Supplementary information

Large-scale preparation and purification of 4-(*R*)-OH-OTA

Incubations were performed with liver microsomes from PCB-treated rats (2 mg/mL), with 275 μM [^{3}H]OTA (2 mCi/mmol), 100 mM Tris HCl buffer (pH 7.4), 3 mM $MgCl_2$, containing a NADPH-generating system consisting of 0.5 mM NADPH, 5 mM glucose-6-phosphate, 0.3 mM $NADP^+$, 2 U of glucose-6-phosphate dehydrogenase/mL, with 5 mM GSH for 3 h at 37°C in a final volume of 8 mL. The reaction was terminated by addition of 2 vol CH_3CN, followed by removal of protein by centrifugation. The supernatant was evaporated under a stream of N_2, and the extract was resuspended in 50 mM $NaHCO_3$ (pH 8.0) and filtered with 0.2 μm Whatman membrane filters. Metabolites were purified by HPLC as described in Materials and Methods, except that 0.1% CH_3CO_2H was used as solvent A and the system was held at 60% B until 26 min instead of 21 min. Metabolites were monitored with a Hewlett-Packard 1090 diode array detector (320 nm), and a Hewlett-Packard 1046A fluorescence detector (λ_{ex} = 326 nm, λ_{em} = 463 nm). Results showed that approx. 30% of OTA (t_R: 22 min) was converted to a major metabolite coeluting with the 4-(*R*)-OH-OTA reference standard (t_R: 18.5 min). Approximately 300 nmol of the putative 4-(*R*)-OH-OTA metabolite was obtained upon HPLC collection and rotary evaporated to dryness. The metabolite was resuspended with ultrapure H_2O, and lyophilized at 0.02 mbar in the presence of P_2O_5. The metabolite was resuspended either in DMSO-d_6 for [^{1}H]-NMR analysis or in CH_3OH/H_2O (1/1 v/v) for LC/ESI-MS analyses.

Spectroscopic characterization of OTA and 4-(*R*)-OH-OTA

LC-ESI-MS analyses were carried out on Finnigan MAT TSQ-7000 mass spectrometer (Bremen, Germany) connected to a Finnigan electrospray API I interface (Bremen, Germany). The electrospray interface was operated with a high voltage of 3.5 kV and a capillary temperature of 180°C. N_2 used as sheath gas at a pressure of 80 psi. The mass spectrometer operated in positive

ionization mode, using the full scan mode from 100 to 500 Da. Tandem MS/MS analyses of OTA and the metabolite were carried out after collision-induced dissociation (CID) of the protonated molecules at a collision energy of 25 eV. Argon was used as the collision gas at a pressure of 1 mTorr. Samples were introduced by infusion with a Harvard syringe pump (Harvard Apparatus, South Natick, MA) at a flow rate of 10 μL/min. In full scan acquisition mode, the protonated molecule of OTA and the metabolite were observed $[M+H]^+$ *m/z* 404 and 420, respectively, indicating that the metabolite contained an additional oxygen atom. The product ion spectrum of OTA displayed prominent fragment ions at *m/z* 358 corresponding to $[M+H-HCCOH]^+$, m/z 341 $[M+H-HCOOH-NH_3$ or $-OH]^+$, and the base peak observed at *m/z* 239 corresponding to [M+H-Phe]$^+$. The product ion spectrum of the OTA metabolite was similar, except that the mass was increased by 16 Da and displayed prominent fragment ions at *m/z* 374 corresponding to $[M+H-HCCOH]^+$, *m/z* 357 $[M+H-HCOOH-NH_3$ or $-OH]^+$, and the base peak observed at *m/z* 255 corresponding to $[M+H-Phe]^+$. These spectra indicate that the site of oxidation is on the oxoisochromane ring and not on the phenylalanine moiety.

All NMR samples were prepared under N_2 atmosphere in 5 mm o.d. WILMAD 528-PP NMR tubes using DMSO-d_6 as the solvent (Dr. Glaser AG, Basel, Switzerland). ^{1}H-NMR spectra were recorded at 22 °C on a Bruker AM-360 spectrometer at 360.13-MHz equipped with a selective 5-mm ^{1}H probehead (Spectrospin AG, now Bruker AG, Fällanden, Switzerland). Typical parameters for basic one-dimensional spectroscopy were: spectral width 7575.8 Hz, free induction decay 64K data points, spectral resolution 0.231 Hz/data point, transmitter pulse 4 μs (ca. 64°), relaxation delay 10s. Nuclear Overhauser effect (NOE) difference spectra were acquired with a selective preirradiation of 7.5 s and a pulse repetition inverval of 12.69 s. Two-dimensional homonuclear correlation experiments (COSY) were acquired with the standard 90°/90° two-pulse sequence with 2k data points in the F_2 dimension, 512 time increments and single zero-filling in the F_1 dimension. The spectral width was 7.5 to 9.3 ppm and the relaxation delay 5 s. Unshifted sine filtering was

applied in both dimension before Fourier transformation and symmetrization along the diagonal. All chemical shifts are reported in ppm downfield from internal tetramethylsilane. The spectra of the samples which had undergone HPLC suffered from a limited signal to noise ratio and usually showed several impurity signals. The ^{1}H-NMR data of two different OTA standard preparations and of 4-(*R*)-OH-OTA in DMSO-d_6 are given in Table S1 and in Figures S1-S3. For ^{1}H-NMR spectroscopy, the synthetic crystalline OTA standard provided by the United States Food and Drug Administration (1 mg) was used as received or following HPLC as described above.

Table S1: 360 MHz ^{1}H-NMR Data of Ochratoxin A and 4-(R)-OH-Ochratoxin A in DMSO-d_6[a]

Proton	OTA crystalline1.0 mg	OTA from HPLC, ca. 0.4 mg	4-(*R*)-OH-OTA from HPLC, ca. 0.1 mg
10-CH_3	1.46, d, J = 6.0, 3H	1.37, d, J ~ 6.2, ~3H	1.31, d, J ~ 6.5, ~3H
4-H_{pro-R}	2.90, d d, J =17.2, 11.7, 1 H	~2.64, m br, ~1 H	-
3'-H_{pro-S}[b]	3.07, d d, J = 13.9, 8.3 Hz, 1 H	~3.00, m br, ~1 H	2.97, d d sl br, J =13.9, 9.0, ~1 H
4-H_{pro-S} or 4-H	3.21, d d, $J \cong$ 16.9, 2.8, 1 H[c]	~3.00, m br, ~1 H	4.45, d sl br, J ~ 4.4, 1 H
3'-H_{pro-R}	3.21, d d, $J \cong$ 14.3, 5.3, 1 H[c]	3.14, d d sl br, J ~ 13.9, 5.1, ~1 H	3.12, d d sl br, J ~ 13.8, 5.2, ~1 H
2'-H	4.71, m sl br, 1 H	4.60, m br, ~1 H	4.54, m br, ~1 H
3-H	4.82, m br, 1 H	4. 49, m br, ~1H	4. 32, "q" sl br, J ~ 6.5, ~1H
4-OH[d]	-	-	~5.44, "d" br (?), J ~ 4-5, 1 H
Phenyl-H	~7.25, m, 5 H	~7.23, m, 5 H	~7.21, m, 5 H
6-H	8.05, s, 1 H	7.85, s, 1 H	7.79, s, 1 H
Exch. H[d,e]	~8.81, very br, ~1H ~12.8, very br,~2 H	- ~12.7, extremely br, ? H	- ~12.0, very br, >1 H

[a] Shifts in ppm from internal TMS. Multiplicity abbreviations used to describe ^{1}H-NMR signals: s = singlet, d = doublet, t = triplet, q = quartet, m = complex multiplet, br = broad, sl = slightly, . ~ = *ca.* Quotes "..." mean approximate description of the coupling pattern. Coupling constants J are given in Hz. The atom numbering of the molecule is adopted from previous literature (1,4)

[b] Since we did not investigate them in detail, the assignments of the pro-S and pro-R positions of the 3'-CH_2 were taken from (2,4), based on shift order and coupling constants.

[c] Near isochronous signals; the coupling constants were determined without line fitting and their precision is limited because of line overlap. This explains the discrepancy with the corresponding J values found for the coupling partners.

[d] These protons were exchanged upon addition of D_2O to the sample.

[e] No individual assignment is given for these D_2O-exangeable protons. For the HPLC preparations, especially for the OTA sample, the detection of the very broad signals was uncertain for S/N and linewidth reasons.

Ochratoxin A

The different shift values of the OTA preparations illustrate the sensitivity of the ochratoxin A proton spectrum in DMSO-d_6 to the conditions of sample preparation. A shift difference of up to 0.33 ppm was noted (e. g. for 3-H), and in one case the shift order was inversed (for 2-H' and 3-$H_{pro\text{-}S}$). Therefore, ^{1}H-NMR identification of ochratoxin A in DMSO-d_6 (and possibly in other solvents) needs to rely on the coupling patterns rather than on exact shift values. Considering this, our results are compatible with earlier work in $CDCl_3$ (1,2).

The assignment of the OTA signals of the crystalline sample (Figure S1) was therefore based mainly on the COSY spectra and on the values of the coupling constants. A COSY spectrum also facilitated unravelling the coinciding 4-$H_{pro\text{-}S}$ and 3'-$H_{pro\text{-}R}$ signals. The two unequal vicinal coupling constants (11.7 and 2.8 Hz) between 3-H and the two 4-CH_2 protons, and NOE difference effects, indicated that the (3-C)- atom is probably puckered *above* the near-planar arrangement of the five atoms -(2-O)-(1-C)-(9-C)-(10-C)-(4-C)- in the non-aromatic 6-ring. The NOE-difference experiments, even though they resulted in low signal/noise spectra, qualitatively indicated the spatial proximity of the 3-H to the 4-$H_{pro\text{-}S}$ proton, as well as the proximity of 3–CH_3 more to the 4-$H_{pro\text{-}R}$ and less to the 4-$H_{pro\text{-}S}$ proton.

The ^{1}H-NMR spectrum of OTA obtained following HPLC, at lower concentration (Figure S2), revealed that another pair of signals coincided: 3'-$H_{pro\text{-}S}$ and 4-$H_{pro\text{-}S}$. For linewidth and S/N reasons, the coupling constants were not as well measurable as with the crystalline sample (see Table S1). However, the Bruker WINNMR program permitted the shifted superposition of the two spectra. Thus, it was possible to graphically verify the assignments by establishing a shape equivalence between the poorly resolved multiplet signals of the HPLC sample and the well resolved coupling patterns of the crystalline sample. Furthermore, all couplings between the non-exchangeable side chain protons, the coupling between 3-CH_3 and the 3-H proton, as well as that

between 4-H_{pro-R} and 4-H_{pro-S} could be corroborated by the COSY spectrum (the S/N ratio was insufficient to show the other correlations).

4-*(R)*-OH-ochratoxin A

The ^{1}H-NMR spectra of 4-*(R)*-OTA (Figure S3) showed the close relationship with OTA pepared by HPLC (Table S1). The shifts of the phenyl protons, 6-H, 2'-H and 3'-CH_2 signals, as well as the 10-CH_3 changed very little, indicating that the corresponding substructures were intact. However, the 4-H_{pro-R} signal at 2.64 ppm was no longer found, and signals appeared at 5.44 (D_2O-exchangeable), 4.45 and 4.32 ppm, which were assigned as 4-OH, 4-H_{pro-S} and 3-H, based on their (weak) COSY cross peaks and the observed coupling constants. NOE-difference experiments meant to elucidate the stereochemistry of that part of the molecule were not successful because of insufficient sample quantity. However, the fact that the coupling constant $^3J_{4\text{-Hpro-R},3\text{-H}}$ of ca. 11.7 Hz was no longer observed, is a strong argument for the replacement of 4-H_{pro-R} by an OH group. Addition of D_2O lead to shift changes, especially in the region of the new hydroxyl group (ca. 0.3 ppm for 3-H, smaller for the other signals), thus illustrating once more the sensitivity of the spectrum to the solvent and preparation conditions. Significant shift differences had therefore to be expected when comparing our results with those of the literature where different solvents were used (3) and Xiao *et al.* (4). This factor taken into account (deviations of up to 0.5 ppm were found), our data are well compatible with those of the literature, and the structure and stereochemistry of 4-*(R)*-OTA is confirmed.

Supplementary references

1. Stormer, F. C., Storen, O., Hansen, C. E., Pedersen, J. I., and Aasen, A. J. (1983) Formation of (4R)- and (4S)-4-hydroxyochratoxin A and 10-hydroxyochratoxin A from ochratoxin A by rabbit liver microsomes. *Appl. Environ. Microbiol.* **45**, 1183-1187.

2. Xiao, H., Marquardt, R. R., Frohlich, A. A., and Ling, Y. Z. (1995) Synthesis and structural elucidation of analogs of ochratoxin A. *J. Agric. Food Chem.* **43**, 524-530.

3. Hutchkinson, R. D., Steyn, P. S., Thompson, D. L. (1971) The isolation and structure of 4-hydroxyochratoxin A and 7-carboxy-3,4-dihydro-8-hydroxy-3-methylisocoumarin from *penicillium viridicatum. Tetrahedron Letters* **43**, 4.33-4036.

4. Xiao, H., Marquardt, R. R., Abramson, D., and Frohlich, A. A. (1996) Metabolites of ochratoxins in rat urine and in a culture of Aspergillus ochraceus. *Appl. Environ. Microbiol.* **62**, 648-655.

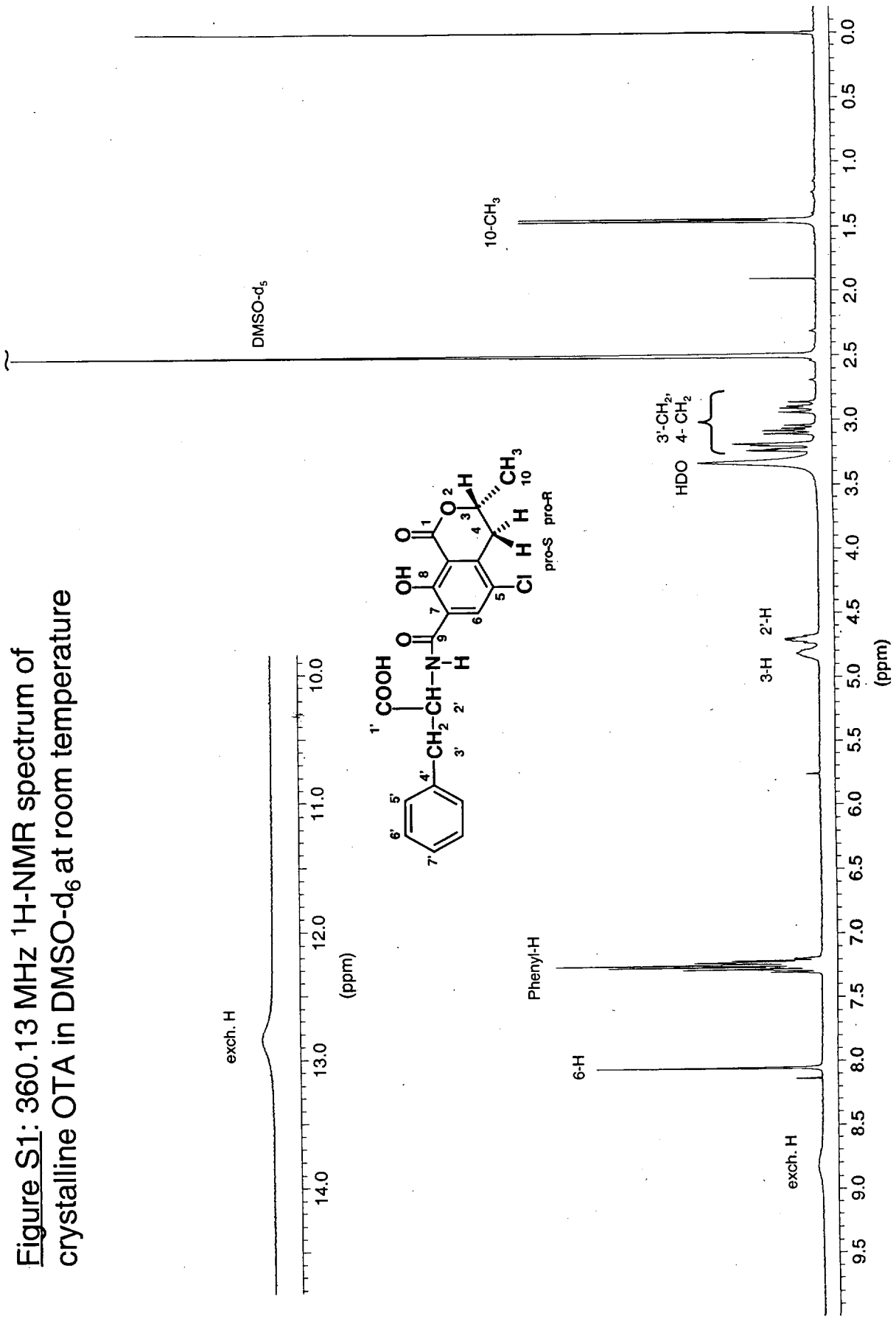

Figure S1: 360.13 MHz ^{1}H-NMR spectrum of crystalline OTA in DMSO-d_6 at room temperature

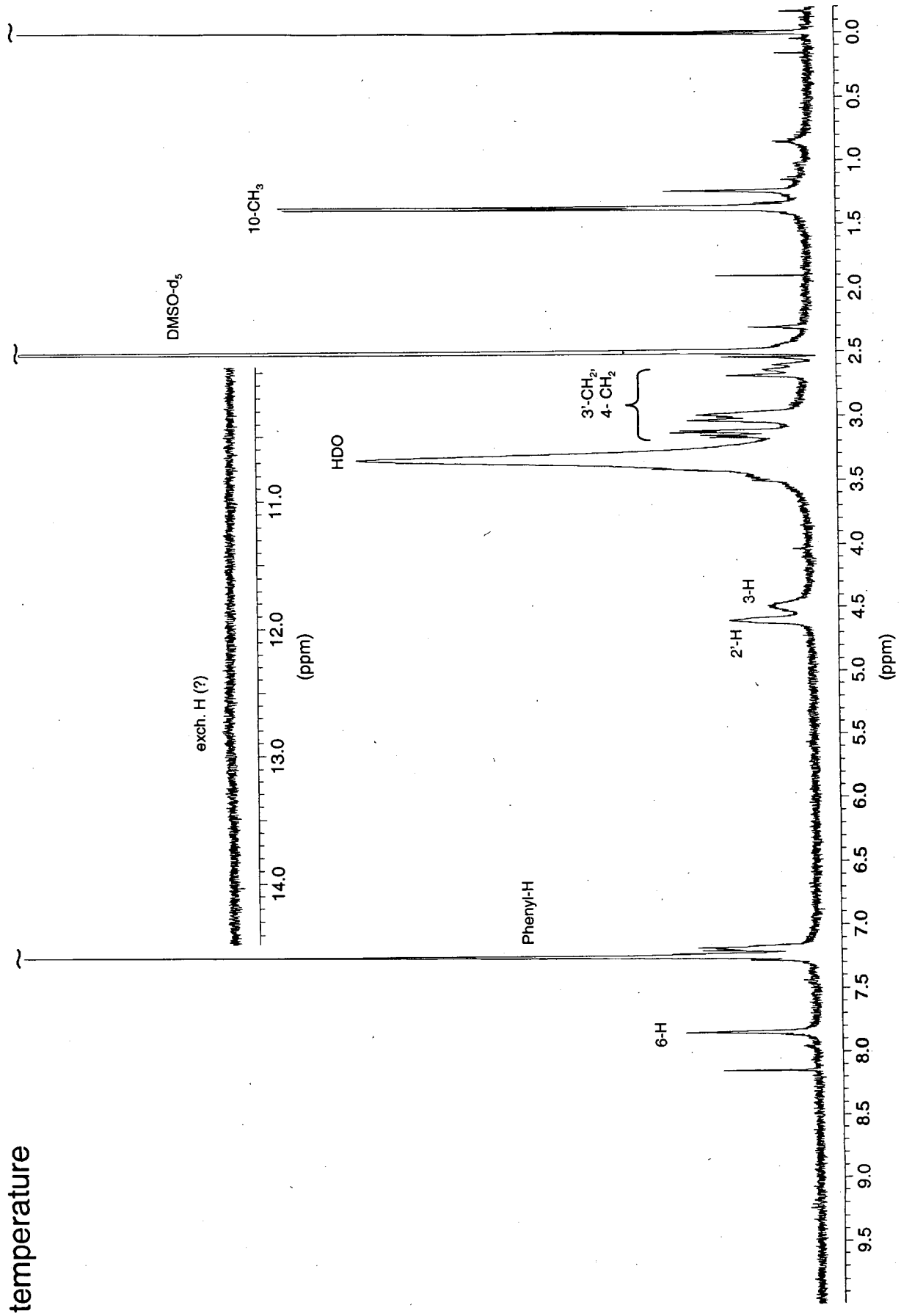

Figure S2: 360.13 MHz ^{1}H-NMR spectrum of OTA following HPLC in DMSO-d_6 at room temperature

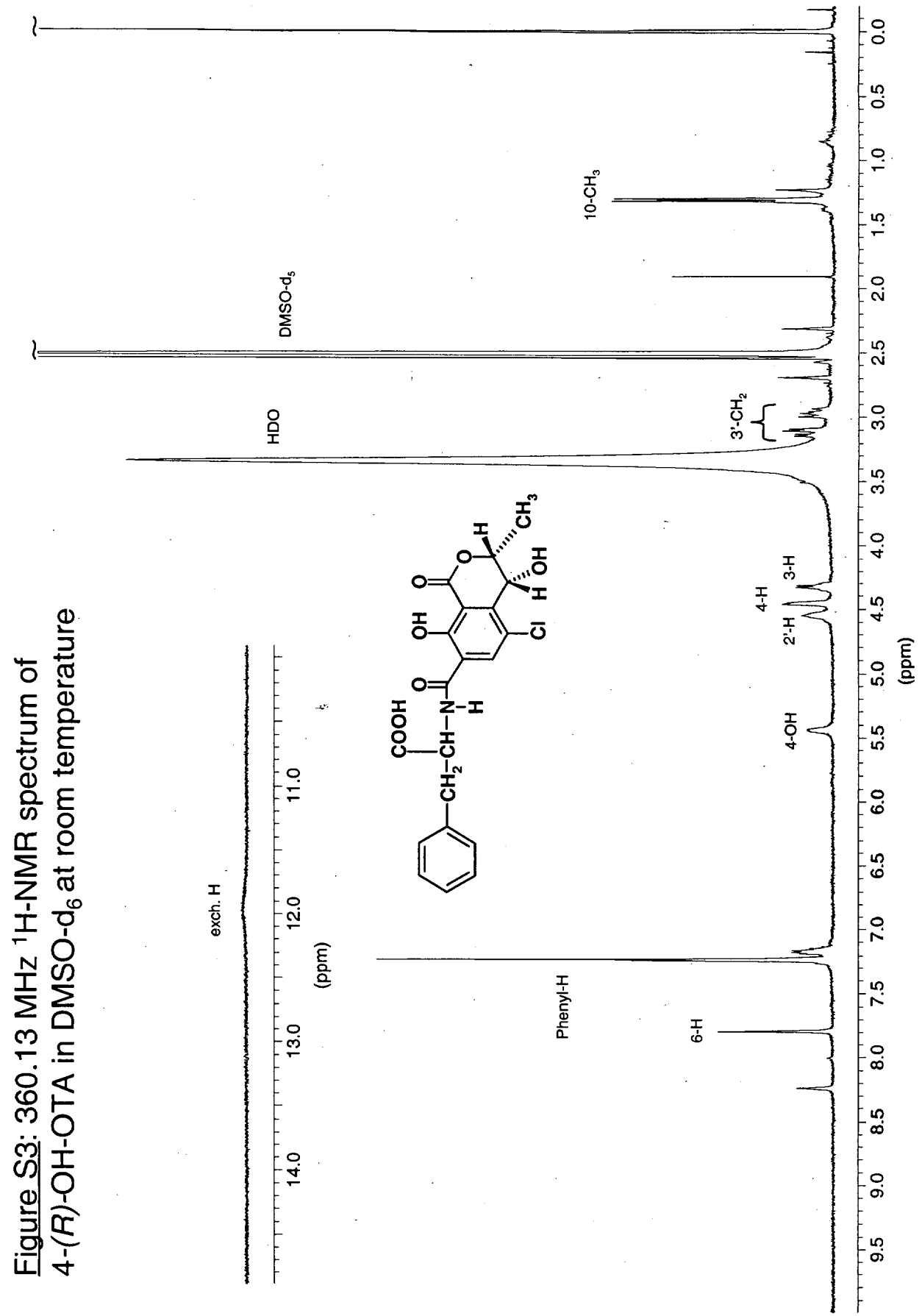

Figure S3: 360.13 MHz ^{1}H-NMR spectrum of 4-*(R)*-OH-OTA in DMSO-d_6 at room temperature