Synthesis and Characterization of a deuterium labeled Stercobilin: A Potential Biomarker for Autism

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Abstract:

Stercobilin is an end-stage metabolite of hemoglobin, a component of red blood cells. It has been found that there is a significantly lower concentration of stercobilin in the urine of people diagnosed with Autism Spectrum Disorders (ASD), suggesting potential utility as a biomarker.

In vitro, we have synthesized stercobilin from its precursor bilirubin through a reduction reaction proceeded by an oxidation reaction. In addition, we have isotopically labeled the stercobilin product with deuterium using this protocol.

Nuclear Magnetic Resonance (NMR) investigations show the products of the unlabeled stercobilin (Rxn 1) and the deuterated stercobilin (Rxn 2) both had a loss of signals in the 5.0-7.0 ppm range indicating proper conversion to stercobilin. Changes in the multiplicity of the sp3 region of the proton NMR suggest proper deuterium incorporation. Mass Spectrometry (MS) studies of Rxn 1 show a difference in fragmentation patterns than that of Rxn 2 proposing potential locations for deuterium incorporation. This isotopologue of stercobilin is stable (> 6 months), and further analysis permits investigation for its use as a biomarker and potential quantitative diagnostic probe for ASD.

Introduction:

Heme is a heterocylic porphyrin that is a cofactor present in red blood cells. When subject to heme oxygenase, heme's rings are cleaved with high regioselectivity, creating a linear tertrapyrrole. One of the downstream metabolic products that results from this cleavage is bilirubin (1,2). Bilirubin is a tetrapyrrole bile pigment that has been recently found to play an important role in helping to defend mammals from oxidative stress (1-4). The body eliminates bilirubin by subsequent gut flora metabolism which results in the formation of stercobilin. Stercobilin (*Scheme 1*) is a bile pigment that is found in the large intestine and is then excreted in urine and feces (4).

To date, there are no intrinsic biomarkers used to diagnose ASD. Current diagnostics rely on the observation of differences in behavior and communication of the child when comparing these traits to behaviors of other children their age (5). Professionals such as psychologists, neurologists, psychiatrists, and speech therapists work in tandem to affirm that the reason for the developmental and behavioral differences can be the result of the child having ASD (5). Children can be diagnosed as early as 18 months old, but some are not diagnosed until they reach school age (6). While these methods of diagnosis are valid, they are not quantifiable making it difficult to diagnose a child before their differences in behavior become apparent. Having a quantitative diagnostic test for autism would provide the opportunity to diagnose a child before any behavioral differences would become evident. With earlier detection, parents and healthcare professionals can be made more aware of how to best support the child with ASD as well as promoting therapy for socialization and speech sooner in the child's life which can serve as a way to provide an easier method of assimilation into society for those children (6). To date, one of the most promising biomarkers for ASD is the ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) in blood plasma; this ratio is higher in the plasma of children with ASD vs.

neurotypical children (7).

Preliminary evidence that stercobilin is depleted in the urine of children with ASD vs. neurotypical children (8) has also recently been shown in the fecal matter of a mouse model of ASD vs. littermate controls (9). This suggests a potential role for stercobilin as a diagnostic tool when analyzing the concentration of stercobilin in the urine of patients using mass spectrometry.

In order to explore the viability of stercobilin as a biomarker, it is necessary to synthesize an isotopically labeled internal standard of the molecule. Previous methods involved labeling the oxygen atoms in the carboxylic acid groups with heavy oxygen, ^{18}O, or derivatizing the carboxylic acid groups into methyl esters (10). Synthesis of a more stable isotopologue is desired and presented in this paper. Incorporation of deuterium occurred through activating the reactive carbon-carbon double bonds by the addition of deuterium atoms across the double bonds (11). The protocol used incorporates an average of deuterium atoms into stercobilin, which increases the molecule's mass by more than 12 mass units.

The method for the synthesis of stercobilin by the eduction and subsequent oxidation of bilirubin was demonstrated by the work of Kay *et. al.* (12) (*Scheme 2*). This method reduces the six C=C double bonds with six equivalents of hydrogen gas (Rxn 1) or deuterium gas (Rxn 2) producing stercobilinogen. (13,14). Stercobilinogen is then oxidized to stercobilin, resulting in a more highly conjugated system by introducing a double bond in between the two pyrrole rings.

Materials and Methods:

Bilirubin alpha and stercobilin hydrochloride were purchased from Frontier Scientific (Logan, Utah). Palladium, (10 wt % on activated carbon) was purchased through Sigma Aldrich (St. Louis, Missouri). Magnesium sulfate (anhydrous), cupric sulfate powder (anhydrous), and chloroform, (spectranalyzed with approximately 0.75% ethanol as

preservative) were purchased from Fisher Scientific. Glacial acetic acid >99.7% was also purchased from Fisher Scientific (Waltham Massachusetts). Hydrogen gas was purchased though Jackson Welding. Deuterium gas and the NMR solvent, DMSO-d6, were purchased through Cambridge Isotopes (Tewksbury, Massachusetts). Acetic acid-d_4, 99.5 atom % D was purchased from Sigma Aldrich.

Stercobilin was synthesized according to *Scheme 2* (12).

Reaction 1 (Rxn1):

Bilirubin, or 3-[2-[[3-(2-carboxyethyl)-5-[(Z)-(3-ethenyl-4-methyl-5-oxopyrrol-2-ylidene) methyl]-4-methyl-1H-pyrrol-2-yl] methyl]-5-[(Z)-(4-ethenyl-3-methyl-5-oxopyrrol-2-ylidene) methyl]-4-methyl-1H-pyrrol-3-yl] propionic acid (0.200g), glacial acetic acid (25 mL), and palladium on carbon (0.200g) were hydrogenated for 1.5 hours at 65.0˚C and at 20.5 psi of hydrogen gas in the dark. The hydrogenation vessel was filled and evacuated three times prior to the final fill of hydrogen gas. The hydrogenation product was filtered via vacuum filtration and combined with 4.0 mL $CuSO_4$(aq) solution (from a stock solution consisting of 1.25 g $CuSO_4$(s) in 25 mL of deionized water) and 5.0 mL water to then be aerated for 1.5 hours at room temperature in the dark. Aeration product was extracted using three 15.0 mL washes of deionized water and three 20.0 mL extractions using chloroform. Product was then filtered with $MgSO_4$(s), and the liquid product was brought to dryness using a rotovap system. The product was collected for analysis.

Reaction 2 (Rxn 2.):

Synthesis of deuterium labeled stercobilin was identical to the procedure outlined in Rxn 1 save for the substitution of deuterium gas and deuterated glacial acetic acid (CD_3COOD).

Mass Spectrometry Analysis:

A 12 Tesla Bruker Daltonics (Billerica, MA) SolariX electrospray ionization Fourier transform ion cyclotron resonance (ESI-FT-ICR) mass spectrometer was utilized to analyze both reaction products, ~1 mg dissolved in a 20/80 mixture of water/acetonitrile. The Rxn. 2 product was subjected to 24 h of back-exchange in H_2O before mass spectrometry analysis to minimize any potential deuteration of the carboxylic acid groups. Full ESI mass spectra were collected in the positive (ESI +4 kV) and negative (ESI -4.5 kV) ion modes at a flow rate of 120 μL/h. Nitrogen drying gas (4.2 L/min, 180 °C) was used to help desolvate droplets generated by ESI. For high resolution, 2 MWord data sets were acquired. Further tandem mass spectrometry (MS/MS) experiments were conducted using collision induced dissociation (CID) at 20 eV using argon collision gas in the positive ion mode. All data analysis was performed using Bruker Data Analysis 4.0 software to extract exact *m/z* and absolute peak intensities.

NMR Analysis:

A Varian 400MR 400MHz NMR spectrometer was utilized to analyze both reaction products. The chemical shifts for ^{1}H NMR that follow are in parts per million downfield from TMS (ppm δ) and were analyzed in a solution of dimethyl sulfoxide-d6 (Cambridge Isotope, 99.5% purity using 0.75 mL per sample). A pulse angle of 90° was used, 512 transients were collected, and a relaxation delay (d1) of 5 s was utilized to maximize signal.

Results and Discussion:

The first reduction step incorporates six equivalents of H_2 (Rxn 1) or D_2 (Rxn 2) into the two end pyrroles, and the subsequent oxidation step incorporates a double bond between the two center pyrroles. This oxidation step introduces methine in the center of the stercobilin molecule which is not present in bilirubin. Bilirubin is a highly conjugated tetrapyrrole that has six double bonds susceptible to reduction, with three residing on each of the two outermost pyrroles (15) (*Scheme 2*). The reduction of bilirubin followed by the oxidation results in the formation of the compound stercobilin which differs in structure as compared to bilirubin by a total of five degrees of unsaturation (16). NMR and MS/MS were employed to analyze, compare, and contrast the products of Rxn 1 and Rxn 2. These two techniques were used in concert to first verify synthesis of stercobilin in Rxn 1, and second to specifically confirm the deuterium incorporation in Rxn 2. The results of these reactions were verified using the NMR and MS/MS of standard stercobilin. Spectra of the standard are included in the *Supplemental Material Section*.

Mass Spectrometry Analysis:

Stercobilin ($C_{33}H_{46}N_4O_6$) has an exact atomic mass of 594.3417 amu for its all-^{12}C isotope. Analysis of the Reaction 1 product has a parent peak at 595.3477 *m/z* (*Figure 1*, top), corresponding to the $(M+H)^+$ of the all-^{12}C peak with -2.2 ppm mass error. The peak at 593.3330 *m/z* is due to mesobilirubinogen (-0.7 ppm error); because of the high mass accuracy of FT-ICR, it is possible to distinguish the peak due to the $^{13}C_2$ isotope of mesobilirubinogen from the monoisotopic peak of stercobilin since it is 0.010 amu lower in mass. Negative ion mode analysis produced a peak at 593.3355 *m/z*, corresponding to the $(M-H)^-$ of the all-^{12}C peak with 1.8 ppm mass error (not shown).

Reaction 2 that had undergone 24 h H_2O back-exchange had a broader distribution of abundance because of the different amounts of deuterium that were incorporated into the structure. Under positive ion ESI conditions shown in the bottom of *Figure 1*, the distribution centered about the most intense peak at 609.4379 *m/z*. By taking the exact mass of each peak in *Figure 1* for both the deuterated and not deuterated forms and dividing by the exact mass difference between deuterium and ^1H, an average of 13.4 hydrogen-deuterium exchanges (HDX) have occurred; this is just slightly lower than the 13.8 HDX in the absence of any H_2O back-exchange. Even after 6 months of storage, HDX only decreased to 12.9, showing high stability of deuterium incorporation. This implies approximately 13 HDX. It should be pointed out, however, that a mixture of exchanged sites ranging from 6 to 18 is produced, although HDX of 13 is the most common. This analysis was repeated in the negative ion mode, where the reaction 2 product produced a distribution centered about the most intense peak at 607.4232 m/z, leading to HDX = 13.1. Clearly, a modest degree of deuteration has occurred in the labelling procedure beyond the expected sites. The distribution proves that this synthesis is an effective method for creating an isotopologue of stercobilin; the average mass is significantly higher than that of pure stercobilin and its HDX level is stable for months, so the isotopologue can be utilized as an internal standard.

To determine if Rxn 1 had indeed produced stercobilin, tandem mass spectrometry using collision induced dissociation (CID) was performed. The CID mass spectra of stercobilin have been previously reported by Quinn *et. al.* (17). For Rxn 1, the parent ion at ca. 595.35 m/z was isolated and dissociated in argon (20 eV); the resultant product ion mass spectrum is shown in *Figure 2 (top)*. Two characteristic fragment ions of stercobilin are detected. First, the fragment at 470.263 *m/z*, accounts for what is remaining after cleavage of one of the terminal pyrroles (remaining is the tripyrrole) (17). The fragment observed at 345.1799 *m/z* indicates that the two center pyrroles remained intact after both of the outer

pyrroles were cleaved off of the molecule (dipyrrole) (17). This strongly supports the conclusion that stercobilin is indeed the product of Rxn 1. As stated above, the study of the stercobilin from Rxn 2 displayed a parent ion of 609.4379 *m/z*; this ion was isolated and subjected to CID in argon at 20 eV and the resultant product ion mass spectrum is shown in *Figure 2 (bottom)*. Fragmentation of this precursor ion from Rxn 2 generated two significant product ions that reveal important clues about deuterium incorporation within stercobilin. The fragment centered at 479.319 *m/z* is 9.056 *m/z* higher than that for Rxn 1 (unlabeled stercobilin). This fragment contains three connected pyrroles (loss of one terminal pyrrole) and averages between 8 and 9 HDX (*Figure 2*). The product ion centered at 348.199 *m/z*, representing the two innermost pyrroles, shows an average HDX between 3 and 4. From this, it can be deduced that, on average, there are 4-5 deuterium atoms in the two terminal pyrroles, while on average only 2-3 reside in the central two pyrrole groups.

Nuclear Magnetic Resonance Analysis:

^{1}H NMR analysis indicated that stercobilin was produced from bilirubin by observing the disappearance of the signals due to the vinyl protons in the 5.0-7.0 ppm δ range in all of the spectra of Rxn 1 and Rxn 2 (*Figure 3*).

Direct comparison of the ^{1}H NMR spectra of the products of Rxn 1 and Rxn 2 indicate that the alkene functional groups seen in the starting bilirubin have been reduced, most clearly seen in the area of 5-7 ppm δ. This is the typical area to detect vinyl protons in ^{1}H NMR spectra, but as can be seen in the ^{1}H NMR spectrum of stercobilin, the original peaks are no longer present in this chemical shift range.

In order to further confirm that deuterium was incorporated into the isotopologue of stercobilin, a deuterium NMR spectrum was acquired. As can be seen in *Figure 5*, the deuterium signals have chemical shifts that correlate to the expected locations (see *Figure 4*) in stercobilin's proton NMR spectrum as previously assigned. (15, 16)

Conclusion:

Stercobilin is a metabolite produced by the breakdown of heme, and it is naturally excreted from the body by way of urine and feces. It is proposed that people with ASD have a lower concentration of stercobilin in their urine compared to people not on the spectrum implicating it as a potential biomarker for the disorder. To further examine stercobilin's potential role as a biomarker, a stable internal standard was synthesized in native and deuterated forms. These two standards will allow for direct quantitative analysis of the stercobilin content in patient's urine by mass spectrometry.

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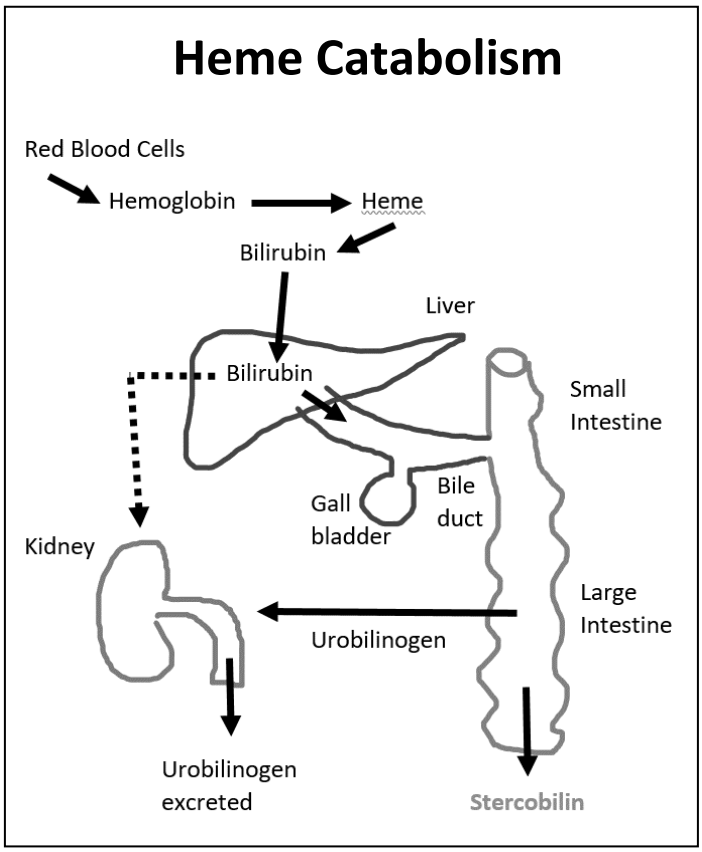
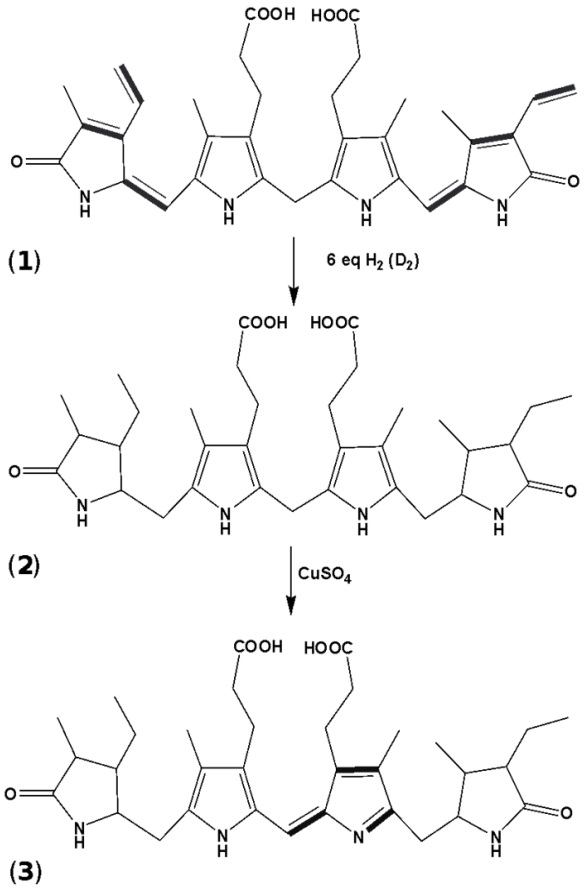


Scheme 1. Mode of conversion of bilirubin to stercobilin in the body. The breakdown of heme to bilirubin occurs in the liver to be transported to the small intestine for conversion to stercobilin. Stercobilin is then released in the urine.



Scheme 2. The method for converting bilirubin into stercobilin in the lab. Bilirubin is converted to stercobilinogen by reducing the double bonds. Stercobilinogen is converted into stercobilin by oxidizing one of the inner pyrroles.

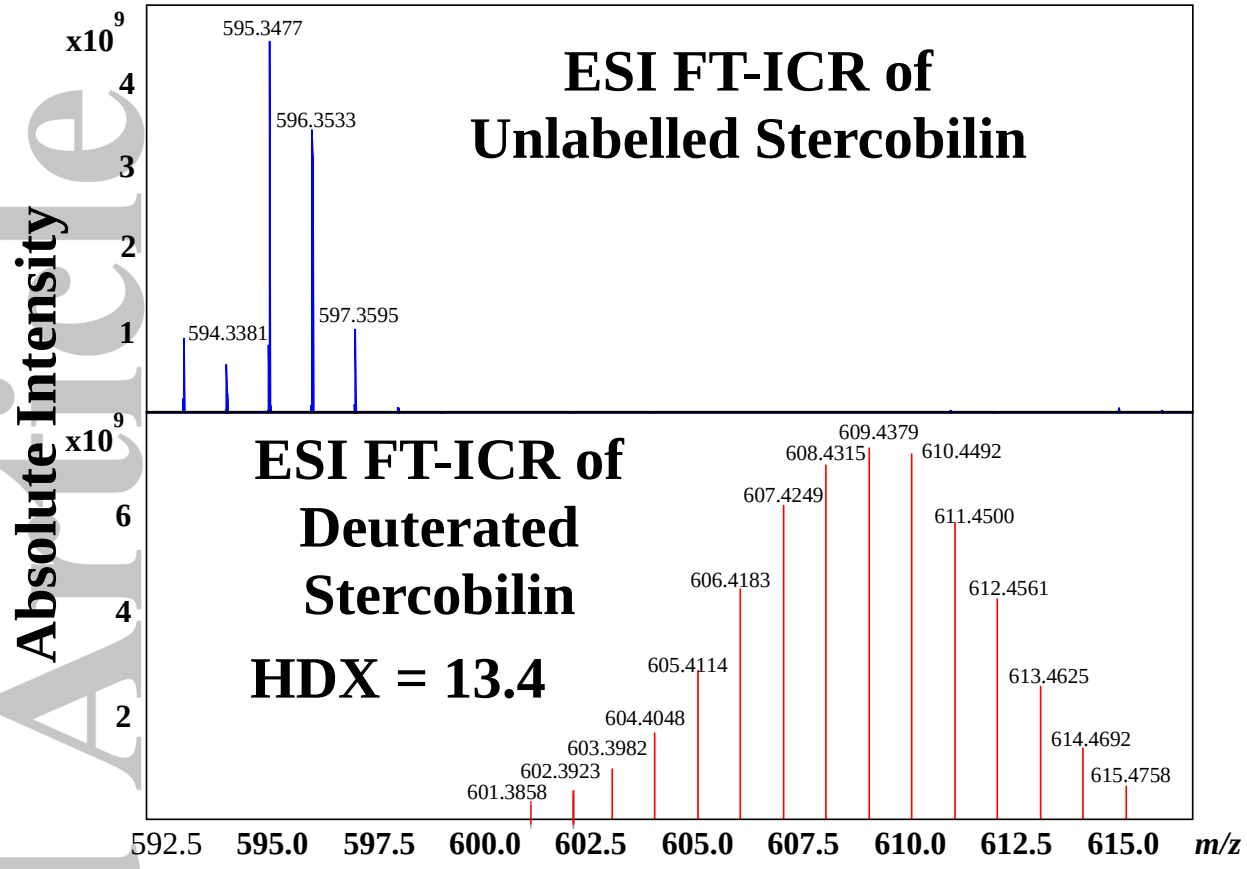


Figure 1. Comparison of the ESI mass spectra of the products from Rxn 1 (top) and Rxn 2 (bottom) to compare the addition of deuterium atoms.

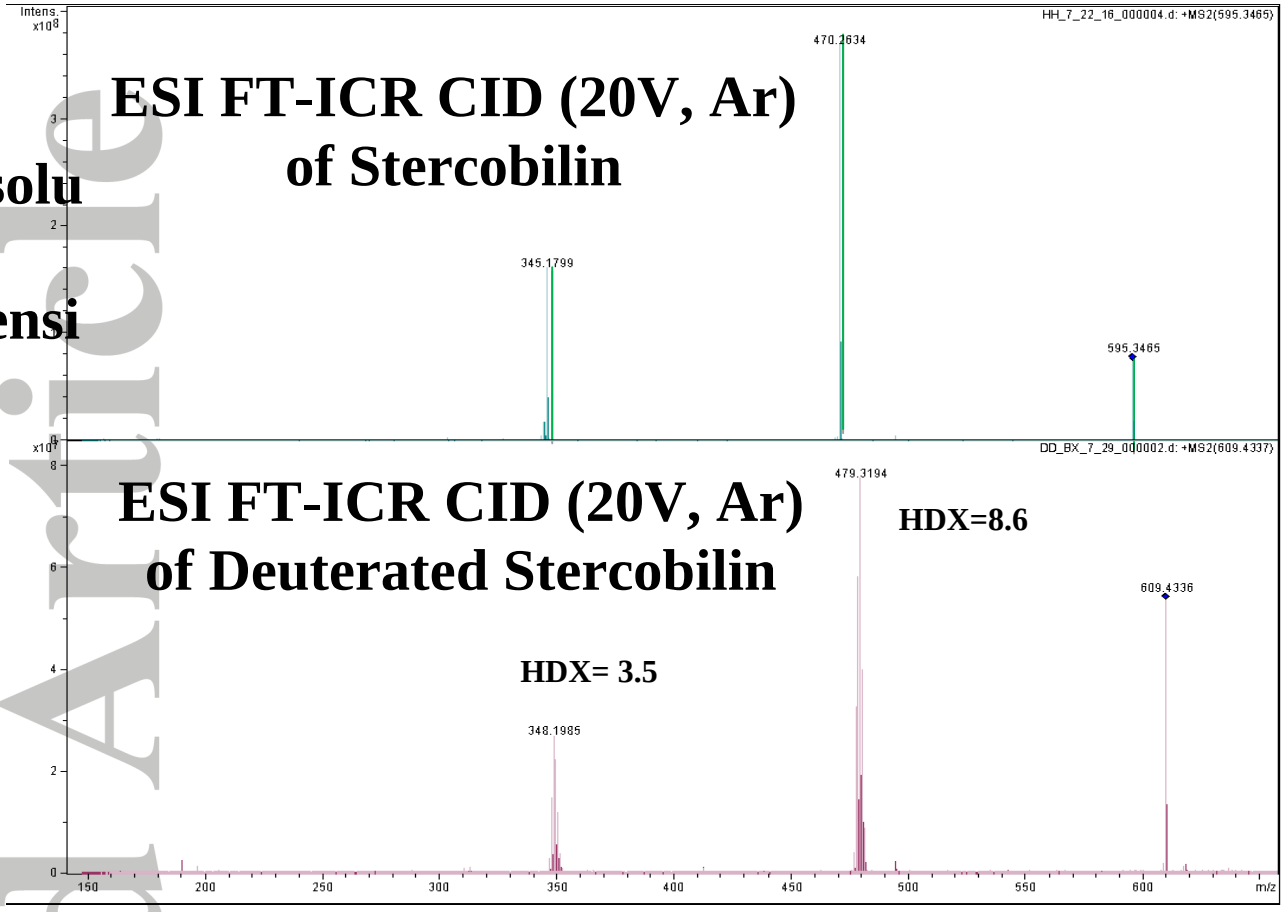


Figure 2. ESI mass spectra fragmentation of stercobilin from Rxn 1 (top) and Rxn 2 (bottom). The HDX numbers specify the average number of deuterium atoms added to each fragment on the deuterated stercobilin molecule.

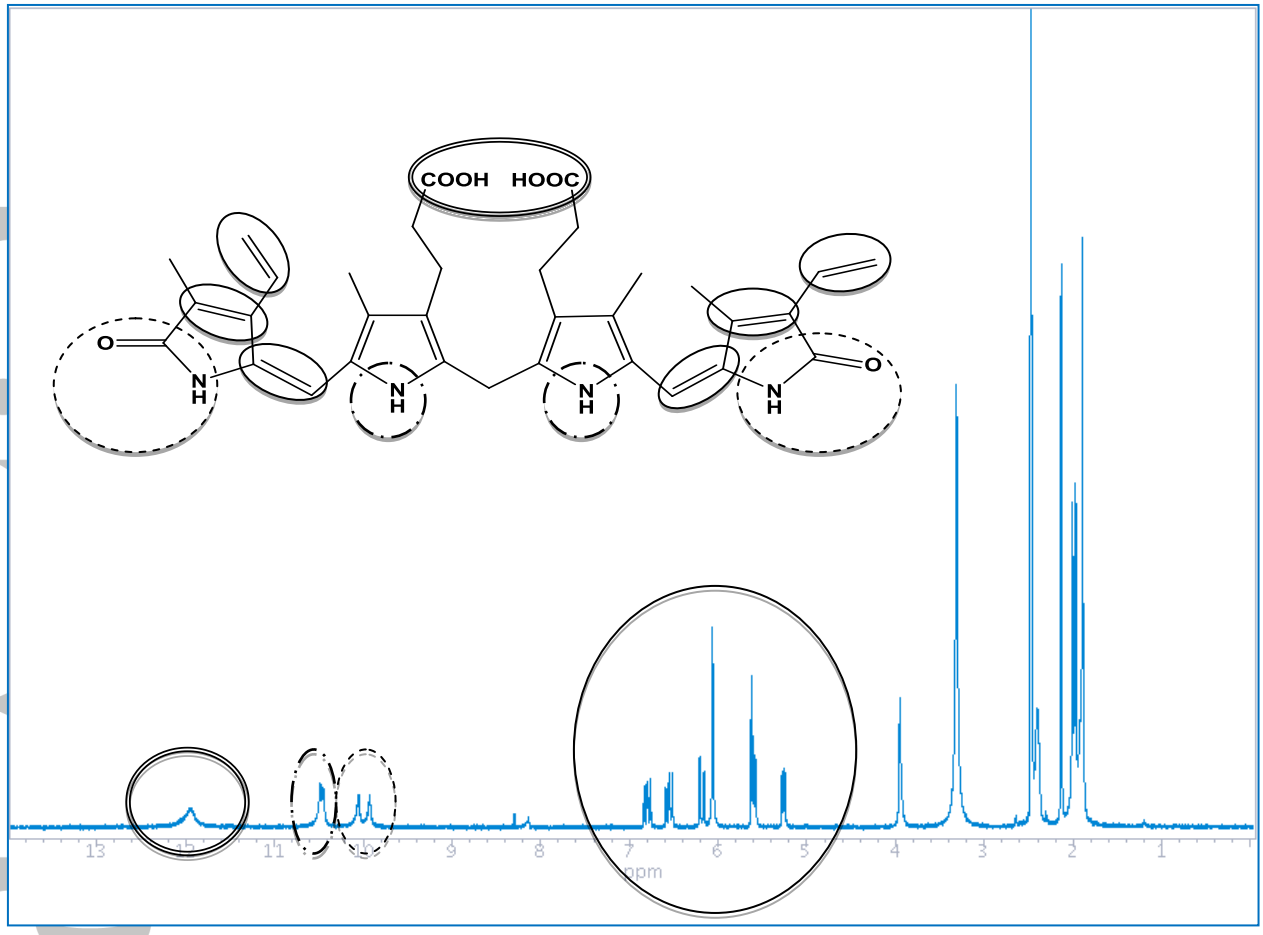


Figure 3a. ^{1}H NMR spectrum of bilirubin. The area circled in blue represents the carboxylic acids. The area in green represents the amines, and the area in red represents the amides in their respective region on the ^{1}H NMR spectra. The area circled in black represents the vinyl protons present only in bilirubin, which are not observed on the stercobilin ^{1}H NMR spectrum.

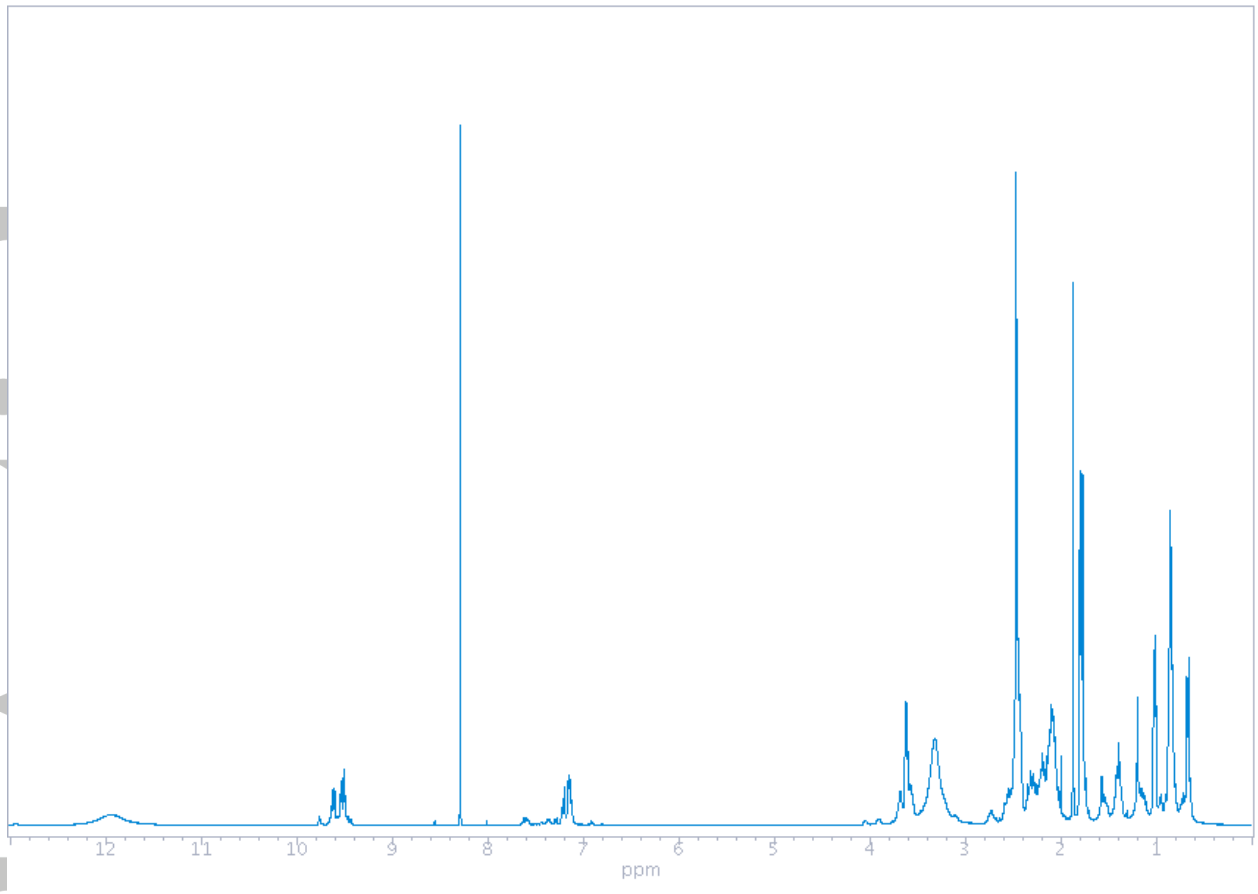


Figure 3b. ^{1}H NMR spectrum of Rxn 1. The absence of peaks in the 5.0-7.0 ppm δ range indicates the double bonds of bilirubin have been reduced. The change in the signals from 0.0-4.0 ppm δ are due to the additional saturated groups after reduction of the carbon-carbon double bonds.

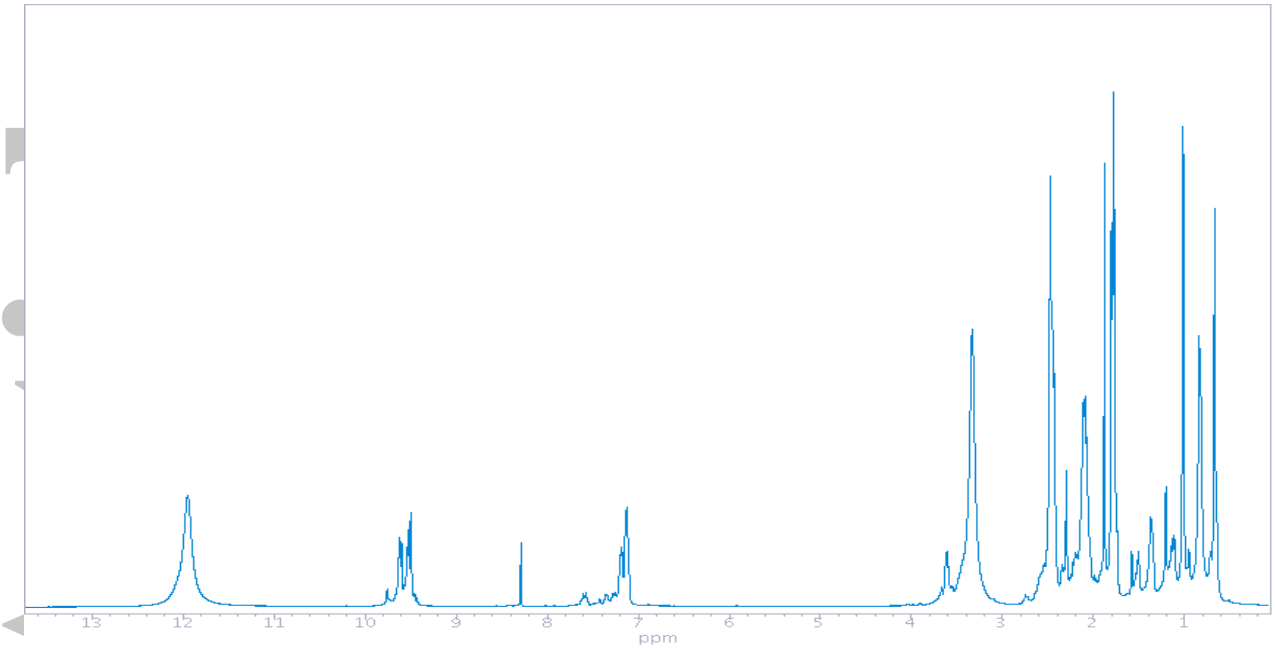


Figure 3c. ^{1}H NMR spectrum of Rxn 2. The absence of peaks in the 5.0-7.0 ppm δ range indicates the double bonds of bilirubin have been reduced. The change in the signals from 0.0-4.0 ppm δ compared to Figure 3b are due to the incorporation of deuterium into the molecule, causing some signals change their multiplicity.

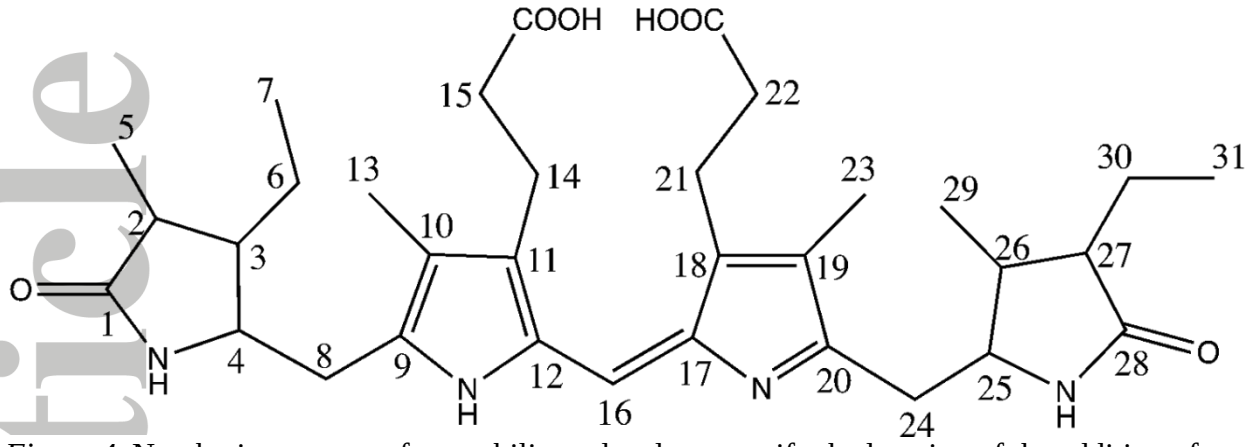


Figure 4. Numbering system of stercobilin molecule to specify the location of the addition of hydrogen or deuterium atoms.

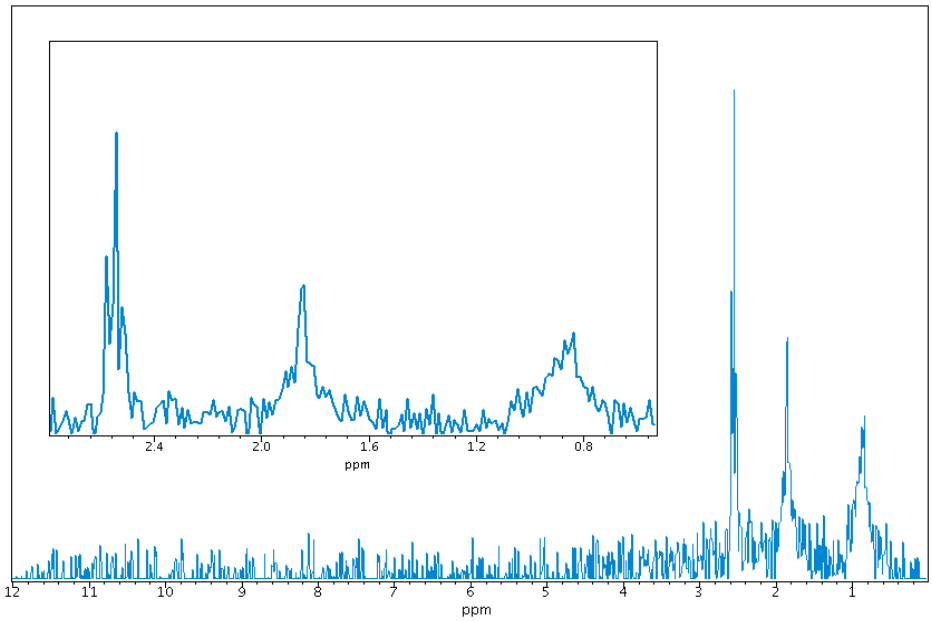


Figure 5. ^{2}H NMR of the isotopologue of stercobilin produced in Rxn 2. The inset enlarges the upfield region of the spectrum, where one would expect to find the deuterium signals on saturated carbons C_2 and C_3, C_6 and C_7, C_4 and C_8, C_{24} and C_{25}, C_{26} and C_{27}, and finally, C_{30} and C_{31}.