 to 61 were unified to CM/HPLC/16/25-36 (3.1 mg) and CM/HPLC/16/46-61 (5.7 mg).
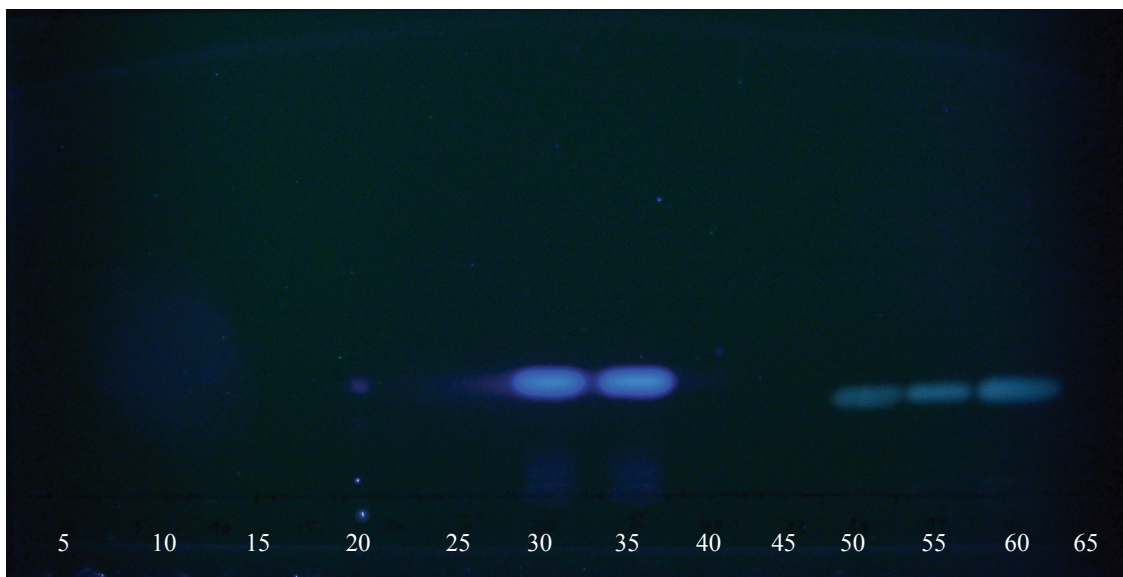


**Figure 24.** TLC screening of the fractionation of CM/HPLC/16, using silica gel plates (KG60 $F_{254}$, 20x10). Zones could be detected under $UV_{366}$ without further derivatisation. From every fifth fraction 20 μL, taken directly from the fractionation vial, were applied on the plate.

Subsequent HPLC analysis revealed that the unified fractions contained one pure component each (Fig. 25, p. 61). Structures were elucidated by NMR to reveal that fraction CM/HPLC/16/25-36 contained osthole (annex V) and fraction CM/HPLC/16/46-61 contained imperatorin (annex VI).
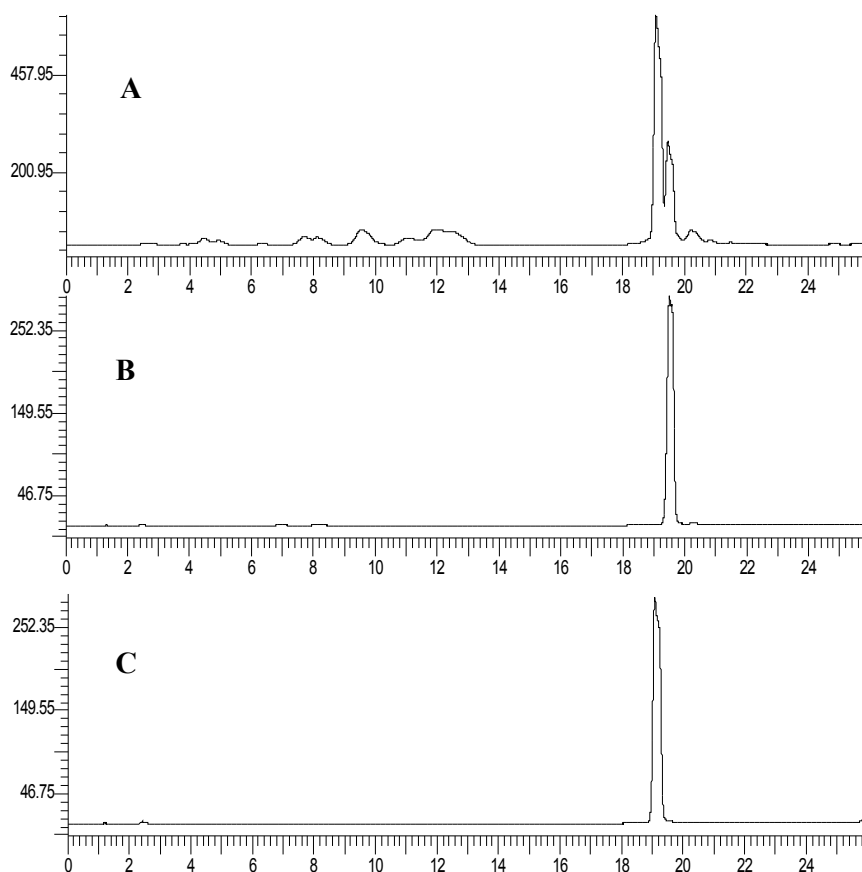
**Figure 25.** HPLC analysis of CM/HPLC/16/25-36 and CM/HPLC/16/46-61 (each 1 mg/mL MeOH, injection volume 10 μL). Instrument 1 and column 1 were employed using an analysis method according to Chen *et al.* (2007a, see chapter 2.4.5.2, p. 27). **(A)** Chromatogram of the *C. monnieri* PE extract for comparison (5 mg/mL MeOH, 20 μL injection volume). **(B)** Chromatogram of fraction CM/HPLC/18/25-36. **(C)** Chromatogram of fraction CM/HPLC/16/46-61.

When testing the pure compounds for $I_{GABA}$ potentiation, both displayed positive modulatory activity. Interestingly, osthole exerted a 2 times higher enhancement of $I_{GABA}$ than imperatorin (125 ± 11 % vs. 54 ± 13 %) when tested in concentrations of 100 μM (Fig. 26, p. 62). This suggests, that the activity of coumarin derivatives is dependent on the basic structure i.e. higher activity for simply coumarins (osthole) compared to (linear) furanocoumarins (imperatorin).
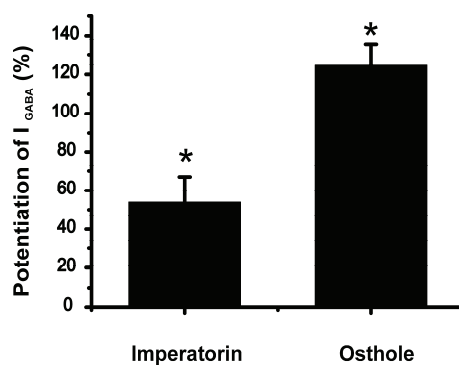
**Figure 26.** Potentiation of $I_{GABA}$ by imperatorin and osthole (100 µM) through $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors expressed in *Xenopus laevis* oocytes. Bars represent mean potentiation ± SEM in percent from at least 3 oocytes from ≥ 2 different batches. Statistical significance (Student's t-test, p=0.05) is indicated with (*).

To investigate this hypothesis, other coumarin derivatives – divided in the groups of simple, linear furanocoumarins and angular furanocoumarins – were screened for their ability to enhance $I_{GABA}$, to uncover the structural requirements for (positive) $I_{GABA}$ modulation by coumarin derivatives.

**3.4 Insights into structural requirements of GABA$_A$ receptor modulating coumarins and furanocoumarins (manuscript)**

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This manuscript was submitted to the ***European Journal of Pharmacology*** on 21$^{st}$ of December 2010. All of the phytochemical work, except NMR measurements and structure elucidation (Prof. Ernst Urban, Department of Pharmaceutical Chemistry, University of Vienna), as well as the activity screening of the 18 coumarins, concentration-response curves, benzodiazepine experiments were done by the author of this thesis. The two components osthruthole and ostruthin were isolated by Mag. Sylvia Vogl (Department of Pharmacognosy). The pharmacophore model was created partly by the author under supervision of Prof. G.F. Ecker (Department of Pharmaceutical Chemistry, University of Vienna), whereas the binary classification tree was created by Prof. G.F. Ecker.

**Abstract**

The coumarins imperatorin and osthole are known to exert anticonvulsant activity. We have therefore analyzed the modulation of GABA-induced chloride currents ($I_{GABA}$) by a selection of 18 coumarin derivatives on recombinant $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors expressed in *Xenopus laevis* oocytes by means of the two-microelectrode voltage clamp technique.

Osthole (EC$_{50}$ = 14 ± 1 µM) and oxypeucedanin (EC$_{50}$ = 25 ± 8 µM) displayed the highest efficiency with $I_{GABA}$ potentiation of 116 ± 4 % and 547 ± 56 %, respectively. $I_{GABA}$ enhancement by osthole and oxypeucedanin was not inhibited by flumazenil (1 µM) indicating an interaction with a binding site distinct from the benzodiazepine binding site. In general, prenyl residues are essential for the positive modulatory activity, while longer side chains or bulkier residues (e.g. geranyl residues) diminish $I_{GABA}$ modulation.

Generation of a binary classification tree revealed the importance of polarisability, which is sufficient to distinguish actives from inactives. A 4-point pharmacophore model based on oxypeucedanin - comprising three hydrophobic and one aromatic feature - identified 6 out of 7 actives as hits.

In summary, (oxy-)prenylated coumarin derivatives from natural origin represent new GABA$_A$ receptor modulators.

## 1. Introduction

The gamma-aminobutyric acid type A receptor (GABA$_A$) is a ligand gated ion channel mediating fast inhibition of neuronal signal transmission (Mody and Pearce, 2004). Binding of GABA to GABA$_A$ receptors induces hyperpolarization of the neuronal membrane due to an increased chloride influx and thus decreases or inhibits ongoing neurotransmission. The GABA$_A$ receptors are heteropentameric proteins, which can assembly from 19 different subunits: $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, $\delta$, $\pi$, $\epsilon$, $\theta$, and $\rho_{1-3}$ and potentially generate a large variety of receptor subtypes (Simon et al., 2004). From theoretically over 150.000 possible GABA$_A$ receptors only a few seem to occur *in vivo* in the mammalian central nervous system (Olsen and Sieghart, 2009). The most abundant receptor subtype consists of 2 $\alpha_1$, 2 $\beta_2$ and 1 $\gamma_{2S/L}$ subunit (McKernan and Whiting, 1996; Sieghart and Sperk, 2002). While binding of GABA opens GABA$_A$ receptor channels, there is also evidence for binding sites interacting with benzodiazepines, general anesthetics, barbiturates and many other therapeutically important drugs (Sieghart, 1995; Korpi et al., 2002; Sieghart and Enna, 2006). In addition to drugs that are in clinical use a variety of structurally diverse natural products have been shown to elicit positive modulatory effects on GABA$_A$ receptors, e.g. borneol (Granger et al., 2005), thymol (Priestley et al., 2003), valerenic acid (Khom et al., 2007; Trauner et al., 2008), piperine (Zaugg et al.), flavonoids (Huen et al., 2003; Fernandez et al., 2008), polyacetylenes (Baur et al., 2005), and various others (Johnston et al., 2006).

Compared to other natural compound classes like flavonoids or monoterpenes, the action of coumarins on GABA$_A$ receptors is largely unknown. However, coumarins often occur in plants that are used as sedatives or spasmolytic agents in traditional medicinal systems worldwide (Murray et al., 1982; O'Kennedy and Thorne, 1997). Furthermore, *in vivo* antiepileptic activity of coumarins was reported by Luszczki and co-workers (Luszczki et al., 2007a; b; Luszczki et al., 2009a; Luszczki et al., 2009b).

Evidence for interaction of coumarins with GABA$_A$ receptors comes also from binding studies suggesting that phellopterin and imperatorin interact with the benzodiazepine binding site of the GABA$_A$ receptor (Dekermendjian et al., 1996; Bergendorff et al., 1997).

In the present study we examine the effects of 18 (furano-)coumarins on chloride currents ($I_{GABA}$) through recombinant $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors expressed in *Xenopus laevis* oocytes and provide first insights into the structural requirement for a positive modulatory effect.

## 2. Material and Methods

### 2.1 Chemicals and substances

γ-Amino butyric acid (GABA) , reagents for ND96 solution, diazepam and flumazenil were purchased from Sigma (Vienna, Austria). Bergamottin, bergapten, bergaptol, coumarin, isobergapten, isopimpinellin, scopoletin and umbelliferone were purchased from Extrasynthese (Lyon, France). Auraptene and isoimperatorin were purchased from LGC Standards (Wesel, Germany). Oxypeucedanin was purchased from Phytolab (Vestenbergsreuth, Germany). Phellopterin was purchased from Sequoia Research Products Ltd. (Pangbourne, UK). Pimpinellin was purchased from Herboreal Ltd. (Edinburgh, UK). Ostruthin (purity ≥ 98 %) and ostruthol (purity ≥ 98 %) were isolated from *Peucedanum ostruthium* L. (Koch) by S. Vogl (Vogl et al., 2011) and imperatorin and osthole were isolated from *Cnidium monnieri* L. as follows: a petroleum ether extract of Cnidium monnieri fruits was first subjected to semi-preparative HPLC using a RP-18 column (Nucleosil 100, Machery-Nagel) and a gradient elution consisting of $H_2$ (solvent A) and acetonitrilee (solvent B) with a concentration of B of 35 % B for 15 min, followed by an increase of B to 80 % in 5 min and a steady concentration of B for 7 min followed by a decrease to 35 % B in 3 min. Flow rate was set at 27.6 mL/min. Fraction 16, which according to literature contains imperatorin and osthole, was subjected to normal phase column chromatography on silica gel (60 x 0.5 cm i.d.) using n-hexane:EtOAc (95:5) as mobile phase (flow rate 10 mL/h, fraction volume: 5 mL). Fractions were screened by TLC on silica gel coated aluminium plates KG60 F254 (Merck, Germany) using n-hexane:EtOAc (90:10) as mobile phase. Fractions 25-36 (blue fluorescent zone in the TLC screening) and fractions 46-61 (brown fluorescent spot in the TLC screening) were unified to yield two cumulative fractions. Their structure was elucidated by 1- and 2-D 1H and 13C-NMR as imperatorin (purity: ≥ 98 %) and osthole (purity: ≥ 97 %), respectively. Purity was determined using HPLC by comparing UV spectra and retention time to reference substances which were purchased from Sigma (Vienna, Austria).

### 2.2 Voltage clamp and fast solution exchange on *Xenopus* oocytes

Preparation of stage V-VI oocytes from *Xenopus laevis* (NASCO, USA) and injection of cRNA were done as previously described (Khom et al., 2006). Female frogs were anaesthetized 15 min prior to surgery using 0.2 % solution of MS-222 (Sigma, Vienna,

Austria) and parts of the ovaries were removed. Remaining follicle membranes were enzymatically digested with 2mg/mL collagenase Type 1 A (Sigma, Vienna, Austria). Synthesis of capped off run-off poly (A+) cRNA transcripts was performed from linearized cDNA templates (pCMV vector). cRNAs were diluted with DEPC-treated water and stored at - 80 °C. Injection of 10 - 50 nL of the different cRNA solutions was carried out on the day of isolation. To ensure the expression of the γ-subunit, cRNAs of $\alpha_1$, $\beta_2$, and $\gamma_{2S}$ were injected in a ratio of 1:1:10 (Boileau et al., 2003; Baburin et al., 2008). Successful expression of the γ-subunit was determined by application of diazepam (300 nM). Injected oocytes were stored at 18 °C in penicillin and streptomycin supplemented ND96 solution, containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2*6H_2O$, 1.8 mM $CaCl_2$ and 5 mM HEPES (pH 7.4) in double distilled water.

Chloride currents through $GABA_A$ receptors were measured by means of the two-microelectrode voltage clamp method making use of a TURBO TEC 03X amplifier (npi electronic, Tamm, Germany) at a holding potential of –70 mV as previously described (Baburin et al., 2006). Current measurements were recorded with pCLAMP 10 data acquisition software (Molecular Devices, Sunnyvale, CA, USA). ND96 was used as bath solution. Microelectrodes (Harvard Apparatus, Kent, UK) with resistances between 1 and 3 MΩ were pulled by means of a microelectrode puller (Narishige, Tokyo, Japan) and filled with 2 M KCl.

GABA and compounds were applied to oocytes by means of the ScreeningTool (npi electronic, Tamm, Germany) fast perfusion system as described by Baburin et al. (2006). Stock solutions of the tested compounds (100 mM) were prepared in DMSO and stored at -20 °C. GABA and test solutions were prepared freshly every day. The DMSO concentration of 1 % in both, the control and test solutions, did not affect GABA-induced chloride current ($I_{GABA}$). In the DMSO-stock solutions (10 mM) and the aqueous test solutions used no precipitates or turbidity was observed and thus the compounds were regarded as fully dissolved.

$I_{GABA}$ modulation was measured at a GABA concentration eliciting between 5 and 10 % of the maximal current amplitude ($EC_{5-10}$), corresponding to 3 - 10 µM GABA. The $EC_{5-10}$ was established at the beginning of each experiment. In the presence of compound concentrations higher than 30 µM wash out periods were extended to up to 10 minutes to exclude effects of receptor desensitization on current amplitudes.

**2.3 Data analysis**

Compound induced changes in chloride current amplitudes were calculated as

$I_{(GABA+compound)}/I_{GABA}-1$, where $I_{(GABA+compound)}$ is the current response in the presence of a given compound and $I_{GABA}$ is the control GABA current.

Concentration-response curves were generated and the data were fitted by nonlinear regression analysis using Origin Software (OriginLab Corporation, Northampton, MA, US). Data were fitted to the equation $1/(1+(EC_{50}/[\text{compound}]^{nH})$, where $EC_{50}$ is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50% of the compound-induced maximum response, and $n_H$ is the Hill coefficient. Responses were graphed as mean ± S.E.M. from at least three oocytes out of $\geq 2$ different batches. Statistical significance (*) was calculated using $t$-test and one-way ANOVA with a confidence interval of $P < 0.05$.


**2.4 Molecular modeling**

Molecules were built using the builder module in MOE 2009.10 and energy minimized using standard conditions (MMFF94x force field, adjust H and LP, gradient = 0.01, calculate forcefield partial charges). A database was built and a small set of physicochemical parameters was calculated. These comprise logP (logP(o/w)), topological polar surface area (TPSA), polarisability (apol), molar refractivity (mr), number of rotable bonds (b_1rotN), as well as number of H-bond donors and –acceptors. These descriptors allow a general description of the physicochemical properties of the molecules and have been successfully applied in classification analyses (Demel et al., 2010). The data set was split into two classes, active/inactive, with a threshold of 10% potentiation. This resulted in 7 active and 11 inactive compounds. Chemical structures, class labels as well as selected physicochemical descriptors are given in Table 1.


**2.4.1. Binary Classification Tree**

A binary classification tree was built using the QuaSAR-Classify tool in MOE 2009.10 and the physicochemical descriptors outlined above. The tree was constructed applying standard conditions (number of test samples: 18; number of cross-validation subsets: 2; cross-validation subset size: 9; random subset selection: on; minimum node size for splitting: 10; the maximum growth depth: 10; classes equally important). Quality of the models was

assessed by identifying the number of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) obtained in leave one out cross validation runs. The overall prediction accuracy (A), the sensitivity (SE) which represents the accuracy on actives, and the specificity (SP), which illustrates the accuracy on inactives, were calculated as follows: A = (TP + TN)/(TP+TN+FP+FN), SE = TP/(TP+FN) and SP = TN/(TN + FP).

## 2.4.2 Creating a pharmacophore model

3D structures were built interactively using MOE 2009.10. The number of conformers generated using the 'best' feature of the program for each substrate was limited within the program to a maximum of 255 with an energy range of 15.00 kcal/mol beyond the calculated potential energy minimum. A 4-point pharmacophore model using the most efficient modulator, oxypeucedanin, as template (fig. 7A), was created using the Pharmacophore Modeling tools implemented in MOE. The final model features 3 hydrophobic regions at the prenyl residue and in position 4 of the carbon skeleton of oxypeucedanin, a position directly opposite to the attachment site of the prenyl residue, as well as one aromatic feature.

## 3. Results

### 3.1. Potentiation of $I_{GABA}$ by osthole and oxypeucedanin
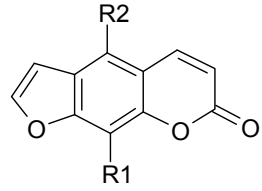
Recombinant $\alpha_1\beta_2\gamma_{2S}$ receptors were expressed in *Xenopus laevis* oocytes and GABA-induced chloride current ($I_{GABA}$) modulation by 18 coumarin derivates (Fig. 1, 2) was investigated by means of two-microelectrode voltage clamp and a fast perfusion technique (see Methods). The 18 tested compounds consist of 6 simple coumarins, 10 linear furanocoumarins and 2 angular furanocoumarins (Fig. 1).



| | R1 | R2 | R3 |
|---|---|---|---|
| **coumarin** | H | H | H |
| **umbelliferone** | H | OH | H |
| **scopoletin** | H | OH | OMe |
| **osthole** | | OMe | H |
| **auraptene** | H | | H |
| **ostruthin** | H | OH | |
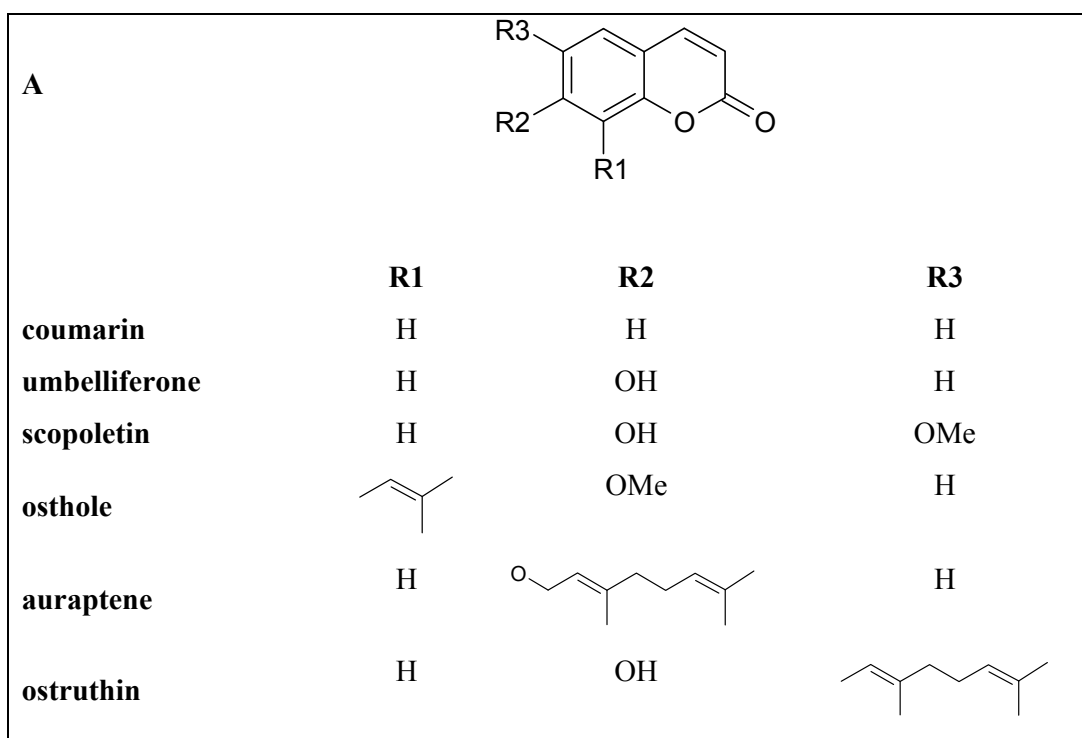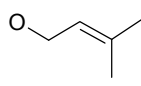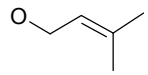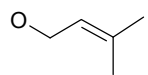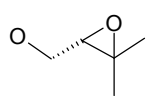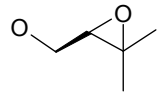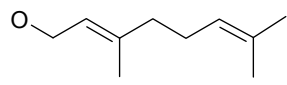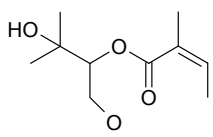
**Fig. 1.** Structure of the selected compounds divided in the three groups: (A) simple coumarins, (B) linear furanocoumarins and (C) angular furanocoumarins (continued on the next page).
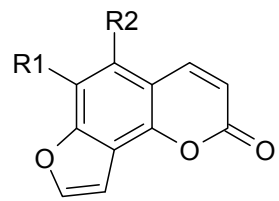
**B**



|  | **R1** | **R2** |
|---|---|---|
| **bergaptol** | H | OH |
| **bergapten** | H | OMe |
| **isopimpinellin** | OMe | OMe |
| **isoimperatorin** | H | (O-prenyl) |
| **imperatorin** | (O-prenyl) | H |
| **phellopterin** | (O-prenyl) | OMe |
| **heraclenin** | (O-epoxide) | H |
| **oxypeucedanin** | H | (O-epoxide) |
| **bergamottin** | H | (O-geranyl) |
| **ostruthol** | H | (O-ester) |

**C**



|  | **R1** | **R2** |
|---|---|---|
| **isobergapten** | H | OMe |
| **pimpinellin** | OMe | OMe |

**Fig. 1.** continued
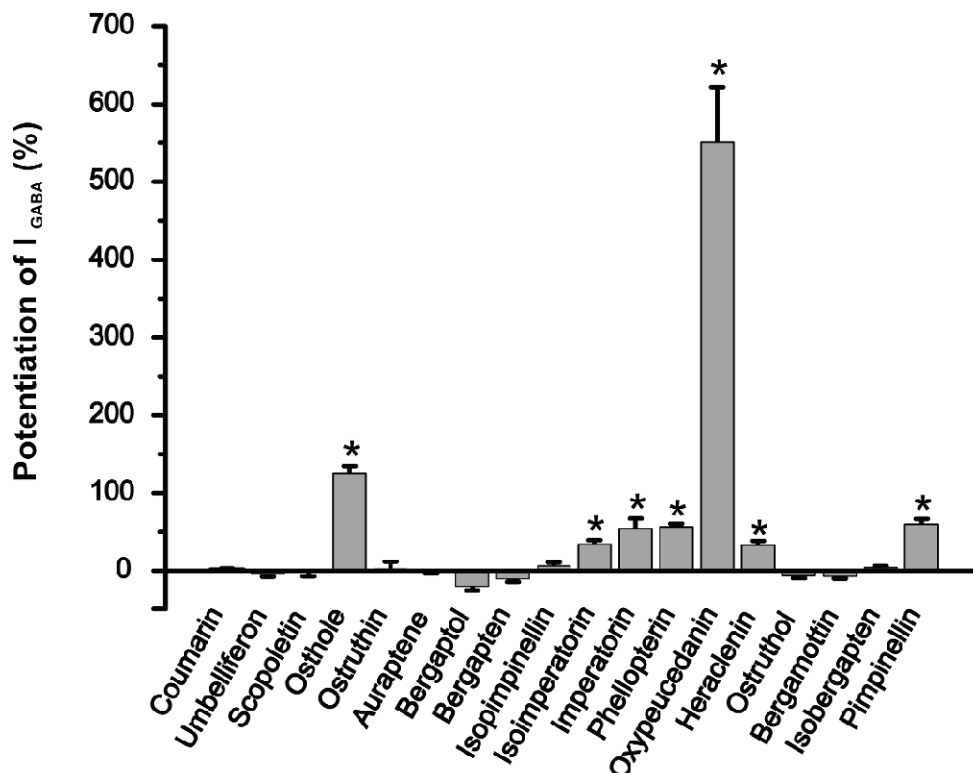
**Fig. 2.** Potentiation of $I_{GABA}$ by different coumarins and furanocoumarins (100 µM) from a selection of 18 coumarin derivatives in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors. The compounds are ordered in following groups: simple coumarins, linear coumarins and angular coumarins. Within these groups the columns are arranged according to the length of the side chain - small (-OH, -OMe), medium (-OC$_5$, -C$_5$) and large (-OC$_{10}$, -C$_{10}$). Statistical significance (*t*-test, $p < 0.05$) is indicated with (*).

Oxypeucedanin and osthole at 100 µM enhanced $I_{GABA}$ most efficiently by $550 \pm 71$ % (n = 5) and $124 \pm 11$ % (n = 5), respectively (Fig. 2, Table 1). The concentration-dependent potentiation of $I_{GABA}$ by osthole (maximum enhancement by $116 \pm 4$ %) is illustrated in Fig. 3. The EC$_{50}$ value was determined as $14 \pm 1$ µM with a Hill coefficient ($n_H$) of $1.4 \pm 0.2$ (n = 4).

The concentration-response curve for oxypeucedanin (EC$_{50}$ of $26 \pm 8$ µM, $n_H$ = $1.2 \pm 0.1$, n = 4) is shown in Fig. 4. Neither of the tested compounds activated the GABA$_A$ receptor in the absence of GABA in concentrations up to 300 µM suggesting an allosteric modulation. The observed enhancement of $I_{GABA}$ was always reversible.
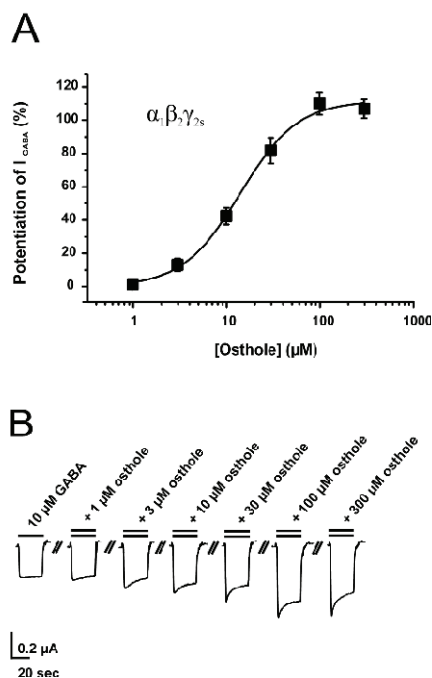
**Fig. 3. (A)** Concentration-response curve for $I_{GABA}$ enhancement by osthole ($EC_{50} = 14 \pm 1$ μM, $n_H = 1.4 \pm 0.3$, n = 4) in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors. **(B)** Representative currents through $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors in the presence of GABA (10 μM, single bar, control) and currents and 1, 3, 10, 30, 100 and 300 μM of osthole (double bar) recorded during co-application of GABA (10μM)
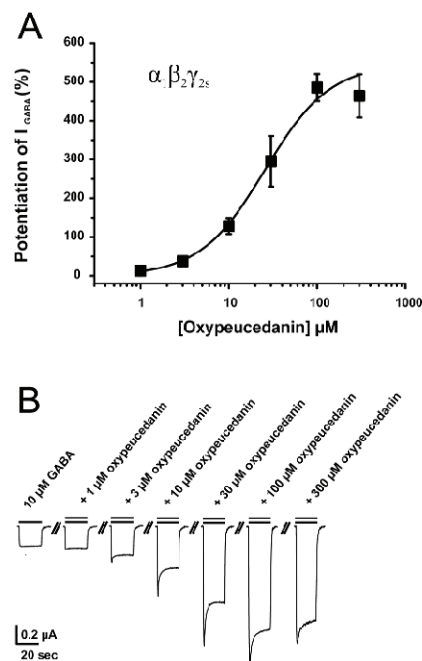
**Fig. 4. (A)** Concentration-response curve for $I_{GABA}$ enhancement by oxypeucedanin (OPD, $EC_{50} = 26 \pm 8$ μM, $n_H = 1.2 \pm 0.1$, n = 4) in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors. **(B)** Representative currents through $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors in the presence of GABA (10 μM, $EC_{5-10}$, single bar, control) and currents recorded during co-application of GABA (10 μM) and 1, 3, 10, 30, 100 and 300 μM of oxypeucedanin (double bar).

## 3.2. Osthole and oxypeucedanin modulate GABA$_A$ receptors not *via* the benzodiazepine binding site

To determine if osthole or oxypeucedanin modulate GABA$_A$ receptors by interaction with the benzodiazepine binding site, $I_{GABA}$ modulation by these two compounds was studied in the presence and absence of flumazenil (1 μM) or diazepam (300 nM). Co-application of flumazenil (1 μM) did neither inhibit osthole- (15 μM) nor oxypeucedanin-induced (20 μM) potentiation of $I_{GABA}$ ($62 \pm 6\%$, n = 4 vs. $68 \pm 12$ %, n = 4, Fig 5A; and $152 \pm 20$ %, n = 5 vs. $153 \pm 24$ %, n = 5, Fig 6A). When osthole (15 μM) and diazepam (300 nM) were co-applied an additive increase in the $I_{GABA}$ amplitude ($195 \pm 38$ %, n = 5) was observed compared to $I_{GABA}$ modulation by diazepam ($129 \pm 19$, n = 4) and osthole ($60 \pm 7$ %, n = 5, Fig. 5C).
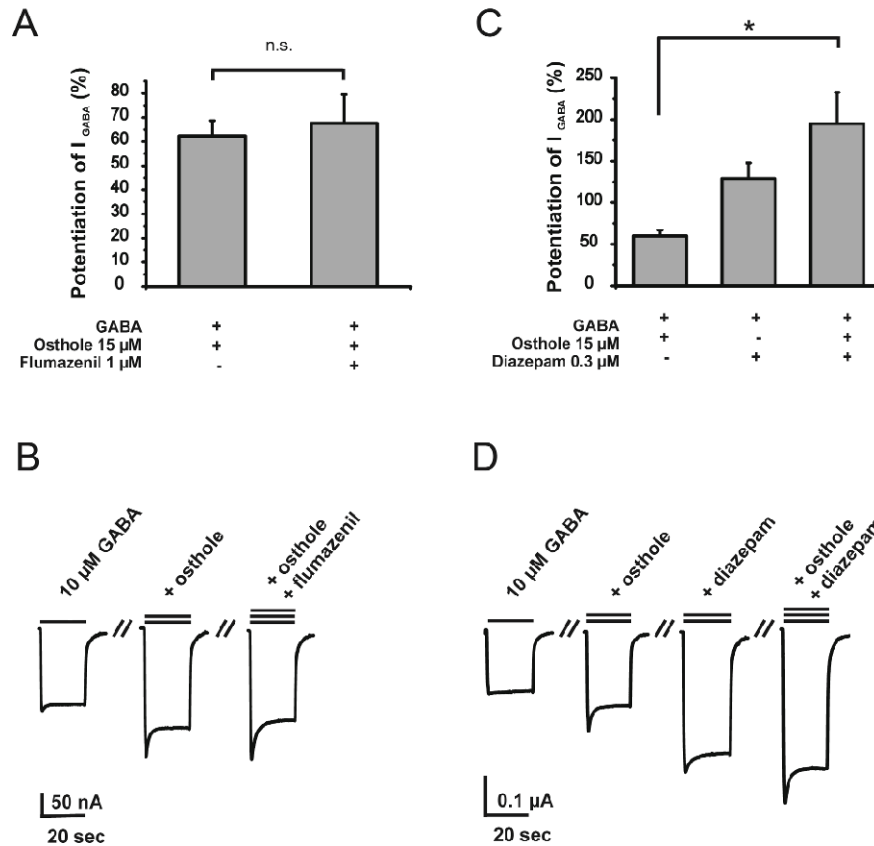
**Fig. 5.** Effect of osthole (OST) on $I_{GABA}$ in the presence of flumazenil (FLZ) and diazepam (DZP) in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors. Statistical significance (one-way ANOVA, $p < 0.05$) is indicated with (*), n.s. = not significant ($p > 0.05$). **(A)** Stimulation of $I_{GABA}$ by osthole in the presence of flumazenil (1 µM).The left bar shows the positive allosteric modulation of the GABA ($EC_{5-10}$)-induced chloride currents by 15 µM osthole. The right bar illustrates that flumazenil (1µM) does not antagonize the osthole-induced enhancement of $I_{GABA}$. **(B)** Representative currents through $\alpha_1\beta_2\gamma_{2S}$ receptors in the absence and presence of the indicated concentrations of osthole or osthole and flumazenil, respectively. The leftmost current represents the GABA control current (10 µM, single bar). **(C)** Additive effects of osthole and diazepam on $I_{GABA}$. The left bar illustrates the enhancement of $I_{GABA}$ by 15 µM osthole, the middle bar by 300 nM diazepam, and the right bar illustrates the enhancement of $I_{GABA}$ by co-application of osthole (15 µM) and diazepam. (300 nM). **(D)** Representative currents through $\alpha_1\beta_2\gamma_{2S}$ receptors in the absence and presence of the indicated concentrations of osthole, diazepam, or osthole and diazepam, respectively. The leftmost current represents the GABA control current (10 µM, single bar).

Similar observations were made for oxypeucedanin (Fig. 6C, 300 nM diazepam: $130 \pm 16$ %, n = 3, 20 $\mu$M oxypeucedanin: $158 \pm 26$ %, n = 5 vs. oxypeucedanin and diazepam co-applied: $366 \pm 80$ %, n = 3).
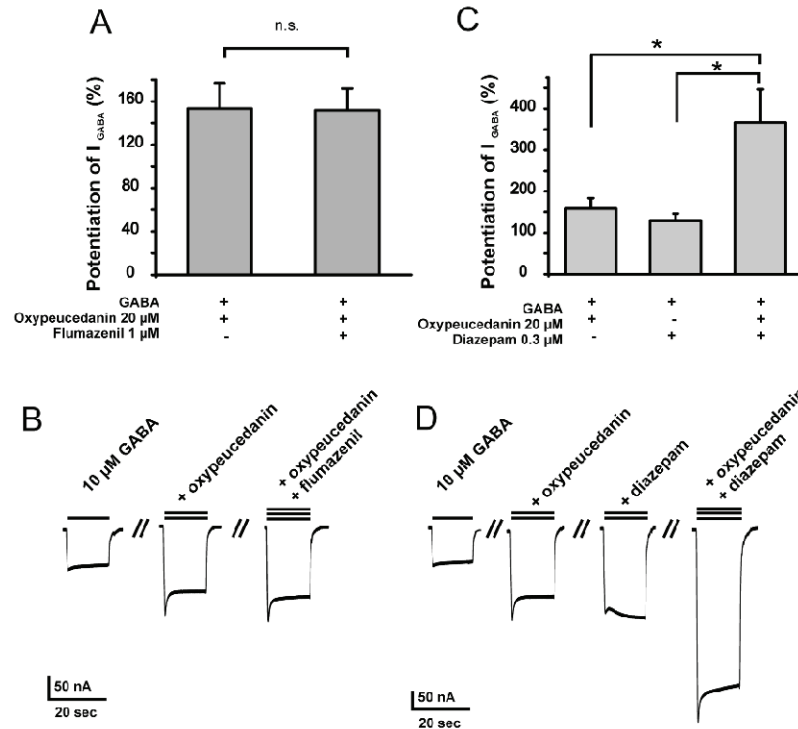


**Fig. 6.** Effect of oxypeucedanin (OPD) on $I_{GABA}$ in the presence of flumazenil (FLZ) and diazepam (DZP) in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors. Statistical significance (one-way ANOVA, $p < 0.05$) is indicated with (*), n.s. = not significant ($p > 0.05$). **(A)** Stimulation of $I_{GABA}$ by oxypeucedanin in the presence of flumazenil (FLZ, 1 $\mu$M). The left bar shows the positive allosteric modulation of the GABA (EC$_{5-10}$)-induced chloride currents by 20 $\mu$M oxypeucedanin. The right bar illustrates that flumazenil (1 $\mu$M) does not antagonize the oxypeucedanin-induced enhancement of $I_{GABA}$. **(B)** Representative currents through $\alpha_1\beta_2\gamma_{2S}$ receptors in the absence and presence of the indicated concentrations of oxypeucedanin, or oxypeucedanin and flumazenil, respectively. The leftmost current represents the GABA control current (10 $\mu$M, single bar). **(C)** Additive effects of oxypeucedanin and diazepam on $I_{GABA}$ The enhancement of $I_{GABA}$ by oxypeucedanin (20 $\mu$M, left bar) or 300 nM diazepam (middle bar) is increased in an additive manner when oxypeucedanin and diazepam are co-applied (right bar). **(D)** Representative currents through $\alpha_1\beta_2\gamma_{2S}$ receptors in the absence and presence of the indicated concentrations of oxypeucedanin, diazepam, or oxypeucedanin and diazepam, respectively. The leftmost current represents the GABA control current (10 $\mu$M, single bar).

## 3.3 Isopentenyl residues are a structural requirement for allosteric modulation of GABA$_A$ receptors

Insights into the structural requirements for GABA$_A$ receptor modulation by coumarins were obtained by comparing the action of 18 different coumarin derivatives (Fig. 2, Table 1). From the 6 simple coumarins only the prenylated osthole (100 µM) significantly potentiated I$_{GABA}$ by 124 ± 12 %. All other coumarins, which contained small hydroxyl or methoxyl groups (coumarin, umbelliferone, scopoletin) as well as the components with bulkier (geranyl/geranyloxy/other) residues (ostruthin, auraptene), did not enhance I$_{GABA}$ when co-applied with GABA at 100 µM (Fig. 2, Table 1).

| Compound | Potentiation (%) | Class | apol | a_acc | a_don | b_1rotN | logP(o/w) | mr | TPSA |
|---|---:|---:|---:|---:|---:|---:|---:|---:|---:|
| Coumarin | 1.5 | 0 | 21.44 | 1 | 0 | 0 | 2.18 | 4.14 | 26.30 |
| Umbelliferone | -4.4 | 0 | 22.25 | 2 | 1 | 0 | 1.90 | 4.26 | 46.53 |
| Scopoletin | -1.8 | 0 | 26.14 | 3 | 1 | 1 | 1.90 | 4.90 | 55.76 |
| Osthole | 124.5 | 1 | 39.47 | 2 | 0 | 3 | 3.20 | 7.05 | 35.53 |
| Auraptene | -0.1 | 0 | 50.52 | 2 | 0 | 6 | 3.85 | 8.85 | 35.53 |
| Ostruthin | 2.6 | 0 | 50.52 | 2 | 1 | 5 | 3.88 | 8.81 | 46.53 |
| Bergaptol | -19.9 | 0 | 26.56 | 2 | 1 | 0 | 2.17 | 5.16 | 59.67 |
| Isopimpinellin | 6.3 | 0 | 33.56 | 3 | 0 | 2 | 2.35 | 6.31 | 57.90 |
| Bergapten | -10.5 | 0 | 29.66 | 2 | 0 | 1 | 2.43 | 5.68 | 48.67 |
| Isoimperatorin | 33.8 | 1 | 40.70 | 2 | 0 | 3 | 3.25 | 7.49 | 48.67 |
| Imperatorin | 54.1 | 1 | 40.70 | 2 | 0 | 3 | 3.25 | 7.49 | 48.67 |
| Phellopterin | 56.5 | 1 | 44.60 | 3 | 0 | 4 | 3.16 | 8.13 | 57.90 |
| Heraclenin | 32.9 | 1 | 41.51 | 3 | 0 | 3 | 2.99 | 7.45 | 61.20 |
| Oxypeucedanin | 550 | 1 | 41.51 | 3 | 0 | 3 | 2.99 | 7.45 | 61.20 |
| Bergamottin | -7.2 | 0 | 54.84 | 2 | 0 | 6 | 4.11 | 9.77 | 48.67 |
| Ostruthol | -6.6 | 0 | 57.24 | 4 | 1 | 6 | 3.06 | 10.15 | 95.20 |
| Isobergapten | 4.6 | 0 | 29.66 | 2 | 0 | 1 | 2.43 | 5.68 | 48.67 |
| Pimpinellin | 65.4 | 1 | 33.56 | 3 | 0 | 2 | 2.10 | 6.31 | 57.90 |

**Table 1.** Class labels and selected physicochemical descriptors of the (furano-)coumarins including mean potentiation of I$_{GABA}$ by selected coumarin derivatives (100 µM). 7 compounds with an I$_{GABA}$ potentiation above 20 % were classified as active (1) while the other 11 components were regarded as inactive (0).

From the linear furanocoumarins (100 µM), only compounds with oxyprenyl residues modulated I$_{GABA}$. The epoxy-group containing oxypeucedanin (100 µM) induced the strongest

potentiation (550 ± 71 %, n = 5, Fig. 2, Table 1). The same concentration of heraclenin induced much less $I_{GABA}$ stimulation (33 ± 6 %, n = 3). Other tested furanocoumarins with an oxyisopentenyl residue - isoimperatorin, imperatorin, and phellopterin (all at 100 µM) - potentiated $I_{GABA}$ by 34 ± 6 % (n = 4), 54 ± 13 % (n = 4) and 57 ± 4 % (n = 3), respectively. None of the other components displayed $I_{GABA}$ enhancement above ≥ + 10 %, e.g. the furanocoumarins with only methoxy or hydroxyl groups e.g. bergaptol (- 20 ± 5%, n = 4), bergapten (- 11 ± 4 %, n = 4) and isopimpinellin (6 ± 5 %, n = 4). The same is valid for the furanocoumarins with bulkier residues such as the oxygeranylated bergamottin (- 7 ± 3 %, n = 4) and the ester compound ostruthol (-7 ± 3 %, n = 3, Fig .2, Table 1). This data correlates with the results of the simple coumarin group (see Fig. 1 for classification). Interestingly, while the angular furanocoumarin isobergapten showed no activity, its two times methoxylated derivative pimpinellin enhanced $I_{GABA}$ by 65 ± 5 % (n = 4).

## 3.4 Molecular modeling

First insights into the structural features necessary for significant $I_{GABA}$ potentiation were further complemented by preliminary computational studies. As more than half of the compounds are inactive, classification algorithms rather than classical QSAR analysis were chosen.
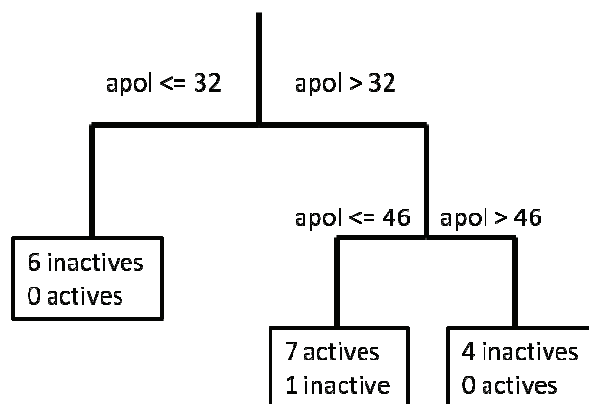


**Fig. 7.** Binary decision tree based on polarisability (apol).

Binary classification tree analysis as implemented in the software package MOE revealed a highly significant model (total accuracy = 88.9 %) based solely on polarisability (apol) as descriptor. Compounds with apol values < 31,6 or > 47,6 are assigned as inactive, whereas those exhibiting values within this range show GABA-modulating potency.

Furthermore, a pharmacophore model was constructed using oxypeucedanin as template. The model consists of three hydrophobic regions and one aromatic feature resulting in 4 false positive and 1 false negative annotations.
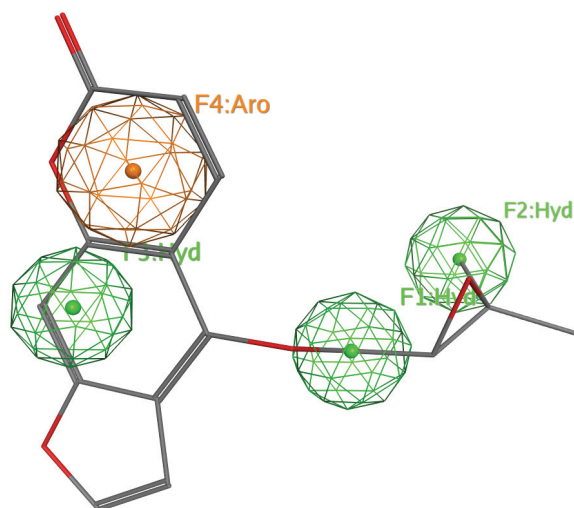


**Fig. 8.** 4-point pharmacophore model for coumarins. Superposition of oxypeucedanin with the pharmacophore query. Green color indicates hydrophobic regions and orange represents the aromatic feature.

All 4 false positives (ostruthin, auraptene, bergamottin, and ostruthol) are compounds with either a geranyl or oxygeranylresidue. To exclude these compounds, several volume exclusion domains were placed along the templates, but no decrease in the number of false positives was achieved. The inclusion of hydrogen acceptor regions (Acc) in the pharmacophore model always led to a reduction of the number of true positives. Further addition of Hyd or AtomQ features in the prenyl residue or the ring system had the same effect. The final model (18 compounds) produced 6 true positives, 7 true negatives, 1 false negative (phellopterin) and 4 false positives, resulting in an overall accuracy of 72 %, a sensitivity of 86 % and a specificity of 64 %.

## 4. Discussion

Coumarins are a class of secondary metabolites commonly found in various plant families. Despite the well known anticoagulant action of the class of 3-substitued 4-hydroxycoumarins (Hirsh et al., 2001; Sadler, 2004; Gebauer, 2007), the pharmacological properties of many natural coumarin derivatives are insufficiently characterized (Yarnell and Abascal, 2009). Previous studies with (furano)coumarins revealed photosensitizing (Abouelzahab et al., 1987; Eisenbrand, 2007), antimicrobial (Widelski et al., 2009; Tsassi et al.), anti-oxidant (Piao et al.,

2004; Kostova, 2006) and cytotoxic activity (Yang et al., 2003; Thanh et al., 2004; Kostova, 2005). There is also evidence for neuroprotective (Epifano et al., 2008) and antiepileptic effects (Luszczki et al., 2007a; b; Luszczki et al., 2009a; Luszczki et al., 2009b) induced by coumarins.

Effects of coumarins and furanocoumarins on $GABA_A$ receptors were first suggested by Bergendorff et al. (1997) and Dekermendjian et al. (1996) who observed [$^3$H]diazepam displacement in the presence of furanocoumarins, especially phellopterin. Direct evidence for potential effects of a furanocoumarin related substance on the $GABA_A$ receptor comes from recent studies, which described a positive allosteric modulation of $I_{GABA}$ by a novel plant derived dihydroisocoumarin (Li et al., 2010) and coumarins from *Angelica pubescens* L. (Zaugg et al. 2011).

We have therefore systematically analyzed 18 structurally diverse coumarin derivatives for $I_{GABA}$ enhancement. A comparison of their activity on $GABA_A$ receptors enabled first insights into their structure-activity relationship. From the tested 18 structurally diverse coumarins, imperatorin, isoimperatorin, phellopterin, osthole, oxypeucedanin, heraclenin, and pimpinellin potentiated $I_{GABA}$ by more than 20 % when applied at 100 µM (Fig. 2). All 7 components, except the angular furanocoumarin pimpinellin, bear either an oxyprenyl or a prenyl residue, while the position of the side chain varies. This indicates that the C5 side chain represents a structural requirement for $I_{GABA}$ modulation. While osthole, the second most active compound, is a simple coumarin, the most efficient substance - oxypeucedanin - represents a furanocoumarin with an epoxylated oxyprenyl residue. The stabilizing effect of the two geminal methyl groups rules out an unspecific effect caused by the chemical reactivity of the epoxide moiety. Interestingly, the regioisomeric heraclenin showed a more than 10-fold loss of activity (31%). Furthermore, both regioisomers of the respective furanocoumarin analogue (isoimperatorin and imperatorin) were almost equally active, which indicates that the different activities of oxypeucedanin and heraclenin could be due to the different configuration of the chiral center rather than to the different position of the side chain. Extending the prenyl side chain of isoimperatorin by one additional isopentenyl moiety (bergamottin) or attaching a large and sterically complex group (ostruthol) completely abolished biological activity. Finally it's worth mentioning that in the group of angular furanocoumarins one additional methoxy group leads to a remarkable increase in $I_{GABA}$ potentiation (pimpinellin *vs* isobergapten). This also accounts for the configurational isomers pimpinellin and isopimpinellin, where the compound with the angular scaffold (pimpinellin) is more active than the respective linear analogue (isopimpinellin).

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In order to gain deeper insights into the molecular features relevant for high biological activity, we also performed preliminary computational studies utilizing both a decision tree algorithm and pharmacophore modeling.

A binary classification tree based on a small set of physicochemical descriptors was able to classify 17 out of 18 compounds correctly, using only polarisability (apol) as descriptor (Fig. 7). The only compound miss-classified in this model was isopimpinellin (FP). However, one needs to consider that the descriptor used (apol) cannot distinguish between the configurational isomeres isopimpinellin (inactive) and pimpinellin (active), giving for both compounds a value of 33.56. In conclusion, this model might be useful for predicting the activity of structurally analogous derivatives.

Finally, a pharmacophore model was constructed which could aid in the understanding of the main pharmacophoric features necessary for $I_{GABA}$ enhancement by coumarins. Using oxypeucedanin as template, 6 out of 7 actives were correctly annotated as active. Having additionally 4 false positives, a total accuracy of 72 % with a sensitivity of 86% and a specificity of 64% was achieved. The high sensitivity indicates that the model would be a versatile tool for in silico screening attempts in order to identify potentially actives out of a coumarin-based compound library. The pharmacophore used consisted of three hydrophobic regions placed at the prenyl residue and opposite the carbon atom linking the basic skeleton with the side chain, and one aromatic feature (Fig. 8). Interestingly, introduction of other typical features like hydrogen-bond acceptor zones or an additional aromatic domain could not improve the model but rather resulted in assignment of active compounds as false negatives.

However, the feasibility of this 4-point pharmacophore for virtual screening should be taken with caution, since these rather unspecific hydrophobic zones will probably lead to a high number of false positives when used for screening. Nonetheless, given that geranylated coumarins are not active *in vitro*, screening of a coumarin database which has upfront been cleaned from such compounds might lead to interesting new hits, especially when combined with the binary classification tree.

Interestingly, neither osthole nor oxypeucedanin induce a current in the absence of GABA, which distinguishes the action of osthole and oxypeucedanin from other modulators like etomidate, or the barbiturates. Furthermore, our data clearly show that the two components do not interact with the binding sites of the benzodiazepines (Fig. 5 and 6). Future studies employing point-mutated receptors will clarify the exact binding site of constituents like osthole and oxypeucedanin on the $GABA_A$ receptor.

The effect of the tested coumarins occurs at very high concentrations which makes a therapeutic application unlikely, although all compounds tested met the Lipinski Rule of Five, indicating a low risk of insufficient bioavailability. However, it is currently not known if such a high concentrations can enter the brain. When referring to their anti-convulsant activity in vivo, the high concentrations needed for $I_{GABA}$ by such compounds as imperatorin suggest that coumarins exert their effects not exclusively via the $GABA_A$ receptor, but may additionally interact with other receptors supporting an anticonvulsive (Luszczki et al., 2007a; b; Luszczki et al., 2009a; Luszczki et al., 2009b).

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**3.5 Isolation of the active principles from *Juncus effusus* L.**

*Juncus effusus* L. (Juncacea), also known as common rush, is a perennial wetland plant growing all over the Northern hemisphere. The evergreen plant can reach heights up to 1 m and the unobtrusive inflorescences are white with a light-brown calyx (Fig. 27A). In East European traditional medicine the rhizomes of *J. effusus* are used as a diuretic agent for dehydration therapies and homeopathic preparations are applied for the treatment of kidney stones (Pahlow, 1999). In CM, the inner part of the stem is used to treat insomnia, especially in children, and as an antipyretic and diuretic agent. The stem pith – dēng xīn cǎo - is dried and then tied into bundles and cut to the same length (Fig. 27B). The drug can also be charred before use, but since the dry material is easily inflammable, this is rarely done. This herbal drug is normally used in dosages between 1.5 to 4.5 g (Bensky et al., 2006).



**Figure 27.** (A) Aerial parts of *J. effusus* downloaded from de.academic.ru on the 20th of December, 2010. (B) Typical bundles of *J. effusus* pith as it is used in CM (photographed by I. Sprinzl, 2007).

Phenanthrenoid compounds seem to represent the major components in *Juncus*. Additionally, some cyclo-artane type triterpenes, flavonoids and phenolic acid derivatives together with essential oil compounds, β-sitosterole and α-tocopherol have been already isolated from this plant and related species (Bensky et al., 2006).

After the preliminary extract screening, which revealed high activity for the EtOAc extract, a freshly prepared EtOAc extract was produced from approximately 2.2 kg of ground drug. Due to its high volume and sponge-like quality, around 200 L EtOAc were needed for the

extraction and this was conducted by portion-wise extraction of 30 - 50 g drug/flask, whereby each flask could hold a volume of 2 L.

Finally, 13.4 g of extract could be gained and 10.0 g of this dark brown residue were dissolved in EtOAc, mixed with ca. 20 g of silica gel and evaporated to dryness under reduced pressure. This mixture was applied on the silica gel column (65 x 3.5 cm i.d.) for VLC. The solvent mixtures (1.5 L each) used for VLC and the respective fractionation yields are given in table 3.

| fraction | solvent | ratio | yield (g) | appearance |
|----------|---------|-------|-----------|------------|
| JE/VLC/1 | PE:EtOAc | 9:1 | 0.33 | white |
| JE/VLC/2 | | 8.5:1.5 | 0.47 | yellow |
| JE/VLC/3 | | 8:2 | 0.31 | orange |
| JE/VLC/4 | | 7.5:2.5 | 0.34 | orange-brown with crystals |
| JE/VLC/5 | | 6.5:3.5 | 0.70 | brown with white crystals |
| JE/VLC/6 | | 6:4 | 0.96 | yellow-brown crystals |
| JE/VLC/7 | | 5:5 | 0.36 | dark brown |
| JE/VLC/8 | | 3:7 | 0.53 | dark brown |
| JE/VLC/9 | | 1.5:8.5 | 0.39 | dark brown |
| JE/VLC/10 | EtOAc | | 0.21 | dark brown |
| JE/VLC/11 | MeOH | | 2.20 | dark brown |
| **TOTAL** | | | **6.80** | |

**Table 3.** VLC fractionation yields for the *J. effusus* EtOAc extract.

From the 11 fractions gained, JE/VLC/1 was considered the pre-run fraction and JE/VLC/11 was the column wash out, thus neither of these two fractions was used for activity screening. TLC analysis was conducted with toluene:EtOAc:MeOH:HAc (35:10:1:2.5) as mobile phase. Plates were sprayed with ASR and heated at 105 °C for 5-10 min. The zones could then be detected under $UV_{366}$ (Fig. 28, p. 86) and in visible light. This TLC system was used for all further analyses.
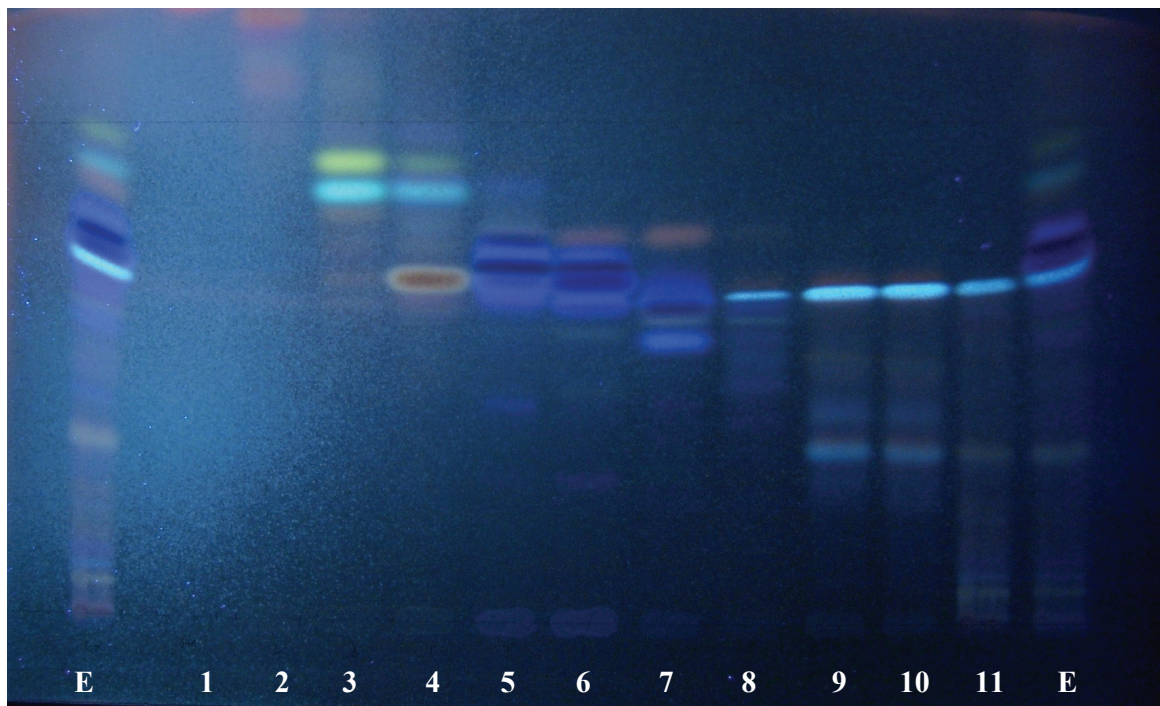
**Figure 28.** TLC screening of the VLC fractions 1-11 gained from *J. effusus* EtOAc extract using silica gel KG60 $F_{254}$ (20x12) as stationary phase and toluene:EtOAc:MeOH:HAc (35:10:1:2.5) as mobile phase. Application volume was 20 µL from solutions of 1mg/mL of each fraction as well as the extract. Extracts were applied for comparison on the first and last band. Zones were detected under $UV_{366}$ after spraying with ASR and heating for 5-10 min at 105 °C.

Fractions JE/VLC/9 and -/10 were unified according to their similar TLC patterns and the unified fraction was used for the activity screening, in which fractions JE/VLC/5 and -/6 displayed highest activity when tested in concentrations of 100 µg/mL (see Fig. S1, chapter 3.6, p.105). As can be seen in Fig. 28, these two fractions contain very prominent dark blue-violet fluorescent zones under $UV_{366}$, which appear dark violet in visible light. These zones could not be detected in the other VLC fractions (JE/VLC/1 to -/4 and JE/VLC/7 to -/10).

Fraction JE/VLC/6 (0.8 g) was then subjected to CC on Sephadex LH-20 using EtOAc as mobile phase. For this, the fraction was dissolved in EtOAc and mixed with 1.6 g of Sephadex LH-20, then evaporated to dryness under reduced pressure. This dry, homogenized residue was applied on the column (80 x 3 cm i.d.) filled with Sephadex, which was previously let to stand for several hours in EtOAc. Fractions of 5 mL each were gained with a flow rate set at 10mL/h to yield a total of 320 fractions. Column flow had to be adjusted repeatedly during the day to give constant fraction volumes. After TLC screening, the fractions were unified according to their TLC pattern to gain 10 fractions in total (Fig. 29, p.

87), whereby fraction JE/VLC/6/291-320 was considered the purge phase.
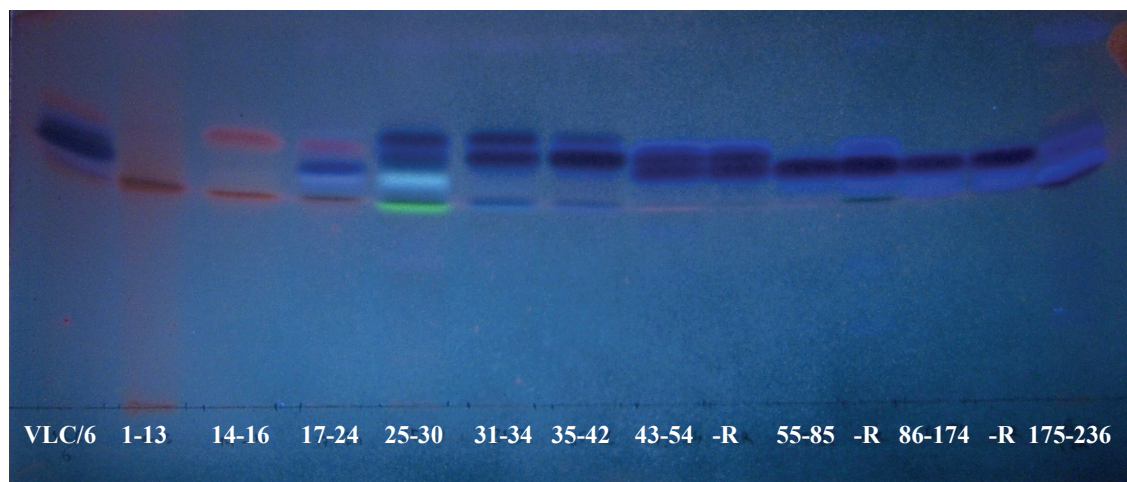


**Figure 29.** TLC screening of the cumulative fractions gained from fractionation of JE/VLC/6 on Sephadex LH-20 using silica gel plates KG60 $F_{254}$ (20x10). Application volume for the fractions was 20 µL, which were directly taken from the fractionation vials. The left- and the rightmost bands show JE/VLC/6 (1 mg/mL) for comparison, whereby 10 µL were applied. -R indicates crystalline residues scraped from the column during this specific fractionation times. All bands indicated with -R are crystalline residues of the before mentioned fraction, which were scraped from the column outlet to avoid "contamination of the subsequent fractions. Zones were detected under $UV_{366}$ after spraying with ASR and heating for 5-10 min at 105 °C.

| fraction | yield (mg) |
|---|---|
| JE/VLC/6/1-13 | 51.7 |
| JE/VLC/6/14-16* | 128.7 |
| JE/VLC/6/17-24 | 41.8 |
| JE/VLC/6/25-30* | 7.2 |
| JE/VLC/6/31-34 | 10.4 |
| JE/VLC/6/35-42* | 22.9 |
| JE/VLC/6/43-54* | 45.9 |
| JE/VLC/6/55-85* | 211.2 |
| JE/VLC/6/86-174 | 48.4 |
| JE/VLC/6/175-236 | 9.0 |
| JE/VLC/6/ 291-320 | 20.4 |
| **total** | **687.4** |

**Table 4.** Fractionation yields of JE/VLC/6 on Sephadex LH-20. Fractions used for further activity screening are indicated with (*).

For the activity screening only the most distinguishable fractions were used, which are marked in table 4 (p. 88) with an asterisk (*).

When screened for their $I_{GABA}$ potentiation, fraction JE/VLC/6/35-42 displayed highest activity (see Fig. S2, chapter 3.6, p. 105). This fraction contained one prominent blue fluorescent zone, and two additional, less prominent zones which can be found in other, less active fractions as well and thus were not considered the active principle (Fig. 29, p. 87). From this active fraction (JE/VLC/6/35-42) only 15 mg were left after the activity screening. Hence this compound was enriched from fraction JE/VLC/5, where this blue fluorescent zone was also one of the main spots visible on the TLC plate (compare Fig. 28, p. 86). Thus 0.6 g of JE/VLC/5 were prepared like JE/VLC/6 and applied on the silica gel column (80 x 3 cm i.d.). Instead of EtOAc, EtOAc:MeOH (95:5) was used as solvent and mobile phase. The column was set to a flow rate of 10 mL/h and fractions of 5 mL were collected to give 290 fractions in total.
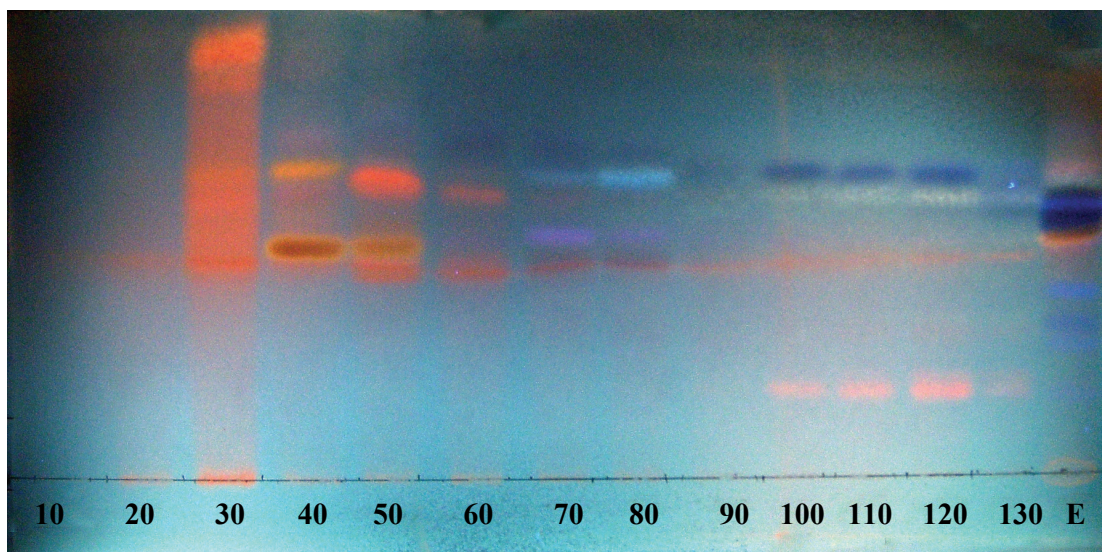


**Figure 30.** TLC screening of the fractionation of JE/VLC/5 using silica gel plates (KG60 $F_{254}$, 20x10). Every $10^{th}$ fraction from fraction 10 to fraction 130. Application volume for the fractions was 20 µL, which were directly taken from the fractionation vial. These fractions were comparable to the VLC fractions JS/421-424, and thus not further investigated. The rightmost band shows a 1mg/mL solution of JE/VLC/5 for comparison. E = extract. Zones were detected under $UV_{366}$ after spraying with ASR and heating for 5-10 min at 105 °C.

As can be seen in figure 30, the fractionation of JE/VLC/5 yielded the same compounds that are contained in the inactive VLC fractions (compare Fig. 28, p. 86): the red fluorescent zones of fraction JE/VLC/1 and 2 are detectable in fractions 10 to 30. The yellow-orange fluorescent zones of JE/VLC/3 and 4 can be seen in fractions 40 to 60 and the yellow-brownish

fluorescent zones of JE/VLC/4 are visible in fractions 70 - 130.  Starting with fraction 140 (Fig. 31), the presumably active blue-violet fluorescent zones are detectable, and in fraction 150 the compound found to be highly active in fraction JE/VLC/6/35-42 seemed to elute as pure substance (14 mg). The fractions were unified according to their TLC pattern (Tab. 5).
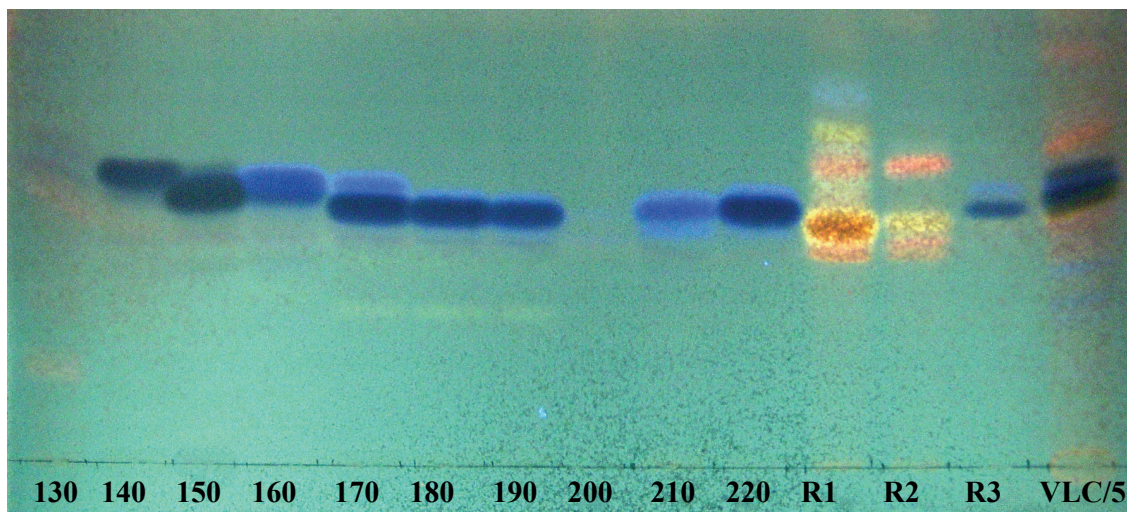


**Figure 31.** TLC screening of the fractionation of JE/VLC/5 using silica gel plates (KG60 F$_{254}$, 20x10). Every 10$^{th}$ fraction (130 - 220) was applied on the plate (20 µL, taken directly from the fractionation vial). The last four bands show residues scrapped from the column outlet (R1-R3) during the fractionation and a 1mg/mL solution of JE/VLC/5 for comparison (10 µL). Zones were detected under UV$_{366}$ after spraying with ASR and heating for 5-10 min at 105 °C.

| fraction | yield (mg) |
|---|---|
| JE/VLC/5/36-47 | 168.5 |
| JE/VLC/5/61-90 | 4.4 |
| JE/VLC/5/91-129 | 10.1 |
| JE/VLC/5/130-140 | 43.6 |
| JE/VLC/5/141-145 | 32.9 |
| JE/VLC/5/146-149 | 21.7 |
| JE/VLC/5/150 | 13.9 |
| JE/VLC/5/151-156 | 50.5 |
| JE/VLC/5/157-160 | 26.3 |
| JE/VLC/5/161-170 | 55.1 |
| JE/VLC/5/171-230 | 87.4 |
| JE/VLC/5/231-290 | 1.1 |
| **total** | **585.6** |

**Table 5.** Yields from the fractionation of JE/VLC/5 on Sephadex LH-20.

Fraction JE/VLC/5/150 and the other blue fluorescent zones were compared to JE/VLC/6/35-42 as well as the initial fractions JE/VLC/5 and -/6 by TLC analysis (Fig. 32) and it was observed that both fractions - JE/VLC/5/150 and JE/VLC/6/35-42 - had the same retention value. Thus these two fractions were unified to yield 29 mg of a pale brown powder. The structure of the compound comprised in this unified fraction was identified by 1- and 2-dimensional $^{1}$H and $^{13}$C-NMR as effusol (purity $\geq$ 96 %, annex VI).
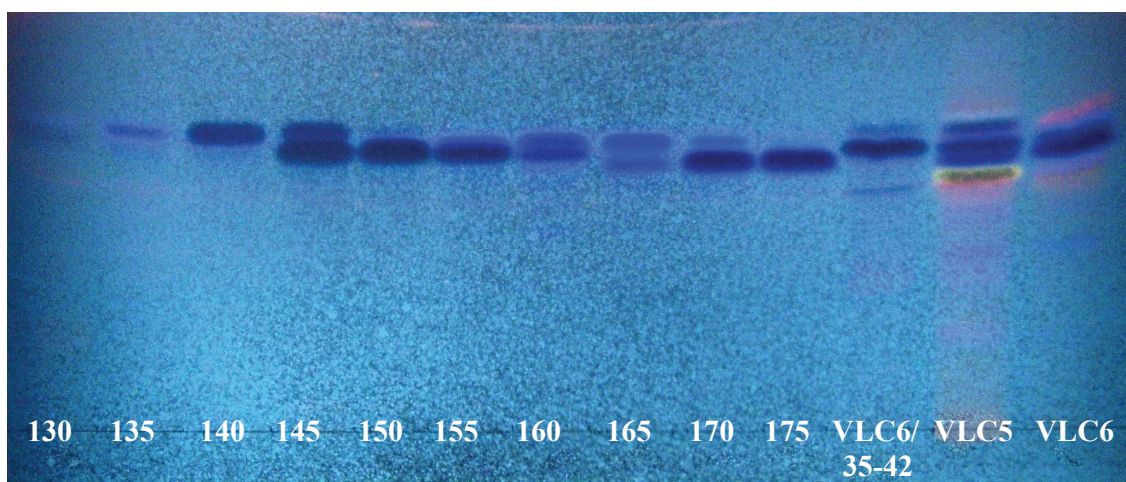


**Figure 32.** TLC screening of the prominent blue fluorescent zones from the fractionation of JE/VLC/5 in comparison with JE/VLC/5, -/6 and JE/VLC/6/35-42. Application volume was 20 µL for the fractions and each solution was directly taken from the fractionation vials. JE/VLC/5, -/6 and JE/VLC/6/35-42, were dissolved in EtOAc (1 mg/mL) and 10 µL were applied on the silica gel plate (KG60 F$_{254}$, 20x10). Zones were detected under UV$_{366}$ after spraying with ASR and heating for 5-10 min at 105 °C.

Following the identification of effusol, another highly prevalent blue fluorescent zone in the TLC screening of both – JE/VLC/6 and JE/VLC/5 by TLC – was isolated (see Fig. 29, p. 87: JE/VLC/6/55-85; and Fig. 31, p. 89: JE/VLC/5-fractions 170, 180 and 190). This component was purified from fraction JE/VLC/6/55-85 by semi-preparative HPLC (instrument 3, column 3). For this, 30 mg of the fraction were dissolved in MeOH (1.5 mL) and 1.2 mL of this solution were injected on the RP-18 column using a gradient consisting of water (solvent A) and MeOH (solvent B) with a starting concentration of 40 % B (see chapter 2.4.5.3, p. 28). In total 36 fractions (13 mL each) were collected. The fractions 17 to 36 were analyzed by analytical HPLC with conditions as described in chapter 2.4.5.3, p. 28), whereby the solutions were directly taken from the fractionation vials (Fig. 33, p. 91).
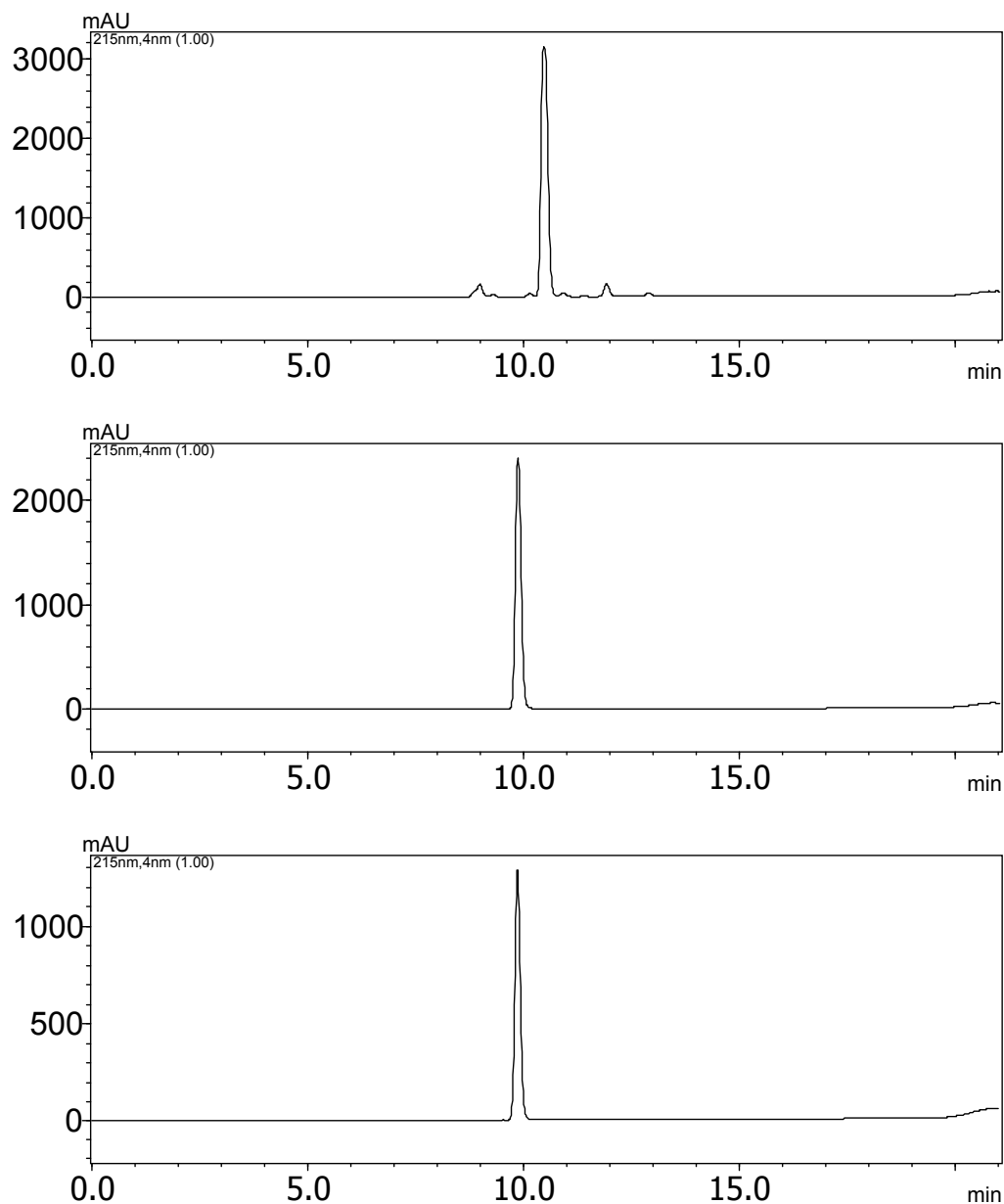
**Figure 33.** Comparison of JE/VLC/6/55-85 (1 mg/mL) with fractions JE/VLC/6/55-85/21 and JE/VLC/6/55-85/22 gained from semi-preparative HPLC using instrument 2 and column 2, employing a gradient elution as described in chapter 2.4.5.3, p. 28. Injection volume was 10 μL and solutions from fraction 21 and 22 were directly taken from the fractionation vial. Detection wavelength: 215 nm.

The main component of fraction JE/VLC/6/55-85 was enriched in fractions JE/HPLC/21 and --/22 (Fig. 33). The two fractions were unified to yield 9 mg of a brown residue. The structure was identified by NMR as dehydroeffusol (purity ≥ 98 %, annex VII), which differs from effusol by an additional double bound in position 9 and 10.

Both compounds were tested in further detail regarding concentration-dependent potentiation of $I_{GABA}$ and probable interaction with the BZ binding site as described in the next chapter (chapter 3.6).

**3.6 GABA$_A$ receptor modulators from the Chinese herbal drug Junci Medulla, the pith of *Juncus effusus* L. (manuscript)**

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This manuscript is prepared for submission**.** All of the phytochemical work except NMR measurements and structure elucidation were conducted by the author of this thesis. Furthermore, all of the pharmacological work except the initial extract screening was conducted by the author. Due to an invention disclosure submitted to the University of Vienna, the manuscript was yet not submitted to a Journal for publication.

The gamma-amino butyric acid (GABA) type A (GABA$_A$) receptor represents a crucial target for clinical agents in the treatment of anxiety and insomnia. Using the two-microelectrode voltage clamp technique on recombinant $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors, effusol (**1**) and dehydroeffusol (**2**) were isolated in a bioactivity-guided approach from the pith of *Juncus effusus* L. Both compounds concentration-dependently enhanced GABA-induced chloride currents (I$_{GABA}$) with maximal potentiation of 188±20% (EC$_{50}$ = 31 ± 8 µM, **1**) and 239±18% (EC$_{50}$ = 27 ± 6 µM, **2**), independent of the benzodiazepine (BZ) binding site. This activity on the GABA$_A$ receptor may explain the traditional use of *J. effusus* as a sedative and anxiolytic agent in Chinese Medicine (CM).

Anxiety and insomnia are central nervous system disorders with high prevalence, especially in developed countries. It is estimated that over 16 % of the world's population suffer from some form of anxiety disorder,[1] and more than 30 % frequently experience sleep disturbance.[2]

A variety of drugs such as the commonly prescribed benzodiazepines (BZs) are available for the treatment of both disorders.[3] Although their use is safe compared to the now mostly obsolete barbiturates, they can generate a range of adverse effects as lethargy, day-time sedation, amnesia or involuntary muscle relaxation. Furthermore, long-term use of BZs, as it would be necessary for the treatment of chronic insomnia or anxiety, leads to tolerance against the anxiolytic and sedative effects. Moreover, dependence and withdrawal symptoms can occur, which further limits their use.[4] This high risk of adverse effects combined with the limited prescription time of BZs and other conventional drugs[5] might be one of the reasons why an increasing percentage of insomnia and particularly anxiety patients rely on herbal preparations.[6] Although the use of herbal drugs is not free of unwanted side effects, they are still considered safe, well-tolerated and effective due to the experience gained from long-term usage. Nonetheless, scientific evaluation of their activity and underlying molecular mechanisms are rare. [7,8]

A Chinese Herbal Medicine (CHM) frequently prescribed against childhood insomnia and night terrors, is Junci Medulla – the pith of *Juncus effusus* L..[9] Several compound classes were detected in *J. effusus*: phenanthrene derivatives, cyclo-artane type triterpenes, flavonoids, phenolic acid derivatives, as well as essential oil components, β-sitosterol and α-

tocopherol could already be isolated from *J. effusus* and related species.[10-16] Studies regarding their pharmacological activity mainly focus on the phenanthrene derivatives, which displayed anti-algal,[17] antimicrobial, phototoxic[18] and cytotoxic activities.[19] Similar compounds derived from other plant families were studied for their anticancer, spasmolytic and anticoagulative activities.[20] Surprisingly, the anti-insomnia and anti-anxiety activities of *J. effusus* have only very recently been investigated *in vivo,*[21] whereas the underlying mechanisms are still unknown.

A key target for sedation and anxiolysis is the γ-amino butyric (GABA) type A (GABA$_A$) receptor, which is the major inhibitory neurotransmitter receptor in the mammalian brain. The GABA$_A$ receptor is a ligand-gated ion channel that transmits its inhibitory signals due to opening of a chloride channel and subsequent hyperpolarization of the neuronal membrane. The heteropentameric GABA$_A$ receptor can be assembled from a range of 19 different subunits ($\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, $\delta$, $\epsilon$, $\pi$, $\theta$, and $\rho_{1-3}$).[22] From more than 150.000 possible receptor assemblies, only some seem to occur *in vivo* in the mammalian brain, with the $\alpha_1\beta_2\gamma_2$ receptor being the most abundant.[23] Interestingly, the GABA$_A$ receptor seems to be a valid target for natural products, since many plant derived compounds are able to influence the GABA$_A$ receptor. [29]

In the present study we investigated the effects of *J. effusus* on recombinant $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors expressed in *Xenopus laevis* oocytes in a bioassay-guided manner. Potentiation of GABA-induced chloride currents ($I_{GABA}$) on the GABA$_A$ receptor was determined using the two-electrode voltage clamp technique by means of an automated fast perfusion system.

In a preliminary screening of 4 *J. effuses* extracts of different polarities, the EtOAc extract displayed the highest ability to potentiate $I_{GABA}$ ($170 \pm 24$ %, n = 3, Fig. 1) and therefore was selected for further bioactivity guided isolation.
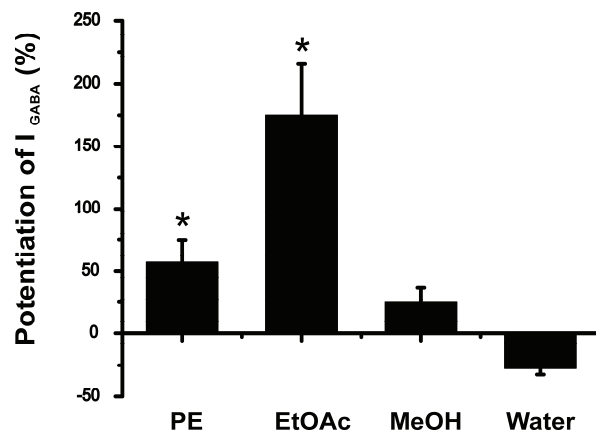
**Figure 1.** Potentiation of $I_{GABA}$ by 4 extracts (100 µg/mL) of different polarity (petroleum ether, EtOAc, MeOH and water) derived from the pith of *Juncus effusus*. Bars represent the mean ± S.E.M from at least 4 oocytes, from ≥ two different batches. Statistical significance ($p < 0.05$, Student's *t*-test) is indicated by (*).

The EtOAc extract was subjected to vacuum liquid chromatography on silica gel, using different mixtures of petroleum ether and EtOAc as mobile phases. The resulting fractions were screened for their activity on the $GABA_A$ receptor, revealing that only VLC fraction 4 and 5 could significantly enhance $I_{GABA}$ to almost similar extent (153 ± 48 %, n = 3; and 127 ± 23 %, n = 3; supporting information, Fig. S1). VLC fraction 4 and 5 were sub-fractionated by column chromatography on Sephadex LH-20 using EtOAc or EtOAc:MeOH (95:5) as mobile phase. Five sub-fractions could be gained from VLC fraction 5 for further activity screening, with sub-fraction 5-3 displaying the highest activity (266 ± 58 %, n = 4, supporting information, Figure S2). TLC screening revealed one major blue fluorescent zone. The same compound was isolated from VLC fraction 4 on Sephadex LH-20 using EtOAc:MeOH (95:5) as mobile phase. The compound was identified by MS, 1D and 2D-NMR experiments as effusol (**1**, Fig. 2) and spectral data was in good accordance with the data published in literature. [30] Additionally, another predominant constituent was isolated from cumulative fraction 5-5, and its structure was determined as dehydroeffusol (**2**, Fig. 2) by MS and NMR experiments and compared to existing spectral data.[31]

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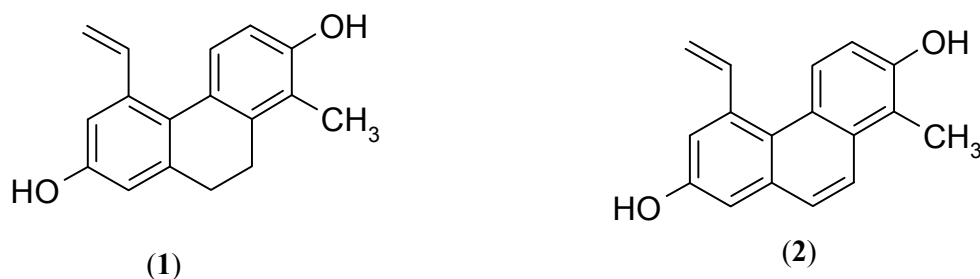
**Figure 2.** Structure of the phenolic phenanthrenes derivatives effusol (**1**) and dehydroeffusol (**2**).

To gain further insight into the molecular mechanism of action, both compounds were investigated for their ability to potentiate $I_{GABA}$. **1** and **2** displayed a concentration-dependent effect with a maximal potentiation of $188 \pm 20$ % ($EC_{50} = 31 \pm 8$ µM, $n_H = 2.5 \pm 0.6$, n = 7) for **1** and $239 \pm 18$ % ($EC_{50} = 27 \pm 6$ µM, $n_H = 1.4 \pm 0.2$, n = 4) for **2** (Fig. 3).
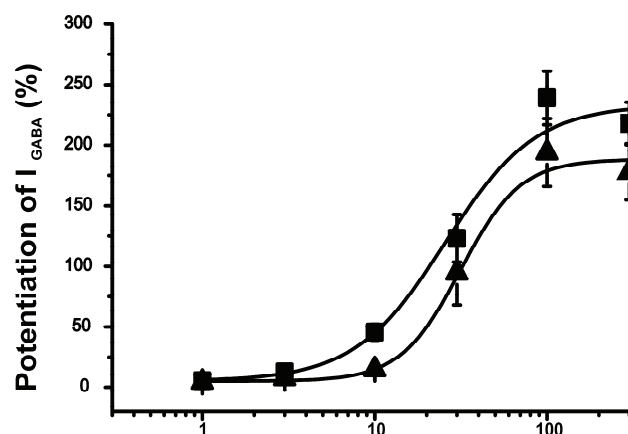


**Figure 3.** Concentration-response curve for **1** (▲) and **2** (■), with a maximum potentiation of $188 \pm 20$ % ($EC_{50} = 31 \pm 8$ µM, $n_H = 2.5 \pm 0.6$, n = 7) for **1** and $239 \pm 18$ % ($EC_{50} = 27 \pm 6$ µM, $n_H = 1.4 \pm 0.2$, n = 4) for **2**, respectively. Each data point represents the mean $\pm$ S.E.M from at least 4 oocytes, from $\geq$ two different batches.

Furthermore, the interaction of **1** and **2** with the benzodiazepine (BZ) binding site was determined, since BZs are the most commonly prescribed drugs used in treatment of sleep and anxiety disorders. $I_{GABA}$ enhancement induced by **2** (50 µM) could not be blocked by co-application of the BZ antagonist flumazenil (1 µM, Fig. 4A, B). Moreover, the co-application of **2** (50 µM) with diazepam (0.3 µM) leads to an additive increase in $I_{GABA}$ enhancement (Fig. 4C, D).
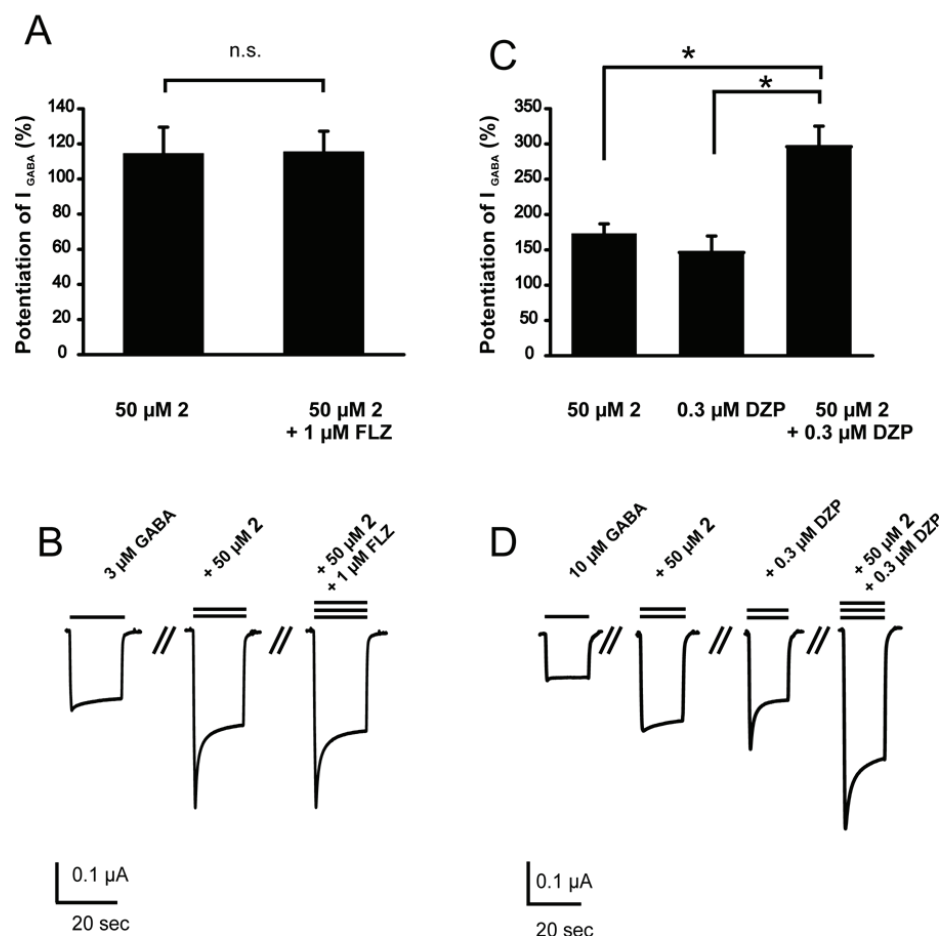
**Figure 4.** Effects of **2** on $I_{GABA}$ in the presence of flumazenil (FLZ) and diazepam (DZP) in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors. Bars represent the mean ± S.E.M from at least 4 oocytes, from ≥ two different batches. Statistical significance ($p < 0.05$, one-way ANOVA) is indicated with (*), n.s = not significant ($p > 0.05$). (A) Potentiation of $I_{GABA}$ (EC$_{5-10}$) by **2** (50 µM) in the absence (left bar) and presence (right bar) of flumazenil (1 µM) is not significantly different ($p > 0.05$), indicating no involvement of the BZ binding site in the positive modulatory activity of **2**. (B) Typical GABA-induced chloride currents in the absence (GABA 3 µM, control, single bar) and presence of the indicated concentrations of **2** (double bar), or **2** and flumazenil (triple bar), respectively. (C)  Additive effects of **2** and diazepam on $I_{GABA}$. The left bar shows the potentiation of $I_{GABA}$ by 50 µM **2**, the middle bar - by 300 nM diazepam, and the right bar illustrates the stimulation of $I_{GABA}$ by co-application of **2** and diazepam. (D) Representative chloride currents induced by GABA (10 µM, control, single bar), by **2** (double bar), by diazepam (double bar) and **2** co-applied with diazepam (triple bar) at the indicated concentrations.

Similar results were obtained for **1** (Fig. S3), indicating that both components do not mediate their $I_{GABA}$ potentiating effects via the BZ binding site. To summarize, we found scientific

evidence for the traditional use of *J. effusus* on the molecular level and, moreover, the ability of **1** and **2** to modulate $I_{GABA}$ may explain its sedative and anxiolytic effects *in vivo*.

**Experimental Section**

**General experimental procedures.** NMR spectra were recorded in $CD_3Cl$ or d6-DMSO on a Bruker Advance 500 spectrometer at 500 MHz ($^1$H NMR) and 125 MHz ($^{13}$C NMR). **ESIMS$^n$** spectra were obtained on a 3D-ion trap mass spectrometer (HCT, Bruker Daltonics) and recorded in positive and negative ion mode. Semi-preparative HPLC of compound **2** was performed using a Shimadzu system consisting of two LC-8A pumps, a SPD-M20A diode array detector, a FRC-10A fraction collector and a CBM-20A interface, engaging a Nucleosil C-100 RP-18 column (250 x 21 mm i.d., 5 μm; Machery Nagel, Germany). **TLC** was carried out on precoated silica gel plates KG60 $F_{254}$ (Merck, Darmstadt, Germany) with toluene:EtOAc:MeOH:acetic acid in a ratio of 35:10:1:2.5 as mobile phase. Zones were detected in visible light and under $UV_{366}$ after spraying with anisaldehyde sulphuric acid reagent and heating for 5 to 10 min at 105 °C.

**Biological material.** Junci medulla, the pith of *J. effusus* L., was purchased from Plantasia (Oberndorf, Austria, lot.: 660558). A voucher specimen (No. JS-07-11-JE) is deposited in the Department of Pharmacognosy, University of Vienna.

**Extraction and isolation.** For a preliminary activity screening, 50 g of ground plant material were extracted successively with solvents of increasing polarity (petroleum ether, EtOAc, methanol and water), with the EtOAc extract showing highest activity. Thus, 2.0 kg of ground drug were extracted exhaustively with EtOAc to yield 13.4 g of a dark brown residue. 10 g of this residue were subjected to vacuum liquid chromatography on silica gel (65 x 3.5 cm i.d.) using petroleum ether:EtOAc mixtures (1 L each) in ratios of 9:1 (pre-run), 8.5:1.5, 8:2,7.5:2.5, 6.5:3.5, 6:4, 5:5, 3:7, 1.5:8.5, 0:10, from which the latter 9 were used for activity testing. Fraction 5 (petroleum ether:EtOAc 6.5:3.5) was further fractionated on a Sephadex LH-20 column (65 x 1.5 cm i.d.) using EtOAc as eluent (5mL fractions, 10 mL/h flow) resulting in six sub-fractions. Subfraction 5-3 displayed one prominent blue fluorescent zone in the TLC screening, which was identified as **1** by MS and NMR experiments and data comparison with literature. The crystalline residue of cumulative fraction 5-5, which was gained from re-crystallization in EtOAc, was purified by semi-preparative HPLC on RP-18. Using a water (solvent A) - methanol (solvent B) gradient from 60 to 80 % B in 15 minutes with a flow rate of 26.7 mL/min, compound **2** (9 mg, purity ≥ 98 %) eluted at 9.9 min. VLC

fraction 4 (petroleum ether:EtOAc 6:4), which also contained **1** and **2**, was fractionated on Sephadex LH-20 (70 x 1 cm i.d.) using EtOAc:MeOH (95:5) as eluent to yield 220 fractions (5mL fractions, ~ 10 mL/h flow). **1** was eluted as pure compound in subfraction 4-150 (14 mg, purity ≥ 96 %).

**Expression of GABA$_A$ Receptors.** Preparation of stage V-VI oocytes from *Xenopus laevis* (NASCO, Fort Atkinson, WI, USA) and injection of cRNA was done as previously described.[34] Briefly, 15 min prior to surgery, female frogs were anaesthetized using a 0.2 % solution of MS-222 (Ethyl 3-aminobenzoate methanesulfonic acid, Sigma, Vienna, Austria). Parts of the ovaries were removed and remaining follicle membranes were enzymatically digested with 2 mg/mL collagenase Type 1A (Sigma, Vienna, Austria). Synthesis of capped off run-off poly (A+) cRNA transcipts was performed from linearized cDNA templates (pCM vector). cRNAs were diluted with DEPC-treated water and stored at -80 °C. Injection of 10 – 50 nL of the different cRNA solutions was carried out on the day of isolation. To ensure the expression of the γ-subunit, cRNAs of α1, β2 and γ2S were injected in a ratio of 1:1:10. Successful expression of the γ-subunit was determined by measuring I$_{GABA}$ after application of diazepam (300 nM). Injected oocytes were stored at 18 °C in penicillin and streptomycin supplemented ND96 solution. ND96 solution contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$ and 5 mM HEPES (pH 7.4). All chemicals for ND96 solution were purchased from Sigma, Vienna, Austria).

**Two-microelectrode voltage clamp technique and drug application.** 1 to 3 days after cRNA injection, GABA$_A$ receptor expressing oocytes were screened for GABA-evoked currents as previously described.[35] Electrophysiological experiments were performed by the two-electrode voltage clamp technique making use of the TEC 03X amplifier (npi electronic, Tamm, Germany) at a holding potential of -70 mV. Current measurements were digitized at 200 Hz and recorded with pCLAMP 10 data acquisition software (Molecular Devices, Sunnyvale, CA, USA). ND 96 buffer was used as bath solution. Glass needles (Harvard Apparatus, Kent, UK) were filled with 2 M KCl (1 – 3 MΩ). Oocytes with maximal current amplitudes ≥ 3 µA were discarded to exclude voltage clamp errors. All experiments were performed at room temperature (23 to 25 °C). Stimulation of chloride currents by extracts, fractions and pure compounds was determined at a GABA concentration eliciting between 5 and 10 % of the maximal current amplitude (EC$_{5-10}$). The EC$_{5-10}$ was established at the beginning of each experiment, corresponding to a concentration between 3 and 10 µM GABA. Stock solutions of extracts and fractions (10 mg/mL) as well as compounds (100

mM) were prepared in DMSO and stored at -20 °C. Reservoirs for control and drug applications contained equivalent amounts of DMSO which did not exceed 1 %. At this concentration, the measurements were not influenced. All stock solutions were diluted with ND96 containing the appropriate amount of GABA to elicit currents of $EC_{5-10}$ immediately before the experiments, whereby the extracts were always diluted to a concentration of 100 µg/mL. Test solutions (100 $\mu$L) were applied to the oocytes at a speed of 300 $\mu$L/s by means of a ScreeningTool (npi electronic GmbH, Tamm, Germany).[3] When compound concentrations higher than 30 µM were used, wash out periods were extended to up to 10 min to avoid effects of receptor desensitization on current amplitudes.

**Data Analysis.** Potentiation of the GABA induced chloride current ($I_{GABA}$) in percent was defined according to the formula $[I_{(GABA+Comp)}/I_{GABA}- 1]*100$, 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. Origin Software (OriginLab Corporation, Northhampton, MA, USA) was used to generate concentration-response curves. Data were fitted by nonlinear regression analysis to the equation $1/(1+(EC_{50}/[compound]n_H)$, where $EC_{50}$ is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50% of the compound-induced maximum response, and $n_H$ is the Hill coefficient. Responses were graphed as mean ± standard error (S.E.M.) from at least three oocytes out of ≥ two different batches. Statistical significance (*) was calculated using Student's *t*-test and one-way ANOVA with a confidence interval of $p < 0.05$.

**Supporting Information.** Activity screening of the VLC fractions (Fig. S1) and the subfractions gained by fractionation of VLC fraction 5 (Fig. S2). The benzodiazepine binding site experiment of (**1**) (Fig. S3). [1]C and ^{13}C- 1- and 2-D NMR data of **1** and **2**. This information is available free of charge via the internet at http://pubs.acs.org.

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## TOC
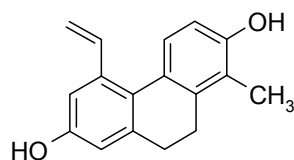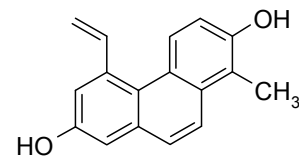
# Supporting information

# GABA$_A$ receptor modulators from the TCM herbal drug Junci Medulla, the pith of *Juncus effusus* L.

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**Figure S1.** Enhancement of $I_{GABA}$ in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors by 9 VLC fractions (100 µg/mL) derived from the EtOAc extract of *Juncus effusus* L. Bars represent the mean ± S.E.M from 3 oocytes, from ≥ two different batches. Statistical significance (p < 0.05, Student's *t*-test) is indicated with (*).
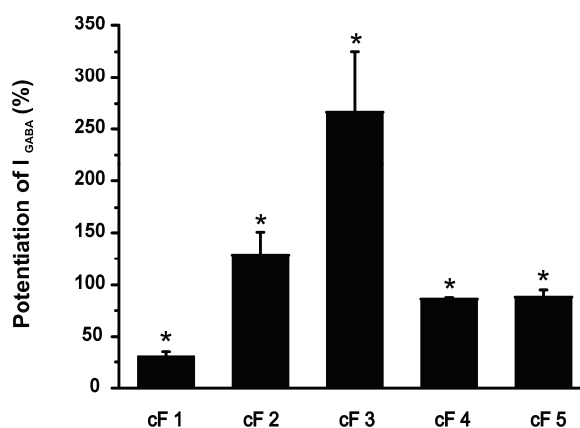


**Figure S2.** Potentiation of $I_{GABA}$ in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors by the cumulative fractions (100 µg/mL) derived from VLC fraction 5. Bars represent the mean ± S.E.M from at least 3 oocytes, from ≥ two different batches. Statistical significance (p < 0.05, Student's *t*-test) is indicated with (*).
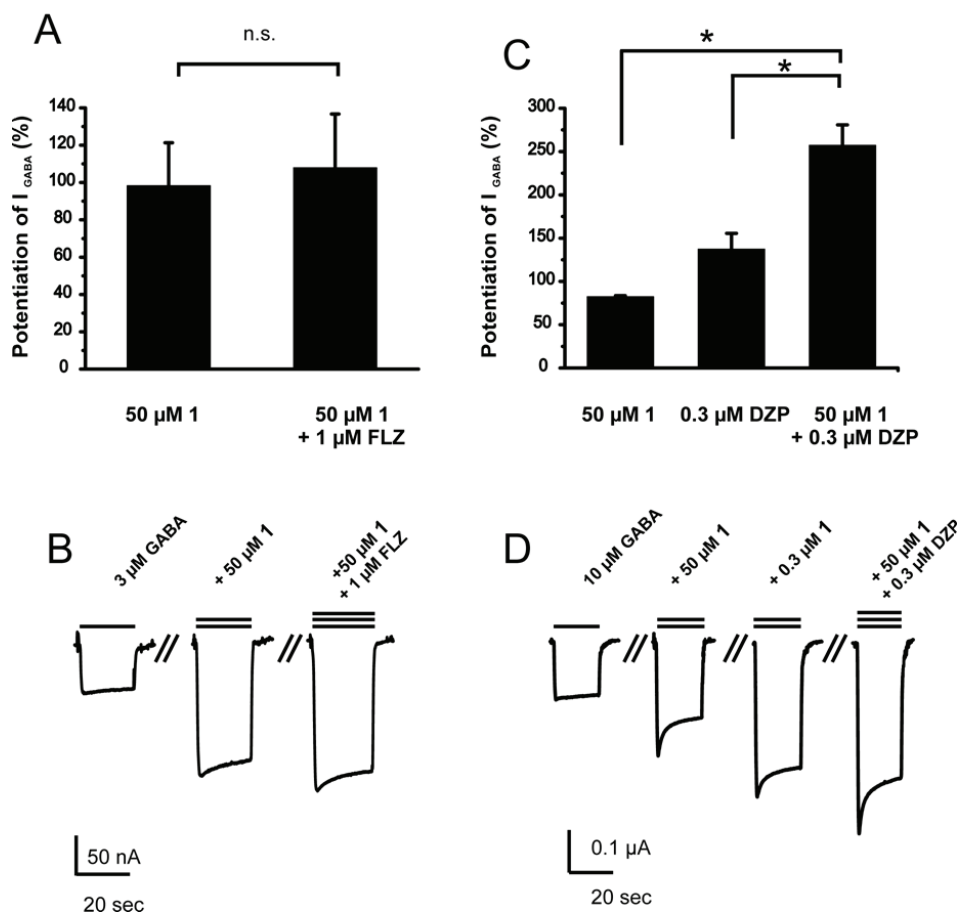
**Figure S3.** Effects of **1** on $I_{GABA}$ in the presence of flumazenil (FLZ) and diazepam (DZP) in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ receptors. Bars represent the mean $\pm$ S.E.M from at least 4 oocytes, from $\geq$ two different batches. Statistical significance (p < 0.05, one-way ANOVA) is indicated with (*), n.s = not significant (p > 0.05). **(A)** Potentiation of $I_{GABA}$ (EC$_{5-10}$) by **1** (50 $\mu$M) in the absence (left bar) and presence (right bar) of flumazenil (1 $\mu$M) is not significantly different (p > 0.05), indicating no involvement of the BZ binding site in the positive modulatory activity of **1**. **(B)** Typical GABA-induced chloride currents in the absence (GABA 3 $\mu$M, control, single bar) and presence of the indicated concentrations of **1** (double bar), or **1** and flumazenil (triple bar), respectively. **(C)** Additive effects of **2** and diazepam on $I_{GABA}$. The left bar shows the potentiation of $I_{GABA}$ by 50 $\mu$M **2**, the middle bar - by 300 nM diazepam, and the right bar illustrates the stimulation of $I_{GABA}$ by co-application of **1** and diazepam. **(D)** Representative chloride currents induced by GABA (10 $\mu$M, control, single bar), by **1** (double bar), by diazepam (double bar) and **1** co-applied with diazepam (triple bar) at the indicated concentrations.

**4 Discussion**

In this work, 14 different CHMs traditionally applied in the treatment of GABA$_A$ receptor-related disease like insomnia or anxiety were investigated for their ability to positively modulate I$_{GABA}$ resulting in the discovery of several new positive GABA$_A$ receptor modulators from natural origin.

After a vast literature research, 14 herbal drugs were selected which due, to their field of application, were expected to exert activity on the GABA$_A$ receptor. Next to herbal drugs mentioned for the usage against insomnia and anxiety, *Arnebia euchroma* was solely selected for its heat clearing effect while *Arisaema* sp. was chosen for its use in the treatment of "epilepsy". None of the selected herbal drugs have been studied for their effects on the GABA$_A$ receptor so far. Nonetheless, some data on the *in vivo* activity of certain CHMs have already been published for *Albizia julibrissin*, *Nelumbo nucifera*, *Polygonum multiflorum* and *Tribulus terrestris* (see chapter 3.2, p 43), and - very recently - *Juncus effusus* (see chapter 3.6, p. 94).

As determined in this thesis, 5 out of 14 herbal drugs displayed promising activity (at least 50 % potentiation of I$_{GABA}$), equaling a hit rate of more than 36 %, compared to an average hit rate in random screenings of 10 % or below (Harvey, 2002). Of course, when using drugs with an ethnomedicinal background time-consuming literature research is necessary to gain basic knowledge on the differences in the descriptions of disease patterns in the alternative medicinal system compared to conventional medicine and to avoid a replication of already published data. CM, for example, insomnia or anxiety can be described as a Yin-Yang imbalance, as Heart Blood deficiency, disruptions in the Heart Yin (Kaptchuk, 2000) or, more generally, as excess and deficiencies of the zang organs (Kidney, Liver, Heart, Lung or Spleen) since they are strongly connected to different emotions (Xinrong, 2003), whereby the terms for these organs used in CM does not necessarily correlate with the ones used in western medicine. Luckily, due to the ever-increasing popularity of CM, attempts are made to translate such symptom patterns into disorders and symptoms observed in conventional medicine. Moreover, we already have access to Chinese Materia Medicas in Western languages. As can be seen in the Chinese Materia Medica compiled by Bensky *et al.* (2006) or by Zhang *et al.* (2007), there is a whole chapter regarding tranquillizing herbs. From this drug-group *Polygonum multiflorum* (stem) and *Albizia julibrissin* (bark and flowers) were

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chosen for the bioactivity screening on GABA$_A$ receptors, while the other drugs contained in this chapter were already tested, currently under investigation or of mineralic origin. None of these three herbs displayed promising activity in the initial screening while herbal drugs not mainly used as "tranquillizers" were more effective on the GABA$_A$ receptor. Thus it is apparently worth the effort to study the literature more vigorously, than just selecting the most obvious herbal drugs when dealing with as complex a medicinal system as CM.

In the beginning, 4 extracts of varying polarity (PE, EtOAc, MeOH, water) were produced from each of the 14 CHMs, giving a total of 56 extracts. Interestingly, all of the active extracts were rather lipophilic while none of the water derived extracts showed promising activity. This is promising, since drug like molecules should be rather lipophilic for better absorption and distribution in the human body. *In vivo* activity of CNS-drugs would require the components to penetrate the blood-brain barrier (BBB). From the active sesquiterpene lactone atractylenolide III it is already known that it can cross the BBB, since it can be found in the brain after oral administration to mice. Due to their high lipophilicity this can also be assumed for the prenylated coumarins or the phenanthrenes, but no specific studies on these compounds have been conducted so far. Nonetheless, for several coumarins like imperatorin, osthole as well as for the phenanthrene dehydroeffsuol a penetration of the BBB is likely, since they displayed anti-convulsive or anxiolytic activity *in vivo* (Luszczki *et al.*, 2007a; Luszczki *et al.*, 2009a; Liao *et al.*, 2011) Moreover, compared to hydrophobic, in hydrophilic extracts the occurrence of GABA is likely and could give false positive results. These extracts would have to undergo an additional separation step to remove GABA from the extract e.g. by using ion-exchange chromatography (Trauner *et al.*, 2008).

After the selection and preliminary activity screening, the most active extracts were further fractionated according to their bioactivity. This was accomplished by either using semi-preparative HPLC, as is the case for *A. macrocephala* and *C. monnieri* PE extracts, or "classical" fractionation by column chromatography as it is was conducted with the EtOAc extract of *J. effusus*. The advantage of a HPLC-aided fractionation clearly lies in the fastness in which first results can be gained. However, since the amount of extract which can be separated on a (semi-)preparative HPLC column is limited, repetitive fractionations are often necessary to yield amounts high enough for activity testing and further isolation. This was true for the PE extracts of *A. macrocephala* and *C. monnieri*. Furthermore, both HPLC methods used MeCN as organic solvent. Although one can attempt to exchange MeCN with

MeOH, latter solvent often does not reach as good a separation and thus the fractionation has to be conducted with MeCN. Regrettably, this solvent is rather expensive and, in addition, carcinogenic as well as environmentally toxic. Moreover, for fractionation by HPLC a suitable method is required and if no method is available the development of one can be as time-consuming as normal column chromatography. Although in a first approach, semi-preparative HPLC was used for the fractionation of the *Juncus* EtOAc extract as well, the yields gained were not high enough for activity testing. Furthermore, the main components seemed to appear as one major peak, since in the subsequent TLC analysis several zones were visible. Although the gradient was changed repeatedly, they could not be separated. Hence a classical column chromatography seemed to be the better choice. For this the EtOAc extract was first roughly fractionated by VLC. This method can be conducted rather fast and when the solvent or solvent mixtures are chosen cautiously, can yield a good separation as well. Moreover, higher quantities of extract can be applied to the column compared to HPLC and thus large enough amounts of fractions for subsequent isolation of the active components will be received. This makes repetitive fractionation unnecessary compared to semi-preparative HPLC. In the end, both methods led to the discovery of new positive $GABA_A$ receptor modulators and are thus equally suitable for bioactivity-guided isolation.

By comparing the activity of the isolated compounds with the $I_{GABA}$ enhancement induced by the extracts, the latter always showed equal or even higher activity although one would expect the fractions to exert significantly stronger $I_{GABA}$ enhancement. For example, the PE extract of *A. macrocephala* potentiated $I_{GABA}$ by ~ 320 %, while the isolated compounds only enhance $I_{GABA}$ by a maximum of around 150 %. The same is valid for *C. monnieri*. Even more so, since in the initial screening, the extract showed very promising activity (~ 250 % potentiation of $I_{GABA}$), while the compounds osthole and imperatorin at concentrations of 100 µM displayed activities of ~ 120 % and ~ 30 %, respectively. Considering the activity of the *Juncus* EtOAc extract, the results are not as disturbingly different. However, the purification of the most active compounds does not lead to a strong increase of $I_{GABA}$ modulation when comparing the amount of extract and the concentration of pure compounds used in the screening, i.e. 170 vs. 250 %. When re-calculating, 100 µg of the EtOAc extract would correspond to approximately 380 µM of pure effusol (250 % maximum potentiation) or even higher concentrations of dehydroeffusol (180 %). Moreover, both compounds were only tested in concentrations up to 300 µM and seemed to reach their maximum effect between 100 and 300 µM. Several explanations are possible for such a "loss" in activity. In an extract

many (related) constituents can be found and although in a bioactivity-guided isolation the constituents mainly responsible for the observed effect are isolated, other compounds that add to this effect are often neglected. This could be shown for *A. macrocephala*, where the HPLC fraction containing atractylenolide I did not show promising activity when the fractions were screened. Atractylenolide I was nonetheless isolated due to its close structural relationship to atractylenolide II and III and displayed around 100 % enhancement of $I_{GABA}$ when applied in the same concentrations. Thus, although it is not as active as atractylenolide II and III, it probably adds to the overall activity of the extract. Moreover, when summing up the activity found in each of the HPLC fractions, a total of only 280 % is reached, if the negative (allosteric) effect of some fractions is included. This loss of activity not only points to additive effects probably caused by related components like atractylenolide I, but also to synergistic effects as well and it seems likely that compounds with low activity on the $GABA_A$ receptor potentiate the effects exerted by the two sesquiterpenes atractylenolide II and III. These additive or synergistic effects are frequently observed for herbal preparations in attempts to find the *one* compound responsible for an observed activity of a distinct extract (Williamson, 2001; Spinella, 2002; Houghton *et al.*, 2007). A very good example for such effects is *Valeriana officinalis*, since it is one of the more thoroughly investigate herbal drugs. Valerian contains several known positive $I_{GABA}$ modulators from different natural compound classes i.e. the sesquiterpene valerenic acid (Trauner *et al.*, 2008), the monoterpenoid borneol (Buchbauer *et al.*, 1992; Granger *et al.*, 2005) and the flavonoids linarin (Fernández *et al.*, 2004) and 6-methylapigenin (Wasowski *et al.*, 2002). Additionally this herbal drug influences other neurotransmitter systems as well (Dietz *et al.*, 2005; Sichardt *et al.*, 2007). This may explain, why extracts and fractions are often more active compared to the isolated components, as it was true for all of the tested herbal drugs and once again shows, that the value of whole-extracts is as high or actually higher than that of single substances. Generally, all of the isolated compounds are highly abundant in the respective extract. It is likely, that the sheer amount of these substances led to their identification as the active ingredients in the preparations. Moreover, it is probable that many structurally related components would exert the same or even higher activity when isolated and applied in the same concentrations. Since they are contained only in traces so that their occurrence bears no meaning in a bio-activity guided isolation, they can not be considered the active principles. Nonetheless, such minor constituents surely play a crucial role in the overall $I_{GABA}$ modulation through the investigated herbal drugs.

Next to the discovery of the bioactivity of certain components, their use as bio-markers for quality control was confirmed or new markers provided. For *A. macrocephala*, the sesquiterpene lactones and for *C. monnieri* the coumarins imperatorin and osthole are quality-determining markers. To the best of my knowledge, no such analytical (HPLC) method is available for  *J. effusus*. Thus, with the discovery of effusol and dehydroeffusol as $GABA_A$ receptor modulators, interesting marker substances for any future attempts to develop such a method were provided. In addition to our results, the anxiolytic activity of dehydroeffusol was confirmed by Liao *et al.* (2011) *in vivo* in the EPM.

Next to minor compounds contributing to the overall activity of an extract, as discussed above, there could be several other reasons for the isolated substances to display lower effects as expected. From a study of Campbell *et al.* (2004), it is known that certain compounds can exert positive/synergistic effects when co-applied with other $GABA_A$ receptor modulators although they actually are antagonists, negative allosteric modulators or essentially inactive. This so-called "second-order modulation" was observed for epi-gallocatechin gallate (EGCG) and apigenin in low concentrations when co-applied with diazepam. In higher concentrations the antagonistic effect of apigenin and EGCG overrode their positive effect on DZP-induced potentiation. Considering the high potentiation reached by the *C. monnieri*-extract compared to the isolated compounds, the loss of 5/6 of the activity is very surprising. Thus several other components contained in the extract could influence imperatorin or osthole like EGCG or apigenin influence the activity of diazepam.

Other possible reasons for such a loss in activity could be based on the methods used, either phytochemical or pharmacological. In general, the GABA concentration used for control and thus is also added to the test solution, can have great impact on $I_{GABA}$ modulation, since compounds often give higher modulations the lower the GABA concentration used. This was convincingly shown e.g. by Granger *et al.* (2005). In the present work, the initial extract screening was conducted with an $EC_{1-10}$, while in any further studies a more confined GABA EC ($EC_{5-10}$) was used. This would result in a significant decrease of $I_{GABA}$ modulation for the isolated substances, if the extracts were tested at lower GABA EC ($EC_{1-5}$).
In addition, fractionation on semi-preparative HPLC was conducted with water/MeCN gradients. By evaporating the fractions, loss of volatile components would be inevitable due to the evaporation of the water-portion in the fractions. This may also explain a loss of activity since many essential oil constituents like linalool (Hossain *et al.*, 2007), thymol

(Priestley *et al.*, 2003), or menthol (Hall *et al.*, 2004a) are known to affect the GABA$_A$ receptor. However, with the HPLC methods used the volatile components are expected to elute with the purge phase and this phase was not included in the activity testing for neither of the two herbal drugs, *Atractylodes* and *Cnidium*. Furthermore, many volatile constituents have already been extensively studied for their I$_{GABA}$ modulation effects (see chapter 1.3, pp. 16 and 17) and thus such compounds were not studied in further detail.

When searching for new drug scaffolds the isolation of major *and* minor compounds is essential when investigating structure activity relationships (SAR). There are several publications in this regard, since many groups search for I$_{GABA}$ modulation in a certain compound class with structurally related constituents. In the present thesis, such preliminary SAR studies were accomplished with a selection of coumarin derivatives commonly found in herbal drugs. During this screening compounds with mono-prenyl residues emerged as the only (positively) active substances. Therefore, these residues seem to be the structural features responsible for the activity on the GABA$_A$ receptor. Interestingly, oxypeucedanin showed a rather high enhancement of I$_{GABA}$ whereas the related heraclenin is a weak modulator of GABA-induced chloride currents. These two components only differ in the position of the epoxylated prenyl side chain and the configuration of the chiral centre. Since we found that the position of the side chain apparently does not interfere with the observed I$_{GABA}$ potentiation, this led us to the assumption that the differences in activity are due to the absolute configuration of the molecules. That such slight changes in structure can have a deep impact on the activity of closely related components was convincingly shown through the GABA$_A$ receptor activity of (+) menthol compared to five of its (inactive) stereoisomers (Corvalán *et al.*, 2009). In another study, flavan-3-ols were found to exert higher activity on the GABA$_A$ receptor, if the 3-position is trans-configurated (Fernández *et al.*, 2008), next to other activity-determining substituents (Mewett *et al.*, 2009). This is a special trait of natural compounds since they - more than synthetic drugs – often posses several chiral centers and it again gives proof of the usefulness of components from natural origin.

When the compounds were studied in further detail by creating a concentration response curve, they displayed rather moderate activity on the GABA$_A$ receptor, ranging from 100 to 550 % I$_{GABA}$ potentiation. Compared to other positive GABA$_A$ receptor modulators, like the anesthetic etomidate, the barbiturates or some natural compounds e.g. acetyl-shengmanole from *Actaea racemosa* L. (Cicek *et al.*, 2010), the activity of the compounds investigated

during this thesis is rather low. However, this does not imply that they are ineffective *in vivo*. If we consider the GABA$_A$ receptor modulators in clinical use, we know that for example the highly potent and efficient barbiturates enhance I$_{GABA}$ to a higher extent as the BZs. This makes the prescription of BZs not less effective but safer than barbiturates, since they can not be that easily overdosed. Furthermore, modulators displaying high activity *in vitro* and *in vivo* are more frequently used as anesthetic agents than for the treatment of insomnia or anxiety. Hence, the use of moderately active modulators, as is the case for many other natural compounds as well (Li *et al.*, 2010b; Zaugg *et al.*, 2010), may be more appropriate to treat anxiety or sleep disorders thereby probably minimizing the risk of unwanted side effects. Although their moderate activity might be more similar to BZs than to e.g. etomidate, none of the compounds studied in more detail (osthole, imperatorin, atractylenolide II, effusol and dehydroeffusol) did exert their activity via the BZ binding site. This was convincingly shown by co-application of the respective compound with the BZ antagonist flumazenil. Moreover, the addition of the BZ agonist diazepam - in concentrations thought to elicit maximum effects - to the test solution always led to a roughly additive increase in I$_{GABA}$. This gives further evidence for a BZ binding site-independent modulation. Interestingly, many publications on natural compounds and the GABA$_A$ receptor use a BZ binding assay were the displacement of radioactively labeled BZs is measured as a determinant for the activity of natural compounds on the GABA$_A$ receptor, as extensively reviewed in chapter 1.3, pp. 16 - 22. For example, in a study of Bergendorff et al. (1997), phellopterin was found to have high affinity to the BZ binding site and thus considered an interesting GABA$_A$ receptor modulator. Controversially, in our functional screening, the same compound showed only weak effects on I$_{GABA}$ (~ 60 % potentiation at 100 µM) and the related coumarins osthole and oxypeucedanin, which displayed higher I$_{GABA}$ enhancement, revealed no dependence on the BZ binding site. Nonetheless, it would be possible that phellopterin is dependent on this binding site while oxypeucedanin is not, but due to their structural similarity this is rather unlikely. Thus, it seems that many of the herbal drugs screened for their "activity on the GABA$_A$ receptor", revealing high affinity for the BZ binding site, are falsely considered highly potent/efficient GABAergic compounds. On the other hand, many such preparations could have produced false negative results and exert high activity on the GABA$_A$ receptor when using an electrophysiological approach, but on a different binding site. Such extracts would have been missed in an affinity screening. Thus, the two-microelectrode voltage clamp technique is probably more suitable for GABA$_A$ receptor activity screens. Furthermore, identification of

binding sites can be accomplished using electrophysiological methods, provided an antagonist for a certain binding site is available. In the case of the BZ-binding assay one can easily revert to flumazenil, as was done in the present work. Nonetheless, binding assays can give additional information on the binding site of a compound and some compounds with high affinity on the BZ binding site also displayed activity on recombinant $GABA_A$ receptors e.g. xenovulene A, a terpenoid structure isolated from *Acremonium strictum* (Thomas *et al.*, 1997). The independence of the investigated compounds on the BZ binding site also suggests, that the effect is not dependent on the γ but on α and β subunits, and thus all $GABA_A$ receptors in the CNS could be affected by these substances, since all of them contain α and β-subunits. This is interesting, since αβδ-subunit containing receptor subtypes, which are insensitive to the classical BZs, are responsible for tonic inhibition, providing the basic activity of GABAergic neurotransmission and seem to be of great importance in sleep disorders (Orser, 2006). Of course, the sensitivity of these receptors for the isolated compounds would have to be confirmed in future studies. Since the activity of our compounds seems to be dependent on α and/or β subunits, binding to the aforementioned extrasynaptic receptors is likely, unless the compounds are selective for $α_1$ subunits, which cannot be found in extrasynaptic receptor subtypes. Finally, a $GABA_A$ receptor modulation without involvement of the BZ binding site suggests that patients under BZ treatment could profit from the intake of herbal remedies due to dose reduction of BZs in a combination therapy and this probably reduces the risk of adverse effects like dependence or tolerance. On the other hand this also indicates a possibility for side effects due to an over-dosage of $GABA_A$ receptor modulating compounds and an unwanted increase in drug activity, if the dosage is not reduced (Carrasco *et al.*, 2009). However, these hypotheses would warrant further studies. Possible future work could also include more-detailed studies on the molecular mechanisms of the compounds on the $GABA_A$ receptor, i.e. determination of the binding site in binding assays or through point mutations and investigations for subunit specifity, to determine the probable activity *in vivo* e.g. anxiolytic, sedative or anticonvulsive. Furthermore, the compounds could be tested in for their ability to cross the BBB and for their metabolism in *in vitro* model. Subsequently, "positive" findings could be confirmed in animal models e.g. EPM or rotarod test. This would also allow an assessment of the compounds' toxicity. Furthermore, it would be interesting to unearth the discrepancy between high activity of extracts and comparably lower activity of isolated compounds by searching for second-order modulation or other synergistic/additive effects. But this would require the isolation of all components in an extract and subsequent

"cross-testing", meaning co-application with the known positive modulators. In a next step, the investigation of the extracts in a battery of first *in vitro,* and then, suitable *in vivo* models would be desirable, to further confirm the synergism concept.

Now that I determined the active principles of some CHMs, I can conclude that

- an ethnopharmacological approach can not only lead to the discovery of new scaffolds for drug development, which then could effectively be used in clinic. It, first and foremost, provides scientific evidence for traditional application of ethnomedicinally used drugs. Thus it was shown clearly in this work, that an ethnopharmacological approach is advantageous over a random screen.

- extracts are often more active as the isolated "active principles", which clearly shows that extracts can have higher value than single compounds.

- although the isolated compounds displayed only moderate activity on the GABA$_A$ receptor, they can still be active *in vivo,* as was already shown for the phenanthrene derivatives from J. effusus, dehydroeffusol and effusol and the coumarin derivatives imperatorin and osthole.

- the two-microelectrode voltage clamp technique is more suitable as a BZ binding assay to screen for GABA$_A$ receptor modulators. Controversially to BZ binding assays, in which only the affinity of a certain compound to one of the numerous binding sites on the GABA$_A$ receptor is determined, the two-microelectrode voltage clamp technique offers the possibility to directly asses the activity of a compound on ion channels.

## 5 Summary

The bioactivity guided screening of 14 different TCM herbal drugs revealed that 5 out of these were active when tested for their positive modulatory activity on $GABA_A$ receptors using of the two-microelectrode voltage clamp technique. For this purpose, 4 extracts of different polarity (petroleum ether, ethyl acetate, methanol and water) were produced from each herbal drug.

The 3 most active drugs were chosen for further investigation: the rhizomes of *Atractylodes macrocephala*, the fruits of *Cnidium monnieri* and the pith of *Juncus effusus*. From the petroleum ether (PE) extract of *A. macrocephala* the sesquiterpene lactones atractylenolide II and III were isolated as the active principles by HPLC-aided fractionation giving a maximum enhancement of $166 \pm 12$ % ($n_H = 1.2 \pm 0.1$, $EC_{50} = 70 \pm 17$ µM) and $157 \pm 12$ % ($n_H = 1.4 \pm 0.4$, $EC_{50} = 99 \pm 20$ µM), respectively. The related atractylenolide I, only differing in an additional double bound in position 8 and 9, enhance $I_{GABA}$ by "only" $96 \pm 3$ % ($n_H = 2.5 \pm 0.2$, $EC_{50} = 12 \pm 1$ µM).

From the petroleum ether extract of *Cnidium monnieri* fruits, two compounds could be isolated in the same manner, revealing that the simple coumarin osthole and the furanocoumarin imperatorin were the compounds with strongest $I_{GABA}$ enhancement ($124 \pm 11$ % and $54 \pm 13$ %). To gain insights into the structural requirements of $I_{GABA}$ potentiation induced by coumarin derivatives, 16 other coumarins were investigated (100 µM). It was shown, that only compounds with (mono-)prenyl or -oxyprenyl residues could significantly enhance $I_{GABA}$. From the tested compounds, oxypeucedanin showed highest maximum potentiation of $550 \pm 17$ % ($EC_{50} = 26 \pm 8$ µM, $n_H = 1.2 \pm 0.1$) when applied in a concentration of 100 µM.

Controversially, the *Juncus* ethyl acetate extract was fractionated by vacuum liquid chromatography followed by the subfractionation of the most active VLC fractions by Sephadex LH-20 column chromatography. After further purification, two compounds could be isolated, the phenanthrene derivatives effusol ($188 \pm 20$ %, $EC_{50} = 31 \pm 8$ µM, $n_H = 2.5 \pm 0.6$) and dehydroeffusol ($239 \pm 18$ %, $EC_{50} = 27 \pm 6$ µM, $n_H = 1.4 \pm 0.2$).

In summary, we isolated the "active principles" of three different CHMs. Interestingly, the compounds isolated belong to three different compound classes - sesquiterpene lactones, phenolic diterpenes and coumarins and did not display dependence on the BZ binding site.

## 6 Zusammenfassung

Ein bioaktivitäts-geleitetes Screening von insgesamt 56 Extrakten (Petrolether, Ethylacetat, Methanol und Wasser) von 14 verschiedenen CHMs konnte zeigen, dass 5 dieser Arzneipflanzen positive modulatorische Aktivität am GABA$_A$ Rezeptor besitzen. Dazu wurde die Zwei-Elektroden-Spannungs-Klemm Technik verwendet. Die höchste Aktivität zeigten die Extrakte aus den Rhizomen von *Atractylodes macrocephala*, den Früchte von *Cnidium monnieri* und dem Stängelmark von *Juncus effusus*. Aus dem Petrolether-(PE) Extrakt von *A. macrocephala* wurden die Sesquiterpenlaktone Atractylenolid II und III als die aktiven Verbindungen isoliert. Die erstellten Konzentrations-Wirkungskurven ermittelten eine maximale Potenzierung von 166 ± 12 % ($n_H$ = 1,2 ± 0,1; EC$_{50}$ = 70 ± 17 µM) für Atractylenolid II und 157 ± 12 % ($n_H$ = 1,4 ± 0,4; EC$_{50}$ = 99 ± 20 µM) für Atractylenolid III. Die strukturell ähnliche Substanz Atractylenolid I, die sich nur durch eine zusätzliche Doppelbindung in Position 8 und 9 unterscheidet, potenzierte I$_{GABA}$ nur zu maximal 96 ± 3 % ($n_H$ = 2,5 ± 0,2; EC$_{50}$ = 12 ± 1 µM).

Aus dem Petrwhere Früchte von *Cnidium monnieri* konnten auf gleiche Weise 2 Substanzen isoliert werden. Es wurde gezeigt, dass das einfache Coumarin Osthol und das Furanocoumarin Imperatorin die aktiven Verbindungen darstellen (124 ± 11 % and 54 ± 13 %). Um bessere Erkenntnisse über die strukturellen Vorraussetzungen für eine I$_{GABA}$ Potenzierung durch Coumarinderivate zu erhalten, wurden weitere 16 Coumarin-derivate getestet (100 µM). Es konnte gezeigt werden, dass nur Komponenten mit (mono-)prenyl oder -oxyprenyl Resten I$_{GABA}$ signifikant modulieren konnten. Von allen getesteten Substanzen zeigte Oxypeucedanin die höchste Wirksamkeit mit einer maximalen Potenzierung von 550 ± 17 % (EC$_{50}$ = 26 ± 8 µM, $n_H$ = 1,2 ± 0,1).

Der *Juncus* Ethylacetatextrakt wurde dagegen zuerst mittels Vakuum-Flüssigchromatographie fraktioniert und die aktivsten Fraktionen auf Sephadex LH-20 subfraktioniert. Nach weiterer Aufreinigung wurden 2 Substanzen isoliert, die Phenanthrenderivate Effusol (188 ± 20 %, EC$_{50}$ = 31 ± 8 µM, $n_H$ = 2,5 ± 0,6) und Dehydroeffusol (239 ± 18 %, EC$_{50}$ = 27 ± 6 µM, $n_H$ = 1,4 ± 0,2).

Letztendlich konnten die aktiven Substanzen von drei verschiedenen CHMs isoliert werden. Interessanterweise gehören diese zu jeweils unterschiedlichen Substanzklassen - Sesquiterpenlaktone, phenolische Diterpene und Coumarin, wobei keine der untersuchten Verbindungen Abhängigkeit von der BZ Bindungsstelle zeigte.

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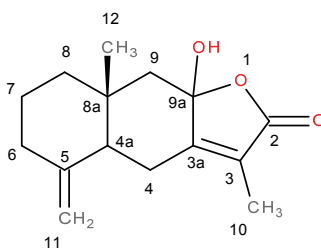
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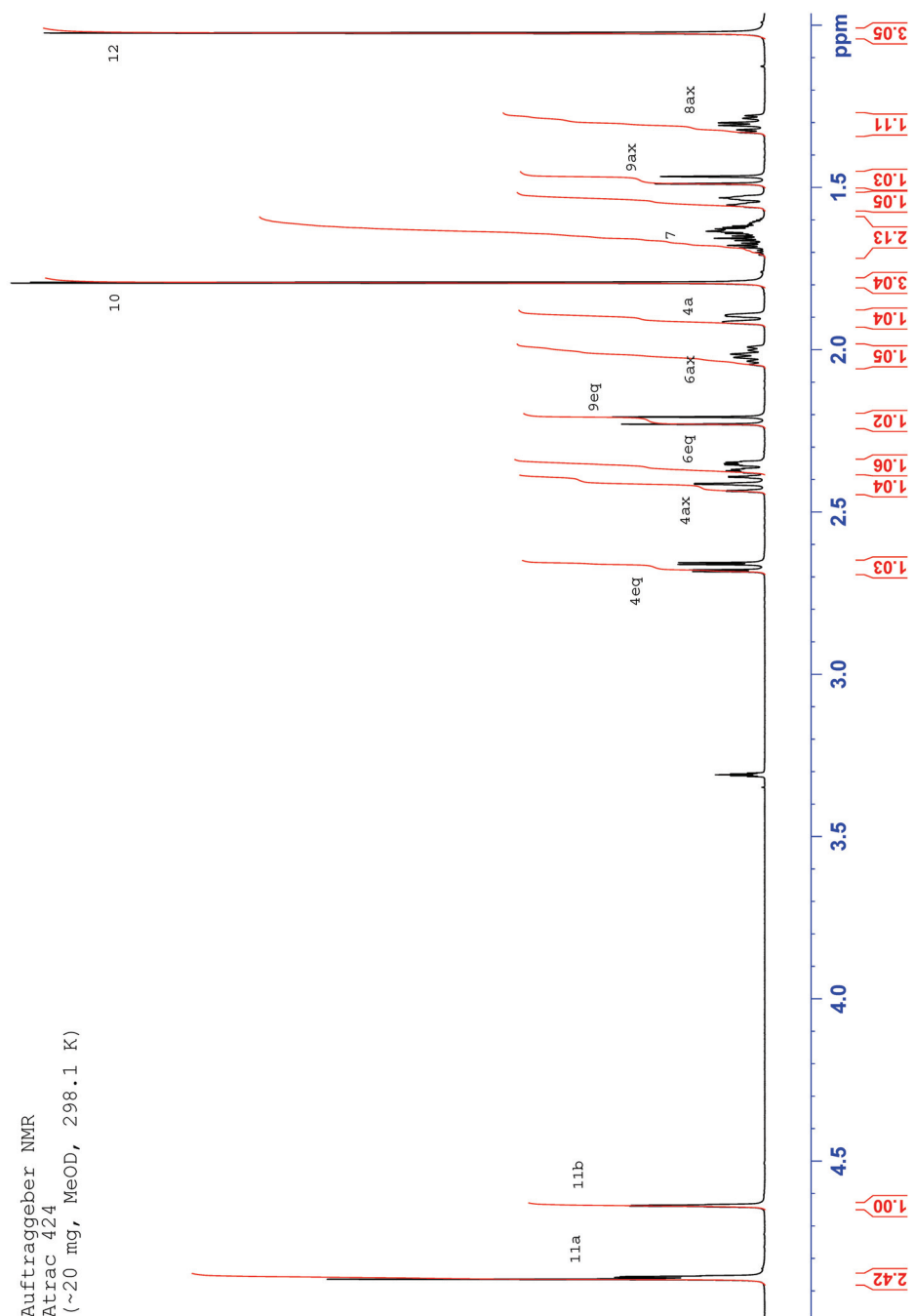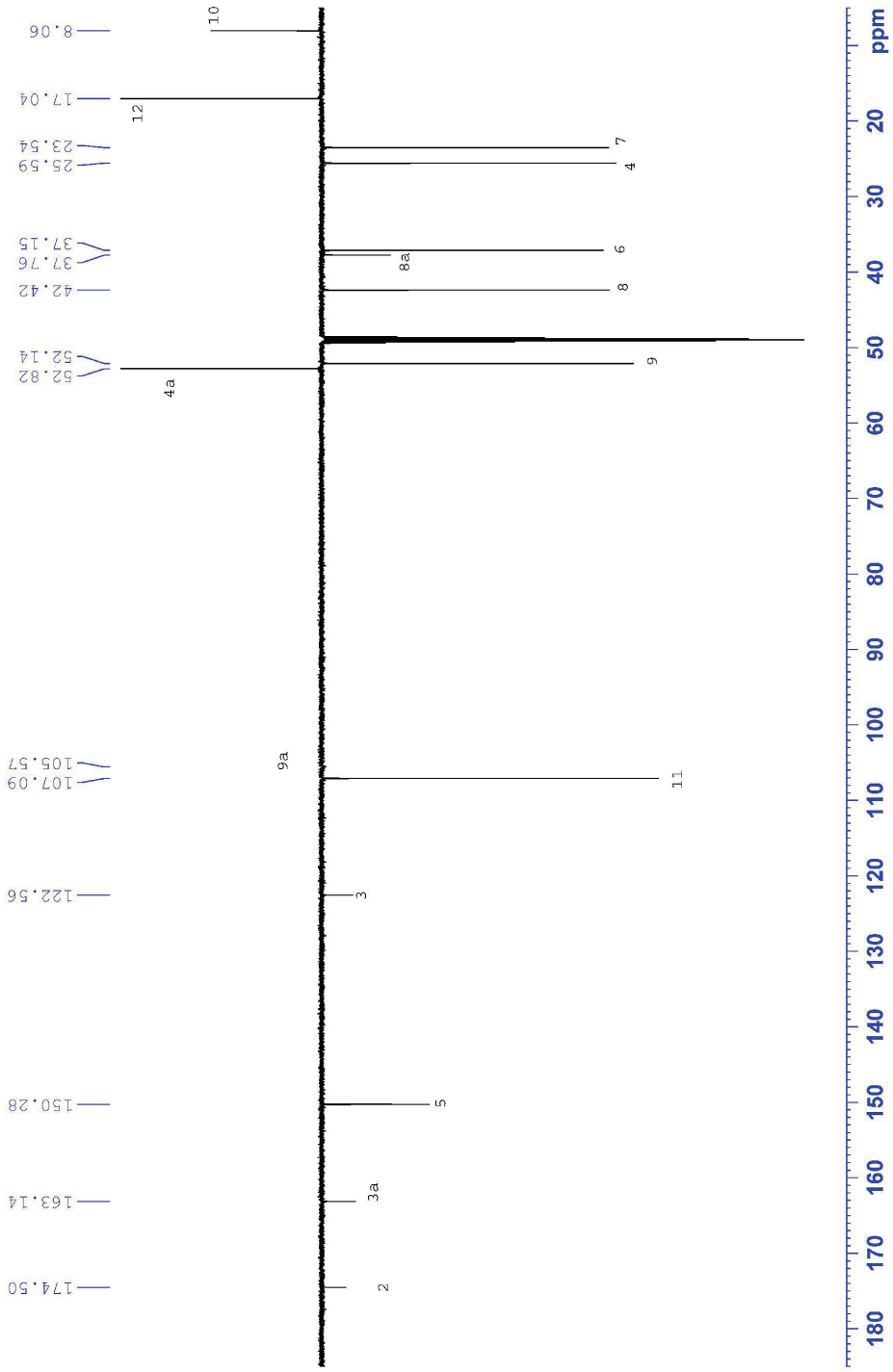
Atractylenolide III (19.7 mg in MeOD)



|  | $^{13}C$ |  | $^{1}H$ | $J_{H,H}$ (Hz) |
|---|---|---|---|---|
| 2 | 174.5 | Cq | | |
| 3 | 122.6 | Cq | | |
| 3a | 163.1 | Cq | | |
| $4_{ax}$ | 25.6 | $CH_2$ | 2.41 | 13.2 ($4_{eq}$), 12.9 ($4a$)/1.4 Hz (10) |
| $4_{eq}$ | | | 2.67 | 13.2 ($4_{ax}$), 3.2 (5) |
| 4a | 52.8 | CH | 1.91 | 12.9 ($4_{ax}$), 3.2($4_{eq}$), 1.5($11a$), 1.5 ($11b$) |
| 5 | 150.3 | Cq | | |
| $6_{ax}$ | 37.2 | $CH_2$ | 2.02 | 13.4 ($6_{eq}$), 13.2 ($7_{ax}$), 6.0 ($7_{eq}$), 1.5 ($11a$), 1.5 ($11b$) |
| $6_{eq}$ | | | 2.36 | 13.4 ($6_{ax}$), 1.9 ($8_{eq}$), 4.2 ($7_{ax}$), 2.1 Hz ($7_{eq}$) |
| | | | | |
| $7_{ax}$ | 23.5 | $CH_2$ | 1.67 | 13.4 ($7_{eq}$), 13.2 ($8_{ax}$), 4.2 ($8_{eq}$), 13.2 ($6_{ax}$), 4.2 ($6_{eq}$) |
| $7_{eq}$ | | | 1.62 | 13.4 ($7_{ax}$), 4.6 ($8_{ax}$), 2.4 ($8_{eq}$), 6.0 ($6_{ax}$), 2.1 ($6_{eq}$) |
| $8_{ax}$ | 42.4 | $CH_2$ | 1.31 | 13.2 ($8_{eq}$), 13.2 ($7_{ax}$), 4.6 ($7_{eq}$), 0.6 (12) |
| $8_{eq}$ | | | 1.54 | 13.2 ($8_{ax}$), 4.2 ($7_{ax}$), 2.4 ($7_{eq}$), 1.9 ($6_{eq}$) |
| 8a | 37.8 | Cq | | |
| $9_{ax}$ | 52.1 | CH | 1.48 | 13.4 ($9_{eq}$), 0.6 (12) |
| $9_{eq}$ | | | 2.22 | 13.4 ($9_{ax}$) |
| 9a | 105.6 | Cq | | |
| 10 | 8.1 | $CH_3$ | 1.79 | 1.4 ($4_{ax}$) |
| 11a | 17.0 | $CH_3$ | 4.86 | 1.5 ($11b$), 1.5 ($4a$), 1.5 ($6_{ax}$) |
| 11b | | | 4.64 | 1.5 ($11a$), 1.5 ($4a$), 1.5 ($6_{ax}$) |
| 12 | 107.1 | $CH_2$ | 1.02 | 0.6 ($9_{ax}$), 0.6 ($8_{ax}$) |

Auftraggeber NMR
Atrac 424
(~20 mg, MeOD, 298.1 K)
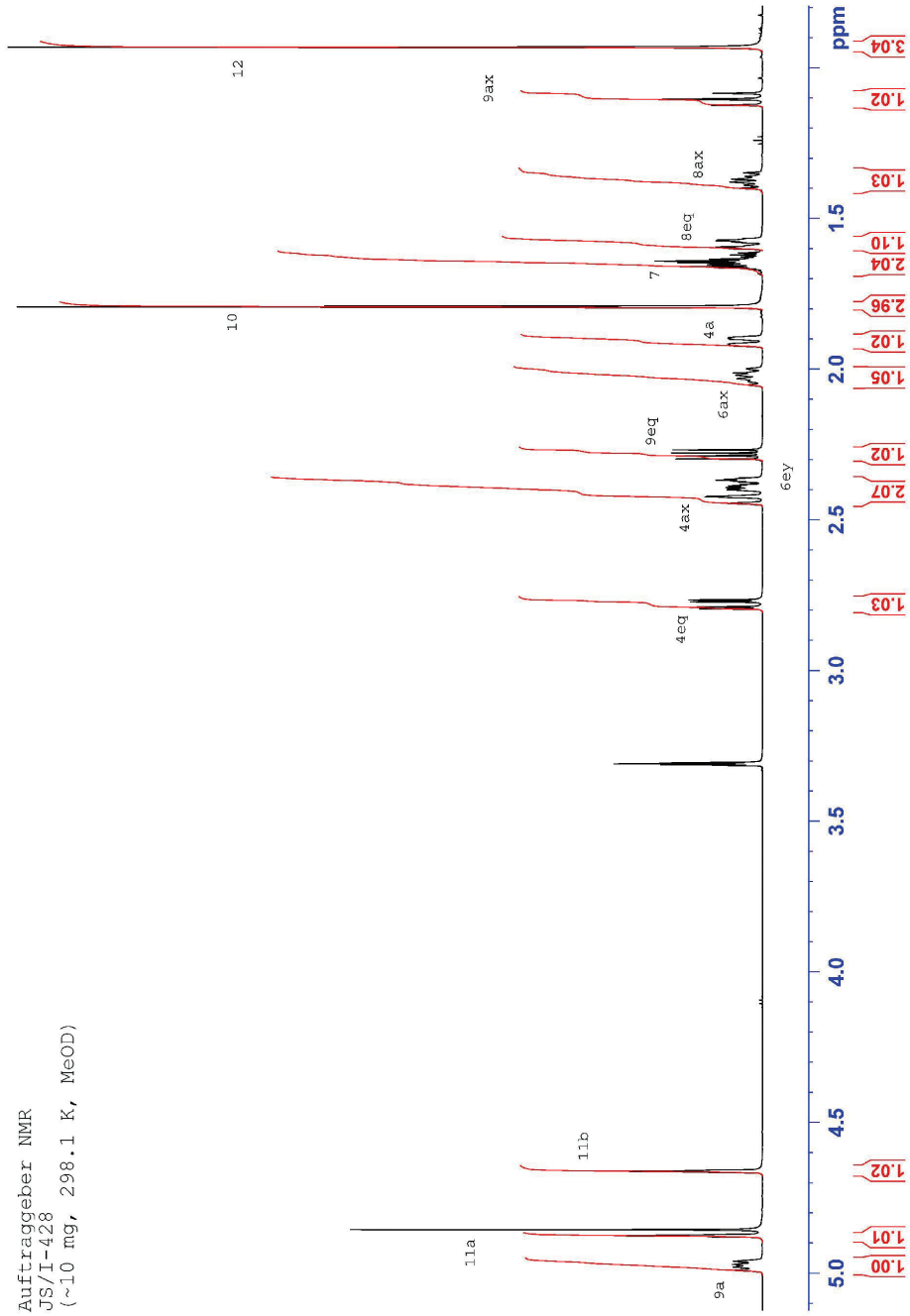
Auftraggeber NMR
Atrac 424
(~20 mg, MeOD, 298.1 K)

8.06 — 10

17.04 — 12

23.54 — 7
25.59 — 4

37.15 — 6
37.76 — 8a
42.42 — 8

52.14 — 9
52.82 — 4a

105.57 — 9a
107.09 — 11

122.56 — 3

150.28 — 5

163.14 — 3a

174.50 — 2

ppm
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20

Atractylenolide II (10.22 mg in MeOD)
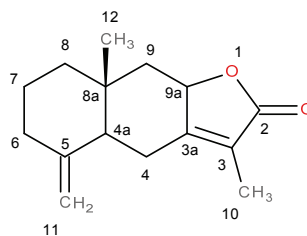


| | $^{13}C$ | | $^{1}H$ | $J_{H,H}$ (Hz) |
|---|---|---|---|---|
| 2 | 177.2 | Cq | | |
| 3 | 120.6 | Cq | | |
| 3a | 165.7 | Cq | | |
| $4_{ax}$ | 26.7 | $CH_2$ | 2.42 | 13.9 ($4_{eq}$), 12.8 (4a), 1.4 (9a), 1.6 (10) |
| $4_{eq}$ | | | 2.78 | 13.9 ($4_{ax}$), 3.7 (4a) |
| 4a | 51.1 | CH | 1.91 | 12.8 ($4_{ax}$), 3.7 ($4_{eq}$), 1.5 (11a), 1.5 (11b), 0.8 ($6_{ax}$) |
| 5 | 150.1 | Cq | | |
| $6_{ax}$ | 37.4 | $CH_2$ | 2.03 | 13.2 ($6_{eq}$), 13.3 ($7_{ax}$), 6.0 ($7_{eq}$), 1.5 (11a), 1.5 (11b), 0.8 (4a) |
| $6_{eq}$ | | | 2.38 | 13.2, 1.6, 4.8, 2.1 |
| $7_{ax}$ | 23.5 | $CH_2$ | 1.64 | 13.4 ($7_{eq}$), 13.5 ($8_{ax}$), 4.2 ($8_{eq}$), 13.3 ($6_{ax}$), 4.8 ($6_{eq}$) |
| $7_{eq}$ | | | 1.65 | 13.4 ($7_{ax}$), 4.4 ($8_{ax}$), 2.4 ($8_{eq}$), 6.0 ($6_{ax}$), 2.1 ($6_{eq}$) |
| $8_{ax}$ | 41.9 | $CH_2$ | 1.38 | 13.2 ($8_{eq}$), 13.5 ($7_{ax}$), 4.4 ($7_{eq}$), 0.6 (12) |
| $8_{eq}$ | | | 1.58 | 13.2 ($8_{ax}$), 4.2 ($7_{ax}$), 2.4 ($7_{eq}$), 1.6 ($6_{eq}$) |
| 8a | 38.0 | Cq | | |
| $9_{ax}$ | 48.7 | CH | 1.10 | 12.0 ($9_{eq}$), 11.6 (9a), 0.6 (12) |
| $9_{eq}$ | | | 2.28 | 12.0 ($9_{ax}$), 6.4 (9a) |
| 9a | 79.9 | Cq | 4.98 | 11.6 ($9_{ax}$), 6.4 ($9_{eq}$), 1.4 ($4_{ax}$), 1.6 (10) |
| 10 | 8.1 | $CH_3$ | 1.79 | 1.6 ($4_{ax}$), 1.6 (9a) |
| 11a | 107.3 | $CH_2$ | 4.88 | 1.5 (11b), 1.5 (4a), 1.5 ($6_{ax}$) |
| 11b | | | 4.66 | 1.5 (11a), 1.5 (4a), 1.5 ($6_{ax}$) |
| 12 | 16.7 | $CH_3$ | 0.93 | 0.6 ($9_{ax}$), 0.6 ($8_{ax}$) |

5a

Auftraggeber NMR
JS/I-428
(~10 mg, 298.1 K, MeOD)
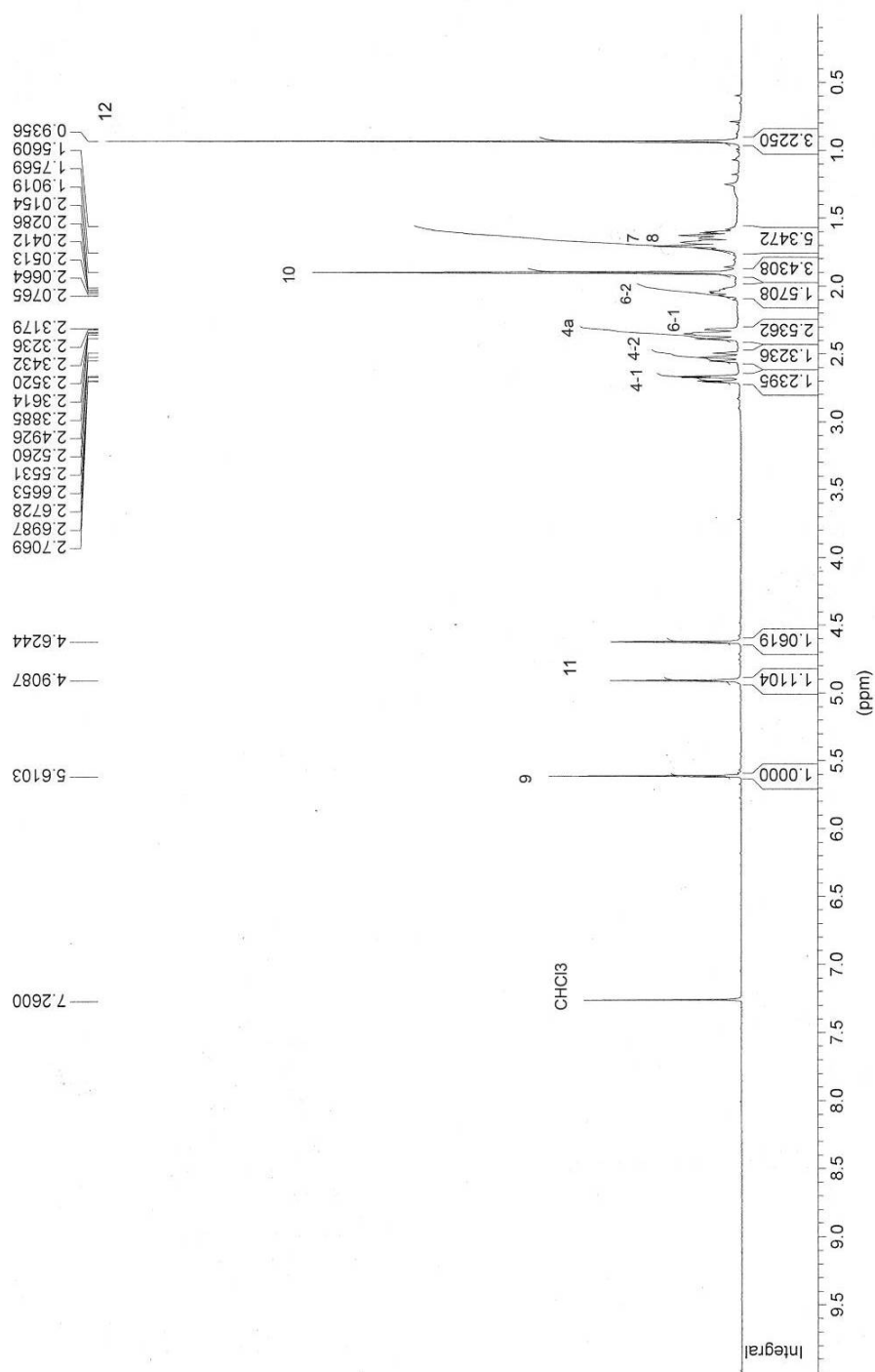
Auftraggeber NMR
JS/I-428
(~10 mg, MeOD, 298.1 K)

177.17
165.73
150.06
120.60
107.33
79.94
51.07
48.66
41.86
38.00
37.35
26.66
23.67
23.56
16.67
8.06

ppm
180  170  160  150  140  130  120  110  100  90  80  70  60  50  40  30  20
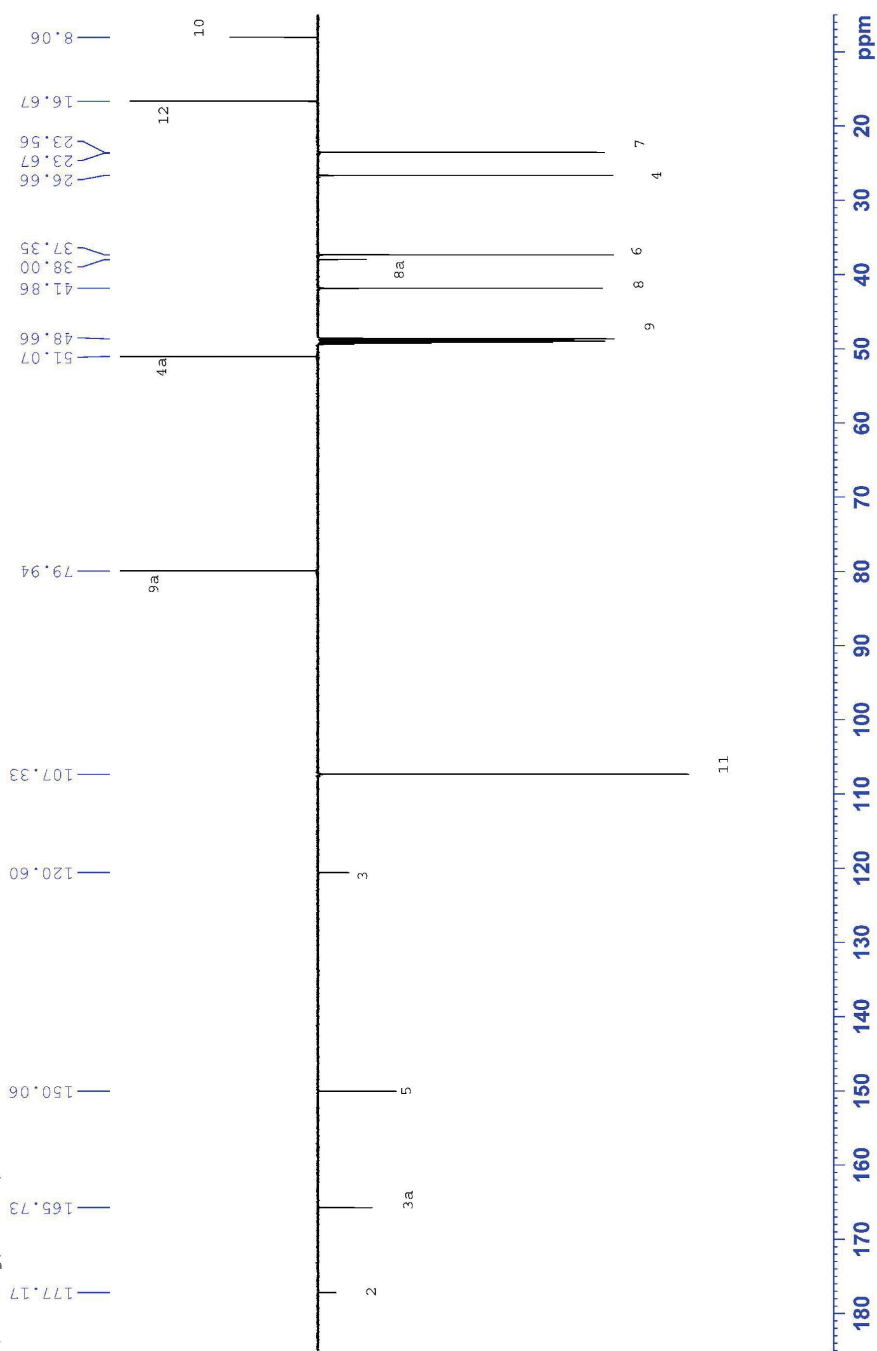
Atractylenolide I (8.96 mg in CDCl$_3$)
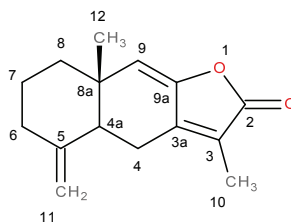


| | | | |
|---|---|---|---|
| 2 | 171.4 | Cq | |
| 3 | 120.5 | Cq | |
| 3a | 148.3 | Cq | |
| 4 | 22.7 | CH$_2$ | 2.69 (dd, J = 17.1, 3.9 Hz, 1 H) |
| | | | 2.53 (dd, J = 17.1, 13.2 Hz, 1 H) |
| 4a | 48.4 | CH | 2.37-2.32 (m, 2 H, H-4a, H-6/1) |
| 5 | 148.1 | Cq | |
| 6 | 36.2 | CH$_2$ | 2.08-2.02 (m, 1 H) |
| | | | 2.38-2.32 (m, 2 H, H-4a, H-6/1) |
| | | | |
| 7 | 23.0 | CH$_2$ | 1.76-1.56 (m, 2H, H-7, H-8) |
| 8 | 39.1 | CH$_2$ | 1.76-1.56 (m, 2H, H-7, H-8) |
| 8a | 38.1 | Cq | |
| 9 | 119.2 | CH | 5.61 (s, 1 H) |
| 9a | 148.0 | Cq | |
| 10 | 8.5 | CH$_3$ | 1.90 (s, 1 H) |
| 11 | 107.5 | CH$_2$ | 4.91 (d, J = 1.5 Hz) |
| | | | 4.62 (d, J = 1.5 Hz) |
| 12 | 18.6 | CH$_3$ | 0.94 (s, 1 H) |

Atrac1 (Atractylenolide I, 8.96 mg) in cdcl3 (Proton) 14.5.2010

Atrac1 (Atractylenolide I, 8.96 mg) in cdcl3 (APT) 14.5.2010

8.4765 — 10

18.5632 — 12
22.6567
22.9876 — 7 — 4

36.1629 — 6
38.1238 — 2a
39.0676 — 8

48.3821 — 4a

77.0000 — 

107.4685 — 11·

119.1853 — 9
120.4476 — 3

147.9869 — 9a
148.0605 — 5
148.3424 — 3a

171.4082 — 2

(ppm)
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
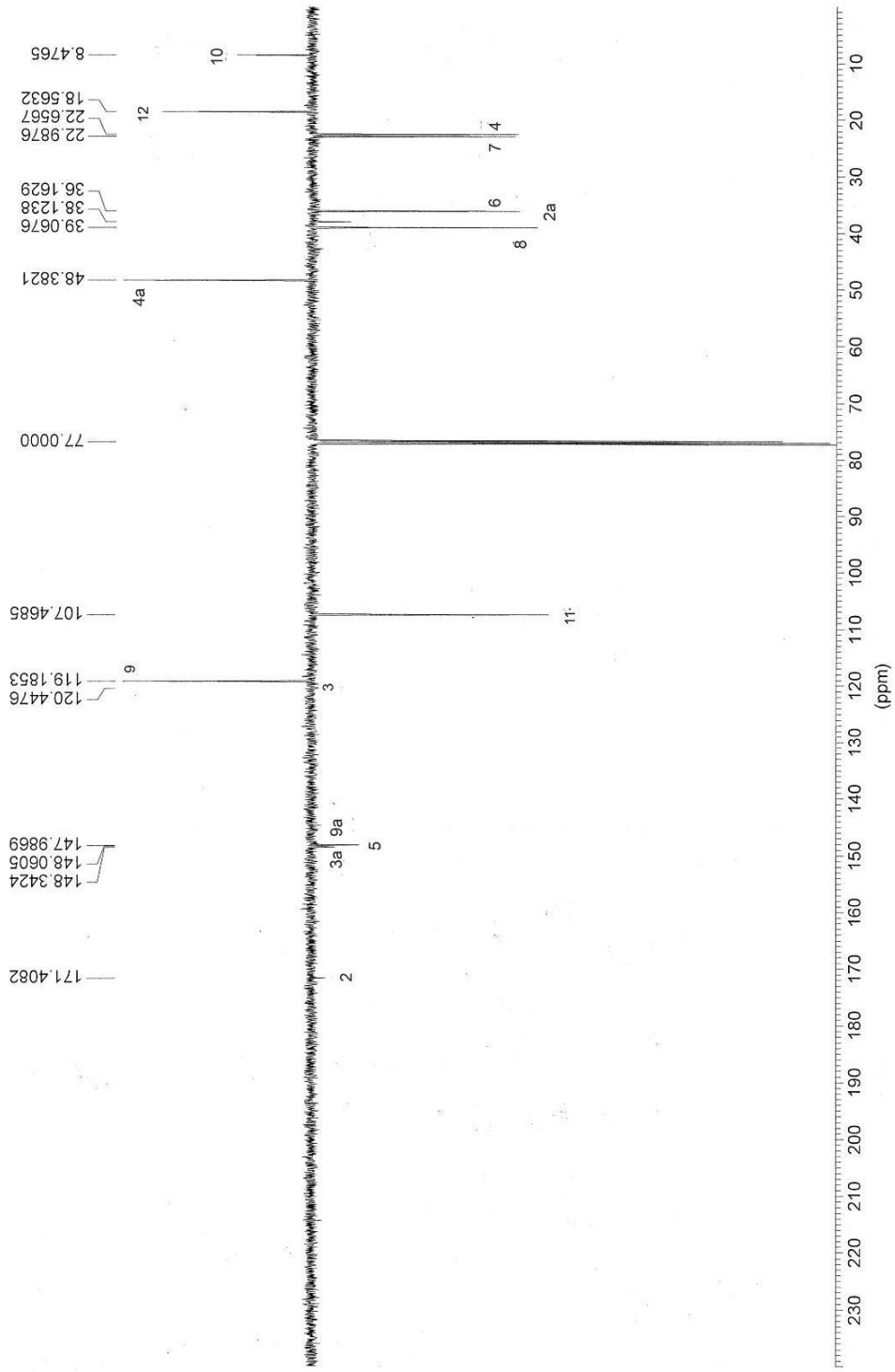
Atrac1 (Atractylenolide I, 8.96 mg) in cdcl3 (COSY 45) 14.5.2010
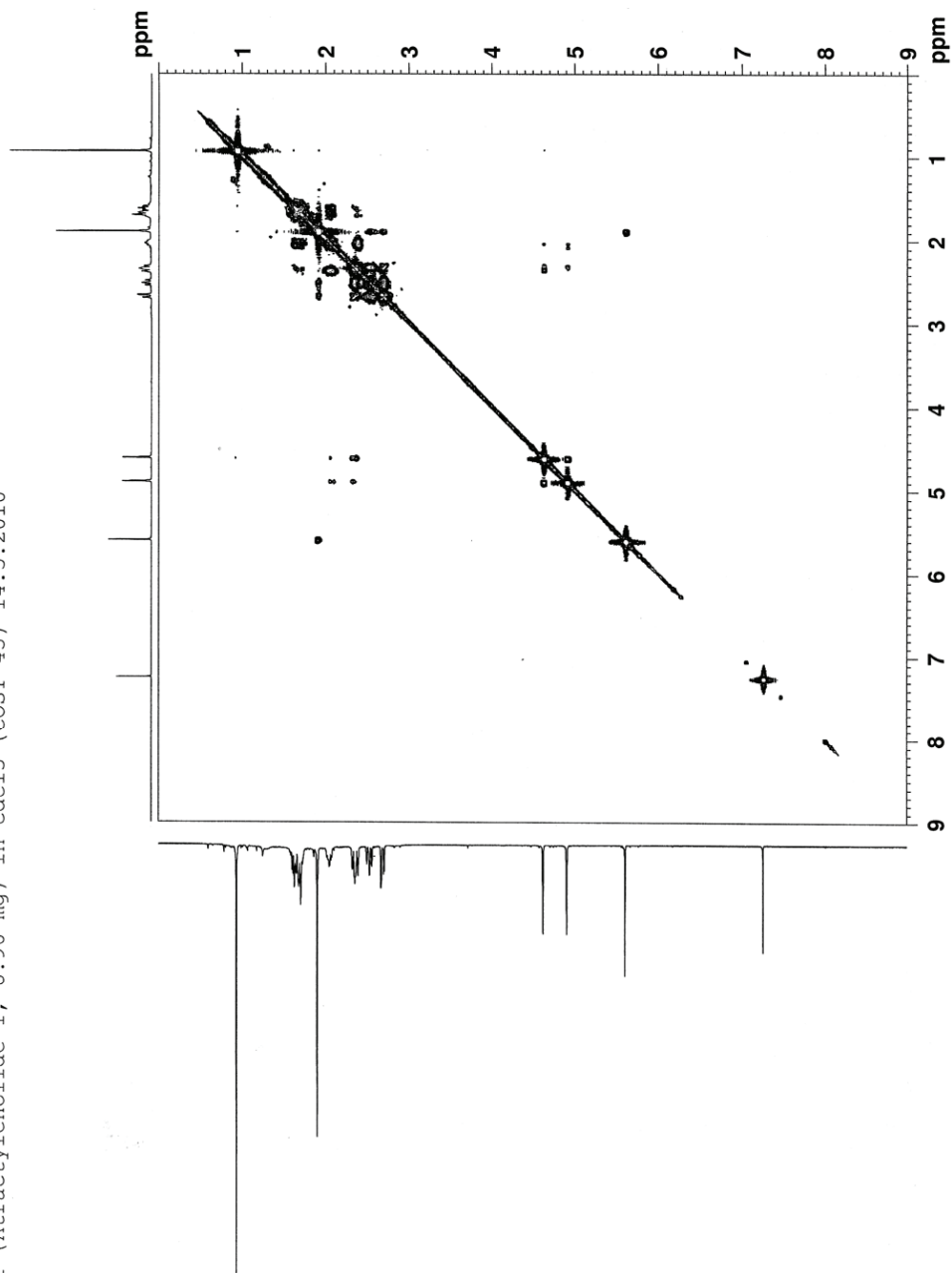
Atrac1 (Atractylenolide I, 8.96 mg) in cdcl3 (HMBC) 14.5.2010
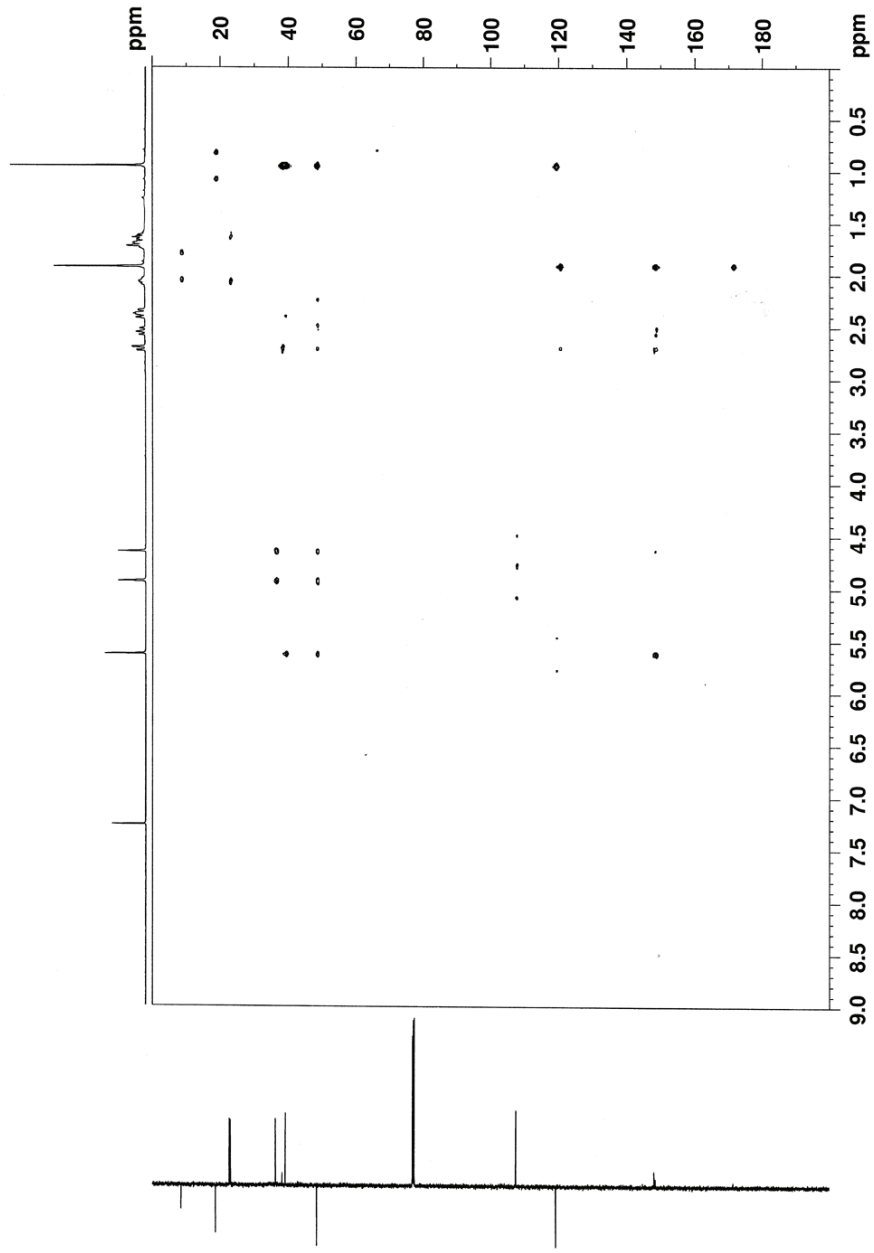
Atrac1 (Atractylenolide I, 8.96 mg) in cdcl3 (HSQC) 14.5.2010
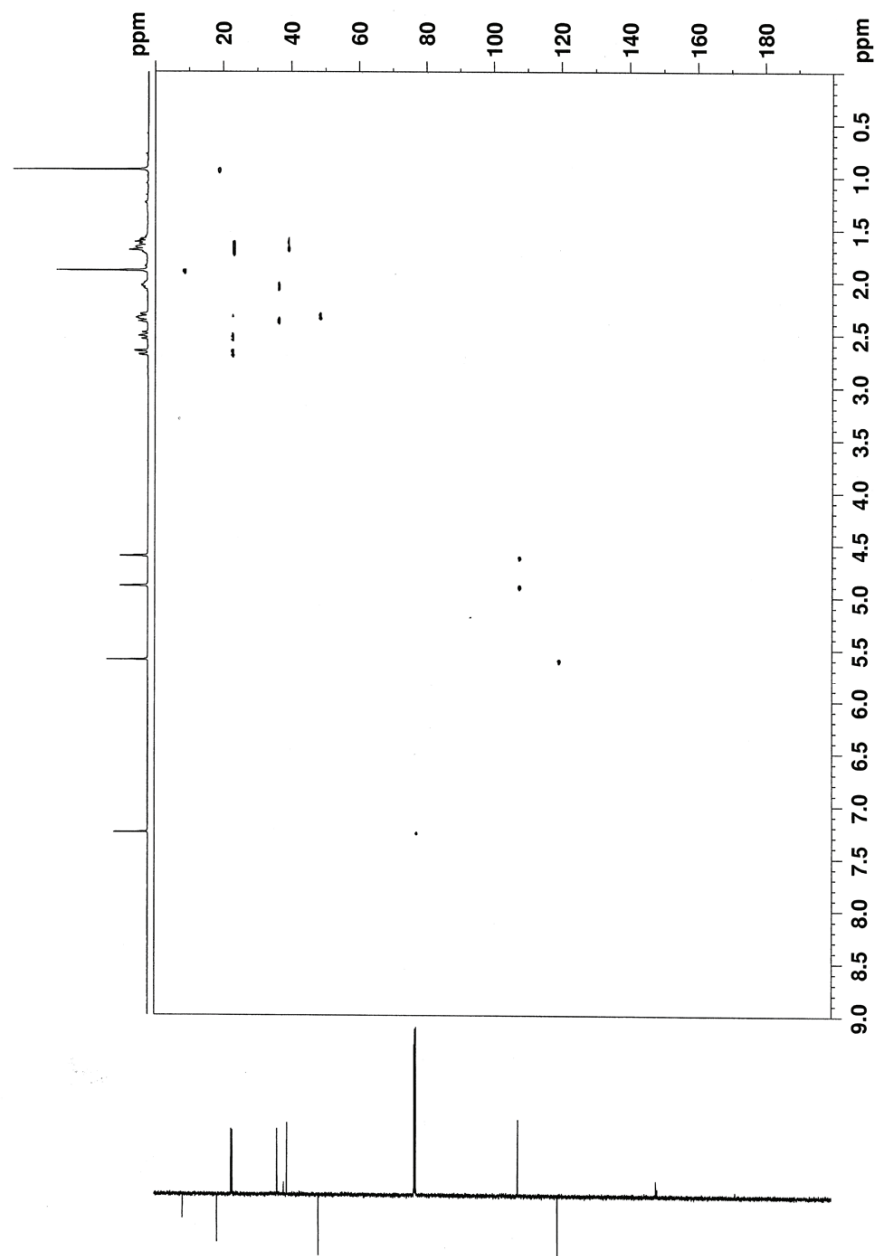
Osthole (1.45 mg in CDCl$_3$)
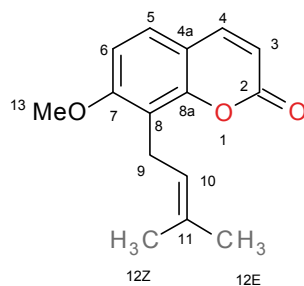


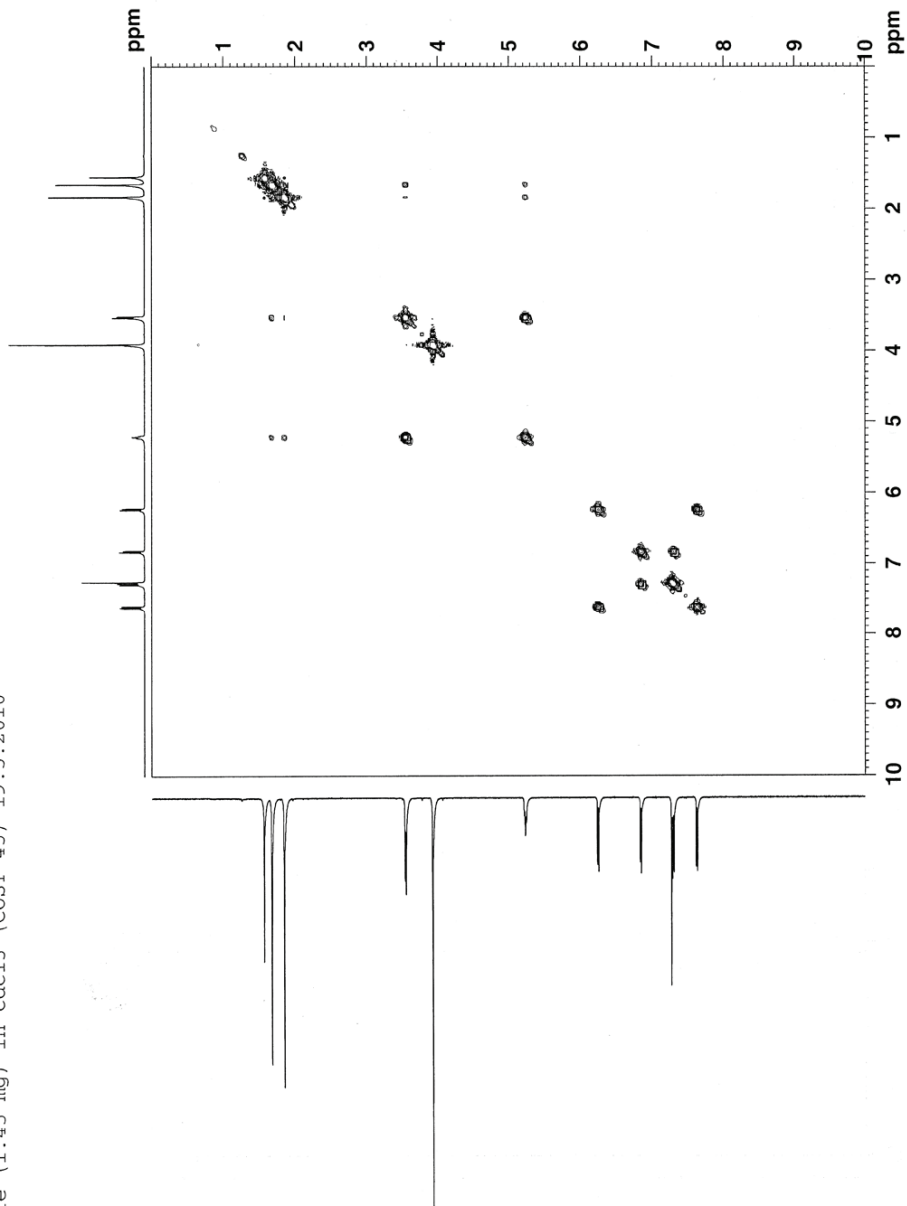| | | | |
|---|---|---|---|
| 2 | 161.4 | Cq | |
| 3 | 113.0 | CH | 6.24 (d, J = 9.5 Hz, 1 H) |
| 4 | 143.8 | CH | 7.61 (d, J = 9.2 Hz, 1 H) |
| 4a | 113.0 | Cq | |
| 5 | 126.2 | CH | 7.29 (d, J = 8.5 Hz, 1 H) |
| 6 | 107.3 | CH | 6.83 (d, J = 8.6 Hz, 1 H) |
| 7 | 160.2 | Cq | |
| 8 | 118.0 | Cq | |
| 8a | 152.8 | Cq | |
| 9 | 21.9 | CH$_2$ | 3.54 (d, J = 7.3 Hz, 1 H) |
| 10 | 121.1 | CH | 5.24-5.21 (m, 1 H) |
| 11 | 132.7 | Cq | |
| 12E | 25.8 | CH$_3$ | 1.67 (s, 1 H) |
| 12Z | 17.9 | CH$_3$ | 1.84 (s, 1 H) |
| 13 | 56.0 | OCH$_3$ | 3.92 (s, 1 H) |

Osthole (1.45 mg) in cdcl3 (Proton) 19.5.2010

Osthole (1.45 mg) in cdcl3 (APT) 19.5.2010

17.9382
21.9091
25.7943
56.0422
77.0000
107.3214
113.0082
117.9842
121.0849
126.1712
132.6791
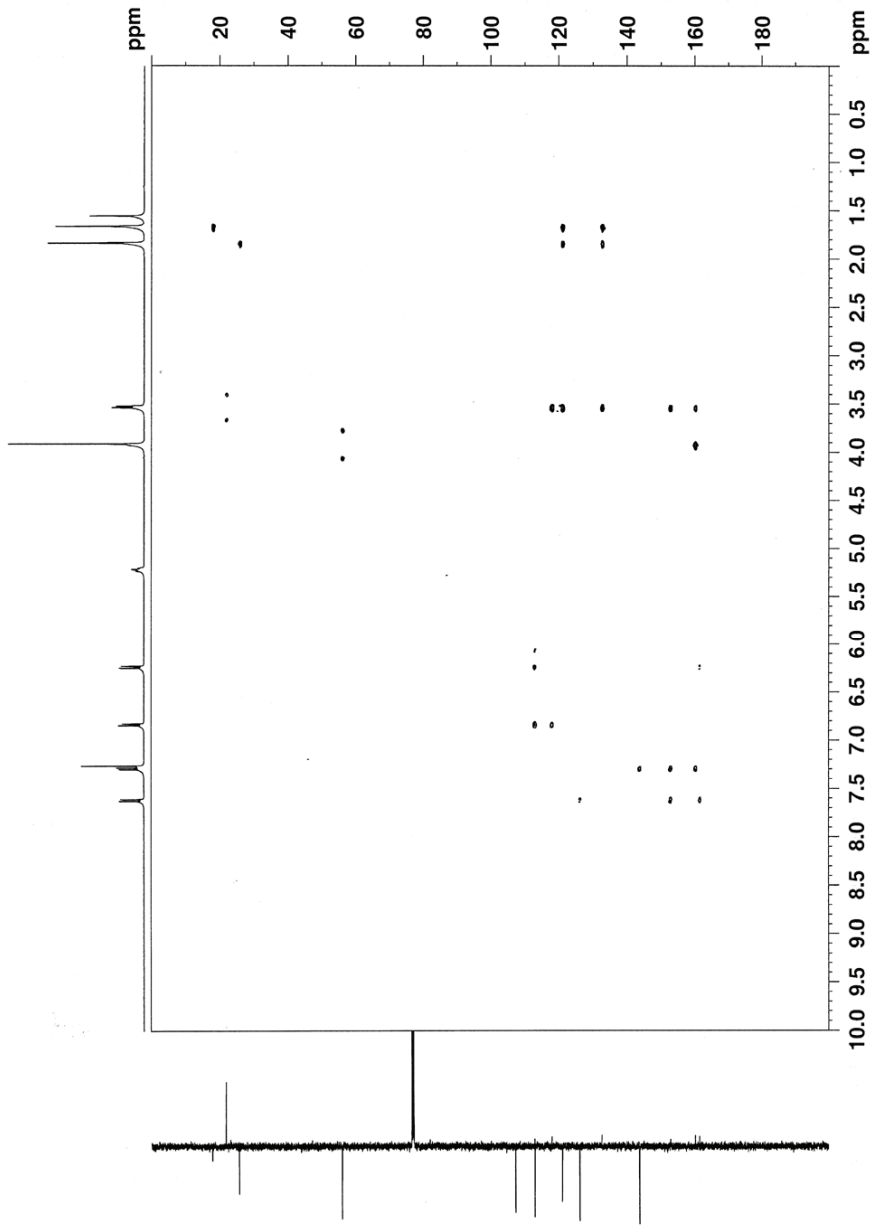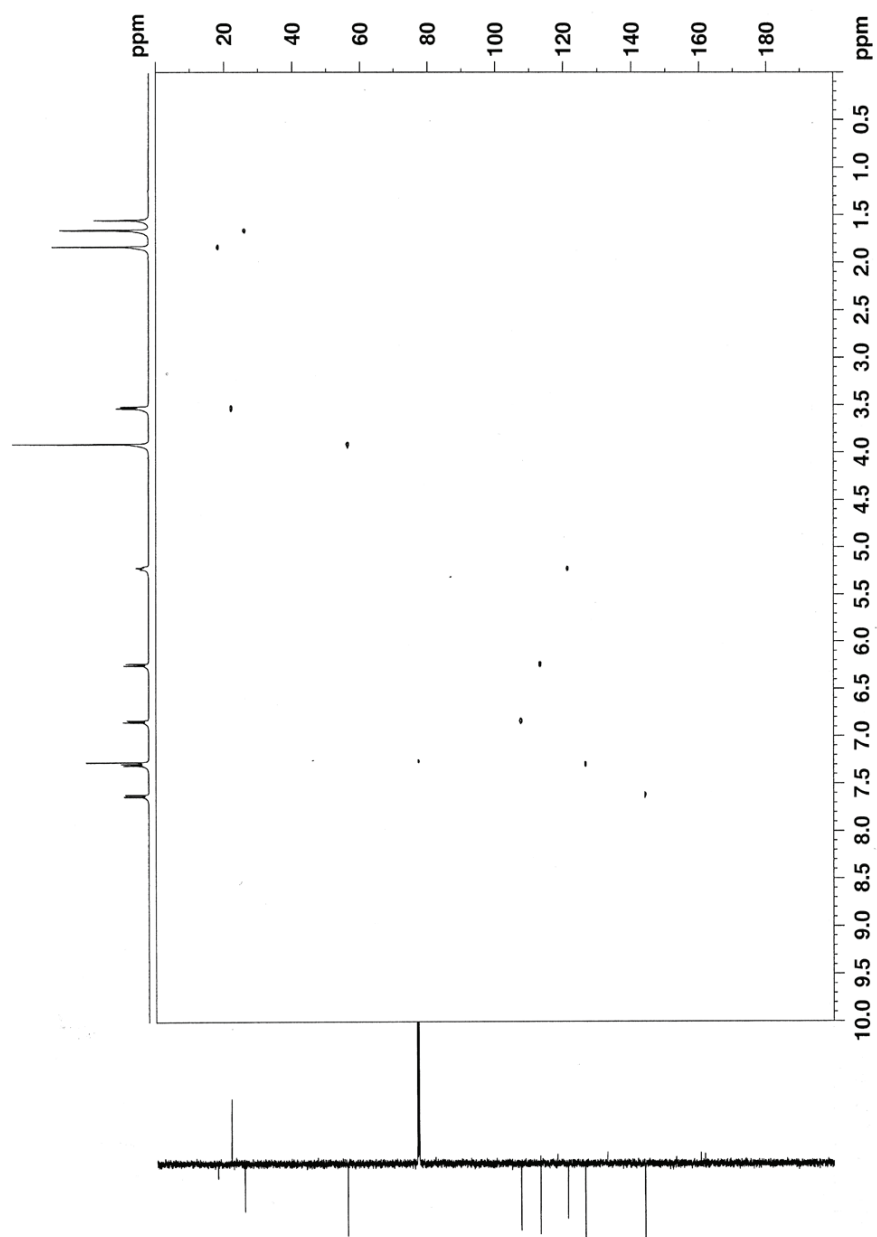143.7463
152.8158
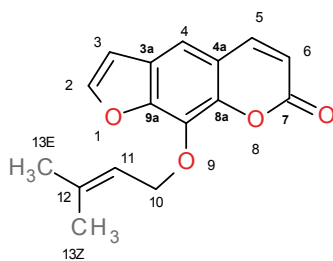160.1939
161.4073
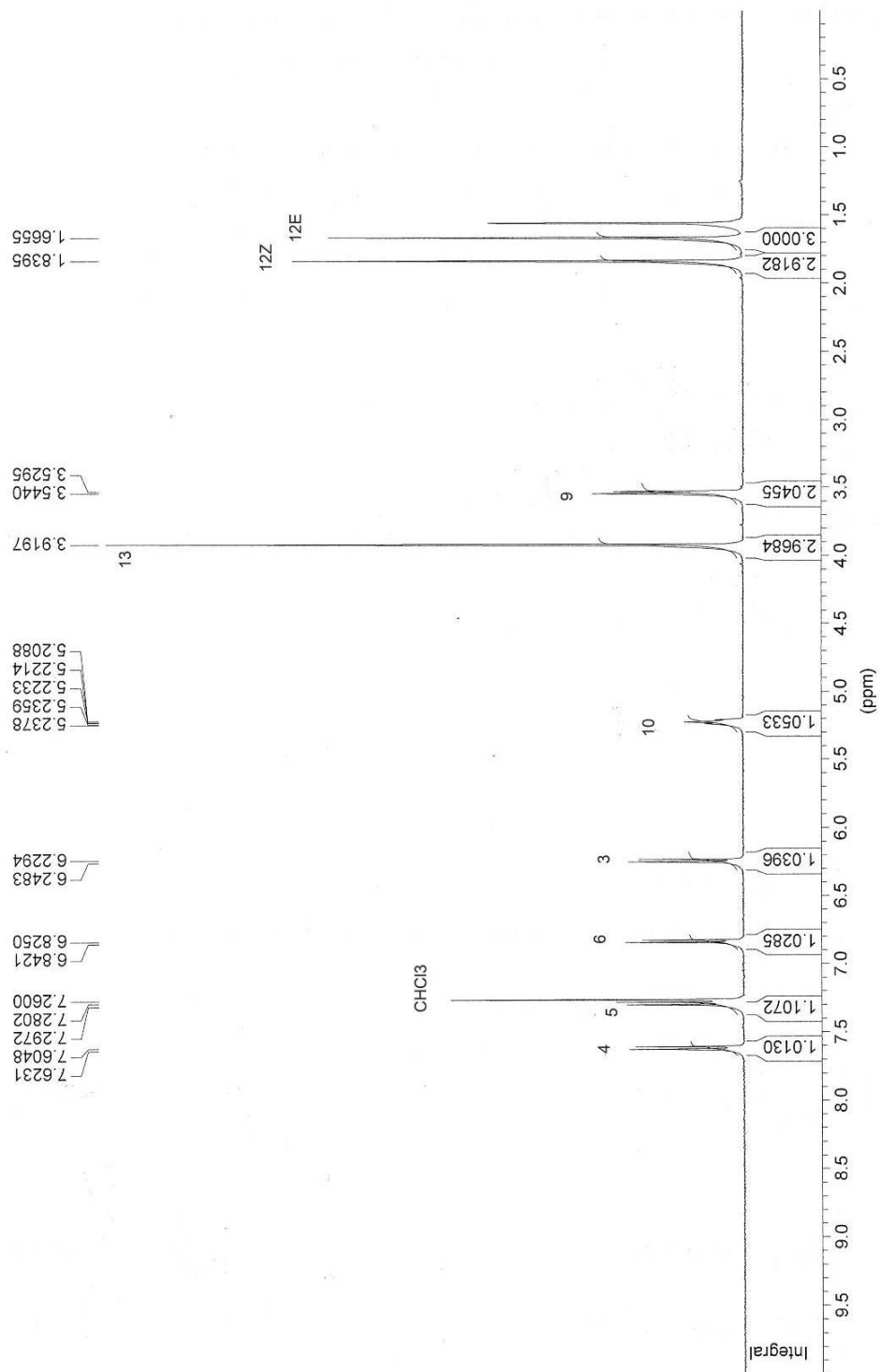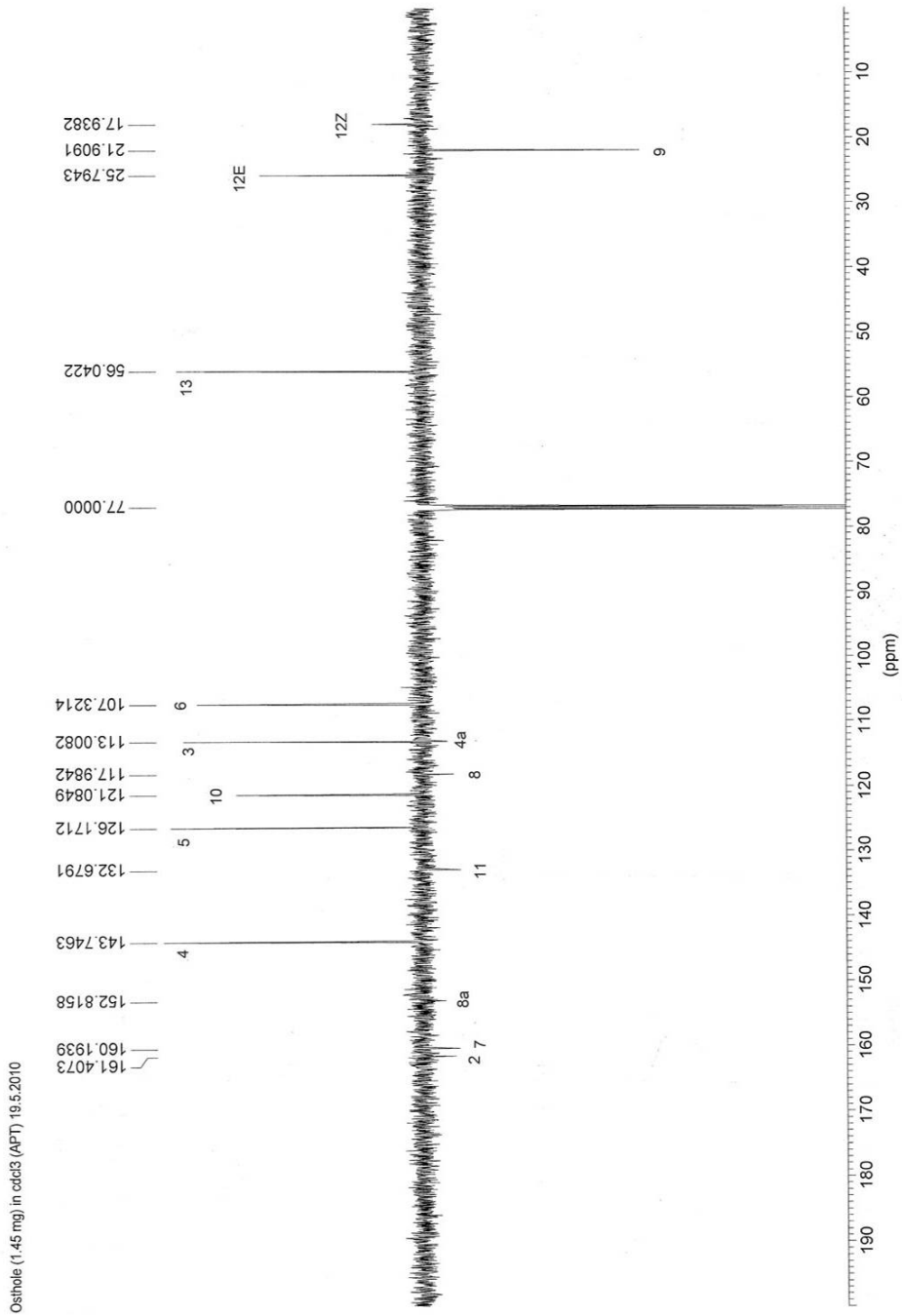
Osthole (1.45 mg) in cdcl3 (COSY 45) 19.5.2010

Osthole (1.45 mg) in cdcl3 (HMBC) 19.5.2010

Osthole (1.45 mg) in cdcl3 (HSQC) 19.5.2010

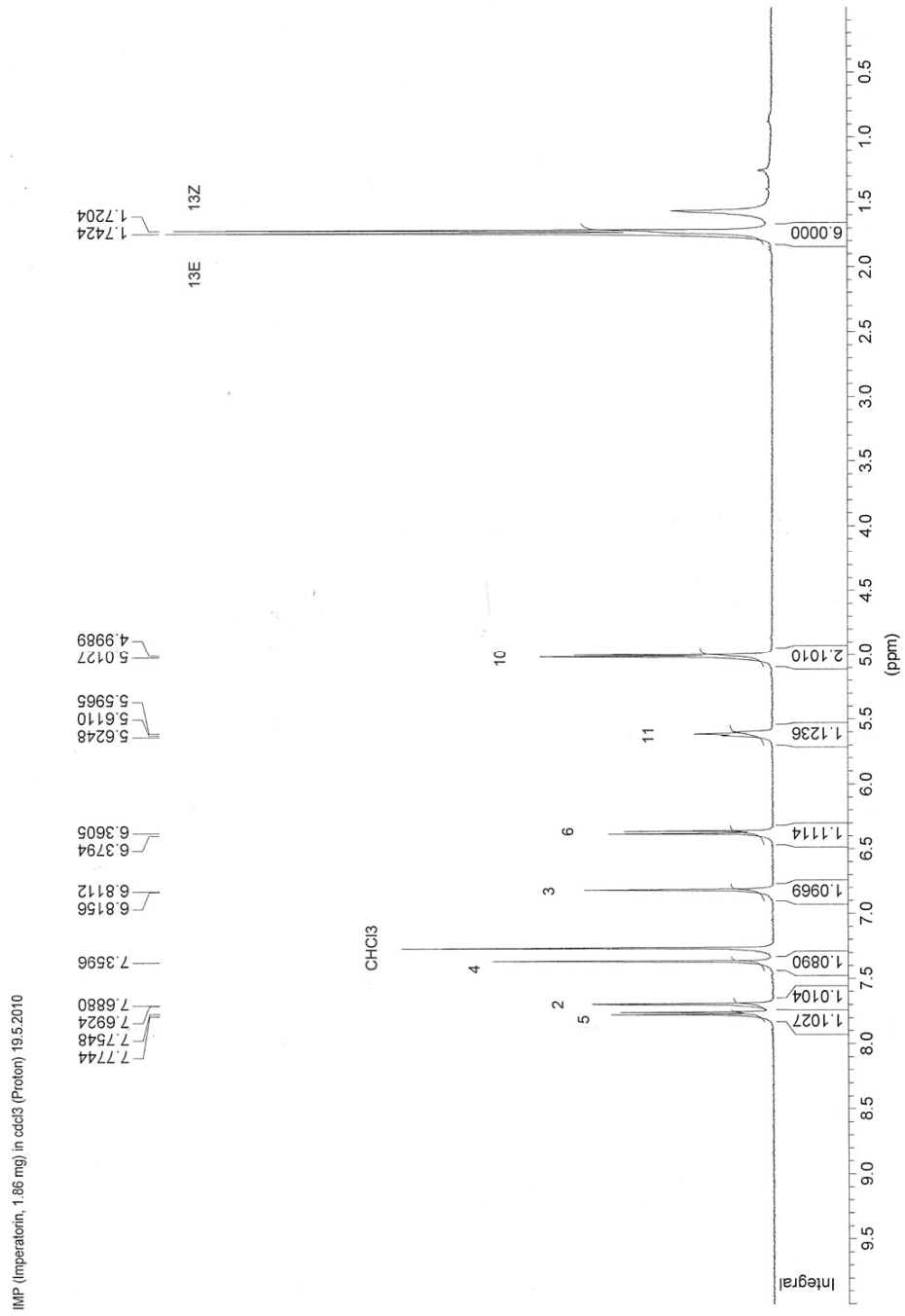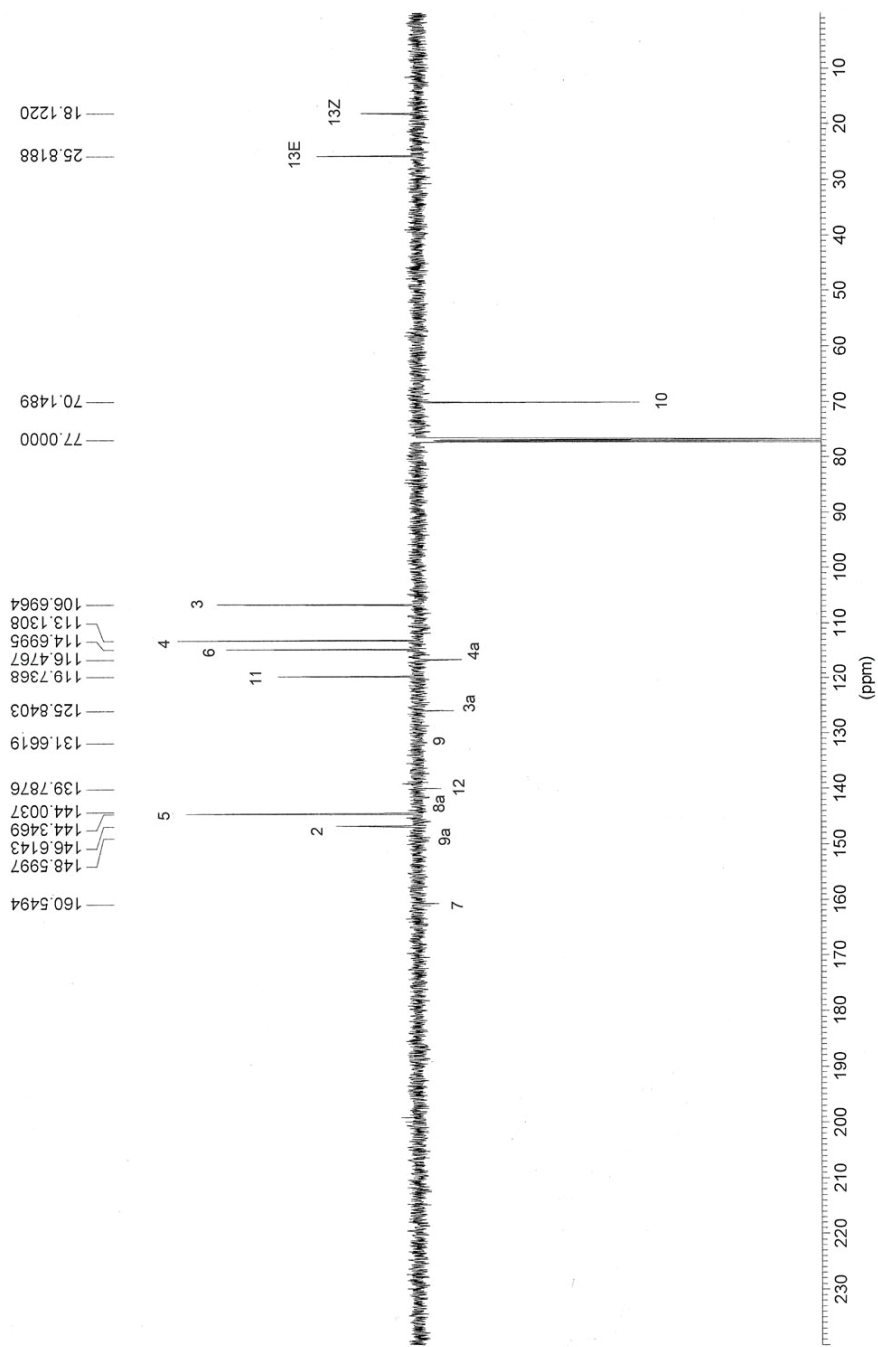Imperatorin (1.86 mg in CDCl$_3$)



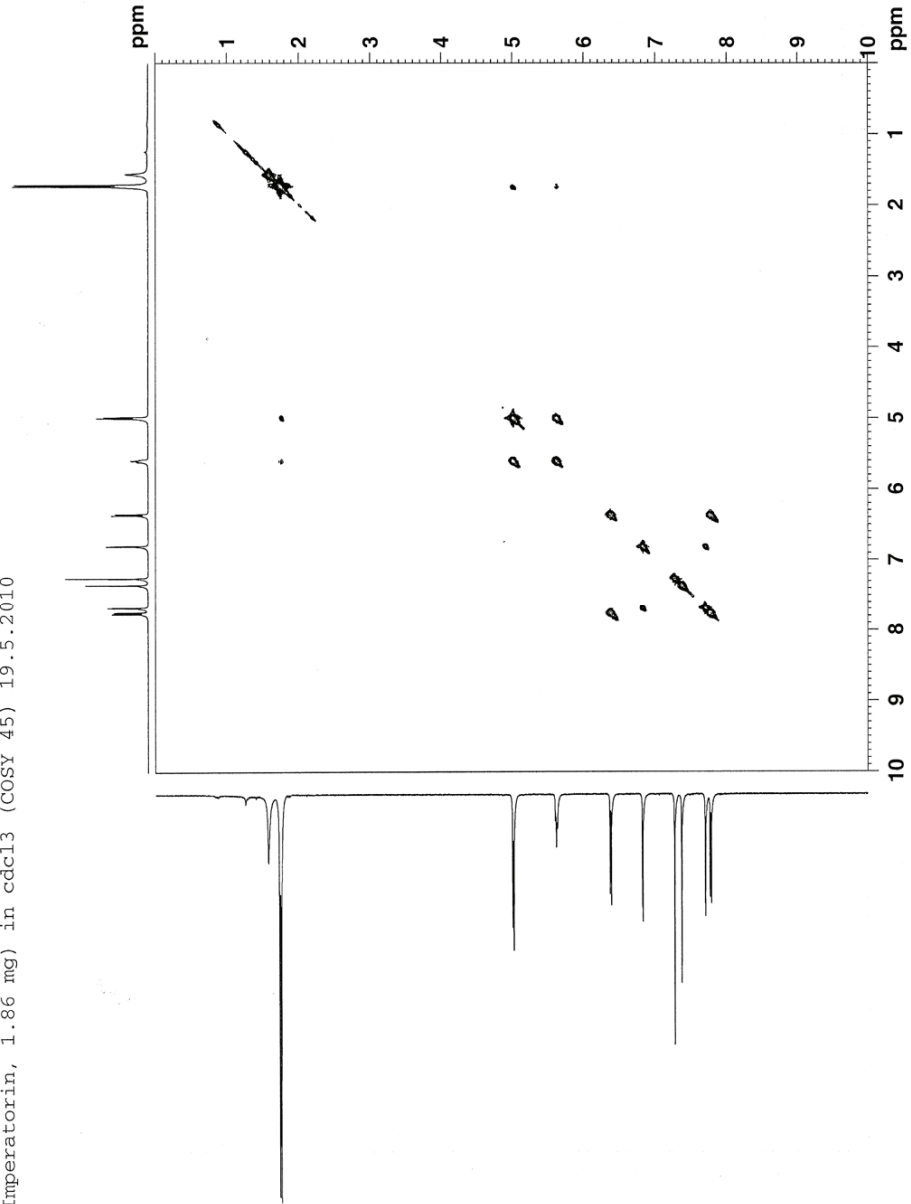| | | | |
|---|---|---|---|
| 2 | 146.6 | CH | 7.69 (d, J = 2.2 Hz, 1 H) |
| 3 | 106.7 | CH | 6.81 (d, J = 2.2 Hz, 1 H) |
| 3a | 125.8 | Cq | |
| 4 | 113.1 | CH | 7.36 (s, 1 H) |
| 4a | 116.5 | Cq | |
| 5 | 144.4 | CH | 7.76 (d, J = 9.8 Hz, 1 H) |
| 6 | 114.7 | CH | 6.37 (d, J = 4.7 Hz, 1 H) |
| 7 | 160.6 | Cq | |
| 8a | 144.0 | Cq | |
| 9 | 131.7 | Cq | |
| 9a | 148.6 | Cq | |
| 10 | 70.2 | CH$_2$ | 5.01 (d, J = 6.9 Hz, 1 H) |
| 11 | 119.7 | CH | 5.61 (dd, J = 14.2, 6.9 Hz, 1 H) |
| | | | 5.60 (dd, J = 7.3, 7.3 Hz, 1 H) |
| 12 | 139.8 | Cq | |
| 13E | 25.8 | CH$_3$ | 1.74 (s, 1 H) |
| 13Z | 18.1 | CH$_3$ | 1.72 (s, 1 H) |

IMP (Imperatorin, 1.86 mg) in cdcl3 (Proton) 19.5.2010

IMP (Imperatorin, 1.86 mg) in cdcl3 (APT) 19.5.2010

18.1220

25.8188

70.1489

77.0000

106.6964
113.1308
114.6995
116.4767
119.7368

125.8403

131.6619

139.7876
144.0037
144.3469
146.6143
148.5997

160.5494

13Z

13E

10

3

4

6

11

4a

3a

9

8a  12

5

2

9a

7

10  20  30  40  50  60  70  80  90  100  110  120  130  140  150  160  170  180  190  200  210  220  230
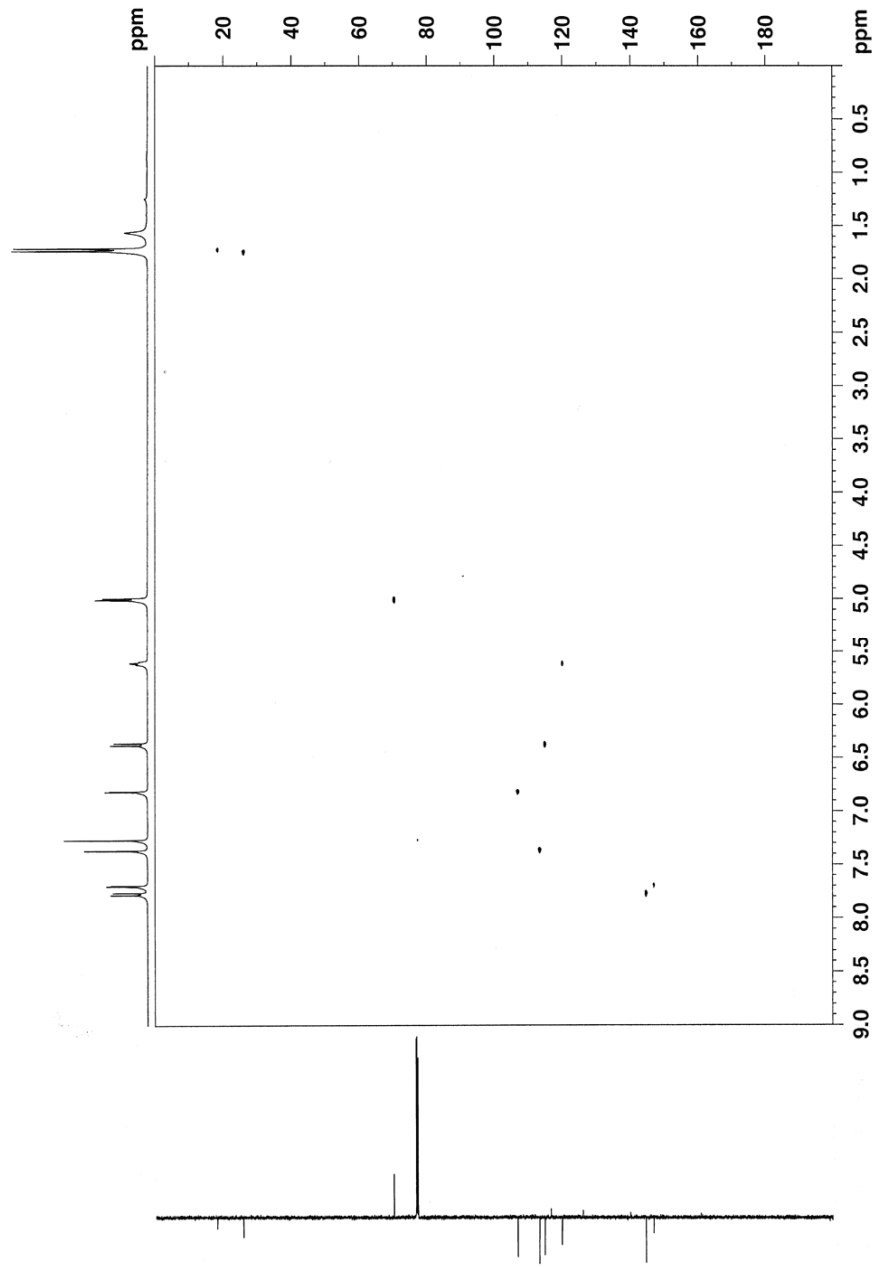
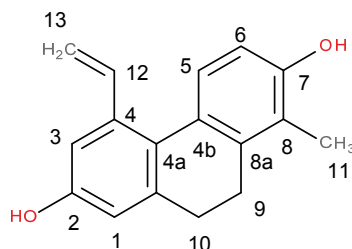(ppm)

IMP (Imperatorin, 1.86 mg) in cdcl3 (COSY 45) 19.5.2010

IMP (Imperatorin, 1.86 mg) in cdcl3 (HMBC) 19.5.2010
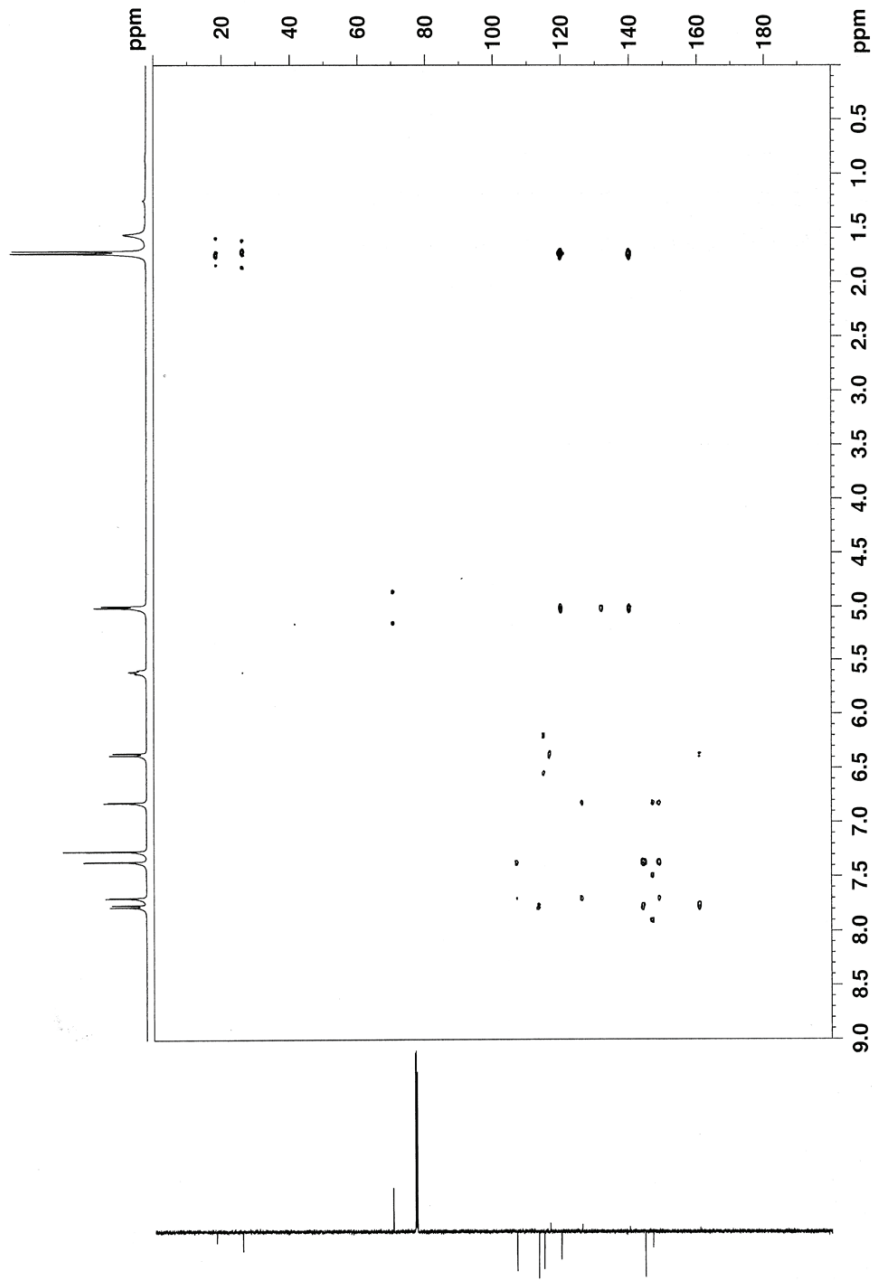
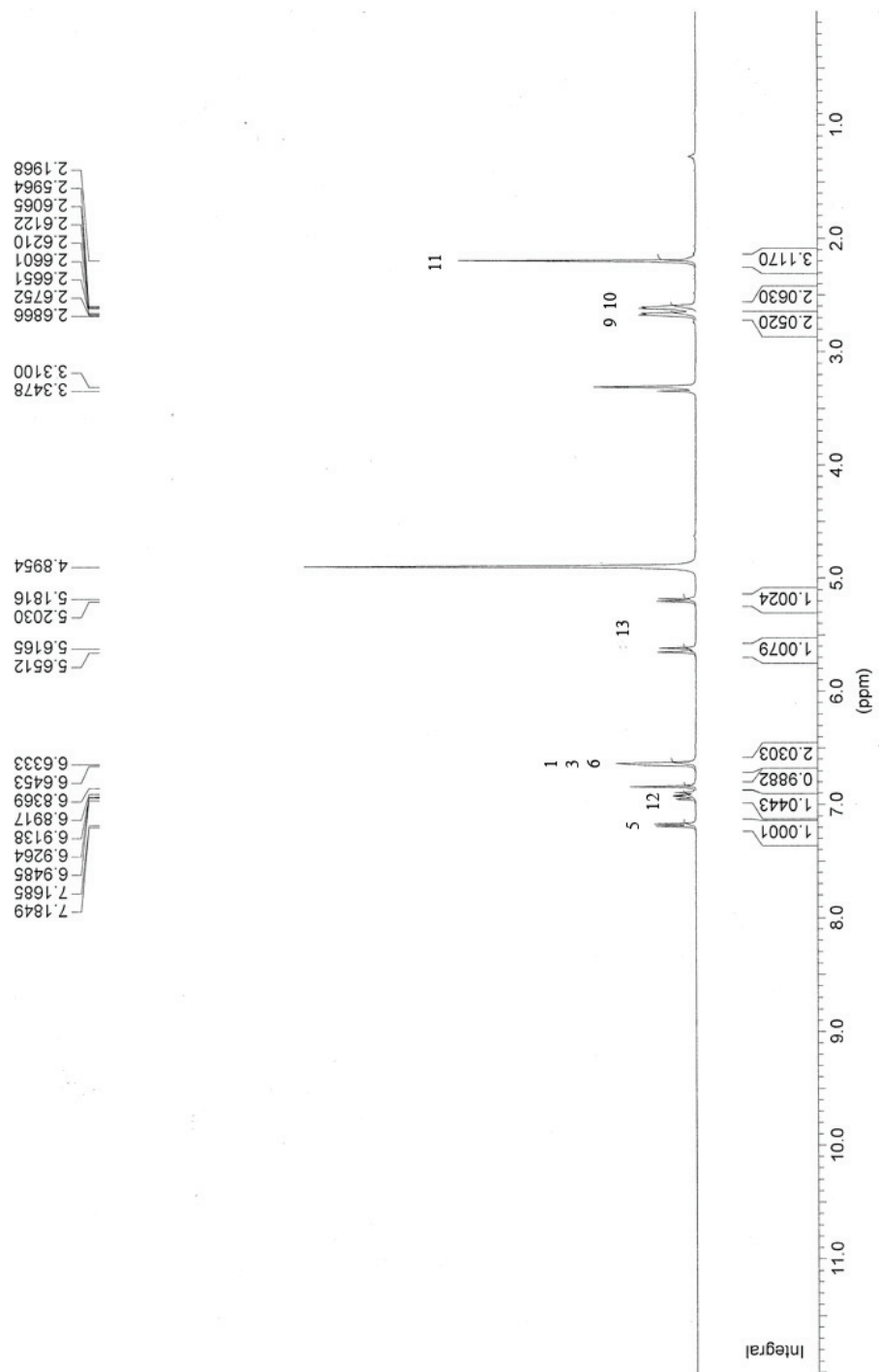IMP (Imperatorin, 1.86 mg) in cdcl3 (HSQC) 19.5.2010
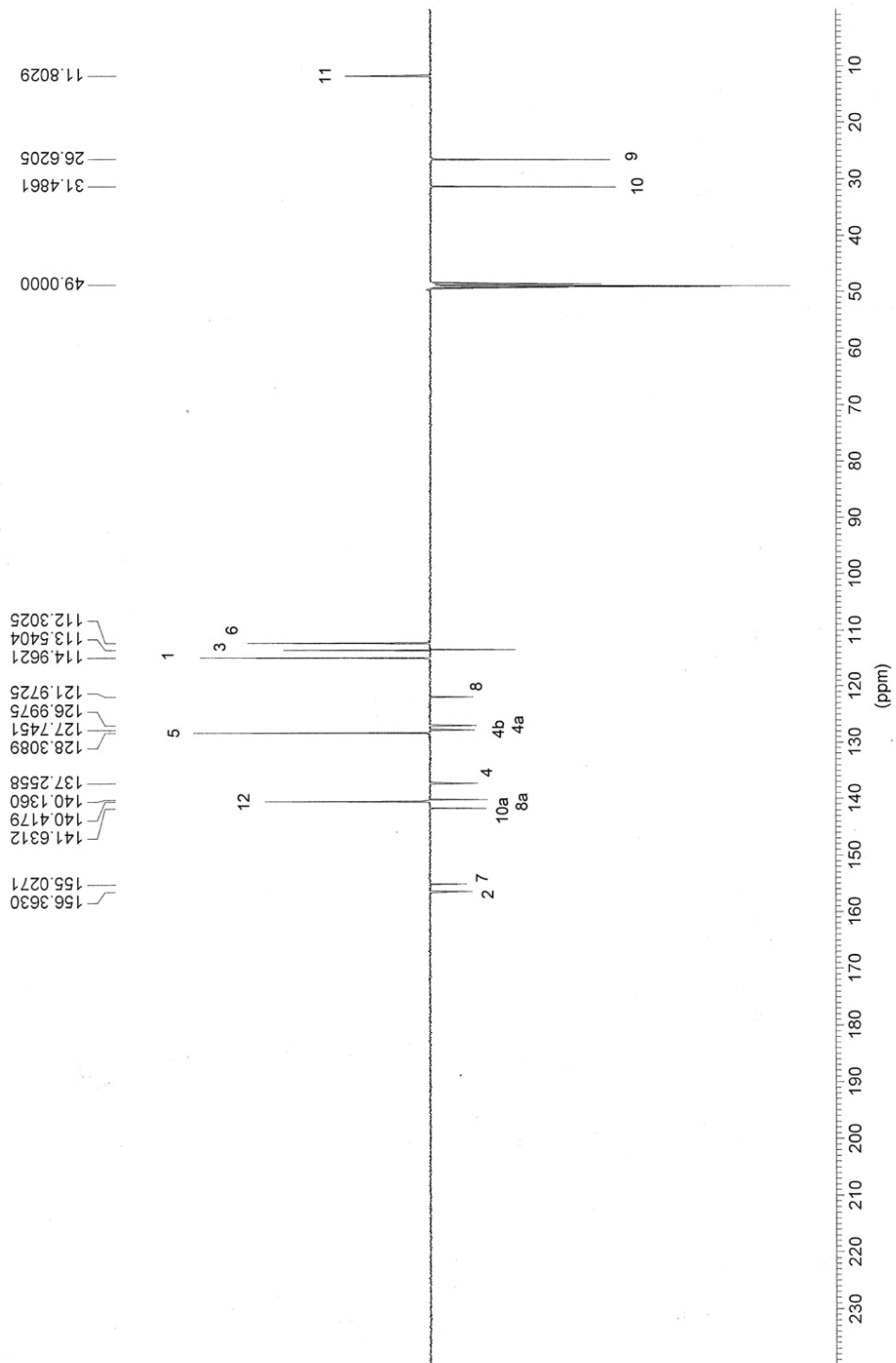
Effusol (8.62 mg in MeOD)



| | | | |
|---|---|---|---|
| 1 | 115.0 | CH | 6.63 (s, 1 H) |
| 2 | 156.4 | Cq | |
| 3 | 113.5 | CH | 6.64 (s, 1 H) |
| 4 | 137.3 | Cq | |
| 4a | 127.0 | Cq | |
| 4b | 127.8 | Cq | |
| 5 | 128.3 | CH | 7.18 (d, J = 8.2 Hz, 1 H) |
| 6 | 112.3 | CH | 6.65 (s, 1 H) |
| 7 | 155.0 | Cq | |
| 8 | 122.0 | Cq | |
| 8a | 140.1 | Cq | |
| 9 | 26.6 | CH$_2$ | 2.68 (dd, J = 10.75, 5.7 Hz, 1 H) |
| | | | 2.66 (dd, J = 7.55; 2.5 Hz, 1 H) |
| 10 | 31.5 | CH$_2$ | 2.62 (dd, J = 7.25, 4.4 Hz, 1 H) |
| | | | 2.60 (dd, J = 7.9, 5.05 Hz, 1 H) |
| 10a | 141.6 | Cq | |
| 11 | 11.8 | CH$_3$ | 2.20 (s, 1 H) |
| 12 | 140.4 | CH | 6.93 (dd, J = 17.35, 11.05 Hz, 1 H) |
| | | | 6.90 (dd, J = 17.35, 11.05 Hz, 1 H) |
| 13Z | 113.5 | CH$_2$ | 5.63 (d, J = 17.35 Hz, 1 H) |
| 13E | | | 5.19 (d, J = 10.7 Hz, 1 H) |

EFF (8.62 mg) in MeOD (Proton) 8.4.2010

2.1968
2.5964
2.6065
2.6122
2.6210
2.6601
2.6651
2.6752
2.6866

3.3100
3.3478

4.8954

5.1816
5.2030

5.6165
5.6512

6.6333
6.6453
6.8369
6.8917
6.9138
6.9264
6.9485
7.1685
7.1849

Integral

1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
(ppm)

11

9 10

13

1
3
6

5
12

3.1170

2.0630
2.0520

1.0024

1.0079

2.0303
0.9882
1.0443
1.0001

EFF (8.62 mg) in MeOD (APT) 8.4.2010

11.8029

26.6205

31.4861

49.0000

112.3025
113.5404
114.9621

121.9725
126.9975
127.7451
128.3089

137.2558
140.1360
140.4179
141.6312

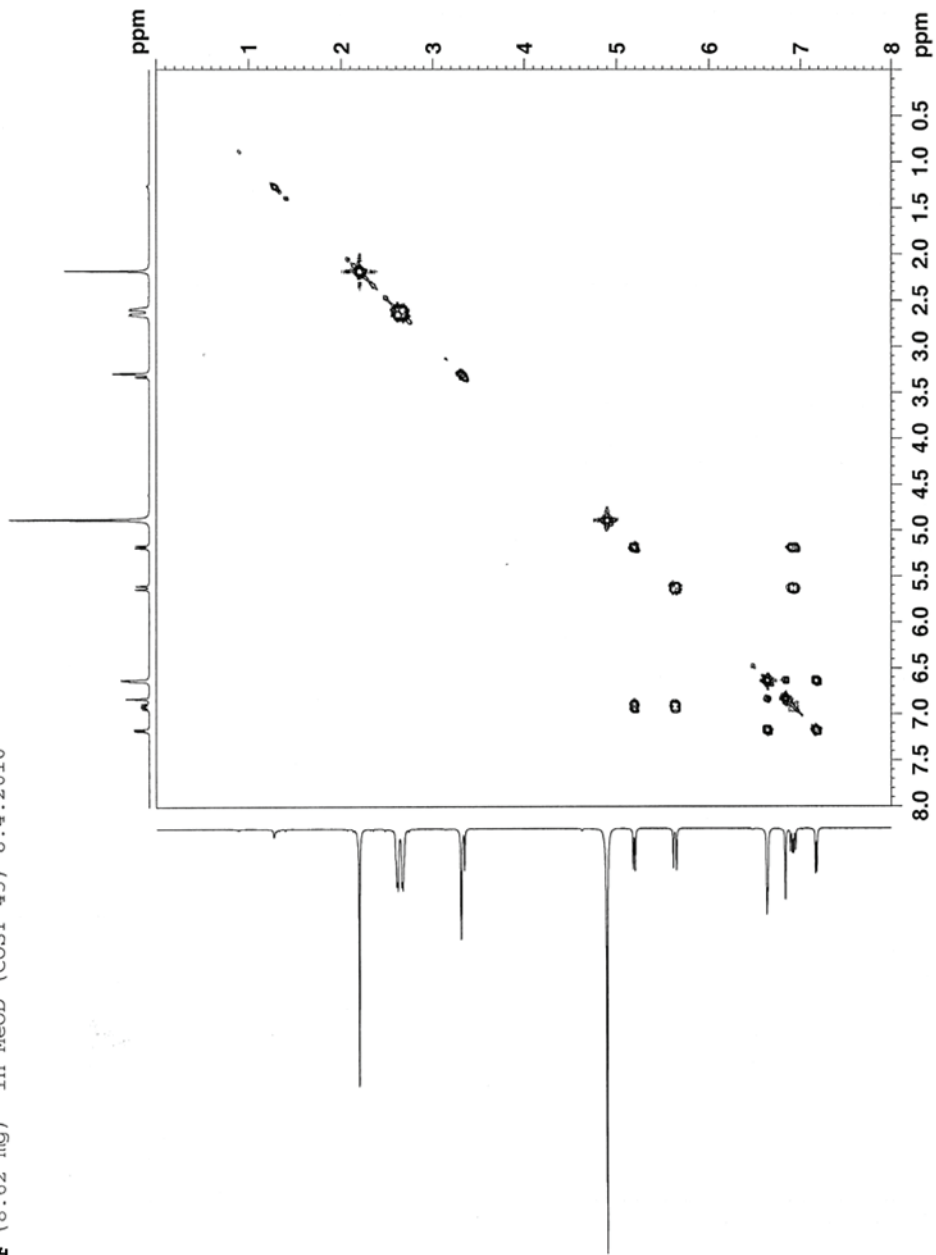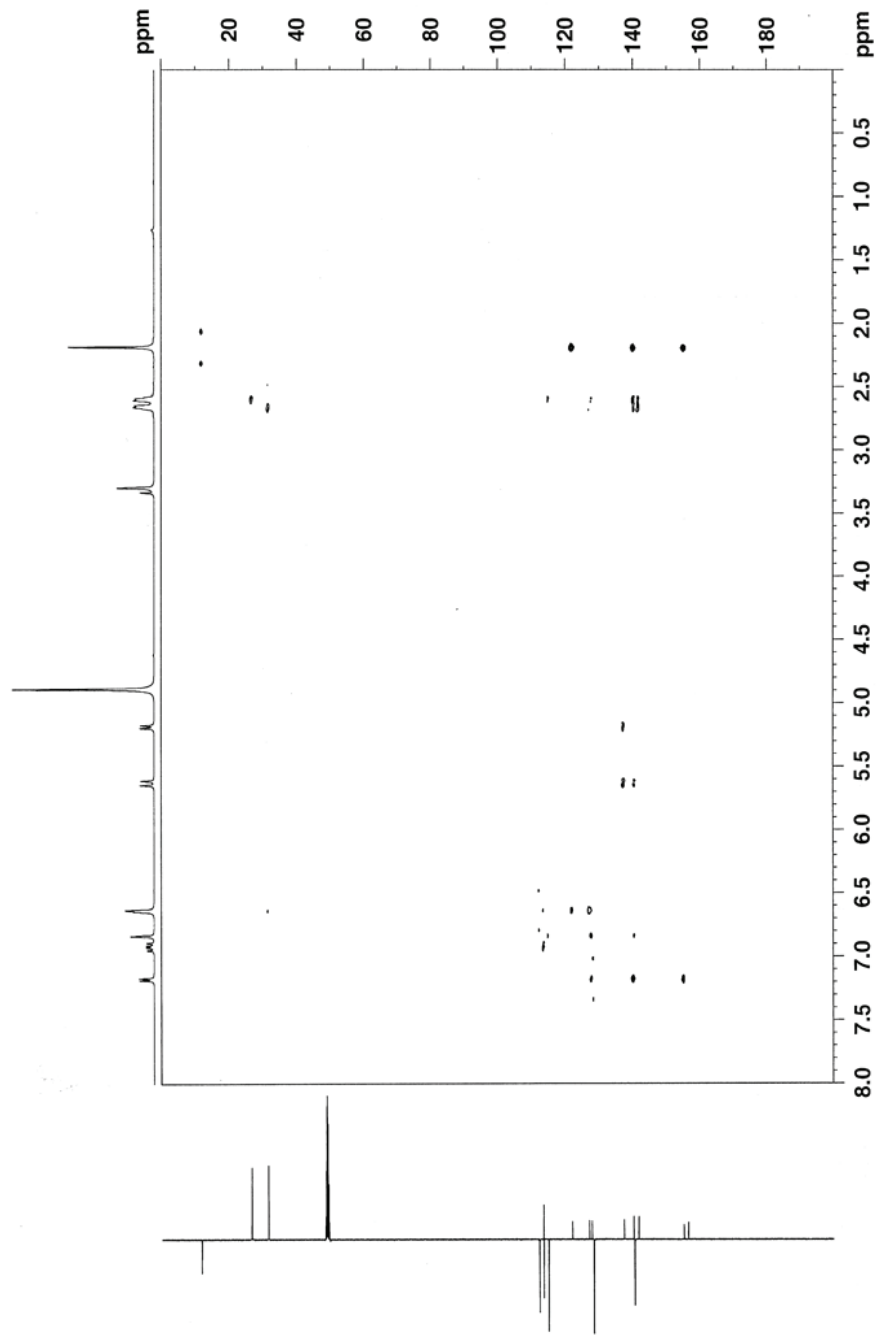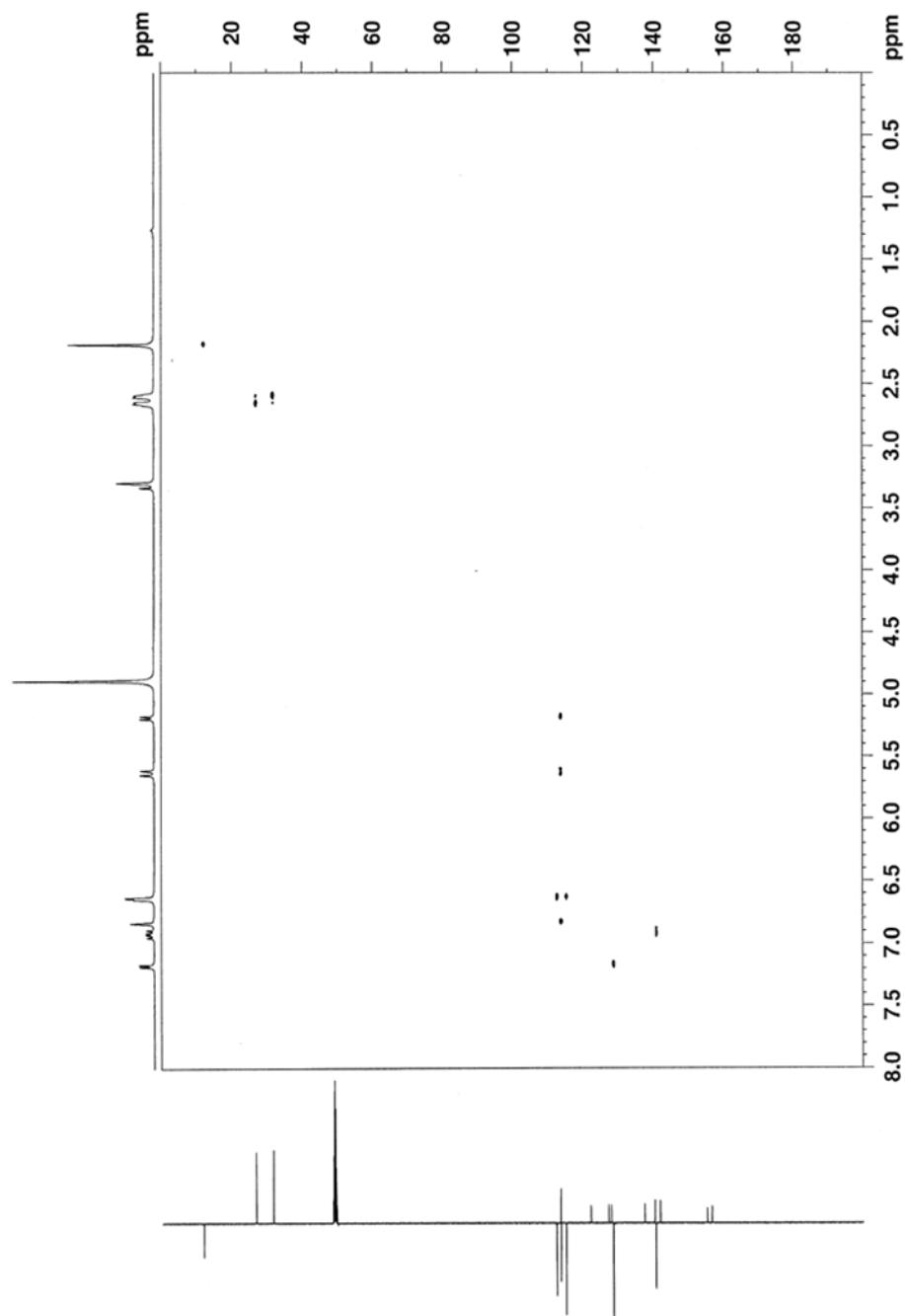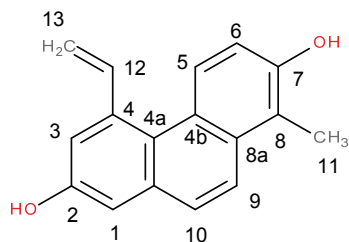155.0271
156.3630

EFF (8.62 mg)  in MeOD (COSY 45) 8.4.2010

EFF (8.62 mg) in MeOD (HMBC) 8.4.2010

EFF (8.62 mg)  in MeOD (HSQC) 8.4.2010

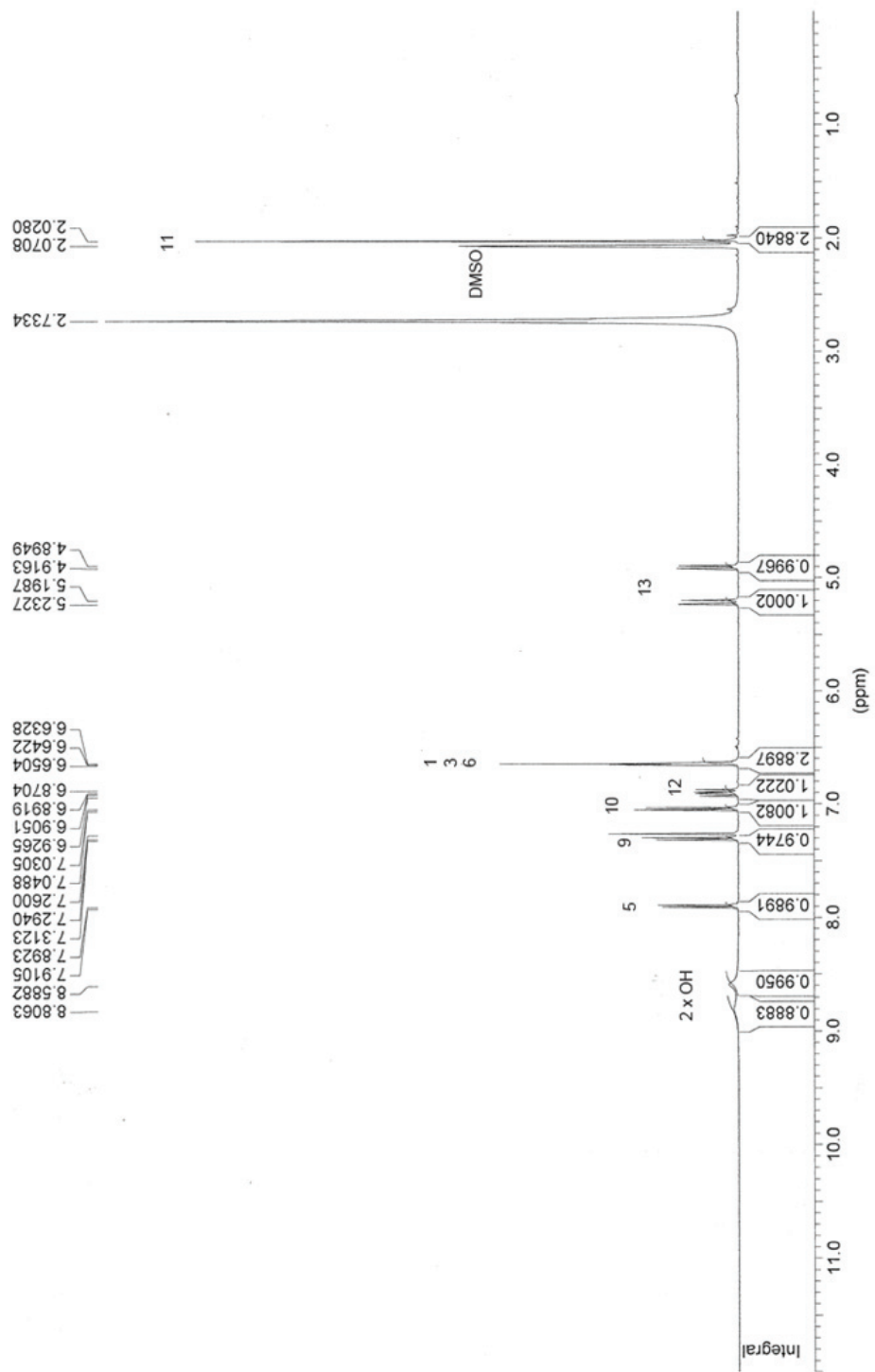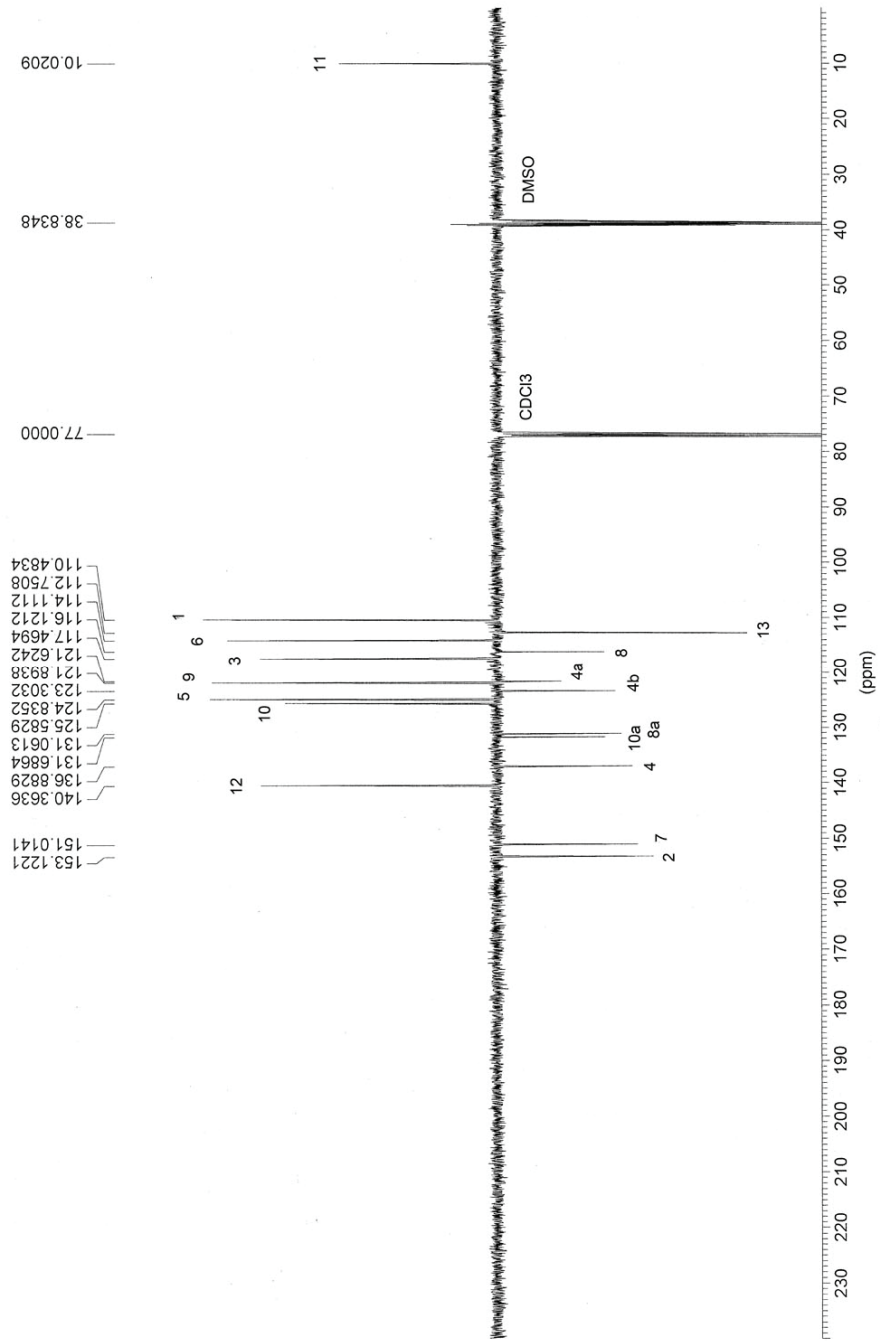Dehydroeffusol (3.40 mg in CDCl$_3$:d6-DMSO in a ratio of 1:1)



| | | | |
|---|---|---|---|
| 1 | 110.5 | CH | 6.63 (s, 1 H) |
| 2 | 153.1 | Cq | |
| 3 | 117.5 | CH | 6.64 (s, 1 H) |
| 4 | 136.9 | Cq | |
| 4a | 121.6 | Cq | |
| 4b | 123.3 | Cq | |
| 5 | 124.8 | CH | 7.90 (d, J =9.1 Hz, 1 H) |
| 6 | 114.1 | CH | 6.65 (s, 1 H) |
| 7 | 151.0 | Cq | |
| 8 | 116.1 | Cq | |
| 8a | 131.1 | Cq | |
| 9 | 121.9 | CH | 7.30 (d, J = 9.15 Hz, 1 H) |
| 10 | 125.6 | CH | 7.03 (d, J = 9.15 Hz, 1 H) |
| 10a | 131.7 | Cq | |
| 11 | 10.0 | CH$_3$ | 2.05 (s, 1 H) |
| 12 | 140.4 | CH | 6.92 (dd, J = 17.3, 10.7 Hz, 1 H) |
| | | | 6.88 (dd, J = 17.35, 11.75 Hz, 1 H) |
| 13Z | 112.8 | CH$_2$ | 4.91 (d, J = 10.7 Hz, 1 H) |
| 13E | | | 5.22 (d, J = 17.0 Hz, 1 H) |

DHE (Dehydroeffusol, 3.40 mg) in d6-DMSO+CDCl3 (Proton) 20.5.2010

2.0280
2.0708
11

2.7334

DMSO

4.8949
4.9163
5.1987
5.2327

13
0.9967
1.0002

6.6328
6.6422
6.6504
1
3
6
2.8897
6.8704
6.8919
6.9051
6.9265
12
1.0222
10
1.0082
7.0305
7.0488
7.2600
7.2940
7.3123
9
0.9744
7.8923
7.9105
5
0.9891
8.5882
8.8063

2 x OH
0.9950
0.8883

2.8840

(ppm)

Integral

DHE (Dehydroeffusol, 3.40 mg) in d6-DMSO+CDCl3 (APT) 20.5.2010

10.0209

38.8348

77.0000

110.4834
112.7508
114.1112
116.1212
117.4694
121.6242
121.8938
123.3032
124.8352
125.5829
131.0613
131.6864
136.8829
140.3636

151.0141
153.1221

DMSO

CDCl3

11

1
6
3
5 9
10
12

13
8
4a
4b
10a
8a
4
2 7

(ppm)

230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
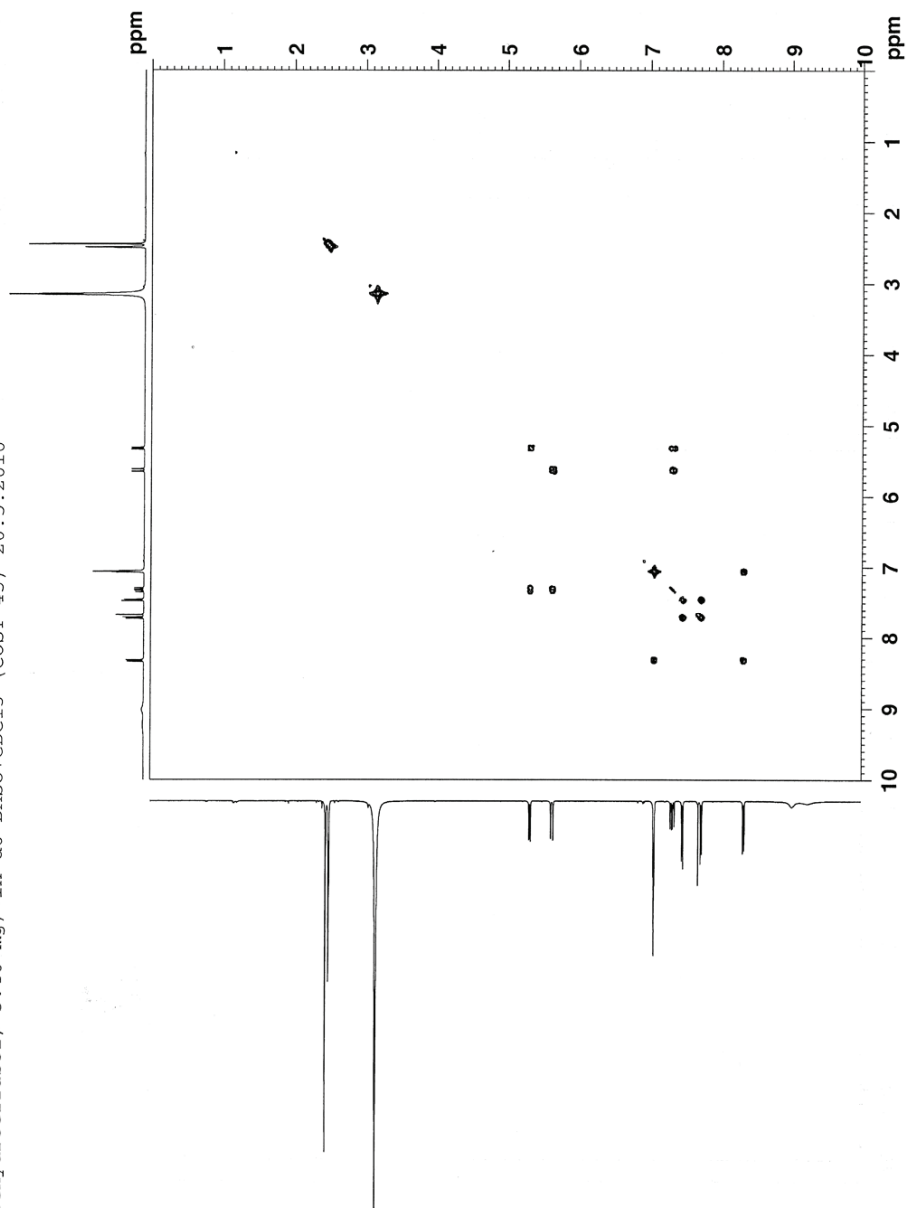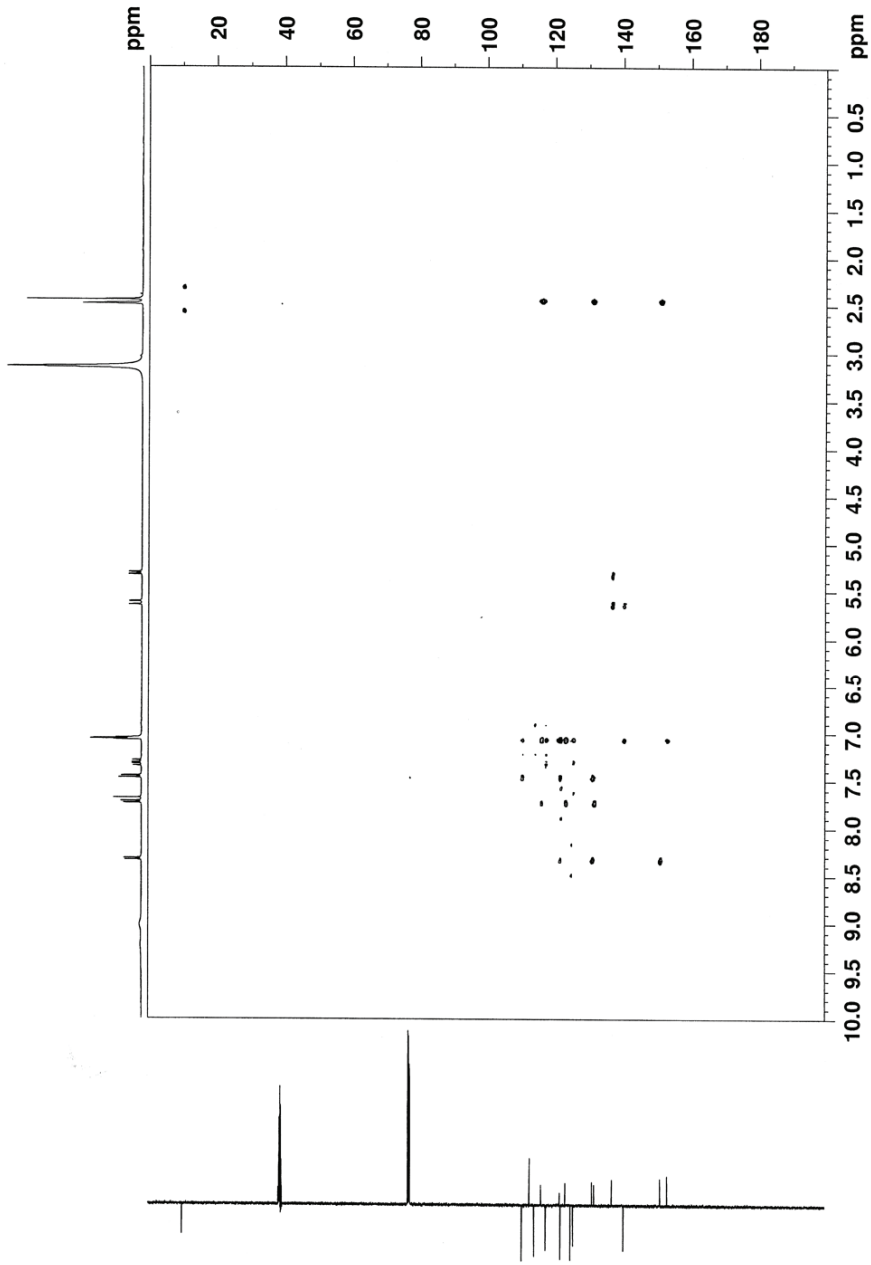
DHE (Dehydroeffusol, 3.40 mg) in d6-DMSO+CDCl3 (COSY 45) 20.5.2010

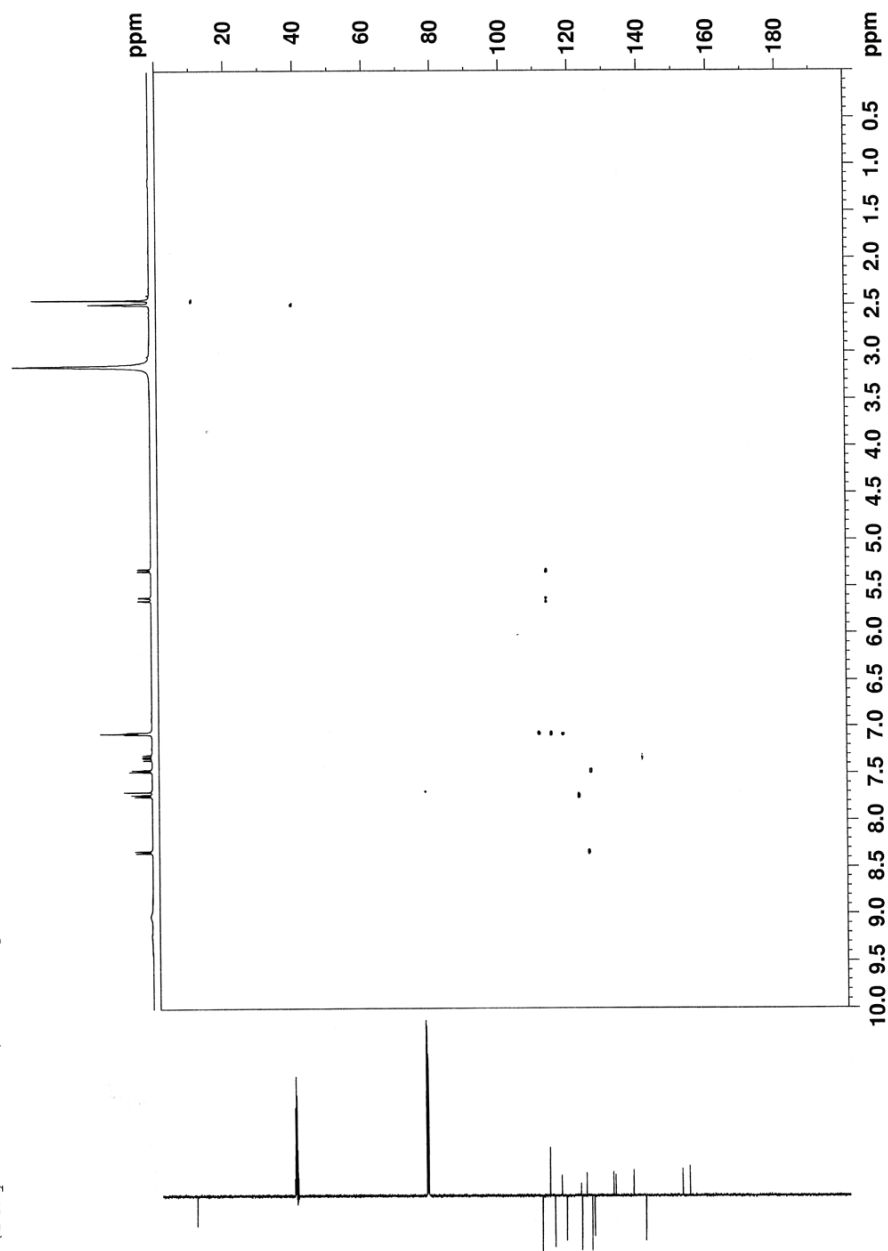DHE (Dehydroeffusol, 3.40 mg) in d6-DMSO+CDCl3 (HMBC) 20.5.2010

DHE (Dehydroeffusol, 3.40 mg) in d6-DMSO+CDCl3 (HSQC) 20.5.2010

# Curriculum Vitae

**Mag. JUDITH SINGHUBER**

Zehetnergasse 41/14
1140 Wien
Austria

**Date and place of birth:** 23.12.1980, in Steyr
**Email:** chum@gmx.net

**School education:**
09/1995 - 06/2000 HBLA for culture and congress managment, Steyr, Upper Austria, Austria

**Universitary Education**

09/2000 - 09/2007 diploma study in pharmacy, University of Vienna, Austria
$1^{st}$ term finished in 06/2003
Diploma thesis at the Department of Pharmacognosy. Promoter: Prof. Brigitte Kopp
Topic: A contribution for quality of *Acontium* species in TCM and *Actaea racemosa* L.

10/2007 - 12/2010 doctoral thesis in pharmacy at the University of Vienna, Department of Pharmacognosy in frame of the PhD college: Initiativkolleg "Molecular Drug Targets".
Promoter: Prof. Brigitte Kopp
Topic: Isolation of positive, allosteric $GABA_A$ receptor modulators from Chinese herbal drugs traditionally used in the treatment of anxiety and insomnia

Practical skills
HPLC, MS, GC analysis
Two-microelectrode voltage clamp technique

**Further activities and interests**

Teaching assistant at the course "Preparation & Analysis of medicinal products of biogenous origin" in winter term **2007, 2008 and 2009** and sommer term **2007/8, 2008/09** and **2009/10**.

TCM Summerschool **2008** in Beijing, China (organized by the University of Graz, Department of Pharmacognosy)

TCM Summerschool **2009** in Chengdu, China (organized by the University of Vienna and the University of Innsbruck, Depts. of Pharmacognosy)

Maintenance of the homepage of the Department of Pharmacognosy (MS Frontpage)

## Publications

**Singhuber J**, Zhu M, Prinz S, Kopp B. *Aconitum* in Traditional Chinese Medicine - a valuable drug or an unpredictable risk? *J Ethnopharmacol* 126(1): 18-30, 2009.  (IF 2.322, Q1):

Özmen A, Bauer S, Gridling M, **Singhuber J**, Krasteva S, Madlener S, Vo TPN, Stark N, Saiko P, Fritzer-Szekeres M, Szekeres T, Askin-Celik T, Krenn L, Krupitza G. *In vitro* anti-neoplastic activity of the ethno-pharmacological plant *Hypericum adenotrichum* Spach endemic to western Turkey. **Oncology Reports** 22(4): 845-852, 2009.  (IF 1.588, Q3

accepted in *Frontiers in Biosciences* (IF 3.736, Q2):
Bauer S, **Singhuber J**, Seelinger M, Unger C, Viola K, Vonach C, Giessrigl, B, Madlener S, Stark N, Wallnöfer B, Wagner K-H, Fritzer-Szekeres M, Szekeres T, Diaz R, Tut FM, Frisch R, Feistel B, Kopp B, Krupitza G, Popescu R. Separation of anti-neoplastic activities by fractionation of a Pluchea odorata extract.

submitted to *European Journal of Pharmacology* (21$^{st}$ of December 2010, IF 2.585, Q2):
Singhuber J, Baburin I, Ecker GF, Kopp B , Hering S. Insights into structure-activity relationship of GABA$_A$ receptor modulating coumarins and furanocoumarins.

in preparation: Singhuber J, Baburin I, Kählig H, Urban E, Hering S, Kopp B. GABA$_A$ receptor modulators from Chinese Herbal Medicines traditionally applied against insomnia or anxiety (*Journal of Ethnopharmacology*, IF 2.322, Q1)

in preparation: Singhuber J, Baburin I, Khom S, Urban E, Hering S, Kopp B. GABA$_A$ receptor modulators from the Chinese Herbal Drug Junci Medulla, the pith of Juncus effusus L. (*Journal of Natural Products*, IF 3.159, Q1)

## Short lectures

**Singhuber J.** *Aconitum* in TCM - a valuable drug or an unpredictable risk? *Congress of the International Society for Ethnopharmacology*, Albacete (Spain), 2010.

**Singhuber J**. Natural coumarins and furanocoumarins as positive GABAergic modulators. Young Researchers' Workshop, *58th International Congress and Annual Meeting of the Society for Medicinal Plant Research*, Berlin (Germany), 2010. Awarded talk.

**Posterpresentations**

**<u>Singhuber J</u>**, Baburin I, Hering S, Kopp B. Natural coumarins and furanocoumarins as positive GABAergic modulators. ***58th International Congress and Annual Meeting of the Society for Medicinal Plant Research***, Berlin (Germany), 2010.

Prinz S, **<u>Singhuber J</u>**, Zhu M, Kopp B. Analysis of Phenolic Acids from *Actaea* spec. by Capillary Electrophoresis. ***54th Annual Congress of the Society for Medicinal Plant Research (GA)***, Helsinki (Finland), 2006.