Detection, Isolation and Characterization of a Novel Impurity from Several Folic Acid Products

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Supporting Information includes the following items:

Materials and methods

Chemicals and materials

The commercial folic acid API product (batch no. 031611001) was obtained from Hebei Jiheng Group Pharmaceutical Co., Ltd (Hengshui, Hebei, China). Reagent-grade folic acid products were purchased from several reagent companies including Shanghai Aladdin Bio-Chem Technology Co., Ltd (batch no. K1908009), Shanghai Macklin Biochemical Co., Ltd (batch no. C11379431), and Shanghai Zhanyun Chemical Co., Ltd (batch no. 201020). Ammonium acetate (CH_3COONH_4), dipotassium hydrogen phosphate (K_2HPO_4) and acetic acid were purchased from Sigma-Aldrich. Methanol and acetonitrile of chromatographic grade were purchased from J&K Scientific Ltd. Deuterated dimethyl sulfoxide (DMSO-d_6) containing 0.03% (v/v) TMS as internal standard and deuterated water (D_2O, purity 99.9%) were purchased from Cambridge Isotope Laboratories, Inc. Distilled water was purchased from A.S. Watson Group (Hong Kong) Ltd. The folic acid impurity D was purchased from European Directorate for the Quality of Medicines & HealthCare (Strasbourg, France). The folic acid impurity G was purchased from Hangzhou Zebon Technology Co., Ltd.

High-performance liquid chromatography (HPLC) analysis of folic acid products

The folic acid products were analyzed in an isocratic mode using the Agilent 1260 Infinity II HPLC system equipped with a model G7112B pump, a G7115A diode array detector (DAD), a SCIEX X500 QTOF mass detector, and the Agilent OpenLAB software. The analysis method was based on the method of the European Pharmacopoeia 9.5 (The European Pharmacopoeia Commission, 2018) with some modifications for avoiding the damaging effect of phosphate buffer on mass detector and shortening analysis time. In brief, an ACE Excel SuperC18 (4.6 × 250 mm, 5 µm) was used for analysis with the column temperature set at 20 ^oC. The folic acid products were dissolved in 0.1% sodium carbonate. The mobile phase was a mixture of an aqueous buffer and methanol (3/1, v/v). The aqueous buffer was 100 mM ammonium acetate at pH 5.8 (adjusted with acetic acid). The flow rate was set at 0.7 $mL{\cdot}min^{-1}$, and the UV-Vis detection wavelength was set at 317 nm. The MS parameters were optimized as follows: ionspray voltage, 5500 V; ion source gas 1 and 2 (nitrogen), 55 psi; ion source temperature, 550 ^oC; CAD gas, 8; ion accumulated time, 0.1s; declustering potential voltage, 80 V; collision energy, 10 V. Accurate mass was calibrated using ESI Positive Calibration Solution for the SCIEX X500 QTOF System. Retention time of folic acid and all the impurities detected by HPLC was calculated according to the results of 4 independent analyses.

Obtaining impurity K

Impurity K was isolated from 100g of the folic acid product from Shanghai Aladdin Bio-Chem Technology Co., Ltd using the Dynamic Axial Compression (DAC) preparative system equipped with a reversed-phase C18 Dynamic Axial Compression column (50 mm diameter, 8 µm), two NP7000 serials pumps, and an NU3000 serials UV/VIS detector. The folic acid product was dissolved in 0.1% (w/v) sodium carbonate and then loaded onto the column. The flow rate was set at 50.0 $mL{\cdot}min^{-1}$. The UV-Vis detection wavelengths were set at 280 and 317 nm. A gradient

was used for sample elution: 0-35 min, 88% phase A; 35-36 min, 88-85% phase A; 36-56 min, 85% phase A. The phase A was 10 mM K_2HPO_4 (pH 9.0) and the phase B was methanol. The impurity K fraction collected at 43-46 min was concentrated under reduced pressure to 20 mL. The pH value of the concentrated elute was adjusted to 5.0 using diluted hydrochloric acid and the impurity K was then precipitated. The precipitate was collected through centrifugation, desalinated with water and freeze-dried to obtain the impurity K sample (4.9 mg, yield 0.005%). The purity of the impurity K sample was determined according to the results of 4 independent HPLC analyses.

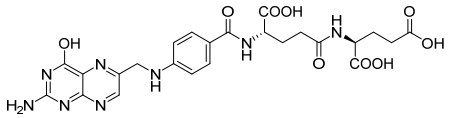
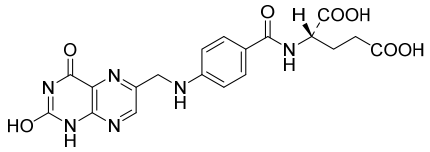
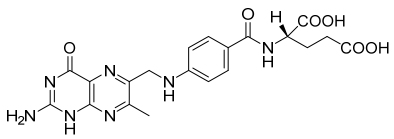
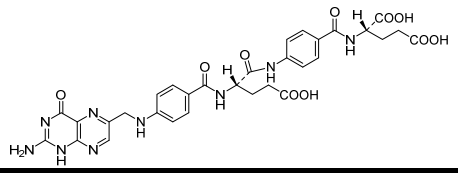
Structural characterization of impurity K

Multidimensional NMR spectroscopic analysis was performed on a Bruker Ascend TM 500M instrument using DMSO-d_6 containing TMS as solvent. The concentration of the impurity K for NMR analysis was 5 mg·mL^{-1}. ^{1}H and ^{13}C NMR experiments were performed at 500 MHz and 125 MHz, respectively. ^{1}H and ^{13}C chemical shifts (δ) were reported in ppm. The data were obtained and analyzed using Bruker ICON software (Bruker Analytik, GmbH, Germany). The H/D exchange experiment was performed by adding several drops of D_2O into the sample-containing NMR tube and recording the NMR spectrum again.

High-resolution mass spectrometric analysis was carried out in the positive ion mode (ES+) on the SCIEX X500 QTOF System equipped with an electrospray ionization (ESI) source. The isolated impurity K sample was dissolved in acetonitrile (0.24 mg·mL^{-1}). Sample injection volume was 1 μL. The HRMS parameters were the same as those described in the above HPLC-UV/Vis-MS analysis. The MS/MS parameters were set as follows: mass range 50-1000 Da; declustering potential 80 V; DP spread 0 V; accumulation time 0.05 sec; collision energy 35 V; CE spread 15 V .

The UV-Vis spectra at 190-400 nm of the impurity K and folic acid were recorded using the G7115A DAD during the HPLC analysis (solvent: 1/3 100 mM CH_3COONH_4 (pH 5.8)/CH_3OH, v/v). Infrared spectroscopy analysis (KBr pellet method) was conducted with an Agilent Cary 630 FT-IR spectrometer (400–4000 cm^{-1}).

Table S1 The identified non-pharmacopeial impurities

Peak No.	RT	Identified structure	*m/z*	Source
1	4.140		$[M+H]^+$517.1903 $[M+Na]^+$593.1746 $[M+K]^+$609.1461 Fragments 295.0939 176.0574	CAS: 19360-00-0 Effectiveness of conjugated forms of folic acid in the treatment of tropical sprue. By Suarez, Ramon M.; Welch, Arnold D.; Heinle, Robert W.; Suarez, Ramon M., Jr.; Nelson, Evelyn M. From Journal of Laboratory and Clinical Medicine (1946), 31, 1294-1304.
2	4.438		$[M+H]^+$443.1309 $[M+K]^+$481.0860 Fragments 296.0779 177.0416 106.0401	CAS: 25663-25-6 Pteridine chemistry. II. The action of excess nitrous acid upon pteroylglutamic acid and derivatives. By Angier, Robert B.; Boothe, James H.; Mowat, John H.; Waller, Coy W.; Semb, Joseph From Journal of the American Chemical Society (1952), 74, 408-11.
3	6.434		$[M+H]^+$456.1620 $[M+K]^+$494.1178 Fragments 309.1086 148.0511	CAS: 6810-75-9 Anthranilic acid as an intermediate in the biosynthesis of tryptophan by Bacterium typhosum. By Rydon, H. N. From British Journal of Experimental Pathology (1948), 29, 48-57.
4	9.422	Isomer of impurity H 	$[M+H]^+$690.7278 $[M+K]^+$728.1836 Fragments 543.1739 378.1327	https://www.pharmaffiliates.com/en/parentapi/folic-acid-impurities

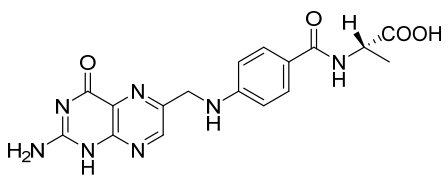
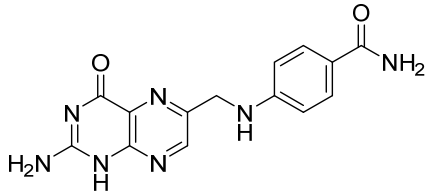
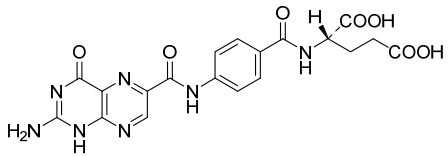
			295.0942	
5	15.433		[M+H]$^+$384.1428 [M+K]$^+$422.0981 Fragments 295.0940 176.0566 120.0449	CAS: 897045-35-1 Analogs of pteroylglutamic acid. IV. Replacement of glutamic acid by other amino acids. By Wright, Wm. B., Jr.; Cosulich, Donna B.; Fahrenbach, Marvin J.; Waller, Coy W.; Smith, James M., Jr.; Hultquist, Martin E. From Journal of the American Chemical Society (1949), 71, 3014-17.
6	16.795		[M+H]$^+$312.1224 [M+K]$^+$350.0754 Fragments 295.0940 176.0566 120.0449	CAS: 109018-57-7 Efficient Syntheses of Pyrofolic Acid and Pteroyl Azide, Reagents for the Production of Carboxyl-Differentiated Derivatives of Folic Acid. By Luo, Jin; Smith, Michael D.; Lantrip, Douglas A.; Wang, Susan; Fuchs, P. L. From Journal of the American Chemical Society (1997), 119(42), 10004-10013.
7	19.302		[M+H]$^+$456.1261 [M+K]$^+$494.0816 Fragments 309.0730 162.0411 121.0290	CAS: 39707-61-4 Synthesis of pteridine-6-carboxamides. 9-Oxofolic acid and 9-oxoaminopterin. By Nair, M. G.; Baugh, Charles M. From Journal of Organic Chemistry (1973), 38(12), 2185-9.

Table S2 The HPLC retention time (min) of folic acid and the detected impurities

Compounds	1st measurement	2nd measurement	3rd measurement	4th measurement	Average	Standard Deviation
Folic acid	4.874	4.872	4.871	5.052	4.917	0.090
Impurity A	3.560	3.560	3.559	3.505	3.546	0.027
Impurity D	12.219	12.232	12.226	12.429	12.277	0.102
Impurity G	10.326	10.338	10.337	10.325	10.332	0.007
Impurity H	7.756	7.777	7.777	7.817	7.782	0.026
Impurity FOP	7.283	7.289	7.289	7.247	7.277	0.020
Impurity K	20.280	20.321	20.311	20.387	20.325	0.045
Others						
1	4.145	4.148	4.148	4.140	4.145	0.004
2	4.374	4.378	4.378	4.438	4.392	0.031
3	6.265	6.275	6.275	6.434	6.312	0.081
4	9.446	9.464	9.465	9.422	9.449	0.020
5	15.452	15.465	15.425	15.423	15.441	0.021
6	16.886	16.890	16.888	16.795	16.865	0.047
7	19.384	19.396	19.390	19.302	19.368	0.044

Table S3 Comparison of the chemical shift data of impurity K and folic acid

Position No.	Impurity K (DMSO-d_6 as solvent)		Folic acid* (DMSO-d_6 as solvent)	
	^1H (δ_H)	^{13}C (δ_C)	^1H (δ_H)	^{13}C (δ_C)
1	11.30 (NH)	/	/	/
2	6.50 (NH_2)	151.90	/	156.16
3	/	/	/	/
4	/	160.73	/	161.27
4a	/	122.34	/	127.95
5	/	/	/	/
6	/	150.74	/	148.61
7	/	144.76	8.75	148.61
8	/	/	/	/
8a	/	151.56	/	153.82
9	2.62	21.69	4.59	45.92
10	8.71 (NH)	/	7.02	/
11	/	145.39	/	150.79
12	7.98	117.34	6.74	111.22
13	7.86	130.04	7.75	129.00
14	/	122.53	/	121.32
15	7.86	130.04	7.75	129.00
16	7.98	117.34	6.74	111.22
17	12.48 (OH)	167.04	/	166.44

* The chemical shift data of folic acid was obtained from the work of Rossi *et al* (Rossi et al., 1992).

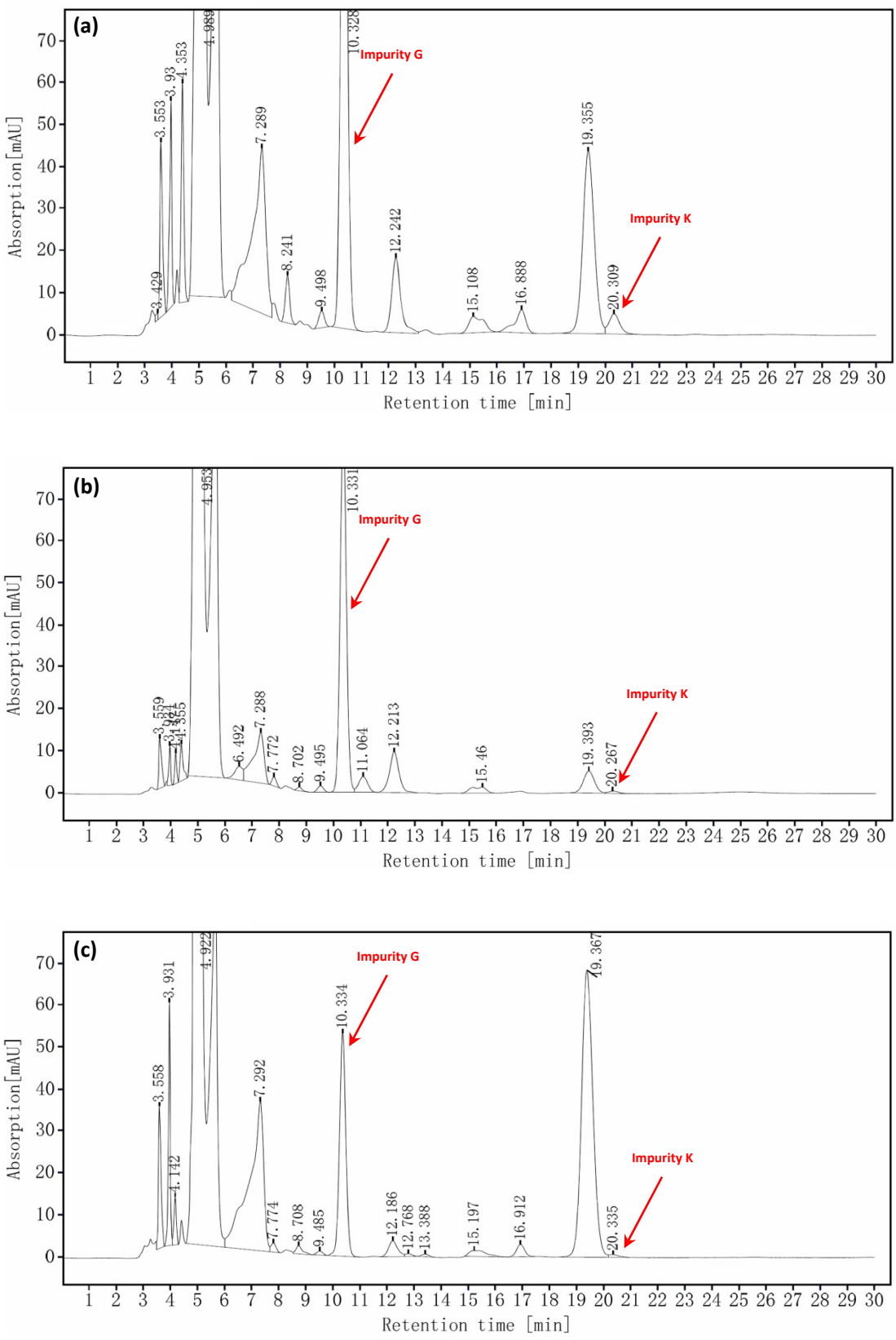


Fig. S1 The detection of the impurities K and G in the folic acid products obtained from (a) Shanghai Aladdin Bio-Chem Technology Co., Ltd; (b) Shanghai Macklin Biochemical Co., Ltd; (c) Shanghai Zhanyun Chemical Co., Ltd.

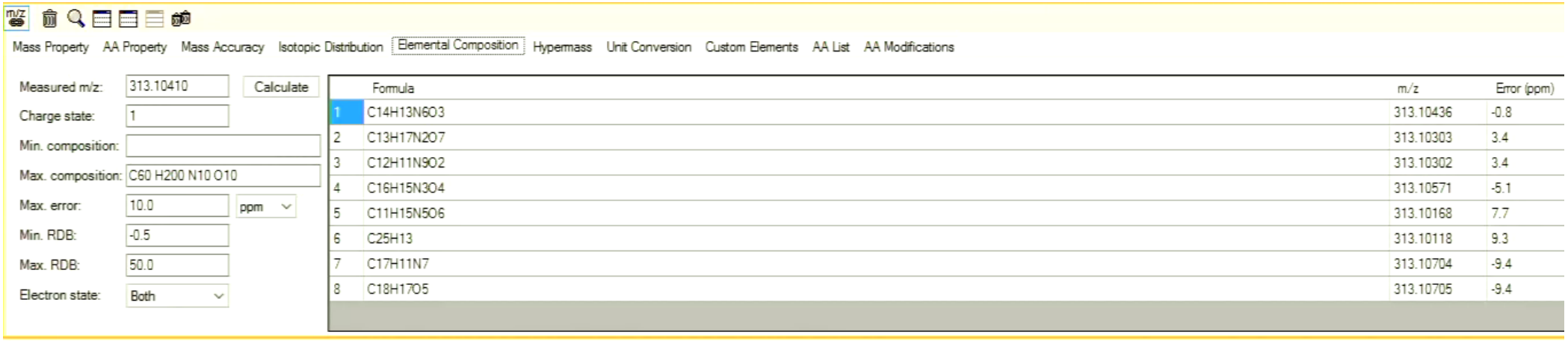


Fig. S2 The molecular formula prediction of the impurity K by the QTOF-MS software.

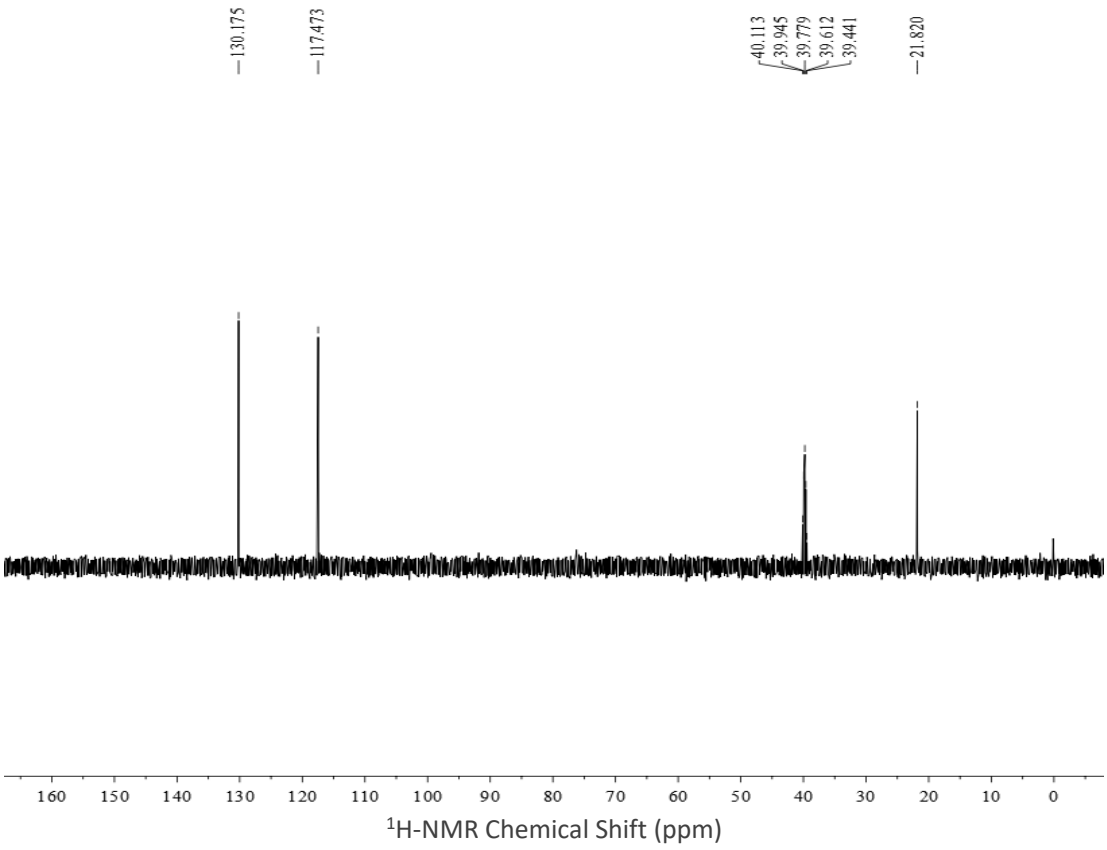
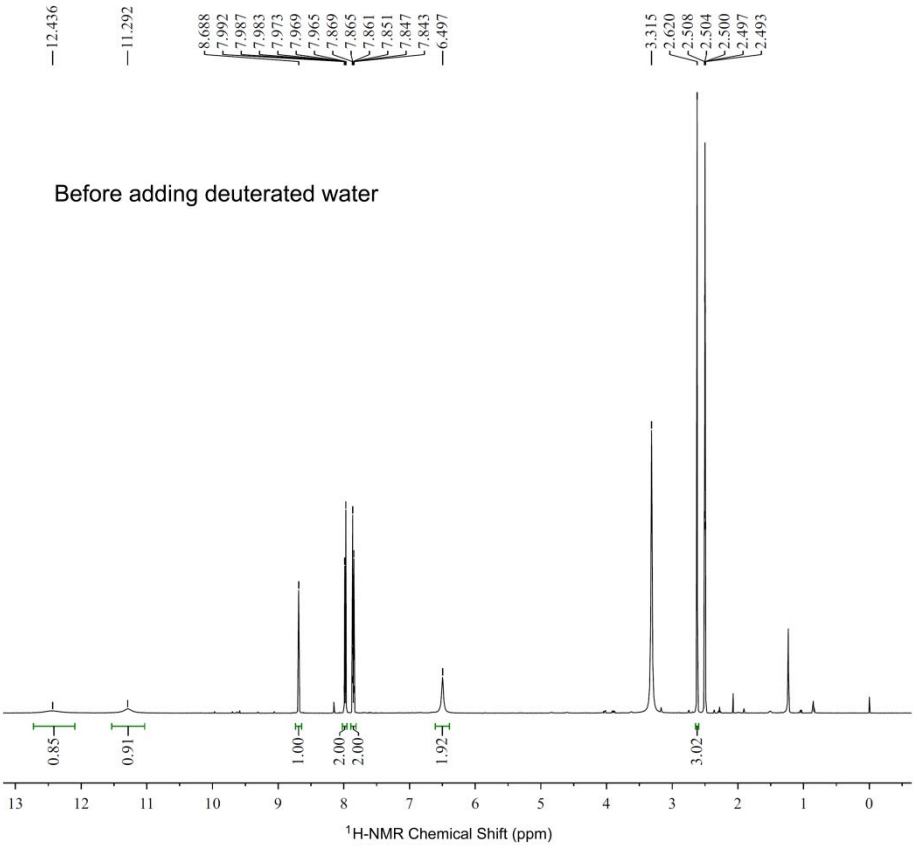


Fig. S3 The DEPT-135 spectrum of the impurity K.



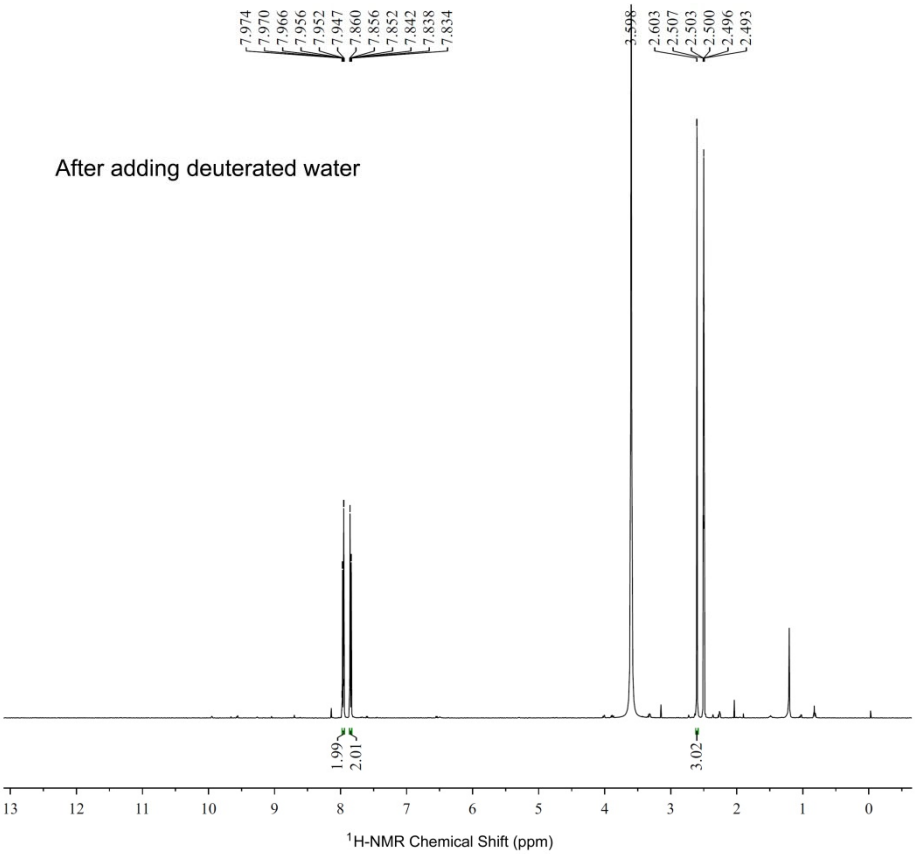


Fig. S4 The H/D exchange experiment for the impurity K.

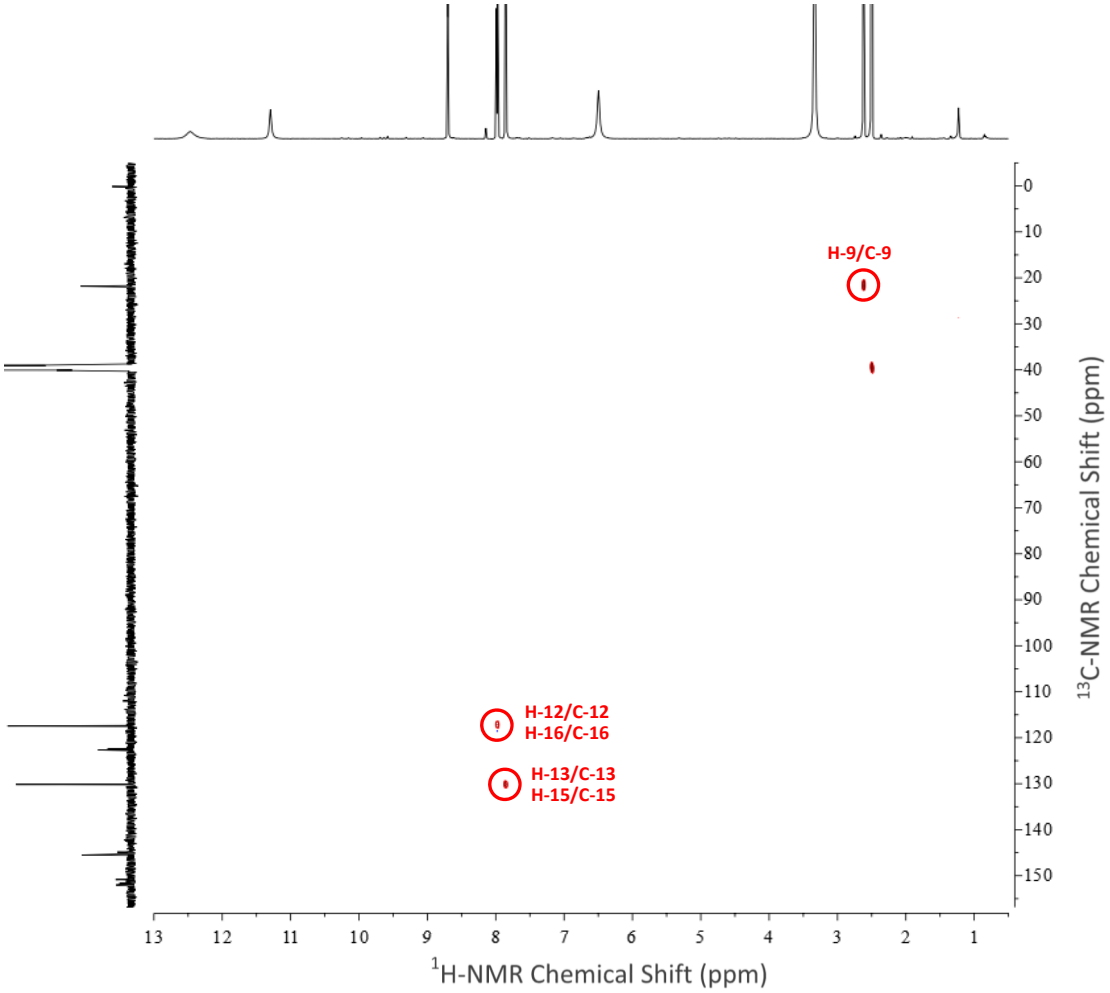


Fig. S5 The HSQC spectrum of the impurity K. The C-H one-bond correlations are marked in red circles.

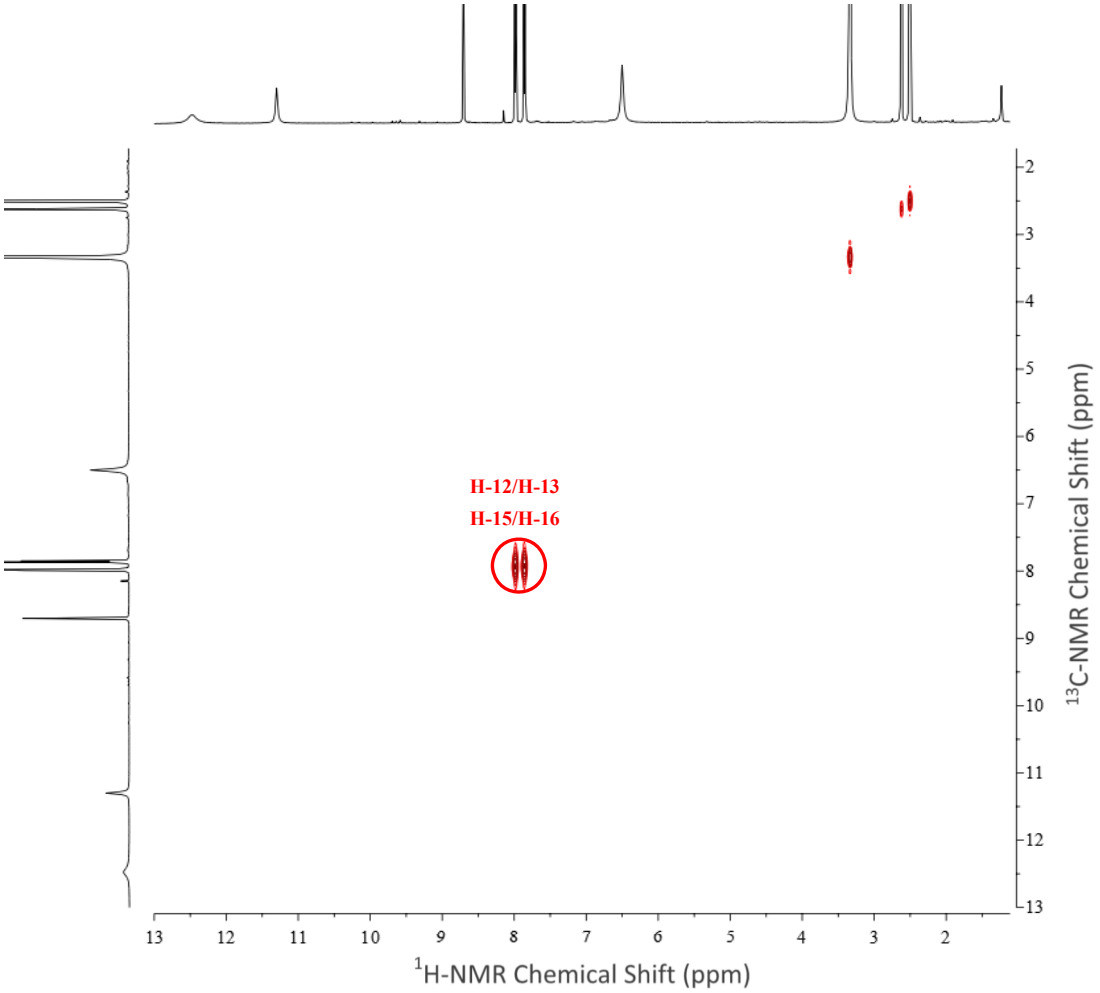


Fig. S6 The ^{1}H-^{1}H COSY spectrum of the impurity K. The H-12/H-13 and H-15/H-16 three-bond correlations are marked in red circles.

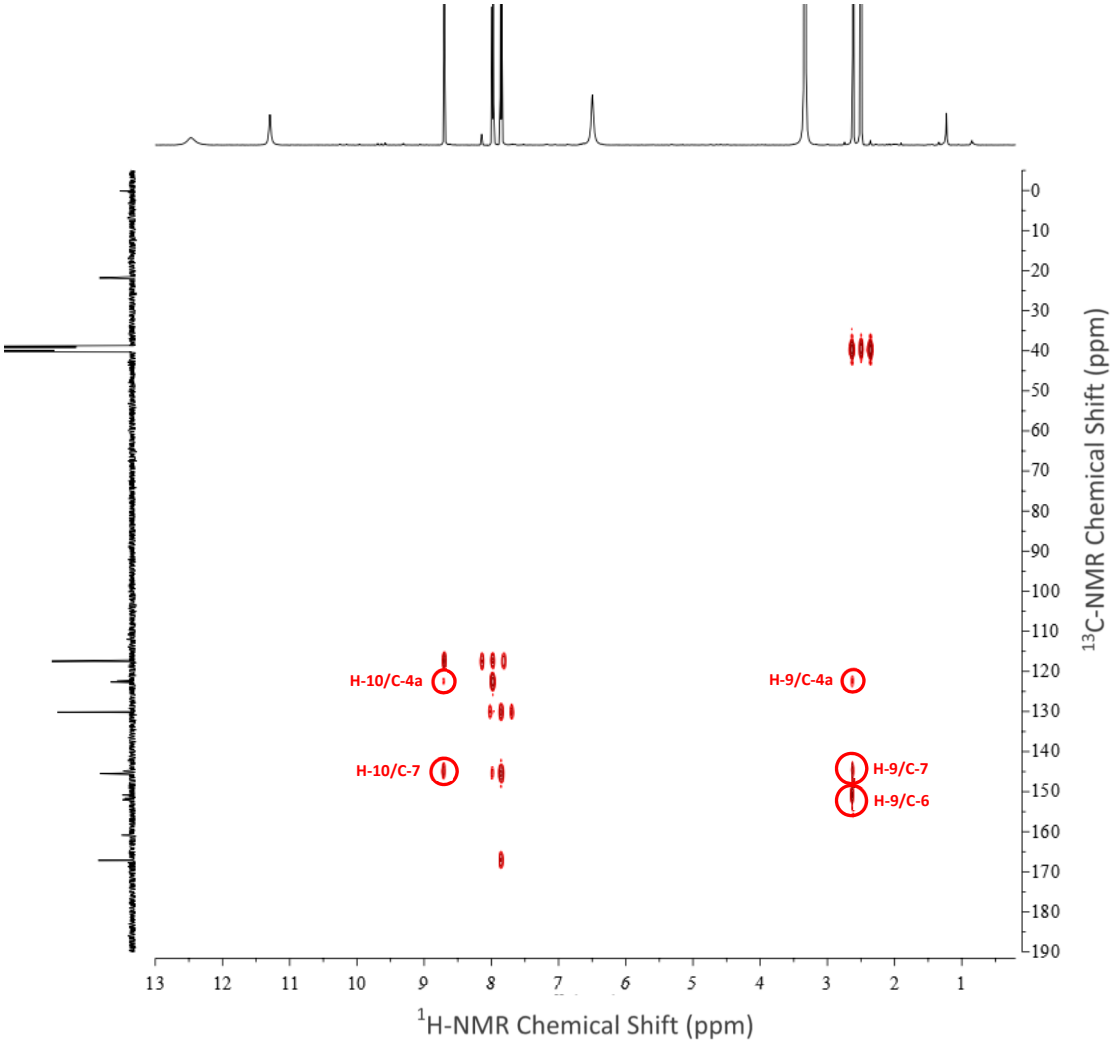


Fig. S7 The HMBC spectrum of the impurity K. The five-bond H-9/C-4a, four-bond H-10/C-4a, three-bond H-10/C-6, three-bond H-9/C-6, and two-bond H-9/C-7 correlations are particularly marked in red circles, which clearly demonstrate the direct linkage of N-10 to C-6 and the direct linkage of C-9 to C-7.

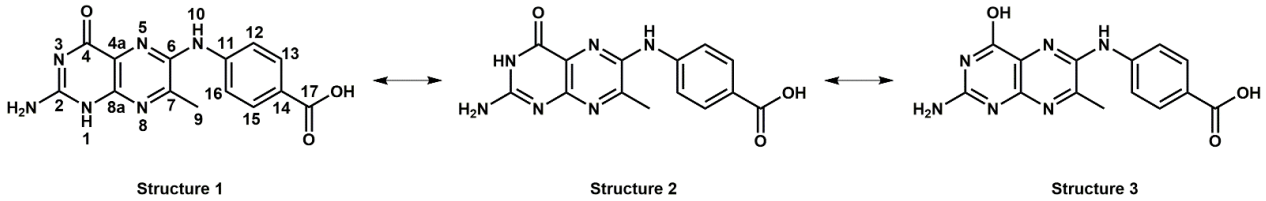


Fig. S8 The chemical resonance structures of the impurity K.

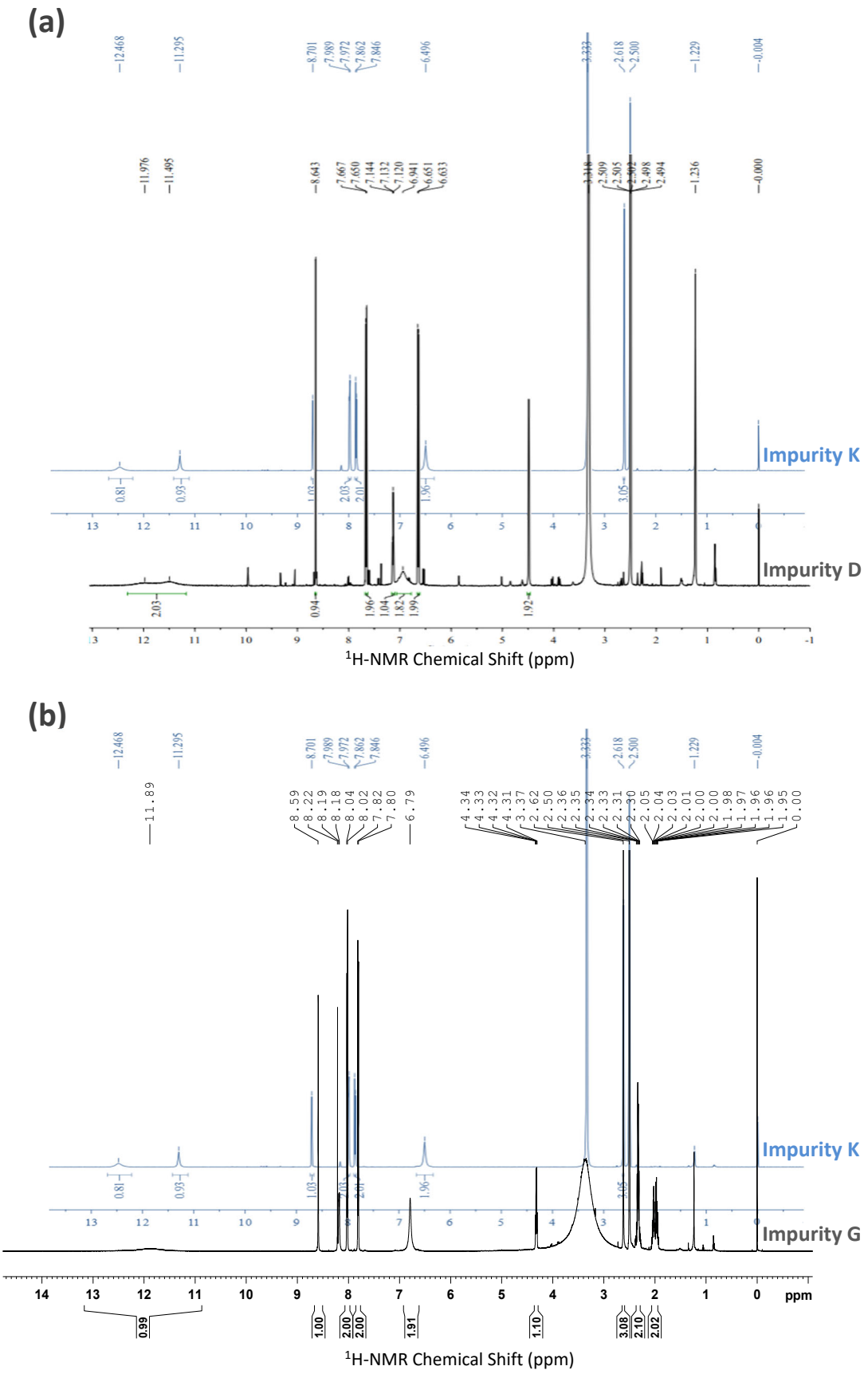


Fig. S9 Comparison of the ^{1}H-NMR spectra of the impurities K, D, and G. (a) Comparison of impurities K and D; (b) comparison of impurities K and G.

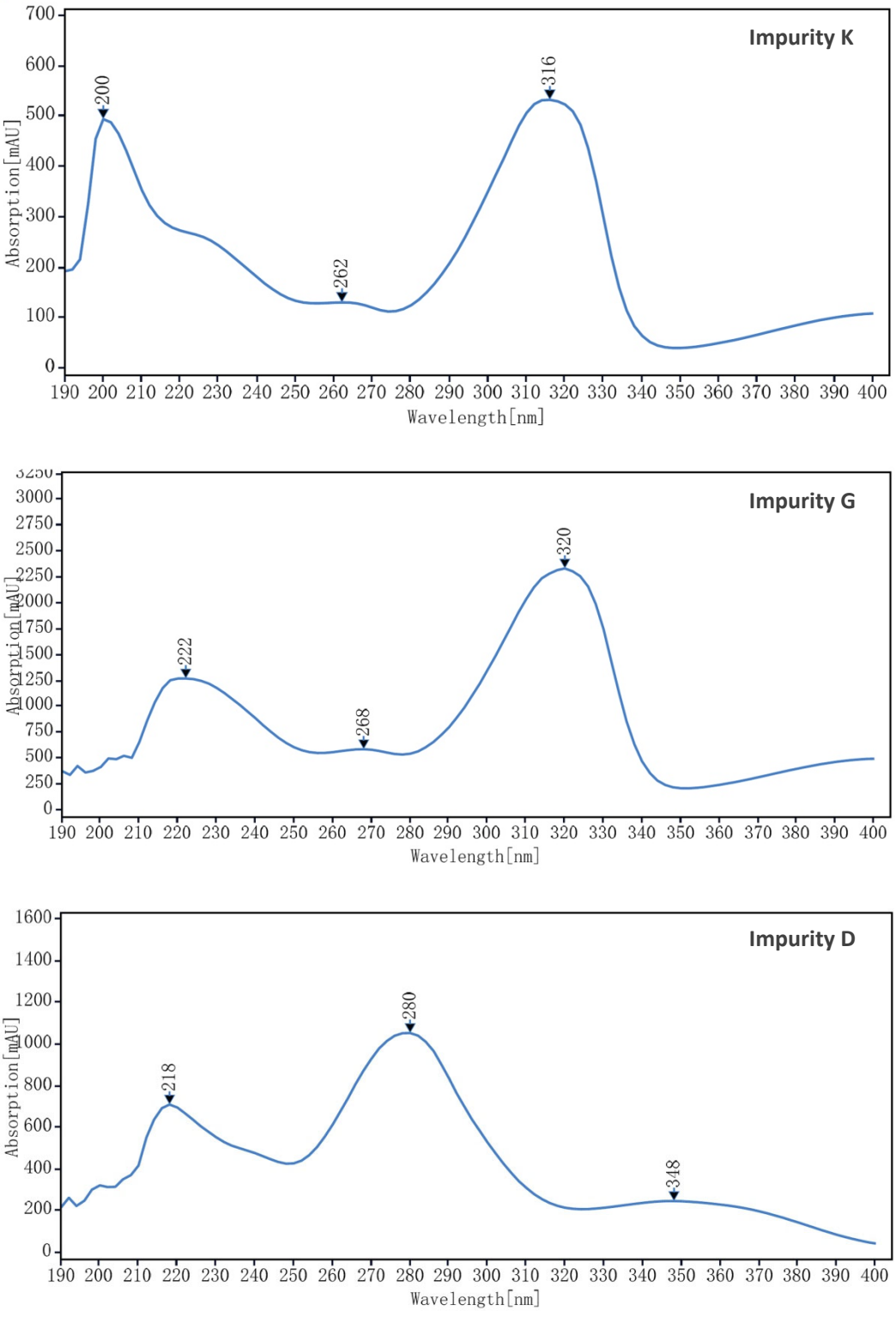


Fig. S10 The UV-Vis spectra of the impurities K, D, and G.

References

Rossi C, Donati A, Ulgiati S, et al., 1992. Structural investigation of folic acid by nmr proton relaxation and molecular mechanics analysis. *Bulletin of Magnetic Resonance*, 14(1-4):181-185.