three times larger for the transitions at 265 nm compared to the transition at 310 nm.

The low circular birefringence of the solvents used for the present investigations rules out the possibility of relevant contributions to ICD of "macroscopic mechanism".

The presence of more than one mechanism for the induction of the optical activity prevents any quantitative analysis of the intensity of ICD signals to obtain information on the geometries and chiral discrimination energies for the two enantiomeric excited species originated by the Jahn–Teller mechanism.

The circular dichroism of the fluorescence of SBF, dissolved in a chiral solvent, which should be dominated by the more stable of the two chiral species is likely to provide more conclusive experimental evidence for the Jahn–Teller mechanism of induction of circular dichroism.

Experimental Section

The CD spectra have been recorded by a Jouan II and a JASCO J 500 dichrograph/DP 500 data processor. The *l*- and *d*-diethyl tartrates, which have been synthetized by the procedure described in ref 20, have $[\alpha]_D$ −7.3 and +6.5° respectively, and the *d*-2-phenylbutanoic acid[21] has $[\alpha]_D$ +102°.

The concentration of DcNa was 0.19 M, well above the critical micellar concentration, in phosphate buffer at pH 7.8. In all cases, the base line of the ICD spectra was obtained by recording the CD spectra of the pure chiral solvent or of solutions of SBF or fluorene in racemic solvents with solute concentrations identical with those used for the ICD measurements. The LCLD spectra have been recorded by the JASCO/LD attachment using a nematic liquid crystal matrix trasparent to the UV radiation (E. Merck—ZLI 1167) oriented by surface coupling agents.[10]

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Reversible Wavelength Shifts of Chlorophyll Induced by a Point Charge

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Abstract: The recently supported point-charge hypothesis of shifts in the optical spectra of the retinal Schiff base in rhodopsin suggests a mechanism for the anomalous red shifts of chlorophyll (Chl) spectra in vivo. This idea is tested here by synthesis of a Chl model compound, 3-demethyl-3-(aminomethyl) Chl *a*, in which a point charge is reversibly induced on the periphery of the Chl macrocycle. Chl(3a-NH_2) is formed from Chl *b* by reductive amination of the formyl group with sodium cyanoborohydride. The structure is confirmed by the Chl *a*-like absorption spectrum (λ_{max} = 661 nm, $\epsilon = 5.67 \times 10^4$ M^{-1} cm^{-1} in $CHCl_3$), by ^1H NMR, and by reversible changes in both absorption and ^1H NMR on adjustment of pH (amine nitrogen pK_a = 7.9). Chl(3a-NH_3^+) displays a reversible blue shift in its red absorption maximum of 4 nm (90 cm^{-1}) relative to Chl(3a-NH_2). Presumably, a negative charge in the same position would give a red shift of the same size; charges located differently on the macrocycle perimeter might give shifts of larger magnitude. Because such charges could be provided by polar amino acids of the Chl-binding proteins in vivo, our results lend support to a point-charge hypothesis of Chl spectral shifts.

The longest wavelength visible (or near-IR) absorbance maxima of virtually all chemical forms of chlorophyll (Chl) or bacteriochlorophyll (Bchl) found in vivo are red shifted relative to corresponding (unaggregated) forms in vitro.[1] These shifts can be as small as 225 cm^{-1} for Chl *a* in the green plant light-harvesting protein complex designated CP-II,[2] or as large as 2740 cm^{-1} for Bchl *b* in the photosynthetic bacterium, *Rps. viridis*.[3] Most of the shift is anomalous: It cannot be attributed to interactions of inter-Chl electronic transition moments (exciton interactions), or to effects analogous to changes of solvent polarity. The first point is most clearly demonstrated by a small Bchl–protein complex, analysis of the spectra of which shows that *none* of the more than 1300-cm^{-1} shift is attributable to exciton effects.[4,5] The second point is best attested by the fact that extremes of solvent polarity cause shifts of no more than 15% of those observed for Bchl *a* in vivo.[3,6,7] It has been proposed that some of the shift can be attributed to inter-Chl charge-transfer interactions,[8] but there is at present no experimental support for this hypothesis.

Recently, in the case of rhodopsin there has been considerable support for a model in which spectral shifts (red or blue) are

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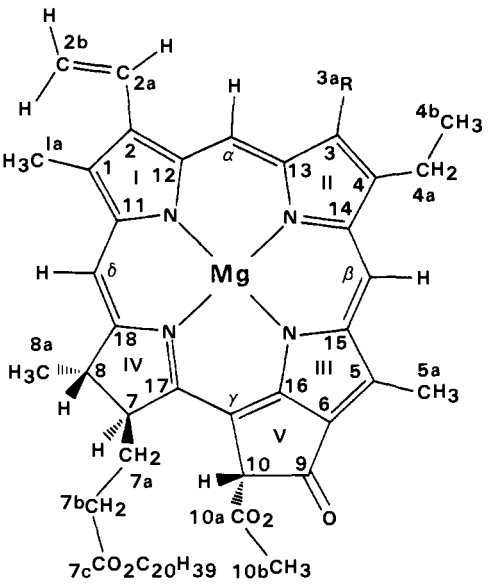

Figure 1. Structure and numbering system for chlorophylls.

induced by interaction of the prosthetic chromophore with charged amino acids of its apoprotein.[9] The possible magnitudes and spatial dependence of charge location for Chl systems, rather than retinal, have not yet been calculated. In spite of this fact, the hypothesis does have appeal and merits careful investigation. It is now known that virtually all Chl in vivo is protein bound.[10-12] The protein hosts of Chl in vivo could supply charged amino acids to perturb the chromophores in a manner analogous to rhodopsin. For this reason we undertook a test of the point-charge spectral-shift hypothesis for Chl. Our approach is to synthesize a suitable model compound in which a point charge can be reversibly induced on the periphery of the Chl macrocycle. This charge, not conjugated with the aromatic macrocycle, is in a position which may not be unreasonable in mimicking charges supplied by the host protein.

We report here the synthesis, characterization, and reversibly titratable absorbance shift of the model compound, 3-demethyl-3-(aminomethyl) Chl *a*, formed by reductive amination of Chl *b* (Figure 1). Chl(3a-NH_3^+) displays a reversible 90-cm^{-1} blue shift relative to Chl(3a-NH_2).

Experimental Section

UV-vis spectra were recorded with a Cary 118 spectrophotometer, using a sample pathlength of 1 cm. Proton nuclear magnetic resonance (^{1}H NMR) data were obtained with a Varian CFT-20 Fourier transform NMR spectrometer. Chemical shifts are given as ppm relative to tetramethylsilane (Me_4Si). pH measurements were made with a Radiometer PM23 pH meter with a semimicro combination electrode. All measurements were performed at room temperature.

Chlorophylls *a* and *b* were purified from spinach leaves according to the method of Strain and Svec.[13] All solvents and reagents used throughout this study were reagent grade or the highest quality commercially available and were used without further purification. Molecular sieves (3 Å) were from Aldrich Chemical Co.; $CDCl_3$ (99.5% D) and CD_3OD (99.9% D) were from Stohler Isotope Chemicals, Waltham, MA; and CF_3COOD was from Peninsular Chemresearch, Inc., Gainesville, FL.

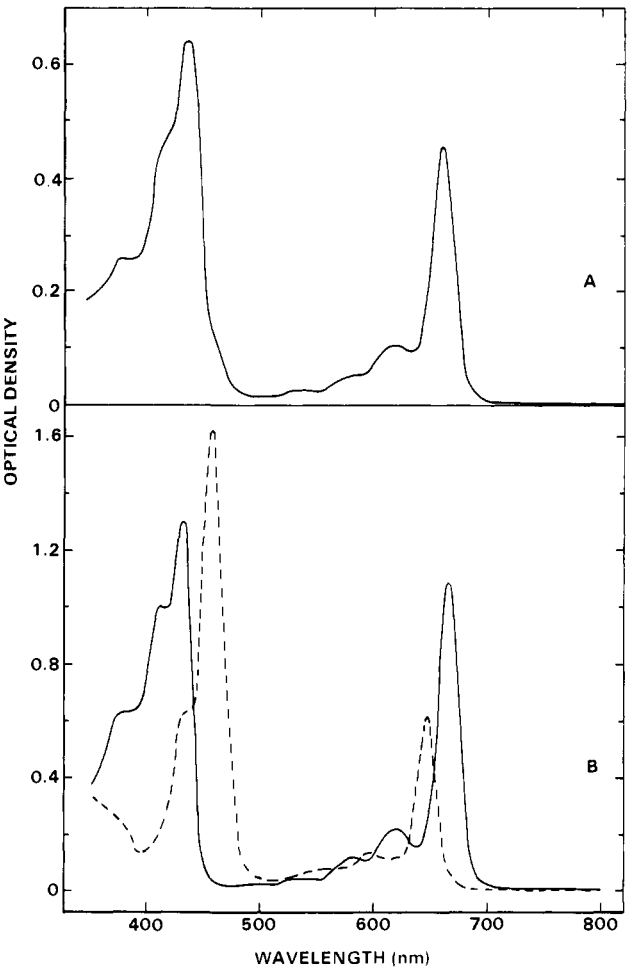

Figure 2. Absorption spectra of chlorophylls in $CHCl_3$. (A) Chl(3a-NH_2). (B) (—) Chl *a*, (- - -) Chl *b*. [Chl(3a-NH_2)] = 7.79×10^{-6} M (based on our measured extinction of 5.67×10^4 M^{-1} cm^{-1} in $CHCl_3$). [Chl *a*] = 1.50×10^{-5} M (extinction from ref 16). [Chl *b*] = 1.08×10^{-5} M (based on extinction of 5.42×10^4 M^{-1} cm^{-1} in $CHCl_3$, determined relative to the reported extinction in diethyl ether[17]).

In the reductive amination, done in the presence of molecular sieves, 26 μmol of Chl *b* was added to 10 mL of a methanol solution that contained 250 mM ammonium acetate plus 50 mM sodium cyanoborohydride.[14] Aliquots were removed and diluted at various times to follow progress of the reaction (by visible absorption spectroscopy). The reaction was judged to be complete when absorption maxima and Soret/red peak ratios no longer changed. This required 24-48 h at ambient temperature with the reaction mixture kept in total darkness. The reaction mixture was diluted with an equal volume of water, then extracted with an equal volume of light petroleum ether. Repeated extractions with more ether removed the unreacted Chl *b*. The product was extracted from the aqueous methanol phase with a small volume of chloroform. The chloroform was evaporated under a stream of argon. The green residue was vacuum desiccated for several hours prior to storage at -20 °C. Yield was ~80%.

The UV-vis absorption spectra of the product dissolved in chloroform are reported in Figure 2. ^{1}H NMR spectral data were obtained with a 10 mg/mL solution of the pigment in 3% CD_3OD in $CDCl_3$.[15] Formation of the charged ammonium salt was attained through addition of

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Table I. Chemical Shifts[a]

proton	Chl *a*	Chl *b*	Chl-(3a-NH_2)	Chl-(3a-NH_3^+)
α	9.25	10.01	9.33	9.15
β	9.51	9.60	9.56	9.61
δ	8.29	8.17	8.26	8.29
1a	3.27	3.21	3.20	3.18
2a	7.93	7.89	7.71	7.71
2b[b]	6.00	6.02	6.00	6.04
3a	3.24	11.18	5.23	5.60
4a	3.77			
4b	1.72	1.70	1.69	1.70
5a	3.61	3.57	3.58	3.59
8a	1.81	1.79	1.79	1.79
7b	2.27	2.32	2.32	2.33
10	6.23	6.30	6.21	6.22
10b	3.98	3.98	3.95	3.95
phytyl[c]	5.07	5.07	5.13	5.16
phytyl[d]	4.27	4.28	4.32	4.34

[a] Assignments according to Closs et al., ref 15 (ppm relative to Me_4Si). [b] Average of the two protons at position 2b. [c] Olefinic hydrogen. [d] Oxygen-bonded methylene.

a stoichiometric amount of CF_3COOD.

Effect of pH on the product was observed by titration of $\sim 10^{-5}$ M pigment dissolved in a methanolic solution (pH 7.3) buffered with 5 mM triethanolamine plus 5 mM triethylamine. pH was then adjusted by addition of either NaOH or HCl (1 N methanolic solution). Visible spectra were recorded immediately following pH adjustment. pH was then measured following dilution of an aliquot of the pigment solution with a 20-fold excess of water.

Results

During the reaction to form Chl(3a-NH_2) it is immediately apparent that the visible absorption spectrum changes from that of the parent molecule Chl *b* (Figure 2). Major differences include the changes in peak wavelength and intensity of red and Soret bands. Overall the absorption spectrum of Chl(3a-NH_2) resembles that of Chl *a*[16] more closely than that of Chl *b*,[17] which is consistent with the presumed loss of the carbonyl function at C-3.

Proton NMR spectroscopy (Figure 3 and Table I) further confirms the formation of Chl(3a-NH_2) by showing the loss of the 3a (formyl) proton, and concommitant appearance of the aminomethylene protons substantially upfield. The α proton, no longer deshielded by the formyl group, also shows a distinct upfield shift to a position similar to the α proton of Chl *a*. The chemical shift of the δ proton, closer to that of Chl *a* than to that of Chl *b*, also indicates a greater structural similarity between Chl(3a-NH_2) and Chl *a*.

Additional evidence for the structure of Chl(3a-NH_2) comes from the chemical shifts of the aminomethylene protons of its neutral and ammonium forms, 5.23 and 5.60 ppm downfield from Me_4Si, respectively. The aminomethylene protons of benzylamine can serve as models for comparison.[18] The 0.37 ppm downfield shift of the Chl(3a-NH_2) aminomethylene protons upon acidification is remarkably similar to the 0.42 ppm downfield shift observed on protonation of benzylamine.[19] The chemical shift of the aminomethylene protons of benzylamine should be corrected for the difference in aromatic ring current deshielding of phenyl and chlorin rings before a comparison is made. The 3a methyl protons of Chl *a* (3.24 ppm) are further downfield than the methyl protons of toluene (2.29 ppm).[20] Consequently, we would predict the aminomethylene protons of Chl(3a-NH_2) to resonate 0.95 ppm downfield from the analogous protons of benzylamine (which are observed at 3.85 ppm downfield from Me_4Si). The predicted and observed chemical shifts of 4.80 and 5.23 ppm, respectively, support our contention.

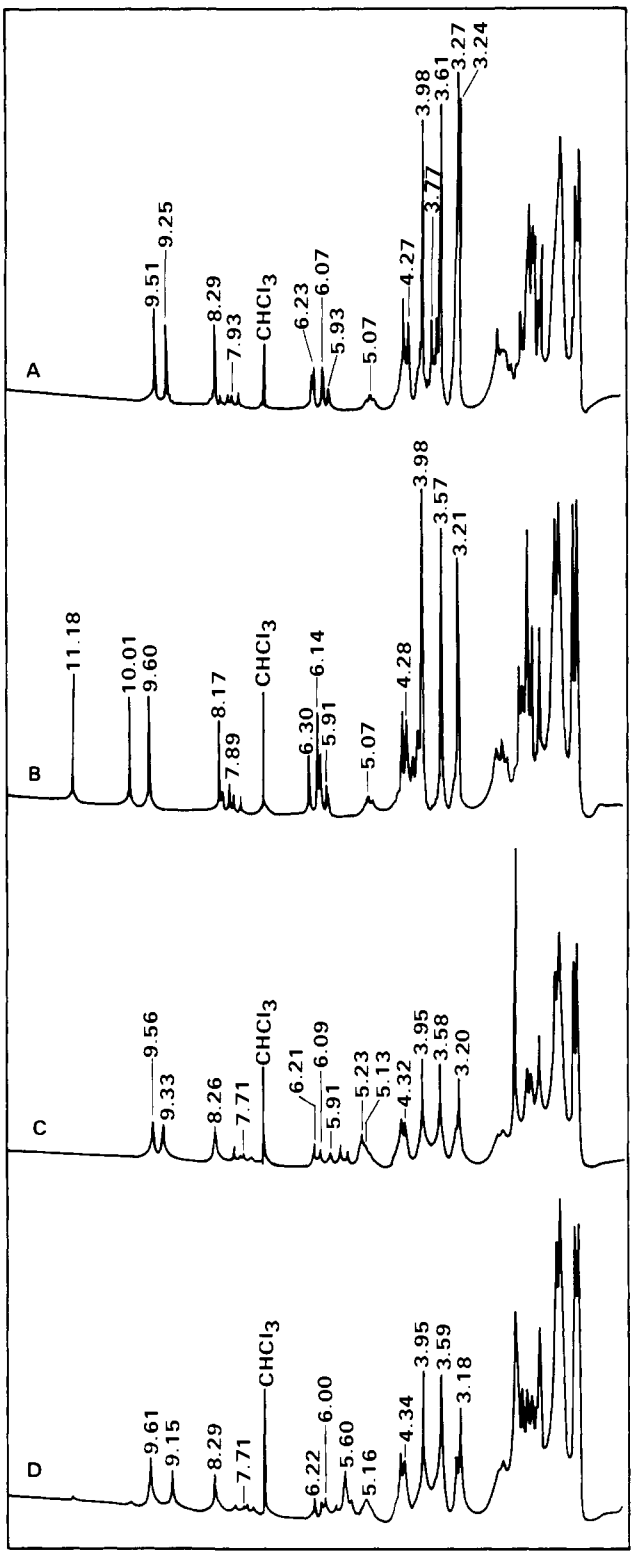

Figure 3. ^1H NMR spectra of chlorophylls in 3% CD_3OD in $CDCl_3$, [Chl] = 1.1×10^{-2} M. A = Chl *a*, B = Chl *b*, C = Chl(3a-NH_2), D = Chl(3a-NH_3^+).

Addition of an equimolar amount of acid to Chl *a* or Chl *b* induced no UV–vis spectral changes but caused loss of the δ proton intensity in NMR (within 10 min). Conversely, addition of equimolar acid to Chl(3a-NH_2) did cause a UV–vis spectral shift, but no apparent loss of δ proton intensity on the same time scale. It is well established[21] that the δ proton can be exchanged in

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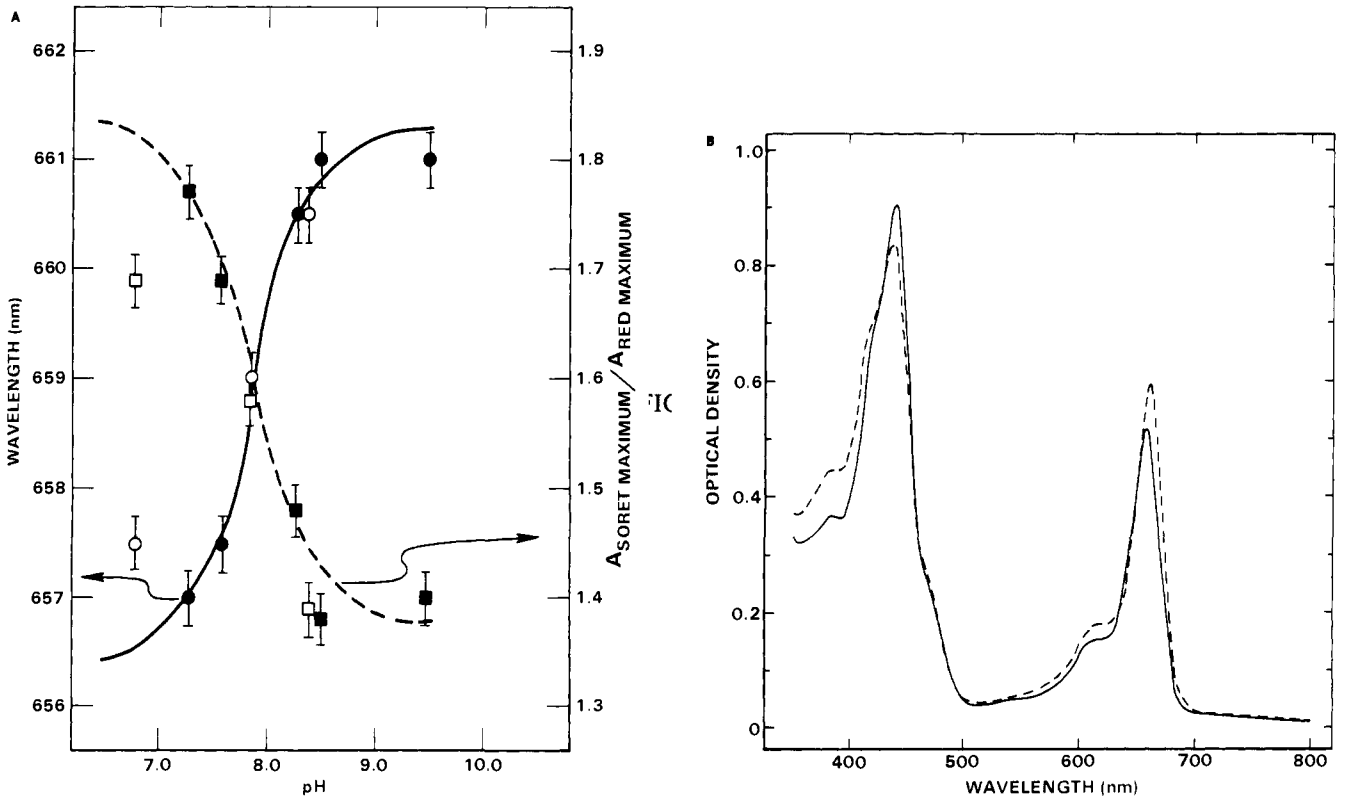

Figure 4. (A) Titration curve reflecting effect of pH on (—) wavelength of red maximum and (- - -) Soret/red absorbance ratio. (■, ●) pH increase from 7.3 to 9.5. (□, ○) Same solution back titrated to pH 6.8. (B) Absorption spectra of (—) Chl(3a-NH_3^+) at pH 7.3, and (- - -) Chl(3a-NH_2) at pH 9.5 (see text).

deuterated solvent. Apparently the process is catalyzed by free acid, present when stoichiometric acid is added to Chl *a* or Chl *b*, but not in the case of Chl(3a-NH_2) where the free amino group complexes the acid.

pH titration of Chl(3a-NH_3^+) in buffered methanol (Figure 4A) indicates that neutralization of the ammonium group by addition of hydroxide induces a reproducible 4-nm (90-cm^{-1}) red shift of the red peak. There is no shift in the Soret maximum, although its intensity drops slightly as the red absorbance increases (Figure 4B). The effect of pH as reflected by the Soret/red absorption ratio substantiates the observation that $pK_a = 7.9$ for the amino group as obtained from the shift in red maximum.

The reversibility of this deprotonation has been demonstrated by back titration of the neutral species by addition of HCl. However, some pheophytinization may occur as the pH drops below neutrality. This slight pheophytinization[7] at low pH is demonstrated both by the shift of the red peak to shorter wavelength and by the initial decrease in Soret/red ratio prior to the blue shift and subsequent increase in Soret maximum.

Discussion

The close similarity of the neutral Chl(3a-NH_2) spectrum to that of Chl *a* suggests that the aminomethyl group of neutral Chl(3a-NH_2) has no appreciable perturbing effect on the macrocycle π-electron structure of Chl *a*. Therefore, when Chl(3a-NH_2) is protonated, it is the introduction of the charge rather than the loss of the nitrogen lone-pair electrons that causes the blue shift of the Chl(3a-NH_2) red peak. This shift is smaller by a factor of 2 or 3 than those of antenna Chl *a* in green plant photosystem II, and in the "wrong" direction.[2] Presumably, a negative charge at the same site would produce the "correct", i.e., red, shift. We chose the C-3 position because of the convenient synthetic route from Chl *b*. We have not carried out any calculations to predict the dependence of the shift of the long wavelength band on the position of the perturbing charge. Charges at other locations might very well induce shifts of opposite sign and/or larger magnitude. In vivo, such charges could be provided by polar amino acids of the Chl-binding proteins. Indeed, the specific conformation of charged groups in a protein complex might increase the magnitude of the shift (relative to those possible in vitro), as appears to be the case for the visual pigment.[9] Increased shifts could come about as a result of the lower dielectric constant at the interior of a protein or the specific localization of counterions. If the shift-inducing charges were negative, their presence could stabilize the Chl cation radical that is a transient photochemical product in photosynthetic reaction centers.

The spectral shift induced by the reversibly titratable charge on the model compound, Chl(3a-NH_2), provides some measure of support for the point-charge hypothesis of Chl spectral shifts in vivo. The hypothesis would be further substantiated if similar model compounds with inducible spectral shifts of larger magnitude and in the "red" direction were synthesized. The technique used here, i.e., reductive amination, is one that may be useful in forming other such compounds. Other types of chemistry that can covalently link charged groups to the periphery of the Chl macrocycle also deserve attention.

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