Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

In vivo and *in vitro* identification of *Z*-BOX C – a new oxidative bilirubin degradation product

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I. General Information

Chemicals

All solvents and compounds were purchased and used without further purification. HPLC gradient grade acetonitrile was obtained from VWR and water was purified with TKA microPure (Thermo Electron. Niederelbert, Germany). ULC gradient grade water and acetonitrile was purchased from Fisher Scientific (United Kingdom and ULC formic acid was obtained from Biosolve B.V. (Valkenswaard, The Netherlands).

Instrumentation

To remove solvents an evacuated centrifuge (Christ SpeedVac RVC 2-25) at 40 °C was used.

NMR data (^{1}H; ^{13}C; ^{1}H,^{13}C-HSQC; ^{1}H,^{13}C-HMBC) were collected on a 400 MHz Bruker Avance I using the residual solvent resonance of the solvent [D_6]DMSO as internal standard for referencing.

ATR-IR was measured with an IR-Affinity-1 from Shimadzu (Duisburg, Germany) and 20 scans per spectrum. Mass spectrometry and HPLC / UHPLC was carried out on the below-mentioned instruments.

Preparative liquid chromatography

For preparative separation a HPLC (Shimadzu LC-8A, Kyoto, Japan) with a HTEC C18-column (5 µm, 250 x 16 mm, Macherey-Nagel, Düren, Germany) equipped with a SPD-10AV UV-VIS detector measuring at 285 nm was used. Solvent A contained 2% acetonitrile in water with 0.1% formic acid and solvent B 100% acetonitrile with 0.1% formic acid. The used gradient (time, vol% solvent B) was as follows: 0 min, 0%; 1 min, 0%, 2 min, 13%, 25 min, 13%; 27 min, 100%; 35 min, 100%; 36 min, 0%; 45 min, 0% with a flow rate of 5 mL/min.

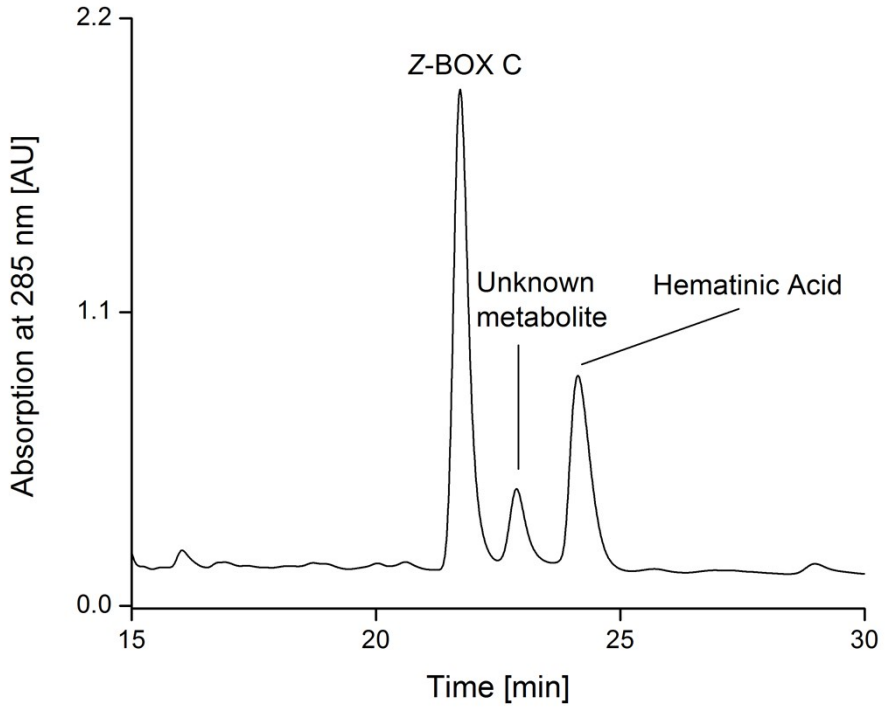


Figure S1. HPLC-UV/Vis profile at 285 nm of the preparative HPLC-separation.

LC-MS for *Z*-BOX C analytics

For reaction monitoring a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Leicestershire, United Kingdom) equipped with an Acquity UPLC BEH C18 column (1.7 µm, 100 × 2.1 mm) was used. The UHPLC was coupled to a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Leicestershire, United Kingdom) and ionization was carried out with electrospray in positive ion mode. Solvent A contained 2% acetonitrile in water with 0.1% formic acid and solvent B 100% acetonitrile with 0.1% formic acid. The following gradient (time, vol% solvent B) was used: 0.0 min, 0%; 0.2 min, 0%; 6.0 min, 18%; 6.5 min, 100%; 7.9 min, 100%; 8.0 min, 0%; 10.0 min, 0% with a flow rate of 0.4 mL/min. For quantification of BOX C in human bile samples and in vitro degradation of PDPs with H_2O_2 the same system was used with the following gradient: 0.0 min, 0%; 0.5, 0%; 1.0 min, 18%; 8.0 min, 18%; 9.0 min, 100%; 10.9 min, 100%; 11.0 min, 0%; 13.0 min, 0% with a flow rate of 0.4 mL/min. External calibration using isolated standards was performed for quantification.

Preparation of biological samples

The bile samples were prepared according to Seidel *et al.*.[1]

II. Oxidative degradation

Bilirubin degradation procedure

The oxidative degradation of bilirubin was carried out based on published protocols.[2,3] Briefly, bilirubin (1 g, 1.72 mmol) was suspended in 0.5 L of 5 M NaOH and stirred for 24 h. The pH was adjusted to 7.5 with conc. HCl before H_2O_2 was added to a final concentration of 10% and stirred for 24 h. After extraction with chloroform to isolate *Z*-BOX A and B, the water phase was subjected to solid phase extraction (6 g Oasis hydrophilic lipophilic balanced cartridges; Waters, Manchester, United Kingdom). The cartridge was washed with brine, prior to the elution of degradation products with water. The water fraction was dried in an evacuated centrifuge and preparative HPLC-UV/vis was carried out to isolate the product. Additionally, an elution with 20% acetonitrile/water was performed to isolate a PDP-rich fraction.

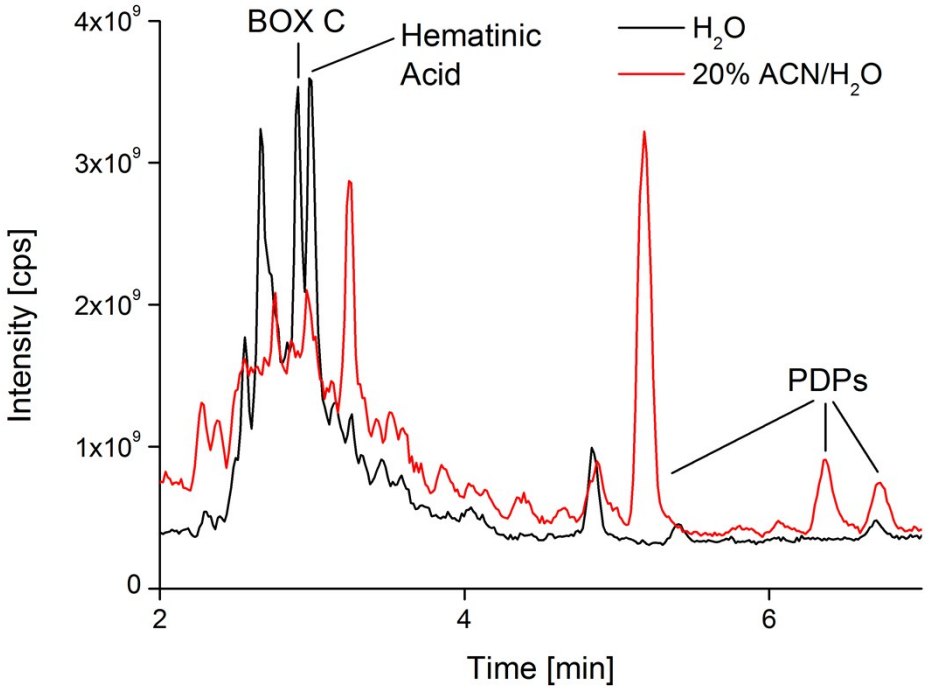


Figure S2. UHPLC-MS profile of the water and the 20% ACN/water fraction.

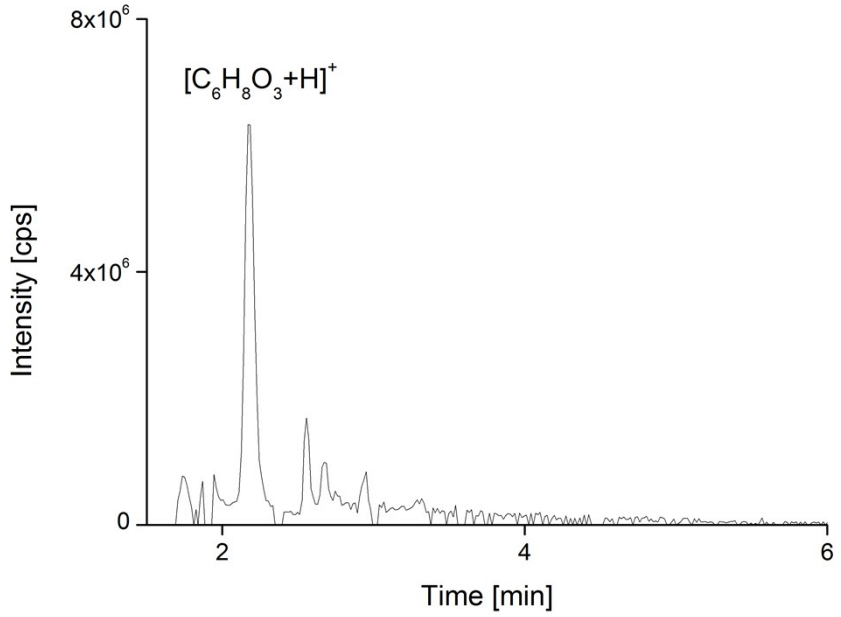


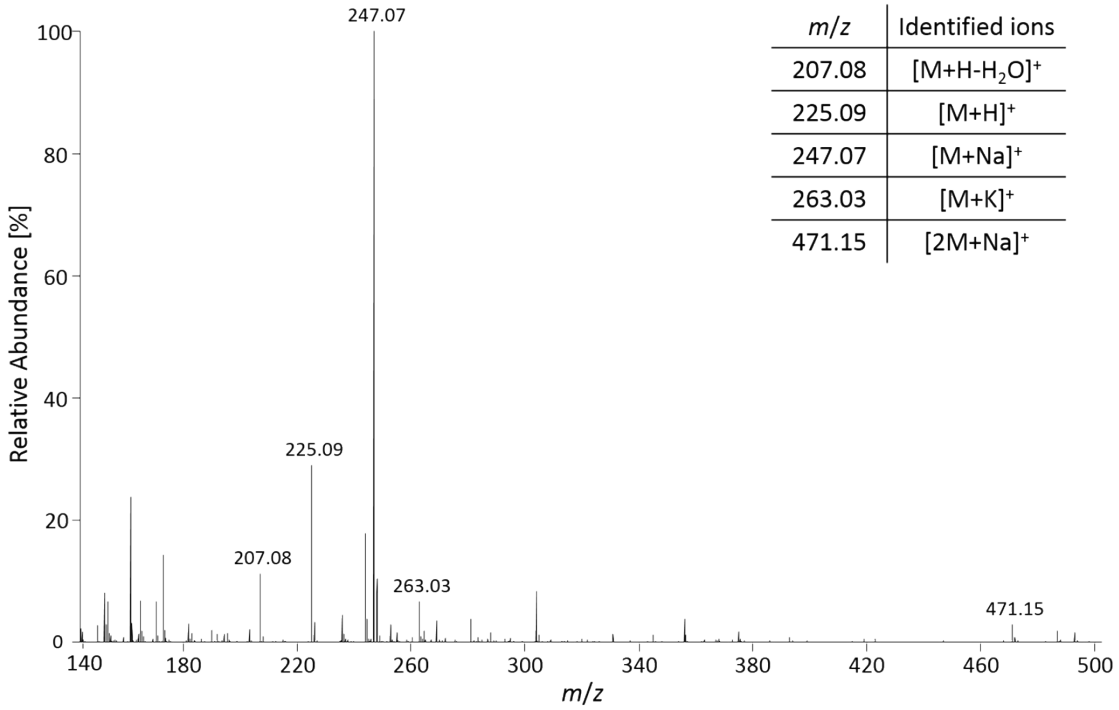
Figure S3. UHPLC-HR-MS profile of 129.05473 amu (+/- 5 ppm) with calculated sum formula of $C_6H_9O_3$ representing the other product of the proposed mechanism.

Degradation of PDPs

PDPs were isolated according to Ritter *et al.*.[4] A one to one mixture of PDP A1 and A2, as well as B1 and B2 was dissolved in water (final concentration 50 µM). These solutions (400 µL) were oxidized with 1% hydrogen peroxide under stirring for six days in the dark and monitored daily with UHPLC-HR-MS.

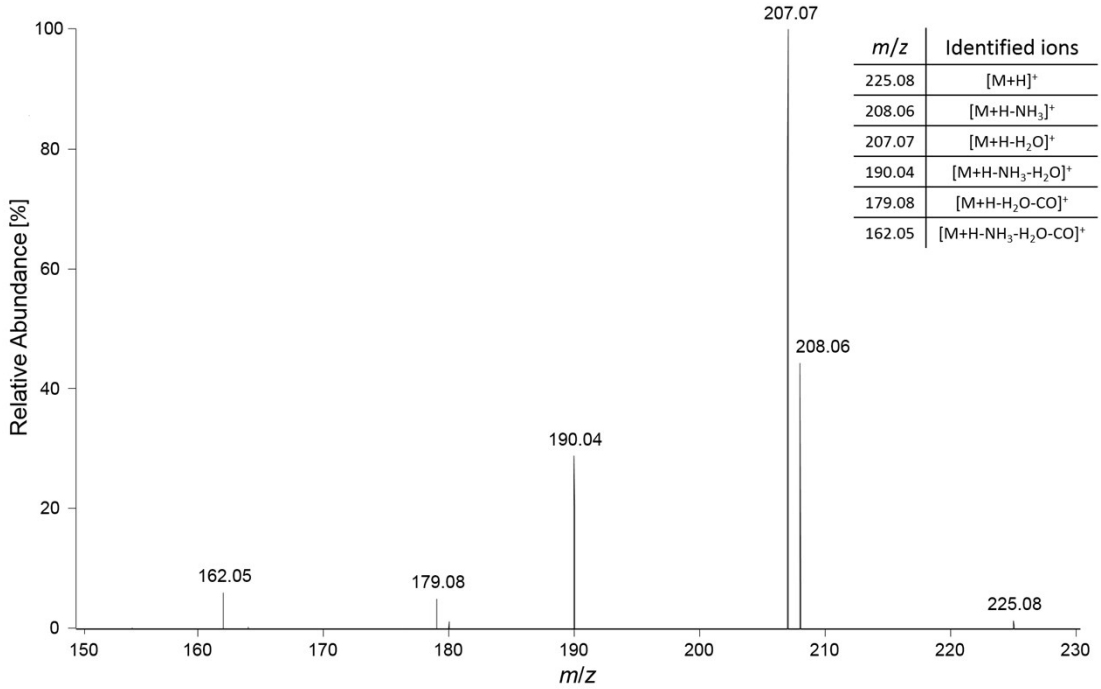
III. Spectrometric and spectroscopic data

MS



m/z	Identified ions
207.08	$[M+H-H_2O]^+$
225.09	$[M+H]^+$
247.07	$[M+Na]^+$
263.03	$[M+K]^+$
471.15	$[2M+Na]^+$

Figure S4. MS spectrum of *Z*-BOX C.



m/z	Identified ions
225.08	$[M+H]^+$
208.06	$[M+H-NH_3]^+$
207.07	$[M+H-H_2O]^+$
190.04	$[M+H-NH_3-H_2O]^+$
179.08	$[M+H-H_2O-CO]^+$
162.05	$[M+H-NH_3-H_2O-CO]^+$

Figure S5. MS/MS spectrum of *Z*-BOX C (collision energy 25 eV).

NMR spectra

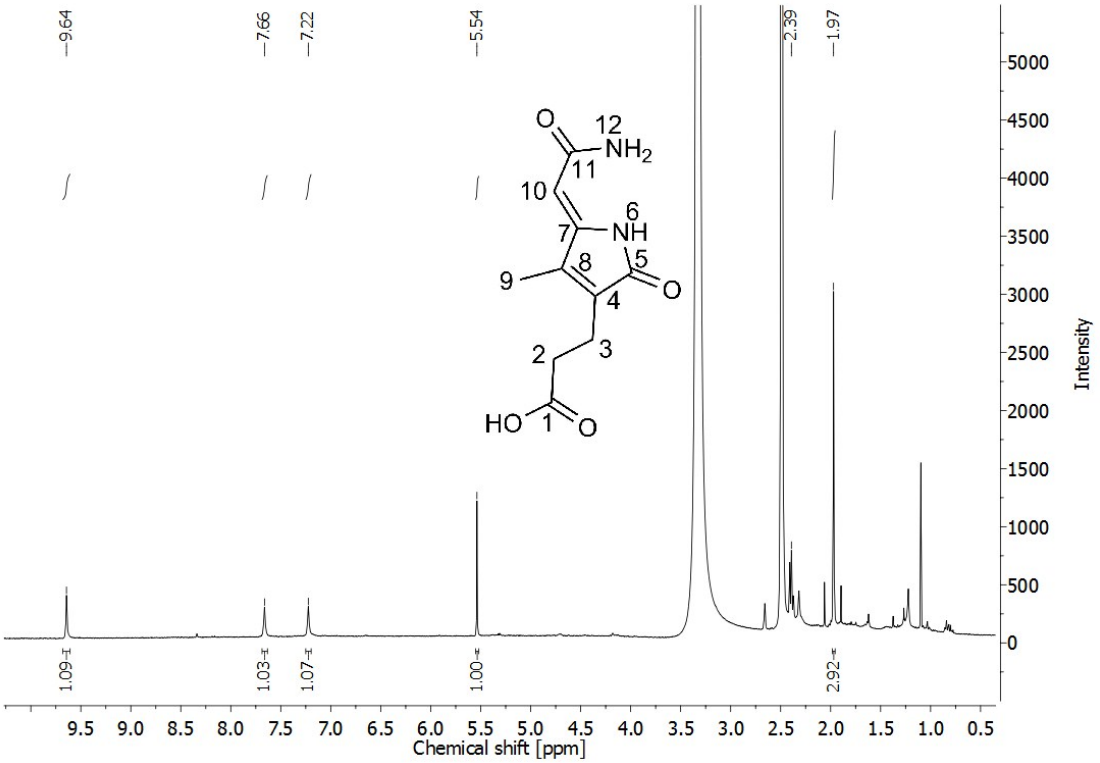


Figure S6: ^{1}H-NMR spectrum (400 MHz) of *Z*-BOX C in [D_6]DMSO.

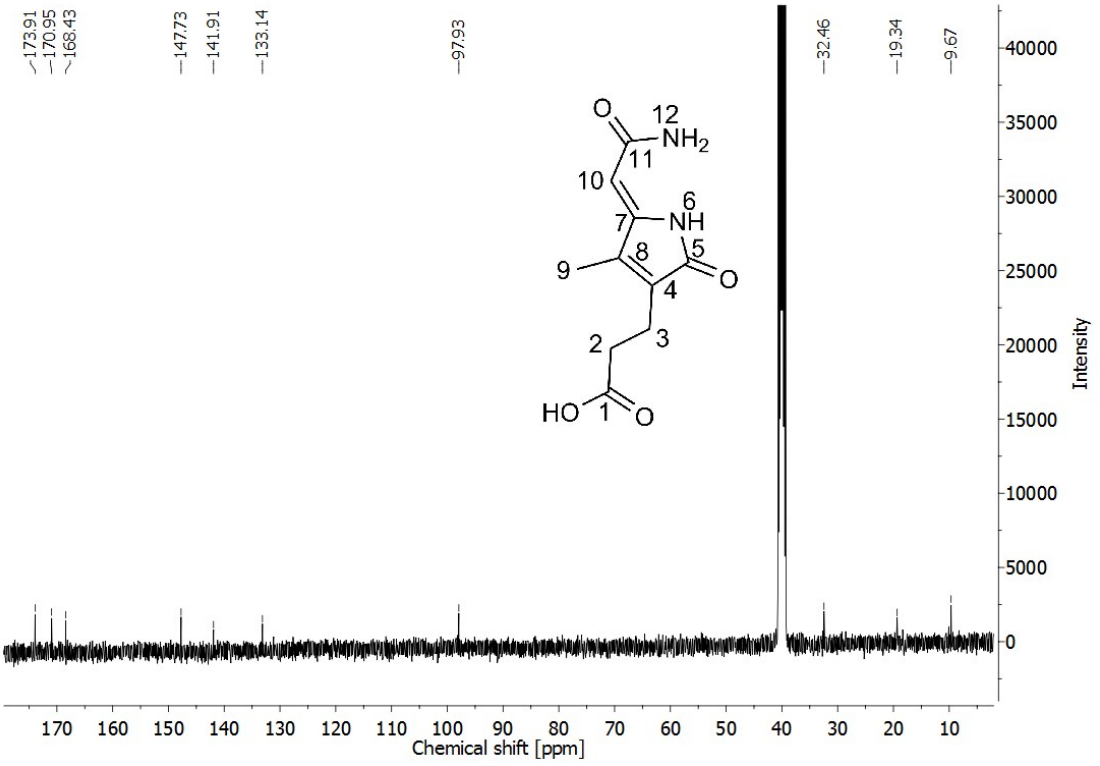


Figure S7: ^{13}C-NMR-spectrum (100 MHz) of *Z*-BOX C in [D_6]DMSO.

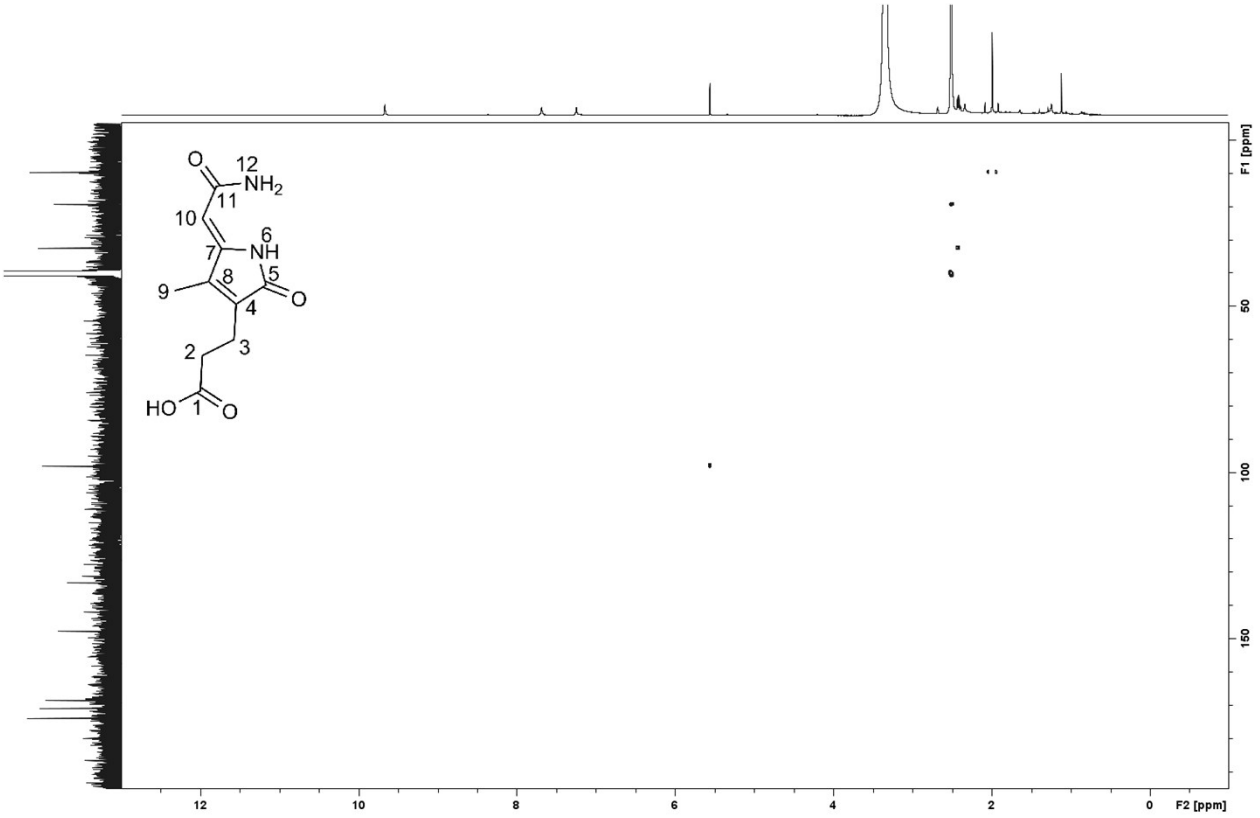


Figure S8: $^{1}H,^{13}C$-HSQC-NMR spectrum (400 MHz) of *Z*-BOX C in $[D_6]$DMSO.

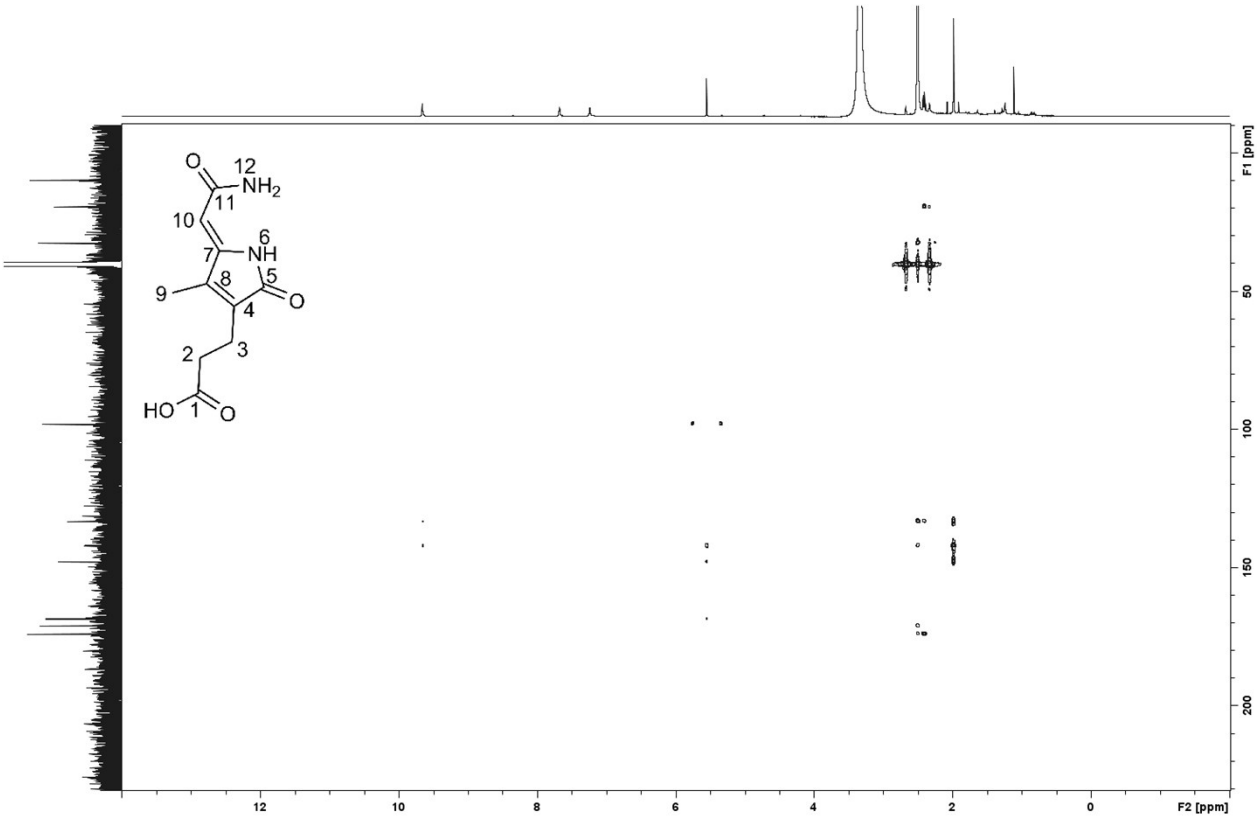


Figure S9: $^{1}H,^{13}C$-HMBC-NMR spectrum (400 MHz) of *Z*-BOX C in $[D_6]$DMSO.

IR spectrum

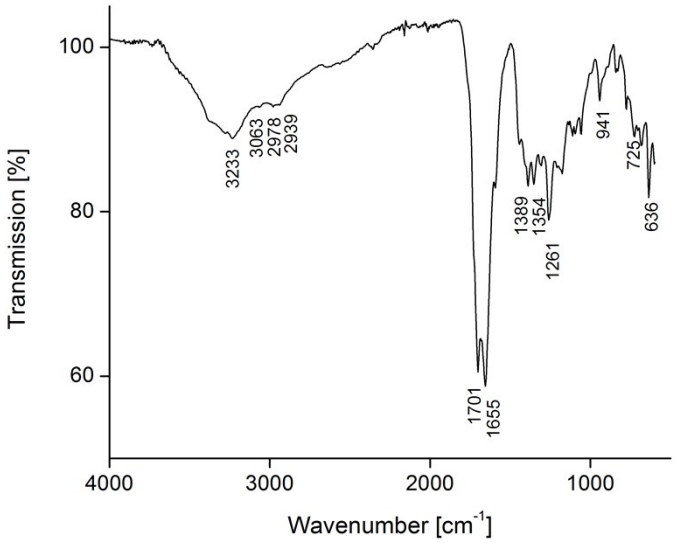


Figure S10: ATR-IR spectrum of *Z*-BOX C.

UV spectrum

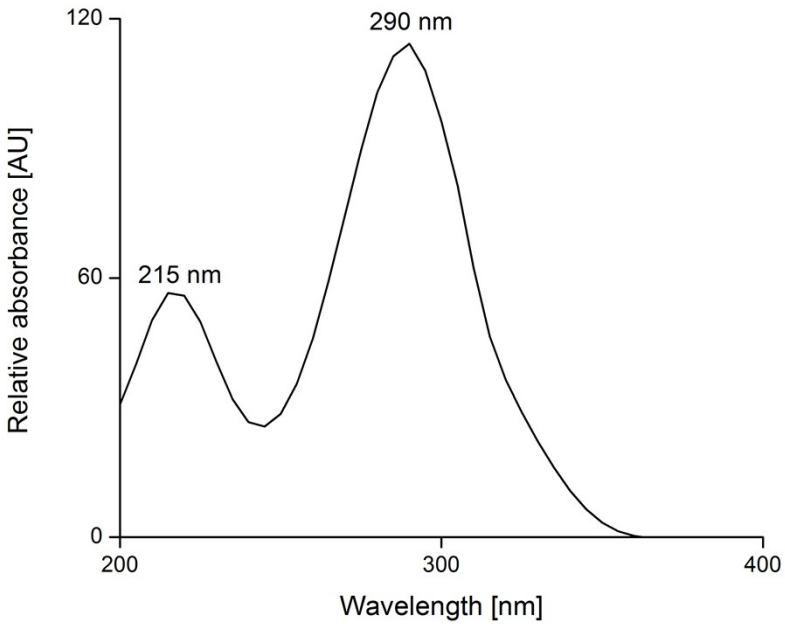


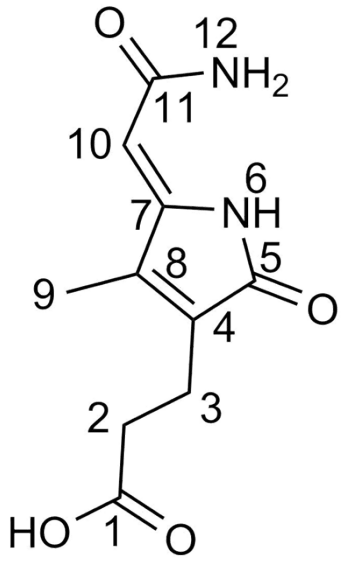
Figure S11: UV plot of *Z*-BOX C.

IV. DFT calculations of NMR shifts

Carbon NMR chemical shifts where calculated using density functional theory (DFT) with Gaussian 09 following the procedures described in [5,6]. An ensemble of 5 diverse conformations of both the E- and the Z-form where generated with RDKit on KNIME 3.4.2, followed by DFT geometry optimization with Gaussian 09 at the b3lyp/6-31+G(d,p) level of theory, accounting for the DMSO solvation with a Polarisable Continuum Model (PCM). DFT calculations of carbon isotropic shieldings where then performed at the mPW1PW91/6-311+G(2d,p) level of theory and Boltzmann-weighted carbon chemical shifts where calculated according to [2] based on the energies calculated in step 1. For the *Z*-form, a fit of experimental and calculated chemical shifts yielded an RMSD of 2.29 ppm and a maximal deviation between experimental and calculated shift (delta_C_max) of 4.6 ppm (Table S1), whereas the E-form had an RMSD of 3.64 ppm and a delta_C_max von 7.67 ppm (Table S2).

Table S1: Comparison of theoretical ^{13}C-shifts of *Z*-BOX C and obtained ^{13}C shifts.

Atom numbers	Gaussian atom numbers	Calculated carbon shift (ppm)	Experimental carbon shift (ppm)	dev	dev^2
1	12	174.38	174.00	0.38	0.14
2	11	32.01	32.40	0.39	0.15
3	9	19.89	19.30	0.59	0.35
4	5	133.80	133.30	0.50	0.25
5	6	170.02	168.40	1.62	2.64
7	3	150.07	147.80	2.27	5.17
8	4	145.23	142.00	3.23	10.43
9	8	9.69	9.80	0.11	0.01
10	2	94.45	97.90	3.45	11.92
11	1	166.29	170.90	4.61	21.21



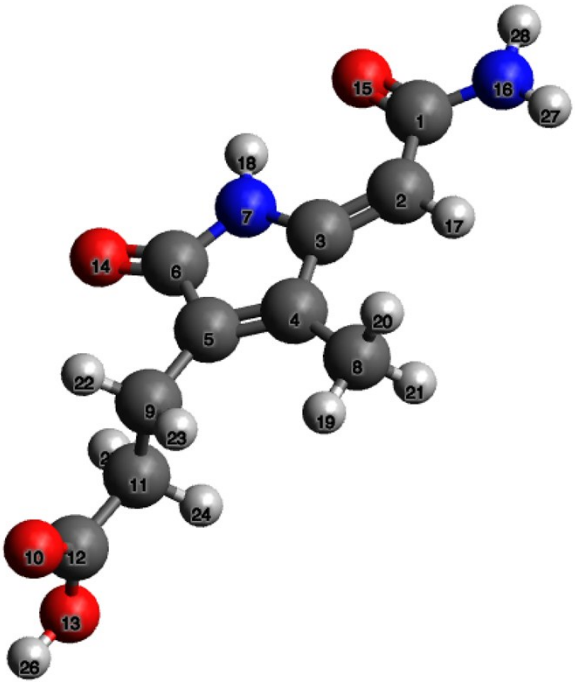
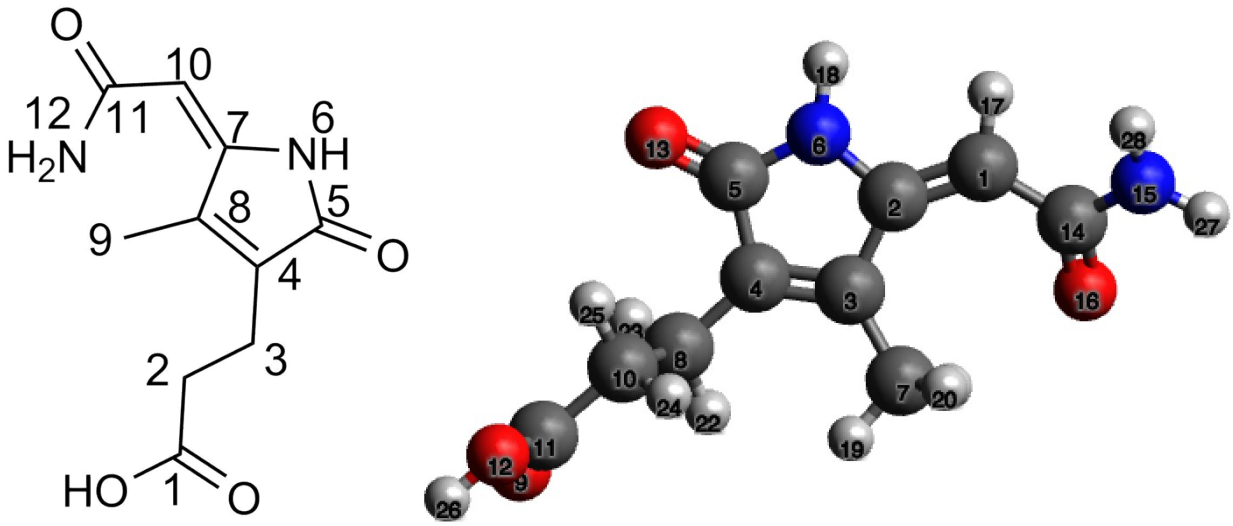


Table S2: Comparison of theoretical ^{13}C-shifts of *E*-BOX C and obtained ^{13}C shifts.

Atom numbers	Gaussian atom numbers	Calculated carbon shift (ppm)	Experimental carbon shift (ppm)	dev	dev^2
1	11	174.45	174.00	0.45	0.20
2	10	32.28	32.40	0.12	0.02
3	8	19.96	19.30	0.66	0.43
4	4	136.02	133.30	2.72	7.38
5	5	168.87	168.40	0.47	0.22
7	2	147.35	147.80	0.45	0.20
8	3	144.84	142.00	2.84	8.05
9	7	14.71	9.80	4.91	24.11
10	1	104.39	97.90	6.49	42.07
11	14	163.83	170.90	7.07	49.99



V. Quantification in human bile samples

Table S3: *Z*-BOX C concentrations of denatured human bile samples at room temperature determined with LC-MS at different storage times. From the data a mean increase of *Z*-BOX C in samples 3, 10, and 11 was calculated (increase factor 3.02 after 9 h and 7.38 after 33 h).

	Concentration of *Z*-BOX C in µM		
Samples	**6 h**	**15 h**	**39 h**
1	0.00	0.00	0.00
2	0.00	**0.23**	**0.87**
3	**0.33**	**1.01**	**2.73**
4	0.00	**0.13**	**0.36**
5	0.00	0.00	0.00
6	0.00	0.00	0.00
7	0.00	0.00	0.00
8	0.00	0.00	0.00

9	0.00	0.00	0.00
10	**0.31**	**0.84**	**2.00**
11	**0.22**	**0.72**	**1.62**
12	0.00	0.00	0.00

Table S4: *Z*-BOX C concentrations in freshly prepared human bile samples. Bile was stored at room temperature and samples were generated at every time point immediately before LC-MS quantification. From the data a mean increase of *Z*-BOX C was calculated (increase factor 2.48 after 9 h and 4.69 after 32 h).

	Concentration of *Z*-BOX C in µM		
Samples	**0 h**	**9 h**	**32 h**
3	0.13	0.44	1.00
10	0.13	0.38	0.58
11	0.07	0.09	0.15

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(1) Seidel, R. A.; Claudel, T.; Schleser, F. A.; Ojha, N. K.; Westerhausen, M.; Nietzsche, S.; Sponholz, C.; Cuperus, F.; Coldewey, S. M.; Heinemann, S. H.; Pohnert, G.; Trauner, M.; Bauer, M. *J. Hepatol.* **2017**, *67* (2), 272–281.

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(3) Kranc, K. R.; Pyne, G. J.; Tao, L.; Claridge, T. D.; Harris, D. A.; Cadoux-Hudson, T. A.; Turnbull, J. J.; Schofield, C. J.; Clark, J. F. *Eur. J. Biochem.* **2000**, *267* (24), 7094–7101.

(4) Ritter, M.; Seidel, R. A.; Bellstedt, P.; Schneider, B.; Bauer, M.; Görls, H.; Pohnert, G. *Org. Lett.* **2016**, *18* (17), 4432–4435.

(5) Buevich, A. V., and Elyashberg, M. E. Magn. *Reson. Chem.* **2017**, *1*, 1–12.

(6) Willoughby, P. H., Jansma, M. J., and Hoye, T. R. *Nature protocols* **2014**, *9*, 643–660.