Singlet oxygen oxidation products of biliverdin IX α dimethyl ester

Sarina J. Dorazio^{a,#}, Steven Halepas^{a,#}, Torsten Bruhn^b, Kathleen M. Fleming^c, Matthias Zeller^c, and Christian Brückner^{a,*}

^a Department of Chemistry, University of Connecticut, Storrs, CT 06269-3060, USA

^b Institute of Organic Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

^c Department of Chemistry, Youngstown State University, 1 University Plaza, Youngstown, OH 44555, USA

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ABSTRACT

Biliverdin IX α is a naturally occurring linear tetrapyrrolic product of the enzymatic oxidative ring cleavage of heme. Evidence is mounting that biliverdin possesses antioxidant properties in mammals but its mode of action is unclear. We present the single crystal X-ray structure analysis of two regioisomeric biladien-1,19-diones-*ab* that are derived from biliverdin IX α dimethyl ester by addition of two vicinal *trans*-methoxy groups to the 4,5- or 15,16-double bonds, respectively. The compounds were likely formed by photosensitized singlet oxygen addition, followed by Lewis acid-catalyzed methanol-induced ring-opening of the intermediate epoxide, and OH-to-OMe substitution. We thus present structural evidence for a possible reaction mechanism by which biliverdin can act as an antioxidant.

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* Corresponding author. Tel.: +1-860-486-2743; fax: +1-860-486-2981; e-mail: c.bruckner@uconn.edu

Contributed equally.

1. Introduction

Biliverdin IX α ¹ (**2a**) is a naturally occurring linear tetrapyrrolic pigment that is the product of the enzymatic oxidative ring cleavage of heme **1aFe** (Scheme 1).² As a regular product of senescent or damaged erythrocytes, biliverdin is responsible for the greenish color seen in bruises—before biliverdin gets rapidly reduced by biliverdin reductase to the yellow pigment bilirubin.³ Biliverdin is also present in, for instance, the healthy blue-green blood or the green bones of some fish,⁴ the serum and eggs of frogs, the turquoise bones of a Cambodian frog, the blood of the tobacco hornworm, or the wings of some moths and butterflies.⁵ It is also the green-blue pigment found in some bird's eggshells, most visible in the dark green emu eggshells or the Robin's blue-colored eggshells of many thrushes.⁶

Scheme 1. Biosynthetic origin of biliverdin IX α .

The role of biliverdin in eggshells may solely be as colorant aiding in crypsis or signaling reproductive health.⁷ Biliverdin IX α is the biosynthetic precursor for the phytochromobilins and phycobilins of plants, algae and cyanobacteria.³ Furthermore, evidence is mounting that biliverdins have more physiological roles in humans than being mere intermediates in heme metabolism.³ For instance, biliverdin was shown to be a HIV-1 protease inhibitor.⁸ Physiologically more important, perhaps, the antioxidant roles of biliverdins are also becoming increasingly apparent.⁹ Biliverdin synthesis is induced in response to oxidative stress and was shown to be an endogenous tissue protector.^{10–11} Biliverdin prevents lipid peroxidation,¹² and quenches singlet molecular oxygen.¹³ The reaction kinetics of radicals with a range of pyrrolic compounds, including bile pigments, were studied.¹⁴ Biliverdins react also with nitric oxide and peroxynitrite.¹⁵ The anti-mutagenic¹⁶ and antioxidant activities^{9d} of bile pigments have been reviewed. However, only scant models for the mode of antioxidant action of biliverdin were proposed.^{9d, 14}

We present here the formation and structure of two regioisomeric biladiene-*ab*-1,19-dione derivatives that were formed by reaction of biliverdin IX α dimethyl ester (DME) with singlet oxygen, a reactive oxygen species (ROS). Thus, these derivatives provide structural proof for a possible reaction pathway along which biliverdin can act as an antioxidant in biological settings. Even though the formation of the compounds was the result of a fortuitous event, we were able to gather convincing evidence to rationalize their mode of formation.

2. Results and Discussion

2.1 The Extraction of Biliverdin IX α from Emu Eggshells

Extraction of the large (10–13 cm long) dark green and commercially available emu (*Dromaius novaehollandiae*) eggshells under acidic conditions (MeOH, H₂SO₄) is a

convenient, hygienic, and cost-effective way to obtain crude biliverdin IX α as its dimethyl ester **2b**.¹⁷ We used a variation of the classic procedure.¹⁷ While the teal biliverdin is the dominant pigment in emu eggshells, it also contains, like the colored eggshells of other bird species, the red-brown pigment protoporphyrin IX **1a**.⁶ This porphyrin, also as its dimethyl ester **1b**, is extracted alongside biliverdin DME **2b**.¹⁸ Careful chromatographic purification of the crude biliverdin DME removes this impurity.

2.2 Formation and Spectral Characterization of Biliverdin IX α DME Dimethoxy Adducts **5** and **6**

When we attempted to form a zinc(II) complex of a crude sample of biliverdin DME **2b** using standard methods in oligopyrrole/porphyrin chemistry (reflux over several hours, CHCl₃/MeOH, zinc acetate),¹⁹ we failed to obtain the zinc complex. Somewhat unconventionally, we thus extended the reaction times to several days. TLC control of the reaction mixtures in several—but not all—experiments showed that after several days reaction time, two similarly auspiciously bright pink- and purple-colored compounds, **5** (pink) and **6** (purple) of lower polarity (*R*_f = 0.32 and 0.30, respectively; silica-CH₂Cl₂/2% MeOH), than the starting material (*R*_f under these conditions < 0.10) were formed in a ~1:3 ratio, and in variable yields. Typically a combined yield of both products of less than ~30% by TLC was recorded, with the remainder unreacted starting material. Intriguingly, using highly purified samples of **2b** as starting material, the compounds did not form. Both products, separable only with difficulty by preparative HPTLC (silica-CH₂Cl₂/1% MeOH, multiple developments), proved to be of identical composition (C₃₇H₄₅N₄O₈ for MH⁺, as determined by HR-ESI⁺ MS; 100% CH₃CN; expected *m/z* 673.3237, found 673.3217 and 673.3238, respectively). Notably, no zinc was incorporated into any of the products. Their composition suggested that an uptake of two methoxy groups by biliverdin DME **2b** had taken place, resulting in the formation of two regioisomeric products. Since the 10-position of biliverdins is susceptible to nucleophilic attack by a range of nucleophiles,²⁰ we first suspected an attack onto this position (see Scheme 2 for the numbering system used).

Both products **5** and **6** possessed similar UV-vis spectra but with slightly different intensity ratios of their two bands (Figure 1). The spectra retained some characteristics of the linear oligopyrrolic pigments, but the observed hypochromic spectra with a significant hypochromic shift, compared to the spectrum of the starting material biliverdin DME **2b**, suggested that a reduction of the number of conjugated pyrrolic moieties had taken place.² The presence of a biladien-1,19-dione-*ab* chromophore that could have resulted from a reduction/addition reaction at the C10-position could be excluded since these bilirubin-like chromophores are characterized by single band UV-vis spectra.^{20–21}

Figure 1. UV-vis spectrum of biliverdin DME **2b** (green trace) (EtOH)²² in comparison to the spectra of the pink biliverdin dimethoxy adduct **5** (red trace; EtOH) and the purple dimethoxy adduct **6** (blue trace; EtOH).

The ¹H NMR spectra (500 MHz, CDCl₃) of compounds **5** and **6** were similar to each other, but with some diagnostic differences (see also ESI). In comparison to the spectrum of **2b**, they clearly showed retention of the two non-equivalent vinyl and ethyl groups, four methyl groups, and the propionic acid ester functionalities. In addition, the spectra of **5** and **6** showed the appearance of two new methoxy groups (s at 2.95 and 3.27, and 2.96 and 3.28 ppm, respectively). Most diagnostic for a possible location of the methoxy groups, one of the three *meso*-hydrogens was shifted by ~2 ppm high-field: for compound **5**, it was the hydrogen on position 15 that was shifted, for compound **6** it was the hydrogen on position 5. This strongly suggested that a double bond involving these positions was modified. For electronic reasons, it seemed most likely that the 15,16 and 4,5 double bonds, respectively, involving the pyrrolone moiety were involved. An NOE correlation between a vinyl proton and a MeO group in **6** also suggested this. However, a fully conclusive spectroscopic assignment of the structure could not be accomplished, particularly not with respect to the relative stereochemistry of the two methoxy-substituted carbons. Crystal structure analysis came to resolve all ambiguities.

2.3 Crystal Structures of Adducts **5** and **6**

Early experiments overlooked that two new compounds were formed since column chromatography did not separate the two products. Dark purple crystals of a mixture of **5** and **6**, grown by slow evaporation of the solvent CDCl₃ from an NMR tube, proved suitable for analysis by single crystal X-ray diffractometry, the result of which is shown in Figure 2.

Figure 2. Stick representation of the molecular structures of the regioisomeric biliverdin dimethoxy adducts **5** and **6**, as determined by single crystal diffractometry. Only one of the enantiomeric pairs present in the crystal is shown; all intramolecular disorder and solvents were omitted for clarity. For details of the crystal structure determination, see ESI.

The crystal proved to contain a 0.25:0.75 mixture of a solid solution of two regioisomeric biliverdin DME dimethoxy adducts, **5** and **6** (Figure 2). As already suspected, the 15,16 and

4,5 double bonds of the starting material are functionalized in products **5** and **6**, respectively. The biladiene-1,19-dione-*ab* regioisomers are likely formed by the *trans*-addition of two methoxy groups to either the 4,5- or the 15,16-double bond of biliverdin IX α DME **2b** (Scheme 2). Like biliverdin itself,² the molecules adopt an overall helimeric conformation, with the remaining double bonds in the *Z,Z*-configuration. An intramolecular H-bond pattern links the two halves of the molecule (see ESI). Since the 4,5- or the 15,16-double bonds are only differentiated by the relative order of the β -substituents on the terminal pyrrolone moieties (methyl-vinyl vs. vinyl-methyl), the solution of the mixture of the two compounds **5** and **6** is visible in the crystal structure only through an apparent 0.25:0.75 mixture of methyl and vinyl substituents at each pyrrolone. The *vic-trans*-addition of the two methoxy groups generates two chiral centers. Therefore, the compounds **5** and **6** each occur as racemic mixtures (**5**: 15-*R*,16-*S* and 15-*S*,16-*R*; **6**: 4-*S*,5-*R* and 4-*R*,5-*S*) in the non-chiral crystal space group (*P* $\bar{1}$) the crystal structure was refined in.

2.4 Mechanism of Formation of Biliverdin IX α DME Dimethoxy Adducts **5** and **6**

What are the likely chemical transformations giving rise to the conversion of biliverdin **2b** to the dimethoxylated biladiene-*ab*-diones **5** and **6**? The *trans*-configuration of the *vic*-dimethoxy functionalities suggests their formation by methanol-induced (and possibly Lewis acid-catalyzed) ring-opening of an epoxide, followed by a substitution reaction (Scheme 2). The alcohols formed in the ring-opening step at both possible carbons are in benzylic positions or α to a nitrogen, respectively, and thus readily exchange for a methoxy group under the current reaction conditions. The epoxide could perceivably be formed by photosensitized generation of singlet oxygen (¹O₂) and subsequent addition to a double bond, a known reaction of ¹O₂.²³ Remarkable in this reaction is the apparent chemoselectivity of this addition to the 4,5 and 15,16-double bonds.

Scheme 2. Likely reaction sequence of formation of the dimethoxy biliverdin adducts **5** and **6**. Formal numbering of compound shown. The stereoisomers and conformations shown correspond approximately to those of the compounds in the crystal (cf. Figure 2).

The most likely photosensitizer to have generated the singlet oxygen were traces of protoporphyrin IX DME **1b**, a known and potent sensitizer.²⁴ The varying quantities of **1b** present in different preparations (and/or the different degrees to which the reaction was exposed to light and oxygen) would explain the varying outcomes of the different reaction runs. Indeed, addition of ~1 mol% of independently prepared **1b** to a reaction of purified biliverdin **2b** and exposing it to a strong light source (45 W compact fluorescent bulb at a distance of less than 10 cm; borax glass reaction vessel) accelerated the formation of **5** and **6**, with complete conversions of **2b** attainable. As the photosensitized reaction eventually leads to a complete bleaching of the reaction mixture, for preparative purposes we do not recommend allowing the reaction to proceed to very high degrees of conversion. Inversely, rigorous exclusion of light (aluminum foil) with or without the presence of the photosensitizer prevented formation of the methoxy adducts. The role of the zinc salt is believed to be a Lewis acid in the epoxide ring-opening and substitution reactions. The absence of zinc salts significantly slows the reactions, but does not entirely prevent them.

Radical oxidations could perceivably provide an alternative mechanistic explanation for the observed oxidations. The regiochemically, and particularly the stereochemically specific outcome of the reaction, in the absence of any stereochemical induction, provides in our eyes a strong argument against a radical pathway. However, we note that the reaction also produces significant quantities of ‘baseline’ material and multiple small fractions of side products, none of which were characterized. Thus, the existence of a radical oxidation pathways parallel to the singlet oxygen addition reaction proposed cannot

be excluded, particularly since open-chain oligopyrroles have been shown to be susceptible to multiple radical-induced reactions.^{9d, 14b}

To find a rationale for the observed regioselectivity, some simple calculations were performed. However, neither the orbital coefficients of the frontier orbitals of a B3LYP-D3/def2-SVP optimized **2b**, nor the computed averaged local ionization energies (ALIEs) of this molecule provided a clear hint (see ESI). From the HOMO and LUMO orbitals, one would expect increased reactivity in all *meso*-position (positions 5, 10, and 15). ALIEs were recently shown to be simple quantitative reactivity predictors of the nucleophilicities of aromatic molecules in cases where steric influences are low.²⁵ ALIE calculations of **2b** indicated that the *meso*-positions are indeed a location of low ionization, but not the lowest: this distinction goes to the vinyl double bonds (see ESI). Thus, there is no solitary origin of an increased reactivity of the 4,5- and the 15,16-double bonds that could explain the high regioselectivity of the observed reaction, indicating that the reaction is much more complex than being controlled by a single parameter. However, the relative computed thermodynamic stability of the products **5** and **6** (B3LYP-D3/def2-SVP energies) match those reflected in the product distribution. Thus, **6** is thermodynamically slightly preferred relative to **5**. In a pure thermodynamically controlled reaction, an 80:20 mixture of **6** and **5** would have been expected, a ratio close to that found experimentally.

3. Conclusions

In conclusion, the connectivity of a new biliverdin DME dimethoxy adduct, a biladien-*ab*-1,19-dione, was deduced to likely have been the result of reaction of the ROS ¹O₂ with biliverdin, followed by alcoholysis of the intermediate epoxide. This study thus provided insight into a possible molecular mechanism of the proposed antioxidant reactivity of biliverdins in general, and its ability to quench singlet oxygen in particular.^{13b} In fact, we are not aware of any other example of a comparable reaction of biliverdin.^{2, 26}

4. Experimental

4.1 General

All solvents and reagents used were reagent grade or better and were used as received. The green emu eggs were ordered via Internet from an emu farm (Floek’s County Ranch, Tucumcari, NM). The analytical TLC plates used were Silicycle ultra pure silica gel 60, provided by Scientific Adsorbents Inc., Atlanta, GA (aluminium backed, 250 μm). As preparative chromatography plates were used Merck, Darmstadt, Germany, nano-DC HPTLC plates (10 × 10 cm, 200 μm silica gel on glass with 2.5 cm concentration zone).

¹H NMR spectra were recorded on a Bruker Avance 500 MHz instrument in the solvents indicated, and were referenced to residual solvent peaks. ¹H,¹H-NOESY spectra were taken on a Varian 600 MHz instrument. UV/Vis spectra were recorded on a Cary 50 spectrophotometer. High resolution mass spectra were provided by the Mass Spectrometry Facility, Department of Chemistry, University of Connecticut.

4.2 X-Ray Diffraction Analysis of **5** and **6**

The structure is a solid solution of two regioisomeric derivatives of biliverdin DME dimethoxy adducts present in a 0.753(2):0.247(2) ratio. This led to an (apparent) methyl vs. vinyl side group disorder in the average structure that was refined.

Some vinyl groups were further rotationally disordered. Details of the data collection and structural parameters for the structure elucidation are available from the Cambridge Crystallographic Data Centre (<http://www.ccdc.cam.ac.uk>), CCDC 1056049.

4.3 Extraction of Biliverdin IX α from Emu Eggshells

Procedure revised from the literature:¹⁷ The dark green emu eggshells (~30 to 50 g, broken into ~1–3 cm³ large pieces) were submersed in a wide-mouthed 500 mL Erlenmeyer flask with 2N aq HCl (~100 mL) for 24 h at ambient temperature. The solids were transferred onto a paper towel and the hard green shell was manually separated from the white under-laying soft membrane (to be discarded) using tweezers. The eggshells were then heated to reflux in MeOH containing 5% v/v H₂SO₄ (75 mL total) for 48 h. The resulting milky teal-colored mixture was filtered through a glass-fritted funnel (M) and the filter cake rinsed with ethyl acetate (~2 × 10 mL). To the combined filtrates were added a saturated aq NaHCO₃ solution and the solution was extracted with ethyl acetate (2–3 × 50 mL). The combined organic extracts were dried over anhydrous Na₂CO₃, filtered, the solvent removed by rotary evaporation, and the residue chromatographed (silica-ethyl acetate). The teal main fraction was isolated, the volume reduced to a few mL, and **2b** was precipitated in the freezer after addition of minimal hexane, yielding between 8 and 15 mg analytically pure **2b**. The product had all the spectroscopic properties previously described.²⁷ See also ESI.

4.3 Biliverdin IX α Dimethoxy Adducts **5** and **6**

To a solution of biliverdin DME **2b** (20 mg, 3.3×10^{-5} mol) dissolved in CHCl₃ (5 mL) was added a solution of Zn(OAc)₂·2H₂O (72 mg, 10 eq) in MeOH (10 mL) and heated to reflux. No measures to exclude air or light were taken. Over the course of 48–72 h, TLC of the reaction mixture showed the consumption of the starting material and the joint appearance of a minor pink product, **5**, and a major and more purple product, **6**. Preparative plate chromatography (silica-CH₂Cl₂/2% MeOH) isolated the products in ~5 and 12% yields, respectively. Pink isomer **5**: R_f (silica-CH₂Cl₂/2% MeOH) = 0.32; UV-vis (EtOH): λ_{max} (log ϵ): 332 (4.59), 564 (4.49) nm; ¹H NMR (500 MHz, CDCl₃, 25°C): δ 6.84 (s, 1H), 6.64 (dd, J = 18, 11 Hz, 1H), 6.40–6.35 (m, 1H), 6.27 (d, J = 18 Hz, 1H), 6.03 (s, 1H), 5.69–5.65 (m, 2H), 5.45 (d, J = 11 Hz, 1H), 4.32 (s, 1H), 3.68 (s, 3H), 3.63 (s, 3H), 3.28 (s, 3H), 2.95 (s, 3H), 2.93–2.89 (m, 4H), 2.55–2.53 (m, 4H), 2.06 (s, 6H), 2.02 (s, 3H), 1.74 (s, 3H) ppm (NH signals not detectable); HR-MS (ESI+, 100% CH₃CN, TOF) m/z calcd for C₃₇H₄₅N₄O₈ (M + H⁺) 673.3238. Purple isomer **6**: R_f (silica-CH₂Cl₂/2% MeOH) = 0.30; UV-vis (EtOH): λ_{max} (log ϵ): 338 (4.64), 570 (4.44) nm; ¹H NMR (500 MHz, CDCl₃, 25°C): δ = ¹H NMR (500 MHz; CDCl₃): δ 6.86 (s, 1H), 6.58 (dd, J = 18, 11 Hz, 1H), 6.40 (dd, J = 18, 2 Hz, 1H), 6.34 (m, 1H), 5.96 (s, 1H), 5.52 (dd, J = 11, 2 Hz, 1H), 5.36 (dd, J = 17, 2 Hz, 1H), 5.26 (d, J = 11 Hz, 1H), 4.31 (s, 1H), 3.69 (s, 3H), 3.68 (s, 3H), 3.27 (s, 3H), 2.99 (s, 3H), 2.95–2.93 (m, 4H), 2.52–2.58 (m, 4H), 2.19 (s, 3H), 2.09 (s, 3H), 1.95 (d, s, 3H), 1.94 (d, s, 3H) ppm (NH signals not detectable); HR-MS (ESI+, 100% CH₃CN, TOF) m/z calcd for C₃₇H₄₅N₄O₈ (M + H⁺) 673.3237; found 673.3217. For a comparison of the NMR spectra of **2b**, **5**, and **6**, see ESI.

4.4 Mechanistic Studies

In parallel, two reaction flasks with solutions of highly purified biliverdin DME **2b** (5.3 mg, 8.7×10^{-6} mol) dissolved in CHCl₃ (1.5 mL) were stirred for ~20–48 h while the following parameters were varied in multiple iterations: Absence/presence of Zn(OAc)₂·2H₂O (27 mg dissolved in 2.5 mL MeOH, 14 eq); absence/presence of a trace of hematoporphyrin IX DME (120 μ L of a 0.7 mM stock solution in CHCl₃, 0.01 eq); running the

reaction in air/following a N₂ purge; excluding light by wrapping the reaction flasks in aluminum foil or irradiating the reaction flask with a 45 W compact fluorescent light bulb from a distance of 5–10 cm. All reactions were monitored by TLC.

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References and Notes

- The Roman numeral specifies the order of ring substituents—biliverdin IX is derived from protoporphyrin IX (IUPAC-IUB Joint Commission on Biochemical Nomenclature, *Pure Appl. Chem.* **1987**, *59*, 779–832.); the Greek letter indicates the particular bridge that is cleaved; in principle, cleavage could occur at any of the four bridge carbons, thereby generating structural isomers, designated α , β , etc.
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