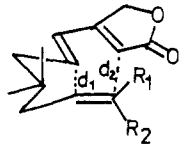
and the data here and others from our laboratories[3a] have established that relatively small changes in this tendency occur upon manipulation of the steric bulk or electronic characteristics of the activating groups. Nonbonded interactions and conformational preferences within the connecting chain appear dominant.[2a] One possible explanation for this effect is that cycloaddition reactions of this type proceed through rather unsymmetrical transition states.[23] In the case of **11**, this would result in enhancement of any nonbonded interactions in the connecting chain at the expense of other nonbonded and electronic (secondary orbital) interactions in other regions of the molecule due to a significantly shorter distance d_1 vs. d_2 in the transition state (**22**).



22

The implications of this analysis remain to be fully tested, and additional efforts are in progress to attempt to further influence the stereochemical outcome of the key intramolecular Diels–Alder cyclization and to develop a good predictive model for stereocontrol in these processes. Results of these investigations will be reported in due course.

Acknowledgment. This investigation was supported by research grants from the National Institutes of Health (GM 25982) and the National Science Foundation (CHE-78-07525), to whom we are extremely grateful.

(23) Cycloaddition by a concerted but nonsynchronous mechanism involving an unsymmetrical transition state has been suggested previously: Houk, K. N. *J. Am. Chem. Soc.* **1973**, *95*, 4092. Bond formation leads at the termini having the largest coefficient, which in our case results in a smaller distance between the reacting centers linked by the connecting chain at the transition state, magnifying any nonbonded interactions arising from atoms in the chain.

(24) (a) Fellow of the Alfred P. Sloan Foundation (1976–1980). (b) Recipient of a Research Career Development Award (CA-00273) from the National Cancer Institute of the National Institutes of Health.

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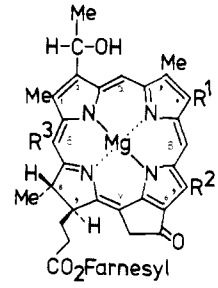
Structures of the Bacteriochlorophyll *c* Homologues: Solution to a Longstanding Problem

Sir:

The bacteriochlorophylls *c* and *d* are major photosynthetic pigments found in certain strains of green sulfur bacteria. There is general agreement about the structures assigned to the homologous group of six pigments in the bacteriochlorophyll *d* series,[1,2] but despite a large amount of skilled work, the structures postulated for the fractions (or bands) which constitute the bacteriochlorophylls *c* have been the subject of continual dispute and controversy since 1965. These bacteriochlorophylls were isolated by Purdie and Holt[3] and then separated as the pheophorbides into six homologous chromatographic bands. Structures



A

BAND	R^1	R^2	R^3	%AGE
1	i-Bu	Et	Et	0.5%
2	i-Bu	Et	Me	0.5%
3	n-Pr	Et	Et	2.0%
4	n-Pr	Et	Me	16.0%
5	Et	Et	Me	71.0%
6	Et	Me	Me	10.0%

B

BAND	R^1	R^2	R^3	CONFIG. AT POSN. 2	%AGE
1	i-Bu	Et	Me	S	4.5%
2	i-Bu	Et	Me	R	≪0.1%
3	n-Pr	Et	Me	S	5.3%
4	n-Pr	Et	Me	R	18.3%
5	Et	Et	Me	R	71.7%
6	Et	Me	Me	R	0.2%

Figure 1. Structural proposals and percentage compositions for the bacteriochlorophylls *c*: (A) due to Holt, Purdie, and Wasley;[5] (B) from the work described in the present paper. Percentage compositions here were obtained from high-performance LC separations,[13] assuming equal extinction coefficients for all the bands at 405 nm.

were assigned[3] after extensive degradative work, but following mass spectrometric determinations,[4] the assignments for bands 1 and 2 and for bands 3 and 4 were interchanged to give those shown in Figure 1A.[5] Bands 1 and 2 and bands 3 and 4 were clearly chromatographically different, so meso ethyl substituents were proposed[6] for bands 1 and 3; since samples of these materials were no longer available, it was not possible to check these proposals by mass spectrometry. However, on the basis of synthetic[7] and biosynthetic[8,9] work, the presence of meso ethyl groups in the bacteriochlorophylls *c* has never been acceptable to us. There is, however, no doubt that the meso alkyl substituent is located[2,5,8,10-12] at the δ position.

As a result of achieving excellent reverse-phase high-performance LC separations of the methyl bacteriopheophorbide *c* mixture[13,14] and a synthesis of optically pure methyl bacteriopheo-

(1) Holt, A. S. In "The Chemistry and Biochemistry of Plant Pigments"; Goodwin, T. W., Ed.; Academic Press: New York, 1965; pp 3–28.

(2) Archibald, J. L.; Walker, D. M.; Shaw, K. B.; Markovac, A.; MacDonald, S. F. *Can. J. Chem.* **1966**, *44*, 345–362.

(3) Purdie, J. W.; Holt, A. S. *Can. J. Chem.* **1965**, *43*, 3347–3353.

(4) Smith, K. M. Ph.D. Thesis, University of Liverpool, 1967.

(5) Holt, A. S.; Purdie, J. W.; Wasley, J. W. F. *Can. J. Chem.* **1966**, *44*, 88–93.

(6) Structural assignments were made[5] on the basis of degradation to maleimides, but the pairs of bands 1 and 2 and 3 and 4 gave the same maleimides. It was therefore deduced that the differences between bands 1 and 2 and 3 and 4 must be at the meso positions which were lost as CO_2 in the CrO_3/HOAc degradation. Hence, meso ethyl groups were deduced for bands 1 and 3.

(7) Cox, M. T.; Jackson, A. H.; Kenner, G. W. *J. Chem. Soc. C* **1971**, 1974–1981.

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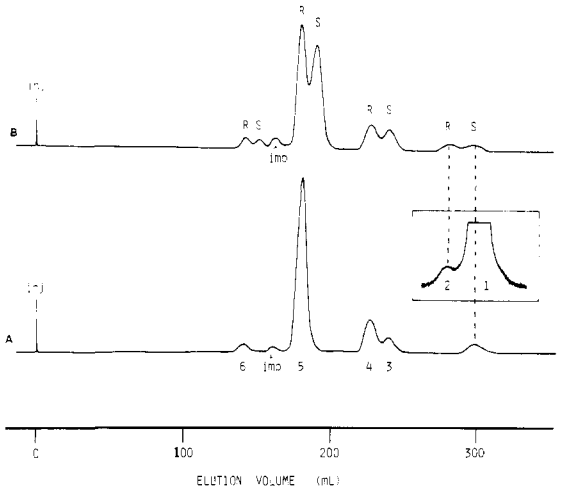

Figure 2. High-performance LC separations[16] of the methyl bacteriopheophrobides from *Chloropseudomonas ethylica*.[15] (A) Intact natural mixture; note that the most retained band is now labeled as band 1 rather than band 2, as previously.[14] The inset shows a scan run at increased detector gain and reveals the presence of a minor amount of band 2. (B) Intact natural mixture after dehydration of the 2-(1-hydroxyethyl) group (to give vinyl) and then rehydration using HBr/HOAc.[14] Numbers beneath peaks refer to band assignments; the peak labeled "imp" is an impurity present in variable amounts from one preparation to another.

phorbide *c* (band 6),[14] we have been able to further study the homologous bands of methyl bacteriopheophorbides *c* from *Chloropseudomonas ethylica*;[15] in this paper we report definitive assignments for all six homologous bands.

Figure 2A shows the high-performance LC separation achieved[14,16] for the methyl bacteriopheophorbide *c* mixture from our strain of bacteria.[15] It is readily apparent that the separation between bands 3 and 4 (which are supposed[5] to differ by a methylene group; see Figure 1A) is much less than that between, for example, bands 6 and 5 or bands 5 and 4 (which do differ by a methylene group[4,13]). Recent mass spectrometric studies have shown[13] that the methyl bacteriopheophorbides *c* from bands 3 and 4 have identical molecular weights and virtually identical fragmentation patterns. Moreover, at 360 MHz, the NMR spectra of these two methyl bacteriopheophorbides are almost identical, indicating that they have the same peripheral substituents. We have previously reported that it is impossible to resolve bands 3 and 4 in the methyl 2-vinylbacteriopheophorbides *c* [where the 2-(1-hydroxyethyl) group has been dehydrated] or in methyl mesobacteriopheophorbides (where the vinyl has been reduced to ethyl), no matter how many times the sample is recycled through the high-performance LC system;[13,16] however, the separations between bands 6 and 5 or bands 5 and 4 are comparable throughout the 2-(1-hydroxyethyl), 2-vinyl, or 2-ethyl series of pigments. The reason for this is now obvious because pure bands 3 and 4 (obtained by high-performance LC separation using multiple injections) give the *same* 2-vinylbacteriopheophorbide *c* (by high-performance LC and 360-MHz NMR spectroscopy). This suggests that the difference between them in the methyl bacteriopheophorbides *c* is in the 2-(1-hydroxyethyl) group, i.e., one is *R* and the other is *S*. However, Brockman and co-workers have shown by Horeau analyses,[17] NMR spectroscopy,[17] and chemical degradation[11,18] that the absolute stereochemistry in the 2-substituent is *R*.[19]

Further confirmation of the *R,S* relationship was obtained as follows: Figure 2B shows the high-performance LC chart from methyl bacteriopheophorbides *c* (complete mixture) after dehydration to 2-vinyl and then rehydration[14] (to give the 2-*R,S* mixture). New companion peaks are apparent in Figure 2B for bands 6, 5, and 2, but *not* for bands 3 and 4, which have merely equalized in intensity as would be expected if bands 3 and 4 have a *R,S* relationship in the 2-substituent. Moreover, if pure band 3 or pure band 4 is dehydrated and then rehydrated as described above, then *both* give the same 1:1 (approximately) mixture of bands 3 and 4, as monitored by the high-performance LC and 360-MHz NMR spectroscopy.

It seems sensible to propose that bands 1 and 2 are likewise related by having *R* or *S* absolute stereochemistry in the 2-(1-hydroxyethyl) substituent. However, from a comparison of Figure 2, A and B, it becomes obvious that the band which we have heretofore referred to as band 2[14] (with band 1 being at some other, greater elution volume) is really band 1 (using Holt's nomenclature).[5] Also, assuming that the *R* diastereomer runs faster than *S* for all bands[20] (Figure 2), we must assign the naturally occurring band 1 to be the *S* diastereomer in the 2-(1-hydroxyethyl) group.[21] Figure 1B shows the newly deduced assignments and the relative proportions in our culture.

It therefore appears that biosynthetic hydration of the 2-vinyl group in precursors for bands 4 (3) and 2 (1) does not give uniquely the *R* diastereomer as was previously thought.[17,18] As the size of the 4-substituent increases, it is apparent that the chirality generated by hydration of the vinyl changes from *R* (completely) to *S* (almost completely).[22] Our conclusions are further reinforced by consultation of literature[5] melting points for δ-phylloporphyrin and pyrroporphyrin degradation products derived from bands 3 and 4.[23]

Acknowledgment. This work was supported by a grant from the National Science Foundation (CHE-78-25557) and by grants from the Research Corporation (which enabled purchase of the high-performance LC system) and the UC Davis NMR Facility.

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(15) We have previously referred to our bacterium as *Chloropseudomonas ethylicum*; it was obtained from Dr. M. C. W. Evans (University College, London). We wish to thank Professor Norbert Pfennig for identifying our culture as a mixture of *Prosthecochloris aestuarii* and the sulfur-reducing symbiont *Desulfuromonas acetoxidans*. We are also grateful to Dr. John M. Olson (Brookhaven) for prompting us to have the culture identified.

(16) A Waters Associates instrument, consisting of a Model 6000A solvent delivery system and a U6K injector, was used for high performance LC. The detector was a Perkin-Elmer LC 55B variable wavelength detector set at 660 nm. Chromatography was performed by using a Waters Associates C18 μBondapak column (30 cm × 7.8 mm i.d.) with 15% H_2O in MeOH at 3 mL/min which created a back-pressure of 2000 psi.

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(18) R. Tacke Dissertation, Braunschweig, 1975, pp 39–46.

(19) However, the Horeau method is not quantitative and would not have detected minor amounts of *S* diastereomer in the mixture if their culture was indeed producing it. A back-calculation from the slightly low rotation obtained[18] from the methyl benzoyllactate degradation product could indicate about 10% contamination with the (*S*)-lactate. Perhaps fortuitously, bands 3 and 1 constitute 9.8% of the mixture produced by our bacterium.

(20) This seems a reasonable assumption, but we are now attempting to obtain enough of pure bands 1 and 3 to carry out a Horeau analysis.

(21) If this is not done we are left with the biochemical problem as to why only band 3 has the (*S*)-2-(1-hydroxyethyl) configuration in the intact natural mixture. On the other hand, assignment of the most-retained high performance LC peak to band 1 suggests a progressive change from *R* to *S* as we go from band 6 through 1, and this is easier to rationalize (but see ref 22).

(22) The inset in Figure 2A shows the high performance LC scan in the band 2,1 region under conditions of high detector gain. This clearly indicates the presence of very small amounts of band 2 in our mixture. Recycling of pure band 5 also reveals a very minor slower running component at the retention volume expected for the *S* diastereomer corresponding to band 5. We are attempting to isolate and characterize these minor components in the natural mixture.

(23) With hindsight it can be seen that the melting points for the pyrroporphyrin methyl ester degradation products from band 3 (mp 205–208 °C) and band 4 (205–208 °C) and the δ-phylloporphyrin methyl esters from band 3 (196–199 °C) and band 4 (195–197 °C)[5] indicate that these pairs of compounds are identical for bands 3 and 4.[24] Apparently, no mixture melting points were ever carried out. Elsewhere in the literature it is possible to find hints about the true identities of bands 1 and 3; Cox et al.[7] found that the X-ray powder photograph for their synthetic band 4 δ-phylloporphyrin methyl ester was similar to that for natural band 3 and declared in frustration that "the obvious conclusion is that the differentiation between fractions 3 and 4 is mythical". Indeed it is, but only for the δ-phylloporphyrin methyl esters and *not* for the natural chlorophylls, which were the subject of their comment.

(24) These melting points are clearly different from those for the band 6 pyrroporphyrin methyl ester (mp 241 °C) and δ-phylloporphyrin methyl ester (209–213 °C) and the band 5 δ-phylloporphyrin methyl ester (215–215.5 °C) degradation products.[5]

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^{13}C-Enriched *S*-Methyl Probe at the Active Site of an Enzyme: [*S*-[^{13}C]Methylmethionine-192]-α-chymotrypsin (MSMC)

Sir:

The serine protease α-chymotrypsin contains two methionine (Met) residues. The side chain of Met-192 lies at the outer edge of the active site crevice[1] where it is more vulnerable to S-alkylation[2] and S-oxidation[3] than the side chain of Met-180, which is buried in the interior of the tertiary structure of the enzyme. Met-192 is mechanistically interesting since it serves as a flexible hydrophobic lid over the active site and interacts with bound substrates and inhibitors.[4]

Others have shown that the facile detection of S-[^{13}C]-methylated Met residues by ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR) may be exploited to gain insights into protein conformational changes induced by a variety of perturbing conditions. Thus far the *S*-methyl probe has been used to study apomyoglobin,[5] basic myelin protein,[6] ribonuclease,[7] and basic pancreatic trypsin inhibitor.[8] We now wish to report its selective incorporation into Met-192 of α-chymotrypsin. Our results show that an enzymatic *S*-methyl probe can be a particularly informative reporter when the labeled Met residue is near or involved in the enzyme's catalytic and binding functions.

Native α-chymotrypsin was S-methylated by stirring 1.0 g of the protein dissolved in 20 mL of pH 4.0 HCl containing 0.1 M KNO_3 with a 100-fold molar excess of 90 atom % $^{13}CH_3I$ for 16 h in the dark. Affinity chromatography of the dialyzed and lyophilized protein on a lima bean trypsin inhibitor—Sepharose column gave three protein peaks. The protein in the first peak ($\sim$10% of the total protein) eluted in the breakthrough fractions with pH 8.0, 0.05 M Tris—probably denatured chymotrypsin and its autolysis products, it was inactive in a rate assay with *p*-nitrophenyl 3-phenylpropionate (PNPP).[9] Amino acid analyses of samples of the inactive protein hydrolyzed in 3 M *p*-toluenesulfonic acid gave a 1.7:0.3 molar ratio of *S*-methylmethionine (SMM) to Met. The protein in the second peak ($\sim$75% of the total protein), eluted with pH 8.0, 0.1 M Tris containing 0.12 M $CaCl_2$ and 0.1 M KCl, exhibited 130% of the activity of the native enzyme in the PNPP assay.[10] Amino acid analyses of its hydrolysates gave equimolar quantities of SMM and Met. The protein in the third peak ($\sim$15% of the total protein), eluted with pH 2.0 HCl containing 0.1 M KCl, had a PNPP activity and amino acid composition identical with that of native α-chymotrypsin.

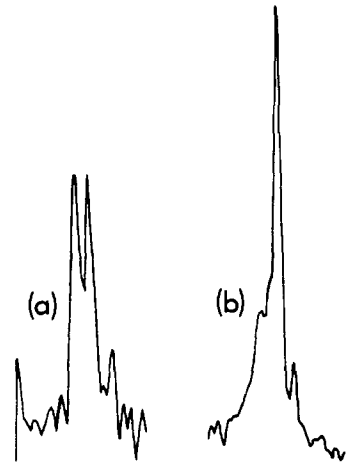

Figure 1. Methyl resonances of *S*-[^{13}C]methyl groups in the proton-decoupled 15.0-MHz Fourier transform ^{13}C NMR spectrum of MSMC after (a) 2 h (1.5×10^4 transients) and (b) 14 h (1.0×10^5 transients). Enzyme concentration 1.5 mM, temperature 30 °C, pH 7.0. Solvent composition is given in the text. Chemical shifts of the two resonances in spectrum a are 24.11 and 23.82 ppm downfield from the methyl carbon of internal acetonitrile, respectively; the resonance in spectrum b is at 23.82 ppm. Conditions of acquisition were 27.7° pulse, repetition rate 0.5 s, 8×10^3 data points, and 0.25-Hz digital resolution.

Chemical evidence confirms that the protein obtained in the second peak is MSMC: the methylated protein fails to react with α-bromo-4′-nitroacetophenone, a chromophoric alkylating agent which specifically alkylates Met-192,[11] and amino acid analyses of hydrolysates of methylated protein treated with H_2O_2 at conditions which give predominant S-oxidation of Met-192 in the native enzyme[12] revealed negligible amounts of methionine sulfoxide.

For ^{13}C NMR studies proteins were dissolved in pH 7.0 0.1 M phosphate buffer in 30% v/v deuterium oxide–water containing 1% v/v acetonitrile. Chemical shifts are referenced to the methyl carbon of the internal acetonitrile. The 15.0-MHz broadband proton-decoupled Fourier transform ^{13}C NMR spectrum of a 1.5 mM solution of MSMC equilibrated with the NMR solvent for 12 h is essentially identical with that of the native enzyme except for a narrow intense resonance of 23.82 ppm attributable to a ^{13}C-enriched SMM residue. No separation of resonances due to diastereotopic methyl groups was observed.[4] A study of the effects of active-site-directed inhibitors on the microenvironment of the *S*-methyl probe was initiated by converting MSMC to its phenylmethanesulfonyl (PMS) derivative. The phenylmethanesulfonylation doubtless occurs at Ser-195 as it does in the native enzyme.[13] The resonance assigned to the ^{13}C-enriched *S*-methyl carbon shifted downfield by 0.28 ppm in the ^{13}C NMR spectrum of PMS–MSMC, indicating an interaction between the probe and the inhibitor.

The ^{13}C NMR spectrum of MSMC taken immediately after dissolution of the lyophilized protein in the NMR solvent is different from that taken after thorough equilibration. For example, the spectrum of the nonequilibrated protein after 2 h of data acquisition (1.5×10^4 scans) exhibited two *S*-methyl absorptions of equal intensity, one at 23.82 ppm corresponding to the peak seen in the spectrum of the equilibrated protein and the other a new, broader absorption at 24.11 ppm (Figure 1). Successive spectra obtained by continuing the data acquisition

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