Journal of Chromatography, 281 (1983) 209–223
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,214

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND STRUCTURAL ASSIGNMENTS OF THE BACTERIOCHLOROPHYLLS-*c**

KEVIN M. SMITH*, G. WAYNE CRAIG and LISA A. KEHRES
Department of Chemistry, University of California, Davis, CA 95616 (U.S.A.)
and
NORBERT PFENNIG
Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz 1 (F.R.G.)
(First received July 18th, 1983; revised manuscript received August 10th, 1983)

SUMMARY

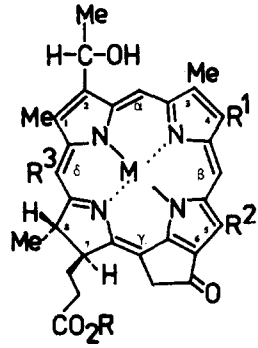
On the basis of high-performance liquid chromatographic retention times, separations, and nuclear magnetic resonance spectroscopy, structural assignments are made for the six fractions in the bacteriochlorophylls-*c* from *Prosthecochloris aestuarii* strain C.e. The origin of the previously unknown structural similarities between chromatographic bands 1 and 2, and between bands 3 and 4, is shown to lie in the nature of the absolute stereochemistry of the 2-(1-hydroxyethyl) group in these pigments, which affords chromatographically separable diastereomers. From pure colonies of the original *P. aestuarii* strain C.e., six strains (Nos. 1–6) are isolated, and these are shown to differ only slightly in the proportions of the six homologous pigments.

INTRODUCTION

Green and brown photosynthetic sulfur bacteria (Chlorobiaceae) produce a remarkable abundance of bacteriochlorophyll (BChl) pigments which are primarily used as light-harvesting antennae[2]. In the case of the green bacteria, two different series of BChls have been identified, and these have been designated[3] as the BChls-*c* and -*d*. Brockmann and co-workers have recently characterized two new sets of BChls, one from *Chlorobium phaeobacteroides* (or *C. phaeovibrioides*)[4,5], known as the BChls-*e*, and another from the green bacterium *Chloroflexus aurantiacus* (Chloroflexaceae)[6]; this last type is closely related to BChl-*c* (compound 1f), differing only from it with regard to the nature of the esterifying alcohol. Both the BChl-*c* and the BChl-*d* series of pigments appear to consist of at least six homologous bands[7,8], two new BChls-*d* having recently been identified to bring their number to eight[9], and the structures for the BChls-*c* and -*d* have been given as (1) and (2), respectively.

* Part of this work has been published as a preliminary communication, see ref. 1.

0021-9673/83/$03.00



(1) M = Mg; R = Farnesyl (2) M = Mg; R = Farnesyl

(3) M = 2H; R = Me (4) M = 2H; R = Me

		R^1	R^2	R^3			R^1	R^2	R^3
a	Band 1	i-Bu	Et	Et	a	Band 1	i-Bu	Et	H
b	Band 2	i-Bu	Et	Me	b	Band 2	n-Pr	Et	H
c	Band 3	n-Pr	Et	Et	c	Band 3	i-Bu	Me	H
d	Band 4	n-Pr	Et	Me	d	Band 4	Et	Et	H
e	Band 5	Et	Et	Me	e	Band 5	n-Pr	Me	H
f	Band 6	Et	Me	Me	f	Band 6	Et	Me	H

The structures of the various homologous fractions composing (1) and (2) have been the subject of controversy over the years[10–12]. Katz *et al.*[13] confirmed that the major esterifying alcohol in the BChls-*c* and -*d* is farnesol, though Caple *et al.*[14] revealed, for the BChls-*c*, that the pigments are esterified with minor amounts of five other alcohols. More recently still, it has been shown[15] that the BChls-*d* are likewise esterified with several different minor alcohols. The proportions of these alcohols appear to depend upon the age of the culture being studied.

In early work, Purdie and Holt[7] separated the methyl bacteriopheophorbide-*d* (BPheo-*d*) homologous mixture (4a–f) into six bands using liquid–liquid partition chromatography on Celite; however, this separation has since been shown to be inefficient[16]. Each compound in the mixture was shown to have a 1-hydroxyethyl substituent, a conjugated carbonyl group, and to belong to the "pyro"-chlorophyll series, *i.e.* to lack the 10-methoxycarbonyl substituent characteristic of Chl-*a*. The BPheo-*d* (4) (obtained by treatment of the BChls-*c* with methanol in sulfuric acid, thereby removing the chelating magnesium atom, and transesterifying the ester with methanol), were converted into the corresponding pyrroporphyrins, and these, as well as the BPheos, were oxidized with chromic acid to give maleimides which were identified by nuclear magnetic resonance (NMR) spectroscopy and gas–liquid chromatography. When all the evidence was analyzed, the structures shown in (2) were derived for the BChl-*d*[7]. These structural assignments were subsequently confirmed by Archibald *et al.*[17,18] and Smith[19], though the chirality of the 2-(1-hydroxyethyl) substituent has recently been readdressed[20].

Purdie and Holt[7] carried out similar work on the BPheo-*c* and separated the mixture again into six homologues (3a–f). The main difference between the BPheo-*c* and the BPheo-*d* lay in the presence of a *meso* alkyl group in the former, and this was assigned as methyl in all cases except for two bands. In these, ethyl was preferred, for no reason other than the fact that this would provide a means of making bands 1 and 2, and bands 3 and 4 different from each other in pairs. Since the maleimide degradation work showed bands 1 and 2 to have the same maleimides, and likewise for bands 3 and 4, it was deduced that the differences within these pairs must lie in some part of the molecule which could not be monitored by the maleimide degradation method, and this must be the *meso* carbon atoms, which were lost as carbon dioxide. Mass spectra run in Liverpool[19] failed to yield any evidence of *meso* ethyl groups, and moreover, indicated that the molecular weights of two of the BPheo-*c* were incorrect. As a result, the structural assignments for bands 1–4 were interchanged to give those shown for the BPheo-*c* in (3)[8]. Furthermore, none of the phylloporphyrin degradation products from the BChl-*c* could be identified with a synthetic *meso*-ethyl phylloporphyrin prepared by Cox *et al.*[21]. Synthetic work, however, did confirm the identities of fractions 5 and 6 [(3e) and (3f), respectively][17,18].

The precise position of the *meso* alkyl group in the BChl-*c* has also been a point of controversy. However, an abundance of evidence, both spectroscopic[4,22–25] and synthetic[17,18,21], firmly places the novel alkyl substituent at the delta-*meso* position.

The absolute stereochemistry in ring IV of the BChls-*c*, -*d* and -*e* has been established by Brockmann *et al.*[4] by way of chromic acid degradation to maleimides. In ring I, the chirality of the 2-(1-hydroxyethyl) has been shown to be *R* by Horeau analyses[25], NMR spectroscopy[25], and chemical degradation[4,26].

In our earlier work[27] the use of high-performance liquid chromatography (HPLC) for separation of the BPheo-*c* (3) was investigated; some separation of the mixture was apparent using normal-phase columns (μPorasil, Waters Assoc.), but this clearly was unsatisfactory for our purposes. Noting that Caple *et al.*[28] and Chow *et al.*[29] had obtained satisfactory preparative scale separations using an ingenious reversed-phase system, a breakthrough was achieved when reversed-phase HPLC separations (Fig. 1A) of the BPheo-*c* mixture from *Prosthecochloris aestuarii* were attempted. Clear separations between bands 6, 5, 4, 3 and 2 were apparent, but band 1 appeared to be absent from our sample of BPheo-*c*. Reversed-phase HPLC separations of the meso-BPheo-*c* (bearing an ethyl group at the 2-position, in place of 1-hydroxyethyl) surprisingly failed to reveal any separation of bands 3 and 4 in this series, and similar inability to separate these same bands was encountered with the 2-vinyl-BPheo-*c* series[27]. However, all other band separations in the 2-vinyl and 2-ethyl series were comparable with those observed for the 2-(1-hydroxyethyl) series (3) (Fig. 1A). At this stage it was not possible to establish why the comparative HPLC characteristics of the modified bands 3 and 4 had altered.

Procedures were developed[30] for the partial synthesis of the band 6 homologue (3f) from chlorin-e_6, a Chl-*a* degradation product. Fig. 1A shows the reversed-phase HPLC separation obtained for the complete mixture of BPheo-*c* homologues and isomers. In Fig. 1B, the trace shows the HPLC separation after spiking of the natural mixture with the synthetic band 6 material. It was anticipated that only the peak

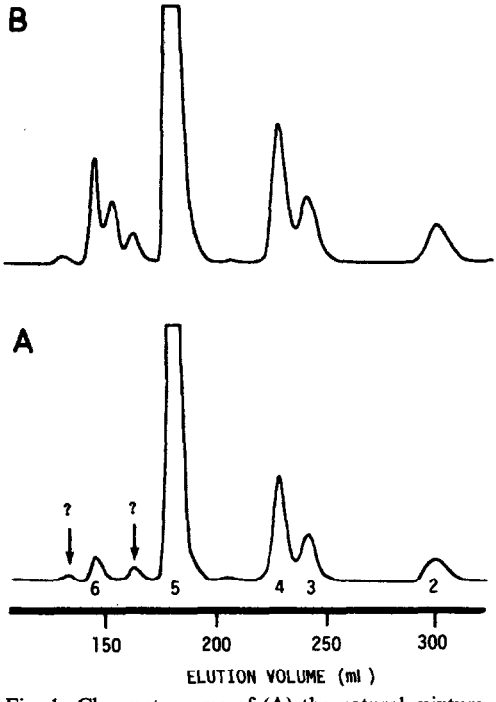
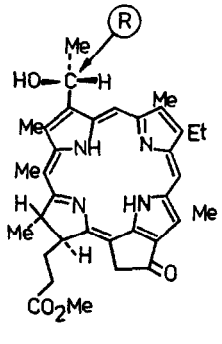
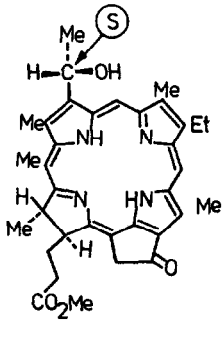


Fig. 1. Chromatograms of (A) the natural mixture of BPheo-*c* from *P. aestuarii* strain C.e.; numbers beneath the peaks represent band numbers as indicated in structure (3); (B) the natural mixture after spiking with synthetic [2-*R*,*S*]BPheo-*c* band 6^{30}. (Note the similar profile of the newly introduced peaks with the band 3/4 profile.) The flow-rate was 3 ml/min of methanol–water (85:15), and two μBondapak columns were used; back pressure was *ca.* 2000 p.s.i.

corresponding to band 6 would have increased in intensity but surprisingly, Fig. 1B shows two newly enhanced peaks. It was decided that the two peaks must be due to formation of the 2-*R* and 2-*S* forms of the 1-hydroxyethyl group during the final hydration of the vinyl in a synthetic precursor. The ease of the HPLC separation is understandable because the hydration affords diastereomers (5) and (6). These diastereomers were separated by HPLC and the 2-*R* diastereomer was satisfactorily iden-



(5)



(6)

tified with the natural material[30]; a similar partial synthesis has also recently been accomplished by Brockmann *et al.*[31].

It was clear that a definitive solution to the structural problem in the BChl-*c* series would require efficient separation of the intact mixture; in this paper we describe an extremely efficient reversed-phase HPLC method which accomplishes this, and which is used to assign structures to all six members of the BChl-*c* series. We also describe initial studies on the homogeneity of *P. aestuarii*, and resulting variability in the BPheo-*c* pigment composition.

EXPERIMENTAL

The bacterial culture was originally obtained from Dr. M. Evans (London) and was known as "*Chloropseudomonas ethylica*". We have identified it as a syntrophic mixture of *Prosthecochloris aestuarii* strain C.e. and the sulfur-reducing acetate-metabolizing non-photosynthetic bacterium *Desulfuromonas acetoxidans*[32]. The syntrophic mixed culture has been grown for *ca.* 15 years in our hands using a medium which was described elsewhere[10].

Typical preparation of BPheo-c (3)

Three 20-l volumes of culture medium[10] were inoculated with 600 ml of growing culture in each of three 20-l carboys. After stirring under fluorescent light tubes at 30°C for 10 days, the cells were collected by centrifugation (Sharpless centrifuge). The cell concentrate (sludge) was stirred in methanol (5 l) for 2 h, then filtered through a pad of sand on a sintered glass funnel. Evaporation of the solvent and treatment of the residue with 3% sulfuric acid in methanol (1 l) for 4.5 h accomplished both removal of the magnesium atom and transesterification of the farnesyl ester to methyl. The mixture was extracted with dichloromethane (1 l) which was washed with water (500 ml). (Note: emulsions invariably form but these can be broken by treatment with ethyl acetate and allowing to stand for several hours in the dark.) The dark purple organic layer was concentrated, dried (anhydrous sodium sulfate), and evaporated to dryness. The residue was chromatographed on alumina (Merck, Neutral; Brockmann Grade III, elution with a gradient of toluene with increasing amounts of dichloromethane) to accomplish separation of the fast-running carotenoids from the BPheo-*c*. Two faster running minor bands [2-(1-methoxyethyl) and 2-vinyl series] were also discarded in the chromatography. The purple eluates were evaporated to dryness, azeotroped with ether, and then foamed under high vacuum, to give 1.5 g of BPheo-*c* (3).

Analytical HPLC was used for the separation and subsequent analysis of milligram amounts of the BPheo-*c*; this was performed on a Waters Assoc. HPLC instrument equipped with a Model 6000A solvent delivery system, U6K injector and a Perkin-Elmer LC55B variable-wavelength detector (set at 660 nm). Two Waters semi-preparative μBondapak C_{18} columns (reversed phase, 300 × 7.8 mm I.D., 10-μm particle size) were employed for earlier separations, followed later by the use of the Waters Radial Compression Module (RCM-100) which gave comparable separations using Radial-Pak C_{18} cartridges (5-μm particle size). The eluent consisted of water–methanol (15:85). The methanol used was Mallinckrodt reagent grade while the water was twice-distilled (Dow-Corning "Megapure" automatic distillation sys-

tem) and then filtered through a Millipore filter (0.45 μm), as was the methanol.

Proton NMR spectra were measured at 360 MHz (Nicolet NT-360) in deuteriochloroform solution (concentration *ca.* 0.4 m*M*) with the residual chloroform peak at 7.258 ppm as internal standard.

Typical dehydration of the 2-(1-hydroxyethyl) group in a BPheo-c

Crude band 5 BPheo-*c* (3e) (50 mg) in benzene (40 ml) was refluxed under nitrogen for 20 min. *p*-Toluene sulfonic acid (270 mg) was added and the mixture was refluxed for a further 35 min before the solution was diluted with chloroform (50 ml) and then washed with saturated sodium bicarbonate solution (3 × 50 ml). The green solution turned brownish-purple, so the organic layer was washed with water (3 × 50 ml) before being dried (anhydrous sodium sulfate) and evaporated to dryness. The residue was dissolved in dichloromethane (10 ml) and treated with excess ethereal diazomethane; after evaporation of the solvents, the residue was chromatographed on alumina (Merck, Neutral, Brockmann Grade III, elution with dichloromethane–methanol, 50:1). The appropriate eluates were evaporated and the

TABLE I

360-MHz PROTON NMR SPECTRA OF BPheo-*c* AND 2-VINYL DERIVATIVES

Chemical shifts are given in δ (ppm); spectra were measured in C^2HCl_3.

	Band 6 (3f)	*Band 5 (3e)*	*Band 4 (3d)*	*Band 3 (3c)*	*Band 1 (3a)*	*Band 5 2-vinyl*	*Band 3(4) 2-vinyl*
β-H	9.50	9.50	9.53	9.52	9.50	9.53	0.52
α-H	9.94	9.86	9.94	9.96	9.95	9.49	9.48
2a-H	6.54	6.43	6.53	6.57	6.54	7.96	7.96
2b-H	–	–	–	–	–	6.28 6.14	6.27 6.13
10-CH_2	5.24	5.24	5.26	5.25	5.24	5.27	5.26
8-H	4.60	4.57	4.59	4.59	4.59	4.60	4.60
7-H	4.21	4.17	4.19	4.20	4.20	4.22	4.22
5a-CH_2	–	4.09	4.10	4.11	4.11	4.12	4.12
δ-CH_3	3.90	3.84	3.90	3.90	3.90	3.91	3.90
4a-CH_2	3.72	3.70	3.68	3.68	3.58	3.72	3.67
5-CH_3	3.69	–	–	–	–	–	–
7d-CH_3	3.64	3.60	3.59	3.58	3.59	3.59	3.59
1-CH_3	3.47	3.47	3.53	3.53	3.53	3.50	3.49
3-CH_3	3.29	3.27	3.29	3.29	3.30	3.27	3.25
2a-OH	2.65	2.60	2.63	2.65	2.64	–	–
7b-CH_2	2.50	2.49	2.51	2.52	2.52	2.53	2.53
7a-CH_2	2.20	2.17	2.18	2.19	2.19	2.21	2.19
4b-CH_2	–	–	2.17	2.17	2.52(CH)	–	2.15
2a-CH_3	2.14	2.11	2.16	2.17	2.20	–	–
5b-CH_3	–	1.96	1.96	1.95	1.96	1.96	1.96
4b-CH_3	1.71	1.71	–	–	–	1.72	–
8-CH_3	1.51	1.47	1.50	1.50	1.49	1.52	1.52
4c-CH_3	–	–	1.23	1.23	1.23	–	1.22
NH	0.88 −1.83	0.90 −1.84	0.90 −1.78	0.87 −1.76	0.88 −1.77	0.88 −1.62	0.87 −1.61

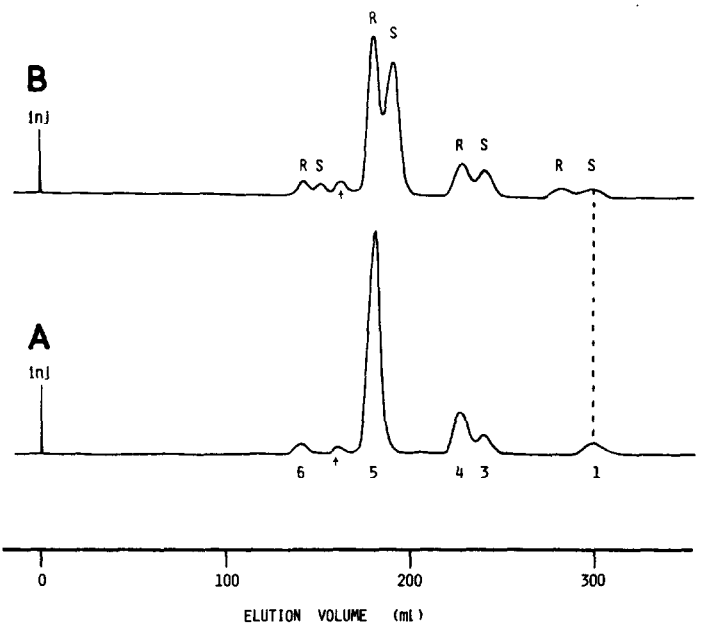


Fig. 2. Chromatograms of (A) the natural mixture of BPheo-*c* from *P. aestuarii* strain C.e. (conditions as in Experimental section). Note that the most-retained peak is now labeled as band 1, rather than band 2 as in Fig. 1 (A); (B) the natural mixture after racemization of the 2-(1-hydroxyethyl) group as described in the Experimental section.

residue was further chromatographed on thick-layer silicagel plates (20 cm × 20 cm × 1 mm thick plates; Merck GF 254; elution with methanol–dichloromethane, 2:98). The faster running band was collected and extracted from the silicagel using methanol–dichloromethane (5:95). The solution was evaporated and gave 10 mg of the required 2-vinyl-BPheo-*c*. The proton NMR spectrum of this material (and that of the corresponding compound from the BPheo-*c* bands 3 and 4) is recorded in Table I. An identical procedure was also used for the racemization of the intact mixture of BPheos-*c* (*cf.* Fig. 2B).

Typical rehydration of a 2-vinyl-BPheo-c

Crude 2-vinyl-BPheo-*c* (20 mg) was treated with 40% HBr–acetic acid (5 ml) and then stirred for 3 h at 55°C under nitrogen in the dark. The mixture was poured into water (100 ml) and then extracted into dichloromethane (3 × 50 ml). Since the aqueous phase was still green, it was treated with solid sodium bicarbonate (5 g) and then re-extracted with fresh dichloromethane (2 × 25 ml). The combined organic extracts were dried (anhydrous sodium sulfate), concentrated, and then treated with excess ethereal diazomethane. After evaporation, the residue was chromatographed on preparative silicagel plates (elution with methanol–dichloromethane, 5:95). The fastest running band (2-vinyl series) was discarded, and the second band was the desired racemized BPheo-*c* (yield typically 25–50%). (The complete dehydration–rehydration procedure can be carried out more efficiently by treatment of the BPheo-*c* with aqueous trifluoroacetic acid[20].)

RESULTS AND DISCUSSION

Structures of the BChl-c components of Prosthecochloris aestuarii strain C.e.

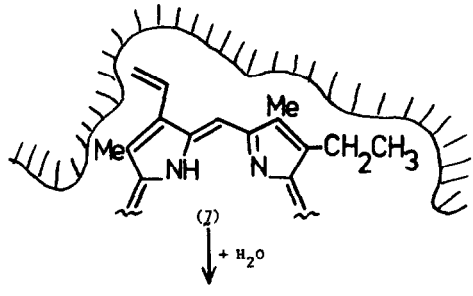
Multiple analytical HPLC injections allowed isolation of milligram amounts of the pure BPheo-*c* (3f–b) which were subjected to standard spectroscopic techniques. For example, Table I shows the proton NMR resonances and assignments of the BPheo-*c* fractions. Assignments of the peripheral substituents were worked out or confirmed by extensive proton decoupling experiments. The NMR work clearly established once more that no fractions bearing a meso ethyl group are present in our samples. Of particular importance is the fact that the proton NMR spectra from BPheo-*c* bands 3 and 4 [*i.e.* (3c) and (3d), using Holt's structural assignments, which are certainly suspect for (3c)], demonstrated that these two bands possess identical peripheral substituents. The conclusions based on the NMR spectra were therefore confusing since it was not clear how a pair of BPheo-*c* compounds could have identical substituents but different chromatographic retention volumes. A solution to the longstanding problem associated with the structural assignments for the BChl-*c* homologues and isomers became possible when a comparison of HPLC retention volumes was combined with a consideration of the NMR results described above. Inspection of the HPLC pattern for the synthetic BPheo-*c* band 6 diastereomeric mixture (Fig. 1B) and comparison of it with the pattern observed for the band 3 and 4 homologues (3c,d) indicated a relationship between the compounds in these pairs. If the BPheos-*c* (3c) and (3d) differed by the presence of an extra methyl group somewhere in the molecule (3c) then one would have expected a greater difference in their HPLC retention volumes; *i.e.*, a difference somewhat akin to that between bands 5 and 4 (Fig. 1A) would have been anticipated. Instead, the difference in retention volume between (3c) and (3d) is comparable to the difference, shown in Fig. 1B, between the diastereomeric pair (5) and (6).

With the assumption that the structural difference between BPheo-*c* (3c) and (3d) rests solely in the chirality of the 2-(1-hydroxyethyl) substituent, the individually purified chromatographic bands 3 and 4 were studied. Dehydration of the 2-substituent (*p*-toluene sulfonic acid in *o*-dichlorobenzene) in each compound gave the corresponding 2-vinyl derivatives, which were subjected to 360-MHz proton NMR spectroscopy. Comparison of two spectra showed the compounds from (3c) and (3d) to be identical; as might have been expected, other spectroscopic data and thin-layer chromatographic characteristics also proved to be identical. Thus, the difference between the band 3 and 4 BPheo-*c* was definitely demonstrated to lie in the nature of the 2-substituent. This conclusion enabled the earlier HPLC work[27] on the 2-vinyl and 2-ethyl BPheo-*c* derivatives to be understood; the reason for the lack of HPLC differences between the band 3 and 4 ethyl (and vinyl) derivatives was because once the vinyl is produced from 1-hydroxyethyl, or is further modified to give ethyl, bands 3 and 4 become identical.

Absolute proof of these new structural assignments was achieved through a simple set of experiments. The intact BPheo-*c* mixture (3) was dehydrated to give the 2-vinyl derivatives and these were rehydrated using Fischer's procedure[33] to give the BPheo-*c* mixture which should consist of approximately equal proportions of 2-*R* and 2-*S* diastereomers for each of the five (or six) chromatographic fractions observed by Holt. (We have recently demonstrated[20] that the 2-(1-hydroxyethyl) can be di-

rectly racemized by heating in aqueous trifluoroacetic acid without the need for stepwise preparation of the 2-vinyl derivative.) Fig. 2A illustrates the reversed-phase HPLC trace of the intact unmodified BPheo-*c* mixture (3). In Fig. 2B the HPLC trace of the racemized mixture is presented. Doubling up (due to the *R,S* racemization) of the peak originally assigned to bands 6 and 5 is immediately obvious. However, no similar doubling of the bands 3 and 4 peaks is apparent. What has happened, however, is that in Fig. 2B, the peaks corresponding to bands 3 and 4 have equalized in size, as would have been expected for a *R* to *S* racemization. This experiment definitively established the relationship between BPheo-*c* (and, by inference, in BChl-*c*) bands 3 and 4.

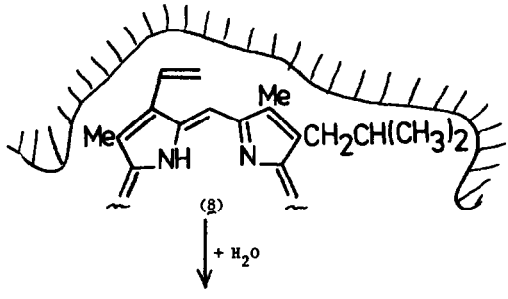
A more surprising conclusion from Fig. 2B can be drawn concerning the chiral nature of the 2-(1-hydroxyethyl) substituent in the BPheo-*c* band 1, 2 pair. It had tacitly been assumed in all of our studies that the most retained compound in the reversed-phase HPLC (*e.g.* Fig. 1A) was due to band 2 in the BPheo mixture, and that band 1 was being produced only in unobservable amounts. What the racemization experiment reveals is that the naturally observed, highly retained peak in Fig. 2A corresponds to BPheo-*c* band 1. Thus, it is band 2 which is absent in the mixture of BChls-*c* from *P. aestuarii*. On the basis of the fact that the *R* -diastereomer always travels faster (on reversed-phase) than the *S*-isomer (Fig. 2B), the conclusion can be made that the 4-isobutyl-5-ethyl-BPheo-*c* which is found in the natural mixture has the unexpected 2-*S*-(1-hydroxyethyl) orientation. This conclusion points to an ex-



(7)

+ H_2O

Bands 6,5,4 and 2
(1f,e,d,b)



(8)

+ H_2O

Bands 3 and 1
(1c,a)

tremely interesting biosynthetic possibility; as the 2-vinyl biosynthetic precursor is enzymically hydrated to give 2-(1-hydroxyethyl), the chirality of the newly generated center changes from *R* when the 4-substituent is small [*e.g.* ethyl (3e,f)] to *S* when the same substituent is more bulky [*e.g.* isobutyl, (3a,b)]. This observation allows us to make some proposals about the spatial requirements in the hydrating enzyme. With the assumption that the substrate chlorin molecule (at a very late stage in the biosynthesis of the BChl-*c*) does not change its orientation on the enzyme surface, the vinyl group must rotate 180° prior to its hydration in the isobutyl series. It must therefore be that the bulky isobutyl group at the 4-position causes, through protein contacts at the enzymatic site, the 2-vinyl to change from the orientation shown in (7) to that in (8) (or *vice versa*). Enzymatic hydration of the vinyls in a biosynthetic precursor would lead to formation of chiral centers with the opposite absolute configuration for (7) compared to (8). An alternative explanation could be that the precursor is present at the active site in an orientation which differs by rotation 180° about the alpha/gamma *meso* axis, and such orientational heterogeneity has been observed in a variety of native and reconstituted heme proteins[34–37]; on balance, however, we favor vinyl rotation rather than reorientation of the whole precursor molecule. Whatever the biosynthetic origin of this difference in chirality, however, it indicates that the 2-(1-hydroxyethyl) group is formed from vinyl *after* and not before the elongated 4- and 5-substituents are fashioned; thus, the side-chain methylation steps in the biosynthesis of the BChls-*c* and -*d* appear to be early[10,23] rather than very late[38,39] events.

A definitive confirmation of the steric dependence of chirality at the 2-(1-hydroxyethyl) has been provided[20] for the BChl-*d* series, in which single-crystal X-ray studies have confirmed that the chirality at the 2-substituent depends upon the size of the group at the 4-position, with a large 4-substituent favoring the 2-*S* absolute stereochemistry.

The above conclusions, with the benefit of hindsight, could have been drawn from literature information. Cox *et al.*[21] found that the X-ray powder photograph for their synthetic band 4 phylloporphyrin methyl ester was similar to that for natural band 3, and commented that "...the obvious conclusion is that the differentiation between fractions 3 and 4 is mythical". These workers were referring to the BChls-*c*, and it is ironic that though there is a difference between BChl-*c* fractions 3 and 4, there exists no difference between the phylloporphyrin derivatives for bands 3 and 4, upon which their X-ray powder photographs were measured. Yet more literature

TABLE II

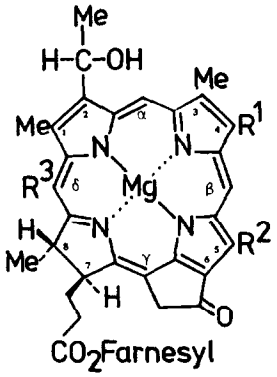
MELTING POINTS OF PHYLLO- AND PYRROPORPHYRIN DEGRADATION PRODUCTS FROM BChl-*c**

Chromatographic fraction	*δ-Phylloporphyrin methyl esters (needles from chloroform–methanol)*	*Pyrroporphyrin methyl esters (needles from diethyl ether)*
Band 3	196–199°C	205–208°C
Band 4	195–197°C	205–208°C
Band 5	215–215.5°C	–
Band 6	209–213°C	241°C

* See ref. 8.

TABLE III

NEW ASSIGNMENTS FOR BChl-*c*



		R^1	R^2	R^3	*Confign. at position 2*
(1a)	Band 1	Isobutyl	Ethyl	Methyl	*S*
(1b)	Band 2	Isobutyl	Ethyl	Methyl	*R*
(1c)	Band 3	*n*-Propyl	Ethyl	Methyl	*S*
(1d)	Band 4	*n*-Propyl	Ethyl	Methyl	*R*
(1e)	Band 5	Ethyl	Ethyl	Methyl	*R*
(1f)	Band 6	Ethyl	Methyl	Methyl	*R*

confirmation is available; Table II shows the melting points for various phylloporphyrin and pyrroporphyrin methyl ester degradation products from the BChl-*c*. Again with hindsight it is possible to see that the phylloporphyrins and pyrroporphyrins (all of which bear a 2-ethyl group and therefore are devoid of the differentiating 1-hydroxyethyl) have almost identical melting points. Presumably Holt *et al.* never carried out mixture melting points for the bands 3 and 4 phylloporphyrins or pyrroporphyrins. For comparison, the melting points for other bands, where available from the literature, are included in Table II.

Table III shows the new assignments for the BChl-*c* homologues and isomers. Assignment of the 2-*S*-absolute configuration to BChl-*c* bands 1 and 3 is contrary to previous degradative and spectroscopic studies[25,26]. However, the Horeau method is not quantitative and would not have detected small amounts of 2-*S*-diastereomer if it had been present.

Studies of pigment composition of six different subcultures of the original P. aestuarii strain C.e.

HPLC analysis following prolonged growth of *P. aestuarii* strain C.e. after 20, 37 and 60 days showed no significant change in the proportions of the homologous bands but rather only a decreased amount of isolated pigment. In order to test the original *Prosthecochloris aestuarii* strain C.e. for homogeneity, an agar shake dilution series was prepared, from which six different well-separated green colonies were isolated and subcultured as strains 1–6 (see Table IV). Each of the six *P. aestuarii* strains

TABLE IV

PERCENTAGES OF BPheo-*c* FRACTIONS (BANDS) OBTAINED FROM STRAINS 1–6 ISOLATED FROM *P. AESTUARII* STRAIN C.e.

Pigment percentages were estimated by integration of the areas beneath HPLC peaks, and assume identical extinction coefficients for all fractions. Where peaks were overlapping, samples were subjected to recycle before integration.

BPheo-c fraction or band	*Holt's value (%)**	*Original C.e. value (%)***	*Strain****					
			1	*2*	*3*	*4*	*5*	*6*
1 (3a)	0.5	4.5	6.0	4.1	7.2	4.0	6.1	4.0
2 (3b)	0.5	<0.1	0.4	0.2	1.7	1.4	0.7	0.2
3 (3c)	2.0	5.3	5.3	6.7	7.0	5.8	6.3	5.4
4 (3d)	16.0	18.3	25.3	17.0	26.7	23.2	25.2	18.1
5 (3e)	71.0	71.7	62.6	71.8	55.7	64.2	61.0	72.1
6 (3f)	10.0	0.2	<0.4	<0.2	<1.7	<1.4	<0.7	<0.2

* See ref. 8.
** See ref. 27.
*** Values are in per cent.

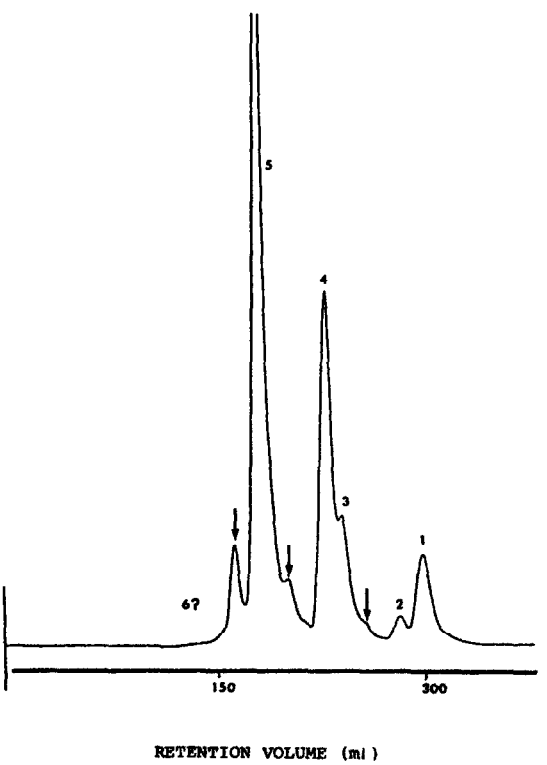


Fig. 3. Chromatogram of the natural mixture of BPheo-*c* from *P. aestuarii*, strain 3. "Impurity" peaks are arrowed.

was inoculated from a pure culture of *Desulfuromonas acetoxidans* strain 5071 in order to reconstitute well-growing syntrophic cultures. Subcultures of these six *P. aestuarii* strains were used as inocula for 20-l carboy cultures from which the BChls and BPheos were isolated by the normal procedure and then subjected to HPLC analysis. It was conceivable that each *P. aestuarii* strain 1–6 might preferentially synthesize one or more of the homologous bands. Analysis by HPLC gave the results shown in Table IV. Comparison with previous data of Holt showed that overall, the present cultures (both strain C.e. and strains 1–6) produced less of band 6 (3f, *i.e.* 4-ethyl, 5-methyl), indicating a more active methylating enzyme system in our culture. Furthermore, comparison with earlier data[27] from this laboratory showed that strain 6 possessed comparable ratios of each of the bands, while strain 3 was "abnormal" in that it was significantly decreased in band 5 (3e, 4-ethyl, 5-ethyl) with concomitant increases in bands 1 (3a, 4-isobutyl, 5-ethyl), 2 (3b, 4-isobutyl, 5-ethyl), and 6 (3f, 4-ethyl, 5-methyl), the first two further implicating a more active methylating enzyme system present.

Further analysis of the BPheos from strains 1–6, as well as the original strain C.e. revealed slight shoulders near the base of each band indicating the presence of another series of homologues. Fig. 3 shows the HPLC trace for strain 3, while Fig. 4 presents the trace, at high loading, for the original strain C.e. mixture. Comparison (Fig. 5) of retention volumes with those of BPheo-*d* by coinjection verified that the

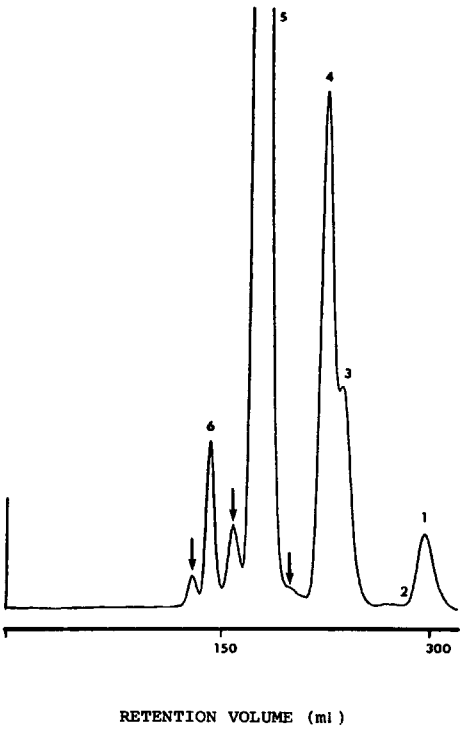


Fig. 4. Chromatogram (heavy loading) of the normal *P. aestuarii* strain C.e. culture. "Impurity" peaks are arrowed.

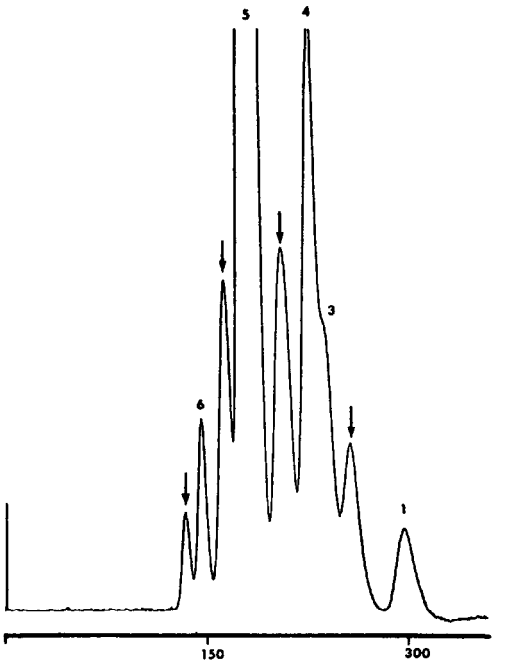


Fig. 5. Chromatogram of the natural mixture of BPheo-*c* from *P. aestuarii* strain 3, after spiking with the BPheo-*d* from *Chlorobium vibrioforme*[20]. Peaks identified with an arrow in Figs. 3 and 4 are increased in intensity, confirming their identity as BPheo-*d*.

base shoulders correspond throughout in each of the six strains and in the strain C.e. (Figs. 3, 4 and 5). Since the individual strains 1–6 originated from single pure colonies, we can eliminate the possibility that *P. aestuarii* strains 1–6 are contaminated with a BPheo-*d* producing strain. Furthermore, isolation of a minor quantity of a neighboring band ahead of band 6 (labeled as impurity in Fig. 1A) and analysis by 360-MHz NMR indicated the presence of three *meso* protons, supporting evidence for a BPheo-*d*[40]. These observations may indicate that BChl-*d* are biosynthetic precursors of the BChl-*c*, and this possibility is currently being tested. Elsewhere, Richards and Rapoport[39] have observed BChl-*d* in their BChl-*c* containing cultures. Attempts to resolve these shoulders by HPLC on recycle were fruitless. It should be noted that the impurity fraction in Fig. 1A does not split into a doublet (Fig. 2B) upon racemization of the 2-(1-hydroxyethyl) group. This result is in accord with earlier observations[30] that HPLC separation of BPheo-*d* diastereomers is very much more difficult than those in the BPheo-*c* series.

ACKNOWLEDGEMENTS

We thank the National Science Foundation (CHE-81-20891) for support of this research and Research Corporation for a grant to purchase the HPLC system. Thanks are also extended to Dr. H. D. Tabba for measurement of proton NMR spectra.

REFERENCES

1 K. M. Smith, L. A. Kehres and H. D. Tabba, *J. Amer. Chem. Soc.*, 102 (1980) 7149.
2 L. P. Vernon and G. R. Seely, *The Chlorophylls*, Academic Press, New York, 1966.
3 A. Jensen, O. Aasmundrud and K. E. Eimhjellen, *Biochim. Biophys. Acta*, 88 (1964) 466.
4 H. Brockmann, Jr., *Phil. Trans. Roy. Soc., London, Ser. B*, 273 (1976) 277.
5 H. Brockmann, Jr., A. Gloe, N. Risch and W. Trowitzsch, *Justus Liebigs Ann. Chem.*, (1976) 566.
6 N. Risch, H. Brockmann, Jr. and A. Gloe, *Justus Liebigs Ann. Chem.*, (1979) 408.
7 J. W. Purdie and A. S. Holt, *Can. J. Chem.*, 43 (1965) 3347.
8 A. S. Holt, J. W. Purdie and J. W. F. Wasley, *Can. J. Chem.*, 44 (1966) 88.
9 K. M. Smith, D. A. Goff, J. Fajer and K. M. Barkigia, *J. Amer. Chem. Soc.*, 105 (1983) 1674.
10 G. W. Kenner, J. Rimmer, K. M. Smith and J. F. Unsworth, *J. Chem. Soc., Perkin Trans. I*, (1978) 845.
11 J. H. Mathewson, W. R. Richards and H. Rapoport, *Biochem. Biophys. Res. Commun.*, 13 (1963) 1.
12 J. H. Mathewson, W. R. Richards and H. Rapoport, *J. Amer. Chem. Soc.*, 85 (1963) 364.
13 J. J. Katz, H. H. Strain, A. L. Harkness, M. H. Studier, W. A. Svec, T. R. Janson and B. T. Cope, *J. Amer. Chem. Soc.*, 94 (1972) 7938.
14 M. B. Caple, H. C. Chow and C. E. Strouse, *J. Biol. Chem.*, 253 (1978) 6730; M. B. Caple, H. C. Chow, R. Burns and C. E. Strouse, *Brookhaven Symp. Biol.*, 28 (1976) 56.
15 D. A. Goff and K. M. Smith, unpublished results.
16 R. A. Chapman, M. W. Roomi, J. C. Norton, D. T. Krajcarski and S. F. MacDonald, *Can. J. Chem.*, 49 (1971) 3544.
17 J. L. Archibald, S. F. MacDonald and K. B. Shaw, *Can. J. Chem.*, 85 (1963) 644.
18 J. L. Archibald, D. W. Walker, K. B. Shaw, A. Markovac and S. F. MacDonald, *Can. J. Chem.*, 44 (1966) 345.
19 K. M. Smith, *Thesis*, University of Liverpool, 1967.
20 K. M. Smith, D. A. Goff, J. Fajer and K. M. Barkigia, *J. Amer. Chem. Soc.*, 104 (1982) 3747.
21 M. T. Cox, A. H. Jackson and G. W. Kenner, *J. Chem. Soc. C*, (1971) 1974.
22 A. S. Holt, D. W. Hughes, H. J. Kende and J. W. Purdie, *J. Amer. Chem. Soc.*, 84 (1962) 2835.
23 G. W. Kenner, J. Rimmer, K. M. Smith and J. F. Unsworth, *Phil. Trans. Roy. Soc., London, Ser. B*, 273 (1976) 255.
24 K. M. Smith and J. F. Unsworth, *Tetrahedron*, 31 (1975) 367; D. N. Lincoln, V. Wray, H. Brockmann, Jr. and W. Trowitzsch, *J. Chem. Soc., Perkin Trans. II*, (1974) 1920.
25 N. Risch and H. Brockmann, Jr., *Justus Liebigs Ann. Chem.*, (1976) 578; T. Kemmer, H. Brockmann, Jr. and N. Risch, *Z. Naturforsch.*, 34b (1979) 633.
26 R. Tacke, *Dissertation*, Braunschweig, 1975, p. 39.
27 K. M. Smith, M. J. Bushell, J. Rimmer and J. F. Unsworth, *J. Amer. Chem. Soc.*, 102 (1980) 2437.
28 M. B. Caple, H. C. Chow, R. M. Burns and C. E. Strouse, *Brookhaven Symp. Biol.*, 28 (1976) 56.
29 H. C. Chow, M. B. Caple and C. E. Strouse, *J. Chromatogr.*, 151 (1978) 357.
30 K. M. Smith, G. M. F. Bisset and M. J. Bushell, *J. Org. Chem.*, 45 (1980) 2218.
31 H. Brockmann, Jr., U. Jürgens and M. Thomas, *Tetrahedron Lett.*, (1979) 2133.
32 N. Pfennig and H. Biebl, *Arch. Microbiol.*, 110 (1976) 3.
33 H. Fischer and H. Orth, *Die Chemie des Pyrrols*, Akademische Verlag, Leipzig, Vol. 2, Part 1, 1937, p. 422.
34 G. N. La Mar, D. L. Budd, D. B. Viscio, K. M. Smith and K. C. Langry, *Proc. Nat. Acad. Sci. U.S.*, 75 (1978) 5755.
35 G. N. La Mar, K. M. Smith, K. Gersonde, H. Sick and M. Overkamp, *J. Biol. Chem.*, 255 (1980) 66.
36 G. N. La Mar, J. S. de Ropp, K. M. Smith and K. C. Langry, *J. Amer. Chem. Soc.*, 102 (1980) 4833.
37 G. M. La Mar, P. D. Burns, J. T. Jackson, K. M. Smith and K. C. Langry, *J. Biol. Chem.*, 256 (1981) 6075.
38 W. R. Richards and H. Rapoport, *Biochemistry*, 5 (1966) 1079.
39 W. R. Richards and H. Rapoport, *Biochemistry*, 6 (1967) 3830.
40 L. A. Kehres, *Thesis*, University of California, Davis, 1982.