Extraction of Biologically Relevant Oligopyrrolic Compounds from Nature

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Extraction of Biologically Relevant Oligopyrrolic Compounds from Nature

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APPROVAL PAGE

Masters of Science Thesis

Extraction of Biologically Relevant Oligopyrrolic Compounds from Nature

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2018
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# Table of Contents

**APPROVAL PAGE** .................................................................................................................. III

**ACKNOWLEDGEMENTS** ......................................................................................................... IV

**LIST OF ABBREVIATIONS** .................................................................................................... VII

**LIST OF INSTRUMENTS** .......................................................................................................... VIII

**LIST OF PUBLICATIONS** ......................................................................................................... IX

**LIST OF FIGURES** .................................................................................................................. X

**LIST OF SCHEMES** ................................................................................................................. XIII

1  **INTRODUCTION TO TETR PYRROLES** ................................................................................ 1

   1.1 **PORPHYRINS AND BILINS** .......................................................................................... 1

   1.2 **OPTICAL PROPERTIES OF TETR PYRROLIC COMPOUNDS** ..................................... 3

   1.3 **NATURALLY OCCURRING TETR PYRROLES** .................................................................. 8

   1.4 **BIOSYNTHESIS OF ANIMAL TETR PYRROLES** .......................................................... 12

   1.5 **REFERENCES** ............................................................................................................... 16

2  **EXTRACTION OF OLGOPYRROLES FROM EGG SHELLS** .................................................. 19

   2.1 **INTRODUCTION** .......................................................................................................... 19

      2.1.1 **Developing an Extraction Protocol** ......................................................................... 19

      2.1.2 **Tinamou Eggshell Extractions** ................................................................................. 22

   2.2 **RESULTS AND DISCUSSION** ...................................................................................... 23

      2.2.1 **Extraction of Biliverdin from Emu Eggshells** ......................................................... 23

      2.2.2 **Characterization of Extracted Biliverdin IXα Dimethyl Ester** ............................... 25

      2.2.3 **Optimization of Extraction Protocol** .................................................................... 28

      2.2.4 **Extraction of Pigments from Tinamou Eggshells** ................................................. 31

          2.2.4.1 **Nothura maculosa** ............................................................................................ 31

          2.2.4.2 **Eudromia elegans** ............................................................................................ 39

   2.3 **CONCLUSION** .............................................................................................................. 42

   2.4 **METHODOLOGY AND INSTRUMENTATION** ................................................................. 42

      2.4.1 **Porphyrin Extraction** ............................................................................................. 42

      2.4.2 **HPLC Analysis of Porphyrinoids** .......................................................................... 43

   2.5 **REFERENCES** .............................................................................................................. 44

3  **PORPHYRINOIDS WITHIN HEDGEHOG QUILLS** ............................................................... 45
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 INTRODUCTION</td>
<td>45</td>
</tr>
<tr>
<td>3.2 RESULTS AND DISCUSSION</td>
<td>48</td>
</tr>
<tr>
<td>3.2.1 Spectroscopic Analysis of Quills</td>
<td>48</td>
</tr>
<tr>
<td>3.2.2 Spectroscopic and Chromatographic Analysis of Quill Extract</td>
<td>51</td>
</tr>
<tr>
<td>3.3 CONCLUSIONS</td>
<td>58</td>
</tr>
<tr>
<td>3.4 METHODOLOGY AND INSTRUMENTATION</td>
<td>58</td>
</tr>
<tr>
<td>3.4.1 Porphyrin Extraction</td>
<td>58</td>
</tr>
<tr>
<td>3.4.2 Chromatography of Quill Extract</td>
<td>59</td>
</tr>
<tr>
<td>3.4.3 Spectrophotometric Analysis</td>
<td>59</td>
</tr>
<tr>
<td>3.5 REFERENCES</td>
<td>60</td>
</tr>
</tbody>
</table>
List of Abbreviations

anhyd  anhydrous
aq    aqueous
conc  concentrated
ESI   electrospray ionization
Fl    Fluorescence
HPLC  high-performance liquid chromatography
HR-MS high-resolution mass spectrometry
\( \lambda \) wavelength
m/z   mass to charge ratio
NMR   nuclear magnetic resonance
r.t.  room temperature
sat   saturated
TLC   thin layer chromatography
UV-vis ultraviolet-visible
## List of Instruments

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-Visible spectrometry</td>
<td>VARIAN CARY 50</td>
</tr>
<tr>
<td>Fluorescence spectroscopy</td>
<td>VARIAN CARY ECLIPSE</td>
</tr>
<tr>
<td>NMR spectroscopy</td>
<td>BRUKER AVANCE III 400</td>
</tr>
<tr>
<td>HR-MS</td>
<td>AB SCIEX Q-STAR-ELITE</td>
</tr>
<tr>
<td></td>
<td>THERMO SCIENTIFIC Q EXACTIVE HF</td>
</tr>
<tr>
<td>HPLC</td>
<td>AGILENT 1100 SERIES</td>
</tr>
</tbody>
</table>
List of Publications

This thesis is based on the following in print or planned publications:

  *Contributions*: SH: Large-scale experiment design; RH: Small-scale experiment design, large-scale optimization; LSED: Undergraduate researcher under the tutelage of RH.

  *Contributions*: LSED: Undergraduate researcher under the tutelage of RH.

- **Hamchand, R.;** Brückner, C. ‘Confirmation of the Presence of Porphyrinoids within Quills of the European Hedgehog *Erinaceus europaeus*’ manuscript in preparation for *PeerJ*, to be submitted fall 2018.

**Other publications with RH co-authorship:**

  *Contributions*: ML: Synthesis of imidazoloporphyrins; JA: Early porpholactam studies; JD: Undergraduate research under the tutelage of ML; DT: Synthesis of porpholactams; RH: Spectrophotometric titrations; MZ: Crystallography.
List of Figures

Figure 1-1. Structure and labeling of porphyrins ........................................................... 2
Figure 1-2. Structure and naming of the three classes of hydroporphyrins .......................... 2
Figure 1-3. Structure and name of linear chain tetrapyrrole ............................................ 3
Figure 1-4. Characteristic UV-vis absorption spectra of free-base porphyrins and free-base bilins ........................................................................................................................................ 4
Figure 1-5. A schematic representation of the two HOMOs and two LUMOs (A,B) and corresponding electronic transitions; porphyrins with high degeneracy and low degeneracy ...... 6
Figure 1-6. Emission spectrum of tetraphenylporphyrin in toluene ..................................... 7
Figure 1-7. Modified Jablonski diagram for a porphyrin photosensitizer ............................ 8
Figure 1-8. Structures of Chlorophyll a and Bacteriochlorophyll a .................................... 10
Figure 1-9. Porphyrins as dyes within animal tissues: Great bustard feather, Clanculus pharaonius, Turaco schalowi .......................................................................................................... 11
Figure 1-10. Structure of the unique porphyrins found within demosponges of coral reefs ...... 12
Figure 2-1. Structures of tetrapyrroles found within eggshells ........................................... 19
Figure 2-2. Tinamou Eggshells of Tinamous major, Eudromia elegans, and Nothura maculosa ........................................................................................................................................ 22
Figure 2-3. Expanded scan of the TLC analysis of purified 2b ............................................. 25
Figure 2-4. UV-vis spectrum of purified 2b ......................................................................... 26
Figure 2-5. Expanded 1H NMR spectrum of recrystallized 2b ............................................ 26
Figure 2-6. HR ESI+MS spectrum of purified 2b ................................................................. 27
Figure 2-7. Color of biliverdin 2b before and after reduction to bilirubin 3b. UV-vis spectrum of purified bilirubin dimethyl ester 3b .............................................................................................................. 28
Figure 2-8. Comparative HPLC chromatograms of brown and emu eggshell extracts to 1b and 2b standards. ................................................................. 30

Figure 2-9. HPLC-UV-vis spectra of 1b and 2b as extracted from emu eggshells and brown eggshells ............................................................................................................. 31

Figure 2-10. UV-vis spectrum of raw *Nothura maculosa* eggshell extract. ......................... 32

Figure 2-11. HPLC chromatograms of *Nothura maculosa* eggshell extract. ......................... 32

Figure 2-12. HPLC-UV-vis spectra of *Nothura maculosa* pigments........................................ 33

Figure 2-13. HR ESI⁺-MS of p1. .................................................................................. 35

Figure 2-14. HR ESI⁺-MS of p3. .................................................................................. 38

Figure 2-15. HPLC chromatogram of blue band of *Eudromia elegans* eggshell extract and HPLC-UV-vis spectrum of major pigment................................................................. 39

Figure 2-16. HPLC chromatogram of green band of *Eudromia elegans* eggshell extract and HPLC-UV-vis spectrum of major pigment................................................................. 40

Figure 2-17. HR ESI⁺-MS of green extract. ....................................................................... 41

Figure 3-1. Structures of biologically relevant porphyrins.................................................. 46

Figure 3-2. American Porcupine quills under ambient light and 365 nm black light .......... 48

Figure 3-3. African Crested Porcupine quills under ambient light and 365 nm black light .... 49

Figure 3-4. European Hedgehog quills under ambient light and 365 nm black light .......... 49

Figure 3-5. Diffuse reflectance fluorescence spectroscopy of light bands of hedgehog quills. .. 50

Figure 3-6. Diffuse reflectance spectroscopy of light and dark bands of American Porcupine quills. .................................................................................................................. 51

Figure 3-7. UV-vis spectrum of the raw hedgehog extract.................................................... 52
Figure 3-8. HPLC chromatogram of hedgehog extract and HPLC-UV-vis spectrum of peak #4. ........................................................................................................................................................................................................................................................................................................................................ 53

Figure 3-9. HPLC of porphyrin standards. .................................................................................................................................................................................................................................................................................................................................................. 54

Figure 3-10. HPLC-F1 emission spectrum of the primary pigment in hedgehog extract. .......... 56

Figure 3-11. Emission spectra of protoporphyrin IX DME, coproporphyrin III TME, and uroporphyrin III OME. ........................................................................................................................................................................................................................................................................................................................................ 56

Figure 3-12. HR ESI⁺-MS spectrum of peak 4 from hedgehog extract. ................................. 57
List of Schemes

**Scheme 1-1.** Oxygen association and disassociation with heme B. ................................................ 9

**Scheme 1-2.** Mammalian biosynthetic pathway of heme B.......................................................... 14

**Scheme 1-3.** Proposed mechanism for UROS catalyzed Urogen III formation from HMB....... 15

**Scheme 2-1.** Biological formation of biliverdin IXα and reduction to bilirubin and *in vitro* formation of bilirubin dimethyl ester from biliverdin IXα dimethyl ester................................. 21

**Scheme 2-2.** Fisher esterification of biliverdin IXα and protoporphyrin IX................................. 24

**Scheme 2-3.** Oxidation of 2b to a tripyrrolic compound............................................................ 36
1 Introduction to Tetrapyrroles

1.1 Porphyrrins and Bilins

Porphyrrins are tetrapyrrolic, fully unsaturated aromatic compounds with characteristically large extinction coefficients.\(^1,2\) The \(\alpha\)-positions of each pyrrole are linked to a methylene carbon, forming a conjugated ring structure (Figure 1-1); these methine carbons are designated as meso-positions.\(^2\) The central cavity of the porphyrinic macrocycle is a potent chelator of cationic metals with the porphyrin serving as a tetradeptate dianionic ligand.\(^3,5,35\) The strong UV-vis absorbance and metal chelating ability of porphyrrins are incredibly important in their biological roles (see section 1.3).

Porphyrrins contain 22 conjugated \(\pi\)-electrons, but only 18 are needed to maintain a closed conjugated aromatic system (Figure 1-1).\(^6\) The remaining 4 \(\pi\)-electrons are in cross-conjugation with the Hückel aromatic system and can therefore be removed without affecting aromaticity.\(^6\) Though aromaticity is conserved upon removal of these cross-conjugated electrons, reduction of the peripheral \(\beta,\beta'\) double bonds affects the symmetry of the macrocycle and thus has profound effects on both the chemical and physical properties of the compound (see section 1.2).
Introduction to Tetrapyrroles

Figure 1-1. Structure and labeling of porphyrins. Bold region indicates 18-π aromatic system.

Reduction of the β,β’ double bonds of a porphyrin results in the formation of hydroporphyrins. These hydroporphyrins fall into three classes: chlorins, bacteriochlorins, and isobacteriochlorins (Figure 1-2). Chlorins, or dihydroporphyrins, have a single β,β’ double bond reduced and have an 18 + 2π aromatic system. Bacteriochlorins and isobacteriochlorins, or tetrahydroporphyrins, have two reduced β,β’ double bonds and 18π aromatic systems.\(^1\) In bacteriochlorins, the reduced pyrroles lie opposite to one another while in isobacteriochlorins the reduced pyrroles are adjacent.\(^1\)

Figure 1-2. Structure and naming of the three classes of hydroporphyrins. Bold regions indicate 18-π aromatic systems.
In addition to porphyrin reductions, there are also porphyrin oxidations. Synthetically, porphyrin oxidations are performed on the pyrrole moieties, exploiting the pseudo-olefinic nature of the $\beta,\beta'$ double bonds; however, Nature performs ring oxidations of porphyrins to form linear tetrapyrroles (Figure 1-3). Linear tetrapyrroles are known as bilins, and their chemical and physical properties vary drastically from their closed-ring counterparts. Bilins, though conjugated, are not aromatic and thus have markedly lower extinction coefficients than porphyrins. Moreover, opening of the ring affects both the orbital overlaps and symmetry of the compound which yields a distinctive UV-vis spectrum (see section 1.2).

![Figure 1-3. Structure and name of linear chain tetrapyrrole. Bold region indicates conjugated system.](image)

1.2 Optical Properties of Tetrapyrrolic Compounds

The 18-$\pi$ electron aromatic system of porphyrins gives them unique electronic and spectroscopic properties. Free-base porphyrins normally contain five characteristic absorption bands: a high intensity band near 400 nm known as the Soret band, and four significantly smaller bands between 500-600 nm known as Q bands. The Soret bands of most porphyrins have molar extinction coefficients of $10^5$ M$^{-1}$ cm$^{-1}$ while the less intense Q bands have coefficients of $10^4$ M$^{-1}$
Moreover, the Q bands of free-base porphyrins decrease in intensity at longer wavelength, following what is known as an etio-type pattern.\textsuperscript{10}

In comparison to free-base porphyrins, linear-chain tetrapyrroles lack both Soret and Q bands and are instead characterized by two broad spectral features (Figure 1-4). Both features normally have molar extinction coefficients of $10^4\text{ M}^{-1}\text{ cm}^{-1}$ and the second feature is usually near half as intense as the first.\textsuperscript{11}

![Normalized Intensity vs Wavelength](image)

**Figure 1-4.** Characteristic UV-vis absorption spectra of free-base porphyrins (left) and free-base bilins (right).

The insertion of a metal into the porphyrin or bilin chromophore has a significant effect on their optical spectra. Upon insertion of a metal into a porphyrin, the symmetry of the chromophore is altered from exhibiting two-fold $D_{2h}$ symmetry to four-fold $D_{4h}$ symmetry, which is reflected in the UV-vis spectra. In the porphyrins spectra, the four Q bands are reduced to two, highlighting the higher level of symmetry, and the $\lambda_{\text{max}}$, along with the Soret band to a lesser extent, hypsochromically shift in comparison to the free-base analogue.\textsuperscript{12-14} Metalation of the bilin chromophore has a less predictable effect.\textsuperscript{15} Depending on the metal inserted, the
spectra can: bathochromically shift, gain additional spectral features, lose the low intensity band, or a mixture of all the above.

The spectroscopic properties of porphyrins were described in the 1950’s by the Gouterman group.\cite{Gouterman1951} The description of the porphyrin spectra was based on the electronic frontier orbital transitions. Gouterman proposed a four-orbital model through which the Soret band resulted from $\pi$ to $\pi^*$ transitions between the $S_0$ and $S_2$ states and the Q bands resulted from $\pi$ to $\pi^*$ transitions between the $S_0$ and $S_1$ states. It also proposed that the longest wavelengths at which porphyrin chromophores absorb light corresponds to the HOMO-LUMO (Highest Unoccupied Molecular Orbital-Lowest Unoccupied Molecular Orbital) energy gap.\cite{Gouterman1951}

Modification to the macrocycle, through alteration to the periphery via the $meso$- or $\beta$-positions, or by insertion of a metal changes the relative energies of the electronic transitions due to the perturbation of the chromophore’s symmetry.\cite{Gouterman1951}

The chromophores of free-base porphyrins typically exhibit $D_{2h}$ symmetry as a result of the opposing nitrogen-bonded inner hydrogens. By defining the $x$-molecular axis as passing through the non-hydrogen bonded nitrogens and the $y$-molecular axis as passing through the hydrogen bonded nitrogens, it is readily apparent that the axes are not equivalent and thus implying that there is no degeneracy resulting from the excited states of the $x$- or $y$- polarized orbital transitions (Figure 1-5). The lack of degeneracy results in spectroscopic transitions split between distinctive $x$ and $y$ bands with Q-allowed transitions.

Furthermore, the degeneracy of the HOMOs and LUMOs for a porphyrin with a $D_{4h}$ point group, such as those observed with a metalloporphyрин, occurs due to the symmetry of the macrocycle and exemplifies the four excited electronic transitions of a porphyrin.\cite{Gouterman1951} The HOMO-LUMO transitions of the $D_{4h}$ point group are split into two high energy transitions (B$_x$}
and B\textsubscript{y}) and two low energy transitions (Q\textsubscript{x} and Q\textsubscript{y}). The high energy transitions are strongly optically allowed while there is a zero probability for a low energy transition. As such, D\textsubscript{4h} porphyrins exhibit two short-wavelength Q bands, but no long wavelength Q bands.\textsuperscript{12-14}

![Figure 1-5](image)

**Figure 1-5.** A schematic representation of the two HOMOs and two LUMOs (A,B) and corresponding electronic transitions (C,D); porphyrins with high degeneracy (A,C) and low degeneracy (B,D).

Moreover, porphyrins are highly fluorescence compounds with large quantum yield values.\textsuperscript{17} The Fl emission spectrum a of a typical porphyrin is shown in Figure 1-6.
The emission spectra of porphyrins exhibit characteristically large Stokes shifts between their Soret and their primary emission peak; a feature that is mirrored in the Fl spectra of hydroporphyrins\textsuperscript{18}, though bilins are typically non-fluorescent. The relative intensity of the two emission bands varies depending on the amount of reduction in the porphyrin’s system.\textsuperscript{19} For porphyrins the intensity ratios can be close to 2:1 while in chlorin systems this ratio is increased to near 5:1. Though high quantum yields have led to the use of porphyrins as fluoresce dyes,\textsuperscript{20} pyrrole-modification of the porphyrin macrocycle can tune fluoresce properties allowing for efficient heat conduction through nonradiative decay\textsuperscript{21} or singlet oxygen generation through Type II photoprocesses.\textsuperscript{22} A modified Jablonski diagram, indicating the potential pathways the absorbed light of a porphyrin can undergo, is included in Figure 1-7.
Figure 1-7. Modified Jablonski diagram for a porphyrin photosensitizer.

1.3 Naturally Occurring Tetrapyrroles

Porphyrinoids are ubiquitous throughout Nature and serve a multitude of roles; though, they are most readily recognized by the biological roles they fulfill. For example, the iron complex of protoporphyrin IX, heme B, is the prosthetic group found tightly associated with hemoglobin and myoglobin proteins. These metalloproteins are found in all vertebrates, with the exception of the fish family Channichthyidae, and are indispensable for survival as they facilitate oxygen transfer throughout the bloodstream of an organism. Though the specifics in which oxygen binds hemoglobin and myoglobin is unclear, it has been shown that in areas of high oxygen partial pressure, oxygen binds axially to the iron of the heme. Meanwhile, in areas of low oxygen partial pressure, oxygen dissociates from the iron complex (Scheme 1-1).
Naturally occurring hydroporphyrins also serve important roles in biology. For example, the green color associated with most leaves and grasses results from the presence of chlorophyll $a$. Chlorophyll $a$, a magnesium chlorin complex, acts as a light harvesting pigment and is essential for photosynthesis in all plants.$^{2,28}$ Photoautotrophic purple bacteria and cyanobacteria also use photosynthesis to sustain themselves; however, they use a bacteriochlorin magnesium complex, bacteriochlorophyll $a$, instead of chlorophyll $a$ (Figure 1-8).$^{28}$

**Scheme 1-1.** Oxygen association and disassociation with heme B.
In addition to the many biological roles porphyrinoids serve, they are also found as pigments throughout the animal kingdom. The color of avian eggs shells, for example, are thought to manifest entirely through the mixture of two tetapyrrolic compounds: biliverdin IXα and protoporphyrin IX (for more details, see chapter 2). Moreover, the feathers of some owls and bustards have been shown to contain the coproporphyrin III, a biological intermediate in the biosynthetic pathway of heme B (see section 1.4).\textsuperscript{29,30} Even more interesting is the appearance of a metalloporphyrin within the red feathers of \textit{Turaco schalowi}. The metalloporphyrin, known as turacin, is a copper uroporphyrin III complex, and remains the only example of a copper porphyrin complex in Nature (Figure 1-9).\textsuperscript{31} \textit{Turaco schalowi} also have feathers which contain the pigment turacoverdin, the only true green pigment used in bird feathers, whose structure remains unknown though it is believed to be an oxidation product of turacin.\textsuperscript{32} In addition to porphyrins appearing as dyes in bird feathers and eggs, they also appear in seashells\textsuperscript{33} and demosponges of coral reefs.\textsuperscript{34} Both uroporphyrin I and uroporphyrin III appear in a variety of red
seashells (Figure 1-9) and sponges of corals have been shown to express unique porphyrins, aptly named corallistins (Figure 1-10).

**Figure 1-9.** Porphyrins as dyes within animal tissues. Top image: great bustard feather, middle image: *Clanculus pharaonius*, bottom image *Turaco schalowi*. Great bustard image taken from Ref 29, all other images are public domain.
1.4 **Biosynthesis of Animal Tetrapyrroles**

Almost all biologically relevant mammalian porphyrins are created through the heme B biosynthetic pathway (Scheme 1-2). The first step in this pathway is the generation of 5-aminolevulinic acid (ALA), the general precursor of all known tetrapyrroles. This formation is catalyzed by 5-aminolevulinic acid synthase (ALAS) and proceeds through the condensation of glycine and succinyl coenzyme A. When enough ALA is generated, two ALA units can combine through a Knorr-type condensation reaction, catalyzed by 5-aminolevulinic acid dehydratase (ALAD), to form pyrrole porphobilinogen (PBG). Four PBG moieties can be
condensed through the enzyme porphobilinogen deaminase (PBGD) to from the linear
tetrapyrrole hydroxymethylbilane (HMB).\textsuperscript{38} Interestingly, PBGD possesses a dipyrromethene (a
dipyrrole) cofactor to which PBG units are added sequentially until a linear hexapyrrole is
formed.\textsuperscript{39} At this point, water cleaves the hexapyrrole yielding free HMB and PBGD-bound
dipyrromethane. HMB binds in a circular conformation to the enzyme uroporphyrinogen III
synthase (UROS) which closes the tetrapyrrole forming uroporphyrinogen III (Urogen III). An
important note is that process is not a simple condensation between HMB’s alcohol and \(\alpha\)-
hydrogen on the adjacent pyrrole; the pyrrole ring across from the alcohol flips during ring
closure.\textsuperscript{40} A proposed mechanism for this reaction is shown in Scheme 1-3.\textsuperscript{40} Urogen III is then
converted into coproporphyrinogen III (Coprogen III) through uroporphyrinogen III
decarboxylase (UROD) catalyzed decarboxylation of the four acetate side chains.\textsuperscript{41} From here,
coproporphyrinogen III oxidase (CPOX) oxidatively decarboxylates the two non-adjacent
propionate side chains to vinyl groups, thus forming protoporphyrinogen IX (Protogen IX).\textsuperscript{42} The
penultimate step of heme B synthesis entails the oxidation of Protogen IX to a highly conjugated,
aromatic porphyrin: protoporphyrin IX.\textsuperscript{42} Protoporphyrin IX serves as the porphyrin base of
heme B, all that is required is the insertion of ferrous iron. This insertion is catalyzed by
ferrochelatase (FECH), an enzyme which works by distorting the porphyrin macrocycle into a
nonplanar conformation, thus relieving steric pressures associated with metal insertion.\textsuperscript{43}
Introduction to Tetrapyrroles

Scheme 1-2. Mammalian biosynthetic pathway of heme B.
**Scheme 1-3.** Proposed mechanism for UROS catalyzed Urogen III formation from HMB.
1.5 References

9. Rita Giovannetti (2012). The Use of Spectrophotometry UV-Vis for the Study of Porphyrins, Macro To Nano Spectroscopy, Dr. Jamal Uddin (Ed.)
2 Extraction of Oligopyrroles from Eggshells

2.1 Introduction

2.1.1 Developing an Extraction Protocol

Work published in 1975 surveyed the pigment composition of eggshells from over 100 different species of birds and found that color variations between eggshells resulted from the mixture of two pigments, the red-brown protoporphyrin IX (1a) and the blue-green biliverdin IXα (2a). Some shells, such as the brown eggshells from domestic chickens, are pigmented solely by 1a while others, such as the blue-green eggshells of emus, are pigmented solely by 2a (Figure 2-1).

![Figure 2-1](image)

Figure 2-1. Structures of tetrapyrroles found within eggshells. Images are public domain.
As an egg is produced in the hen’s reproductive system, the egg is built from the inside out. Porous layers of calcite, a white, crystalline form of calcium carbonate, mixed with structural proteins, form the eggshell structure. The eggshell dyes are exclusively found embedded in the cuticle, a thin protein matrix layer on the outside of the eggshell. The cuticle is generated by dedicated glands that also produce the pigments in the final hours before the eggs are laid. The property of the calcium carbonate and cuticle to dissolve under strongly acidic conditions, thus releasing acid-stable tetrapyroles, enables the extraction of the pigments.

Classically, the extraction of these eggshell pigments was performed by covering upwards of 500 g of whole eggshells with a 5% methanolic acid solution and allowing the mixture to sit for two days at room temperature. Though usable, the time frame of the procedure, along with its reliance on large amounts of materials, makes it inaccessible to researchers working with rare materials or hoping to quickly determine the pigment composition of an eggshell. Described in this work is a modification of the classical extraction protocol, utilizing high-performance liquid chromatography (HPLC), in which pigments from 100 mg samples can be reliably and accurately extracted and analyzed.

The utility of this technique was highlighted in an undergraduate laboratory experiment published in the *Journal of Chemical Education* in which we described two experiments centered around the extraction of tetapyrrolic compounds from eggshells. The first is a 3-hour procedure regarding the extraction of biliverdin IXα from emu eggshells as its dimethyl ester. This experiment is designed to show students the parallels between biological and *in vitro*
Extraction of Oligopyrroles from Eggshells

transformations of tetrapyrroles (Scheme 2-1) and give students experience with natural product isolation and characterization.

The second procedure details an optimized extraction protocol for small samples (< 100 mg) in which extraction and isolation take less than one hour and characterization is achieved by HPLC and absorbance spectroscopy.

Scheme 2-1. Biological formation of biliverdin IXα and reduction to bilirubin and \textit{in vitro} formation of bilirubin dimethyl ester from biliverdin IXα dimethyl ester.
2.1.2 Tinamou Eggshell Extractions

The notion that all eggshell colors result as a mixture of 1a and 2a has become dogmatic in recent years with over 140 years of literature supporting the claim\(^1,7,8\) and color-blending computer models predicting that the mixing of these two pigments can generate almost all naturally occurring eggshell colors.\(^9\) Be this as it may, there are some natural eggshells, with unknown pigment concentrations, which have colors that fall outside the model-predicted ranges.\(^9\) Examples of this are the eggshells from several species of tinamous. In total, there are 47 species of tinamou spanning Mexico, Central America, and South America.\(^10\) Tinamou eggs are easily identified through their distinctive porcelain-like gloss, a property conferred by the calcite and calcium phosphate matrices of the shell cuticle.\(^11\) Moreover, tinamou eggs range through a spectrum of colors including green, purple, violet, turquoise, steel grey, chocolate, lemon-yellow, and even white. Most of these colors, like the turquoise eggs of \textit{Tinamus major}, fall within possible blends of 1a and 2a; however, the purple eggs of \textit{Nothura maculosa} and the green eggs of \textit{Eudromia elegans} do not (Figure 2-2).

![Figure 2-2. Tinamou Eggshells of Tinamous major (a), Eudromia elegans (b), and Nothura maculosa (c). Image taken from Ref 11.](image-url)
A 2015 study looked at using Raman spectroscopy as a non-destructive tool to analyze eggshell pigments. The study examined the green eggs of *Eudromia elegans*, among others, and concluded that the principal pigment was 2a; a statement they further confirmed through mass spectrometry, though they do not show a mass spectrum in the paper or in the supporting information. Though it is expected that 2a is within these eggshells to some degree, using Raman spectroscopy to verify the presence of a pigment does not exclude the possibility of other pigments residing in the cuticle as well. As such, the work presented here details the extraction and analysis of tinamou eggs from *Eudromia elegans* and *Northura maculosa* utilizing our optimized extraction protocol.

### 2.2 Results and Discussion

#### 2.2.1 Extraction of Biliverdin from Emu Eggshells

In the preparatory phase of the extraction, the dark green emu eggshells (30 – 50 g) are broken into ~2-4 cm³ large pieces and submersed in 3 N hydrochloric acid. The acid begins to etch the surface of the shells, dissolving the cuticle, and loosens the adhesion between the shell and the thick inner membrane. Following decantation, the shells are dried, the inner membrane is removed, and the membrane-free shells are grinded into a powder using a coffee grinder. 100 mL of 2.0 M methanolic H₂SO₄ is added to the powder in a round bottom flask and the mixture in gently refluxed, under constant stir, for 1 hour. Though the primary focus of the acid digest is to release the biliverdin from the egg’s carbonate matrix, the conditions also serve to esterify the compound into its corresponding dimethyl ester (Scheme 2-2), biliverdin IXα dimethyl ester (2b).
**Scheme 2-2.** Fisher esterification of biliverdin IXα (top) and protoporphyrin IX (bottom).

Following reflux, the solution is adjusted to a near neutral pH through addition of sat. sodium bicarbonate and vacuum filtered. Care is taken to not allow the solution to become too basic to avoid possible saponification. Extraction of 2b is achieved by partitioning between water and ethyl acetate, collecting the organic phase, and drying with anhyd. sodium carbonate. After filtration, the resulting sample is dried to a film through rotary evaporation. This crude product can then be dissolved in minimal ethyl acetate and purified through silica gel flash chromatography. If desired, the pure compound can also be recrystallized into a powder through solvent exchange between methylene chloride and hexanes.
2.2.2 Characterization of Extracted Biliverdin IXα Dimethyl Ester

2b is a deep blue is solution which makes it optimal for naked eye characterization techniques. For example, the purity of the extract can easily be discerned by checking the \( R_f \) value of the pigment through think-layer chromatography (Figure 2-3).

![TLC analysis of purified 2b](image)

**Figure 2-3.** Expanded scan of the TLC analysis of purified 2b obtained in extraction.

Moreover, 2b, (and open-chain tetapyrrolic pigments in general), display broad, two-band UV-vis spectrum. The UV-vis spectrum of the purified extract (Figure 2-4) displays these features and conforms to the published spectra of 2b in particular. Further instrumental characterization, through techniques such as NMR (Figure 2-5) and HR-MS (Figure 2-6) also indicate the extraction of pure 2b from the emu eggshells.
Figure 2-4. UV-vis spectrum (CH₂Cl₂) of purified 2b.

Figure 2-5. Expanded ¹H NMR spectrum (CDCl₃, 400 MHz) of 12 mg recrystallized 2b.
Extraction of Oligopyrroles from Eggshells

Figure 2.6. HR ESI-MS spectrum (CH$_3$CN, cone voltage 30 V) of purified 2b.

Chemical Formula: \( C_{35}H_{39}N_4O_6 + \)

Exact Mass: 611.2864

Molecular Weight: 611.7185

Max. 8035.6 counts.
If laboratory students do not have access to characterization techniques such as NMR and HR-MS, they can instead confirm the extraction of 2b by mimicking the biological reduction of biliverdin to bilirubin (3a), \textit{in situ}. As shown in Scheme 2-1, biology degrades 2a to 3a through an enzymatic process. Students can mimic this through sodium borohydride reduction in methanol and monitor the reaction qualitatively through color, or through absorbance spectroscopy, to confirm the conversion of 2b to bilirubin dimethyl ester (3b) (Figure 2-7).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Color of biliverdin 2b (in EtOAc) before and after reduction to bilirubin 3b (left). UV-vis spectrum (CH$_2$Cl$_2$) of purified bilirubin dimethyl ester 3b (right).}
\end{figure}

\subsection{Optimization of Extraction Protocol}

The procedure detailed above takes roughly 3 hours to complete, meaning this experiment is ill-fit for shorter lab sections of 1.5-2 hours. Though the experiment gives students valuable experience in natural product isolation and characterization, this time commitment is a shortcoming. To counter this, and in a general sense make such extractions more convenient to researchers, we designed a streamlined version of this protocol in which pigment extraction takes only an hour and characterization only 20 minutes. Moreover, this optimized protocol can be
performed on samples of less than 100 mg, making it ideal for the extraction of valuable materials.

This procedure disregards the etching and de-membraning phases of the procedure as, with low mass samples, the egg inner membrane does not complicate extraction or filtration. Moreover, only 5 mL of a 1.8 methanolic acid solution is used during reflux as reduced sample size means there is less calcium carbonate is solution to react with free acid. Reduced acid volume and sample size allow both neutralization and filtration to be completed within minutes. The rest of the procedure mirrors that of the one described previously, but with extraction volumes greatly decreased and column chromatography performed in a pipette.

Depending on the concentration of pigment within the egg extracted, nanograms to micrograms of tetrapyrrolic compounds can be isolated. The low yield makes purification through recrystallization and analysis through NMR effectively obsolete. Instead, HPLC equipped with UV-vis detectors are used for both purification and characterization. HPLC chromatograms comparing standards of $2b$ and protoporphyrin IX dimethyl ester ($1b$) to those extracted from emu eggshells and brown chicken eggshells, respectively, are shown in Figure 2-8.
Figure 2-8. Comparative HPLC chromatograms of brown and emu eggshell extracts to 1b and 2b standards. 404 nm detection wavelength for brown egg extract and 1b standard. 380 nm detection wavelength for emu egg extract and 2b extract. See section 2.4 for detailed HPLC protocol.

The elution protocol described (see section 2.4) cleanly separates 1b and 2b when run on a normal phase silica column. The elution times of the two compounds are one facet through
which the pigments can be characterized, though they can more readily be discerned by their distinctive UV-vis spectra (Figure 2-9).

![Figure 2-9. HPLC-UV-vis spectra of 1b (left) and 2b (right) as extracted from emu eggshells and brown eggshells, respectively.]

2.2.4 Extraction of Pigments from Tinamou Eggshells

2.2.4.1 Nothura maculosa

The purple eggs of *Nothura maculosa* are one example of a color not predicted by blending of 1a and 2a. As such, we obtained several eggs from a distributor in South America and extracted their pigments using our optimized extraction protocol. The UV-vis spectrum of the raw extract is shown in Figure 2-10, chromatograms of the extracts are shown in Figure 2-11, and HPLC-UV-vis spectra of the highlighted peaks are shown in Figure 2-12.
Figure 2-10. UV-vis spectrum of raw *Nothura maculosa* eggshell extract.

Figure 2-11. HPLC chromatograms of *Nothura maculosa* eggshell extract. Chromatogram with detection wavelength of 400 nm (left) and chromatogram with detection wavelength of 475 nm (right). * indicates column artifacts. See section 2.4 for details of the elution protocol.
Extraction of Oligopyrroles from Eggshells

In Figure 2-10 we see a UV-vis spectrum that greatly resembles that of an open-chain tetrapyrrole.\textsuperscript{13} Given the precedence of 2a being used as a dye in eggshells, the main pigment of the extract is likely 2a. It should be noted however that there is spectral feature near 500 nm. This feature is not observed in the UV-vis spectrum of pure 2b, thus indicating that there is at least one additional pigment within the extract.

From Figure 2-11, four peaks are identified through HPLC of the eggshell extract. The 18.0 min peak (p1) has the characteristic UV-vis spectrum of an open-chain tetrapyrrrole.\textsuperscript{13} The HPLC retention time and the absorption maxima of p1 correlate almost precisely to that 2b, though there seems to be a splitting feature in the highest absorption band of the compound. This splitting disappears when the extract is diluted or when less sample is injected into the chromatogram indicating that the splitting results from detector saturation, rather than as a
characteristic of the pigment. Mass spectrometry of \( p1 \) indicates the presence of mono-, di-, and trimeric version of \( 2b \) (Figure 2-13). Dimeric biliverdin is commonly found when biliverdin is run through an ESI\(^+\) protocol, in fact, performing the same analysis on standard \( 2b \) also affords dimeric biliverin as the most intense peak. The presence of trimeric biliverdin is surprising, though not completely absurd. Naturally occurring porphyrins and bilins are composed of various hydrogen-bond acceptors and donors which allow for the formation of polymeric structures.\(^{14}\) This said, when running standard \( 2b \) against this protocol, trimeric biliverdin was never observed. It should be noted, however, that running \( p1 \) multiple times did not consistently produce the trimer peak, indicating that the trimer may indeed be formed in the mass spectrometer rather than in the eggshell.

The 19.4 min peak \( (p2) \) somewhat resembles an open-ring pyrrolic compound; however, it exhibits optical features drastically different from that of \( 2b \). For example, \( p2 \) shows only a single absorption peak centered at 391 nm, which is a 13 nm bathochromic shift from the main absorbance band of \( 2b \). Unfortunately, even after several extractions and pooling together \( p2 \) isolates, ESI\(^+\) HR-MS does not afford a distinguishable parent ion to be analyzed. As such, further study is needed to identify the structure of the compound. Nevertheless, observation of the pigment leads one to believe it is distinct from both \( 1b \) and \( 2b \).
Extraction of Oligopyrroles from Eggshells

**Figure 2.13** HR ESI-MS (CH₃CN, cone voltage 30 V) of p1.

- TOF MS: 3.203 to 5.523 min from Sample 3 (RH-A-Bili) of RH_BRUCKNER_20180423.wiff

Chemical Formula: C₃₅H₃₉N₄O₆⁺
Exact Mass: 611.2864
Molecular Weight: 611.7185

Chemical Formula: C₇₀H₇₇N₈O₁₂⁺
Exact Mass: 1221.5655
Molecular Weight: 1222.4295

Chemical Formula: C₁₀₅H₁₁₅N₁₂O₁₈⁺
Exact Mass: 1831.8447
Molecular Weight: 1833.1405

Max. 58.1 counts.
The 21.1 min peak (p3) also somewhat resembles an open-ring pyrrolic compound, but exhibits features different from that of 2b. Amazingly, rather than a deep-blue color like 2a or a light-pink color like 1a, the p3 extract is deep-red color. Like p1, there are two absorption bands in the UV-vis spectrum; however, these bands have absorbance maxima at 325 nm and 495 nm which significantly differ from those of 2b (378 nm and 651 nm). Large hypochromic shifts such as these have been recorded for tripyrrolic oxidation products of 2a. Indeed, ESI+ HR-MS of p3 gives strong evidence for the presence of the tripyrrolic compound (Figure 2-14). The most abundant peak in the mass spectrum occurs at 516.2041 m/z and correlates to the sodium adduct of a tripyrrolic 2b oxidation product (Scheme 2-3).

**Scheme 2-3.** Oxidation of 2b to a tripyrrolic compound.

There is also a secondary peak at 494.2131 m/z that correlates with the monoprotonated form of this tripyrrole. The species correlating to 683.3461 m/z and 727.3813 m/z are difficult to assign, though it should be noted that these peaks have been observed in mass spectra unrelated to this project and may thus be something like a column contaminant. A minor peak at 1009.5711 m/z is also observed and correlates well to the sodium adduct of the tripyrrolic dimer species.
Given the UV-vis and HR-MS spectra, we believe this compound to be the tripyrrinedione uroerythrin (Scheme 2-3, dimethyl ester form). Uroerythrin is a red pigment found in urine and is often found in elevated concentration in individuals with metabolic disorders.\(^{16}\) Uroerythrin is also known to form through the breakdown of heme, though the details of this process are unknown. The UV-vis spectrum of our red isolate match closely with those recorded for tripyrrindiones\(^{17}\) and uroerythrin in particular.\(^{18}\)

The finding that extraction of both emu eggshells and biliverdin standards do not result in degradation, oxidative or otherwise, of the resulting 2\(b\), leads us to conclude that this compound, uroerythrin, is found in the eggshells rather than formed as a result of the extraction protocol.

The peak at 21.8 min (\(p4\)) is the most difficult to isolate as it is difficult to achieve baseline separation between it and \(p3\). From its UV-vis spectrum, it seems to be a mixture of \(p3\) and a biliverdin type compound. It has four spectral features occurring at 321 nm, 370 nm, 474 nm, and 627 nm. All of these features are hypsochormically shifted from their corresponding \(p1\) or \(p3\) peaks indicating that \(p4\) may not result from the coelution of two compounds as this would not modulate the absorption peaks. Unfortunately, not enough sample has yet to be collected for meaningful analysis by HR-MS. As such, further study is needed to identify the structure of the compound.
Extraction of Oligopyrroles from Eggshells
2.2.4.2 *Eudromia elegans*

The green eggs of *Eudromia elegans* also fall into the class of egg colors not predicted by computer-generated blending models. Our group was not the first to become interested in studying the pigment composition of these eggshells; indeed, it was our collaborators at Yale University who approached us about studying this egg. We received two eggs from the Prum ornithology group and extracted their pigments through our optimized extraction protocol. It is important to note that when the raw extract was run on a silica gel pipette column two bands were readily separated. The first was a blue band, reminiscent of 2b, that moved quickly in ethyl acetate; however, the second was an incredibly polar light-green band which only began to move with a solvent composition of 90% ethyl acetate:10% methanol. In comparison, both 1b and 2b move readily in ethyl acetate and neither are green in color. HPLC was performed on each band, individually. The HPLC chromatogram and UV-vis spectrum of the blue band is shown in Figure 2-15 and the HPLC chromatogram and UV-vis spectrum of the green band is shown in Figure 2-16.

![HPLC chromatogram of blue band of *Eudromia elegans* eggshell extract (left) and HPLC-UV-vis spectrum of major pigment (right). For protocol details, refer to section 2.4.](image-url)

**Figure 2-15.** HPLC chromatogram of blue band of *Eudromia elegans* eggshell extract (left) and HPLC-UV-vis spectrum of major pigment (right). For protocol details, refer to section 2.4.
Figure 2-16. HPLC chromatogram of green band of Eudromia elegans eggshell extract (left) and HPLC-UV-vis spectrum of major pigment (right). For protocol details, refer to section 2.4.

Observing Figure 2-15, both the retention time and the UV-vis spectrum of the blue isolate indicate that it is 2b. This is unsurprising given biliverdin’s prevalence in eggshells. Figure 2-16 shows the green band to have both polarity and optical properties unique from 1b and 2b. The green isolate is incredibly polar compared to 2b and does not move in hexanes or ethyl acetate. As such, the HPLC protocol had to be altered to incorporate methanol in order to elute the compound. Moreover, the UV-vis spectrum of the green isolate looks non-tetapyrrolic; there is a single absorption band, compared to the two bands of linear tetapyrroles, at 433 nm. The overall vibrionic structure of the absorption band is similar to that of p2, though they differ in absorption maxima by 42 nm. Surprisingly, HR-MS of the green extract shows clear indication of 2b being the primary compound present (Figure 2-17); exhibiting both the mono- and dimeric masses. The presence of 2b, given the anomalous UV-vis spectrum of the green extract, is strange, though it should be noted that conformational variations in the compound, or even complexation with a protein could affect the UV-vis spectrum.
Extraction of Oligopyrroles from Eggshells

Figure 2-17. HR ESI+ MS (MeOH, cone voltage 30 V) of green extract.

Chemical Formula: C35H39N4O6+
Exact Mass: 611.2864
Molecular Weight: 611.7185

Chemical Formula: C70H77N8O12z+
Exact Mass: 1221.5655
Molecular Weight: 1222.4295
2.3 Conclusion

In this chapter, we have highlighted a procedure for the extraction of porphyrinoids from avian eggshells. Recognizing the inherent flaws in the procedure, such as time and material, we designed an optimized version in which samples as small as 100 mg could be accurately and reliably extracted and analyzed. Utilizing our optimized protocol, we analyzed the exotic eggshells of the Tinamou family; shells which had the potential to challenge the dogmatic notion that all eggshells colors are produced through a mixture of biliverdin IXα and protoporphyrin IX. Analysis of eggs from both *Nothura maculosa* and *Eudromia elegans* revealed inherent pigments with spectrophotometry properties inherently different from both 1b and 2b. Moreover, through UV-vis and mass spectra analysis, one of the primary pigments within *Nothura maculosa* was tentatively identified as the tripyrrindione oxidation product of 2b, uroerythrin. Overall, our procedure has been shown to be of use in analyzing these precious samples and has provided us reason to more deeply analyze these Tinamou eggshells. From here, the goal is to continue collecting and pooling pigments together in hopes of gather enough material for NMR analysis.

2.4 Methodology and Instrumentation

2.4.1 Porphyrin Extraction

100 mg of eggshell was weighted and grinded into a fine powder with a pestle. The powder was placed in a 25 ML RBF equipped with a magnetic stir and to this was added 5 mL of a 1.8 M methanolic H₂SO₄ solution. The RBF was equipped to a reflux condenser and heated to a gentle reflux for 45 minutes. The pH of the cooled solution was adjusted to between 6-8 by solution addition of sat. aq. NaHCO₃ (~3.5 mL) until effervescence stopped. The suspension was
then filtered through a small (8 cm diameter) Büchner funnel and the filter cake was washed with ethyl acetate (10 mL). The filtrate was transferred to a 50 mL separatory funnel containing 10 mL of water. The aq. layer was extracted with ethyl acetate (2 x 5 mL). The combined organic layers were dried over anhyd. Na$_2$CO$_3$ (~ 1 g), and the drying agent was removed by gravity filtration. The solution was concentrated by evaporation and passed through a short silica gel pipette column. The faintly colored band(s) were collected, and the fractions evaporated to dryness.

2.4.2 HPLC Analysis of Porphyrinoids

*Nothura maculosa* extracts and the blue band of *Eudromia elegans* were dissolved in minimal (~ 1 mL) ethyl acetate and analyzed using an Agilent 1100 series HPLC equipped with a Grace analytical normal-phase Apollo silica column (4.6 x 250 mm, 5 µm). The mobile phase employed a gradient delivery of hexanes and ethyl acetate: linear gradient of pure hexanes to 70:30 v/v hexanes:ethyl acetate over 6 min, then isocratic delivery of 70:30 v/v hexanes:ethyl acetate over 7 min, followed by linear gradient to pure ethyl acetate over 2 min, all with a flow rate of 1.5 mL/min. The detection wavelengths of the UV-vis detector were set to 400 nm and 475 nm. The green band of *Eudromia elegans* were dissolved in minimal (~ 1 mL) methanol and analyzed using an Agilent 1100 series HPLC equipped with a Grace analytical normal-phase Apollo silica column (4.6 x 250 mm, 5 µm). The mobile phase employed an isocratic delivery of 100% methanol. The detection wavelength of the UV-vis detector was set to 400 nm.
2.5 References

3 Porphyrinoids within Hedgehog Quills

3.1 Introduction

Porphyrins, in the form of their iron complexes (hemes), are the prosthetic groups of a large number of electron transfer and oxygen transfer, storage, or activation enzymes in essentially every organism.\(^1\) 7,8-Dihydrotoporphyrins, as their magnesium(II) complexes, are the basic porphyrinoid framework of the chlorophylls, Nature’s primary photosynthetic pigment.\(^1\)

The tetrapyrrolic, fully conjugated macrocycle of the porphyrins and chlorins is the origin of their deep color, characterized by strong absorption bands in the visible region of the electromagnetic spectrum. Irrespective of their ubiquity and excellent dye properties, Nature uses them only most sparingly as coloring agents.\(^2\)

The primary pigments used in bird feathers, for example, are melanins and carotenoids, or most frequently colors are generated by physical means.\(^3\) The use of a metalloporphyrins as pigments in bird feathers is confirmed in only a single case, namely the use of the deep red pigment turacin ([uroporphyrinato III]copper(II), \(\text{1Cu}\)), in the red wing patches of the turacos (Musophagidae), a family of pigeon-sized arboreal birds endemic to sub-Saharan Africa, where they live in forests, woodland and savanna.\(^4\) Some blue-green [chlorinato]Nickel(II) complexes of unknown biological function were isolated from the blue-green Caribbean tunicate Trididemnum solidum.\(^5\) While their function is unknown and their involvement in reduction catalysis was suspected, they possibly include utilization as blue-green pigments.\(^5\)
Free base porphyrins—porphyrins not carrying a coordinated metal ion in their central cavity—are intermediates in the biosynthesis of hemes.\textsuperscript{6-8} Outside of these pathways, their occurrence in biology as (functional) dyes is restricted to few examples.\textsuperscript{2,4,9,10,11,17-19} In a family of pathologies of the heme biosynthetic pathway, collectively known as porphyrias, free base porphyrins are found in the skin, urin, or feces.\textsuperscript{9} Purple porphyrin-derived pigments (corallistins \textit{a-e}) were found in a New Caledonian marine sponge of the \textit{Corallistes sp.}\textsuperscript{10,11} Perhaps the most common utilization of the purple-brown protoporphyrin IX (2), together with the deep blue-green protoporphyrin degradation product biliverdin IX\textsubscript{a}, is as eggshell dyes in the colored eggshells of birds.\textsuperscript{12} The coloration plays roles in crypsis of the eggs from predation or intra-
species signaling, it has also been proposed to play a role in the physical stability of the eggshell, or as a photoantimicrobial agent. Porphyrin 2 is also found as a brown pigment in the shells of select marine snails.

Most important in the context of this contribution, free base porphyrins, such as uroporphyrin III (1), protoporphyrin IX (2), or coproporphyrin III (3) were also found as fluorescent pigments in the light-colored or pink plumulaceous barbs of the contour feathers of turacos (Musophagidae), young owls (Strigiformes), nightjars (Caprimulgiformes), bustards (Gruiformes), and a kite (Elanus caeruleus). Their role is not entirely clear, though thermoregulatory functions were discussed. A hypothesis was recently advanced that the light pink coloring by porphyrins 2 and 3 in the bustards is used as a short-lived and irreversible–because photodegradable–optical signal in mate selection, particularly for the signaling of likely virginity. Not discussed in the literature are possible origins of these porphyrins from porphyrin- (or porphyrinogen-) expressing yeasts.

Quills are another inert integumentary structure that is primarily colored by melanins. Curiously, a 1925 report firstly mentioned that the stems of young pigeon feathers and the quills of the European hedgehog (Erinaceus europaeus) contain a fluorescent component that was, based on the red color of the emission that was extinguished upon treatment with strong acid, suspected to be a porphyrin. We are not aware that the presence of porphyrins in hedgehog quills was confirmed since the original report, or if the porphyrin(s) had been analyzed. These aspects are the aim of this report. The only primary work that mentions fluorescent quills in hedgehogs is the case study of a sole inbred African hedgehog (Atelerix albiventris) diagnosed with a congenital porphyria and that also showed much elevated levels of type I uro- and
coproporphyrin in the urine and feces. Additionally, there have been spurious reports of domesticated hedgehogs fluorescing on the internet.

### 3.2 Results and Discussion

#### 3.2.1 Spectroscopic Analysis of Quills

Before we attempted the extraction of hedgehog quills, we aimed to confirm the observations of red fluorescence within these, and related, quills. We examined the quills of three specimen: the American porcupine (*Erethizon dorsatum*), the African (crested) porcupine (*Hystrix cristata*), and the European hedgehog (*Erinaceus europaeus*). Inspection of either of the porcupine quills under black light ($\lambda = 365$ nm) revealed them not to possess any visible fluorescence (Figure 3-2, Figure 3-3). In contrast, the hedgehog quills exhibited a red fluorescence (Figure 3-4). This fluorescence was observed only along the light sections of the quill and it was noted that some quills fluoresced brilliantly while others did not. This finding of non-ubiquitous fluorescence matches the online reports. The red fluorescence indeed suggests the presence of porphyrin as many naturally occurring dyes do not fluoresce at this wavelength.

![Figure 3-2](image). American Porcupine quills under ambient light (left) and 365 nm black light (right).
Figure 3-3. African Crested Porcupine quills under ambient light (left) and 365 nm black light (right).

Figure 3-4. European Hedgehog quills under ambient light (left) and 365 nm black light (right).

To determine the spectral features of the fluorescence emission of the quills, we performed a diffuse reflectance fluorescence emission spectroscopy and recorded diffuse excitation-emission spectra (Figure 3-5).
Figure 3-5. Diffuse reflectance fluorescence spectroscopy of the light bands of hedgehog quills. Emission spectra, with corresponding excitation wavelengths shown on the left, and excitation spectra with the corresponding emission wavelengths recorded, shown on the right.

Examining the emission spectra, we see two key features: a peak with a $\lambda_{\text{max}}$ near 450 nm that red-shifts significantly as the excitation wavelengths shift to longer wavelengths, and a peak with a $\lambda_{\text{max}}$ near 650 nm but that red-shifts much less with the excitation wavelengths. Both of these features are most intense when excited at 400 nm, indicating that they absorb strongly at this wavelength. The low-wavelength feature corresponds to the presence of melanin, a pigment characteristically found in dark patches of keratinaceous tissues. This pigment was also identified in the light and dark bands of the porcupine quills, though no porphyrinic fluorophore was observed (Figure 3-6).
Figure 3-6. Diffuse reflectance spectroscopy of the light (left) and dark (right) bands of American Porcupine quills.

The high-wavelength emission feature past 650 nm in the Fl emission spectra is typical for a porphyrin. Each excitation spectrum shows an intense band near 400 nm, the porphyrin-diagnostic Soret band, and multiple long wavelength bands, the four Q-bands that are diagnostic of free base porphyrins. The fact that the $\lambda_{\text{max}}$ Q-band is the lowest intensity band of the four Q-bands suggest the presence of a porphyrin and excludes a hydroporphyrin. Besides the fact that many biologically relevant metalloporphyrins (manganese and iron(II) and (III), copper(II), nickel(II), for example) are non-emissive (though zinc(II) and manganese(II) porphyrins are), the presence of four Q-bands exclude the presence of a metalloporphyrin. Because of the similarity of the spectra of the various naturally occurring porphyrins, the data do not allow an assessment to be made whether one or more porphyrins are present or whether a (non-emissive) metalloporphyrin (or other pigment) is present.

3.2.2 Spectroscopic and Chromatographic Analysis of Quill Extract

We thus extracted the quills and analyzed the extract by HPLC. Extraction of the porphyrins from the keratin matrix of the finely ground quills was accomplished by reflux in a
methanolic $\text{H}_2\text{SO}_4$ solution. The acid afforded a softening of the keratinaceous matrix of the quill, releasing the porphyrin into solution, while also catalyzing a Fischer esterification of any acid functionalities on the porphyrin with methanol to yield the corresponding methyl ester(s). The esterified derivatives allow the porphyrins to be more readily extracted from aqueous media using organic solvents, thus greatly facilitating their concentration in the organic phase.

A UV-vis spectrum of the raw quill extract (Figure 3-7) shows again a clear indication of the presence of a free base porphyrin, with a sharp Soret band (414 nm) and distinguishable (albeit not very well defined) four Q bands (with a $\lambda_{\text{max}}$ at 648 nm).

![UV-vis spectrum](image)

**Figure 3-7.** UV-vis spectrum (ethyl acetate) of the raw hedgehog extract.

Chromatographic analysis of the quill extract was performed through HPLC utilizing UV-vis detection. The chromatogram of the quill extract indicates the presence of several compounds which absorb at 418 nm (Figure 3-8).
Figure 3-8. HPLC chromatogram (see section 3.4 for chromatography details) of hedgehog extract (left) and HPLC-UV-vis spectrum of peak #4 (right). Sample dissolved in ethyl acetate prior to injection.

The chromatogram shows several compounds eluting (peaks #1 through #5) from the raw hedgehog extract. All peaks generated UV-vis spectra typical of free base porphyrins (inter alia, Soret band between 414-418 nm), but only the most abundant compound in the chromatogram (peak #4) was concentrated enough to generate base-line separated Q bands.

Given Nature’s precedence for using 1, 2, and 3 as dyes in keratinaceous tissues, and the fact that these are the porphyrin intermediates formed in the biosynthetic pathway of heme B in non-photosynthetic eukaryotes, we compared the hedgehog extract to the HPLC traces and UV-vis spectra of the esterified standards of 1, 2, and 3 (Figure 3-9).
Figure 3-9. HPLC of porphyrin standards. Chromatograms of porphyrins (detection wavelength of 404 nm) (left) and HPLC-UV-vis spectra (right). Protoporphyrin IX dimethyl ester (top), coproporphyrin III tetramethyl ester (middle), uroporphyrin III octamethyl ester (bottom). See section 3.4 for HPLC details.
Porphyridoids within Hedgehog Quills

Comparing the chromatogram of the hedgehog extract to those of the standards we see that the extract does clearly not correspond to any of them. While peak #4 of the hedgehog extract eluted at 5.6 minutes, all of the porphyrin standards elute past 10 minutes. This difference in retention time indicates that peak 4 is notably less polar than the esterified standards. Furthermore, the UV-vis spectrum of peak #4 is markedly different than those of the standards. The Soret band of the quill extract is centered at 418 nm while the Soret bands of the esterified protoporphyrin, coproporphyrin, and uroporphyrin standards are 405 nm, 398 nm, and 402 nm, respectively. Slight variations in UV-vis spectra between porphyrins are expected as, though they share the same $18 +4\pi$ conjugated $\pi$ system, the substituents at their $\beta$-positions vary. For example, protoporphyrin IX exhibits a 7 nm bathochromic shift in the Soret band compared to coproporphyrin III because two propionic acid moieties are replaced with two cross-conjugated vinyl groups which serve to slightly extend the $\pi$-system and thus red-shift the spectrum. Also, though peak #5 of the hedgehog extract and protoporphyrin dimethyl ester have similar retention times, the Soret band of peak #5 centers at 418 nm and is thus not protoporphyrin.

An HPLC-Fl spectrum was performed on the main hedgehog pigment (Figure 3-10). In comparison the biological porphyrins which exhibit emission peaks near 630 nm with spectral shoulders near 695 nm (Figure 3-11), peak #4 of the hedgehog extract shows an emission peak at 648 nm, with a shoulder at 717 nm.
Figure 3-10. HPLC-Fl emission spectrum ($\lambda_{\text{excitation}} = 414$ nm) of the primary pigment in hedgehog extract. Sample prepared in ethyl acetate.

Figure 3-11. Emission spectra of protoporphyrin IX DME (left), coproporphyrin III TME (middle), and uroporphyrin III OME (right). Excitation wavelength set to 404 nm for all spectra and samples prepared in ethyl acetate.

To glean more insight into possible structures for the main pigment in the extract, mass spectrometry was performed (Figure 3-12). Of the two major peaks, only $m/z$ 659.2962 produces hits for analogues of biologically relevant porphyrinoids; however, these compounds either do
not have a biosynthetic pathway for formation or have optical properties distinctly different from those of the hedgehog extract.\textsuperscript{24}

\textbf{Figure 3-12.} HR ESI\textsuperscript{+}-MS spectrum (CH\textsubscript{3}CN, cone voltage 30 V) of peak 4 from hedgehog extract.
3.3 Conclusions

Taken as a whole, this work shows the red fluorescence of European hedgehog quills is due to the presence of free base porphyrin(s) and details the extraction and isolation of said porphyrinoid pigment(s). Moreover, through chromatographic and spectrophotometry analysis of the pigment, we have found that it is not one of the porphyrins most commonly found as dyes in keratinaceous tissues: protoporphyrin IX, coproporphyrin III, or uroporphyrin III. Further study on this pigment is forthcoming and is currently hindered by availability of the quills; however, once procured in suitable amounts (likely 50-100 grams), this work will continue by analyzing the structure and determining a molecular formula of the compound by utilizing NMR.

3.4 Methodology and Instrumentation

3.4.1 Porphyrin Extraction

10 quills which exhibited strong red fluorescence under 365 nm black light were selected for extraction. The quills were placed in a mortar alongside crushed dry ice and the mixture was crushed with a pestle until a slightly powdery sample remained. Once all residual dry ice had sublimed, the crushed quills were transferred to a 10 mL round bottom flask to which a stir bar and 5 mL of a 10% (v/v) methanolic acid solution was then added. The solution was heated to reflux gently, and under constant stirring, for 1 h. The resulting light-brown solution was brought to a pH of 7 by careful addition of a sat. NaHCO₃ solution (~3.5 mL) and filtered. The neutralized filtrate was extracted with 10 mL/10 mL of water and ethyl acetate, and the aqueous phase was washed with an additional 5 mL of ethyl acetate. The organic phases were isolated and combined, dried over anhyd. Na₂SO₄, filtered, and evaporated to a film through rotary evaporation.
3.4.2 Chromatography of Quill Extract

HPLC chromatography of the quill extract was performed on an Agilent 1100 Series high-performance liquid chromatograph equipped with a Grace analytical normal-phase Apollo silica column (4.6 × 250 mm, 5 μm). The mobile phase employed a gradient delivery of hexanes and ethyl acetate: linear gradient of pure hexanes to 70:30 v/v hexanes:ethyl acetate over 6 min, the isocratic delivery of 70:30 v/v hexanes:ethyl acetate over 7 min, followed by linear gradient to pure ethyl acetate over 2 min, all with a flow rate of 1.5 mL/min. Samples were dissolved in ethyl acetate prior to injection. Detection wavelength set to 400 nm for porphyrin standards and 418 nm for hedgehog extract.

3.4.3 Spectrophotometric Analysis

Diffuse Reflectance Spectroscopy of the intact hedgehog and porcupine quills was performed on a Fluorolog-3 with the quills placed in a 3 mm ID cylindrical quartz tube. Absorption spectroscopy was performed on a Varian Cary Spectrophotometer with samples, dissolved in ethyl acetate, in 1 × 1 cm quartz microcuvette. FL spectroscopy was performed on a Varian Cary Fluorescence Spectrophotometer with samples in a 1 × 1 cm quartz microcuvette.
3.5 References


23. 2012, Sept. *My Hedgehog is FLUORESCENT!* Retrieved from