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Phytochemistry of Cimicifugic Acids and Associated Bases in *Cimicifuga racemosa* Root Extracts

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Abstract

Introduction—Earlier studies reported serotonergic activity for cimicifugic acids (CA) isolated from *Cimicifuga racemosa*. The discovery of strongly basic alkaloids, cimipronidines, from the active extract partition and evaluation of previously employed work-up procedures has led to the hypothesis of strong acid/base association in the extract.

Objective—Re-isolation of the CAs was desired to permit further detailed studies. Based on the acid/base association hypothesis, a new separation scheme of the active partition was required, which separates acids from associated bases.

Methodology—A new 5-HT_7 bioassay guided work-up procedure was developed that concentrates activity into one partition. The latter was subjected to a new 2-step centrifugal partitioning chromatography (CPC) method, which applies pH zone refinement gradient (pHZR CPC) to dissociate the acid/base complexes. The resulting CA fraction was subjected to a second CPC step. Fractions and compounds were monitored by ^{1}H NMR using a structure based spin-pattern analysis facilitating dereplication of the known acids. Bioassay results were obtained for the pHZR CPC fractions and for purified CAs.

Results—A new CA was characterized. While none of the pure CAs was active, the serotonergic activity was concentrated in a single pHZR CPC fraction, which was subsequently shown to contain low levels of the potent 5-HT_7 ligand, N_ω–methylserotonin.

Conclusion—This study shows that CAs are not responsible for serotonergic activity in black cohosh. New phytochemical methodology (pHZR CPC) and a sensitive dereplication method (LC-MS) led to the identification of N_ω–methylserotonin as serotonergic active principle.

Keywords

Cimicifuga (Actaea) racemosa; cimicifugic acids; counter-current chromatography (CCC); centrifugal partition chromatography (CPC); ^{1}H NMR full spin analysis; N_ω–methylserotonin; pH zone refinement counter-current chromatography (pHZR-CCC); serotonergic activity

INTRODUCTION

In the past decade, a variety of the composite molecules known as cimicifugic acids (CAs) have been isolated from *Petasites* and *Cimicifuga* species and their structure elucidations

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reported (Takahira *et al.*, 1998a; Takahira *et al.*, 1998b; Kruse *et al.*, 1999; Kusano *et al.*, 2001; Zhao *et al.*, 2002; Nuntanakorn *et al.*, 2006). The initial compound of this series of dimeric phenylpropanoic acid derivatives was named fukinolic acid (FA) after the common name of its plant source, the Japanese "fuki" (*Petasites japonicus*) (Sakamura *et al.*, 1973). However, most congeners were first reported from the genus *Cimicifuga* (syn. *Actaea*). *C. racemosa* (*Ranunculaceae*) also known as black cohosh or black snakeroot, had already been used by Native Americans and received a monograph in the first US pharmacopoeia in 1820. It was introduced into Europe in the early 18th century. In the 19th century, it emerged as an important treatment for numerous female-related conditions, and it is currently being used for alleviating menopausal symptoms that impact the quality of life, such as hot flashes, night sweats and mental disorders (Foster, 1999; Viereck *et al.*, 2005). In spite of the known beneficial effects of *C. racemosa* root extract, identification of the active principle of *C. racemosa* has represented a quite elusive challenge. Hence, a variety of *in vitro* assays have been employed to evaluate the activity of *C. racemosa* root extracts. Even though the plant is primarily being used to alleviate symptoms of menopause in women and as an alternative treatment to hormone replacement therapy (for a review see (Viereck*et al.*, 2005)), evidence for an interaction with the estrogen receptors (ER) is scarce and controversial. For example, in a previous study, an *iso*-propanol extract of *C. racemosa* had no effect on uterine weight or estrogen-like specific cell differentiation in rats (Burdette *et al.*, 2003). Moreover, it has been reported that methanol, *iso*-propanol, ethanol or hydrolyzed ethanol extracts fail to bind to either recombinant human ER-α or -β receptors (Dixon-Shanies and Shaikh, 1999; Liu *et al.*, 2001; Bodinet and Freudenstein, 2002; Zierau *et al.*, 2002; Jarry *et al.*, 2003). However, an ethanol extract of *C. racemosa* was shown to displace estradiol from unspecified binding sites in human endometrium cytosolic preparations (Jarry *et al.*, 2003), and a recent report described agonistic effects of the ethyl acetate-soluble fraction of a hydro-alcoholic extract (*C. racemosa* root/rhizome, Res Pharma, Trezzo sull'Adda, MI-Italy) on ER-α expressed in yeast (Bolle *et al.*, 2007).

In contrast to HT (hormone-replacement therapy), which is known to increase the risk of developing breast cancer, as shown in a recent randomized controlled trial by the Women's Health Initiative (Rossouw *et al.*, 2002), *C. racemosa* extracts exhibit antiproliferative effects on MCF-7 and T47D breast cancer cells *in vitro* (Liu *et al.*, 2001). In a study employing a phytochemical procedure, Hostanska *et al.* (Hostanska *et al.*, 2004) showed that the induction of apoptosis in MCF-7 cancer cells of an *iso*-propanolic roots extract of *C. racemosa* can be attributed to both its triterpene as well as its cinnamic acid ester partition. In an attempt to mimic liver metabolism that would occur *in vivo* after ingestion of the extract, the authors demonstrated that the activity remained unaffected by incubation with a hepatic microsomal fraction (Hostanska *et al.*, 2004). Competitive binding of an ethanol (70% v/v) extract to the dopamine D_2 receptor and to serotonin 5-HT_{1A}, 5-HT_{1D} and 5-HT_7 receptors by *iso*-propanol (40% v/v), ethanol (75% v/v), and methanol (100%) extracts has been observed (Burdette *et al.*, 2003; Jarry *et al.*, 2003). The 5-HT receptor subtypes 1A and 7, found in the hypothalamus, have been associated with the generation of hot flashes (Burdette *et al.*, 2003; Sipe *et al.*, 2004), and in several clinical trials (Joffe *et al.*, 2007; Zanardi *et al.*, 2007) the efficacy of selective serotonin reuptake inhibitors (SSRI) in the treatment of hot flashes has been demonstrated (for a review see (Albertazzi, 2006)).

Considering the above and previous studies, a 5-HT_7 receptor-binding assay-guided fractionation scheme was employed to further investigate the extracts and identify the serotonergic compound(s) from *C. racemosa* roots. A liquid-liquid partition of the extract that contained the phenolic compounds such as cinnamic acid esters showed the highest 5-HT_7 receptor binding affinity and, thus, was used for further isolation of single compounds. Furthermore, as CAs represent unique *Cimicifuga* phytochemicals, another goal was to assess their role in the 5-HT_7 binding affinity of black cohosh. Because CAs are commercially

unavailable, isolation was necessary. The method was expected to separate the acidic constituents from co-occurring, associated bases (e.g., cimipronidines) (Fabricant *et al.*, 2005) in the first step, and then provide enrichment of the acids in a second step. To this end, a liquid-liquid chromatographic method was developed that provides purified CAs in a two-step scheme. A new CA derivative **1** was identified in the course of developing a ^{1}H NMR spin pattern analysis method aimed at identifying CAs in mixtures such as enriched fractions. As part of this effort, a unified nomenclature was developed that was consistent with the structural properties of the subunits of the molecules.

EXPERIMENTAL

Materials

Authentic *C. racemosa* (L.) Nutt. roots/rhizomes were acquired through Naturex (formerly Pure World Botanicals), South Hackensack, NJ, USA (lot#: 9-3999). A voucher specimen has been deposited at the Field Museum of Natural History Herbarium, Chicago, IL, USA. All chemicals and solvents were purchased from Fisher Scientific (Hampton, NH, USA) or Sigma–Aldrich (St. Louis, MO, USA).

Extraction and fractionation

The dried rhizomes/roots (1 kg) of *C. racemosa* were milled, homogenized, and exhaustively extracted with methanol at room temperature. The crude organic extract was concentrated in vacuo (< 40° C) to yield 173 g of syrupy residue. The residue was reconstituted in deionized water (250 mL) and partitioned with ethyl acetate (20 × 400 mL; 42 g residue). The water partition was subjected to column chromatography on Amberlite XAD-2 (1.735 kg) equilibrated with water. Elution with water (19 L) and methanol (9 L) yielded 2 fractions of 120 g and 6 g dry weight, respectively. Of the methanol-soluble fraction, 1.5 g were further subjected to centrifugal partitioning chromatography (CPC) using the solvent system ethyl acetate-butanol- water (1:4:5), which shows a volume ratio upper phase to lower phase of 54/46. After separating the two phases, the lower phase was used as mobile phase, adding 0.3% (v/v) of a 28% aqueous ammonia solution. Trifluoroacetic acid (TFA) [0.1 % (v/v)] was added to the stationary phase prior to loading the column. The sample was dissolved in 5 mL of stationary phase and injected. Immediately afterwards, mobile phase was pumped through the column at a flow-rate of 4 mL/min and 1200 rpm in a head to tail mode. The stationary phase retention volume ratio, S*f*, was 0.38 (70 mL). The eluate was monitored at 354 nm and 254 nm, fractions (4 mL = 1 min) were collected starting 15 min after injection using a fraction collector and combined according to TLC analysis, pH and UV profile. Test tubes 44–73 (K = 0.9–2.5) contained the CAs and were further subjected to CPC in a HEMWat +5 system [*n*-hexanes-ethyl acetate-methanol-water (3:7:3:7)] (Friesen and Pauli, 2007a). TFA [0.2 % (v/v)] was added to both phases prior to separation. The column was spinning at 1100 rpm and allowed to equilibrate before injecting the sample. The stationary phase retention volume ratio was 0.57. The sample was dissolved in 4 mL of a mixture of both phases and run in a tail to head mode using the lower phase as the stationary phase. The eluate was monitored at 354 nm, the flow-rate was 4 mL/min, and fractions (4 mL) were collected using a fraction collector. The separation was run in Elution-Extrusion CPC mode (Berthod *et al.*, 2007), and the solvents were switched to extrusion at K = 3 (392 mL).

MS and NMR spectrometry

LC-MS analyses were carried out using negative ion electrospray on a Thermo Scientific (Waltham, MS, USA) LTQ linear ion trap mass spectrometer. Product ion spectra were obtained using helium as a collision gas using normalized collision energy of 35% Activation q was set at 0.25. One dimensional (1D) ^{1}H and ^{13}C spectra as well as homo- and heteronuclear 2D NMR experiments (gCOSY, gHSQC, and gHMBC) were measured on a Bruker (Karlsruhe,

Germany) AVANCE-400 NMR spectrometer, equipped with a auto-tune broad band probe, in 5 mm NMR tubes with the samples dissolved in methanol-d_4. The temperature was maintained at 20 °C. Chemical shifts were expressed relative to tetramethylsilane (TMS) using the solvent signal (δ_H = 3.210 ppm, δ_C = 49.2 ppm relative to TMS) as internal standard. NMR data were processed and simulated (NS mode) with NUTS (Acorn NMR Inc.; www.acronnmr.com). Line resolution was enhanced by Lorentz-Gauss (LG) transformation using individually determined parameters (WV mode). Digital resolution was better than 0.0004 ppm and 0.008 ppm for ^{1}H and ^{13}C, respectively, and chemical shifts are reported with three and two decimal places on the ppm scale, respectively, to appropriately reflect relative chemical shifts of signals.

Chromatography

Counter-current chromatography (CCC) was carried out using a 185 mL centrifugal partition chromatography (CPC) column (FCPC, CPC; Kromaton, Angers, France), an HPLC solvent pump (Series III, LabAlliance, State College, PA, USA), a dual-wavelength UV detector (Shimadzu, Columbia, MD, UDA; SPD-10A VP, UV Vis Detector), a pH meter (Navi F-51, Horiba; Irvine, CA, USA), and a LabAlliance Foxy Jr. fraction collector. EECCC chromatograms and K values were calculated using previously published calculations and reciprocal symmetry plots (Friesen and Pauli, 2007b). For fractionation monitoring, analytical thin layer chromatography (TLC) was performed at room temperature on precoated silica gel 60 F254 glass plates (20 × 20 cm; Merck, Darmstadt, Germany). Fractions were monitored under UV light of 254 and 365 nm, before and after spraying with Naturstoff reagent (1% 2-aminoethyl-phenylborinate in methanol).

Serotonergic Evaluation

Briefly, evaluation of serotonergic activity was performed in a competitive serotonin receptor-binding assay using a Chinese hamster ovary (CHO) cell line stably transfected with the human 5-HT$_7$ serotonin receptor. The tested fractions and pure CAs competed with [^{3}H] LSD (lysergic acid diethylamide, 5.67 nM), and receptor binding inhibition was measured. Details will be published separately (Powell *et al.*, 2008).

RESULTS AND DISCUSSION

While *C. racemosa* root extracts have known beneficial effects on menopausal complaints, *in vitro* results indicate that there is little or no estrogenic activity depending on the type of assay employed, leaving open the questions of the active principle and mode of action. Based on previous studies, a plausible approach is to assess alternative modes of action by elucidating the serotonergic activity of black cohosh extracts. In this respect, CAs seemed interesting phytochemical targets, since they are abundant in the highly polar serotonergic fraction (Fabricant, 2006) and had previously shown 5-HT$_7$ activity (Burdette, 2003) and antioxidant effects (Burdette *et al.*, 2002). In addition, reports in the literature, as well as our own data, provided strong support for the hypothesis that in the extract acidic and basic constituents exist as associated complexes (molecular recognition complexes) (Shefter, 1969; Martin *et al.*, 1986; Chen *et al.*, 2002; Blondeau *et al.*, 2007). Based on this phytochemical behavior, one working hypothesis was that this interaction might play a role in explaining the previously reported 5-HT$_7$ activity of CAs (Burdette, 2003). Accordingly, it was important to use isolation conditions capable of dissociating acid-base complexes. This was achieved by designing a liquid-liquid based isolation method, which employs a pH gradient to affect the elution of compounds according to their pK_A values. In addition to using an innovative separation approach, characterization of a new CA (**1**) was achieved by employing a ^{1}H NMR fingerprinting method, which facilitates rapid dereplication of both known and unknown CAs. A systematic nomenclature (Figure 1) is introduced for congeneric CAs, which covers the new

compound (**1**) and previously described CAs studied herein, as well as CAs that are likely to exist but remain to be characterized.

Isolation of CAs and Identification of CA KS (1)

According to preliminary TLC analysis, the most 5-HT_7 active partition [3.5% (m/m)] of the total methanol extract contained primarily phenolic components such as monomeric cinnamic acid derivatives, including CAs. The isolation of acidic compounds presents a number of challenges: Acidic molecules have been shown to associate with nitrogen-containing, basic phytoconstituents such as cimipronidine (Fabricant *et al.*, 2005). This was corroborated by the observation of several NOESY cross-peaks between CA and cimipronidine signals (Figure 2): In addition to the expected *intra*molecular NOE interactions within the respective observed molecules, additional *inter*molecular cross-peaks were observed between the aromatic signals belonging to the CAs and peaks in the high-field region belonging to cimipronidine. The COSY spectrum (S5, Supplementary Material), showing through-bond correlations between protons, reveals only cross-peaks within each molecule. In contrast, the NOESY experiments reveal spatial proximity of the protons, which are “linked” by cross-peaks that arise between protons in the CAs and cimipronidine and thus provide experimental evidence supporting the hypothesis of their complex-formation in the extract. The formation of molecular recognition complexes has been previously described in the literature (Shefter, 1969; Martin *et al.*, 1986). It should be further noted that well-established alkaloid isolation procedures (Svoboda, 1961; Mukhopadhyay *et al.*, 1981) have taken association effects into account by drastically altering the pH of the extract and subsequently extracting with organic solvents. Successful isolation of both the acids and the N-containing bases requires dissociation of the recognition complexes as a component of the isolation process. Interestingly, prior to the report of the isolation and identification of cimipronidine from *C. racemosa* (Fabricant *et al.*, 2005), earlier published isolation schemes employed acidic conditions for this purpose (Takahira *et al.*, 1998b; Kruse *et al.*, 1999; Kusano *et al.*, 2001; Stromeier *et al.*, 2005).

Extending the hypothesis that bases associated with the CAs were responsible for the observed 5-HT_7 activity, a fractionation methodology was required, which would preferably be loss-free and readily enable bioassay of resulting fractions. The application of a pH gradient to the chromatography would likely address the dissociation of acid-base associates. Therefore, a pH zone refined liquid-liquid chromatography method (Ito, 1996), utilizing the hydrostatic variant, pHZR-CPC, was developed that meets the challenge of full sample recovery and separation of the acids from their basic complex-partners. The pHZR CPC step yielded 7 combined fractions that eluted from the column according to pH starting from acidic around pH 1.5 to basic around pH 9.5. Fractions were combined according to their pH, TLC, and UV profiles, and subsequently tested in the 5-HT_7 competitive binding assay (Figure 3) (Powell *et al.*, 2008). Only fraction 7 exhibited activity. Surprisingly, in contrast to previous reports on their activity in the respective assay (Burdette, 2003), the fraction containing the CAs was found to be inactive. Using LC-MS analysis of the active fraction 7 eventually led to the identification of N_{ω}-methylserotonin (syn. N-methyl-5-hydroxytryptamine, Fig. 3) as the active component of the extract with a calculated content of 31 ppm in a 75% ethanol extract. Its identity was determined by means of LC-MS versus an authentic reference standard (Powell *et al.*, 2008).

Since the CAs had previously been found to be active, isolation was still desirable in order to be able to subject pure compounds to 5-HT_7 binding assays and verify the observed (in)activity of the crude CA fraction. Fraction 4, which primarily contained the CAs, was subjected to a second, pH-uncontrolled CPC isolation step to afford quantities of the known compounds **4** (K = 9.6–23.0) (23 mg), **2** (K = 3.4–3.7) (8 mg), **3** (K = 4.8–6.0) (110 mg), **5** (K = 1.3–1.4) (3 mg) and **6** (K = 2.2–2.5) (12 mg). The structures of the isolates were dereplicated by TLC and ^{1}H NMR analysis and comparison with published (Takahira *et al.*, 1998a; Takahira *et*

al., 1998b) and deposited digital NMR reference data from previous studies (Kruse, 2000; Stromeier, 2003). Along with the known compounds, 6 mg of a composite fraction (K = 6.4–8.7) were obtained, from which the new derivative, **1**, could be unambiguously identified by ^{1}H NMR spin pattern analysis, subsequent 2D NMR (S1, S2, S3; Supplementary Material), and LC-MS analysis.

During the structural analysis of **1**, it became evident that 1D ^{1}H NMR spectra alone can serve as highly characteristic fingerprints and are conclusive of the structure of each individual CA. As indicated in the spectrum of **1** (Figure 4), there are three distinct spectral regions: a singlet for H-2 appearing at about δ 5.5, two doublets for H-4a and 4b appearing around δ 3 (Table 1), and two doublets for the olefinic protons, H-2'' and H-3'', at about δ 6.4 and δ 7.7, respectively, which are shifted downfield when compared to other cimicifugic acids (Table 2 and Table 3). This gave a first indication that the B ring of **1** possesses a greater electron deficiency than the other CAs. Moreover, the aromatic ring of moiety A, Ar_A (Figure 1A), showed the typical *m*,*p*-dihydroxylated AMX signal pattern: a doublet (J = 1.9 Hz) at δ 6.639 assigned to H-2', a doublet for H-5' at δ 6.540 (J = 8.1 Hz), and an up-field doublet of doublets for H-6' at δ 6.490 (J = 1.9 Hz and J = 8.1 Hz), coupled to both H-2' and H-5', respectively (Figure 5). For the other aromatic moiety, Ar_B, only one singlet accounting for two protons at δ 6.880 and one methoxy singlet at δ 3.800 accounting for two methoxy groups were observed. The lack of COSY cross-peaks between the B-ring protons suggested a symmetric substitution of Ar_B, as it found in sinapic acid (Figure 1CC), and was further supported by the symmetry of the δ_H and δ_C shifts of Ar_B (Table 1). HMBC cross peaks observed between H-2'''/H-6''' and C-6'''/C-2''', respectively, but also with C-4''' and the olefinic C-3'' provided further evidence for an S-type substitution pattern for Ar_B (Figure 4). Ignoring any substituent chemical shift (s.c.s.) effects from esterification, the ^{1}H and ^{13}C NMR data for the aromatic B-moiety of the molecule were found to be in good agreement with the published NMR data for sinapic acid (Ternai and Markham, 1976;Bashir *et al.*, 1993).

Since the structure elucidation of **1** was conducted on an enriched fraction, which also contained CA KI (**3**) and small amounts of CA KK (**4**), LC-MS analysis was performed to provide further support for the structural assignment. During negative ion electrospray, **1** formed an abundant deprotonated molecule of *m/z* 477. Upon collision-induced dissociation, the major fragment ion was detected at *m/z* 253, corresponding to the elimination of neutral molecule of sinapic acid. Complementary fragment ion representing deprotonated sinapic acid was observed at *m/z* 223 (S6, Supplementary Material). This fragmentation pattern is similar to that observed for other cimicifugic acid analogs (Li *et al.*, 2003). Based on the 1D and 2D NMR and LC-MS experiments, compound **1** was identified as the fukiic acid ester of sinapic acid (syn. 2-*O*-sinapoyl fukiic acid, 2-*O*-sinapoyl-3-hydroxy-4-(3',4'-dihydroxyphenyl)-3-carboxybutyric acid), for which the name cimicifugic acid KS is proposed (Figure 1B). While a detailed evaluation of the 5-HT_7 activity will be published elsewhere (Powell *et al.*, 2008), it can be summarized that the CAs show only moderate serotonergic activity. Thereby, the cimicifugic acid KK (syn. fukinolic acid) showed a slightly higher 5-HT_7 inhibition (IC_{50} = 100 μM) than KF (syn CA A), KI (syn. CA B), which are esters of fukiic acid (3',4'-dihydroxybenzyl tartaric acid) and PF (syn. CA E), PI (syn. CA F), which represent esters of piscidic acid (4'-hydroxybenzyl tartaric acid) (IC_{50} > 500 μM).

Spin Pattern Analysis

From a ^{1}H NMR spin analysis perspective, CAs constitute a class of compounds with the conserved structural element of a linker between the two aromatic rings, consisting of H-2, H-4a, H-4b, H-2'', and H-3''. The substituted aromatic rings are more variable, but still clearly laid out (Figure 1A). All congeneric CAs have in common the presence of a singlet for H-2 at δ 5.53, two doublets for H-4a at δ 2.81 and H-4b at δ 2.93, and two doublets for the olefinic

protons H-2'' at δ 6.45 and H-3'' at δ 7.69 (Figure 45). It is mainly the substitution patterns on the aromatic rings that serve to distinguish the CAs and give rise to spectral differences. The aromatic substitution pattern exerts overall only minor influences on the chemical shifts of the remaining parts of the molecule, resulting in conserved NMR sub-spectral elements, which exhibit only minor differences from compound to compound. However, these subtle changes were found to be structurally characteristic. For example, the chemical shifts of the methylene protons H-4a and H-4b are effected by the Ar_A substitution pattern, whereas the olefinic protons H-2'' and H-3'' are very sensitive to the substitution of the B-ring, permitting conclusions about the number of hydroxyl-groups on the rings and the occurrence of methoxylation (Figure 5; Table 2; Table 3). Of all protons in the molecule, proton H-2 has the most unaffected chemical shift: it is the most remote, separated from both sites of structural variation in the aromatic rings and, thus, can serve as an internal reference signal when making comparisons of the ^{1}H-NMR spectra of CAs (Figure 5; Table 2; Table 3).

Similarly substituted aromatics are recognized by their similar ^{1}H-NMR signal patterns. E.g. the A-rings of monooxygenated CAs give rise to an AA'XX' resonance pattern that is distinctly different from the pattern of their *m*,*p*-dioxygenated AMX A-ring counterparts (Ar_A, Figure 5). The position of methoxylation as opposed to hydroxylation has a characteristic effect on the chemical shifts of the signals of the AMX spin system, as can be seen for the signals of the aromatic ring of moiety B (Ar_B, Figure 5). Finally, the signals of the methoxy protons themselves exhibit shift variation that is indicative of their position in the aromatic ring (Ar_B, Figure 5).

As is shown in Table 2 and Table 3, the shift differences between, e.g., the pair of H-2'/−3' in the isoferulic **3** and **6** versus the ferulic acid derivatives **2** and **5** are subtle. Therefore, in order to unambiguously identify a CA, the comparison of very small differences in chemical shift values requires a reference. It is our observation that mixtures of two or more CAs can provide reference for one another. In order to compare dereplicated ^{1}H-NMR spectra with a set of previously recorded spectra, it is advantageous to have access to raw NMR data for all, which can be processed in the same way and using the H-2 signal as internal reference. The latter could be illustrated for CA PK, of which an original ^{1}H NMR FID was made available by the authors of a previous study (Stromeier, 2003). The consistency of the NMR spectra was remarkable, even though the FIDs had been collected on a different spectrometer and in a remote laboratory (Stromeier, 2003). Since the observed chemical shift differences between any two CAs are generally small, it is crucial to reference the spectra to the same signal. Suitable references are either tetramethylsilane (TMS) and/or the H-2 signal as noted earlier. The lack of a uniform chemical shift reference may explain why CA NMR data originating from the literature may not readily lend themselves for direct comparison of chemical shift values. As can be seen for the data of CA KP and CA KD, their chemical shifts do not appear consistent with all the other compounds (Table 2 and Table 3). This may partly be attributed to the reduced precision resulting from reporting of chemical shifts to only 2 decimal points and, in the case of CA KD, also to ambiguous referencing of the spectrum. This underlines the role of proper reporting of the digital resolution of ^{1}H NMR spectra (see Materials and Methods), in particular with regard to *relative* chemical shifts.

In addition, it should be noted that the values for the ^{1}H NMR shifts depend on many factors including the temperature of the sample, its solution concentration and the intermolecular interactions of molecules in mixed samples which can result in slightly different shift values when compared to the chemical shifts of the pure compounds in the same solvent. However, our findings indicate that at concentrations of 20 mM, which were used in this study, no decisive chemical shift effects are observed on the ^{1}H NMR spectrum. In order to evaluate the influence of sample concentrations on the proton chemical shifts of CAs, a solution of CA KI was examined at five concentrations (Table 4). As can be seen, the shift differences are negligible

over the measured range of concentrations. Only at higher concentrations above 40 mM shift migrations could be observed, which are likely due to intermolecular interactions in solution (Figure 6). This indicates π-stacking, which has been shown to occur as a result of increased electron densities in the aromatic ring-systems (Hunter *et al*., 1990). Hence, systems with low electron density carrying electronegative substituents will be attracted to systems with high electron density. This phenomenon has been discussed as the cause of chemical shift changes in interacting portions of aromatic molecules (Hunter *et al.*, 1990). A study by Mitra *et al.* demonstrated that conventional π-stacking has no impact on concentration dependent shift variations of substituted quinoline systems (Mitra *et al.*, 1998). In contrast, a study conducted by Katsuyama *et al.* attributed the observed concentration-dependent shift variations of alicyclic, conjugated alkaloids to the π-stacking phenomenon (Katsuyama *et al*., 2003). These apparent contradictions may suggest that π-stacking does not generally occur for all π-systems, but depends on the electron densities of the molecules. In order to explain the observed small concentration dependent chemical shifts of CAs, electron density mapping studies would be required to determine the precise nature of the interactions of these molecules. For practical purposes, it can be concluded that for comparative analyses decisive shift variations can be avoided when working with sample concentrations below 40 mM.

Since congeneric CAs are frequently present as mixtures, an additional study was conducted to show whether different CAs present in the same sample influence each other's chemical shifts. Samples, prepared by mixing solutions of CA KI and CA KS in varying ratios were examined (Table 5). This study was performed at a concentration of 19 mM, well below the previously observed 40 mM threshold for intermolecular interactions. The chemical shift values observed for any of the mixtures of CA KI and CA KS (Table 5) did not show any significant variability according to differences in ratio. However, with increasing sample concentrations (Table 6) shift variation tendencies similar to those observed for the pure CA KI sample became apparent. Therefore, it can be concluded that CAs do not interfere with each other at a low concentration. At concentrations above 40 mM, however, similar interactions take place as observed before for CA KI alone at comparable concentrations. This suggests that it has little impact, whether the sample is a mixture of acids or a pure CA.

Finally it must be mentioned that temperature represents another important variable that can exert and influence on the ^{1}H shift values (Mitra *et al*., 1998). Clearly, the temperature should be noted when making shift assignments, especially if comparison of datasets is intended. Routinely, the default temperature setting of the spectrometer is more or less constant, and in the case of the present study was 20° C.

Structure Based Nomenclature for CAs

Since the initial discovery of fukinolic acid (FA), historical nomenclature has evolved by giving FA congeners isolated from *Cimicifuga* species the names of cimicifugic acids (CAs), adding alphabetical letters in the order of their discovery. Structurally, FA/CAs they are condensation products (Figure 1) of a glycoloyl phenylpropanoid acid moiety, such as fukiic acid, and a cinnamoyl moiety, such as caffeic acid, which in the given example yields FA. The two main building blocks can exhibit variations in the substitution pattern of their respective aromatic rings. Therefore, a number of resulting structural combinations are possible (Figure 1). As yet, only a relatively small number of these molecules have been reported in the literature and a far greater number remains to be discovered.

Prompted by the discovery of the new CA, CA KS (**1**), and in order to directly connect structural features of CAs with observed NMR spectral properties, a systematic naming scheme was developed for CAs. According to this scheme, two letters are added to the name of the compound class CA, to indicate the substitution pattern of the two aromatic rings Ar_A and Ar_B, respectively (Figure 1B). Each letter originates from the abbreviation of the trivial name

of the similarly substituted phenylpropanoic moiety that forms the respective part of the molecule (Figure 1C). The proposed nomenclature reflects the structure of the dimeric molecules and simplifies their spectroscopic characterization in the future.

While the biosynthetic pathway of the CAs in plants still requires further investigation, Hasa and Tazaki. have shown in a ^{13}C labeled feeding study with *Petasites japonicus* that the A moiety of CA KK is derived from tyrosine, which is conjugated with acetic acid or acetyl-CoA to form piscidic acid. The product further condensates with the cinnamoyl B-portion of the final molecule, which is derived from phenylalanine (Figure 1A) (Hasa and Tazaki, 2004). The B part, a cinnamic acid derivative, appears to be biosynthetically more variable. Cinnamic acid derivatives are widely dispersed in the plant kingdom and exhibit considerable variation in the substitution pattern of the aromatic ring. In contrast, only two of the possible variations of the A moiety of CAs, namely the *p*-hydroxylated and the *m,p*-dihydroxylated derivatives of the glycoloyl-phenylpropanoic acid moiety, piscidic acid and fukiic acid, have so far been reported. Translated into the new nomenclature scheme, this means that only the P and the K series are known and, thus, illustrates the potential for further discovery (Figure 1B and C). Another benefit of the proposed nomenclature is that it is universally applicable to other composite polyphenols such as rosmarinic acids, cimiciphenols and cimiciglycolates, of which the latter two are also found in *Cimicifuga* species (Figure 1C).

The newly developed pHZR CPC method is fit for the purpose of separating the highly polar serotonergic fraction of black cohosh extract into 7 sub-fractions according to their pK_A values. This led to the concentration of almost all of the 5-HT_7 activity (Powell *et al.*, 2008) in the most basic fraction (Figure 3), and resolved of *Cimicifuga* phenolic acids from bases such as cimipronidines and tryptamine alkaloids. From the concentrated CA fraction, purification and identification of one new CA (**1**) and 5 previously identified CAs free of interference with alkaloids was accomplished. Using **1** as an example, structural dereplication by systematic analysis of full ^1H NMR spin pattern demonstrated the value of high-resolution ^1H NMR fingerprints and led to the implementation of a straightforward, structure-based CA nomenclature. In addition, it was shown that the ^1H NMR chemical shifts of CAs are independent of whether the sample is a mixture of CAs or a pure CA, and are only mildly affected at sample concentrations above 40 mM. These findings support the application of ^1H NMR full spin analysis (NMR fingerprinting) for the rapid dereplicate of known CAs and future elucidation of presently unknown CAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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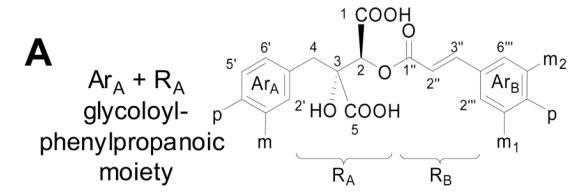
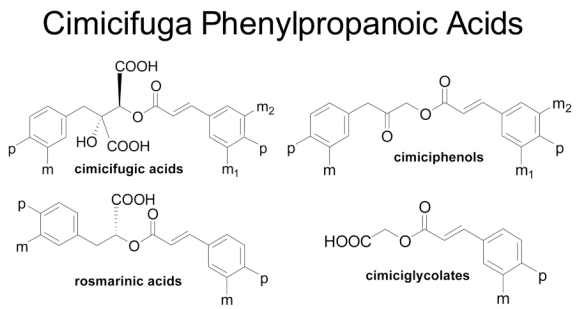


B

	A_A Substitution		A_B Substitution			New Nomenclature			1st Given Name	1st Citation
	p	m	p	m_1	m_2		A&R_A	A&R_B		
P series 1	OH	-	OH	-		CA -	P	P		
P series 2	OH	-	OCH_3	-		CA -	P	M		
P series 3	OH	-	OH	OH		CA -	P	K	CA D	Takahira *et al.*, 1998
P series 4	OH	-	OCH_3	OH		CA -	P	I	CA F	Takahira *et al.*, 1998
P series 5	OH	-	OH	OCH_3		CA -	P	F	CA E	Takahira *et al.*, 1998
P series 6	OH	-	OCH_3	OCH_3		CA -	P	D		
P series 7	OH	-	OH	OCH_3	OCH_3	CA -	P	S		
K series 1	OH	OH	OH	-		CA -	K	P	CA C	Takahira *et al.*, 1998
K series 2	OH	OH	OCH_3	-		CA -	K	M		
K series 3	OH	OH	OH	OH		CA -	K	K	FA	Sakamura *et al.*, 1973
K series 4	OH	OH	OCH_3	OH		CA -	K	I	CA B	Takahira *et al.*, 1998
K series 5	OH	OH	OH	OCH_3		CA -	K	F	CA A	Takahira *et al.*, 1998
K series 6	OH	OH	OCH_3	OCH_3		CA -	K	D	CA G	Nuntanakorn *et al.*, 2006
K series 7	OH	OH	OH	OCH_3	OCH_3	CA -	K	S		



C

aromatic ring substitution			trivial name of respective 3-benzyl-2,3-dihydropropionic acid	
p	m_1	m_2		abbreviation
-	-	-	cinnamic acid	C
OH	-	-	p-coumaric acid	P
OCH_3	-	-	p-methoxicinnamic acid	M
OH	OH	-	caffeic acid	K
OCH_3	OH	-	isoferulic acid	I
OH	OCH_3	-	ferulic acid	F
OCH_3	OCH_3	-	3,4-dimethoxycinnamic acid	D
OH	OCH_3	OCH_3	sinapic acid	S

Figure 1.

Key to atom numbering of the biosynthetic moieties (A) and proposed nomenclature (B) for cimicifugic acids (CAs) and congeneric molecules (C) typically found in *Cimicifuga* species, but also present in other plant genera.

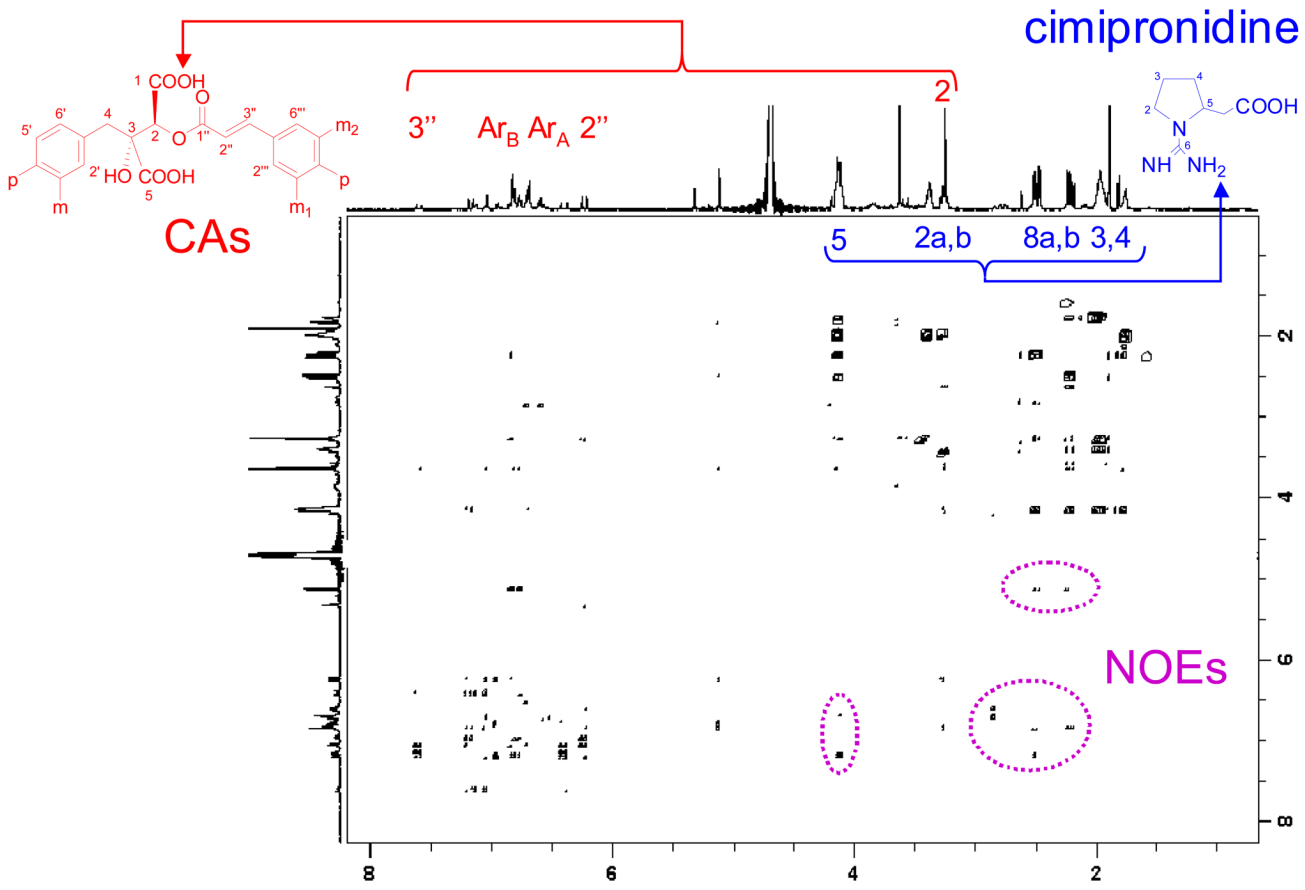


Figure 2.

The association of CAs and cimipronidine-type guanidine alkaloids in solution is consistent with the observation of several intermolecular correlations in a gNOESY spectrum of a sample fraction containing both phytochemical entities (in D_2O, reference to HDO at 4.700 ppm; diagonal and solvent signals suppressed).

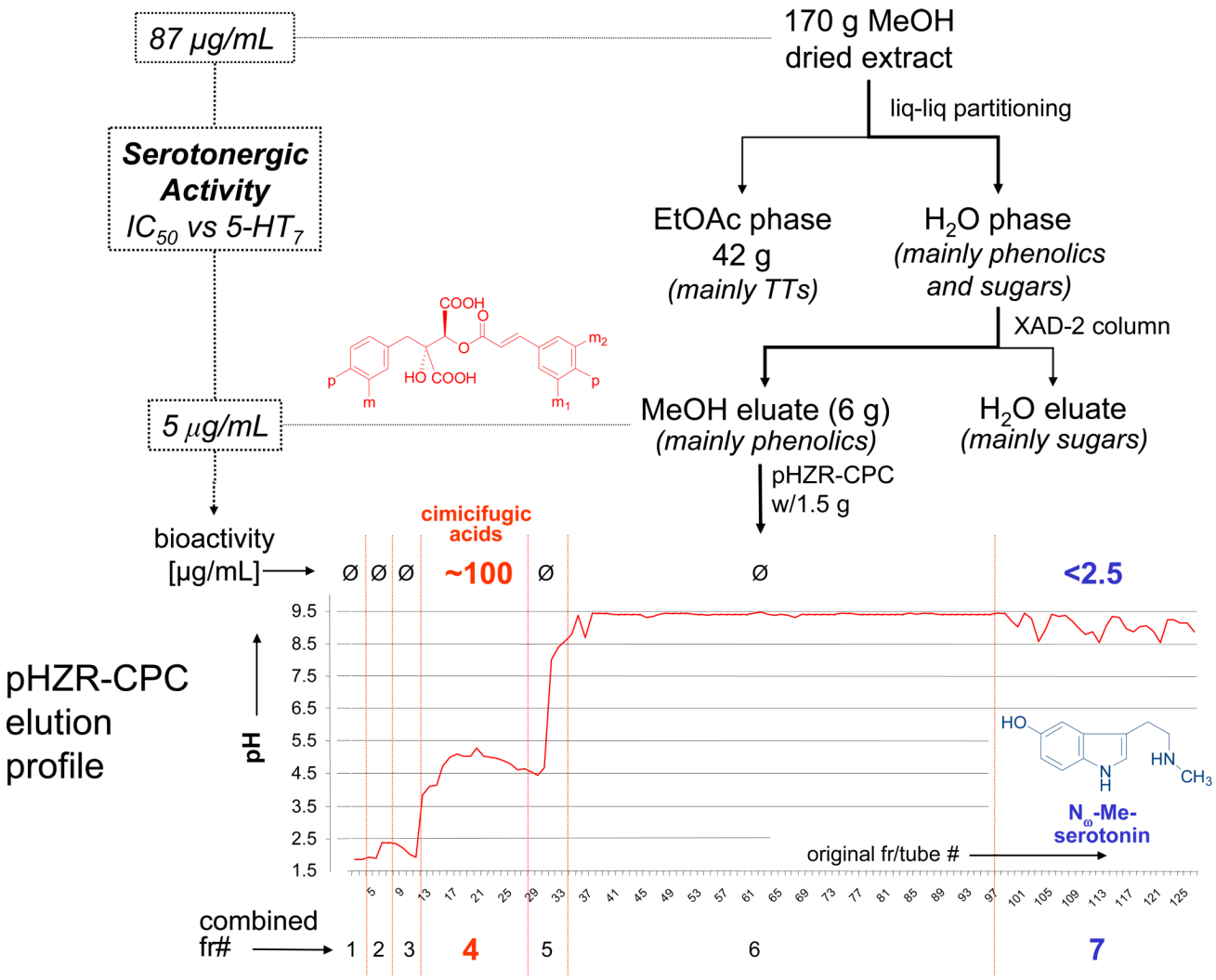


Figure 3.

5-HT$_7$ bioassay guided fractionation scheme (Ø means inactive with IC$_{50}$ > 150µg/mL) using pH Zone Refined CPC, yielding 7 fractions; subsequent LC-MS/MS analysis led to identification of N$_\omega$–methylserotonin in active fraction 7.

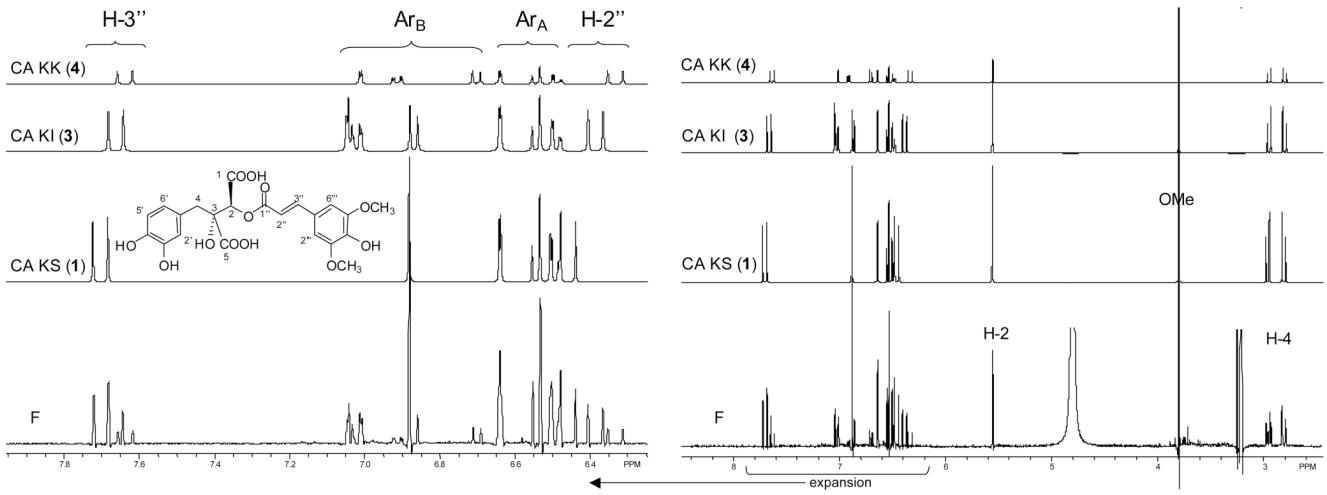


Figure 4.

A: ^{1}H NMR resonances of the CA fraction containing CA KS (**1**) as main component and simulated spectra of the constituents **1**, CA KI (**3**), and CA KK (**4**) (8:5:1 ratio; chemical shift references set to residual methanol-d_3 at 3.210 ppm, relative to TMS at 0.000 ppm as external standard)

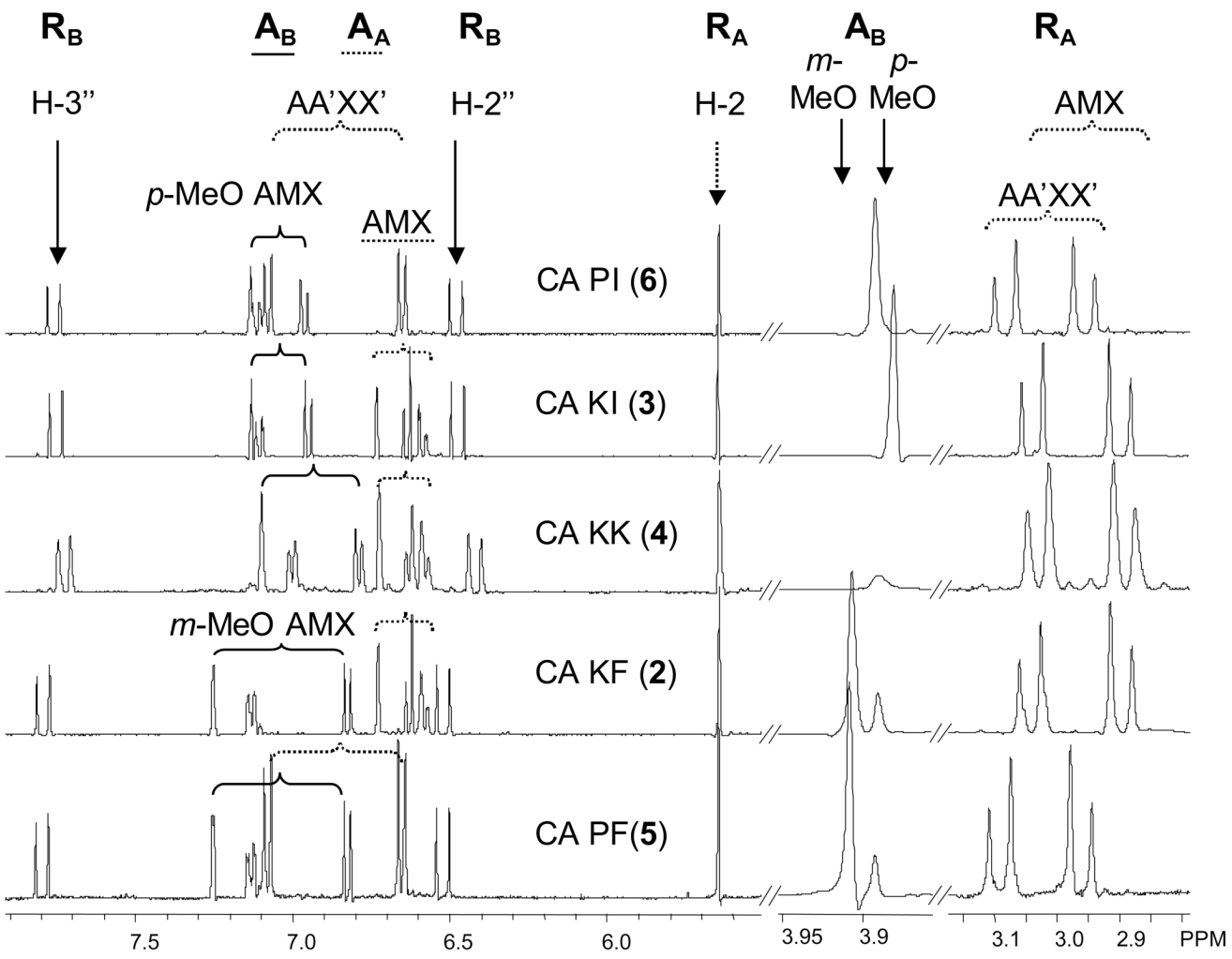


Figure 5.

^{1}H NMR spin pattern of cimicifugic acids: CA KF (**2**), CA KI (**3**), CA KK (**4**), CA PF (**5**) and CA PI (**6**) (400 MHz, CD_3OD, chemical shift reference set to 3.210 ppm for residual methanol-d_3, relative to TMS signal at 0.000 ppm as external standard)

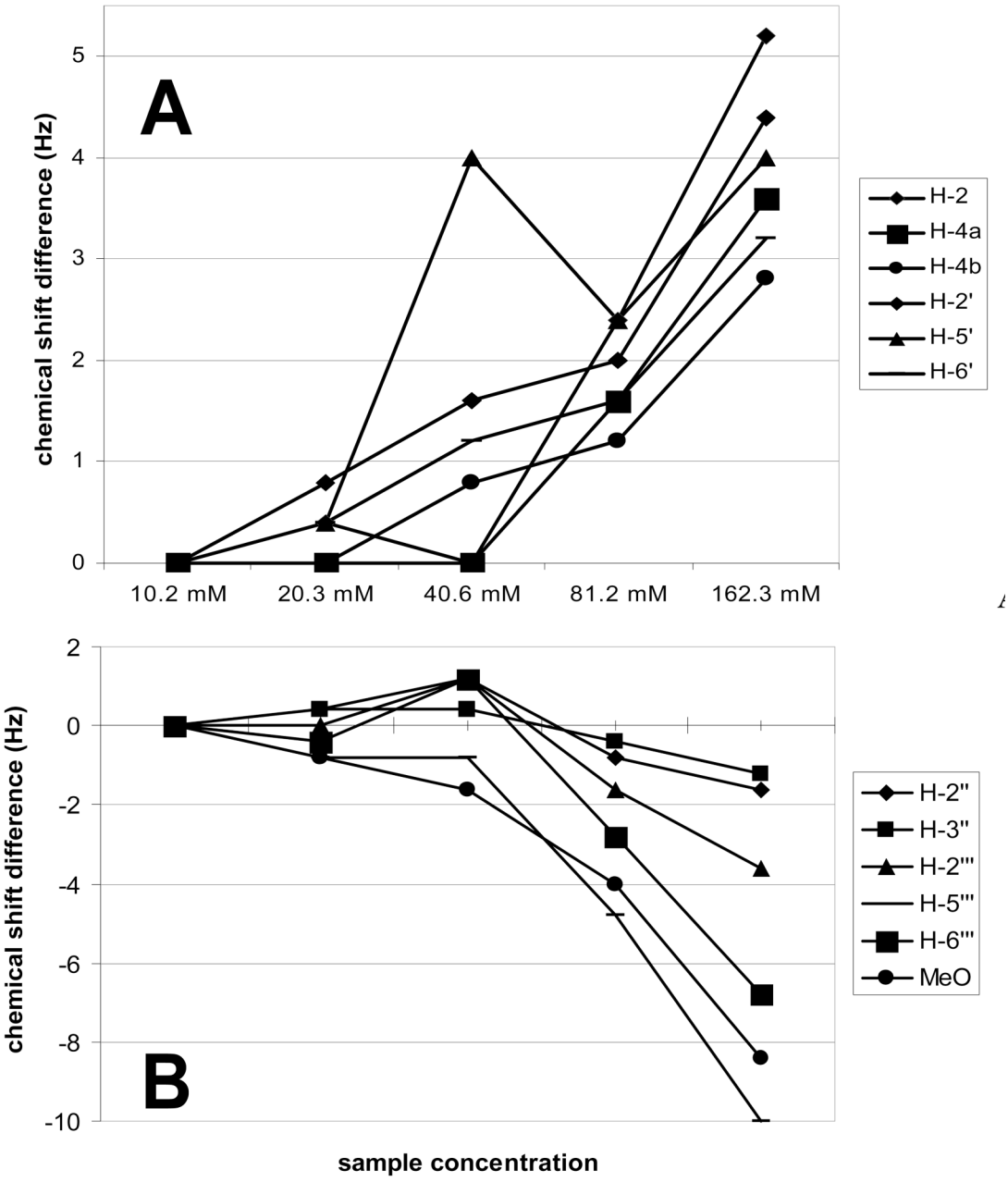


Figure 6.

A: Concentration dependence of ^{1}H NMR chemical shift differences (ΔHz) for moieties K_A and Ar_A of CA KI (**3**); B: concentration dependence of ^{1}H NMR chemical shift differences (ΔHz) for moieties K_B and Ar_B of CA KI (**3**) (positive values for ΔHz indicate down-field shift in comparison to shift values observed at 10.2 mM)

Table 1

NMR spectroscopic data of cimicifugic acid KS (**1**) (400 MHz, CD_3OD)

			CA KS (1)			
	moiety	**position**	**^1H [a] (ppm)**	**COSY correlation**	**^{13}C [b] (ppm)**	**HMBC correlation**
glycoloyl phenylpropanoic moiety	R_A	1	-		172.03	
		2	5.531		78.08	C-1’’, 1
		3	-		80.38	
		4	2.814 (*d*, *J* = 13.7 Hz)	H-4b	42.55	C-2, 6’, 2’, 1’
			2.933 (*d*, *J* = 13.7 Hz)	H-4a	-	C-6’, 2’, 1’
		5 COOH	-		175.21	
cinnamic moiety	Ar_A	1’	-		128.28	
		2’	6.639 (*d*, *J* = 1.9 Hz)		119.04	C-4, 6’, 4’
		3’	-		145.87	
		4’	-		145.45	
		5’	6.540 (*d*, *J* = 8.1 Hz)	H-6’	116.12	C-1’, 3’
		6’	6.490 (*dd*, *J* = 1.9, 8.1 Hz)	H-5’	123.27	C-2’, 4’
	R_B	1’’	-		168.39	
		2’’	6.449 (*d*, *J* = 16.0 Hz)	H-3’’	115.31	C-1’’’
		3’’	7.685 (*d*, *J* = 16.0 Hz)	H-2’’	148.48	C-2’’’/6’’’, 1’’
	Ar_B	1’’’	-		126.85	
		2’’’	6.880		107.28	C-6’’’, 4’’’, 3’’
		3’’’	-		149.69	
		4’’’	-		134.00	
		5’’’	-		149.69	
		6’’’	6.880		107.28	C-2’’’, 4’’’, 3’’
		MeO-m_1	3.800		57.29	C-3’’’
		MeO-m_2	3.800		57.29	C-5’’’

[a] Chemical shift reference: residual methanol-*d3* signal at 3.210 ppm

[b] Chemical shift reference: MeOD signal at 49.20 ppm

Table 2

^{1}H-NMR chemical shifts δ (in ppm) of the K-series of cimicifugic acids, *J* in Hz (400 MHz, CD_3OD)

moiety	position	CA-KF (2)[a]	CA-KI (3)[a]	CA-KK (4)[a]	CA-KP [b]	CA-KD [c]
R_A	2	5.559	5.555	5.553	5.65	5.66
	4	2.956 (*d*, *J* = 13.6)	2.943 (*d*, *J* = 13.9)	2.940 (*d*, *J* = 13.9)	3.04 (*d*, *J* = 13.8)	3.06 (*d*, *J* = 13.8)
		2.810 (*d*, *J* = 13.6)	2.807 (*d*, *J* = 13.9)	2.800 (*d*, *J* = 13.9)	2.92 (*d*, *J* = 13.8)	2.94 (*d*, *J* = 13.8)
Ar_A	2’	6.642 (*d*, *J* = 1.9)	6.643 (*d*, *J* = 1.8)	6.639 (*d*, *J* = 1.9)	6.75 (*d*, *J* = 2.3)	6.76 (*d*, *J* = 1.8)
	3’					
	5’	6.504 (*d*, *J* = 8.3)	6.544 (*d*, *J* = 8.5)	6.541 (*d*, *J* = 8.2)	6.65 (*d*, *J* = 8.2)	6.66 (*d*, *J* = 8.1)
	6’	6.494 (*dd*, *J* = 8.3,1.9)	6.495 (*dd*, *J* = 1.8,8.5)	6.507 (*dd*, *J* = 1.9,8.2)	6.60 (*dd*, *J* = 2.3,8.2)	6.61 (*dd*, *J* = 1.8,8.1)
R_B	2’’	6.428 (*d*, *J* = 18.8)	6.384 (*d*, *J* = 16.2)	6.333 (*d*, *J* = 15.8)	6.48 (*d*, *J* = 16.0)	6.58 (*d*, *J* = 15.9)
	3’’	7.702 (*d*, *J* = 15.8)	7.662 (*d*, *J* = 16.2)	7.639 (*d*, *J* = 15.8)	7.79 (*d*, *J* = 16.0)	7.82 (*d*, *J* = 15.9)
Ar_B	2’’’	7.156 (*d*, *J* = 1.9)	7.043 (*d*, *J* = 1.8)	7.010 (*d*, *J* = 1.9)	7.52 (*d*, *J* = 8.4)	7.30 (*d*, *J* = 1.8)
	3’’’				6.83 (*d*, *J* = 8.4)	
	5’’’	6.739 (*d*, *J* = 8.6)	6.866 (*d*, *J* = 8.5)	6.702 (*d*, *J* = 8.2)	6.83 (*d*, *J* = 8.4)	7.02 (*d*, *J* = 8.4)
	6’’’	7.039 (*dd*, *J* = 1.9,8.6)	7.018 (*dd*, *J* = 1.8,8.5)	6.913 (*dd*, *J* = 1.9,8.2)	7.52 (*d*, *J* = 8.4)	7.25 (*dd*, *J* = 1.8,8.4)
	m-MeO	3.814				3.89
	p-MeO		3.795			3.91

[a] Chemical shift reference: residual methanol-*d3* signal at 3.210 ppm (relative to TMS signal at 0.000 ppm as external standard)

[b] Data from (Takahira *et al.*, 1998b) (relative to TMS signal at 0.000 ppm as internal standard)

[c] Data from (Nuntanakorn *et al.*, 2006) (chemical shift reference undocumented)

Table 3

^{1}H-NMR chemical shifts δ (in ppm) of the P-series of cimicifugic acids, *J* in Hz (400 MHz, CD_3OD)

		CA-PF (5) [a]	CA-PI (6) [a]	CA-PK [d]
moiety	**position**			
R_A	2	5.558	5.560	5.56
	4	3.002 (*d*, *J* = 13.6)	2.995 (*d*, *J* = 13.9)	2.988 (*d*, *J* = 13.9)
		2.872 (*d*, *J* = 13.6)	2.870 (*d*, *J* = 13.9)	2.875 (*d*, *J* = 13.9)
Ar_A	2’	6.990 (*d*, *J* = 8.5)	6.992 (*d*, *J* = 8.5)	6.991 (*d*, *J* = 8.3)
	3’	6.565 (*d*, *J* = 8.5)	6.566 (*d*, *J* = 8.5)	6.569 (*d*, *J* = 8.3)
	5’	6.565 (*d*, *J* = 8.5)	6.566 (*d*, *J* = 8.5)	6.569 (*d*, *J* = 8.3)
	6’	6.990 (*d*, *J* = 8.5)	6.992 (*d*, *J* = 8.5)	6.991 (*d*, *J* = 8.3)
R_B	2’’	6.432 (*d*, *J* = 16.2)	6.389 (*d*, *J* = 15.9)	6.330 (*d*, *J* = 15.9)
	3’’	7.707 (*d*, *J* = 16.2)	7.669 (*d*, *J* = 15.9)	7.639 (*d*, *J* = 15.9)
Ar_B	2’’’	7.162 (*d*, *J* = 1.8)	7.045 (*d*, *J* = 1.8)	7.010 (*d*, *J* = 2.2)
	3’’’			
	5’’’	6.737 (*d*, *J* = 8.5)	6.869 (*d*, *J* = 8.2)	6.912 (*dd*, *J* = 2.2,8.3)
	6’’’	7.042 (*dd*, *J* = 1.8,8.5)	7.020 (*dd*, *J* = 1.8,8.2)	6.705 (*d*, *J* = 8.3)
	m-MeO	3.817		
	p-MeO		3.798	

[a] Chemical shift reference: residual methanol signal at 3.210 ppm (relative to TMS signal at 0.000 ppm as external standard)

[d] Data from (Stromeier, 2003) (relative to TMS signal at 0.000 ppm as internal standard: methanol-*d3* peak bserved at 3.210 ppm; reprocessing of original FIDs identical to other spectra [a])

Table 4

Concentration dependence of ^{1}H-NMR chemical shifts δ (in ppm) in CA KI (**3**), *J* in Hz (400 MHz, CD_3OD)

		CA KI (3)					
		^{13}C *a*	^{1}H *b*				
moiety	**position**	**40.6 mM**	**10.2 mM** *c*	**20.3 mM** *c*	**40.6 mM** *c*	**81.2 mM** *c*	**162.3 mM** *c*
R_A	1	170.798					
	2	77.855	5.5**47**	5.5**48**	5.5**47**	5.5**53**	5.5**60**
	3	80.375					
	4	42.371	2.9**40** (*d*, *J* = 13.7)	2.9**40**	2.9**40**	2.9**44**	2.9**49**
			2.8**09** (*d*, *J* = 13.7)	2.8**09**	2.8**11**	2.8**12**	2.8**16**
	5 [COOH]	174.963					
Ar_A	1’	128.127					
	2’	119.018	6.6**40** (*d*, *J* = 2.0)	6.6**42**	6.6**44**	6.6**45**	6.6**51**
	3’	145.862					
	4’	145.459					
	5’	116.191	6.5**46** (*d*, *J* = 8.1)	6.5**47**	6.5**56**	6.5**52**	6.5**56**
	6’	123.259	6.4**94** (*dd*, *J* = 8.1,2.0)	6.4**95**	6.4**97**	6.4**98**	6.5**02**
R_B	1’’	168.388					
	2’’	115.322	6.3**86** (*d*, *J* = 16.2)	6.3**87**	6.3**89**	6.3**84**	6.3**82**
	3’’	148.134	7.6**61** (*d*, *J* = 16.2)	7.6**62**	7.6**62**	7.6**60**	7.6**58**
Ar_B	1’’’	128.975					
	2’’’	115.060	7.0**49** (*d*, *J* = 2.1)	7.0**49**	7.0**52**	7.0**45**	7.0**40**
	3’’’	148.175					
	4’’’	151.965					
	5’’’	112.757	6.8**76** (*d*, *J* = 8.2)	6.8**74**	6.8**74**	6.8**64**	6.8**51**
	6’’’	123.401	7.0**26** (*dd*, *J* = 8.2,2.1)	7.0**25**	7.0**29**	7.0**19**	7.0**09**
	MeO-m_1	56.617	3.**802**	3.**800**	3.**798**	3.**792**	3.**781**

a Chemical shift reference: MeOD signal at 49.2 ppm (relative to TMS signal at 0.000 ppm as external standard)

b Chemical shift reference: residual methanol-*d3* signal at 3.210 ppm (relative to TMS signal at 0.000 ppm as external standard)

[c] Splitting and coupling constants are only mentioned once but apply for respective signals at all concentrations

Table 5

Concentration dependence of ^{1}H-NMR chemical shifts δ (in ppm) in a mixture of CA KI (**3**) and CA KF (**2**) (splitting and coupling information not shown, 400 MHz, CD_3OD)

CA KF (2)		**^{1}H chemical shifts at a sample concentration of 19 mM[a]**					
		ratios of CA KF (2) with varying amounts of (3)					
moiety	**position**	**100:0 (pure 2)**	**46:54**	**31:69**	**23:77**	**15:85**	**pure 3**
R_A	2	5.53**6**	5.55**1**	5.55**0**	5.55**0**	5.54**9**	
	4	2.93**4**	2.95**0**	2.95**0**	2.95**0**	2.94**8**	
		2.81**5**	2.80**9**	2.80**9**	2.80**9**	2.80**9**	
Ar_A	2’	6.64**4**	6.64**1**	6.64**2**	6.64**2**	6.64**2**	
	5’	6.54**6**	6.54**8**	6.54**8**	6.54**9**	6.55**2**	
	6’	6.49**7**	6.49**4**	6.49**5**	6.49**5**	6.49**6**	
R_B	2’’	6.42**2**	6.42**9**	6.42**9**	6.42**8**	6.42**9**	
	3’’	7.68**9**	7.70**3**	7.70**2**	7.70**2**	7.70**2**	
Ar_B	2’’’	7.15**6**	7.16**1**	7.16**0**	7.15**9**	7.15**9**	
	5’’’	6.74**0**	6.74**0**	6.74**0**	6.74**0**	6.74**3**	
	6’’’	7.04**0**	7.04**3**	7.04**2**	7.04**2**	7.04**4**	
	MeO	3.815	3.816	3.816	3.815	3.815	
				----------> adding increasing amounts of **3** to **2**			

CA KI (**3**)		^{1}H chemical shifts at a sample concentration of 19 mM[a]					
		ratios of CA KI (**3**) with varying amounts of (**2**)					
moiety	position	pure **2**	54:46	69:31	77:23	85:15	100:0 (pure **3**)
R_A	2		5.551	5.550	5.550	5.549	5.549
	4		2.942	2.942	2.942	2.941	2.940
			2.809	2.809	2.809	2.809	2.809

CA KF (2)		^{1}H chemical shifts at a sample concentration of 19 mM[a]					
		ratios of CA KF (2) with varying amounts of (3)					
moiety	**position**	**100:0 (pure 2)**	**46:54**	**31:69**	**23:77**	**15:85**	**pure 3**
Ar_A	2’		6.641	6.642	6.642	6.642	6.642
	5’		6.548	6.548	6.549	6.552	6.547
	6’		6.494	6.495	6.495	6.496	6.495
R_B	2’’		6.388	6.388	6.387	6.389	6.387
	3’’		7.664	7.663	7.663	7.663	7.662
Ar_B	2’’’		7.049	7.048	7.049	7.051	7.049
	5’’’		6.874	6.873	6.873	6.874	6.874
	6’’’		7.027	7.025	7.025	7.027	7.025
	MeO		3.800	3.800	3.799	3.799	3.800
			adding increasing amounts of **2** to **3** <----------				

[a] Chemical shift reference: residual methanol-*d3* signal at 3.210 ppm (relative to TMS signal at 0.000 ppm as external standard)

Table 6

Concentration dependence of the ^{1}H chemical shifts in mixtures of CA KI (**3**) and CA KF (**2**), (splitting and coupling information not shown, 400 MHz, CD_3OD)

		^{1}H chemical shifts of CA KF (2) [a]			^{1}H chemical shifts of CA KI (3) [a]		
	final sample concentrations:	**27.9 mM**	**37.2 mM**	**55.8 mM**	**27.9 mM**	**37.2 mM**	**55.8 mM**
		ratios (2):(3)			**ratios (3):(2)**		
	position	**31:69**	**23:77**	**15:85**	**69:31**	**77:23**	**85:15**
	2	5.551	5.551	5.549	5.551	5.551	5.549
R_A	4	2.950	2.950	2.949	2.943	2.942	2.941
		2.809	2.810	2.810	2.809	2.810	2.810
	2’	6.642	6.642	6.645	6.642	6.642	6.645
Ar_A	5’	6.548	6.548	6.556	6.548	6.548	6.556
	6’	6.495	6.495	6.498	6.495	6.495	6.498
R_B	2’’	6.429	6.428	6.429	6.388	6.386	6.389
	3’’	7.701	7.701	7.702	7.662	7.662	7.662
	2’’’	7.159	7.158	7.154	7.048	7.048	7.051
	5’’’	6.740	6.740	6.744	6.873	6.872	6.871
Ar_B	6’’’	7.041	7.040	7.044	7.024	7.023	7.027
	MeO				3.799	3.798	3.795
	MeO	3.815	3.814	3.811			

[a] Chemical shift reference: residual methanol-*d3* signal at 3.210 ppm (relative to TMS signal at 0.000 ppm as external standard)