Supporting Information

Isolation and identification of intermediates of the oxidative bilirubin degradation

Marcel Ritter,† Raphael A. Seidel,†,§ Peter Bellstedt, †, Bernd Schneider,#
Michael Bauer,§ Helmar Görls† Georg Pohnert*†

† Institute of Inorganic and Analytical Chemistry, Friedrich Schiller University,
Lessingstrasse 8, D-07743 Jena, Germany,

‖ Institute of Inorganic and Analytical Chemistry, Friedrich Schiller University,
Humboldtstrasse 8, D-07743 Jena, Germany,

§ Department of Anesthesiology and Intensive Care Medicine/Center for Sepsis Control and
Care, Jena University Hospital, Erlanger Allee 101, D-07747 Jena, Germany,

# Max Planck Institute for Chemical Ecology, Beutenberg Campus,
Hans-Knöll-Str. 8, D-07745 Jena.

Table of Contents

| I. General information | S2 |
| II. Oxidative degradation | S3 |
| III. Spectroscopic and spectrometric data | S4 |
| IV. X-ray diffraction measurements | S17 |
| V. Standard addition | S18 |
| References | S18 |
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). UHPLC gradient grade water and acetonitrile was purchased from Fisher Scientific (Loughborough, United Kingdom) and UHPLC formic acid was obtained from Biosolve B.V. (Valkenswaard, Netherlands).

Instrumentation
To remove solvents an evacuated centrifuge (Christ SpeedVac RVC 2-25) at 40 °C was used.
NMR data ($^1$H, $^{13}$C-DEPT135, $^1$H,$^{13}$C-HSQC, $^1$H,$^{13}$C-HMBC, $^1$H,$^1$H-ROESY) was collected at room temperature with a 400 MHz Bruker Avance I, a 600 MHz Bruker Avance III and a 700 MHz Bruker Avance III HD using the residual resonance of the solvent [D$_6$]DMSO as internal standard for referencing.
ATR-IR was measured with an IR-Affinity-1 from Shimadzu (Duisburg, Deutschland) 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 280 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 (flow rate of 6 mL/min) was as follows: 0 min, 18% B; 38 min, 18% B; 43 min, 25% B; 50 min, 25% B; 51 min, 100% B; 61 min, 100% B; 62 min, 18% B; 72 min, 18% B.
LC-MS for PDP-analytics

For reaction monitoring a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Leicestershire, United Kingdom), with a Kinetex C18 column (1.7 µm, 50 × 2.1 mm, Phenomenex, Aschaffenburg, Germany) coupled with a UV/Vis detector (180-800 nm) and with a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Leicestershire, United Kingdom) with electrospray ionization in positive ion mode was used. Solvents were used as described above with a flow rate of 0.4 mL/min and the following solvent program: 0.0 min, 0% B; 0.5 min, 0% B; 1.0 min, 12% B; 7.5 min, 17% B; 8.0 min, 100% B; 9.0 min, 100% B; 9.1 min, 0% B; 10.0 min, 0% B. For quantification of PDPs the same solvent system was used with an Acquity UHPLC BEH C18 column (1.7 µm, 100 × 2.1 mm) and the following gradient was used: 0.0 min, 0% B; 0.5 min, 0% B; 1.0 min, 18% B; 8.0 min, 18% B; 9.0 min, 100% B; 10.9 min, 100% B; 11.0 min, 0% B; 13.0 min, 0% B with external standards.

Preparation of biological samples

The bile and gallstone 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

Bilirubin (5 g, 8.6 mmol) was suspended in 2.5 L 5 M NaOH solution and stirred for 24 h. The pH was adjusted to 7.5 with conc. HCl and H2O2 was slowly added (4 h) to a final concentration of 1% and stirred for
20 h. After extraction with chloroform to isolate BOXes, the water phase was subjected to solid phase extraction (6 g-Oasis- hydrophilic lipophilic balanced cartridges; Waters, Manchester, United Kingdom). The cartridges were washed with water, prior to the elution of the degradation products were with 20% acetonitrile/water. Fractions were dried in an evacuated centrifuge and preparative HPLC-UV/vis was carried out to isolate the products. Due to the fast isomerization of the products it was necessary to freeze the fractions immediately with liquid nitrogen. After drying four different PDPs were obtained.

Further degradation of PDPs

PDPs in equilibrium were dissolved in water (50 µM, 400 µL) and oxidized with 1% hydrogen peroxide under stirring for six days and measured daily with UHPLC-MS.

III. Spectrometric and spectroscopic data

MS measurements

Figure S2. MS spectra of the four fractions with the following proposed fragments: m/z = 301.12 as [M+H-H2O]+, m/z = 319.13 as [M+H]+, m/z = 341.11 as [M+Na]+, m/z = 357.07 as [M+K]+, m/z = 659.23 as [2M+Na]2+ and m/z = 675.19 as [2M+K]2+.
NMR spectra

Figure S3: $^1$H-NMR spectrum (600 MHz) of PDP A1 in [D$_6$]DMSO.

Figure S4: $^1$H-NMR spectrum (600 MHz) of PDP B1 in [D$_6$]DMSO.
Figure S5: $^1$H-NMR spectrum (600 MHz) of PDP A2 in [D$_6$]DMSO.

Figure S6: $^1$H NMR spectrum (600 MHz) of PDP B2 in [D$_6$]DMSO.
Figure S7: $^{13}$C-DEPT135 NMR-spectrum (150 MHz) of PDP A1 in [D$_6$]DMSO.

Figure S8: $^{13}$C-DEPT135-NMR spectrum (150 MHz) of PDP B1 in [D$_6$]DMSO.

Figure S9: $^{13}$C-DEPT135-NMR spectrum (150 MHz) of PDP A2 in [D$_6$]DMSO.
Figure S10: $^{13}$C-DEPT135-NMR spectrum (150 MHz) of PDP B2 in [D$_6$]DMSO.

Figure S11: $^1$H, $^{13}$C- HSQC-NMR spectrum (600 MHz) of PDP A1 in [D$_6$]DMSO.
Figure S12: $^1$H,$^{13}$C-HSQC-NMR spectrum (600 MHz) of PDP B1 in [D$_6$]DMSO.

Figure S13: $^1$H,$^{13}$C-HSQC-NMR spectrum (600 MHz) of PDP A2 in [D$_6$]DMSO.
Figure S14: $^1\text{H},^{13}\text{C}$-HSQC-NMR spectrum (600 MHz) of PDP B2 in [$D_6$]DMSO.

Figure S15: $^1\text{H},^{13}\text{C}$-HMBC-NMR spectrum (700 MHz) of PDP A1 in [$D_6$]DMSO.
Figure S16: $^1$H,$^{13}$C-HMBC-NMR spectrum (700 MHz) of PDP B1 in [D$_6$]DMSO.

Figure S17: $^1$H,$^{13}$C-HMBC-NMR spectrum (700 MHz) of PDP A2 in [D$_6$]DMSO.
Figure S18: $^1$H,$^13$C-HMBC-NMR spectrum (700 MHz) of PDP B2 in [D$_6$]DMSO.

Figure S19: $^1$H,$^1$H-ROESY-NMR spectrum (700 MHz) of PDP A1 in [D$_6$]DMSO.
Figure S20: $^1$H,$^1$H-ROESY-NMR spectrum (700 MHz) of PDP B1 in [D$_6$]DMSO.

Figure S21: $^1$H,$^1$H-ROESY-NMR spectrum (700 MHz) of PDP A2 in [D$_6$]DMSO.
Figure S22: $^1$H,$^1$H-ROESY-NMR spectrum (700 MHz) of PDP B2 in [D$_6$]DMSO.

IR spectra

Figure S23: ATR-IR spectrum of PDP A1.
Figure S24: ATR-IR spectrum of PDP B1.

Figure S25: ATR-IR spectrum of PDP A2.
Figure S26: ATR-IR spectrum of PDP B2.

UV plots

Figure S27: UV plots of PDP A1/2 and B1/2.
IV. X-ray diffraction measurements

The intensity data were collected on a Nonius KappaCCD diffractometer, using graphite-monochromated Mo-K$_\alpha$ radiation. Data were corrected for Lorentz and polarization effects; absorption was taken into account on a semi-empirical basis using multiple-scans.$^{[4-6]}$

The structure was solved by direct methods (SHELXS$^{[7]}$) and refined by full-matrix least squares techniques against Fo$^2$ (SHELXL-97$^{[7]}$). All hydrogen atom positions were included at calculated positions with fixed thermal parameters. The crystals of PDP A1 were extremely thin and of low quality, resulting in a substandard data set; however, the structure is sufficient to show connectivity and geometry despite the high final $R$ value. We therefore only report the structure as conformation of the suggested structure, but not as crystallographic data deposited the data in the Cambridge Crystallographic Data Centre.

MERCURY was used for structure representations$^{[8]}$.

![Motive of the structure of PDP A1 via X-ray diffraction.](image)

Crystal Data for PDP A1 : C$_{16}$H$_{18}$N$_2$O$_5$, Mr = 318.32 g mol$^{-1}$, colourless prism, size 0.124 x 0.068 x 0.055 mm$^3$, monoclinic, space group P 2$_1$/n, a = 12.2016(9), b = 7.6651(6), c = 16.3094(13) Å, $\beta$ = 101.125(5)$^\circ$, $V$ = 1496.7(2) Å$^3$, T= -140 $^\circ$C, Z = 4, $\rho_{\text{calc}}$ = 1.413 g cm$^{-3}$, $\mu$ (Mo-K$_\alpha$) = 1.06 cm$^{-1}$, multi-scan, transmin: 0.6387, transmax: 0.7456, F(000) = 672, 13199 reflections in $h(-15/15)$, $k(-7/9)$, $l(-21/20)$, measured in the range $2.31^\circ \leq \Theta \leq 27.48^\circ$, completeness $\Theta_{\text{max}} = 98.9\%$, 3392 independent reflections, largest difference peak and hole: 0.363 / -0.374 e Å$^{-3}$. 

17
V. Standard addition

To proof that the obtained crystal is PDP A1 it was diluted in water and solution, a control of all four PDPs and a standard addition of both was measured via LC-MS.

Figure S29: UHPLC-MS trace of solutions of the crystal, a control with all four PDPs and a combination of both to proof that the crystal was PDP A1.

References


(4) COLLECT, Data Collection Software; Nonius B.V., Netherlands, 1998


(6) SADABS 2.10, Bruker-AXS inc., 2002, Madison, WI, U.S.A
