Physicochemical Properties of Berberine and its Metabolites: Relationship with their Plasma Levels Evaluated after Administration to Human Subjects

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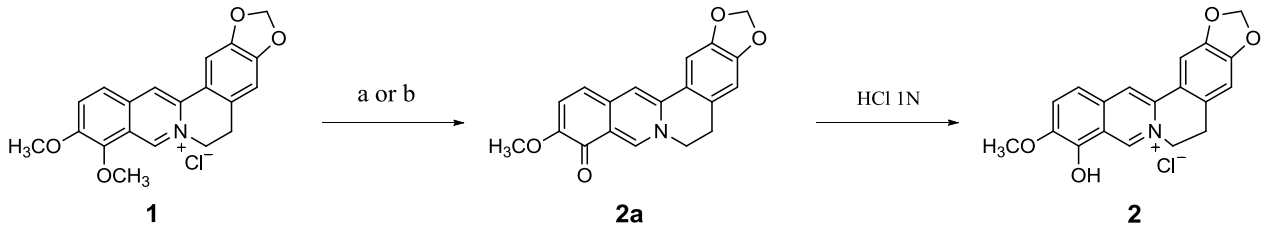
Supporting Information Contents:

Chemistry

General chemical methods

Reaction progress was monitored by TLC on precoated silica gel plates (Kieselgel 60 F254, Merck) and visualized by UV254 light. Flash column chromatography was performed on silica gel (particle size 40-63 Mm, Merck). Unless otherwise stated, all reagents were obtained from Sigma- Aldrich and used without further purification. Compounds were named relying on the naming algorithm developed by CambridgeSoft Corporation and used in Chem-BioDraw Ultra 11.0. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively. Chemical shifts (ppm) are reported relative to TMS as internal standard. Coupling constants (J), when given, are reported in Hertz (Hz). Mass spectra were recorded on a V.G. 7070 E spectrometer or on a Waters ZQ 4000 apparatus operating in electrospray (ES) mode. Reactions involving microwave irradiation were performed using a focused single-mode microwave synthesis system (CEM Discover® SP, 2.45 GHz, maximum power 300 W), equipped with infrared temperature measurement.

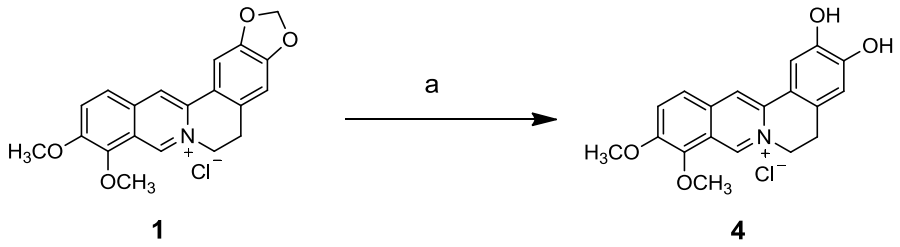
Scheme 1



1 **2a** **2**

Reagents and conditions. (a) 1, 190-200° C, 30-40 mmHg, 15-20 min; **(b) 1**, MW, 250 W, 200°C, 10-15 min

Scheme 2



1 **4**

Reagents and conditions. (a) 1, H_2SO_4 60%, phloroglucin, 2h, r.t.

Berberrubine 2a, procedure a[1]

Berberine chloride (1 g, 2.7 mmol) was heated at 195°-200° C for 10-15 min under vacuum (20-30 mmHg) to afford dark wine solid, which was washed with Et_2O dry and filtered, to give compound **2a** (0.7 g, yield 81%), like quinoid form. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.10 (t, 2H, *J*= 6.0 Hz), 3.79 (s, 3H), 4.54 (t, 2H, *J*= 6.0 Hz), 6.16 (s, 2H), 6.41 (d, 1H, *J*= 8 Hz), 7.02 (s, 1H), 7.27 (d, 1H, *J*= 8 Hz), 7.67 (s, 1H), 8.04 (s, 1H), 9.14 (s, 1H). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 27.5, 52.3, 55.7, 100.6, 101.5, 104.7, 108.2, 117.1, 120.0, 121.2, 121.8, 129.2, 132.0, 133.2, 145.7, 147.3, 148.3, 149.7, 167.3; MS (ES): m/z 322 ($M+H^+$).

Berberrubine 2a, procedure b[2]

Berberine chloride (0.1 g, 0.27 mmol) was taken in a vessel and the sample was irradiate at 250 W, 200°C for 10-15 min to afford dark wine solid, which was washed with Et_2O dry and filtered, to give compound **2a** (0.06 g, yield 70%) like quinoid form (0.06 g, yield 62%). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.10 (t, 2H, *J*= 6.0 Hz), 3.79 (s, 3H), 4.54 (t, 2H, *J*= 6.0 Hz), 6.16 (s, 2H), 6.41 (d, 1H, *J*= 8 Hz), 7.02 (s, 1H), 7.27 (d, 1H, *J*= 8 Hz), 7.67 (s, 1H), 8.04 (s, 1H), 9.14 (s, 1H). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 27.5, 52.3, 55.7, 100.6, 101.5, 104.7, 108.2, 117.1, 120.0, 121.2, 121.8, 129.2, 132.0, 133.2, 145.7, 147.3, 148.3, 149.7, 167.3; MS (ES): m/z 322 ($M+H^+$).

Berberrubine 2

The compound **2a** was treated with HCl 1N to obtain berberrubine like chloride salt **2**. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.25 (t, 2H, *J*= 6 Hz), 4.10 (s, 3H), 4.96 (t, 2H, *J*= 6 Hz), 6.23 (s, 2H), 7.12 (s, 1H), 7.76 (d, 1H, *J*= 8 Hz), 7.84 (s, 1H), 8.15 (d, 1H, *J*= 8 Hz), 8.89 (s, 1H), 9.97 (s, 1H), 11.32 (br s, 1H). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 26.5, 54.9, 57.1, 102.0, 105.4, 108.4, 117.6, 118.1, 119.8, 120.7, 125.5, 130.5, 132.4, 136.6, 143.7, 145.4, 145.8, 147.7, 149.6.

Demethyleneberberine 4[1]

To a stirred solution of 60% H_2SO_4 (50 mL), phloroglucin (2.5 g, 20 mmol) was added portionwise to form a colorless solution. Berberine chloride (2.5 g, 6.7 mmol) was added portionwise and the resulting system was stirred at 90-95°C for 10-15 min. Then the mixture was poured into brine and the resulting mixture was stirred at room temperature for 2 h and cooled down to precipitate completely. The crude product was placed on a short pad of silica gel and eluted with CH_2Cl_2/ CH_3OH to afford to the desired compound **4** (0.97 g, yield: 40%). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.18 (t, 2H, *J*= 6.0 Hz), 4.12 (s, 3H), 4.15 (s, 3H), 4.96 (t, 2H, *J*= 6.0 Hz), 6.91 (s, 1H), 7.60 (s, 1H), 8.11 (d, 1H, *J*= 8 Hz), 8.23 (d, 1H, *J*= 8 Hz), 8.82 (s, 1H), 9.45 (br s, 1H), 9.89 (s, 1H), 10.23 (br s, 1H). ^{13}C-NMR (DMSO-d_6, 100 MHz): δ 25.8, 55.6, 57.1, 61.9, 112.7, 114.9, 117.8, 119.3, 121.2, 123.5, 126.7, 127.2, 133.3, 138.3, 143.5, 145.1, 145.6, 149.2, 150. MS (ES): m/z 324 ($M+H^+$).

NMR studies of 2, 2a, 2b forms

In the past years several spectroscopic studies concerning protoberberine alkaloids were performed (UV-VIS[3,4,5], NMR[6,7]). Some of these paper reported important information about **2**. In most cases berberrubine was represented as structure **2**[1,5,6,9] (Figure S1) with hydroxyl group in position C13, otherwise it was suggested either quinoid structure[2,8] **2a** (Figure S1) or zwitterionc form[3,8] **2b** (Figure S1) as result of tautomerization process[8]. In this work our efforts were directed to investigate the interconversion between the three forms: **2**, **2a** and **2b**. NMR analysis of the crude product **2a**, exhibited a CO signal at 167.3 ppm, corresponding to C13 position, consistent with structure **2a**. By treatment of **2a** with HCl, **2** was obtained and its structure was confirmed by ^{1}H-NMR spectrum showing a broad signal at 11.32 ppm corresponding to the –OH proton of C13. Resonance assignments and structures of both forms **2** and **2a** were verified by 1D Proton and

Carbon NMR spectra combined with 2D HSQC and HMBC[6] (Figures S2-S5). We propose the existence of keto-enol tautomerism between **2** and **2a**, and quinoid-zwitterion resonance for **2a** and **2b** (Figure S1). In order to prove the equilibrium between **2** and **2a**, we performed ^{1}H-NMR analysis on a sample consistent of equimolar mixture of both forms. The spectrum displayed only one average set of signals demonstrating the existence of fast equilibration between this two tautomeric forms. To further validate the existence of a possible equilibrium between the tautomeric forms **2** and **2a**, we carried out for both structures a ^{1}H-^{15}N HMQC (Figures S6 and S7) correlation analysis, recorded at 40° C in DMSO-d_6. Considering form **2**, correlations (Figure S8) were observed from H8 triplet and the H11 singlet to the N10 resonance at 193 ppm. Regarding structure **2a**, the same correlations (Figure S8) from H8 triplet and the H11 singlet to the N10 were obviously observed, but in this case the N10 resonance was found at 164 ppm. Different chemical shifts for the two ^{15}N resonances, are quite reasonable based on a different electron density due to the presence of quaternary N atom in **2** with respect to tertiary N atom in structure **2a**. These data were consistent with the existence of these two tautomeric forms. Regarding the equilibrium involving quinoid **2a** and zwitterionic **2b** forms of **2** (Figure S1), we suggested a quinoid-zwitterion resonance in agreement with Suau et al.[9] that proposed an analogue betaine-quinoid resonance for the protoberberine alkaloid 7,8-dehydrocaseamine. We envisaged that the aprotic environment determined by the solvent DMSO-d_6, used to collect NMR spectra, should stabilized form **2a**, while the presence of water in this deuterate solvent may promote the zwitterion formation **2b**. To gain more insight into the water's effect, NMR titration (1D proton and carbon) was performed. Increasing amounts of water were added to a sample of **2a** in DMSO-d_6. NMR spectra recorded at increasing of water content showed shifted peaks assigned to the nuclei most involved in the structure rearrangement such as: H16, C16, C13 (Table S1). These chemical shift values δ were chosen as markers to follow the process (Figures S9, S10 and S11).

Figure S1. Three possible forms describing **2**

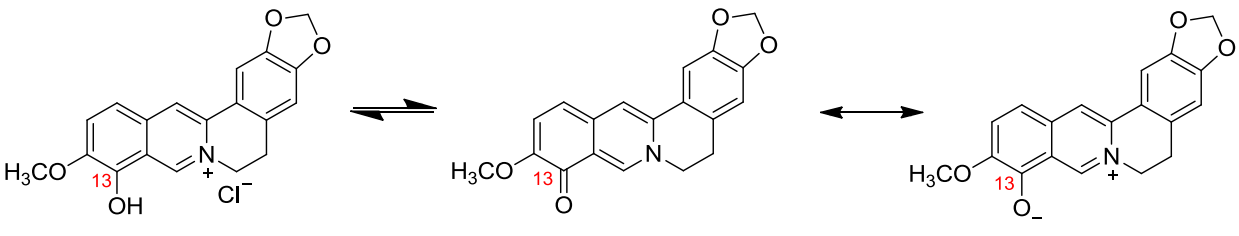


Figure S2. 400 MHz HSQC NMR Spectrum of **2** in DMSO-d_6

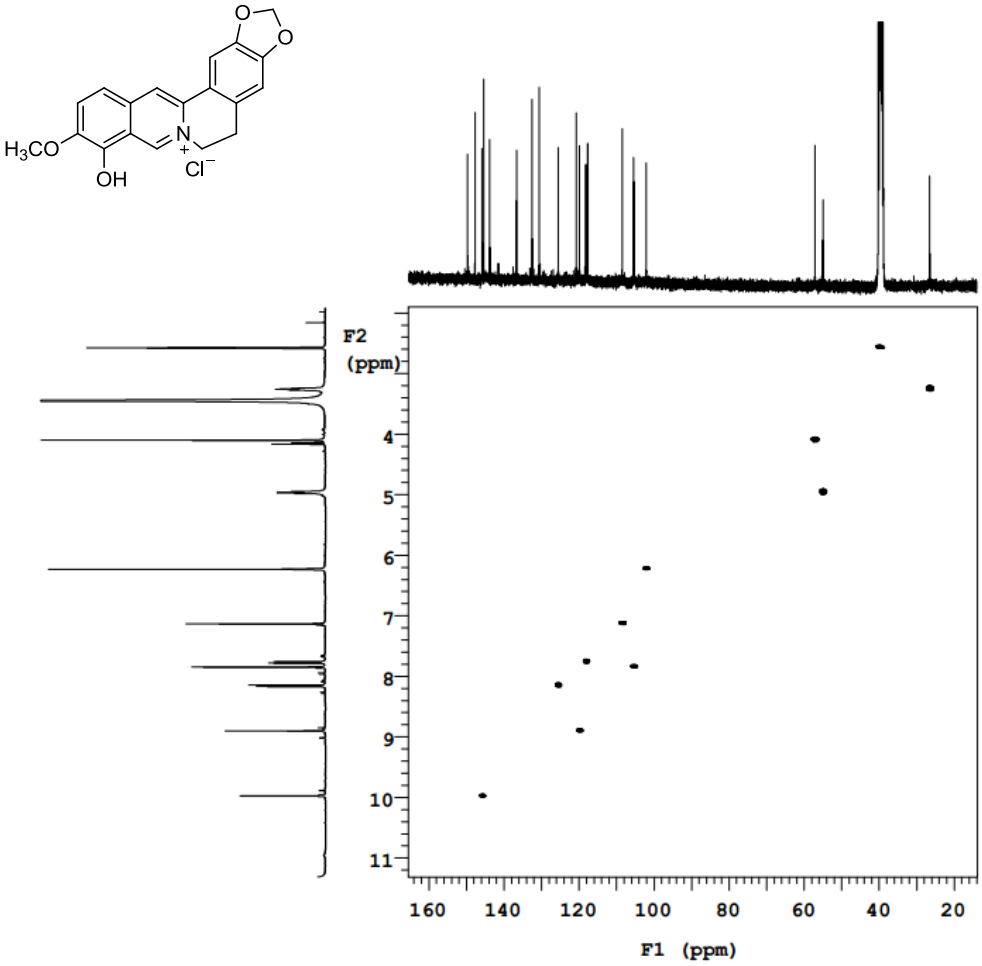


Figure S3. 400 MHz HMBC NMR Spectrum of **2** in DMSO-d_6

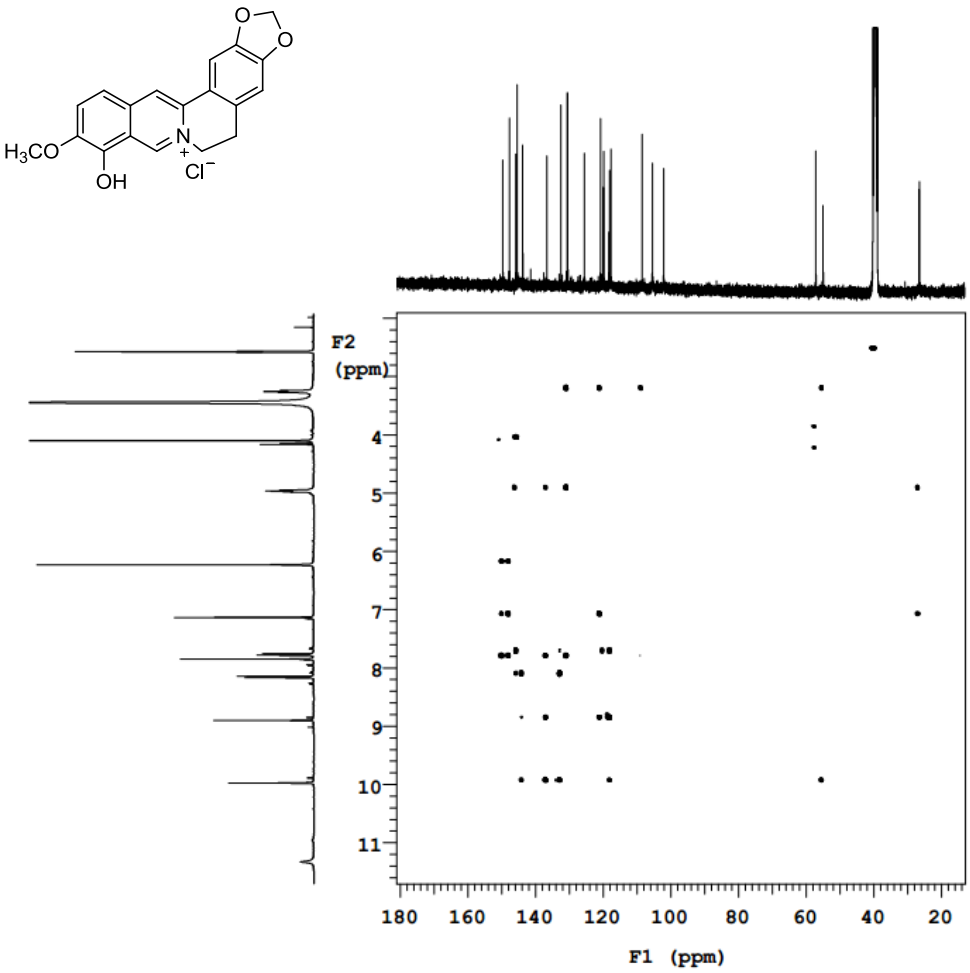


Figure S4. 400 MHz HSQC NMR Spectrum of **2a** in DMSO-d_6

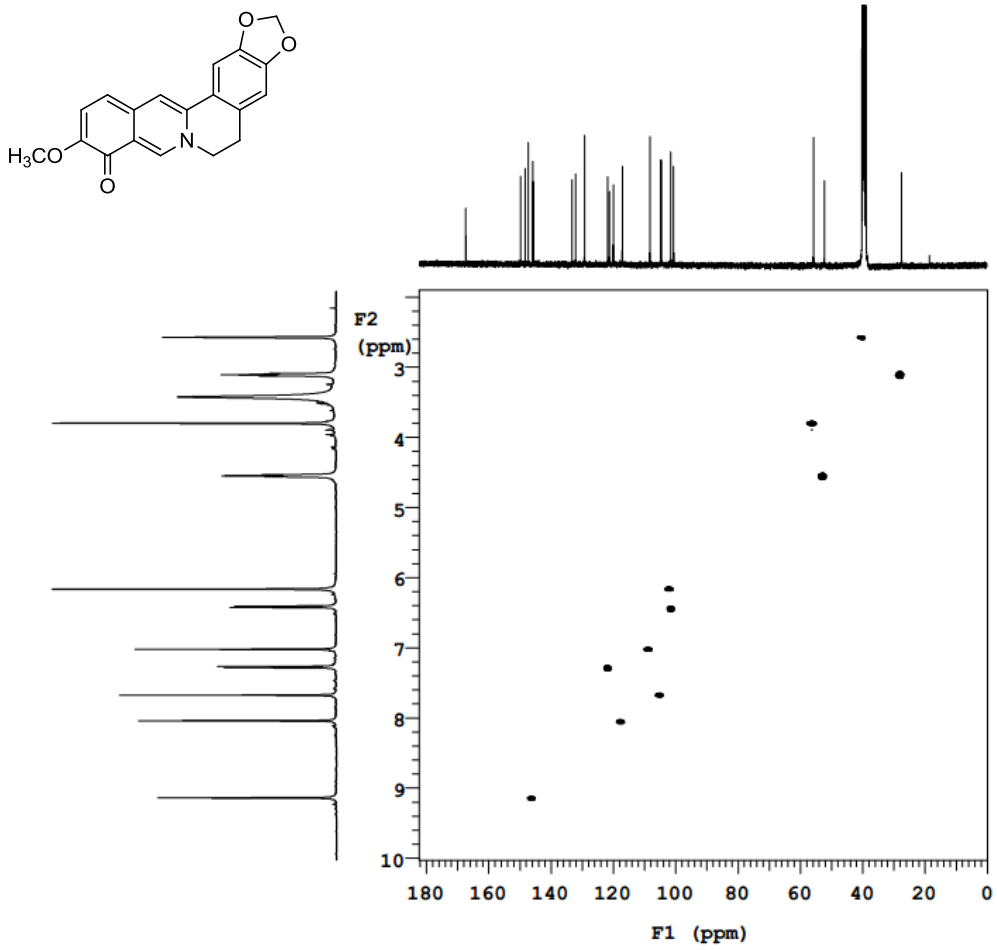


Figure S5. 400 MHz HMBC NMR Spectrum of **2a** in DMSO-d_6

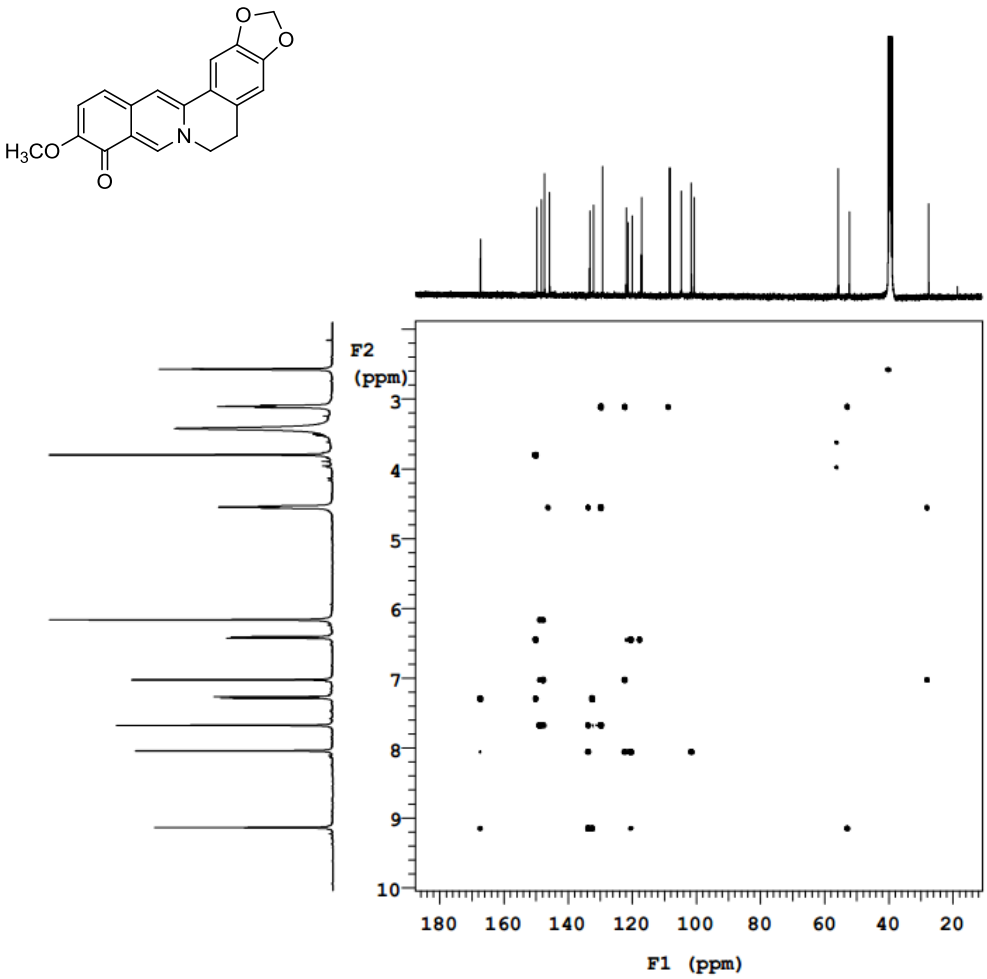


Figure S6. 600 MHz HMQC NMR Spectrum of **2** in DMSO-d_6

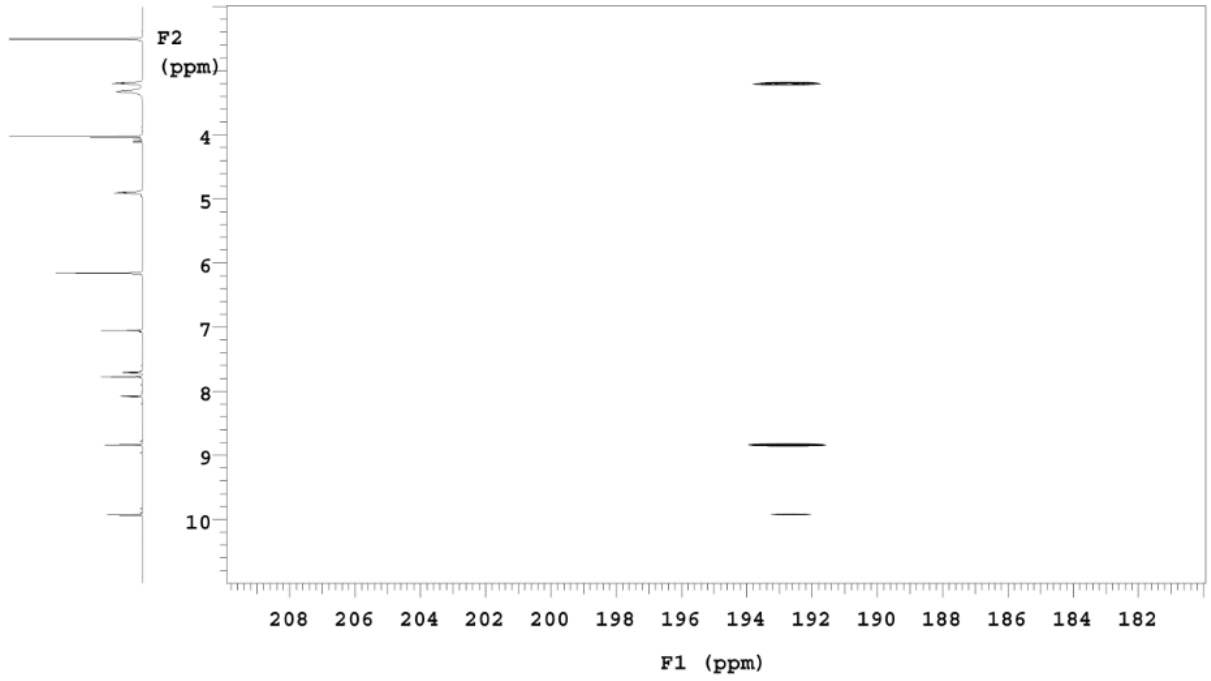


Figure S7. 600 MHz HMQC NMR Spectrum of **2a** in DMSO-d_6

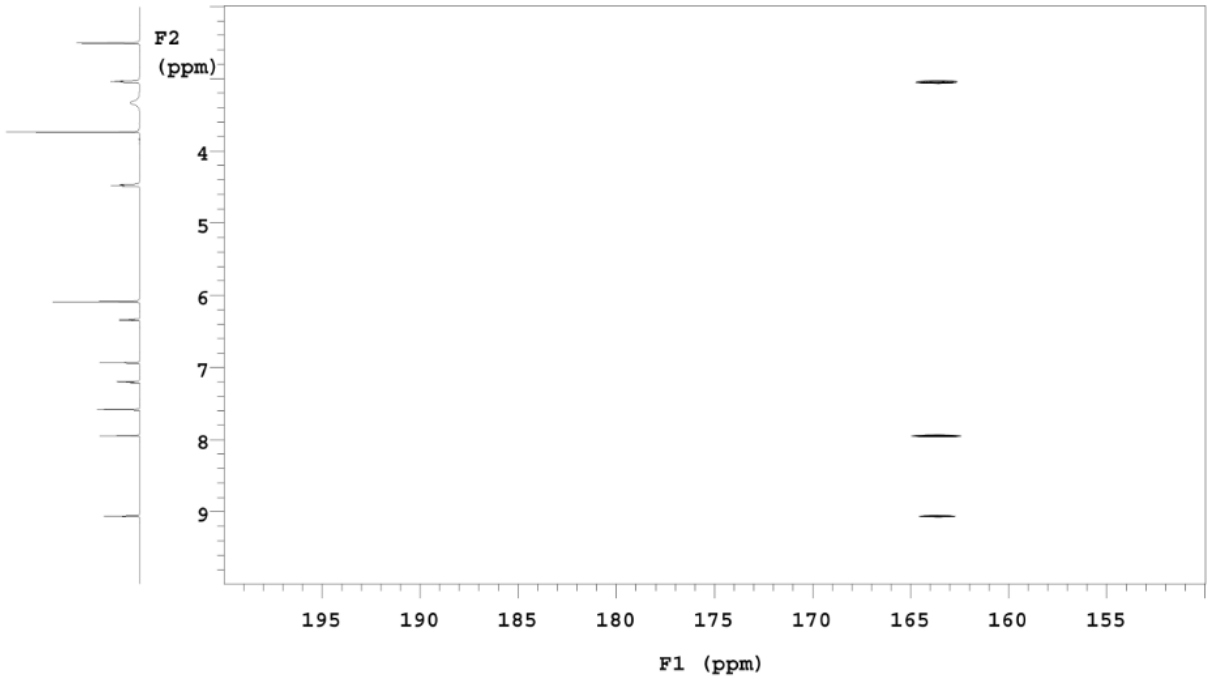
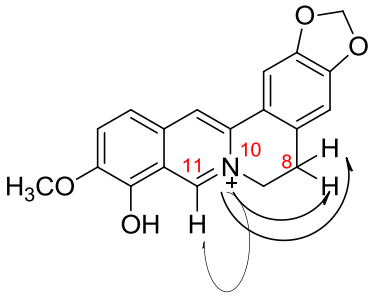
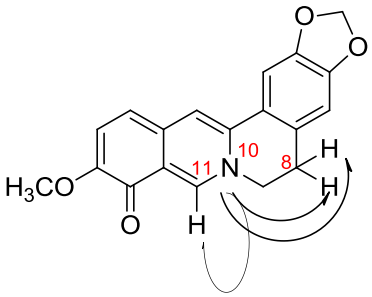


Figure S8. ^{1}H-^{15}N HMQC correlations for the keto-enolic forms of Berberrubine



2

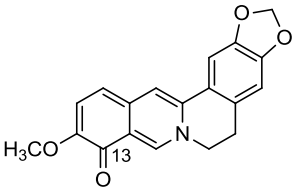


2a

Table S1. ^{1}H and ^{13}C NMR Data for Berberrubine (**2**, **2a**, **2b**) in DMSO-d_6

	compound					
	2		**2a**		**2b**	
position						
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	102.0	6.23, s	101.5	6.16, s	101.9	6.13, s
4	149.6		148.3		148.8	
5	147.7		147.3		147.6	
6	108.4	7.12, s	108.2	7.02, s	108.6	7.00, s
7	130.5		129.2		129.7	
8	26.5	3.25, t (6)	27.5	3.10, t (6)	27.7	3.11, t (6)
9	54.9	4.96, t (6)	52.2	4.54, t (6)	53.2	4.54, t (6)
11	145.8	9.97, s	145.7	9.14, s	146.3	9.14, s
12	117.6		120.0		120.2	
13	143.7		167.3		166.1	
14	145.4		149.7		150.0	
15	125.5	8.15, d (8)	121.2	7.27, d (8)	122.0	7.35, d (8)
16	118.1	7.76, d (8)	100.6	6.42, d (8)	102.9	6.67, d (8)
17	132.4		132.0		132.3	
18	119.8	8.89, s	117.1	8.04, s	117.8	8.07, s
19	136.6		133.2		133.7	
20	119.8		121.8		122.2	
21	105.4	7.83, s	104.7	7.67, s	105.0	7.62, s
22		(-OH) 11.32, s br				
24	57.0	4.10, s	55.7	3.79, s	56.2	3.79, s

Figure S9. The ^{13}C chemical shifts of signal C13, (**2a**), measured at 100 MHz in DMSO-d_6 as function of water content



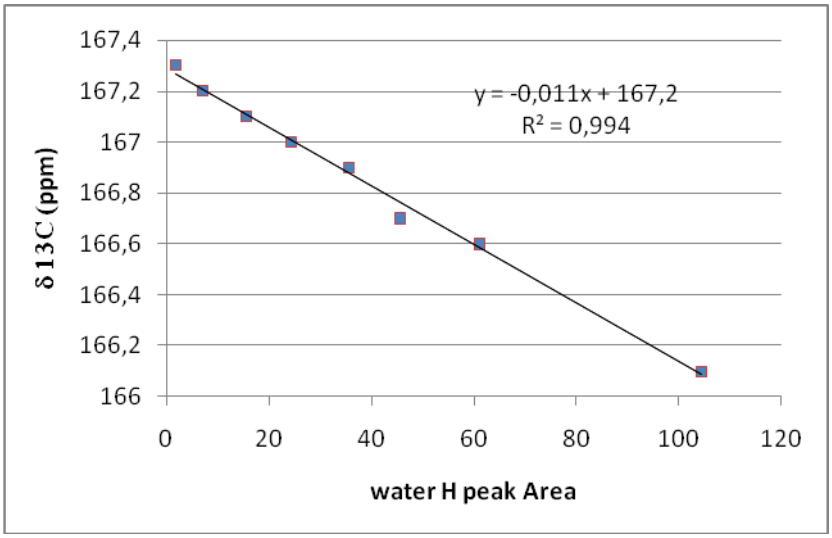
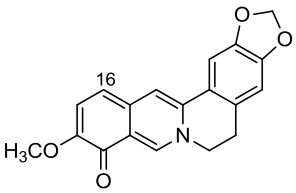


Figure S10. The ^{1}H chemical shifts of signal H16, (**2a**), measured at 400 MHz in DMSO-d_6 as function of water content



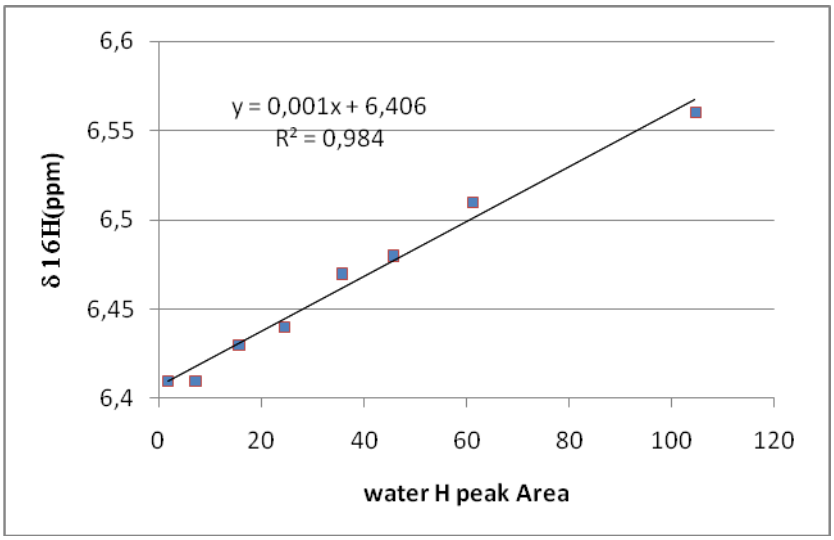
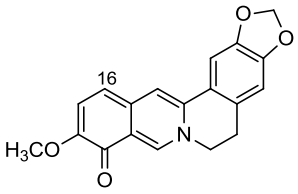
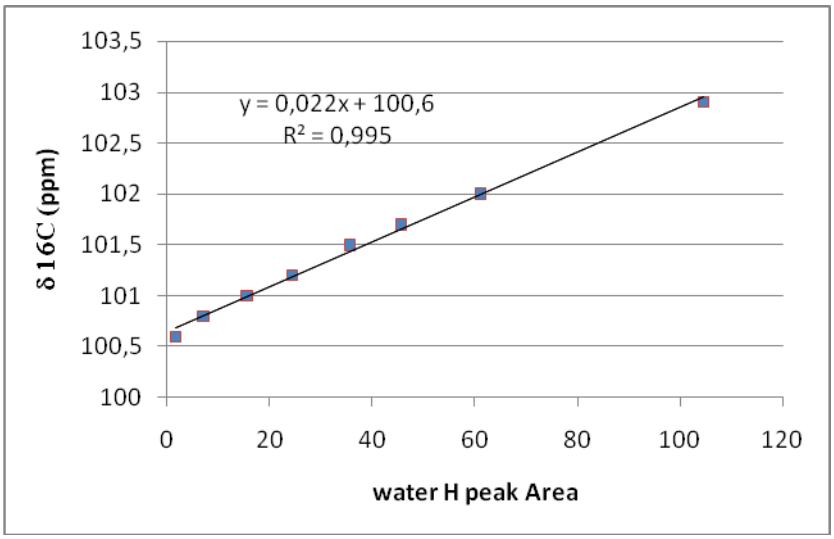


Figure S11. The ^{13}C chemical shifts of signal C16, (**2a**), measured at 100 MHz in DMSO-d_6 as function of water content





Equation S1. General equation used for the calculation of LogD

$$logD = logP + log\left(\frac{1}{1 + 10^{pH - pK_a}}\right)$$

Equation S2. Stern Volmer modificated equation

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_B} \cdot \frac{1}{Q} + \frac{1}{f_a}$$

where:

F_0 – fluorescence intensity in absence of quencher

ΔF – difference in fluorescence in absence and presence of quencher

f_a – fraction of accessible fluorescence

K_B – effective quenching constant for the accessible fluorophores

Q – concentration of quencher

In vivo Study protocol

Study name and code. "The evaluation of biologic effects of Berberine on biliary and cholesterol metabolism in 12 dyslipidemic patients" - Va.Li.Co.-09 - S.Orsola-Malpighi Hospital Review Board No. 7-2209-U-SPER, approved April, 21st 2009.

Study design. Open label, single arm study on 12 subjects of both sex, age ranging between 18-70 year-old; all enrolled patients received 15 mg/kg daily of Berberine for three months.

Population.

Inclusion criteria: age 18 – 70 year-old; c-LDL > 130 mg/mL and < 190 mg/mL; fasting glucose < 100 mg/mL; written informed consent.

Exclusion criteria: abnormal liver function tests and or history of liver disease; presence of biliary stone (on trans-abdominal ultrasound evaluation) or known biliary disease; obesity (BMI > 30); familial hypercholesterolemia; history of thyroid or other endocrine disease; alcohol consumption > 20 mg/day; medication with drugs active on glucose, lipid or biliary metabolism; any serious medical conditions.

Treatment. Berberis Vulgaris 250 mg cps (Registration code: 939673796; KOS s.r.l. Comeana (Po), Italy); patients received 15 mg/kg daily for three months. Min. daily dose prescribed was 750 mg (3 cps/day); max. daily dose prescribed was 1500 mg (6 cps/day).

Visits and evaluation: Before enrollment (T0), after 1 months (T1), after 2 months (T2) and at the end of the study period (T3) we evaluate routine serum lab. tests, total cholesterol, c-LDL, c-HDL, triglycerides, (reported in Table S2).

Table S2. Comparison between Basal vs. After treatment (V0 vs. V3) of cholesterol (total, LDL and HDL) and total triglycerides

		Total-Cholesterol (mg/dL)		**LDL** (mg/dL)		**HDL** (mg/dL)		**Total Triglycerides** (mg/dL)	
	patient	**V0**	**V3**	**V0**	**V3**	**V0**	**V3**	**V0**	**V3**
	1	268	233	173	138	65	59	134	179
	2	238	251	162	181	50	51	119	87
	3	193	205	133	138	35	36	113	156
	4	196	147	131	90	51	46	63	48
	5	211	187	136	115	45	43	133	132
	6	211	200	132	111	69	70	47	87
	7	201	188	126	111	48	51	120	118
	8	241	227	174	158	39	37	126	162
	9	276	267	194	197	48	46	168	110
	10	229	226	145	141	56	58	128	123
	11	213	182	137	124	38	37	190	95
	12	225	210	149	137	49	49	122	118
paired t test	**p**	0.016		0.025		0.312		0.753	

Results are expressed for each patient for the comparison between Basal vs. After treatment (V0 vs. V3)

Pharmacokynetic study. After a single 500 mg dose of Berberine in 10 healthy subject, plasma samples were collected after 0, 1, 2, 3, 4, 6, 8, 24 h from oral administration.

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