Review

Heme-Derived Bilins

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Abstract: Living things on Earth depend on heme – the iron-cyclic tetrapyrrole complex that harnesses iron's oxidizing powers. Heme is toxic, but Nature has evolved ways to control it. One way is breaking it with heme oxygenase which lowers its levels and begins the formation of linear tetrapyrroles called bilins. Bilins occur in many variations, often colorful, sometimes in abundance, and in animals, plants and microbes. Contrary to early notions, bilins are not only waste products of heme degradation. They are increasingly appreciated for their diverse roles such as sensing and gathering light, regulating growth and aging, responding to inflammatory conditions, and influencing behavior. The diverse functions of bilins are exploited with discoveries and uses of bioactive bilins for salutary benefits in medicine and agriculture. Opportunities for finding new bioactive bilins and applications will grow as knowledge of bilin biology and capabilities for producing bilins continue to expand.

Keywords: antioxidants · bilins · biliverdin · heme · mesobiliverdin

1. Introduction. The Heme Enigma and Bilins

The Fe-cyclic tetrapyrrole complex, heme (Fe-protoporphyrin IX) (**1**, Figure 1) is ubiquitous in Nature.

It emerged early in evolution to exploit Earth's second most abundant metal, Fe.[1] Heme's functions are beneficial and critical to life. But heme is also toxic[2] and therefore an enigmatic problem for life. Many of heme's beneficial functions are conferred when bound to apoproteins,[3] and a variety of hemeprotein complexes catalyze critical physiological functions. Among them are gas exchange, storage and transport as performed by hemoglobin, leghemoglobin and myoglobin. Also, hemeproteins function in electron transport, oxidation reduction, drug metabolism and detoxification. In contrast, free heme Fe initiates Fenton chemistry to create reactive oxygen species that inflict damage to cell lipids, proteins, and nucleic acids.[2] Nature has evolved ways to deal with heme toxicity. Mammalian animals degrade free heme and excrete a large portion of the catabolic metabolites to lower its concentration. Other animals, plants, and microbes utilize heme for growth and survival or carefully regulate the levels of biosynthetic heme to below toxic levels. In all cases, linear t etrapyrroles called "bilins" (Figure 1) that are end products of heme catabolism are generated. The bilins are typically pigmented, lack toxicity, and often accumulate in abundance.

The chemistry and biology of bilins have long been popular research subjects. In addition to being heme catabolic waste products in mammals, well-known roles include photomorphogenesis, energy harvesting and transfer and effects on animal behavior. More recently, it has become evident that certain bilins can be bioactive with beneficial physiological effects. Future discoveries of the bioactivities of bilins are anticipated. This mini-review attempts to briefly introduce the biological and chemical scope of heme-derived bilins and then focus on currently known bioactive bilins and future directions for bilin discovery and applications as useful natural products.

2. Heme Oxygenase and Pathways toward Bilins

All known heme degradation systems begin with cleavage of one of four heme methene bridges by heme oxygenase (EC 1.14.99.3) (Figure 2). The enzymatic products after three successive oxygenation steps are linear tetrapyrroles (biliverdins), CO and Fe. Biliverdin isomers (**2-5**, Figure 1) are produced depending on whether the α, β, γ or δ methene bridge is cleaved by heme oxygenase (Figure 2).[4] The most common variation is cleavage at the α position by canonical heme oxygenase-1 (HO-1) which occurs in animals, higher plants, algae, fungi and bacteria to give the bilin, biliverdin (**2**, Figure 1).

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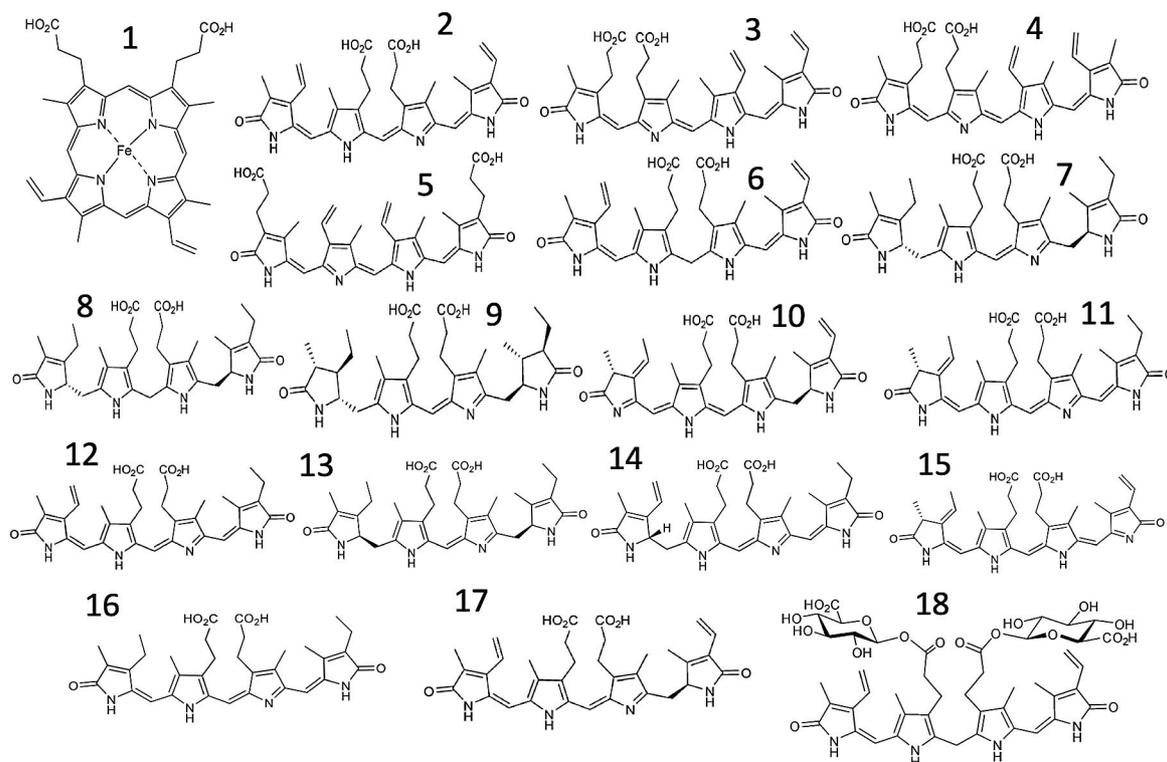Department of Biology, Utah State University, Logan, Utah 84322-5305 U.S.A

Figure 1. Structures of heme and heme-derived bilins discussed. **1**, heme (Fe protoporphyrin IX); **2**, biliverdin (biliverdin IXα); **3**, biliverdin IXβ; **4**, biliverdin IXδ; **5**, biliverdin IXγ; **6**, bilirubin (bilirubin IXα); **7**, urobilin (urobilin IXα); **8**, urobilinogen (urobilinogen IXα); **9**, stercobilin (stercobilin IXα); **10**, phycoerythrobilin; **11**, phycocyanobilin; **12**, $18^1,18^2$-dihydro-biliverdin IX; **13** phycouribilin; **14**, phycoviolobilin; **15**, phytochromobilin; **16**, mesobiliverdin (mesobiliverdin IXα); **17**,15,16-dihydro-biliverdin IXα; **18**, bilirubin diglucuronide.

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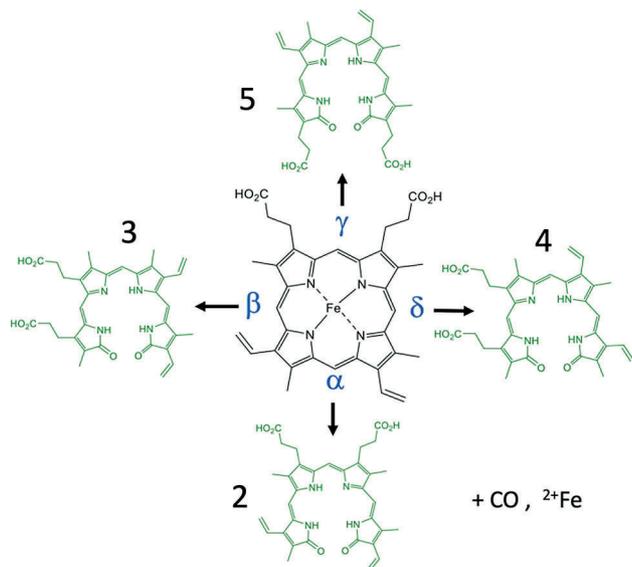
Figure 2. Heme and biliverdin isomer products after methene bridge cleavage by HO-1 at the α, β, γ and δ positions. The bold numerals refer to the numbered bilins in Figure 1.

2.1 Mammalian Animal Bilins

In animals, biliverdin comprises > 90 % of the bilins generated by HO-1 and HO-2. In red-blooded animals, biliverdin is rapidly reduced via biliverdin reductase A (BVRA) (EC 1.3.1.24) in the liver to bilirubin (**6**) which may be toxic when accumulated (although this notion is disputed.[4b] Liver bilirubin is glycosylated with glucuronic acid by uridine 5′-diphosphate glucuronosyl transferase (UGT) (E.C. 2.4.1.17) to make a more hydrophilic conjugated form, bilirubin diglucuronide (**18**, Figure 1) for easier excretion in bile and urine (Figure 3). The production and excretion of conjugated bilirubin is important for animals with oxygen-carrying circulatory systems containing hemoglobin-rich erythrocytes that release heme upon erythrocyte scenescence and resulting in hemoglobin degradation at relatively high rates. Heme catabolism to conjugated bilirubin reduces levels of free heme and unconjugated bilirubin. Before entry into bile and urine, a portion of the conjugated bilirubin is directed to the intestine where it is deglycosylated and transformed largely by gut microbes into additional linear tetrapyrroles (Figure 3) - mainly urobilin, urobilinogen, and stercobilin (**7**, **8**, and **9**, Figure 1, respectively), and minor derivatives such as mesobiliverdin (**16**, alternate name glaucobilin). Animal HO-1 levels are regulated and induced with oxidative stress. The second animal heme oxygenase, HO-2, is constitutively expressed and abundant in testis, endothelial cells, and brain.[5] In contrast to mammalian adults, bilirubin IXβ, γ and δ isomers produced in the fetus by BVRB from corresponding biliverdin isomers[6] tend to accumulate in the fetus since they do not undergo glycosylation. Also, being more hydrophilic than bilirubin IXα, the BVRB-generated isomers are less

capable of crossing the placental barrier contributing further to bilirubin accumulation (hyperbilirubinimea or jaundice) in the fetus and neonates.[4b,7] Such accumulation is speculated to have a protective role against streptococcal sepsis due to the antibacterial action of the bilirubins.[8] Interestingly, BVRB is also expressed in hepatocellular carcinomas and prostate cancer cells.[9]

2.2 Non-Mammalian Animal Bilins

Many non-mammalian animals accumulate heme-derived bilins that impart color, and a large fraction are biliverdins, mostly the biliverdin IXα form.[10] The bluish or greenish colors of avian and dinosaur egg shells,[10–11] fish,[12] and lizards,[13] and frogs[14] are attributed to biliverdin. Other biliverdin isomers and bilins occur in insects,[15] helminths,[16] and marine snails.[17] In response to threats, bilins described as biliverdins rapidly accumulate in circulating blood of skinks, veiled chameleons[18] and certain frogs.[14] The reasons for rapid bilin appearance in these small animals are debated[11] and range widely from a defense mechanism against predator attack to innate immunity.[13,19] Despite the ability of many non-mammalian animals to accumulate biliverdin, BVRA still occurs as evidence by the occurrence of bilirubin in these animals.[20]

2.3 Plant, Cyanobacterial, and Algal Bilins

In the photosynthetic organisms, plants, cyanobacteria, and algae, HO-generated biliverdin is biosynthetically converted via biliverdin reductases to pigmented bilins that sense and transmit light energy.[21] In the model plant *Arabidopsis thaliana*, 4 HO's are expressed with the majority of HO transcripts from HO-1.[22] In cyanobacteria, red algae, and glaucophytes, the phycobilins phycoerythrobilin and phycocyanobilin (**11** and **12**, Figure 1, respectively), and in some cases phycourobilin (**13**, Figure 1) and phycoviolobilin (**14**, Figure 1), are synthesized, covalently bound to apoproteins, and large bilin-protein complexes, called phycobilisomes, are assembled to harvest light energy for photosynthetic metabolism. In cyanobacteria, separate biosynthetic pathways lead to the phycobilins and phytochromobilin (**15**, Figure 1). The enzymes PebA and PebB catalyze consecutive reduction steps of substrate biliverdin to phycoerythrobilin via15,16- dihydrobiliverdin (**17**, Figure 1), PcyA-catalyzed reduction to phycocyanobilin via mesobiliverdin, and phytochromobilin synthase reduction to phytochromobilin (Figure 4).

Brown algae do not have phycobilisomes, and instead use fucoxanthin-chlorophyll a/c-protein complexes for light harvesting. Distinct from light-harvesting function, plants and cyanobacteria possess bilins that are covalently bound to phytochrome and cyanobacteriophytochrome, respectively, to assume signaling, regulatory, and oxidative detoxification roles.[23] Phytochrome with phytochromobilin (**12**, Figure 1)
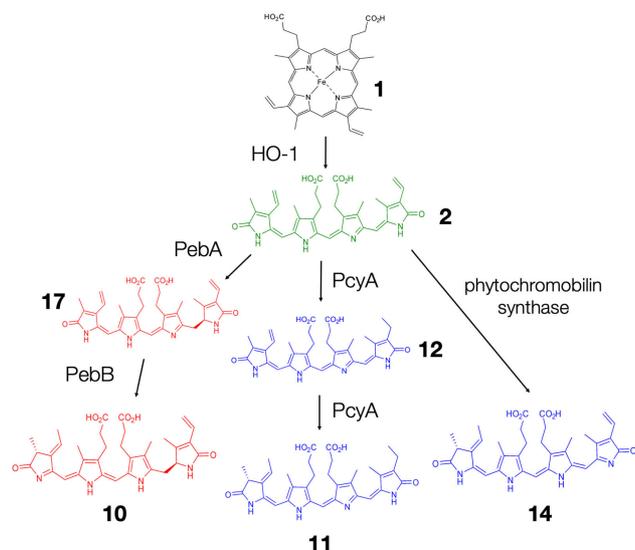
Figure 3. The heme catabolism pathway in red blooded mammals. Bold numbers refer to the numbered bilins listed in Figure 1. Open arrows indicate bilin excretion from an organ (e.g. intestine and kidney). Dashed arrows indicate major (long dashes) and minor (short dashes) transport rates of bilins to and from bile of the gall bladder. Abbreviations are: HO-1, heme oxygenase-1; BVRA, biliverdin reductase A; UGT, uridine 5′-diphosphate glucuronosyl transferase.

and cyanobacteriophytochrome with biliverdin, phycocyanobilin or phycoviolobilin (**14**, Figure) are bilin -protein complexes that initiate and regulate red/far-red light photochromic responses related to growth, development, and circadian clocks.[23–24] Light-dark cycles, red/blue-light sensing, photomorphogenesis, pigment synthesis and a myriad of other processes are controlled at the transcriptional levels by phytochrome and cyanobacteriophytochrome signaling. The green alga *Chlamydomona reinhardtii* has biosynthetic pathways leading to biliverdin and phycocyanobilin but does not express phytochrome nor phycobilisome protein homologs.[25] Interestingly, these *C. reinhardtii* bilins were shown to possess global gene regulatory roles for chlorophyll synthesis and oxidative stress responses as well as unique roles in non-phytochrome light-dependent greening and phototrophic growth.[26] The orange pigmentation of the flower seed arils of

Strelitzia reginae (bird of paradise) is due to bilirubin, but interestingly, the highly visible orange coloration of the 3 upright sepals of this flower is not due to bilirubin though present in low concentrations.[27] This is the only known case of bilirubin in a plant flower.

2.4 Non-Photosynthetic Bacterial and Fungal Bilins

Non-photosynthetic bacteria and fungi have evolved efficient Fe uptake and exclusion mechanisms and highly regulated heme and hemeprotein biosynthetic pathways that guard against the enigmatic problem of heme and Fe accumulation.[1b] They nevertheless express HOs, and in the absence of a need for harvesting light for photosynthesis, the generation of bilins via HO contributes to bacteriophytochrome produc-

Figure 4. Bilin biosynthesis from heme in plants, algae, and cyanobacteria. Bold numerals refer to the numbered bilins in Figure 1.

tion. Generally, canonical HO's provide the biliverdin IXα isomer as chromophore for bacteriophytochromes. An exception may be *Pseudomonas aeruginosa* that when iron-starved expresses a divergent non-canonical heme oxygenase, PigA, that produces biliverdin isomers β and δ[28] (Figure 2). A variant bacteriophytochrome with the biliverdin IXδ isomer was detected but in minor amounts compared to the variant with biliverdin IXα.[28b] Fungal bacteriophytochromes appear to specifically use biliverdin IXα.[29] While the plant phytochrome and cyanobacteriochrome chromophores, phytochromobilin and phycocyanobilin, respectively, bind to a conserved cysteine within the apoprotein GAF domain, the bacterial and fungal biliverdins of the bacteriophytochromes bind a cysteine of the loop region preceding the PAS domain.[30]

3. Bioactive Heme-Derived Bilins Relevant to Medicine and Agriculture

A large body of literature suggests that modulation of the enzymatic producer of bilins, HO, has therapeutic anti-inflammatory, anti-oxidant, and cytoprotective outcomes.[31] However, the mechanisms of HO modulation and HO-modulated therapeutic outcomes are complex and are being intensively explored.[32] Only two human HO-1 deficiency cases with disease manifestations are documented.[33] Alternative approaches that employ the catalytic products of HO, i.e. bilins and CO either alone or in combination have been proposed,[31b] and they have shown promise for a number of therapeutic and agricultural purposes. To date, relatively few of the many heme-derived bilins described above in section 2. are known to possess bioactivities relevant to medical

therapeutics or agricultural applications. Among the most prominent of these are reviewed below.

3.1 Biliverdin

There are many published reports and review articles on the therapeutic bioactivities of biliverdin (for reviews see.[31b,34] They include reports of salutary effects of biliverdin administration for organ (heart, kidney, liver, intestines, and lungs) and tissue allograft transplantation procedures, ischemic reperfusion injury, wound-healing, endotoxic shock, viral infections, Alzeihmer's disease, diabetes, and intestinal bowel diseases. At least three capabilities (either singly or in combination) of biliverdin account for these salutary effects: 1) activation of BVRA cell signaling, 2) anti-oxidation of reactive oxygen and nitrogen species, and 3) conversion to bilirubin by action of BVRA. BVRA is a highly multifunctional and cell regulatory molecule.[34a,b] Its enzymatic reduction of biliverdin into bilirubin is coupled to BVRA activation and initiation of signaling pathways leading to cytoprotective and anti-inflammatory responses and modulation of cell growth, tumorigenesis, and apoptosis.[35] Both biliverdin and bilirubin are strong anti-oxidants[36] and the former is a more effective scavenger of peroxyl radicals than the latter.[37] When combined, both bilins provide broad capabilities for scavenging reactive oxygen and nitrogen species in both hydrophilic and hydrophobic environments. The effects of biliverdin administration against intestinal bowel diseases are particularly well studied. Reported are suppression of colitis,[38] gastroenteritis,[39] sepsis-induced intestinal inflammation and dysmotility,[40] and intestinal ischemic/reperfusion injury.[41]

With plants, exogenous addition of biliverdin stimulates root growth of rice[42] and protects soybean nodules from cadmium-induced stress.[43] In both cases, biliverdin induces expression of HO-1 at the transcriptional level.

3.2 Bilirubin

Many textbooks portray bilirubin as a waste product that results from the degradative turnover of erythrocytes, hemoglobin and heme with no mention of physiological benefit. Outside of textbooks, however, there is ample and growing recognition of bilirubin's numerous beneficial functions and bioactivities (for reviews see.[34a,44] In experimental animal models, administration of bilirubin confers protection against cardiac ischemic/reperfusion injury, neointimal hyperplasia vascular injury, smooth muscle proliferation, acute lung injury, pulmonary fibrosis, renal injury, diabetic nephropathy, and graft rejection in organ and tissue transplantation. Some of these protective effects mirror those of biliverdin's due to the BVRA substrate -product relationship between these two bilins. High levels of bilirubin in human serum are correlated with lower risks of cardiovascular disease, obesity, cancers, diabetes, and lung disease.[44b] Many of the bilirubin pharma-

cologic and serum level-disease correlative effects are directly or indirectly due its antioxidant scavenging capabilities against reactive oxygen and nitrogen species. Also, bilirubin binds to the peroxisome proliferator-activated receptor-α (PPARα) which promotes the uptake, utilization, and catabolism of fatty acids to suppress hyperlipidemia and fatty liver disease. The PPARα mediated effects account for at least partly the anti-obesity and anti-diabetic effects of bilirubin.[44a] Bilirubin is also anti-bacterial, and this property was recently shown to provide natural protection against prenatal sepsis.[8]

For plants, Lv et al.[45] reported intriguing bilirubin effects on boron-stressed *Arabidopsis thaliana.* Boron is a critical plant nutrient but toxic at high levels in the soil. Bilirubin and CO partially rescued boron toxicity-induced inhibition of primary root growth and promoted tolerance to boron by transcriptional activation of boron efflux transporter *BOR4* in turn induced by HY1 – the HO-1 homolog in *A. thaliana.* These finding raise possibilities that HO-1 and bilirubin (and perhaps other bilins) are molecular links in regulating plant abiotic stress responses.

3.3 Gut Bilins – Urobilin, Urobilinogen and Stercobilin

Along with protoporphyrin, urobilin and stercobilin caused significant comet formation in intestinal cancer and hepatocarcinoma cell lines, Caco-2 and HepG2, respectively, implicating their roles in DNA-damage and apoptosis in cancer cells of the digestive system.[36a] Also, in the presence of S9 rat liver extracts, urobilin and stercobilin displayed anti-oxidant activities that were higher than or matched those of bilverdin and bilirubin.[46] These activities have significant implications for the role of these bilins in gut health and disease. The antioxidative capabilities of urobilinogen are also known. Its oxygen radical scavenging activity is higher than those of α-tocopherol, bilirubin and α-carotene, and it suppresses formation of hydroperoxides of linoleic acid.[47]

3.4 Phycocyanobilin

Phycocyanobilin and phycocyanin extracted from the cyano-bacterium, *Arthrospira (Spirulina) platensis*, are reported to suppress diabetic nephropathy.[48] Phycocyanobilin protected against oxidative stress and renal dysfunction in inbred *db/db* mice, a rodent model for Type 2 diabetes. In this study, antioxidant effects were also observed with oral administration of phycocyanobilin, phycocyanin, bilirubin or biliverdin, and phycocyanobilin inhibited NADPH dependent superoxide production in cultured renal mesangial cells.[48] In another study, *A. platensis* cell extracts and phycocyanobilin were reported to inhibit pancreatic cancer cell lines and their proliferation in xenotransplanted nude mice.[49] Phycocyanobilin decreased generation of mitochondrial ROS and glutathione redox status *in vitro* and *in vivo.* These studies reveal the therapeutic potential for using this cyanobacterial bilin

against oxidative stress induced conditions and diseases. Both studies report *in vitro* anti-oxidant activities for phycocyanobilin, but BVR-mediated cytoprotective effects are unlikely since this bilin is not a substrate for BVR.[50] Similar studies with the closely related cyanobacterial light-harvesting phycoerythrobilin are not evident.

3.5 Mesobiliverdin

Ito et al.[50] reported that mesobiliverdin and its analog biliverdin each conferred cytoprotection against pancreatic islet degradation when excised pancreata from healthy rats were infused with these bilins followed by islet recovery and allograft transplantation into recipient diabetic rats. Islets from mesobiliverdin or biliverdin-infused pancreata were more viable compared to controls, and restored insulin function in allograft recipient diabetic rats at higher rates than controls. Also, it was observed that mesobiliverdin served as a substrate for human BVRA and as well as biliverdin. However, phycocyanobilin was a poor substrate for this enzyme (Figure 5). Substrate site binding and BVRA activation are requisites for suppression of downstream inflammatory responses and anti-pro-inflammatory effects by biliverdin.[51] In addition, mesobiliverdin's anti-oxidant capacity to suppress lipid peroxidation is equivalent to that of biliverdin (Figure 6). Therefore, it may be speculated that the pancreatic islet cytoprotective effects of mesobiliverdin are attributed to a combination of inflammatory response suppresssion and anti-oxidation against free radicals. By similar reasoning, the cytoprotective effects of phycocyanobilin described above in section 3.4 may be due mainly to anti-oxidative capabilities and not by suppression of inflammatory responses. These findings suggest that mesobiliverdin has potential as a cytoprotectant similar to biliverdin with anti-inflammatory capabilities. The mesobiliverdin used in this study was obtained by isomerization of the cyanobacterial bilin phycocyanobilin which allows for its production in scalable quantities.[50]

4. Discovery Strategies for New Bioactive Bilins

Among the natural heme-derived bilins identified in animals, algae, plants, and bacteria (section 2), only a few have been examined for bioactivities of therapeutic value (section 3). Ample opportunities exist for discovering bilins and derivatives with previously unknown bioactivities. Such pursuits are attractive in light of recent and growing knowledge about bilin mechanisms of action. The few and better known bioactive heme-derived bilins display two of three molecular activities that are important for therapeutic applications: 1) scavenging of free radical reactive oxygen and nitrogen species, 2) substrate activation of BVRA, and 3) and activation of PPARα. Bilirubin is capable of 1) and 3), and biliverdin of 1) and 2). Therefore, a reasonable bilin discovery approach is to
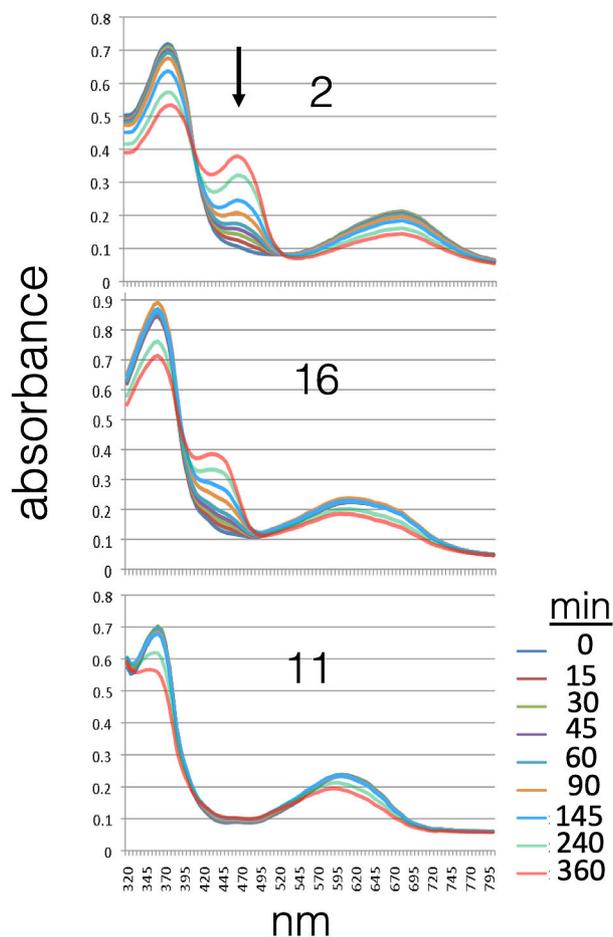
Figure 5. BVRA activities with biliverdin (**2**), mesobiliverdin (**16**), or phycocyanobilin (**11**) as substrate. Bold numbers refer to bilin structures shown in Figure 1. Absorbance spectra were determined at indicated times after addition of bilin to the enzyme reaction mixture. The arrow marks absorbance peaks (440–480 nm) indicative of BVRA enzymatically reduced products bilirubin and mesobilirubin generated from **2** and **16**, respectively. BVRA did not reduce **11** to phycocyanorubin. Data are from Ito et al (2013).[50]

Figure 6. Linoleic acid lipid peroxidation induced by 2,2'-azobis (2,4-dimethylvaleronitrile) and suppression by mesobiliverdin (**16**) and biliverdin (**2**). The methods of Stocker et al. (1990).[35] Data shown with error bars are means $+/-$ SD from three separate experiments.

screen previously untested bilins for these three activities. Bilins that possess, inhibit or activate any of these activities would be candidates for therapeutic development.

New bilin discovery efforts should also be directed toward activity screening against newly discovered roles and modulatory functions in diseases. For example, recent findings that BVRB is over-expressed in hepatocellular carcinomas and prostate cancer cells[9] suggest searches for bilins or derivatives that inhibit BVRB that could be prototypes for new anti-cancer drugs. Increasingly, recognition of roles for bilins in the progression of Alzheimer's disease are emerging.[52] Oxidative stress is charecteric for this neurologic disease, and among the most consistent responses are increases in HO-1 and BVRA. Also, a clearer picture of the intestinal bilin composition generated and modulated by microbes is coming into view[53] as knowledge of the gut microbiome continues to
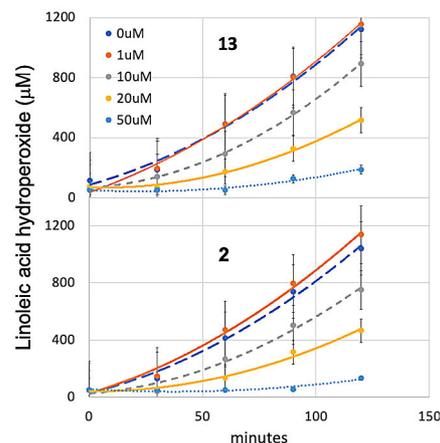
expand. The new information should offer more opportunities to discover bilins with salutary bioactivities against various inflammatory intestinal bowel diseases. Tolerances of higher plants to abiotic stresses imposed by high soil levels of boron and cadmium are improved with exposure to bilirubin[47] and biliverdin,[43] respectively. Such findings should excite plant pathologists to explore novel ways to exploit bilins to combat plant diseases and increase crop yields.

New bioactive bilin discovery efforts are hampered by the scarcity of pure material and the inability to produce it in sufficient amounts. Capabilities for high purity and quantity are necessary for research and development of bilins aimed at medical and agricultural applications, and they also provide incentives to commercially promote them. Strategies to overcome the quality/quantity barriers include semi-chemical synthetic production and exploitation of microbes for production. Mesobiliverdin represents an example of semi-synthetic product from phycocyanobilin extracted from cyanobacteria – a feedstock source which is abundantly available. Biliverdin which does not accumulate due to BVRA, is more difficult to obtain in pure and large quantities. It is now mainly produced semi-synthetically for commercial purposes by oxidation of animal bilirubin – though purity could be a problem when prepared this way.[54] Other examples of the semi-synthetic bilins (mainly bilirubin analogs) are described by Lightner.[55] For use of microbial systems, the production of biliverdin was achieved using *Candida albicans*[56] and *Escherichia coli*[57] both by heterologous expression of HO-1 genes. Total chemical synthesis approaches offer possibilities for high purity and quantity in the production of bilins. However, the requisite multistep procedures for total chemical synthesis of bilins compromise the feasibility for large scale bilin production at reasonable cost. Nevertheless, the total syntheses of bilirubin and biliverdin were achieved ~75 years ago[58] (for review and commentaries see[55]). These historical achieve-

ments admirably demonstrate that it is technically possible to produce bilins de novo in the chemistry laboratory – which is valuable to know for future research and applications of heme-derived bilins.

5. Summary and Outlook

Chemists, biologists, and medical practioners have long had interests in heme-derived bilins. Initially, these closely related compounds were viewed solely as waste products in red-blooded mammals generated to avoid the detrimental effects of heme accumulation. Later, it became evident that heme-derived bilins occur widely in nature and play a variety of biological roles. Growing interest and knowledge of mechanisms and beneficial effects have led to the realization that bilins are capable of protecting and suppressing multiple acute and chronic oxidative stress conditions in humans – although some knowledge of this already existed in Chinese societies centuries ago.[59] Recent and exciting findings include protection against abiotic stress and growth promotion in higher plants. Thus far, only a handful out of many natural product heme-derived bilins known to exist have been examined for potential medical and agricultural applications. With new strategies for bioactivity screening and scalable production of bilins, ample opportunities exist for uncovering salutory benefits of additional bilins and derivatives and for discovering new bilin bioactivity targets.

Acknowledgements

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