Structures and Transformations of the Bacteriochlorophylls *e* and Their Bacteriopheophorbides

Daniel J. Simpson and Kevin M. Smith*

Contribution from the Department of Chemistry, University of California, Davis, California 95616. *Received July 10, 1987*

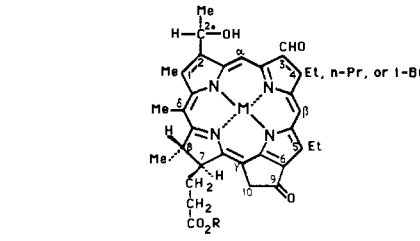
Abstract: Methyl bacteriopheophorbides *e* (Bpheo *e*) isolated from *Chlorobium phaeovibrioides* (NCIB no. 4832 and strain Bl-28) and *Br. zenitani* were investigated by reversed-phase high-performance liquid chromatography. Structures of individual Bpheo *e* homologues were examined by NMR spectroscopy and were found to be consistent with previous literature proposals, but with one exception. The stereochemistry at the chiral 2-(1-hydroxyethyl) position was found to change from primarily (*R*) for the 4-ethyl-5-ethyl homologue to primarily (*S*) for the 4-isobutyl-5-ethyl compound. This observation is consistent with what has been reported for the Bpheo *c* and Bpheo *d*. Identification of the 2-(1-hydroxyethyl) group in the Bpheo *e* was facilitated by their transformation into the Bpheo *c* without loss of diastereomeric purity.

The green and brown sulfur bacteria (Chlorobiaceae) are found in stagnant ponds, lakes, and estuarine habitats having vertical gradients of light (from above) and hydrogen sulfide (from below). They regularly form the lowermost layer of phototrophic organisms in the muddy or sandy sediment or stratified water underneath the layers of purple sulfur bacteria and algae.[1] These phototrophic bacteria carry out anoxygenic photosynthesis using only one photosystem. Therefore, they require electron donors of a lower redox potential than water, such as reduced sulfur compounds. Instead of oxygen the corresponding oxidized product (dehydrogenated donor) is sulfur or sulfate. These bacteria, among other things, play an important role in the detoxification of anaerobic decomposition material by converting toxic sulfide to sulfate.

The main light-harvesting antenna of green and brown sulfur bacteria are the bacteriochlorophylls (Bchls) *c*, *d*, and *e*, also known as the *Chlorobium* chlorophylls.[2] These Bchls, like other chlorophylls (Chls), are coordinated to a central magnesium ion. However, they differ from Chl *a* and *b* in several respects. They exist as mixtures of different alkyl homologues in the 4- and 5-positions (Bchl *e* contains *only* Et in the 5-position) and are generally (but not exclusively)[3] found with farnesol as the esterifying alcohol. Bchl *c*, *d*, and *e* also differ from the plant and algal Chls in that they lack a 10-methoxycarbonyl group, and the 2-position bears a chiral 1-hydroxyethyl group instead of the vinyl found in the plant and algal Chls. Bchl *c* and *e* differ further in that they feature a methyl group in the δ-meso position; in the Bchl *d*, as with other Chls, this position is unsubstituted. These compounds are generally characterized as the methyl bacteriopheophorbide (Bpheo) derivatives, which are obtained by treatment of the BChl with methanolic acid to transesterify the farnesyl ester and concomitantly replace the central magnesium with two protons.

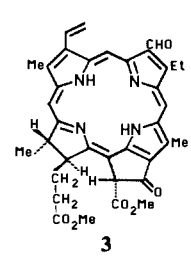
Like Chl *a*, the Bchls also contain three asymmetric centers, the 7-(*S*) and 8-(*S*) centers, which possess the same absolute stereochemistry[4] as the plant and algal Chls, and the 2-(1-hydroxyethyl) group, found only in these bacterial pigments. This functionality arises by enzymatic hydration of the 2-vinyl group, though reduction of a 2-acetyl (present in the Bchls *a* and *b*) is also possible. Initial reports on the structure of the Bchl *c*, *d*, and *e* found the absolute configuration at this center to be (*R*) for all homologues. However, recent work from our laboratory[5,6] has

Chart I



1, M = Mg, R = Farnesyl (BChl *e*)

2, M = 2H, R = Me (Bpheo *e*)



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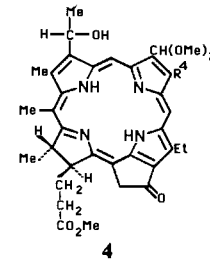

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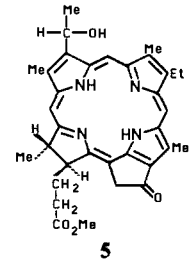

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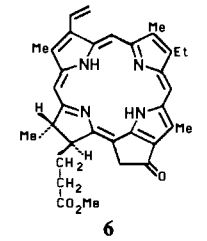

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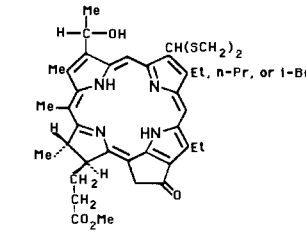

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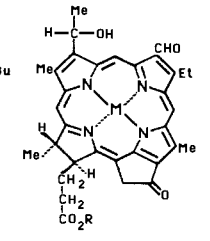

8 M = 2H, R = Me

9 M = Mg, R = Farnesyl

(1) Clayton, R. K.; Sistrom, W. R. *The Photosynthetic Bacteria*; Plenum: New York, 1978; pp 6-8.

(2) Vernon, L. P.; Seely, G. R., Eds. *The Chlorophylls*, Academic: New York, 1966.

(3) Caple, M. B.; Chow, H. C.; Strouse, C. E. *J. Chromatogr.* **1978**, *151*, 357-362; *J. Biol. Chem.* **1978**, *353*, 6730-6737.

(4) Brockmann, H., Jr. *Philos. Trans. R. Soc. London, Ser. B* **1976**, *273*, 277-285. Risch, N.; Brockmann, H., Jr. *Liebigs Ann. Chem.* **1976**, 578-583. Risch, N.; Kemmer, T.; Brockmann, H. Jr. *Liebigs Ann. Chem.* **1978**, 585-594.

(5) Smith, K. M.; Craig, G. W.; Kehres, L. A.; Pfennig, N. *J. Chromatogr.* **1983**, *281*, 209-223.

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shown that for Bpheo *c* and *d* the chirality of the 2-(1-hydroxyethyl) changes from (*R*) when the 4-substituent is small (e.g., R^4 = Et) to (*S*) when the substituent is more bulky (e.g., R^4 = *i*-Bu). With the assumption that the substrate chlorin molecule does not change its orientation on the enzyme surface, it is hypothesized that the vinyl group must rotate 180° prior to its hydration in the isobutyl series. This indicates that side-chain methylation steps in the biosynthesis of these Bchls precede vinyl hydration (or acetyl reduction) to give the 2-(1-hydroxyethyl).

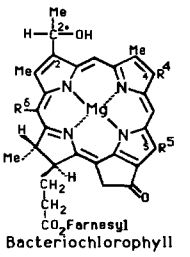
Structures of Bacteriochlorophylls *e*

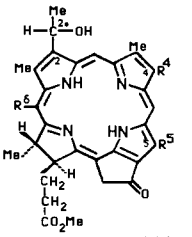
Bchl *e* (**1**; Chart I) isolated from *Chlorobium phaeobacteroides* and from *Chlorobium phaeovibrioides*, were first characterized by Brockmann in 1977.[4,7] The structure was determined primarily by spectroscopic techniques. Bpheo *e* (**2**) possessed an optical spectrum and chromatographic behavior quite different from those of the Bpheo *c* and *d* series pigments. The visible spectrum of Bpheo *e* was very similar to that of methyl pheophorbide *b* (**3**), taking into account the expected discrepancies due to presence of the δ-meso methyl group and absence of the 2-vinyl (replaced by hydroxyethyl in the Bpheo *e*). Mass spectrometry revealed that Bchl *e* was a mixture of at least three homologues, and farnesol was identified as the major esterifying alcohol. The outstanding feature of Bchl *e* was the formyl group in the 3-position. With respect to this group Bchl *e* is related to Bchl *c* in the same way Chl *b* is related to Chl *a*. The presence of a formyl group was confirmed by an IR absorption at 1655 cm^{-1}, a ^{13}C NMR signal at 188.0 ppm, and an aldehyde proton resonance at 11.01–11.07 ppm.

For additional structural confirmation, the 3-formyl group in Bpheo *e* was reduced to give a 2a,3a-diol. This diol when treated with Raney nickel gave a homologous series of compounds that were identical with the mixture of compounds obtained by Raney nickel reduction of Bpheo *c*. Bpheo *e* was also separated[4] by medium-pressure liquid chromatography and was further characterized by NMR and mass spectroscopy of the individual homologues. The stereochemistry of the 7- and 8-positions [(*S*),(*S*)] of Bpheo *e* were determined by comparing ORD and CD spectra of the 2-ethyl-3-methyl-Bpheo *e* derivative with the same series of derivatives obtained from Bpheo *c* of known absolute stereochemistry. The chirality of the 2-(1-hydroxyethyl) was reported as (*R*) for all Bpheo *e* based on the results of a modified Horeau analysis of a mixture of homologues.

Bearing in mind that the Bpheo *c*[5] and *d*[6a] both showed the same pattern of changing stereochemistry at the 2-(1-hydroxyethyl) group and the relative insensitivity of the Horeau analysis for small amounts of opposing stereoisomers, we decided that a more precise investigation of the stereochemistry at the 2-(1-hydroxyethyl) group in the Bpheo *e* was desirable. Using reversed-phase high-performance liquid chromatography (HPLC), as previously done with Bpheo *c*, it was our intention to determine whether or not the Bpheo *e* (and therefore the Bchl *e*) followed the same trends in stereochemical pattern as the Bpheo *c* and *d* (Figure 1).

Initial reversed phase HPLC work using Bpheo *e* (**2**) isolated from *Cb. phaeovibrioides* clearly showed six bands (Figure 2A), and not three bands as was expected.[8] The chromatogram appeared as a major series of three bands and a minor series of three bands (arrowed in Figure 2). However, subsequent HPLC work on other bacterial subcultures of the same strain showed that the relative intensities of the HPLC bands were not reproducible. As shown in Figure 2B the major and minor series initially observed were reversed with only trivial modifications of workup. Thus, the three additional bands could not be simple diastereomers of the existing homologues since one would not expect such random





	A *d* Series				B *c* Series			
	R^4	R^5	R^6	Config at 2a	R^4	R^5	R^6	Config at 2a
a	Et	Me	H	R	Et	Me	Me	R
b	Et	Et	H	R	Et	Et	Me	R
c	n-Pr	Me	H	R	n-Pr	Et	Me	R
d	n-Pr	Et	H	R	n-Pr	Et	Me	S
e	i-Bu	Me	H	S	i-Bu	Et	Me	R
f	i-Bu	Et	H	S	i-Bu	Et	Me	S
g	neoPn	Me	H	S				
h	neoPn	Et	H	S				

Figure 1. Structure of (A) the bacteriochlorophylls *d* and methyl bacteriopheophorbides *d* and (B) the bacteriochlorophylls *c* and methyl bacteriopheophorbides *c*.

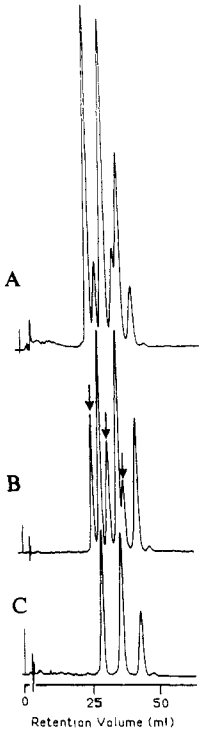


Figure 2. Reversed-phase HPLC chromatograms (conditions: Waters Associates RCM-100 module, 5-μm C-18 μBondapak cartridge, 12% water in methanol, 2.0 mL/min flow rate, detector set at 660 nm) of (A) methyl bacteriopheophorbides *e* from *Chlorobium phaeovibrioides*; (B) the same as in (A) but isolated at a different time, and (C) methyl bacteriopheophorbides *e* isolated from strain B1-28 (see text). This material was also identical with material from (A) or (B) which had subsequently been treated with aqueous acid. Variability of proportions of acetal seen are due to minor unavoidable and unintentional variations in procedures used for isolation of the pigments, and not to any difference in reactivity of the products. Arrowed peaks represent the homologous mixture of dimethyl acetals **4**; the small peak with largest retention volume is believed to be the 4-neopentyl-5-ethyl homologue.

diastereomeric ratios. Meanwhile our attention turned to a new strain of *Cb. phaeovibrioides* (strain B1-28) from the Ivory Coast, obtained via Professor Norbert Pfennig (Konstanz). The Bpheo *e* (**2**) extracted from this new strain showed, by HPLC, only three

(6) (a) Smith, K. M.; Goff, D. A. *J. Chem. Soc., Perkin. Trans. 1* **1985**, 1099–1113. (b) For a preliminary communication on the current Bchl *e* work, see: Smith, K. M.; Simpson, D. J. *J. Chem. Soc., Chem. Commun.* **1987**, 14–16.

(7) Brockmann, H., Jr.; Gloe, A.; Risch, N.; Trowitzsch, W. *Liebigs Ann. Chem.* **1976**, 566–577.

(8) Craig, G. W. Ph.D Dissertation, University of California, Davis, 1982.

bands (Figure 2C) which were base line resolved and had the same retention volumes as the less mobile series of bands (determined by co-injection) discussed earlier (Figure 2A,B). Milligram quantities of each of these three bands were collected by HPLC separation for NMR study (see Experimental Section), which identified these compounds, and thus the less mobile series of bands in the earlier work, as genuine Bpheo *e*.

Furthermore, when the six-band mixture (Figure 2A) was treated with 40% TFA/H_2O and then analyzed by HPLC, only three bands appeared. These three bands were shown by HPLC and NMR spectra to be genuine Bpheo *e*. Thus, the unidentified bands appeared to be an acid-labile artifact of the Bpheo *e* extraction process.

In the preparation of Bpheo *e*, crude Bchl *e* was treated with 3% sulfuric acid/methanol to demetalate and to transesterify the farnesyl ester. These reaction conditions should be sufficient to partially form the dimethyl acetal **4** of the 3-formyl group. The ^{1}H NMR spectrum, after HPLC separation, of the first and third of the unknown bands did not show any resonances in the aldehyde proton region but did show a new resonance in the methoxyl region that could be tentatively identified as the methyls of a dimethyl acetal. Because these spectra were not clean, further identification was necessary. Several attempts to form the dimethyl acetal of Bpheo *e* using conditions similar to the methanolysis conditions failed to yield any recognizable acetal, giving back only the original starting aldehyde. This problem was circumvented by a newer method for acetal formation.

A recent report[9] of a selective acetalization of formyl groups (with ethylene glycol) of several aromatic (and nonaromatic) keto aldehydes prompted us to attempt selective protection of the aldehyde with methanol and acidic alumina as a catalyst. We found that refluxing Bpheo *e* in a minimum amount of chloroform and excess methanol produced a marked change in the optical spectrum of Bpheo *e* in the 400–600-nm region, giving a spectrum similar to that of Bpheo *c*. Monitoring the reaction by infrared (IR) spectroscopy showed the aldehyde carbonyl band (1663 cm^{-1}) to gradually disappear during the course of the reaction, while no change in the 9-keto band, or the rest of the spectrum, was observed. The reaction as monitored by HPLC did not go to completion, probably because no steps were taken to remove water formed in the reaction mixture. The HPLC of the crude acetal product showed two series of bands identical in retention time with those observed in the original HPLC work (Figure 2A). The ^{1}H NMR spectrum of the crude product clearly suggested the dimethyl acetal product due to the diminished aldehyde protons (11.24, 11.21, 11.18 ppm), as well as the new α-meso protons (10.50, 10.45, 10.42 ppm), β-meso protons (9.60, 9.58, 9.57 ppm), and three protons (6.59, 6.55, 6.50 ppm) tentatively assigned as the 3a-H of the acetal. Thus, the most mobile series of HPLC bands is the result of acetal formation during the isolation of Bpheo *e* (Figure 2A,B) and is in no way related to the stereochemistry of the 2-(1-hydroxyethyl) group.

The stereochemistry of the 2-(1-hydroxyethyl) group in Bpheo *d* (Figure 1A) was determined[6a] by HPLC separation and X-ray crystallography. From these data it was established that in the separation of 2a-(*R*) and 2a-(*S*) diastereomers by reversed-phase HPLC, the (*R*) diastereomer is more mobile than the (*S*). This fact, along with high-field ^{1}H NMR spectroscopy, supplied the basis for the absolute configuration assignments for Bpheo *c* (Figure 1B).[5] It was in this same manner that we intended to determine the relative configuration of the Bpheo *e*.

The reversed-phase HPLC of Bpheo *e* was investigated under several different solvent conditions as well as with different reversed-phase columns, but in no instance was there any evidence of diastereomeric separation of any of the homologues. Furthermore, this was also shown not to be necessarily indicative of the absence of diastereomers because no separation was achieved when Bpheo *e* was previously treated under racemization conditions (80% TFA/water, made more acidic with H_2SO_4; vide infra). It was at this point that our attention turned away from HPLC separation and toward NMR as a means for elucidating the stereochemical problem.

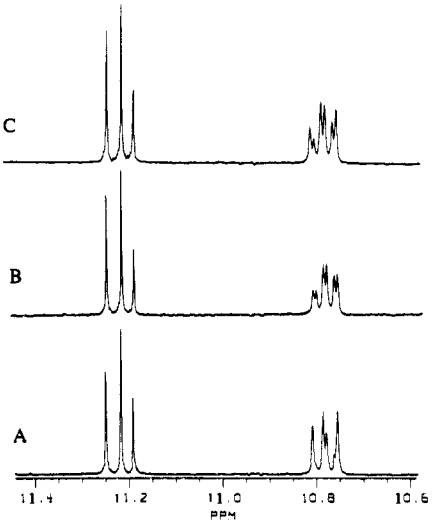


Figure 3. 360-MHz ^{1}H NMR spectra (in $CDCl_3$) of the aldehyde and α-meso protons in methyl bacteriopheophorbides *e*: (A) natural homologous mixture **2**; (B) as in (A) but racemized at the 2a center with aqueous acid; (C) as in (B) but spiked with natural mixture from (A).

Unlike with Bpheo *c* and Bpheo *d* (Figure 1A,B), the meso proton region of the NMR spectrum of a homologous mixture of Bpheo *e* (**2**) is sufficiently resolved at 360 MHz (Figure 3) to allow (*R*) and (*S*) assignments to be made. We were thus able to determine under what conditions the 1-hydroxyethyl group would racemize and, prior to racemization, if any of the homologues existed as more than one diastereomer, without separating individual homologues. The α-meso protons, being closest to the stereocenter of interest, were the most diagnostic for determining differences in chirality. Racemization was accomplished by treating Bpheo *e* (**2**) with 80% TFA/water which by necessity was made more acidic by adding sulfuric acid (approximately 10%). As seen in Figure 3B, the α protons were split into doublets for all three homologues in the NMR spectrum. Under the same conditions, 80% TFA in water alone was not sufficient to effect complete racemization, as evidenced by NMR. These results also demonstrated that at least two homologues (R^4 = Et and *n*-Pr) exist as a mixture of diastereomers. However, without HPLC separation of the diastereomers, these NMR data were inconclusive regarding the absolute stereochemistry. Attempts to crystallize Bpheo *e* (R^4 = Et) failed to produce crystals of sufficient quality for X-ray analysis, so our attention turned to synthetic methods.

Synthetic Conversion of Bpheo *e* (2) into Bpheo *c*

Because of chromatography problems and the lack of suitable crystals for crystallography, the only way left to determine the stereochemistry of the 2-substituent in the Bpheo *e* was by synthetic conversion of the Bpheo *e* into the Bpheo *c*, with complete retention of configuration at the site of interest. While the conversion only requires transformation of the 3-formyl into methyl, for which many standard procedures are available, this partial synthesis was severely limited by the other sensitive functionality present in the molecule.

It has been reported[4] that the 3-formyl group in the Bpheo *e* could be selectively protected with 1,2-ethanedithiol and *p*-toluenesulfonic acid. However, the subsequently performed Raney nickel reduction was intentionally done under conditions severe enough to cause transformation of the 2-(1-hydroxyethyl) into ethyl, with corresponding loss of the chiral center. Previous work[10] on the desulfurization of thioethers suggested that conditions much milder than those used by Brockmann[4] may be sufficient to effect selective reduction of a dithioacetal derivative of Bpheo *e*.

(9) Kamitori, Y.; Hojo, M.; Masuda, R.; Yoshida, T. *Tetrahedron Lett.* **1985**, *26*, 4767–4770.

(10) Smith, K. M.; Goff, D. A.; Simpson, D. J. *J. Am. Chem. Soc.* **1985**, *107*, 4946–4954.

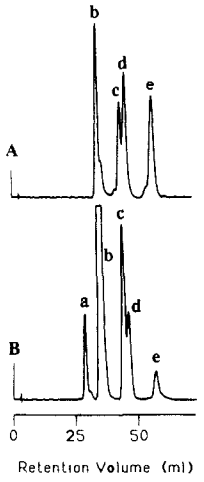


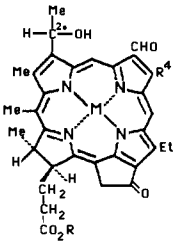
Figure 4. Reversed-phase HPLC chromatograms (conditions: Waters Associates RCM-100 module, 5-μm C-18 μBondapak cartridge, 12% water in methanol, 1.5 mL/min flow rate, detector set at 670 nm) of (A) methyl bacteriopheophorbides *c* produced by partial synthesis from the methyl bacteriopheophorbides *e* and (B) natural methyl bacteriopheophorbides *c* isolated from *Prosthecochloris aestuarii*.[5] Peak assignments: (a) 2a-(*R*),4-Et,5-Me Bpheo *c*; (b) 2a-(*R*),4-Et,5-Et; (c) 2a-(*R*),4-*n*-Pr,5-Et; (d) 2a-(*S*),4-*n*-Pr,5-Et; (e) 2a-(*S*),4-*i*-Bu,5-Et.

Therefore, we felt a two-step conversion, formyl through dithioacetal to methyl, would be the most effective procedure. We began by studying the reaction of a model compound, methyl 2-(1-hydroxyethyl)pyropheophorbide *a* (**5**),[11] to assess a limit to Raney nickel reduction conditions (time and temperature) for which the 2-(1-hydroxyethyl) group could be selectively retained. Compound **5** was heated at 40 °C in tetrahydrofuran with 20 mass equivalents of Raney nickel and monitored periodically by thin-layer chromatography. After 2 h, less than 10% of the hydroxyethyl group had been cleaved. Assuming that a dithioacetal reduces under similar conditions to a thioether, this model suggested that selective reduction was entirely plausible at temperatures of 40 °C or below.

Thus, Bpheo *e* (**2**) was treated with 1 equiv of 1,2-ethanedithiol and a total of 2.2 equiv of boron trifluoride etherate. This Lewis acid was chosen in place of *p*-toluenesulfonic acid because we were concerned that the latter might cause racemization of the 2-(1-hydroxyethyl) group. With boron trifluoride etherate, 2.2 equiv was necessary as there was no reaction with a stoichiometric amount. The crude dithioacetal **7** resulting from this reaction appeared to suffer decomposition during chromatography on silica gel, so it was characterized without purification. The visible absorption and IR spectra were similar to those of the dimethyl acetal **4** of Bpheo *e* (**2**), and the 360-MHz ^{1}H NMR spectrum showed the crude material to be only one product. There were no aldehyde protons, and a new set of signals at about 6.9 ppm was tentatively assigned as the 3a-H protons of the dithioacetal homologous mixture. Close examination of the α-meso proton resonances show that the original stereochemistry of the hydroxyethyl group has been retained such that no racemization with the Lewis acid had occurred.

The crude dithioacetal was converted into synthetic Bpheo *c* by treating with Raney nickel for 1 h at 40 °C. The product was purified on silica gel and characterized by spectrophotometry, IR, NMR, and mass spectroscopy, and HPLC. The 360-MHz NMR spectrum was consistent with the spectrum of Bpheo *c*. Conversion to Bpheo *c* was greater than 95% as judged by NMR spectroscopy, but a small amount of the dithioacetal was still present and could not be separated. Treating this mixture a second time with Raney nickel did not help and only decreased the yield. Because of coincidental chemical shifts of the α-meso protons no information about the stereochemistry could be inferred from the NMR spectra.

(11) Methyl 2-(1-hydroxyethyl)devinylpyropheophorbide *a* (**5**) and Bpheo *f* (**8**) were prepared from methyl pyropheophorbide *a* (**6**) and *b* (see **3**), respectively, by treatment with 30% HBr/acetic acid, followed by hydrolysis with 5% hydrochloric acid. Both compounds were characterized by NMR spectroscopy.



1, M = Mg, R = Farnesyl (BChl *e*)

2, M = 2H, R = Me (Bpheo *e*)

R^4	2a-(R)	2a-(S)
Et	95%	5%
n-Pr	40%	60%
i-Bu	2%	98%

Figure 5. Structural assignments of the bacteriochlorophylls *e* and methyl bacteriopheophorbides *e*. Percentages of 2a-(*R*) and 2a-(*S*) diastereomers are estimated from HPLC (assuming approximately equal extinction coefficients for the individual bands) and proton NMR spectroscopy and are considered accurate to within ±2%.

The reversed-phase HPLC of a natural mixture of Bpheo *c* clearly shows 2a-(*R*) and 2a-(*S*) separation for the R^4 = *n*-Pr homologue. When previously racemized, Bpheo *c* shows clear separation of 2a-(*R*) and 2a-(*S*) diastereomers for all homologues. The HPLC of the partially synthetic Bpheo *c* (Figure 4A) confirms that for Bpheo *e* (**2**) the ethyl homologue has, almost entirely, the 2a-(*R*) configuration. However, as evidenced by the ^{1}H NMR spectrum, the ethyl compound does exist with a small amount (<5%) of the 2a-(*S*) configuration. The *n*-propyl homologue of Bpheo *e* (as judged by the partially synthetic material) is approximately 40% 2a-(*R*) and 60% 2a-(*S*), unlike the Bpheo *c*, in which this pair is reversed. The isobutyl homologue is greater than 98% 2a-(*S*) as would be expected from the Bpheo *e* ^{1}H NMR spectrum and the stereochemistry of naturally occurring Bpheo *c* (R^4 = isobutyl). While we cannot rule out the 2% of 2a-(*R*) [*i*-Bu,Et] homologue being a result of racemization during methanolysis of crude Bchl *e* (**1**), this does establish an upper limit of the racemization for this step. Figure 5 shows the structures determined for the Bchl *e* and Bpheo *e* in the current study.

A small band (>1%) was consistently observed eluting after the 4-isobutyl-5-ethyl homologue in HPLC separations (Figure 2); this is believed to be the 4-neopentyl-5-ethyl homologue of the Bpheo *e*, a substituent pattern previously known [6a] only in the Bpheo *d* series. Work is under way to establish the identity of this fraction. After firmly establishing the final structural features of Bpheo *e* produced by strain B1-28, we investigated two additional strains (NCIB no. 4832 and *Br. zenitani*) also obtained from Professor Pfennig in Konstanz. The Bpheo *e* isolated from both additional strains were identical by HPLC (determined by coinjection) with that from strain B1-28. In addition, the 4-ethyl-5-ethyl homologue of strain NCIB 4832 was separated and found, by NMR spectroscopy, to be identical with the same homologue isolated from strain B1-28.

The expanded meso region of the high-field ^{1}H NMR spectra of Bpheo *e* (**2**) isolated from *Chlorobium phaeobacteroides* showed a significant resonance at 8.49 ppm. When the NMR sample was spiked with synthetic Bpheo *f* [Et,Me][11] (**8**), enhancement of this 8.49 ppm peak was observed. This observation led to speculation about the putative Bchl *f* (**9**), particularly since samples of the Bchl *c* have been shown to exist with trace contamination with Bchl *d*[6a,12] and because of recent observations concerning external environmental effects that induce meso methylation in the Bchl *d*/Bchl *c* pigments.[13] Contamination of Bpheo *d* might be responsible for the observed NMR signal, but this seems unlikely

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Table I. Growth Media for Bchl *e* Bacteria[a]

Basic Salts		Trace Element Solution	
KH_2PO_4	0.5 g	$ZnCl_2$	70 mg
NH_4OAc	0.73 g	$MnCl_2 \cdot H_2O$	100 mg
$MgCl_2 \cdot H_2O$	2.0 g	H_3BO_3	62 mg
NaCl	20.0 g	$CoCl_2 \cdot 6H_2O$	190 mg
$CaCl_2 \cdot H_2O$	0.1 g	$CuCl_2 \cdot 2H_2O$	17 mg
distilled H_2O	950 mL	$NiCl_2 \cdot H_2O$	24 mg
trace element solution	1 mL	$NaMoO_4 \cdot H_2O$	36 mg
		25% HCl	1.3 mL
iron solution	1 mL	distilled H_2O	1000 mL
vitamin B_{12} (2 mg/100 mL)	1 mL	Bicarbonate Solution	
		5% $NaHCO_3$	40 mL
Iron Solution		Sulfide Solution	
$FeCl_2 \cdot H_2O$	1.5 g	5% $Na_2S \cdot H_2O$	12 mL
25% HCl	6.5 mL		
distilled H_2O	1000 mL		

[a] The basic salt, bicarbonate, and sulfide solutions are autoclaved separately. After cooling they are combined and the pH is adjusted to 6.7–6.8. For cultures containing *D. acetoxidans*, absolute ethanol (1.0 g/L) is added, followed by innoculation.

when one considers that the separation of Bpheo *d* (R_f 0.38) and Bpheo *e* (R_f 0.30) by preparative TLC is trivial. Thus, while the 8.49 ppm resonance is not likely to be due to Bpheo *d* contamination, more evidence for the existence of Bchl *f* is necessary before any firm conclusions can be drawn. This work is in hand.

Conclusion

The Bpheo *e* (**2**) [and by analogy the Bchl *e* (**1**)] (Figure 5) from *Chlorobium phaeovibrioides* exist as three homologues, each of which are composed of different mixtures of diastereomers following the general pattern previously described for the 2a stereochemistry of the Bpheo *c* and Bpheo *d*.

Experimental Section

General. Electronic absorption spectra were measured on a Hewlett-Packard 8450A spectrophotometer using solutions in dichloromethane, and mass spectra were obtained by chemical ionization (methane, conditions give $[M + H]^+$) on a Finnigan 4510 mass spectrometer, interfaced with an Incos 2300 data system at the Woods Hole Oceanographic Institution (W.H.O.I.). IR spectra were likewise obtained on a Mattson Sirus 100 FT-IR spectrometer, run as thin films at 4-cm^{-1} resolution. ^1H NMR spectra were obtained at 360 MHz on a Nicolet NT-360 spectrometer; the chemical shifts are reported relative to $CHCl_3$ at 7.260 ppm. The phase "dried and evaporated" indicates drying with sodium sulfate, followed by evacuation with a Büchi rotary evaporator under house or oil pump vacuum.

Reactions were monitored by thin-layer chromatography (TLC) using cut strips (approximately 2 cm × 6 cm) of E. Merck silica gel 60 F254 precoated (0.25-mm thickness) plastic-backed sheets. Two types of packing material were employed in column chromatography: E. Merck neutral alumina (70–230 mesh) and Merck silica gel 60. The alumina was deactivated with either 6% H_2O (Brockmann grade III) or 15% H_2O (Brockmann grade V) before use. A 250-mL J. T. Baker column was used for flash chromatography. Preparative silica gel TLC was performed on 20 × 20 cm glass plates coated (1-mm thickness) with Kieselgel 60 PF254 (Merck). Analytical high-performance liquid chromatography (HPLC) was performed on a Waters Associates instrument equipped with a Model 6000A solvent delivery system, a Valco Model C6U injector, and a Perkin-Elmer LC55B variable-wavelength detector. A Waters RCM-100 module system equipped with a 5-μm C-18 μBondapak reversed-phase cartridge was used. The solvent systems used are specified where appropriate. All solvents were reagent grade and were filtered through a 0.45-μm Millipore filter before use. Deionized water was purified on a Mega-pure distillation apparatus prior to filtration.

Bacteria Growing Procedure. *Chlorobium phaeovibrioides* (Bchl *e*) and *Cb. phaeobacteroides* (Bchl *e*) were grown in salt solutions (Table I), prepared fresh each time. This method was utilized for subculture of 10-mL stab-cultures obtained from Professor N. Pfennig (Konstanz). The trace element solution was prepared separately and then combined with the basic salt ingredients (see Table I). The bicarbonate and sulfide solutions were prepared separately and these three salt solutions were autoclaved for 10–15 minutes (120 °C) and then cooled to room temperature. The bicarbonate and sulfide solutions and absolute ethanol were added to the basic salt solution with thorough mixing. The pH was adjusted to 6.75 with 25% sulfuric acid followed by the addition of growing bacteria. Growth on a large scale (20-L carboys) was carried out similarly except that the solutions were not autoclaved, thus necessitating the use of separate carboys for each of the different species of bacteria.

Table II. 360-MHz ^1H NMR Assignments (in $CDCl_3$) of Bpheo *e* Homologues (Strain B1-28) after Isolation by HPLC[a]

	4-Et,5-Et	4-*n*-Pr,5-Et	4-*i*-Bu,5-Et
3-CHO	11.20 (11.19)[b]	11.20 (11.21)[b]	11.20 (11.25)[b]
α-H	10.74	10.80	10.84
β-H	9.67	9.67	9.67
2a-H (q)	6.70	6.70	6.70
10-CH_2 (q)	5.24	5.27	5.26
8-H (q)	4.60	4.59	4.61
7-H (d)	4.20	4.20	4.20
5-CH_2	4.10 (m)	4.11 (q)	4.12 (q)
4-CH_2	4.10 (m)	4.06 (q)	3.98 (q)
δ-Me	3.88	3.88	3.88
7-OMe	3.60	3.60	3.60
1-Me	3.57	3.57	3.57
7-CH_2CH_2 (m)	2.55, 2.25	2.55, 2.27	2.54, 2.30
4a-CH_2 (m)		2.27	
2a-Me (d)	2.19	2.20	2.20
5a-Me (t)	1.96	1.97	1.97
4a-Me (t)	1.83		
4b-H (m)			2.30
8-Me (d)	1.53	1.55	1.50
4b-Me		1.27 (t)	1.25 (d)
NH	−1.57	−1.50	−1.50

[a] Chemical shifts are relative to $CHCl_3$ at 7.260 ppm. [b] Chemical shifts measured as component of homologous mixture.

Bacteriopheophorbide *e* Isolation Procedure. In general, the isolation of the Bpheo *e* required demetalation and transesterification. On a large scale (>20 L), the cells were conveniently obtained by Sharples centrifugation or on a small scale (2 L) by Sorvall centrifugation. After the wet cells were collected, they were combined with methanol and stirred (12 h) in the dark. The cell debris was then filtered and the crude "methanol extract" was evaporated to dryness with the aid of toluene as a chaser. The solid was dissolved in methylene chloride and then filtered to give a "methylene chloride extract", which was washed with brine and then dried over anhydrous sodium sulfate. After filtration and evaporation of solvent, the mixture was dissolved in 3–4% sulfuric acid in methanol and stirred for a period of 2–5 h (25 °C) in the dark. The mixture was then diluted with methylene chloride, washed with aqueous sodium bicarbonate and brine, and dried over anhydrous sodium sulfate. Filtration and evaporation gave a residue that could be purified by silica gel TLC (elution with 5% tetrahydrofuran–methylene chloride) developed over 2–5 h in the dark (small scale) or by Brockmann grade III alumina gravity column chromatography (large scale, elution with toluene–methylene chloride and tetrahydrofuran–methylene chloride). Generally, following column chromatography, the fractions containing the major BPheo *e* were purified again on silica TLC (5% tetrahydrofuran in methylene chloride) to give Bpheo *e*. Vis (relative absorbance): 444 nm (1.00), 538 (0.203), 576 (0.191), 604 (0.194), 660 (0.287) NMR (360 MHz, $CDCl_3$): see Figure 3 for aldehyde and meso proton region. Table II shows the 360-MHz ^1H NMR assignments (in $CDCl_3$) of the Bpheo *e* homologues (strain B1-28) isolated by HPLC (conditions as in Figure 2). IR ν(C=O) (relative absorbance): 1734 (0.56, ester), 1696 (1.0, 9-keto), 1663 cm^{-1} (0.81, 3-CHO). MS: *m/e* (%), $(M + 29)^+$ 637 (65), 651 (13), 665 (7); $(M + H)^+$ 609 (100), 623 (90), 637 (65); $[(M + H) - 18]^+$ 591 (40), 605 (33), 619 (12).

Methyl 3-(Dimethoxymethyl)bacteriopheophorbide *e* (3). Methyl bacteriopheophorbide *e* (**2**) (30 mg) was dissolved in chloroform (3 mL) and methanol (5 mL). Acidic alumina (25 mg) was added and this mixture was refluxed under nitrogen. After 6 h the product was cooled and the alumina was filtered off on a sintered-glass funnel. The product was obtained as a blue solid (23 mg) from *n*-hexane initially without purification. IR ν(C=O) (relative absorbance): 1735 (0.54, ester), 1693 cm^{-1} (1.00, 9-keto). After the crude material was examined by HPLC and IR, a portion of the product was purified by chromatography on preparative silica gel TLC (elution with 3% methanol–methylene chloride) and then reexamined by HPLC and NMR. Vis (relative absorbance): 420 nm (1.00), 524 (0.165), 556 (0.179), 608 (0.143), 644 (0.396). NMR (360 MHz, $CDCl_3$): 10.50, 10.46, 10.44, 10.42 (s, α-meso H); 9.60, 9.58, 9.57 (s, β-meso H); 6.59, 6.55, 6.50 (s, 3a-CH); 6.58 (m, 2a-CH); 3.72, 3.70 (s, 3b-OMe); −1.70 ppm (broad s, NH); the remaining assignments are analogous to methyl bacteriopheophorbide *e* discussed above.

Racemization of the 2a Chiral Center in Methyl Bacteriopheophorbide e (2). Methyl bacteriopheophorbide *e* (**2**) (15 mg) was dissolved in trifluoroacetic acid–water (9 mL/2 mL), and sulfuric acid (1 mL) was added. This mixture was stirred at 50 °C for 1 h under nitrogen. The reaction mixture was then diluted with methylene chloride and slowly poured into aqueous saturated sodium bicarbonate. The organic layer was further washed with bicarbonate and water and was dried over anhydrous sodium sulfate. The crude dried product was treated with excess ethereal diazomethane and then purified on alumina (Brockmann grade V; elution with methylene chloride). Vis (relative absorbance): 444 nm (1.00), 538 (0.199), 574 (0.182), 604 (0.181), 660 (0.268). NMR (360 MHz, $CDCl_3$): the meso proton region of this spectrum is shown in Figure 3B. The remaining assignments were entirely consistent with Bpheo *e* given above.

Ethanedithiol Ketal (6) of Methyl Bacteriopheophorbide e. Methyl bacteriopheophorbide *e* (**2**) (50 mg) was dissolved in chloroform (5 mL) and cooled to −15 °C (ethylene glycol/dry ice). Ethanedithiol (8 μL, 1.1 equiv) was added via a syringe followed by boron trifluoride etherate (10 μL, 1.1 equiv), which was also added dropwise by syringe. The reaction mixture was then allowed to warm to room temperature and stir overnight under nitrogen. With no apparent change in the visible absorption spectrum, the mixture was again cooled to −15 °C, 1 additional equiv of boron trifluoride etherate (10 μL) was added, and the reaction mixture was again stirred overnight under nitrogen. At this time spectrophotometry showed a dramatic hypsochromic shift of the Soret band from 444 to 426 nm. The reaction mixture was diluted with methylene chloride, washed with water, dried, and evaporated to dryness. Vis (relative absorbance): 426 nm (1.00), 528 (0.078), 562 (0.093), 608 (0.0739), 664 (0.286). IR ν(C=O) (relative absorbance), 1733 (0.56, ester), 1694 cm^{-1} (1.00, 9-keto). MS, *m/e* (%), $(M + 29)^+$ 713 (36), 727 (10), 741 (5); $(M + H)^+$ 685 (42), 699 (40), 713 (36); $[(M + H) - 18]^+$ 667 (55), 681 (40), 695 (25). NMR (360 MHz, $CDCl_3$): 10.92, 10.88, 10.85, 10.83, 10.81 (s, α-meso H); 9.54, 9.52, 9.49 (s, β-meso H); 6.95, 6.91, 6.88 (s, 3a-CH); 6.50 (m, 2a-CH); 5.22 (m, 10-CH_2); 4.58 (q, 8-H); 4.25–4.05 (m, 7-H, 4a- and 5a-CH_2); 3.85 (s, δ-meso Me); 3.78 (m, 3-SCH_2CH_2S); 3.60 (s, 7d-OMe); 3.50, 3.51 (s, 1-Me); 2.83 (broad s, 2a-OH); 2.50, 2.20 (m, 7-CH_2CH_2, 4b-CH_2, 4c-CH, 2b-Me); 1.95 (t, 5b-Me); 1.75 (t, 4b-Me); 1.50, 1.25, 0.85 (m, 8-Me, 4c-Me, 4d-Me); −1.60 ppm (broad m, NH).

Methyl Bacteriopheophorbide c. The dithioacetal derivative **6** (27 mg) was dissolved in methanol–tetrahydrofuran (30 mL/5 mL), to which was added Raney nickel (540 mg, 20 mass equiv) in a pH 10 slurry. This flask was sealed and heated while stirring for 1 h in the dark. The Raney nickel was filtered off on Celite and the solution was evaporated to dryness. The dried product was dissolved in methylene chloride (50 mL) and washed with water (2 × 50 mL), dried, and again evaporated to dryness. The product was purified by preparative TLC on silica gel (elution with 3% methanol–methylene chloride) and obtained as a solid from methylene chloride–*n*-hexane. Vis (relative absorbance): 414 nm (1.00), 520 (0.110), 552 (0.152), 612 (0.100), 670 (0.457). IR ν(C=O) (relative absorbance), 1735 (0.56, ester), 1689 cm^{-1} (1.00, 9-keto). MS, *m/e* (%), $(M + 29)^+$ 623 (60), 637 (13), 651 (10); $(M + H)^+$ 595 (85), 609 (100), 623 (60); $[(M + H) - 18]^+$ 577 (15), 591 (18), 605 (10). NMR (360 MHz, $CDCl_3$): 9.96, 9.95, 9.94 (s, α-meso H); 9.54, 9.52, 9.50 (s, β-meso H); 6.56 (m, 2a-CH); 5.26 (m, 10-CH_2); 4.59 (q, 8-H); 4.21 (d, 7-H); 4.12, 3.72 (m, 4a- and 5a-CH_2); 3.90 (s, δ-meso Me); 3.58 (s, 7d-OMe); 3.54 (s, 1-Me); 3.30 (s, 3-Me); 2.61 (broad s, 2a-OH); 2.54, 2.18 (m, 7-CH_2CH_2, 4b-CH_2, 4c-CH, 2b-Me); 1.96 (t, 5b-Me); 1.71 (t, 4b-Me); 1.50, 1.22 (m, 8-Me, 4c-Me, 4d-Me); −1.76, −1.78 ppm (broad s, N H).

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Rapid Protein Sequencing by the Enzyme–Thermospray LC/MS Method

Krystyna Stachowiak, Cheryl Wilder, Marvin L. Vestal, and Douglas F. Dyckes*

Contribution from the Department of Chemistry, University of Houston, Houston, Texas 77004.
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Abstract: A rapid on-line system for the analysis of protein sequences has been developed. This system uses a combination of immobilized enzymes, liquid chromatography columns, and a thermospray LC/MS. A denatured protein injected into this system is subjected to endopeptidase proteolysis, and the resultant fragments are separated chromatographically and analyzed by mass spectrometry, all in a continuous process. Sequence information on the endopeptidase fragments is generated by inserting a column of immobilized exopeptidase on-line, following the LC column. This causes the fragment in each chromatographic peak to be further digested as it emerges from the LC column and results in a set of sequence peptides. These peptides, which result from the loss of one, two, or more terminal amino acid residues, can all be logically related to the original fragment, and the partial sequence deduced. Through multiple combinations of endopeptidases, exopeptidases, and LC columns, it is possible to determine large portions of the protein sequence. This method is demonstrated for basic pancreatic trypsin inhibitor as the substrate and trypsin, chymotrypsin, aminopeptidase M, and carboxypeptidases B and Y as the immobilized peptidases. The positions of all of the tryptic and chymotryptic fragments of the trypsin inhibitor can be deduced, and fully one-half of the amino acid residues can be assigned to their correct positions on the basis of a series of ten experiments, none of which require over an hour of instrument time.

The determination of the sequence of a protein is the first step in a comprehensive understanding of how it works. The amount of information required and the frequent scarcity of material have made protein sequence determination a continuing challenge.

Edman's automation[1] of his classical chemical sequencing approach[2] was the first major step toward the development of rapid protein sequencing. The Edman method has now been extended to the picomolar level[3] and remains the principal technique for quick, sensitive, and reliable determination of sequence information from proteins. An entirely different approach, of sequencing proteins indirectly, through sequencing their genes,[4,5] has made it possible to generate massive amounts of primary sequence data,

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