Bactobilin: Blue bile pigment isolated from Clostridium tetanomorphum

(3-bilatriene/urobiline-related/enzymatic formation/purification/characterization)

PHILLIP J. BRUMM*†, JOSEF FRIED*‡, AND HERBERT C. FRIEDMANN*§

Departments of *Biochemistry and †Chemistry, The University of Chicago, Chicago, Illinois 60637

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ABSTRACT A blue bile pigment, possessing four acetic and four propionic acid side chains has been isolated from extracts of the anaerobic microorganism Clostridium tetanomorphum and in smaller amounts from Propionibacterium shermanii. The compound could be prepared in larger amounts by incubation of C. tetanomorphum enzyme extracts with added 8-aminolevulinic acid. The ultraviolet-visible, infrared, and proton magnetic resonance spectra of the pigment indicate a chromophore of the biliverdin type. Field-desorption mass spectrometry of the purified methyl ester showed a strong molecular ion at m/e = 962. This corresponds to the molecular weight expected for the octamethyl ester of a bilatriene type of bile pigment structurally derived from uroporphyrin III or I. Of the five possible structures, two could be eliminated by proton magnetic resonance spectroscopy. The name bactobilin is proposed for this previously unreported bile pigment.

The open-chain tetrapyrrole compounds known as bile pigments are widely distributed. They are found free or bound to protein in mammals, birds, amphibians, reptiles, fish, molluscs, and insects and in algae and higher plants (for reviews, see refs. 1-9). As side chains or β-carbon ring substituents, all bile pigments described thus far have four methyl groups, two propionic acid groups, and two vinyl groups, one or both of which can be isomerized to ethylidene (4, 10, 11) or reduced to ethyl (6, 10, 11). These substituents are arranged in the sequence found in protoporphyrin IX; in fact, all these bile pigments are formed from protoheme (8, 12-14), whose oxidative breakdown to biliverdin is catalyzed by the enzyme heme oxygenase (15-17). Bile pigments thus far have not been detected in prokaryotes. The present paper reports the isolation and in vitro formation of a bile pigment from the bacterium Clostridium tetanomorphum. This anaerobic organism makes uroporphyrinogen, the precursor of vitamin B-12, but not heme, protoporphyrinogen, or coproporphyrinogen. The isolated bile pigment is of interest not only because of its detection in a prokaryote, but also because its β-carbon ring substituents, four acetic acid and four propionic acid groups, correspond to those of a uroporphyrin and not of protoporphyrin.

MATERIALS AND METHODS

Supplies. 8-Aminolevulinic acid was obtained from Sigma; biliverdin IXα dimethyl ester and the fully esterified methyl esters of uroporphyrin III, coproporphyrin III, protoporphyrin IX, and heptacarboxyproporphyrin I were from Forpynch Products (Logan, UT). The n-hexane used for flash chromatography was "95 + %" (Aldrich), whereas the n-hexane for thin-layer chromatography was 99 mol % pure (Fisher). Other chemicals used were reagent grade or better. Silica gel for flash chromatography and a flash chromatography column, 3.4-cm diameter, were obtained from J. T. Baker. Whatman high-performance silica thin-layer chromatography plates with a preadsorbent spotting area (type LHP-K, 10 × 20 cm) and E. Merck precoated cellulose thin-layer chromatography plates were obtained from Anspec (Ann Arbor, MI). For fluorescence detection, a lamp emitting at 365 nm was used. C. tetanomorphum cells (ATCC 15920) were grown on a medium containing yeast extract and monosodium glutamate (based on medium 163, American Type Culture Collection) as described (18) and were collected by centrifugation. Uroporphyrin and C-methylated mono-, di-, and trimethylisobacteriochlorins (Factors I, II, and III) were isolated as their octamethyl esters by standard methods (19, 20). Sirohydrochlorin (Factor II) octamethyl ester was given by A. I. Scott (College Station, TX), dimethyl- and trimethylisobacteriochlorin dilactone octamethyl esters were given by V. Koppenhagen (Braunschweig-Stockheim, Federal Republic of Germany).

Enzyme Preparation. An acetone powder was prepared from the bacteria as follows. Immediately after centrifugation, 170-180 g of packed cells (obtained from 56 liters of growth medium) were uniformly suspended in 170-180 ml of ice-cold 2% 2-mercaptoethanol with the help of a nonaerating stirrer (Kraft Apparatus, distributed by Glas-Col Apparatus, Terre Haute, IN). The cold suspension was added slowly with stirring to 1.7-1.8 liters of acetone at −10°C; the stirring was continued for 5 min at about 0°C. The material, collected by suction filtration, was resuspended uniformly in 1.7-1.8 liters of cold acetone with nonaerating stirring, collected again by filtration, and transferred to a round-bottom flask. The remaining acetone was removed in vacuo at room temperature with the aid of a rotary evaporator. The powder could be stored over a desiccant at −20°C for at least 3 months without significant activity loss. The average yield was 50 g (i.e., about 29% of the packed cell weight). For enzyme extraction, 30 g of powder was stirred for 10 min at room temperature without aeration in 300 ml of 100 mM Tris-HCl buffer, pH 8.0/0.2% 2-mercaptoethanol. The suspension was centrifuged for 20 min at 48,000 × g.

Preparation of Bacterial Bile Pigment. To the clear supernatant solution, 60 mg of 8-aminolevulinic acid dissolved in 10 ml of 100 mM Tris (pH 8.0) was added with gentle swirling. The mixture was incubated 18 hr in the dark at 37°C. Precipitated protein was removed by centrifugation for 20 min at 48,000 × g. The uroporphyrins and related anionic substances in the supernatant solution were esterified after retention on an anion exchanger by a modification of the method of Bergmann et al. 1

1 Present address: Moffett Tech. Ctr., Corn Products, P.O. Box 345, Summitt-Argo, IL 60501.

§ To whom reprint requests should be addressed.
RESULTS

The present work resulted from studies of intermediates in vitamin B-12 biosynthesis. After incubation of enzyme extracts of *C. tetanomorphum* with δ-aminolevulinic acid at pH 8 in the presence of 2-mercaptoethanol and workup by conventional methods (19), it was found that when the reaction products in the form of their methyl esters were submitted to thin-layer chromatography on silica plates in a widely used solvent (24), a certain amount of pigmented material remained near the origin. Thin-layer chromatography of this material in chloroform containing from 2% to 3% methanol showed small amounts of a striking hitherto unreported nonfluorescent blue material. The first indication that this substance is a bile pigment and not a cyclic tetrapyrrole was provided by the fact that it gave a positive Gmelin reaction (multiple, gradually developing colors upon careful addition of concentrated nitric acid) (described by TieDEMANN and Gmelin in 1826; for recent references see refs. 2, 25, and 26). This conclusion was confirmed by the absorption spectrum of the compound in chloroform, which indicated a close relationship to biliverdin methyl ester. The data are summarized in Table 1. Both substances show a broad absorbance band above 640 nm and a much sharper and more intense band in the near ultraviolet region. The close similarity between the two sets of maxima, which is also true of the inflection seen in the two absorption curves, indicates that the two chromophoric systems are essentially identical. The slight shift in the sharper band to a lower wavelength may be due to the absence of the two vinyl groups in the new substance. Neither of the absorption bands showed a Cotton effect, demonstrating the absence of chirality in the molecule. The position of the strong infrared

<table>
<thead>
<tr>
<th>Substance</th>
<th>UV-Vis Ratio</th>
<th>IR Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biliverdin IXα</td>
<td>1,732 (ester C=O)</td>
<td>1,730 (amide C=O)</td>
</tr>
<tr>
<td>dimethyl</td>
<td>376, 642-670</td>
<td>3.76, 1,695</td>
</tr>
<tr>
<td>Bacterobilin</td>
<td>1,735 (ester C=O)</td>
<td>0.76</td>
</tr>
<tr>
<td>octamethyl</td>
<td>369, 644</td>
<td>3.56, 1,705 (amide C=O)</td>
</tr>
<tr>
<td>chloroform</td>
<td>1,27</td>
<td></td>
</tr>
</tbody>
</table>

Vis, visible.

**Table 2.** Chromatographic behavior of various methyl esters

<table>
<thead>
<tr>
<th>Compound</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoporphyrin IX</td>
<td>0.81</td>
<td>0.91</td>
<td>0.67</td>
<td>0.96</td>
</tr>
<tr>
<td>Coproporphyrin III</td>
<td>0.69</td>
<td>0.78</td>
<td>0.59</td>
<td>0.78</td>
</tr>
<tr>
<td>Uroporphyrin III</td>
<td>0.31</td>
<td>0</td>
<td>0.33</td>
<td>0</td>
</tr>
<tr>
<td>Heptacarboxylporphyrin</td>
<td>0.44</td>
<td>—</td>
<td>0.40</td>
<td>—</td>
</tr>
<tr>
<td>Factor I</td>
<td>0.37</td>
<td>0.52</td>
<td>0.39</td>
<td>0.45</td>
</tr>
<tr>
<td>Factor II</td>
<td>0.35</td>
<td>0.57</td>
<td>0.39</td>
<td>0.59</td>
</tr>
<tr>
<td>Factor III</td>
<td>0.34</td>
<td>0.54</td>
<td>0.39</td>
<td>0.46</td>
</tr>
<tr>
<td>Factor II dialactone</td>
<td>0.18</td>
<td>0.30</td>
<td>0.27</td>
<td>0.07</td>
</tr>
<tr>
<td>Factor III dialactone</td>
<td>0.19</td>
<td>0.28</td>
<td>0.29</td>
<td>0.05</td>
</tr>
<tr>
<td>Biliverdin IXα</td>
<td>0.29</td>
<td>0.78</td>
<td>0.30</td>
<td>0.59</td>
</tr>
<tr>
<td>Bacterobilin</td>
<td>0.05</td>
<td>0.50</td>
<td>0.06</td>
<td>0</td>
</tr>
</tbody>
</table>

* Solvent: 1 (on silica), benzene/ethyl acetate/methanol (85:13.5:1.5, vol/vol); 2 (on silica), n-hexane/2-propanol/methanol (50:20:10, vol/vol); 3 (on silica), chloroform/methanol (100:1, vol/vol); 4 (on cellulose), n-hexane/chloroform/1-propanol/2-propanol (94:4:2:0.5, vol/vol).
absorption peaks supports the conclusion that the two compounds are structurally closely related. The fact that the isolated bile pigment differed from biliverdin was confirmed by thin-layer chromatography in four different systems (Table 2). The compound moved as one band in all of the solvent systems tried. If one assumes a biliverdin-like bilatriene having the side chains found in uroporphyrin III, one arrives at the four isomeric structures, I–IV (Fig. 1), with a molecular weight of 962 for the octamethyl ester. Indeed, mass spectrometry of the methyl ester of the compound gave rise to a strong molecular ion, \( m/e = 962 \).

The most detailed information regarding the structure of the bile pigment was obtained from the 500-MHz proton NMR spectrum of the methyl ester in both \( C_5HCl_3 \) and \( C_4H_2Cl_2 \). The spectra in the two solvents were nearly identical. The spectrum in \( C_5HCl_3 \) showed seven separate methylecarboxylate signals, the one at chemical shift \( \delta 3.653 \) having twice the intensity of the others. This observation indicated the presence of eight ester groups. A comparison of the chemical shifts of these groups and those of the methine and methylene protons, with the corresponding signals shown by biliverdin IXa dimethyl ester, is given in Table 3. Three low-field signals at \( \delta 6.784, \delta 6.015 \), and \( \delta 5.976 \) corresponded closely to the three meso-proton signals in biliverdin dimethyl ester.

**DISCUSSION**

The new compound, whose chromophore structure and molecular weight indicate the presence of oxygen attached to the two outer rings, qualifies as a bile pigment by the definition of Rüdiger (2). We have considered two names for this substance: bacterovin, in analogy to biliverdin, and bacteria, in analogy to the bound phycobilins (ref. 1; see p. 88 of ref. 27) from certain algae and all higher plants and to pterobilin from Lepidoptera (28). The former name, although it indicates a 5,10,15-trilatriene, was discarded in order not to add to eccentricity and confusion (7) because, like biliverdin, this substance is not green but blue. Hence, we propose the second name.

The presence of but three methine singlets in the 500-MHz spectrum provides strong evidence that the methyl ester of the purified substance constitutes a single product and not a mixture of isomers produced by cleavage at more than one of the methine bridges. The interpretation of the NMR spectra was greatly aided by the work of Bonnett and McDonagh (29), who prepared all four possible isomers of biliverdin and reported their properties. From the available data, it was not possible to deduce unambiguously which of the four possible isomers had been formed. However, structures III and IV (Fig. 1), corresponding to cleavage at the \( \gamma \) and \( \delta \) methine carbons, could be ruled out on the basis of the following considerations. Bonnett and McDonagh showed that the propionyl methylene protons \( \alpha \) to the pyrrole rings exhibited significantly different chemical shifts, depending on whether they were attached to an \( \alpha \) position (i.e., \( \alpha \) to the amide carbonyl group) or to an \( \alpha \) (i.e., at all other positions of the pyrrole rings). The methylene signal in biliverdin IXy, which possesses both propionyl groups in \( \alpha \) positions, appeared at \( \delta 2.51 \), whereas the other biliverdins that possess \( \alpha \)-propionyl substituents showed this methylene signal at \( \delta 2.51 \). The signal for the methylene groups \( \alpha \) to the carboxyl group always appeared at \( \delta 3.0 \).

In the octamethyl ester of the new metabolite, all eight propionyl methylene signals appeared as triplets. Three of these were centered at \( \delta 2.923, \delta 2.847 \), and \( \delta 2.818 \), while the fourth methylene group was represented by one of the triplets found at higher field between \( \delta 2.62 \) and \( \delta 2.49 \), together with the signals for the four methylene protons \( \alpha \) to the carboxyl groups. This indicates the presence of one \( \alpha \)- and three \( \alpha \)-propionyl groups.

Of the four possible structures, I–IV (Fig. 1), whose side chains correspond to those of uroporphyrin III, only structures I and II (corresponding to cleavage at the \( \alpha \) and \( \beta \) methine carbons) possess one \( \alpha \)- and three \( \alpha \)-propionyl groups, as indicated by the presence of three lower-field and one higher-field methylene signals. Structure III would require two lower-field methylene signals, and structure IV, none. The NMR evidence therefore rules out these latter two structures, and bilovin hence may represent either structure I or II.

Structure I corresponds to that of the common biliverdin, designated as IXa. Although the vast majority of bile pigments are derived by cleavage at the \( \alpha \) methine carbon of heme, there are biological precursors for bile pigments cleaved at other methine carbons. Thus, the pigment pterobilin from Lepidoptera (28, 30–32) has been shown to be biliverdin IXy (33, 34);
biliverdin IXβ has been reported in hepatic catalase (35); traces of bilirubin IXβ and IXβ have been reported in pig bile (36); and the δ, β, and γ isomers have been reported in man, pig, dog, and rat bile (37).

The above discussion assumes that the sequence of side chains in biliverdin corresponds to that found in uroporphyrin III. However, under the conditions of the present experiments (incubation of cell-free extracts with added δ-aminolevulinic acid), the alternating sequence of side chains, corresponding to those of uroporphyrin I, cannot be excluded. Analysis of the isolated uroporphyrin by high-performance liquid chromatography showed it to be a mixture of about equal parts of uroporphyrins I and III. Analogous conclusions have been reached with uroporphyrin prepared from suspensions of Propionibacterium shermanii fortified with δ-aminolevulinic acid (38). If the side chains are indeed arranged as in uroporphyrin I, then only one product (structure V, Fig. 1) would result, regardless of which one of the four bridge carbons is eliminated. Structure V cannot be excluded from the NMR data. On the other hand, the fact that biliverdin could be obtained from C. tetanomorphum or, in smaller amounts, from P. shermanii that had been grown without δ-aminolevulinic acid makes it unlikely that this substance is related to uroporphyrin I. Uroporphyrin III apparently free from uroporphyrin I could be isolated from another Clostridium grown without added δ-aminolevulinic acid (39). If the compound would be formed by a random nonenzymatic cleavage of cyclic tetrapyrroles, it would be possible to observe more than one bile pigment in P. shermanii because, under those circumstances, coproporphyrinogen or coproporphyrin III and the corresponding hepta- and hexacarboxylic tetrapyrroles could be expected to be cleaved as well.

The mechanism of formation of biliverdin is still unknown. Biliverdin IXα formation from protoheme IX, as catalyzed by the enzyme heme oxygenase, requires the participation of molecular oxygen, NADPH-cytochrome c reductase, and NADPH (8, 16, 17, 40); a reactive oxygen radical first forms an α-hydroxylheme (41, 42), and further oxygen is added to this complex with resulting ring cleavage, carbon monoxide loss, and iron release (see, for example, refs. 8 and 17). In control experiments on the formation of biliverdin, however, the compound was still formed when the reaction was initiated under strictly anaerobic conditions (2-mercaptoethanol present as usual, but tubes filled to the top), so that the red fluorescence indicative of the liquid to air was completely absent. The clostridial extracts, again, do not contain cytochrome c. They are active after dialysis without added NADPH (unpublished data). It appears likely, hence, that the present enzyme differs from heme oxygenase. A further difference between the formation of bilirubin and biliverdin lies in the intriguing likelihood that bilirubin, unlike biliverdin, is derived from a metal-free tetrapyrrole. The substrate specificity of heme oxygenase has been thoroughly investigated (17, 43, 44). This enzyme does not react with metal-free protoporphyrin (44). Iron coproporphyrin III is a poor substrate (43). At this point it is not known whether a metal complex of uroporphyrin III, such as uroheme, is changed enzymatically to bilirubin. Preliminary experiments have shown that under the conditions used, the present enzyme does not convert uroporphyrin III or I to bilirubin, or protoheme IX to biliverdin. Although the initial enzyme incubation was carried out under anaerobic conditions, it is possible that biliverdin is formed from an anaerobically produced enzyme product during workup in the presence of oxygen. Biliverdin octamethyl ester is obtained also when boron trifluoride methanol is used instead of methanol sulfuric acid for esterification. The nature of the compound leading to bilirubin remains unknown, and the presence of bilirubin in vivo has not been demonstrated.

There is one well-established process associated with the anaerobic ring opening and carbon loss of a cyclic tetrapyrrole. This process occurs during the so-called ring contraction, when a porphyrin type of ring structure is changed to the corin type of ring structure. A two-carbon compound, namely acetic acid, is expelled from a repeatedly C-methylated intermediate (45, 46), but the mechanism, the enzymology, and the exact substrate of this reaction are unknown. It is possible that some analogies exist between the first step in bilirubin and in corrin ring formation.

The physiological significance of bilirubin remains unknown. It may be more than a coincidence that one organism contains apparently two separate systems for the anaerobic cleavage of cyclic tetrapyrroles, but further work is needed to shed light on this puzzle.

We thank Dr. Alan B. Batterby, Cambridge University, for the field desorption mass spectrum and stimulating discussions during his tenure as Kharasch Visiting Professor in the Department of Chemistry; Dr. Jerry C. Bommer, Porphyrin Products (Logan, UT), for the high-performance liquid chromatography; and Mr. Barry Williams for obtaining the various spectra. This work was supported by National Institutes of Health Grants AM-09134 to H. C. F. and KO6 AM-21846 to J. F. and by The Burroughs Wellcome Fund. Funds provided by the National Science Foundation (GP 33116), the National Institutes of Health (Cancer Center Grant CA 14999), and the Louis Block Fund to purchase the NMR equipment used in this work are gratefully acknowledged.


Table 3. 500-MHz proton magnetic resonance signals in C2HCl3

<table>
<thead>
<tr>
<th>Chemical shifts, δ</th>
<th>CO2CH3</th>
<th>bridge-CH*</th>
<th>CH3CH2CO2CH3</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biliverdin IXα dimethyl ester</td>
<td>3.674</td>
<td>6.799, 6.070, 6.015</td>
<td>2.933 (m, *J = 7.5 Hz, 2H)</td>
</tr>
<tr>
<td></td>
<td>3.688, 3.688, 3.659</td>
<td></td>
<td>2.818 (t, *J = 7 Hz, 2H), 2.651 (t, *J = 7 Hz, 2H)</td>
</tr>
<tr>
<td></td>
<td>3.653</td>
<td>2.62–2.49 (m, 6H)</td>
<td></td>
</tr>
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</table>
Biochemistry: Brumm et al.