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Cimipronidine, a Cyclic Guanidine Alkaloid from *Cimicifuga racemosa*†

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A new cyclic guanidine alkaloid, cimipronidine (**1**), together with the known compound fukinolic acid (**2**), was isolated from the *n*-BuOH-soluble fraction of *Cimicifuga racemosa* roots that showed 5-HT_7 receptor binding activity. Structure elucidation of **1**, a minor constituent, presented unique challenges based on its polarity, but was accomplished with the use of a combination of one- and two-dimensional NMR as well as MS analyses. The relative configuration was established by analyzing the H,H-coupling constants and the results of the 2-D gradient NOESY spectrum. The previously reported serotonergic (5-HT_7), highly polar, *n*-BuOH-soluble fraction was characterized by HPLC-ELSD and was shown to be a mixture containing the following compounds: cimicifugic acids A, B, and F, fukinolic acid, ferulic acid, isoferulic acid, and compound **1**, potentially significant as a marker compound of *C. racemosa*.

Higher plants are known to provide a diverse range of secondary metabolites.[1,2] Despite the breadth of chemodiversity, relatively few guanidine compounds have been reported from higher plants (Figure 1).[3−5] One of the earliest reported examples was galegine (**3**), which was first isolated from *Verbena encelioides* (Cav.) Benth. & Hook and later identified as the toxic component in *Galega officinalis* L. (Goat's Rue). Galegine (**3**) provided the template for the synthesis of metformin and stimulated interest in the synthesis of other biguanidine-type antidiabetic drugs.[6] Five hypotensive guanidine compounds, caracasanamides G1 (**4**), G3 (**5**), G5 (**6**), G6 (**7**), and G7 (galegine, **3**) were isolated from extracts of the leaves of *Verbesina caracasana* Rob. & Greenman. All of these guanidine compounds lowered blood pressure in rats (iv). Caracasandiamide G2 (**8**), the truxinic semisynthetic dimer of **4**, exhibited the greatest activity in lowering blood pressure and increasing cardiac inotropism.[7−10] Diverse biological activities have been reported for cyclic guanidines derived from plants, including the estrogenic activity of segetalin H (**9**) from *Vaccaria segetalis* Garcke ex Asch. and the leaf-closing activity of *p*-coumaroylagmatine (**10**) from *Albizzia julibrissin* Durazz.

In previous studies on the chemical characterization and standardization of *Cimicifuga racemosa* (L.) Nutt. (black cohosh), we reported the isolation of 40 compounds.[11−13] In the course of this work, we investigated crude black cohosh extracts, the polar *n*-BuOH-soluble fraction being shown to exhibit significant *in vitro* 5-HT_7 binding activity.[14] To successfully fractionate the polar *n*-BuOH fraction and to better separate polar constituents, nonsilica chromatographic stationary phases were used,[15−17] and C_{18} phases were considered for the final purification step only. The known constituents in the *n*-BuOH-soluble fraction, namely, cimicifugic acids A, B, and F, fukinolic acid, ferulic acid, and isoferulic acid, were identified by HPLC with in-line PDA and ELSD detection. Herein, we report the isolation and structure elucidation of a new plant-derived guanidine alkaloid, {1-[amino(imino)methyl]pyrrolidin-2-yl}acetic acid, a minor constituent trivially named cimipronidine (**1**), from this polar fraction, together with the known constituent fukinolic acid (**2**).

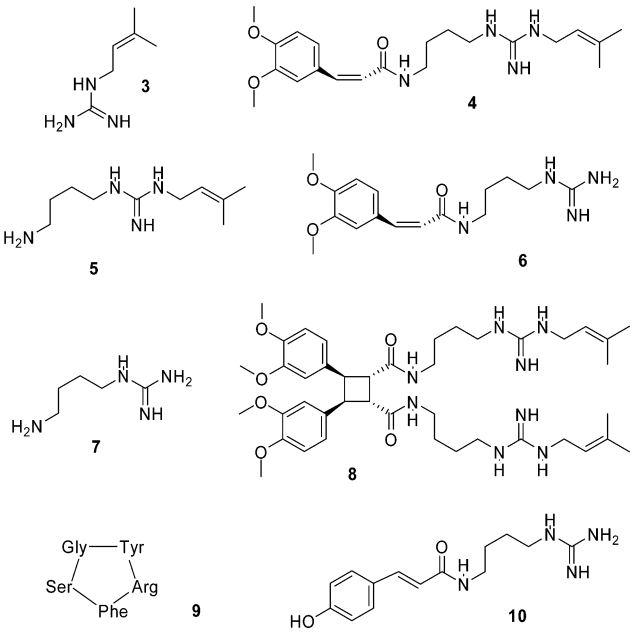

Figure 1. Naturally occurring guanidine derivatives.

In the course of our previous 5-HT_7 binding studies,[14] the *n*-BuOH-soluble fraction from black cohosh showed significant biological activity (IC_{50} 4.98 μg/mL). To better characterize the activity of this fraction, a sizable quantity was used for isolation work from wild-collected, botanically authenticated *C. racemosa* roots. The plant material was exhaustively extracted with MeOH, and the aqueous organic extract was concentrated and subsequently partitioned with $CHCl_3$/2-propanol (3:2) and *n*-BuOH. Liquid chromatography of polar organic compounds on normal-phase silica gel often yields strong adsorption of the polar

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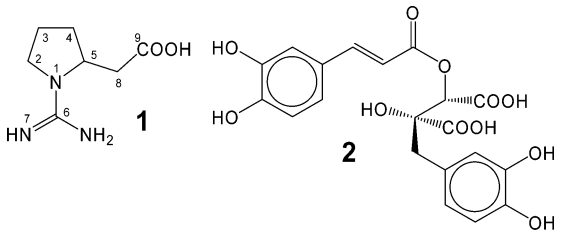

constituents. Therefore, consideration in the selection of chromatographic stationary phase and isolation strategy was necessary.[15−17] The polystyrene resin XAD-2 was selected for the second level of separation following the liquid−liquid partitioning of the *n*-BuOH-soluble fraction. The resultant fractions were further separated with MCI gel CHP20P, the third level of separation. Compounds **1** and **2** were subsequently isolated by semipreparative RP-HPLC, the fourth level of separation. Isolation of **1** occurred when eluted with 10% MeOH. Compound **2** was also isolated with 10% MeOH, but eluting approximately 25 min *after* **1**. The minimal retention of **1** by semipreparative HPLC presents a standardization problem. Many polar compounds and plant extracts are routinely separated by RP-HPLC with small volumes of acid or base (0.1−1.0%) added to improve retention time and peak shape of pH-sensitive analytes such as polyphenolics and alkaloids, respectively;[18,19] in this instance, added TFA can reduce the retention time of the highly polar material. However, the nature of this chemical behavior could be fully understood only *after* the structure had been determined.

One important piece of evidence for the structure elucidation came from the high-resolution exact mass measurement of **1**, providing a protonated molecule of *m/z* 172.1078, indicating a molecular formula of $C_7H_{14}N_3O_2$ (calculated 172.1086, error 4.7 ppm) and, therefore, a molecular formula for the zwitterion of $C_7H_{13}N_3O_2$. This molecular formula possesses a double-bond equivalency of three. Consistent with the exact mass measurements, ^{13}C NMR (D_2O) confirmed the presence of seven resonances, which included a single carboxylic acid functionality (δ_C 179.83) and a single imine functionality (δ_C 154.57). The presence of the latter was further confirmed by the fragment ion of *m/z* 130.0909 $[MH - CN_2H_2]^+$. Both of the low-field ^{13}C NMR signals, as expected, were quaternary carbon resonances. This accounts for two of the three double-bond equivalency sites. The remaining five signals, all protonated carbons (APT, gHSQC), consisted of one methine and four methylene resonances. Since there are no additional sites of unsaturation, the third double-bond equivalency requires a ring. The HMBC data obtained for **1** (D_2O) failed to reveal the presence of all of the correlations necessary for full assignment of the structure. Contrary to expectations, no HMBC correlations were observed between C-5 and the protons attached to C-2, C-3, or C-4. In addition, the expected correlations between C-2 and H-3 and H-4 were also undetected. Missing HMBC correlations have previously been noted in the course of elucidation of guanidine structures.[20] However, the absence of the expected correlations in the present case combined with the slightly broadened resonances in the proton NMR spectrum, with the exception of the signals for H-8a and H-8b, presented a difficult problem for structure elucidation. On the basis of the elucidated structure of **1**, the most probable retrospective explanation for the absence of certain cross-peak correlations from the gHMBC spectrum of **1** may be the following: (i) rapid fluctuations associated with five-membered ring dynamics, which are known to occur and which can lead to broadening of the proton resonances with concomitant introduction of short T_2's that affect the efficiency of polarization transfer from proton to carbon; (ii) the fact that typical $^2J_{H,C}$ couplings are generally reduced in magnitude relative to three-bond couplings; (iii) for the expected three-bond correlations C-5/H-2, C-5/H-3, C-4/H-2, and C-3/H-5 the dihedral angle between these protons and the indicated carbon atoms in the ring approach a dynamically averaged value of 90°, and, therefore, expected correlations are too weak to be observed or are completely absent (effective coupling is zero). This latter alternative is probably the most reasonable explanation.

Appropriate HMBC correlation cross-peaks were observable between the methylene protons at C-8 and carbons C-5 (two-bond) and C-4 (three-bond), while the remaining HMBC correlation cross-peaks predictable for the five-membered ring were absent. These observations are consistent with the proposed structure. Gradient HSQC data further confirmed the carbon and proton shift assignments of C/H-3 as well as C/H-4. The C-4 protons appear as two broadened multiplets in the 1D-^1H experiments (D_2O) at ~1.97 and ~2.20 ppm and are obscured by the two-proton multiplet around 2.15 ppm of the C-3 protons. The $^1H-^1H$-COSY data showed the following correlations, indicating abundant long-range coupling in the molecule and, thus, confirming a cyclic structure: H-2 (a,b) with both H-3 (a,b) and H-4 (a,b); H-3 (a,b) with H-2 (a,b), H-4 (a,b), and H-5; H-4 (a,b) with H-2 (a,b), H-3 (a,b), and H-5; H-5 with H-3 (a,b), H-4 (a,b), H-8a, and H-8b; and H-8a and H-8b with H-5.

The gradient NOESY results (D_2O) provided further evidence consistent with structure **1**. In addition to the presence of strong cross-peaks between the C-2, C-3, C-4, and C-8 geminal protons, correlations consistent with vicinal relationships were observed between H-5 and both C-8 protons, as well as between H-5 and the C-4 protons. This is consistent with structural features inferred from the observed gHMBC data. In addition, there were strong cross-peaks between both of the C-2 protons and the 2H multiplet of the H-3 protons (δ_H ~2.15). Weaker cross-peaks were observed between one of the C-8 protons (the higher field doublet-of-doublets at δ_H 2.410) and both C-4 protons. H-5 exhibited a weak cross-peak in the gNOESY spectrum to the *high*-field C-2 proton multiplet (δ_H 3.594). Its geminal partner, the *low*-field multiplet at δ_H 3.467, showed a weak cross-peak to the *low*-field C-8 proton (doublet-of-doublets at δ_H 2.692). A summary of the observed NOESY data obtained in D_2O, except for the geminal NOE correlations, is shown in Figure 3 and is consistent with proposed structure **1**. The ^1H NMR data for **1** in D_2O permitted observation of all the protons except for overlapping C-3 protons and the low-field multiplet of one of the C-4 protons, which could be differentiated by the gHSQC, gNOESY, and gCOSY results.

Additional ^1H NMR experiments were performed on a dilute solution of **1** in DMSO-d_6, in order to provide supporting evidence for the presence of the guanidine moiety. The spectrum showed two broadened singlets, each integrating for two protons at δ_H 9.29 and 7.83 (Figure 2), respectively, and were attributed to the protons associated with the guanidine zwitterion. A ^1H NMR spectrum of dilute **1** in DMSO-d_6 (~1 mg/mL) with added TFA showed a broad singlet at δ_H 13.58 assigned to the TFA, water, and four guanidinium protons.

Compound **1** was given the trivial name cimipronidine to reflect its plant source, its amino acid-like heterocycle, and its guanidine partial structure. The novel β-amino acid type structure present exhibiting zwitterionic behavior is

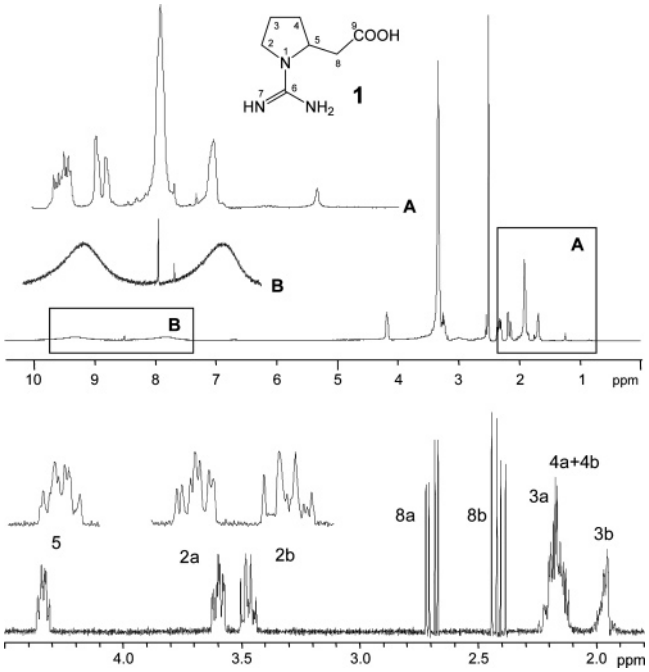

Figure 2. ^{1}H NMR spectra of **1** in DMSO-d_6 (top) and D_2O (bottom) demonstrating the presence of guanidine protons (DMSO-d_6) and the proton multiplicities of all resonances (D_2O), respectively. The data also illustrate why D_2O is the preferred NMR solvent for dereplication of **1** and its congeners.

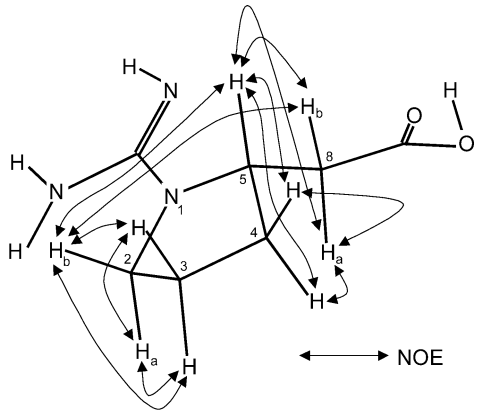

Figure 3. NOESY correlations of **1** (t_{mix} = 1.5 s, D_2O, 400 MHz) except for the observed geminal NOE interactions (not shown).

not typical of known guanidine structures. Most of the reported plant-derived guanidines possess longer aliphatic side chains.[3−5] Relevant NMR data (^{1}H and ^{13}C) for **1** is summarized in Table 1.

The routine isolation of plant-derived natural products, when polyphenolic constituents are present, requires the use of acids.[18,19] However, zwitterionic compounds such as **1** react predominantly basic. Therefore, they are pH-sensitive and with the addition of acid may introduce significant variability in the preparative HPLC retention profile, depending on their isoelectric point. This may also result in a complexation of the zwitterionic components with the polyphenolic constituents that are typically found in the polar extracts.[22,23]

The isolated constituents from the 5-HT$_7$-active *n*-BuOH-soluble fraction, which include cimicifugic acids A (t_R 42.63 min), B (43.26), and F (45.91), fukinolic acid (39.31), ferulic acid (34.58), isoferulic acid (35.58), and cimipronidine (21.12), were analyzed individually by HPLC-ELSD to determine their relative reversed-phase HPLC retention times. Individual UV spectra were recorded using a photodiode array (PDA) detector to confirm the identity of the known constituents. The HPLC-ELSD chromatogram also demonstrates the high polarity of the active fraction (Figure 4) and the chromatographic fingerprint of **1** in the context of known constituents of the active fraction.

The 5-HT$_7$-active, polar *n*-BuOH-soluble fraction of *C. racemosa*, as previously reported, can be partially characterized by the presence of the known constituents: cimicifugic acids A, B, and F, fukinolic acid, ferulic acid, and isoferulic acid, in addition to cimipronidine (**1**). The bioactivity of these isolates and related compounds will be reported in due course, as well as the synthesis of **1**.

The isolation and characterization of guanidines such as cimipronidine (**1**) has only been reported within a limited number of plant genera,[3−5] but this is the first report from a member of the Ranunculaceae. Furthermore, guanidine alkaloids may have the added significance of being marker compounds for the identification and/or standardization of black cohosh products.[24,25] The use of **1** as a marker compound together with the potential discovery of other related guanidine analogues (four methylated and one dehydrated analogue; preliminary LC-MS analyses provided with the Supporting Information), which might possess novel structures, may have significance for future research. Considering that **1** is the first representative of small nitrogenous molecules (< ~200 amu) with unprecedented occurrence in higher plants, it is reasonable to hypothesize that **1** and its congeners are unique to the family Ranunculaceae[24] and possibly are significant markers even at the genus level. Finally, together with the few references to other guanidine work, the present work may serve as an aid in providing solutions to future guanidine isolation and structure elucidation efforts. This applies in particular with regard to the use of non-silica-based stationary phases in the chromatography and the choice of solvents and parameters in the NMR spectroscopy of this class of compounds, respectively.

Experimental Section

General Experimental Procedures. Optical rotations were obtained with a Perkin-Elmer 241 polarimeter (Perkin-Elmer, Inc., MA). UV spectra were taken with a Beckman DU-7 spectrophotometer. FTIR spectra were run on a Jasco 4390 equipped with a Golden Gate-ATR. The NMR data was acquired on Bruker DMX-360, DPX-400, AVANCE-400, and AVANCE-500 MHz spectrometers (Bruker, Billerica, MA). Spectra were referenced internally to TMS (DMSO-d_6) or externally to TSP (D_2O). The digital resolution was better than 0.1 Hz, equivalent to 0.0002 ppm (e.g., 16K real data points, 4 ppm spectral width (SW) for ^{1}H NMR of **1** in D_2O), in the ^{1}H and 1.2 Hz, equivalent to 0.008 ppm (32K real datapoints, 250 ppm SW), in the ^{13}C domain. For 2D NOESY, the relaxation delay (d1) was 7.0 s, and the mixing time (d8) 1.5 s. Exact mass measurements were carried out using positive ion electrospray with a Micromass (Manchester, UK) Q-TOF-2 hybrid mass spectrometer. Column chromatography was performed on MCI gel CHP20P and Amberlite XAD-2 (Sigma). Analytical and semipreparative RP-HPLC were carried out using a YMC-ODS analytical column (4.6 × 250 mm, 5 μm) and a YMC-ODS semipreparative column (20 × 250 mm, 10 μm) on a Waters 600 Delta system equipped with a PDA (λ 210−410 nm for analytical detection, λ 210 nm for semipreparative isolation) and in-line Sedex 75 (Sedere, France) ELSD (N_2 2.9 bar, temperature 61 °C). Thin-layer chromatography (TLC) was performed on precoated 0.25 mm thick Merck (EM Science, Germany) silica gel F_{254} glass-backed plates. TLC fraction control utilized visualization under UV light (254 and 365 nm) and spraying of the plates with a solution of 10% (v/v) H_2SO_4 in EtOH or anisaldehyde/H_2SO_4 reagent (Sigma).

Table 1. ^{1}H and ^{13}C NMR Data of Cimipronidine (**1**) (400/500 and 125/100 MHz, respectively, D_2O and DMSO-d_6)

position	δ_C[a] (D_2O)	mult [C]	δ_H[b] (D_2O)	δ_H (DMSO-d_6)[b]	mult. [H] (D_2O)	J [Hz] (D_2O)	^{1}H−^{1}H COSY (D_2O)
2	47.31	CH_2	3.594 (2a)	3.341 (2a)	ddd/m[c]	2.5 (3), 7.6 (3), 10.4 (2b)	3a, 3b, 4a, 4b
			3.467 (2b)	3.252 (2b)	ddd/m[c]	~1 (3), 9.3 (3), 10.4 (2a)	
3[b]	31.13	CH_2	2.17 (3a)	1.91 (3a)	m		2a, 2b, 4a, 4b, 5
			2.13 (3b)	1.87 (3b)			
4[b]	22.61	CH_2	2.20 (4a)	1.92 (4a)	m		2a, 2b, 3a, 3b, 5
			1.97 (4b)	1.68 (4b)			
5	56.99	CH	4.331	4.163	dddd/m[c]	~1 (4), ~5.4 (8a), ~7 (4), 8.4 (8b)	
6	154.57	C					
8	41.02	CH_2	2.692 (8a)	2.272 (8a)	dd	5.4 (5a), 15.1 (8b)	5
			2.410 (8b)	2.127 (8b)	dd	8.4 (5b), 15.1 (8a)	
9	179.83	C					

[a] H,C correlations established by HSQC experiment. [b] The determination of δ_H from the 1D spectrum was hampered by the broadening of the resonances and by the overlap in the AB-type signal of protons H-3 and H-4. However, the HSQC data gave correlations that permitted the determination of the chemical shifts of these protons. [c] Apparent ddd and dddd multiplicities, respectively, under first-order assumptions. However, additional long-range coupling and higher order effects give rise to a more complex signal multiplicity.

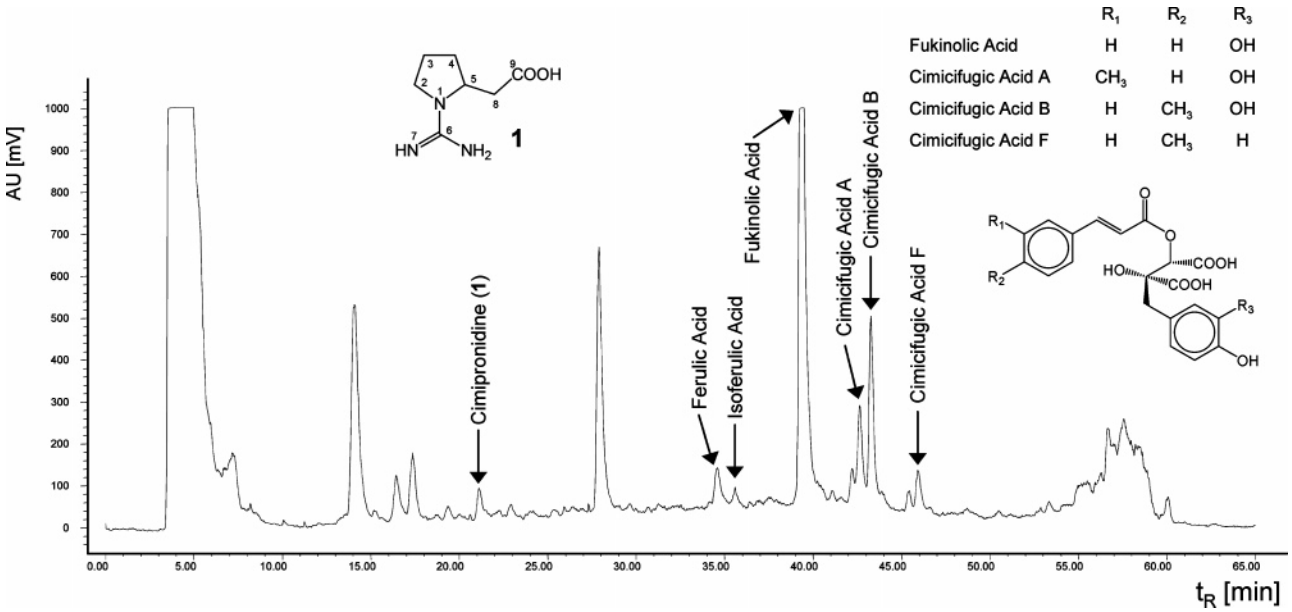

Figure 4. HPLC-ELSD trace of the BuOH-soluble fraction containing **1** (solvent A: 0.05% TFA/5% MeOH/H_2O; solvent B: 0.05% TFA/MeOH; elution gradient: 0.0 min A = 97%, 50.0 min A = 25%, 61.0 min A = 25%, 62.0 min A = 10%).

Plant Material. The roots of *C. racemosa* were collected in Great Smoky Mountain National Park, Rockbridge, Virginia, 6-28-99 GPS coordinates 37 48.27 N × 79 18.67 W. Voucher specimens are deposited in the Field Museum of Natural History Herbarium, Chicago, Illinois, and the Ramsey-Freer Herbarium, Lynchburg College, Lynchburg, Virginia.[11−13] Permits for collections were issued through the National Park Service (Permit Number BLRI-99-028). Identity of *C. racemosa* was confirmed at the collection site by Dr. Gwynn Ramsey.

Extraction and Isolation. The dried roots (4.0 kg) of *C. racemosa* were milled, homogenized, and exhaustively extracted with MeOH. The crude organic extract was concentrated in vacuo (<40 °C) to yield 1.1 kg of a syrupy residue. The residue was reconstituted in *ddi* water (1:9, 2000 mL) and subsequently partitioned with $CHCl_3$/2-propanol (3:2, 4 × 2000 mL) and *n*-BuOH (4 × 2000 mL), to give >500 and 150 g of $CHCl_3$-soluble and *n*-BuOH-soluble extract, respectively. The *n*-BuOH-soluble extract (150 g) was subjected to column chromatography consisting of Amberlite XAD-2 (1.735 kg) equilibrated in H_2O with increasing concentrations (5%) of MeOH and washed with acetone to yield 10 major fractions. Fractions F-5 through F-7 were combined (9.6 g) and further purified by flash column chromatography using MCI gel CHP20P (200 g), equilibrated with H_2O with increasing concentrations (5%) of MeOH to afford 35 (G1−35) fractions. Separation of fraction G-15 by isocratic semipreparative RP-HPLC (YMC-ODS 20 × 250 mm, 10 μm) eluting with 10% MeOH in 0.05% TFA in H_2O gave compounds **1** (t_R 10.0 min; 15.0 mg; 1.5 ppm dry plant material) and **2** (t_R > 35 min; 50.0 mg; 5 ppm). Cimicifugic acids A, B, and F, ferulic acid, and isoferulic acid were previously isolated by the UIC/NIH Center for Botanical Dietary Supplements Research.[26] These standards along with compounds **1** and **2** were each weighed out to 1.0 mg and individually dissolved in 10 mL of HPLC grade MeOH (Fisher Scientific). A 1:10 dilution of each standard was made in MeOH. Aliquots (10 μL) of each were injected onto the HPLC in triplicate. Solvent A: 0.05% TFA/5% MeOH in water. Solvent B: 0.05% TFA in MeOH. A linear gradient was used from 3 to 75% solvent B over 50.0 min followed by isocratic 75% solvent B for 11 min.

Cimipronidine (1): off-white powder (H_2O); $[\alpha]^{20}_D$ 36.21° (*c* 0.1), H_2O; UV (H_2O) λ_{max} (log ϵ) 285 (1.04), 333 (1.62) nm; IR (ITR-neat) ν_{max} 3365, 1631 cm^{-1}; ^{1}H and ^{13}C data for **1** in D_2O and DMSO-d_6, see Table 1; MW calcd for $C_7H_{13}N_3O_2$ 171.1078; high-resolution electrospray MS/MS^2 *m/z* 172.1086 $[M + H]^+$ (20), 154.1006 (6), 130.0909 (18), 112.0929 (29), 94.0789 (7), 70.0881 (100).

Fukinolic Acid (2). ^{1}H and ^{13}C data were in agreement with reported values.[27−30]

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Supporting Information Available: Two-dimensional NMR and HR-MS data of cimipronidine (**1**) and LC-MS data of the serotonergic fraction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Cimipronidine: A cyclic guanidine alkaloid from *Cimicifuga racemosa* (L.) Nutt.[$]

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Supplementary Data

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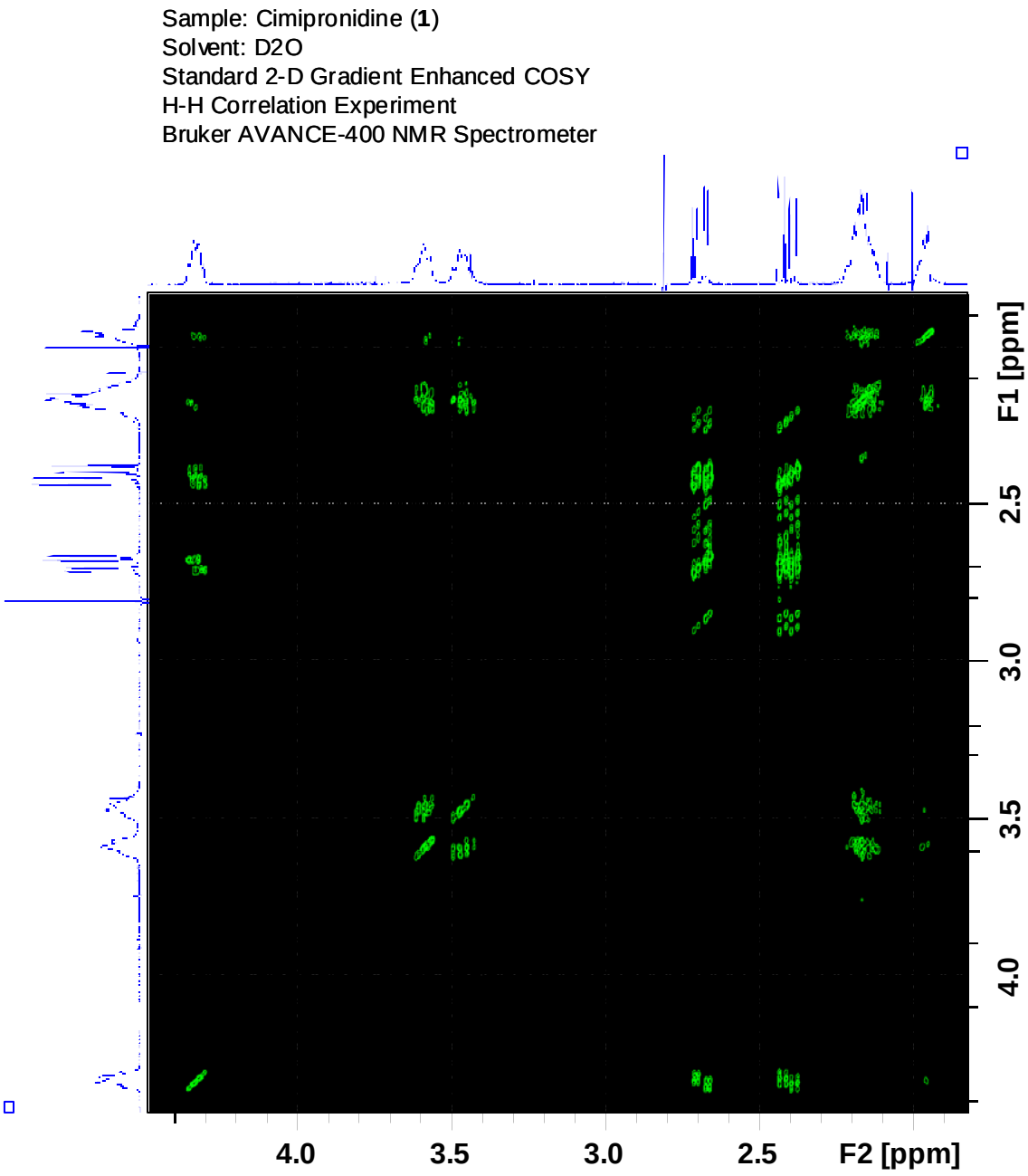
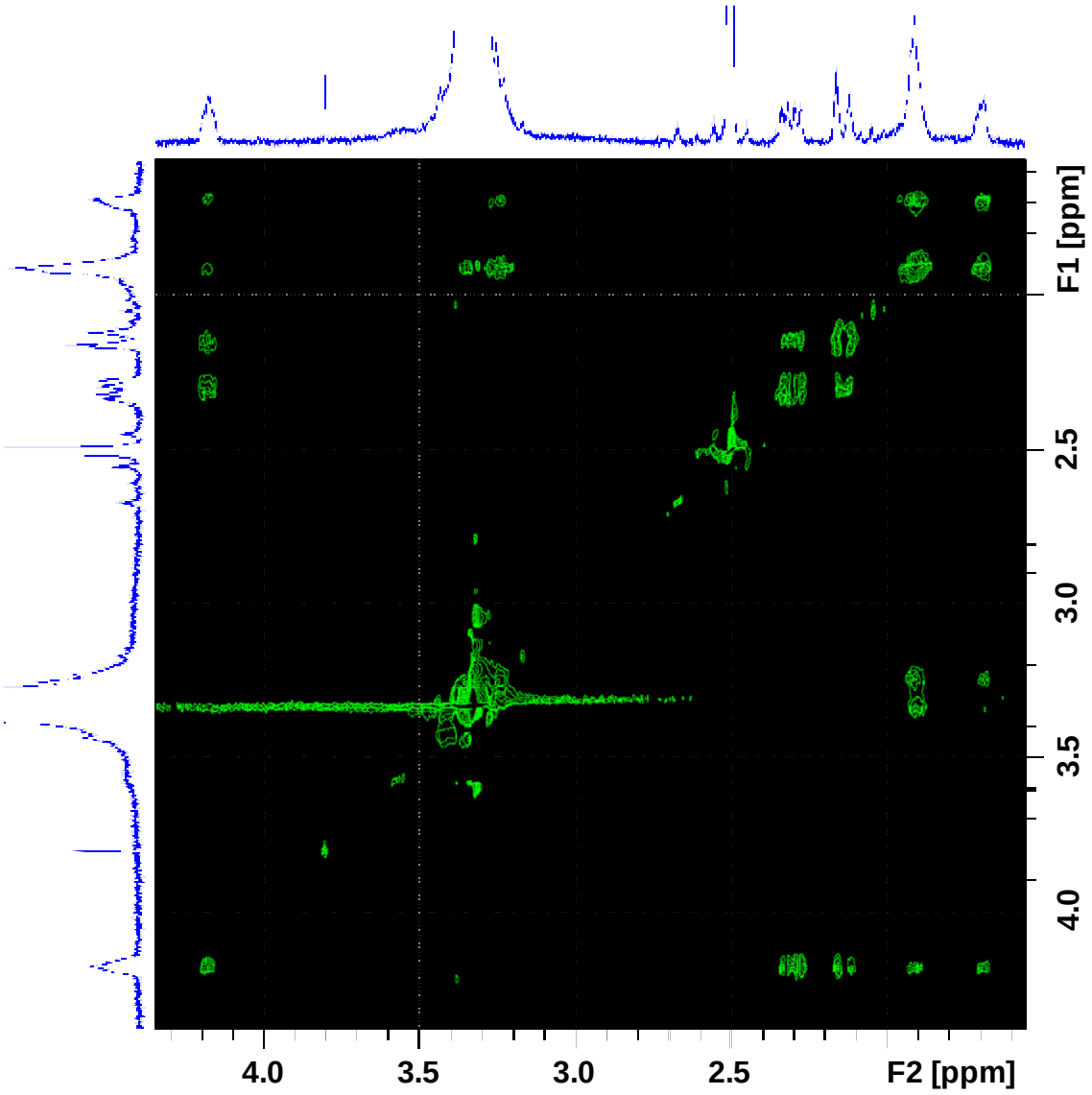
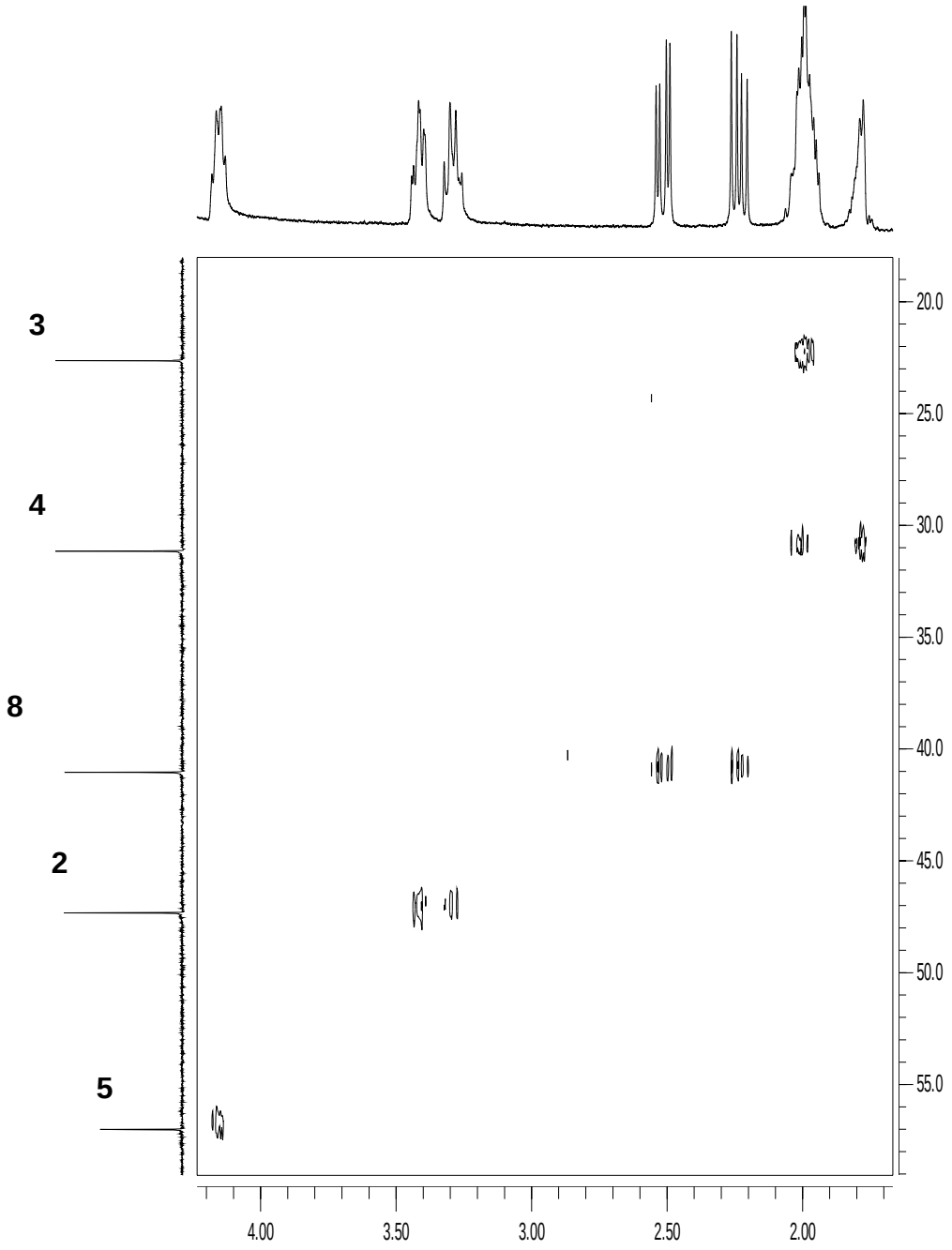


S1. ^{1}H-^{1}H COSY of **1** in D_2O (400 MHz)

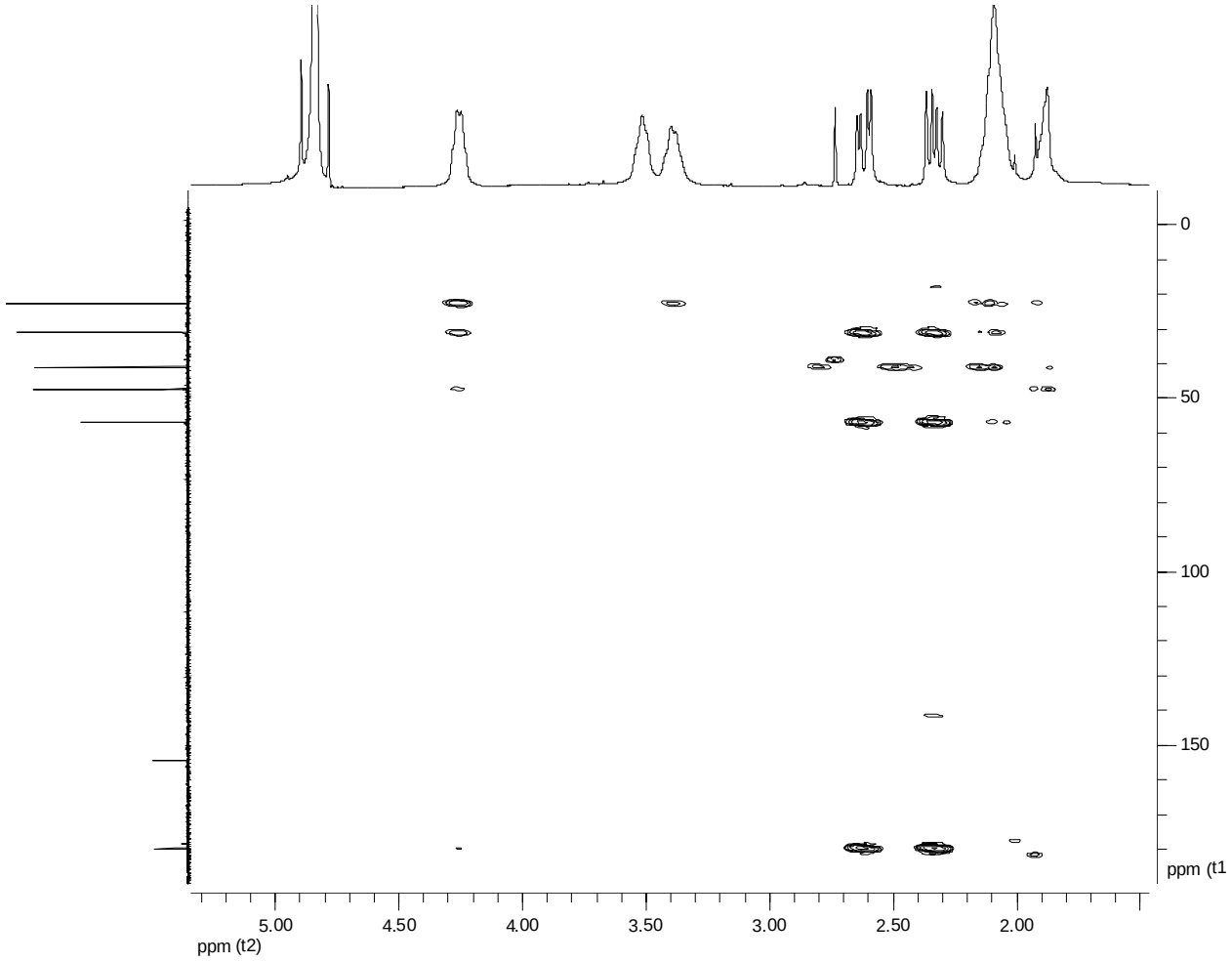
Sample: Cimipronidine (**1**)
Solvent: DMSO solution / ~1 mg
2-D TOCSY (MLEV-17, Phase Sensitive)
Using the 'mlevph' Pulse Sequence
H-H Correlation Experiment Mix Time(d9) 80 ms
Bruker AVANCE-400 NMR Spectrometer
D.Lankin



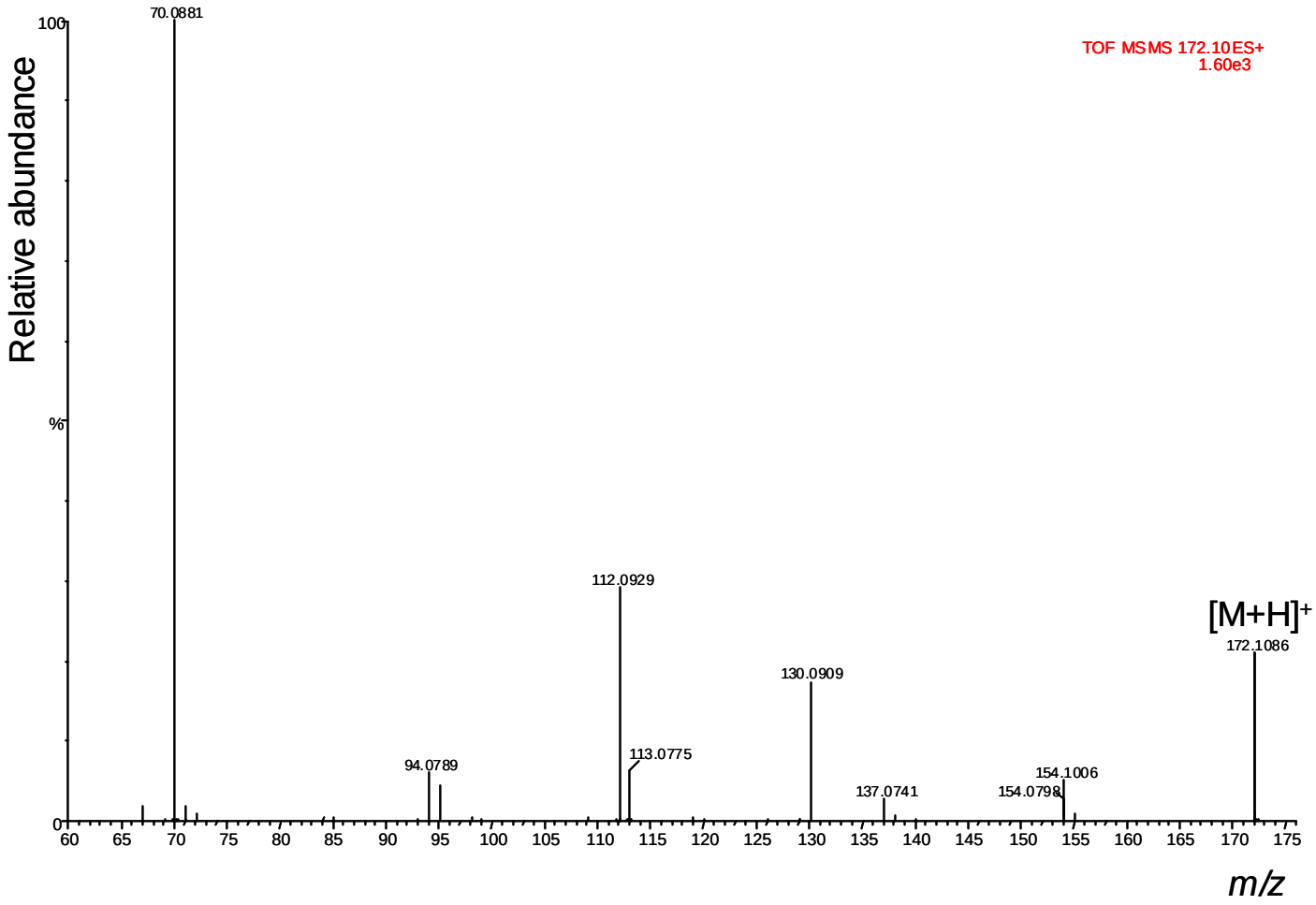
S2. ^{1}H-^{1}H TOCSY of **1** in D_2O (400 MHz)



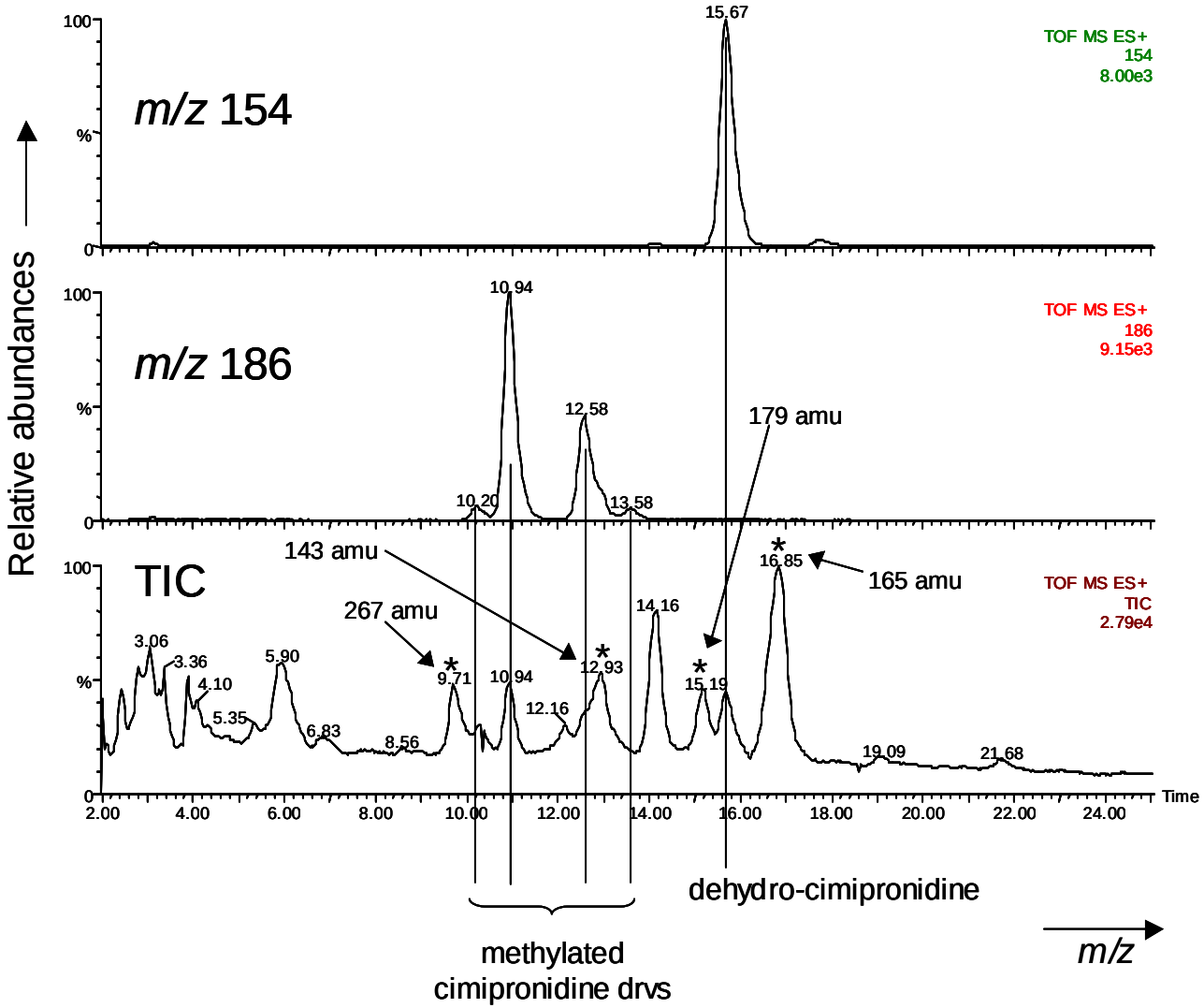
S3. HSQC of **1** in D_2O (500 MHz)



S4. HMBC (8.5 Hz) of **1** in D_2O (500 MHz)



S 5. Positive ion electrospray high resolution tandem mass spectrum of **1**. The precursor ion of *m/z* 172.1086 was used as a lock mass.



S 6. Positive ion electrospray LC-MS of a 5-HT_7 active fraction containing **1** and its analogues. Based on their protonated molecules and MS-MS fragmentation pattern, at least four methylated (*m/z* 186) and one dehydrated (*m/z* 154) cimipronidine (**1**) analogue are contained in the active fraction. Compounds with no obvious structural correlation are marked with *