Compound **800**, a Natural Product Isolated from Genetically Engineered *Pseudomonas*: Proposed Structure, Reactivity, and Putative Relation to Heme d_1[†]

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ABSTRACT: Genetically engineered strains of *Escherichia coli* and *Pseudomonas aeruginosa* were prepared harboring the gene cluster *nirFDLGH* from *Pseudomonas stutzeri* substrain ZoBell on a high copy plasmid. These genes have been previously implicated as being essential for the biosynthesis of heme d_1, the prosthetic group of dissimilatory nitrite reductases in anaerobic, denitryfying bacteria. Tetrapyrroles detectable at steady-state levels were identified from both organisms, and cell-free extracts from each were also used to transform uroporphyrinogen in vitro. *E. coli* does not naturally produce d_1, and the engineered strain failed to produce d_1 or any tetrapyrrole foreign to *E. coli*. Therefore, while *nirFDLGH* may be necessary for d_1 biosynthesis, it is not sufficient. In the denitrifier *P. aeruginosa*, the results were more positive. The presence of the plasmid led to increased levels of d_1. In addition, a previously unidentified tetrapyrrole was detected. This compound was characterized by visible absorption spectroscopy, infrared spectroscopy, X-ray photoelectron spectroscopy, mass spectrometry, and NMR, and a tentative structure was proposed for this compound. The tetrapyrrole has structural features similar to sirohydrochlorin (as precorrin-2 or sirotetrahydrochlorin, a known intermediate of d_1) and d_1 itself. The most unusual substituents are epoxide and sulfoxide moieties. When this tetrapyrrole was treated with strong mineral acid and heat, it was converted into natural d_1.

Heme d_1 (Scheme 1) is the unique prosthetic group of bacterial dissimilatory nitrite reductases termed cytochromes *c*d_1, which reduce nitrite to nitric oxide during the anaerobic respiration of denitrifiers. It is biosynthesized under anaerobic conditions during growth on nitrate or nitrite. Bacterial denitrification, the role of nitrite reductases, and the history of heme d_1 have been recently reviewed (*1*, *2*). Heme d_1 has atypical substituents, the acrylate and oxo groups, and long standing questions have remained about how it is biosynthesized, especially how the oxo groups are introduced during anaerobic growth. It has been shown that the general route (Scheme 1) to d_1 is uroporphyrinogen III to precorrin-2 (sirotetrahydrochlorin III) to d_1 by labeling studies (*3*) and the fact that the stereochemistry in d_1 at the stereocenters C2 and C7 is the same as in precorrin-2, siroheme heme, and vitamin B_{12} (*4*). The multiple steps necessary to convert precorrin-2 to d_1 are not known.

Gene identification, DNA sequencing, and phenotype studies have provided information on the genes involved in d_1 expression. The situation is complex, and some of the evidence is ambiguous or conflicting, so only the barest highlights are recounted here, while a full account of the genetic background has been reviewed elsewhere (*2*). Genes *nirFDLGH* are essential for d_1 biosynthesis (*5*). In the organism *Pseudomonas stutzeri* substrain ZoBell, they are contiguously aligned behind a common promoter in the *nir* locus, which contains all of the genes necessary for the phenotype of nitrite respiration, including *nirS*, the structural gene for cyt *c*d_1. The protein encoded by *NirF* is highly homologous to the heme d_1 binding domain of *nirS*, and thus *NirF* could readily code for a protein with a tetrapyrrole-binding site. *NirE* is homologous to genes in other organisms that code for *S*-adenosylmethionine to uroporphyrinogen methyl transferase (SUMT),[1] the enzyme responsible for methylating uroporphyrinogen to precorrin-2. This activity is essential for d_1 biosynthesis but is not exclusive to such, because precorrin-2 has other fates as siroheme and vitamin B_{12}. *NirJ* is likely to be involved in d_1 biosynthesis (*2*). It has recently been shown to be a member of the "Radical SAM" superfamily of enzymes (*6*), which catalyze a large array of diverse reactions, none of which is immediately obvious as being related to d_1 biosynthesis.

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[1] Abbreviations: ALA, aminolevulinic acid; FAB, fast atom bombardment; SUMT, *S*-adenosyl methionine to uroporphyrinogen methyl transferase; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single-quantum correlation spectroscopy; IPTG, isopropyl *β*-D-thiogalactopyranoside; LSIMS, liquid secondary ion mass spectrometry; ROESY, rotating-frame nuclear Overhauser spectroscopy; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy; XPS, X-ray photoelectron spectroscopy or spectrum.

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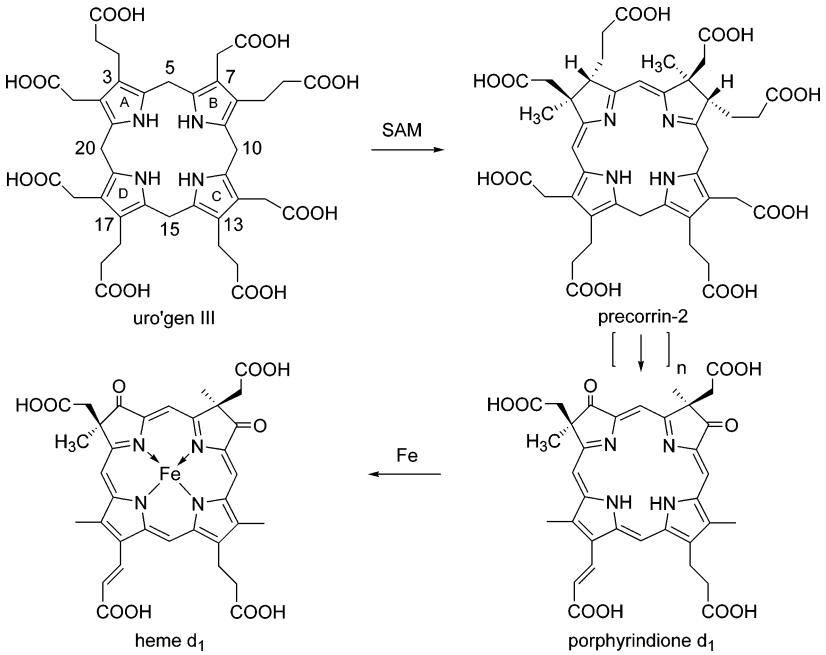
Scheme 1: Known Precursors to Heme d_1



This paper reports studies in which the gene cluster *nirFDLGH* was overexpressed in *Pseudomonas*. The engineered strain was shown to produce a previously uncharacterized tetrapyrrole, whose structure suggests that it is an intermediate along the pathway from precorrin-2 to d_1 or is related to an intermediate. This tetrapyrrole is designated as compound **800** after the unique mass of its methyl ester derivative.

MATERIALS AND METHODS

Cultures and Conditions. Escherichia coli strain DH5α grown in Luria−Bertani broth at 37 °C was used for recombinant DNA manipulations and expression experiments. *P. aeruginosa* (ATCC 19429) was grown anaerobically at 37 °C on a nitrate-containing medium (*7*) supplemented with additives as described in the Results. *Desulfovibrio vulgaris* was obtained from Prof. J. LeGall of the University of Georgia and grown at 37 °C on a lactate−sulfate medium (*8*). *D. vulgaris* SUMT was partially purified by the method of Ishida et al. (*9*) through the first ion exchange step.

Plasmid Construction. A 1.8 kb fragment with the 3′ end of *Pseudomonas* ZoBell *nirB*, the entire *nirMC* transcriptional unit, and the initial 75 bp of *nirF* on a Bluescript (Strategene) cloning vector pBSM21 (*10*) was obtained from Prof. Walter G. Zumft, Universität Karlsruhe. A *Bgl*II−*Kpn*I subfragment (*nirMCF*′) was subcloned into the *Bgl*II−*Kpn*I site of ptacTrpA immediately downstream of a tac promoter used previously for the overexpression of the α subunit of Trp synthase to generate ptacnirMC (*11*). This was digested at a unique *Eco*RV site within 5 bp of the *nirC* termination codon. A *Bgl*II linker was ligated into the former *Eco*RV site to yield the construct ptacnirMb$_2$. *Bgl*II digestion of this plasmid now cut out *nirM* and most of *nirC*. Religation of the backbone produced ptacnirF′, which contains the initial 75 bp of *nirF* now directly behind the tac promoter. Prof. Zumft provided another 4.45 kb *Kpn*I−*Kpn*I fragment on the same Bluescript vector containing the remainder of *nirF*, all of *nirDLGH*, and *norCB*. *norCB* are the structural genes for the subunits of the ZoBell nitric oxide reductase. This plasmid pNIRD1 had been constructed by Zumft's group during their sequencing of the *nir* and *nor* denitrification loci and used in establishing an essential role for the genes in d_1 biosynthesis (*5*). The fragment was removed by *Kpn*I digestion, purified, and inserted into the unique *Kpn*I site on ptacnirF′ to generate ptacnirD, with intact *nirFDLGH* behind a tac promoter. This vector can only be propagated in *E. coli*, and for reasons discussed, it was desired to also reintroduce this gene cluster back into *Pseudomonas*. The plasmid pUCP19 (American Type Culture Collection) is a high copy broad-host-range vector that can be introduced and stably maintained in both *E. coli* and *Pseudomonas* species (*12*−*15*). *Hind*III digestion of ptacnirD produced a 3.4 kb fragment containing the tac promoter and all of *nirFDLGH*. This was inserted into the unique *Hind*III site of pUCP19, 2.3 kbp from the ampicillin resistance gene, to yield pUCP19D1 and pUCP19D2, which differ with respect to the orientation of the insert. Orientation was determined by restriction mapping based on *Acc651* digestion. D1 has the insert oriented the same as amp^R, while in D2 it is opposite. Both are expected to express *nirFDLGH* gene products with their own tac promoters. For unknown reasons, D2 had a higher transformation efficiency in *Pseudomonas* and was used in the majority of the remaining experiments to be discussed.

Tetrapyrrole Purification. The preparation of cell-free extracts, tetrapyrrole esterification, and tetrapyrrole isolation from cells have been described (*16*). Compound **800** was purified from crude esterified tetrapyrroles as follows. Crude esters were redissolved in chloroform and chromatographed on a small column (1 × 15 cm) of silica gel (200−400 mesh, packed in chloroform) eluted with chloroform. This crude column is not sufficient for resolving individual tetrapyrrole esters but is very helpful in removing other background compounds that bind very tightly to silica and interfere with HPLC. The broad band with characteristic tetrapyrrole color

and fluorescence was collected, dried under vacuum, and further purified by HPLC on a 5 μm silica gel column (4.6 $\times$ 250 mm) eluted at 1 mL/min with 8:2 methylene chloride/ethyl acetate, containing 0.025% (v/v) pyridine. The methyl esters of coproporphyrin III, d_1, and **800** eluted successively with capacity factors of 2.27, 2.45, and 2.86, respectively. The fraction containing **800** was further purified by size-exclusion chromatography on Sephadex LH-20 eluted with methanol, reverse-phase thin-layer chromatography (Whatman C18, 5 $\times$ 20 cm plates, 200 μm thick) eluted with methanol, and silica gel column chromatography eluted with 9:1 benzene/ethyl acetate.

Spectroscopic Techniques. Tetrapyrroles were identified by their unique masses using MALDI−TOF mass spectrometry either matrix free or on matrixes that inevitably produce the $(M + H)^+$ parent ion (*17*). The exact mass of the protonated molecular ion for **800** was critical evidence toward the proposed structure. It was measured by liquid secondary ion mass spectrometry (LSIMS, also known as fast atom bombardment or FAB) using a Cs ion source on a triple sector Autospec high-resolution mass spectrometer and by MALDI ionization on an IonSpec Ultima FT mass spectrometer.

Routine one-dimensional and TOCSY spectra were recorded at 500 MHz on a Bruker spectrometer. ROESY (*18*) and HSQC (*19*) experiments were recorded on a Bruker 600 MHz spectrometer equipped with a cryogenically cooled probe.

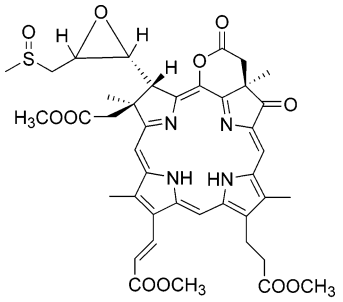
Infrared spectra were recorded for **800** deposited from a methylene chloride solution as a dried film on a solid gold surface using a Mattson FTIR spectrometer with a Harrick external-reflection sample holder. X-ray photoelectron spectra (XPS) were similarly recorded for dried films on gold on a Kratos Axis 165 spectrometer.

The molecular model for **800** was calculated using the software package SYBYL from Tripos Associates.

RESULTS AND DISCUSSION

This research began with the assumption that the *nirFDLGH* locus was not only necessary for d_1 biosynthesis but might also be sufficient in the proper host. The locus from *P. stutzeri* ZoBell was cloned downstream of a high copy promoter under control by IPTG into a broad host vector pUCP19 (*12*−*15*) to give what we termed the pUCP19D2 plasmid. The plasmid was introduced into *E. coli*, which does not produce d_1, and transformants were screened in several ways to see if d_1 was now produced or if some other nonendogenous tetrapyrrole was now present. Tetrapyrroles were isolated from broken cells, purified, and identified. Broken-cell extracts were incubated anaerobically with uroporphyrinogen plus SAM in an attempt at using the enzymes present to promote in vitro biosynthesis that could have made higher than steady-state levels of any putative new tetrapyrroles, a strategy that has been successfully employed previously (*16*). Only well-known tetrapyrroles, such as protoporphyrin IX, coproporphyrin, and decarboxylated porphyrins derived from uroporphyrin (*20*), were detected. Note that SUMT activity was not encoded on pUCP19D2. It had been initially assumed that the endogenous SUMT in *E. coli*, coded by the gene *cysG*, would have been sufficient to methylate uroporphyrinogen. However, SUMTs often have low turnover numbers and are subject to severe product inhibition (*21*), and the chromosomal copy may have been limiting (*22*). Dr. N. M. Kredich kindly donated an engineered strain of *E. coli* carrying overexpressed *cysG*. Broken-cell extracts of this strain were used along with *E. coli* harboring pUCP19, but again, no unexpected tetrapyrroles were detected. In earlier studies on tetrapyrrole biosynthesis in *D. vulgaris*, it had been noted that the *D. vulgaris* SUMT was more active than the *E. coli* SUMT and not susceptible to product inhibition (*16*). SUMT was prepared from cells of *D. vulgaris* (*9*) and used to supplement broken-cell extracts of *E. coli* with pUCP19D2. With this supplement, added uroporphyrinogen was completely consumed and high levels of factor I (monomethylated uroporphyrinogen), sirohydrochlorin, and decarboxylated isobacteriochlorins derived from sirohydrochlorin were now found; therefore, clearly, SUMT activity was not limiting. Unfortunately, no new tetrapyrroles related to d_1 were found. The conclusion was that SUMT plus the *nirFDLGH* was not sufficient for d_1 biosynthesis in *E. coli*.

The broad host vector was also used to introduce multiple copies of *nirFDLGH* into *Pseudomonas*, which already has chromosomal copies of all genes required for d_1 biosynthesis. The baseline content of d_1 in wild-type *P. aeruginosa* was 0.7 nmol per gram wet weight of cells. *P. aeruginosa* transformed with pUCP19D2 also expressed d_1 at the same level when grown in standard rich media. However, broken-cell extracts of transformed cells supplemented with *D. vulgaris* SUMT and incubated anaerobically with uroporphyrinogen and SAM produced d_1 at levels of 2.4−3.4 nmol/g (average = 3.0 nmol/g). More importantly, when the repertoire of expressed tetrapyrroles was surveyed, a pigment not previously characterized was discovered. This was designated compound **800** after the molecular mass of the methyl ester derivative. The compound was present only at very low levels, ca. 0.1 nmol/g. However, because it only appeared in transformed cells when overproducing d_1, it became a candidate for a previously unrecognized intermediate on the d_1 pathway that would normally be present at steady-state levels too low to detect.



800

Conditions were investigated to increase the production of the new tetrapyrrole. The best and operationally most convenient condition was to culture transformed cells on a rich medium supplemented with aminolevulinic acid (ALA) (20 mg/L) and SAM (10 mg/L) and isolate the endogenous tetrapyrroles without broken-cell in vitro biosynthesis. The use of ALA to stimulate tetrapyrrole biosynthesis is well-established (*20*), but it was surprising that external SAM acted as a stimulator, because to the best of our knowledge

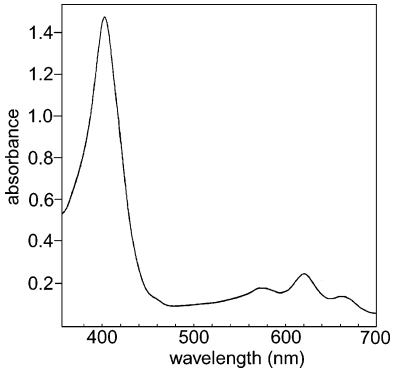


FIGURE 1: Visible absorption spectrum of compound **800** in methylene chloride.

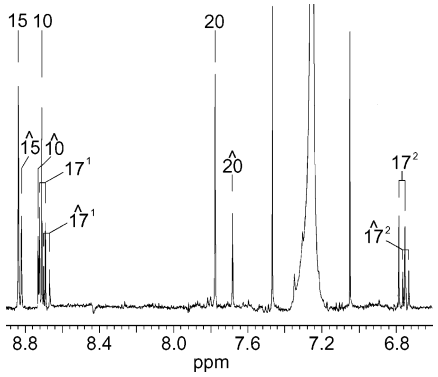


FIGURE 2: Aromatic region of compound **800** in deuterated chloroform showing the major and minor isomers. Meso and acrylate protons are labeled with the standard IUPAC numbering scheme given in Scheme 1, with the minor isomer resonances indicated by the caret ∧ mark.

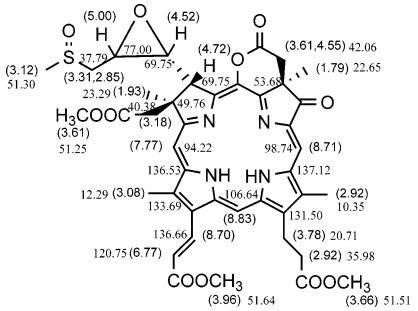


FIGURE 3: Proton (in parentheses) and ^{13}C (no parentheses) chemical shifts for **800**. All carbons with attached protons have been assigned, but not all other carbons could be assigned because of missing or weak peaks in the two-dimensional correlation spectrum.

there are no reports in the literature of bacterial cell uptake of this complicated molecule. For transformed cells so far cultured, d_1 production rose to 12−17 nmol/g and **800** production rose to 0.7−1.0 nmol/g. Both ALA and SAM were required for these production levels. Without either, production fell to the wild-type baseline level. Increasing the concentrations did not further benefit production. While the **800** production level was low, it was possible over time to accumulate enough material to characterize spectroscopically this new pigment and propose a tentative structure. The rest of this section will discuss the evidence that led to this proposed structure.

The visible absorption spectrum (Figure 1) is similar to d_1 for this green-hued material. The Soret band of d_1 (421 nm) is shifted to 408 nm in **800**, and the strongest other band, 608 nm in d_1, is shifted to 620 nm in **800**. A weak 565 nm feature in d_1 becomes stronger and shifts to 578 nm in **800**. The optical evidence is not conclusive, but does support that **800** has isobacteriochlorin character.

FTIR spectroscopy provided critical evidence. A 1717 cm^{-1} carbonyl mode was detected as a shoulder on the intense 1737 cm^{-1} mode; this is highly diagnostic of the oxo group in d_1 (*2*). Epoxides have characteristic modes at 950−810 cm^{-1} and the "12 micron band" at 840−750 cm^{-1} (*23*). Modes at 781, 843, and 864 cm^{-1} are present in the vibrational spectrum of **800** but are not present in d_1.

The presence of sulfur was confirmed by XPS. The ratio of equivalents of nitrogen/sulfur was 4:1. XPS is also sensitive to the oxidation state of sulfur and can differentiate organosulfur compounds such as thioethers, sulfoxides (R−S(O)−R), and sulfones ($R-S(O)_2-R$) by their binding energy (*24*). The observed value of 166.14 eV for **800** clearly indicates that sulfur is present as a sulfoxide.

The exact mass of **800** was determined by high-resolution mass spectrometry. The protonated molecular ion, observed at 801.280 Da by FAB-MS and 801.281 Da by FT-MS, shows a significant mass defect for a C,H,O,N-containing organic molecule and suggests the presence of a heteroatom. The formula $C_{41}H_{45}O_{11}N_4S$ (calculated mass 801.281 Da) agrees well with the observed mass. The sodium adduct was also observed by FT-MS at 823.263 Da, which agrees with the formula $C_{41}H_{44}O_{11}N_4SNa$ (calcd 823.263 Da).

Interpretation of ^1H NMR spectra was initially complicated by the fact that the samples were not homogeneous. Compound **800** was purified by a multistep procedure, and while each step helped, the starting milieu is so complex and **800** is present at such low levels (0.1−1 nmol/g) that residual nontetrapyrrole impurities comigrate. Because there are unavoidable losses and decreasing yields, one must accept this nontetrapyrrole background to have enough **800** to study. Over a period of many independent preparations, NMR spectra were examined and resonances that were constant in intensity could be attributed to **800**, while the impurities were variable in intensity.

800 was a single peak on HPLC detected at 408 nm, a single fluorescent spot on TLC, and a single molecular ion in mass spectrometry with no high (>600 Da) molecular weight contaminants. However, ^1H NMR revealed the tetrapyrrole was composed of two isomers. A major isomer dominated but was accompanied by a minor isomer, which had resonances at very similar chemical shifts. This is indicated in Figure 2. The amount of minor isomer varied from preparation to preparation and was in the range of 14−45%. The spectral homology suggests that these are diastereomers. Sirohydrochlorin is known to spontaneously form epimers (*25*) by inversion of the substituents at C3 and/or C8; such epimerization at C3 could explain the **800** situation. Future discussion will focus on the major isomer. It is important to stress that meso protons for tetrapyrroles, which appear in the aromatic region of NMR spectra are the distinctive fingerprint of any tetrapyrroles. The residual impurities in **800** preparations do not give rise to detectable meso resonances and hence are not tetrapyrroles.

Proton (in parentheses) and ^{13}C (no parentheses) chemical shifts and their assignments are summarized in Figure 3. Scalar coupling patterns were determined by TOCSY or

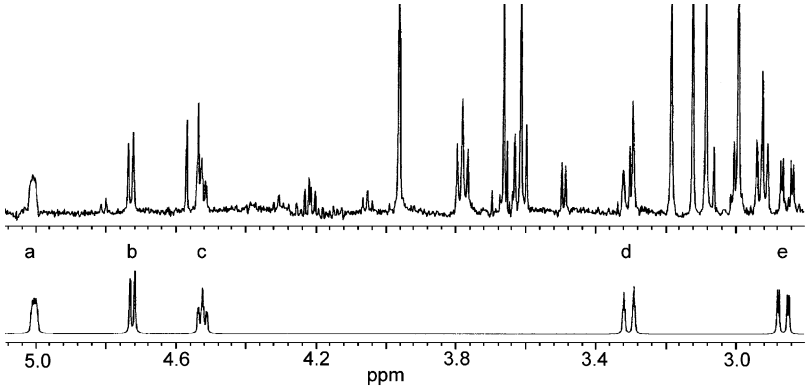


FIGURE 4: Aliphatic region of the ^{1}H NMR spectrum of **800** (upper trace) and a simulation of the coupled spin subsystem of the proposed epoxide substituent (lower trace). Spin-coupling constants in hertz used in the simulation are as follows: $J_{ab} = 0.9$, $J_{ac} = 5.2$, $J_{bc} = 6.9$, $J_{cd} = 1.7$, $J_{de} = 14.3$, $J_{ad} = 0.9$, and $J_{ae} = 3.4$.

direct decoupling irradiation experiments, and the values of coupling constants were confirmed by coupling simulations. ^{13}C spectra were obtained by the inverse-detection experiments HSQC and long-range HSQC (*19*).

Common unsymmetrical tetrapyrroles have four meso proton resonances. However, in the NMR spectrum of **800** in $CDCl_3$, only three meso protons were seen (Figure 2). The compound was examined in acetone-d_6 to ensure that the fourth meso proton was not under the residual $CHCl_3$ resonance. Only three meso protons appeared (9.12, 8.58, and 8.06 ppm) in the acetone solvent spectrum, confirming that the fourth meso proton of compound **800** was substituted by another functional group.

Two characteristic doublets in the NMR spectrum were found at 8.70 and 6.77 ppm with a 16.3 Hz coupling constant. These resonances come from the trans double bond of an acrylate group just as was found on pyrrole ring D of heme d_1. The spin coupled triplets ($J = 7.7$ Hz) at 3.78 and 2.92 ppm come from the southern propionate group. From homology to both d_1 and sirohydrochlorin, the methyl singlets at 3.08 and 2.92 ppm are assignable to southern unsaturated pyrrole rings and those at 1.93 and 1.79 ppm are on northern saturated pyrroles. Therefore, **800** is an isobacteriochlorin, the southern half of which is indeed just like d_1, while the northern half has features suggestive of a sirohydrochlorin derivative.

The acetate methylene apparent singlet at 3.18 ppm is within reasonable range of the corresponding substituents in sirohydrochlorin at 2.7−2.9 ppm. The geminal doublets at 3.61/4.55 ppm ($J = 16.3$ Hz) are in the approximate range for an acetate substituent on d_1, but the dispersion of almost 1 ppm between them is highly unusual. A six-membered lactone ring could arise from condensation of a normal acetate substituent and a hydroxyl group at C5. Spontaneous formation of five-membered lactones by the proximity of hydroxyl and carboxylate functional groups has been well-documented in the case of hemes d_{cis} and d_{trans} (*26*, *27*). A six-membered ring becomes rigid and twists with respect to the main macrocycle plane. A molecular model of compound **800** was calculated starting with the atomic coordinates of natural heme d_1 from the crystal structure of cytochrome *c*d_1 (*28*). The position of the six-membered lactone ring with respect to the tetrapyrrole plane places the two methylene protons in distinct positions where they will experience different ring currents, and this could account for their chemical-shift dispersion. While five-membered lactones such as those found in hemes d_{cis} and d_{trans} have characteristic high-frequency carbonyl modes (*26*, *27*), the absorption for the six-membered lactone may be buried in the strong 1737 cm^{-1} band observed for **800**. The proposed lactone might be considered as a vinyl ester and hence could have an expected frequency higher than 1737 cm^{-1}. However, the distortion from the mean tetrapyrrole plane would diminish the vinyl ester effect.

The spin subsystem of the C3 substituent was difficult to interpret because it is novel and there are no known precedents for this group in tetrapyrroles. However, its uniqueness was also a major key to deciphering the structure. From the known empirical formula minus the structure elements that were readily assignable, one was left with a highly limited number of atoms to place, including the lone sulfur and two oxygen atoms. The doublet at 4.72 ppm has a chemical shift analogous to similarly placed β-pyrrolic protons in sirohydrochlorin. The remaining chemical shifts are reasonable considering the ring current and the effects of the electronegative heteroatoms.

Figure 4 displays the aliphatic region of **800** and a spin-coupled simulation of the epoxide−sulfoxide substituent. For convenience, the simulated peaks have been labeled a−e. The experimental portion was not from the most pure sample ever prepared, but it did show most clearly the doublet of doublets (peak e) at 2.85 ppm. The triplet at 4.52 ppm (peak c) overlaps the 7^{1a} acetate doublet. The 3.31 ppm doublet (peak d) was unfortunately always contaminated with an impurity peak, although this peak was variable in intensity in independent preparations. The coupling constants are reported in the figure caption. Vicinal couplings between the protons at 4.72 (b), 4.52 (c), and 5.00 (a) ppm were typical at 6.9 and 5.2 Hz. Long-range coupling (0.9 Hz) between the 5.00 ppm proton (a) and the 4.72 ppm proton (b) is not always expected in nonaromatic systems but is possible. Within the frame of the **800** structural hypothesis, a cis epoxide would seem most likely. The coupling constant 5.2 Hz for the assigned $J_{a,c}$ is in accordance with J_{cis} but not J_{trans}, because typical vicinal coupling constants in epoxides are 4.5 and 3.1 for J_{cis} and J_{trans}, respectively. Couplings between the 5.00 ppm proton (a) and the 2.85 ppm proton (e) (3.4 Hz) and 5.00 and 3.31 ppm (d) (0.9 Hz) were unusual in that both values were low. In consideration of the usual Karplus dihedral-angle relations, it is readily possible for one

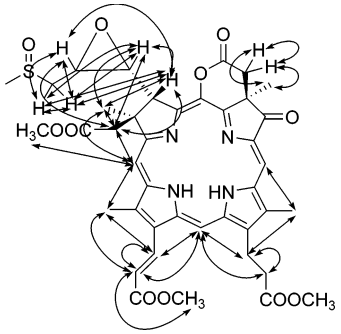
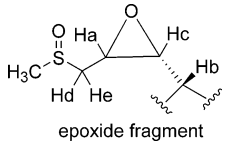


FIGURE 5: Summary of the nuclear Overhauser enhancements observed in the rotating-frame dipolar correlation or ROESY two-dimensional spectrum. The double-headed curved arrows indicate that an NOE was observed between those two protons. In the majority of cases, the requisite cross peak was observed on both sides of the diagonal, but in a few cases, it was observed only on one side because of the peak being weak or obscured by noise.

coupling to be small, but then the other is expected to be large. This anomaly is the weakest link in the spectroscopic-structural interpretation. There are currently no good model compounds for this type of spin subsystem, but distorted geometry between the epoxide and the sulfoxide might be responsible.

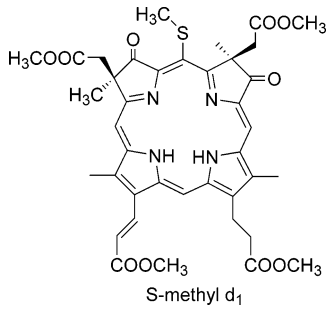


ROESY spectra were obtained to provide nuclear Overhauser enhancement data between nearest neighbor protons. Figure 5 summarizes the observed enhancements, which confirm the assignments originally made on the basis of chemical-shift homologies. The absence of NOEs from either the 1.79 ppm singlet or the 3.61/4.55 ppm geminal doublets to any other resonance fixes an oxo group at C8. Another oxo group cannot be accommodated by the empirical formula from the exact mass. The "missing" meso proton is relegated to the 5 position by placement of meso protons at the other positions via NOEs.

The ^{13}C chemical shifts for common substituents among **800**, d_1, and sirohydrochlorin match closely (*25*, *29*). The new ^{13}C features are consistent with the unusual epoxide−sulfoxide assigned to ring A. The *S*-methyl ^1H and ^{13}C shifts are slightly high compared to a simple model like dimethyl sulfoxide (2.5 and 40 ppm), but this could be due to the tetrapyrrole ring current. The geminal protons at 2.85/3.31 ppm are bonded to a carbon at 37.8 ppm, consistent with this methylene carbon being adjacent to electronegative groups (compare the acetates at 40−42 ppm and the propionate at 36 ppm). The epoxide carbons at 69.7 and 77.0 ppm are high compared to simple alkyl-substituted epoxides, but the increase could be due to the tetrapyrrole core and the sulfoxide.

A series of derivatization reactions on **800** provided additional evidence about its structure. When the molecular ion for the normal protic methyl ester derivative is compared to the molecular ion after esterification in deuterated methanol, it is possible to count the number of carboxylic acids present in the original tetrapyrrole (*17*). This experiment performed on **800** indicated that three carboxylic acids were present.

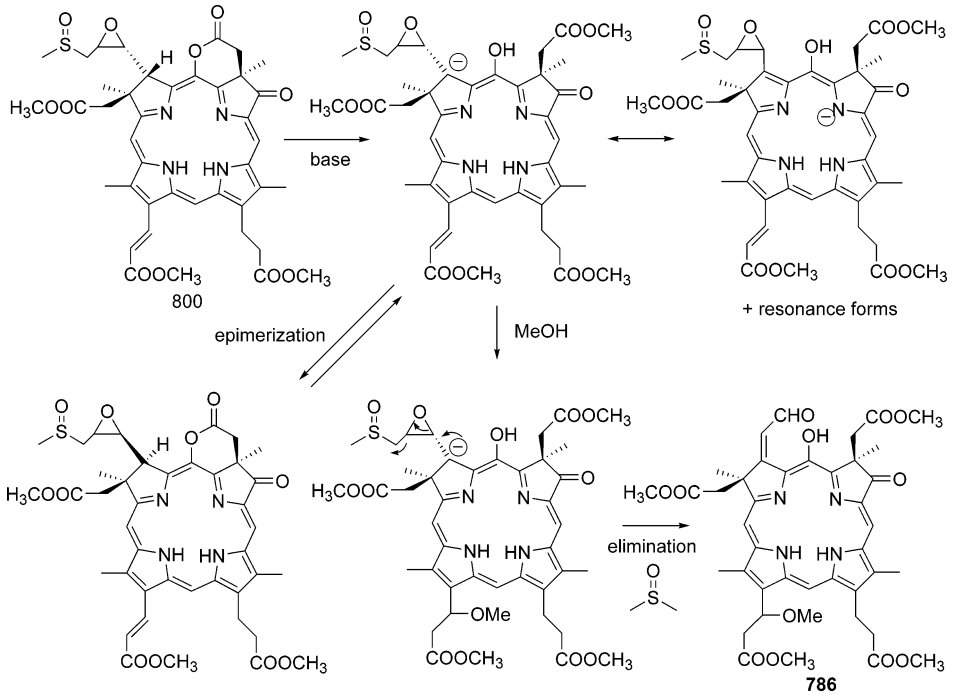
A small amount of **800** was treated with 3% hydrogen peroxide in methanol at room temperature for 20 h. This strong oxidant degrades the tetrapyrrole, but enough remained to afford a mass spectrum. This showed not only an 801 *m*/*z* peak for the unreacted **800** compound (as $M + H^+$), but also an intense 817 *m*/*z* peak corresponding to oxidation of the sulfoxide to the sulfone state. This reaction was patterned after a derivatization that had been instrumental in establishing the structure of the "des-meso" form of d_1. This naturally occurring form of d_1 was discovered during large-scale isolations of native d_1 and was subsequently shown to be d_1 with an *S*-methyl substituent replacing the five meso proton (*30*). In the case of *S*-methyl d_1, the peroxide treatment led to strong molecular ions 16 and 32 Da larger than the starting compound because of sulfoxide and sulfone formation. At the present time, it is impossible to see a clear chemical connection between *S*-methyl d_1 and **800**, but the commonality of *S*-methyl groups and substituted five meso positions suggests that there is such a connection.



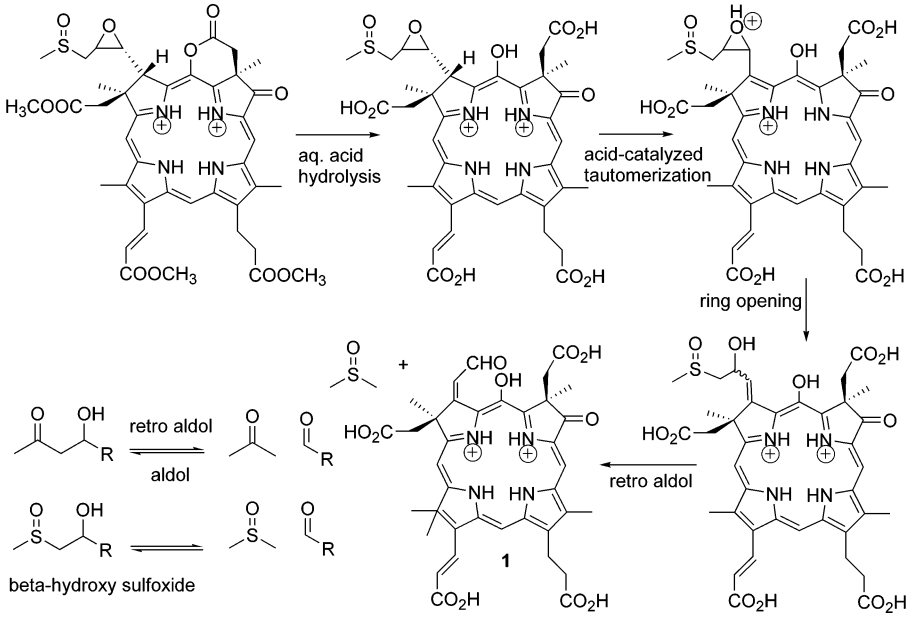
Refluxing **800** with a base in methanol (2% of 2.5 M NaOH (aq) and 98% methanol) destroyed most of the tetrapyrrole, but a new derivative was formed (crude estimated yield ca. 5−10%). Chromatography showed that the new compound was more hydrophobic that **800**. Mass spectrometry gave its molecular mass as 786 Da. Unfortunately, this harsh treatment destroyed most of the tetrapyrrole content, and the derivative product **786** could only be detected by high-sensitivity mass spectrometry. Scheme 2 rationalizes the appearance of this new compound. Base-catalyzed ring opening of the epoxide followed by subsequent retro-aldol condensation led to the α,β-unsaturated aldehyde shown as **786**. Although the geometry of the newly generated double bond is unknown, it was tentatively assigned *Z* (vide infra). The harsh conditions also led to Michael addition of methanol to the acrylate. When d_1 itself was subjected to the same methanol/base conditions, chromatography and mass spectrometry confirmed the addition of methanol.

Epoxides are generally labile toward acid. The current proposed ring must be moderately stable because compound **800** was prepared by mineral acid esterification of a mixture of natural carboxylic-acid-containing tetrapyrroles. The stability of the proposed epoxide to harsher acidic conditions was investigated with astounding results. Compound **800** was refluxed under anaerobic conditions in 3 M aqueous acid for 24 h (Scheme 3). These conditions also hydrolyze the esters, and the resulting acidic tetrapyrrole is difficult to analyze by chromatography and mass spectrometry. Therefore, after removing the aqueous acid, the product was re-esterified by mineral acid in methanol at room temperature. The resulting material had a visible absorption spectrum that

Scheme 2: Proposed Conversion of **800** in Basic Methanol



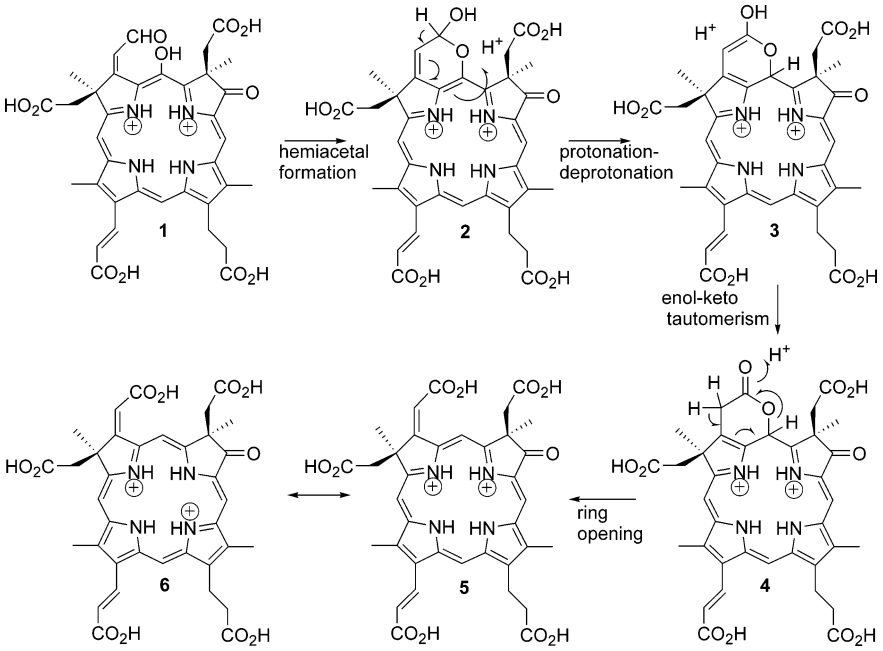
Scheme 3: Proposed Conversion of **800** in Hot Aqueous Acid



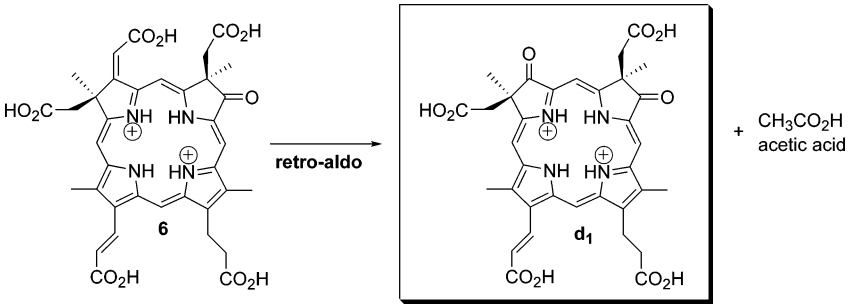
was nearly indistinguishable from that of native d_1 ester. Chromatography showed two products, one comigrating with natural d_1 ester and the other comigrating with **800**. Mass spectrometry gave two molecular ions corresponding to the protonated forms of d_1 methyl ester and **800**. In light of this major surprise, a significantly large relative portion of the available compound **800** was subjected to acid hydrolysis and re-esterification to obtain an NMR spectrum of the product. The spectrum was weak and contaminated in the aliphatic region, but the aromatic region showed the characteristic peaks of the meso and acrylate protons unique to d_1 and thus confirmed the conversion of **800** into d_1. This result was the basis for assigning the C2 and C7 stereocenters in **800** to the d_1 configuration.

Epoxides undergo ring opening in the presence of strong acid, and this was the initial and sole expectation for compound **800**. The complete transformation into d_1 was totally unexpected, but it can subsequently be rationalized by the following schemes. The pK_a values for the formation of the monocation of a tetrapyrrole and then the dication are expected to be in the range of 3−6 and 0.5−4, respectively (*31*). Therefore, in 3 M HCl, the dication is expected to be the dominant species, and the schemes reflect this. The first step is assumed to be hydrolysis of the six-membered lactone to the corresponding acid and alcohol. Acid-promoted elimination affords the same α,β-unsaturated aldehyde as postulated for compound **786**. While there is no evidence for the α,β-double-bond geometry shown, this

Scheme 4: Proposed Conversion of Aldehyde−Hydroxyl Compound into an α,β-Unsaturated Carboxylic Acid



Scheme 5: Conversion to d_1



would avoid steric interactions with C2 substituents and position the aldehyde for the next crucial steps to be discussed below.

Scheme 4 postulates one variation for the conversion of the α,β-unsaturated aldehyde into an α,β-unsaturated carboxylic acid. The first step is internal hemiacetal formation to **2** between the aldehyde and hydroxyl at C5 in **1**. In acidic medium, the proton shift results in migration of the diene leading to attachment of a hydrogen at C5 in **3**. Because extensive π delocalization of the tetrapyrrole is temporarily interrupted, this would likely be a slow step. Subsequent acid-catalyzed ring opening of **4** results in deoxygenation at C5 to afford **5**/**6**, where π delocalization is restored.

The α,β-unsaturated carboxylic acid **6** can then undergo retro-aldol conversion to acetic acid and the dication of d_1 as shown in Scheme 5. Removal of the allylic strain present in **6** could be the driving force for the retro-aldol reaction. Formation of the two very stable products (d_1 and acetic acid) may also help overcome other less favorable energetics in earlier steps.

Under physiological conditions in an anaerobic environment, enzymatic transformations could take the place of these steps or even follow a different reaction mechanism. For example, an α,β-unsaturated carboxylic acid might be formed as a result of the action of redox transformations linked to cofactors such as NAD^+/NADH and so forth. Regardless of the biomimetic merit of Schemes 4 and 5, they are presently offered to rationalize the observed conversion. To these authors, one of the most startling aspects of the conversion was the transformation of the meso hydroxyl group back to a meso proton. Normally, one would consider this as a redox reaction requiring a reducing agent. Strong aqueous acid is not typically considered a reducing agent. The apparent dilemma can be circumvented by the following simplistic view. The epoxide/sulfoxide is really a hidden or protected aldehyde, while the lactone is the same for a hydroxyl. The essence of the schemes is the net conversion of an aldehyde and hydroxyl into a carboxylic acid and water, which formally, could be considered as an internal redox reaction or disproportionation.

It is difficult to envision a sequence of nonenzymatic side reactions that would produce compound **800** from excess native d_1. It is possible that under this engineered situation a new tetrapyyrole was produced by a metabolic shunt. However, the astounding fact that it can be converted into d_1 albeit under nonphysiological conditions does suggest that it could be an intermediate produced from precorrin-2 on the pathway to d_1, whereby appropriate enzymatic activity takes the place of strong aqueous acid. Alternatively, the carboxylic acid form of **800** may not be the actual intermediate itself but is related to an authentic intermediate. For example, the lactone is likely to have formed spontaneously

from the proximity of the acetic acid and the meso hydroxyl group. This presumed accidental reaction probably traps the compound and allows us to isolate **800** in the first place. When the lactone forms, it blocks conversion into the unsaturated carboxylic acid **6** and stops d_1 formation. This could be why an **800**-like compound with ring B affected was not also found; the methyl group at C12 cannot form a lactone.

At the current time, it is difficult to hypothesize a sequence of enzymatically catalyzed events that would lead to compound **800**; therefore, in that sense, the question of how d_1 is biosynthesized is still not settled. However, the discovery of **800** now establishes a firm landmark, and all routes to d_1 must pass through or near this novel tetrapyrrole.

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