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Isolation and Identification of Intermediates of the Oxidative Bilirubin Degradation

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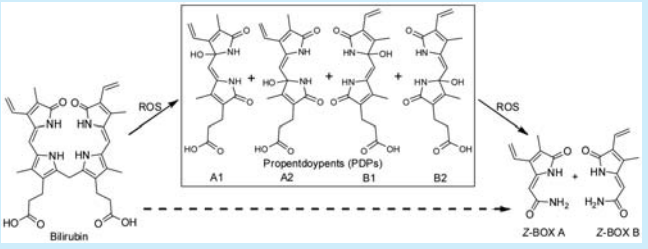
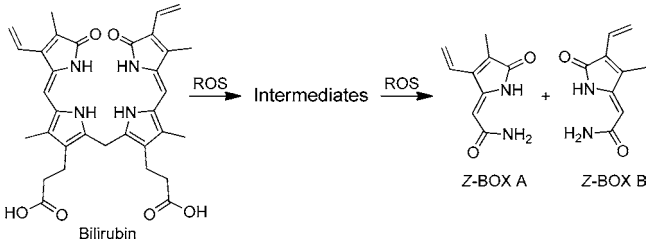
Supporting Information

ABSTRACT: Four endogenous products of oxidative bilirubin degradation were isolated and fully characterized. The constitutional isomers belong to the propentdyopents (PDPs). Their structures and further oxidative transformations to biologically active bilirubin oxidation end products (Z-BOXes) are reported. Using liquid chromatography−mass spectrometry protocols, PDPs were detected in human bile and gallstones. Given the recent interest in BOXes as effectors in cerebral vasospasms and liver dysfunction, co-occurring PDPs represent an additional potentially active compound class to be considered.



Degradation of heme, the prosthetic group of hemoproteins such as hemoglobin, can occur via different pathways. Heme oxygenases catalyze the formation of ferrous iron, carbon monoxide, and biliverdin (BV), which is subsequently reduced by biliverdin reductases to bilirubin (BR). After conjugation with glucuronic acid, BR is eliminated into bile.[1] In addition, the nonenzymatic degradation of heme, BV, and BR with reactive oxygen species (ROS) leads to a series of products. Among these the monopyrroles Z-BOX A and Z-BOX B belong to a family of presumable "bilirubin oxidation end products" (BOXes; Scheme 1).[2]

Scheme 1. Oxidative Degradation of Bilirubin



These compounds are suspected contributors to cerebral vasospasm,[3,4] a severe complication after subarachnoid hemorrhage. In addition, BOXes are enriched in the liver and bile and affect hepatic excretory function.[5] Reports on the activity of BOXes have recently generated much interest in BR oxidation products. Several often poorly characterized di- and tripyrroles that could represent intermediates to BOXes were introduced as oxidative BR degradation products.[2,6−8] Here, we specifically address the isolation and identification of such intermediate molecular weight BR oxidation products to answer open questions about the degradation pathway. We also introduce an analytical method that allows monitoring this compound class *in vitro* and *in vivo*.

To identify possible intermediates of oxidative BR transformation we modified a method for the generation of BOXes by Clark et al., in which bilirubin is oxidized with 10% H_2O_2 for 24 h at room temperature. After extraction with chloroform and preparative high performance liquid chromatography (HPLC) separation, Z-BOXes were isolated in low yields.[2] We optimized the reaction parameters to generate higher amounts of potential intermediates. When bilirubin was oxidized with 1% H_2O_2 the extraction with chloroform led again to a mixture of BOXes, but more abundant higher molecular weight degradation products were detected in the remaining aqueous phase (Figure 1 A). With the help of solid-phase extraction using a hydrophilic−lipophilic balanced resin (HLB), additional degradation products could be enriched from this aqueous phase. Elution with 20% acetonitrile/water recovered a fraction with the dominant BR oxidation products (Figure 1 B). This fraction was further purified with preparative reversed-phase HPLC, which led to four distinct products (Supporting Information Figure S1). As the analysis of the isolated metabolites using

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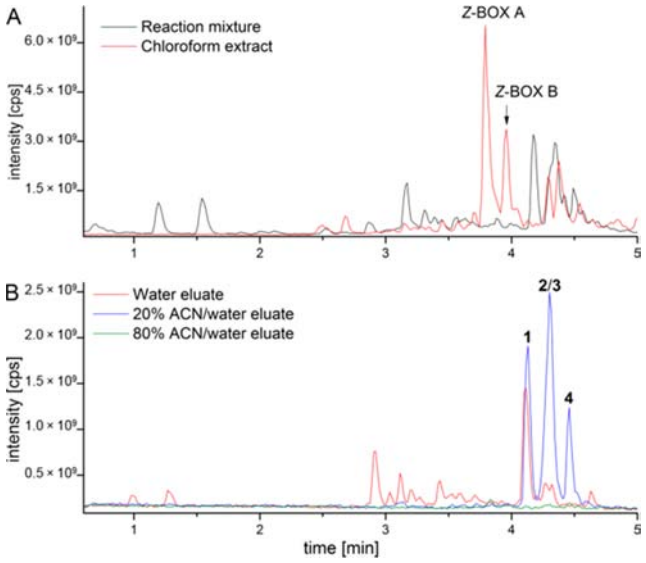

Figure 1. (A) Ultrahigh performance liquid chromatography−mass spectrometry (UHPLC-MS) profiles (total ion current) of the reaction mixture (black) of bilirubin and hydrogen peroxide as well as of the corresponding chloroform extract (red) after 20 h. (B) HLB-SPE extracts of the aqueous phase obtained after BR oxidation eluted with different solvents.

liquid chromatography coupled to high-resolution mass spectrometry revealed identical masses (319.12857 u $[M + H]^+$), we hypothesized these four products to be isomers (Figure S2). The calculated molecular formula ($C_{16}H_{18}O_5N_2$) suggested that they contain two pyrrole moieties of the starting material bilirubin. Potential structures were suggested in the literature and summarized under the group of propentdyopents (PDPs). These compounds were initially discovered as products of the photo- and oxidative degradation of BR.[9−11] PDPs, first described in 1940,[12] have a "long and complicated" history, and several structural proposals have been published over the years.[13] Different approaches using synthetic model compounds or derivatization including methylation were carried out leading to several proposed structures.[13,14] Structures with varying degrees and patterns of substitution were put forward, but no conclusive description of the four isomers is available.[15,16] After photo-oxidation of BR and methylation, Bonnet et al. found three isomers that would be in accordance with the mass spectra of the products reported here.[15] Schaefer et al. oxidized heme and protoporphyrin and ended up with four PDPs, of which two were also described by Bonnet et al.[16] The assignment of defined structures to the isolated compounds proved to be difficult since the studies were not comparable. We concluded that the lack of a clear structural proof as well as the isolation of only some of the possible isomers after derivatization in previous studies were motivation for further in-depth investigations. Insights in the intermediates of oxidative BR transformation would help to clarify *in vitro* oxidation mechanisms and would further facilitate determination of these compounds in patient samples. Structure elucidation was problematic since the compounds isomerized in aqueous solution with equilibria between **1** and **3** as well as **2** and **4**. To prevent isomerization, HPLC fractions needed to be immediately frozen with liquid N_2 and freeze-dried. As solids, the compounds were stable toward isomerization.

The isolated compounds were characterized by MS/MS experiments, in which the m/z = M-18 fragment suggested the presence of a hydroxyl group that can readily result in a neutral loss of water. NMR studies were complicated by the poor solubility of the compounds. ^{1}H- and ^{13}C-DEPT135-NMR confirmed the backbone composed of a methine-bridged system of two substituted pyrroles that is in accordance with PDPs reported in earlier studies (Table 1, Supporting Information).[15,16]

Table 1. NMR Data of PDP A1 (1) in $[D]_6$ DMSO

C/H[a]	#H	δ_H (ppm)	δ_C (ppm)	HMBC	ROESY
1	NH	8.75		2, 3, 4, 5	6, 15
2	C		171.4		
3	C		130.1		
4	C		150.0		
5	C		87.6		
6	CH	4.93	108.9	5, 7, 8	1, 14, 15, 16
7	C		138.8		
8	C		142.1		
9	C		130.5		
10	C		170.7		
11	NH	8.77		7, 8, 9, 10	14, 15
12	CH_3	1.80	8.9	2, 3, 4	13, 14, 15
13	CH	6.53	127.1	3, 4, 5, 14	12, 14, 15
14	CH_2	5.45/5.61	122.0	4, 13	6, 11, 12, 13, 15
15	OH	6.89		5, 6	1, 11, 12, 14, 16
16	CH_3	1.92	9.9	7, 8, 9, 10	15, 17, 18
17	CH_2	2.45	19.4	8, 9, 10, 18	16, 18
18	CH_2	2.38	32.8	9, 17, 19	16, 17
19	C		174.2		

[a]Numbering according to Figure 2.

^{1}H NMR, ^{13}C-DEPT135, ^{1}H,^{13}C-HSQC, and ^{1}H,^{13}C-HMBC revealed the substitution patterns of **1** to be in accordance with a methyl at C3 and C8 as well as a vinyl at C4 and a propionic acid at C9. NMR signals for **1** indicated the presence of the vinyl group with characteristic shifts of δ_H 6.53, 5.61, and 5.45 that could be assigned to the pyrrole (C2−C5) by the HMBC signals (Table 1). The shift of the propionic acid side chain protons δ_H 2.45 and 2.38 and the HMBC data allowed assigning the signals from the second pyrrole (C7−C10). Methyl groups δ_H 1.8 and 1.92 could be assigned to the respective pyrrole units using HMBC data. The remaining methine bridge (C6) showed direct HMBC couplings to C5 and C7. The same substitution pattern of the vinyl and propionic acid groups could be assigned for **3** (Supporting Information). NMR shifts of **2** and **4** were evaluated in the same manner and could be assigned to isomers with interchanged methyl and the vinyl groups at C3 and C4 (Figure 2, Supporting Information). Detailed ^{1}H,^{13}C-HMBC, and ^{1}H,^{1}H-ROESY NMR analysis using a 1.7 mm microcryoprobe at 700 MHz was required to solve the position of the remaining OH groups in **1**−**4**. ROESY couplings of the OH protons indicated that the OH group is located next to the central methine carbon. In combination with HMBC couplings, the OH position could be clearly assigned to C5 (**1** and **2**) and C7 (**3** and **4**), respectively (Figure 2). Thus, the hemiaminal-structure element could be unambiguously identified. Following up the nomenclature of BOXes, the two compounds with the substitution pattern of BOX A are named PDP A1 and A2, whereas the other two isomers with the substitution pattern of

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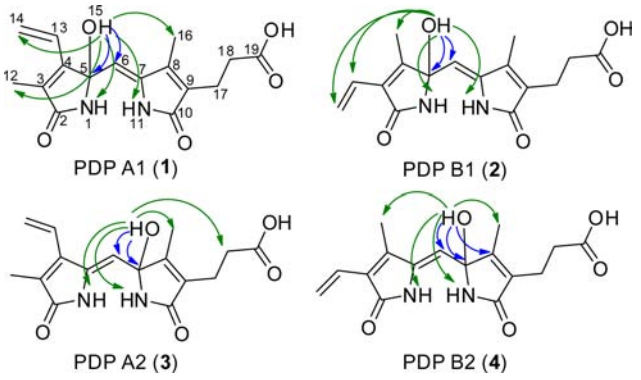

Figure 2. ROESY (green) and HMBC (blue) couplings of **1**−**4**.

BOX B are named PDP B1 and B2. The numbering 1 indicates the OH-group on the vinyl pyrrole and the 2 at the propionic acid pyrrole. The proposed structure of PDP A1 was supported by X-ray diffraction (Figure S28).

The equilibrium between each of the two isomers was further investigated in aqueous solutions. Under acidic conditions (pH = 2.7 in 0.1% formic acid) isomerization reaching equilibrium after 20 h was observed, whereas at neutral pH or in aprotic solvents the reaction was much slower. LC-MS analysis revealed the equilibrium ratio between PDP A1 and A2 to be 4:1 and for PDP B1 and B to be 2:3.

To find out if PDPs are intermediates of the BR oxidation to BOXes, we undertook an LC-MS monitoring of the reaction of the respective isomer pairs with 1% H_2O_2. After 2 days, PDP A1 and A2 were degraded to the corresponding *Z*-BOX A and PDP B1 and B2 to *Z*-BOX B, respectively (Figure 3).

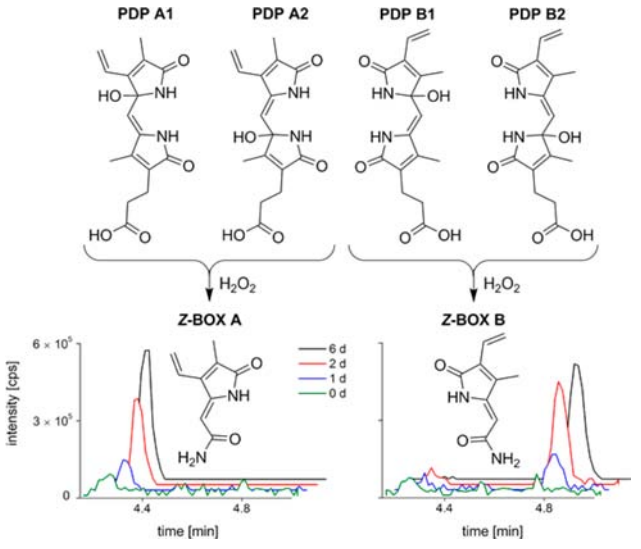

Figure 3. Oxidation of PDP A1/2 and B1/2 leading to *Z*-BOX A and B, respectively. UHPLC-MS of BOXes mass trace over 6 days.

This demonstrates that the PDPs are intermediates in the production of BOXes from BR. As the oxidation of the isomers yielded only the *Z* isomers of BOX A and B, we conclude that like BOXes all four isomers retain the *Z* configuration of their precursor bilirubin. However, compared to BR oxidation under identical conditions, the yield of BOXes from PDPs is lower, indicating that additional pathways might feed the pool of BOXes.[17]

Especially due to recent reports on the activity of BOXes in several disease models, we were interested in the *in vivo* prevalence of these PDPs in comparison to BOXes, which were determined via HPLC-MS/MS according to Seidel et al.[18] Therefore, we developed a UHPLC-MS method to quantify PDPs with external standards (see Supporting Information). Following up previous reports on the occurrence of PDPs in bile concrements,[12] we determined the concentrations of PDP A1, A2, B1, and B2 in human gallstones obtained upon cholecystectomy. We found concentrations in the micromolar range that were ∼22-fold higher than those of BOXes determined by Seidel et al.[5] (Table 2).

Table 2. Determined Concentrations of PDP A1/2, B1/2 Compared to *Z*-BOX A/B in Human Gallstone Samples (SEM = Standard Error of the Mean)

	concentration [(μM) in gallstone extract (0.1 mg/μL)]			
sample	PDP A1/2	PDP B1/2	*Z*-BOX A[a]	*Z*-BOX B[a]
1	1.61	0.81	0.05	0.04
2	0.78	0.38	0.05	0.03
3	2.26	1.11	0.09	0.06
4	2.56	0.89	0.10	0.05
5	0.24	0.26	0.02	0.01
6	1.94	0.80	0.06	0.03
7	1.97	1.12	0.04	0.02
8	2.53	1.15	0.11	0.08
9	0.91	0.41	0.03	0.02
10	2.85	1.06	0.14	0.09
mean	1.77	0.80	0.07	0.04
SEM	0.27	0.11	0.01	0.01

[a]Concentrations of BOXes are taken from Seidel et al.[5]

Furthermore, we measured PDP concentrations in bile samples of the same patient cohort. PDPs were found with a mean concentration of 45 μM (PDP A1/2) and 15 μM (PDP B1/2) and were thus ∼88-fold (PDP A1/2) and ∼34-fold (PDP B1/2) more prevalent compared to BOXes that were determined in the same samples by Seidel et al.[5] (Table 3).

These higher concentrations of PDPs in patient samples are in accordance with the results from the above-mentioned *in vitro* degradation studies, where PDPs were also more

Table 3. Determined Concentrations of PDP A1/2, B1/2 Compared to *Z*-BOX A/B in Human Bile Samples

	concentration (μM)			
sample	PDP A1/2	PDP B1/2	*Z*-BOX A[a]	*Z*-BOX B[a]
1	50.29	3.79	0.86	0.64
2	25.38	6.73	0.58	0.52
3	115.97	17.14	0.68	0.50
6	0.89	0.33	0.07	0.05
8	74.48	27.91	0.28	0.24
9	1.37	0.38	0.12	0.07
11	30.44	32.45	0.55	0.52
12	15.75	13.17	0.57	0.53
13	82.86	27.86	0.31	0.22
14	55.84	19.95	0.86	0.79
15	41.30	11.50	0.75	0.73
mean	44.96	14.66	0.51	0.44
SEM	10.79	3.44	0.08	0.08

[a]Concentrations of BOXes are taken from Seidel et al.[5]

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prevalent. This indicates that a similar pathway might be occurring *in vivo*.

In conclusion, four isomeric degradation products of bilirubin were isolated and characterized as PDPs A1/2 and B1/2. Although the structures were partially elucidated before, the four isomers could never be fully assigned. We show that these PDPs are intermediates between *Z,Z*-BR and *Z*-BOXes. Their high prevalence in bile samples compared to BOXes and their similar structural motifs call for the investigation of their activity using assays in which BOXes are active. Further experiments are needed to assess the occurrence and biological activity in other compartments, in particular the liver and the brain.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02287.

Experimental procedures and spectral data (PDF)

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Notes

The authors declare no competing financial interest.
Bile and gallstones samples were collected after approval by the ethics-committee of Friedrich Schiller University Jena (4406-04/15).

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