chymotrypsin, dimethyl sulfoxide is by far the least harmful.[2] Furthermore, the catalytic pathway established for the enzyme in aqueous solutions is not changed by the addition of dimethyl sulfoxide,[15] nor does the conformation of the active site appear to be affected.[16]

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Stoichiometric Determination of Chlorophyll a–Water Aggregates and Photosynthesis. Symbiotic Roles of the Magnesium Atom and the Ring V Cyclopentanone Group in the Structural and Photochemical Properties of Chlorophyll a Monohydrate and Dihydrate

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Abstract: X-ray photoelectron spectroscopy has been employed to probe the water content of aggregates of pheophytin a and chlorophyll a. The O 1s spectra of these compounds show a distinct shoulder at 533.7 eV when water is present, which is used to calculate the number of bound waters of hydration of the aggregate. We have been unable to observe anhydrous chlorophyll, but have characterized both Chl a·H_2O and Chl a·2H_2O. The latter occurs in a crystalline form of Chl a that absorbs light at 743 nm. For pheophytin a, however, water-free films are readily prepared. Since the Mg in chlorophyll a is replaced by two hydrogen atoms in pheophytin a, we conclude that the water in monohydrate Chl a is bound directly to the Mg atom. The probable role of the Chl a Mg atom in plant photosynthesis is discussed in terms of current and earlier considerations of the apparently symbiotic functions of Mg and the ring V cyclopentanone ring in the Chl a molecule.

The study of chlorophyll a–H_2O interactions is of current interest because of the probable role of Chl a–H_2O aggregates in photosynthesis[3-10] and in the photochemical splitting of water in in vitro solar conversion.[4k,l,11,12] There has been much research activity in probing the possibility that the in vivo P700 reaction center may be one of several plane-parallel dimers of Chl a, interlinked by C_2 symmetrical bonding interactions with H_2O molecules.[3-10] A comparison[4g,i,10] between the properties of the in vitro 700-nm absorbing Chl a dimers with those of the in vivo P700 aggregate and the delineation of the differences in the physical[4g] and photochemical[4i] properties of the C-10 C=O···H(H)O···Mg[3,4] linked and the C-9 C=O···H(H)O···Mg[4g,6,7,9] linked dimers led to the conclusion that the symmetrical dimer A of Chl a monohydrate provides a reasonable
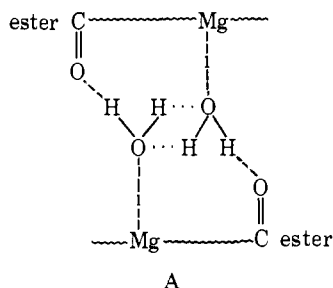
model for P700.[4g,i,13] Chlorophyll a is stable as the monohydrate[4c,d] at temperatures up to 120 °C,[4e] which accounts for the earlier observations that the water content of nonpolar solutions containing 10^{-6}–10^{-4} M Chl a cannot be reduced to a level lower than that of the order of the chlorophyll concentration.[14-17] The presence of excess water in nonpolar Chl a solutions results in the precipitation of the crystalline material which has a characteristic red absorption maximum at 743 nm (P743).[4h] It has been assumed[4c,d] that the structure of P743 is in accord with the x-ray diffraction-determined structure of the ethyl chlorophyllide dihydrate,[5] a dimeric segment B of which is given as:

A

B
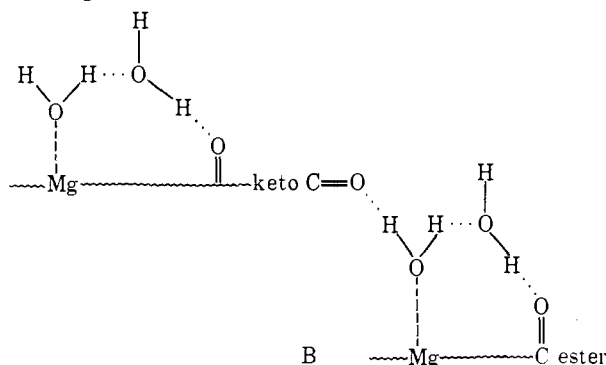
in which one of the two water molecules, datively bonded to the central Mg atom, is hydrogen bonded to the second water molecule attached to the C-10 carbomethoxy group. It is further assumed that the remaining water in Chl a·H$_2$O is associated with the Mg atom.

C
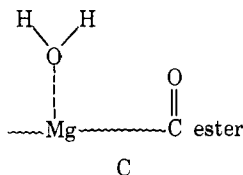
It appears that the Mg atom in Chl a is stable in the pentavalent state. This tightly bound water molecule may be removed at room temperature by the presence of excess polar groups or molecules such as acetone or the keto or carbonyl groups of another Chl a molecule. We have suggested that much of the chemistry of Chl a–H$_2$O aggregation may be understood based on the simple hypothesis that the dihydrate and monohydrate forms of Chl a are interconvertible through heating at 80 °C under vacuum or treatment with an excess of water, respectively.[4c,d,g,11b] In particular, it was noted[4g] that the specific bonding interactions found in Chl a dimeric aggregates are determined by the extent to which the Chl a is hydrated. The plane-parallel structures of hydrated Chl a dimers of the types proposed by Fong[4] and Boxer and Closs[6] have recently been confirmed by Clarke and his associates,[9] using optically detected magnetic resonance techniques.[9] An important point that has remained unsettled is the stoichiometry of the P743 Chl a aggregate. The 743-nm absorbing polycrystalline chlorophyll, known earlier as the dihydrate polymer (Chl a·2H$_2$O)$_n$ after the elemental analysis work of Holt and Jacobs over 20 years ago,[18] was assigned by Katz a monohydrate stoichiometry, (Chl a·H$_2$O)$_n$, in the late 1960's.[19] The Katz assignment, having been based on extensive analytical and IR spectroscopic determinations, became generally accepted,[20] and was parturient to the unsymmetrical semihydrate "special pair" model,[21] (Chl a·H$_2$O·Chl a), for the P700. In 1975, the experimental basis for the (Chl a·H$_2$O)$_n$ assignment was reexamined, and the dihydrate stoichiometry of P743 repristinated.[4c,d] In 1976, the unsymmetrical semihydrate model for P700 was retracted by Katz and co-workers.[7a] Even so the (Chl a·H$_2$O)$_n$ assignment for P743 has persisted in some recent publications.[22]

It would seem possible to establish the stoichiometric composition of P743 by a comparative study of Chl a and its Mg-free derivative pheophytin a (Ph a), in which the Mg atom is replaced by two protons. The absence of the bound water molecule in Ph a observed under conditions identical with those under which Chl a·H$_2$O is stable would thus establish the Mg atom as the binding site for the water molecule in Chl a·H$_2$O. The observation of a doubling in the water content in the P743 chlorophyll compared with the monohydrate chlorophyll would establish the dihydrate origin of the P743. We have employed the technique of x-ray photoelectron spectroscopy (XPS or ESCA) to detect the presence of water in Chl a aggregates since the binding energy of the O 1s electron in water is approximately 1.5 eV higher than that of the other oxygens in Chl a.[4e] One purpose of this paper is to establish, using XPS measurements on Chl a aggregates and Ph a, that the proposed structures B and C are consistent with direct experimental observables.

Experimental Section

High-purity chlorophyll was isolated from fresh spinach. The water content of the Chl a solutions was rigorously monitored[4c] in order to minimize possible ambiguities due to lack of sample quality control. Our procedure for Chl a extraction and purification differs slightly

from the conventional method.[23] One-hundred grams of pulverized leaves was dissolved in 250 mL of acetone. The acetone extract was filtered through four layers of cheesecloth in a conventional Buchner. The filtrate was diluted with about 62.5 mL of water and was passed through a 5 cm long polyethylene powder column covered by 1 cm of glass beads. A pale yellow filtrate that separated from the bulk of deep-green fraction at the top of the column was discarded. The column was dried by aspiration for about 15 s and subsequently covered with ethyl ether. Gentle suction was applied with a suction flask along with periodic addition of small amounts of ether until the bulk of the chlorophyll was washed down into the flask. The ethereal solution was swirled with water in a separatory funnel to remove the remaining traces of acetone. The water was then drawn off and the *n*-pentane solution was evaporated to dryness.

The residue thus obtained was redissolved in a minimum volume of *n*-pentane. The solution was poured onto a powdered sugar column. The column was then eluted with 0.5% 1-propanol in *n*-pentane. The first fraction of yellow pigments was discarded. The green band that followed, containing a mixture of Chl a, Chl a′, and pheophytin a, was collected. The chlorophyll eluent was shaken with water several times to remove the 1-propanol, and was then evaporated to dryness.

The residue from the preceding procedure was redissolved in a small quantity of 4:1 *n*-pentane-ether. The solution was poured onto a second sugar column. The narrow band, about 2–4 cm in length, was washed with small portions of *n*-pentane and eluted with 0.5% 1-propanol in *n*-pentane. The chlorophyll-containing sugar column was transferred into a beaker containing 600 mL of 4% ethanol in *n*-pentane. The sugar was filtered from the solution and washed with several portions of the ethanolic pentane. The resulting solution, about 1 L in volume, was evaporated down to about 100 mL. The solution was shaken with water in a separatory funnel to remove the ethanol. On cooling to 0 °C, the chlorophyll precipitated out as the crystalline P743. Further purification was accomplished by reprecipitation. Due to the stereospecific nature of Chl a–H$_2$O interactions, the preparation of the chlorophyll in the crystalline dihydrate form appears to be a convenient method to minimize any contamination of Chl a by Chl a′. Chl a and Chl a′ are epimeric about the asymmetric center at C-10. The present procedure for Chl a isolation and purification has evolved from earlier procedures as a convenient means for directly obtaining the chlorophyll in the 743-nm absorbing polycrystalline form. The average yield is 40 mg of Chl a per 100 g of spinach.

The purities of the Chl a samples were monitored by optical spectroscopy[4c] and liquid chromatography (useful in detecting contamination by Chl a′). The P743 crystals were readily converted to the monohydrate[4e] by heating under vacuum conditions at 80 °C for several hours. The nonpolar solvent employed in the present study was a 1:1 mixture of *n*-pentane and methylcyclohexane. The hydrocarbon solvents were rigorously dried by distillation over lithium aluminum hydride.[4c] The pheophytin was prepared from Chl a in the usual manner.[24]

Film preparation for analysis was accomplished on an atomically clean gold foil following the previously reported procedure.[4e] Contamination from adventitious sources containing oxygen was avoided by carrying out the procedure in an N$_2$ drybox attached to the inlet system of the Hewlett-Packard 5950A ESCA spectrometer.[25] Binding energy values referenced to the Fermi level of gold (or an Au 4*f*$_{7/2}$ value of 83.8 eV) were obtained by preparing very thin films which did not exhibit charging as determined by the lack of a peak shift on irradiation with the electron flood gun.

Results and Interpretations

In Figure 1 and in Table I, the data are summarized for the O 1s spectra of Ph a and two different hydrated Chl a aggregates at the ambient XPS probe temperature (50 ± 5 °C). Figure 1a shows the O 1s peak from a Ph a film which was prepared from a rigorously dried solution of *n*-pentane. The peak has a full width at half-maximum (fwhm) of 1.4 eV and is quite symmetrical. The O 1s spectrum of the monohydrate Chl a, which was prepared under identical conditions as the Ph a film, is shown in Figure 1b. The sample-handling procedures were identical for these two samples. The peak is somewhat broader (fwhm = 1.5 eV) and slightly unsymmetrical, evincing a small shoulder on the higher binding energy side assigned to a single bound water of hydration.[4e] In Figure 1c,
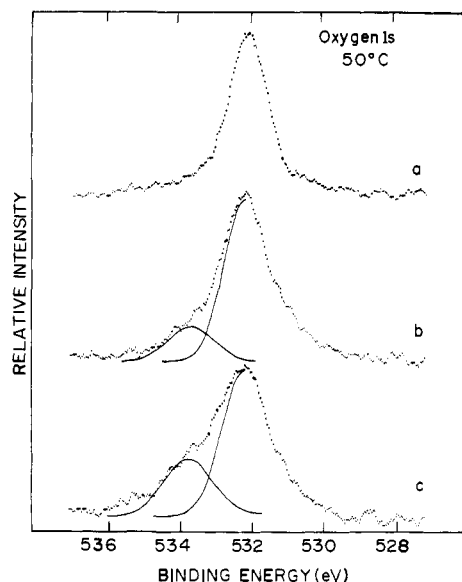
Figure 1. Oxygen 1s spectra of (a) pheophytin a, (b) dried chlorophyll a, and (c) hydrated chlorophyll a. These results establish the Mg atom as the binding site of the water of hydration in Chl a·H_2O, and the dihydrate origin of the 743-nm absorbing polycrystalline chlorophyll. The lack of any shake-up transitions that accompany the O 1s excitation of the five oxygen atoms in the chlorophyll is manifested by the nearly Gaussian shape of the Ph a spectrum in Figure 1a, making possible a meaningful determination of the water content of the samples in Figures 1b and 1c. The present determination of P743 as the dihydrate polycrystal, (Chl a·$2H_2O)_n$, is in agreement with the x-ray diffraction work by Strouse et al.[5] on the 743-nm absorbing polymer of ethyl chlorophyllide dihydrate. The assignment of the monohydrate stoichiometry in b is consistent with the earlier observation[4e] of the tightly bound water in Chl a monohydrate at temperatures less than 120 °C.

Table I. Oxygen 1s and Nitrogen 1s XPS Binding Energies for Pheophytin a and Chlorophyll a[a]

sample	main O 1s peak[b]	shoulder O 1s peak[b]	N 1s peak	obsd[c] shoulder area, %	calcd shoulder area, %
Ph a	532.1		398.2, 400.0		
Chl a	532.1	533.7	398.0	17.8 ± 3.2	16.6 (Chl a·H_2O)
Chl A743	532.1	533.7	398.0	29.5 ± 1.3	28.6 (Chl a·$2H_2O$)

[a] Values reported in eV. [b] Error limits are estimated at ±0.2 eV by averaging the values obtained from different films prepared under identical conditions. [c] Error limits computed from the deconvolution of three spectra taken from films prepared under identical conditions.

we show the O 1s peak from the 743-nm absorbing chlorophyll. A film of this species, suitable for XPS analysis, was prepared in the N_2 drybox by evaporating the solvent from an *n*-pentane suspension of the P743 aggregate at room temperature. The XPS peak shows considerable broadening (fwhm = 1.8 eV) and is decidedly unsymmetrical, with a broad shoulder clearly apparent on the higher binding energy side.

Computer deconvolution of the spectra in Figures 1b and 1c reveals that the shoulder arises from a fairly broad peak centered at 533.7 eV in both cases, with respective contributions to the total area under the curve of 18 and 29%. It should also be noted that the major peak occurs, within experimental error, at the same binding energy (532.1 eV) in all three spectra. In view of the experimental results in Table I, we
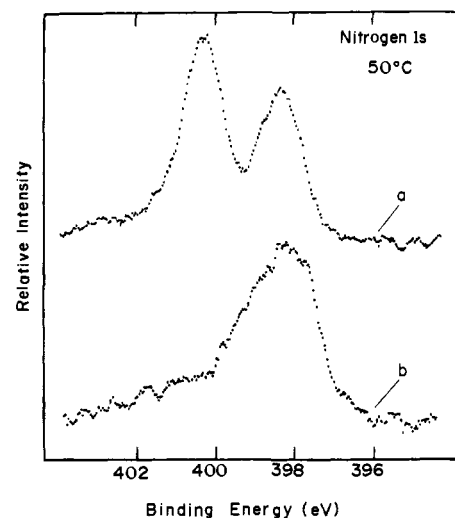
Figure 2. Nitrogen 1s spectra of (a) pheophytin a and (b) chlorophyll a. The deviation from the expected 1:1 area ratio for the two peaks is attributable to a $\pi \rightarrow \pi^*$ shake-up transition accompanying the lower binding energy N 1s excitation.

conclude that the empirical formula of the P743 chlorophyll species is Chl a·$2H_2O$, that Chl a exists as a monohydrate of empirical formula Chl·H_2O, and that Ph a exists as an anhydrous species under these same experimental conditions.

The N 1s spectra for these compounds are shown in Figure 2. For Ph a, the expected N 1s doublet is observed, characteristic of the porphyrin ring structure without a complexed metal ion.[26] The peaks apparently arise from the protonated and unprotonated nitrogens, demonstrating the localized, rather than bridged, nature of the N–H bond. The deviation from the expected 1:1 area ratio for the two peaks has been observed previously on other free base porphyrins[27,28] and is thought to arise from a $\pi \rightarrow \pi^*$ shake-up transition which accompanies the lower binding energy N 1s excitation and which overlaps with the higher binding energy N 1s peak.

Incorporation of the Mg atom into the nitrogen core induces dramatic alterations in the N 1s spectrum as illustrated for the Chl a aggregates in Figure 2b. The Ph a doublet collapses into a skewed singlet as the electron density on all four nitrogens becomes nearly indistinguishable, indicative of more or less degenerate canonical structures for the π-electronic resonance in the macrocycle of Chl a. The electron density on the four nitrogens is not expected to be exactly identical, however, which may be the reason for the observed skew. Such differences have been observed qualitatively using ^{15}N NMR techniques.[29] Both the mono- and dihydrates of Chl a exhibit identical N 1s spectra, suggesting that the hydration process does not significantly alter the nitrogen electron density. The obvious differences between the spectra in Figures 2a and 2b have also proven useful in providing an analytical check for the decomposition of Chl a samples to Ph a.

These XPS measurements yield direct spectroscopic information concerning the state of hydration in Chl a aggregates. The assignment of structure A or C and structure B to the monohydrate and dihydrate, respectively, on the basis of independent experimental observables[4c,d,g,11,12] appears to be completely consistent with the observed O 1s intensity from the bound water in Chl a. Furthermore, the lack of any observable water signal from films of Ph a prepared in an identical manner with that of the Chl a films confirms the role of Mg in the remarkable affinity of Chl a for water.

Discussion

The role of the chlorophyll a in photosynthesis has ensorcelled chemists for the greater part of the present century. It

was once speculated that a bound carbon dioxide molecule transferred an oxygen atom to the carbonyl oxygen to form a peroxide and then molecular oxygen.[30] It was then postulated that the C-10 hydrogen of the triplet Chl a molecule reduced phosphoglyceric acid while the C-9 oxygen formed oxygen via "the enzyme".[31] Recently, it was suggested that the cyclopentanone ring may provide a mechanism for oxygen formation through a dioxolium ion.[32] The similarity of the in vivo spectrum of chlorophyll to that of aggregates[33-36] and of monolayers[37-39] of Chl a was noted by a number of workers. Sherman and Wang investigated the long-wavelength shift of chlorophyll aggregates under the influence of water.[40]

It is generally believed that the Mg ion in Chl a is critical to its photochemistry. The replacement of Mg by transition metal ions quenches this photochemistry.[41] Mauzerall and co-workers[42-44] have shown that the presence of closed shell divalent cations in the chlorin makes the excited state a sufficiently strong reductant to bring carbon dioxide to the free-energy level of the carbohydrates. It was also noted[45,46] that the ground-state redox potential of chlorophyll is >0.4 V more oxidizing than simple magnesium chlorins, suggesting that it is the ring V cyclopentanone group that renders the Chl a radical cation a powerful oxidant. The present determination of the role of the Mg atom and the recent focus[3-10] on the ring V cyclopentanone ring in the binding of waters of hydration thus appear to reinforce Mauzerall's observations and emphasis on these two Chl a functional groups.[42-46]

It has recently been noted[4i,11,12] that, among known aggregates of chlorophyll,[3-9] (Chl a·H_2O)$_2$ and (Chl a·$2H_2O$)$_{n\geq2}$ are distinguished by their ability to be photooxidized in the presence of water, giving rise to well-characterized photogalvanic behavior.[11,12] Significantly, it has been found that the photooxidized dihydrate aggregate, (Chl a·$2H_2O$)$_n^+$, not (Chl a·H_2O)$_2^+$, is sufficiently strong an oxidant to be readily reduced by water.[4j,47] The photochemical activity of Chl a–H_2O aggregates has been attributed to photoactivated proton shifts[4a,b,11] between the Mg-bound water molecule and the carbonyl group to which it is hydrogen bonded, resulting in the acquisition by the Mg atom of a negative charge.[3,4a,b,11] The Mg atom being conjugated to ring V, the negative charge it acquires during photoactivation is expected to be readily transmitted via π-electron resonance to the C-9 keto group, which is presumably the site at which the electron leaves both aggregates A and B during the primary light reaction. We may thus rationalize the apparently symbiotic roles of the Mg atom and the ring V cyclopentanone ring in the photochemical activity of A and B. We note that the charge-transfer mechanism described here appears to be feasible only in A and B. In Chl a–H_2O dimers in which the C=O···H(H)O···Mg interactions involve the C-9 keto group,[4g,6,7] as in the dimer of Chl a polyhydrate[4g] or in covalently linked dimers[7b] in which the C-10 C=O···H(H)O···Mg interactions are sterically forbidden,[4g,h] delocalization of the negative charge, acquired by the Mg atom in photoactivation, to the C-9 keto carbonyl is expected to be stabilized by the water proton to which that carbonyl group is hydrogen bonded. It has been reported that, unlike (Chl a·H_2O)$_2$ and (Chl a·$2H_2O$)$_n$, the C-9 C=O···H(H)O···Mg linked dimer can only be photooxidized in the presence of an added electron acceptor, such as tetranitromethane.[7b]

The experimental determination of the water content of the two lowest states of Chl a hydration and the delineation of the specificity of bonding interactions in Chl a complexes in terms of these hydration states provide the basis for a systematic approach to understanding the experimental behavior of Chl a–H_2O aggregates. Traditionally, the C-9 keto carbonyl has been thought to play the dominant role in Chl a aggregation.[21] It was recently shown that, in rigorously dried nonpolar solutions of Chl a, the dimer structure A is obtained. However, under excess water conditions, the C-10 ester carbonyl group

D
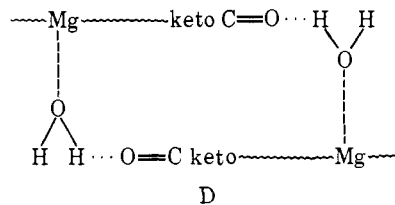
becomes engaged in cluster interactions with the H_2O molecules, leaving the C-9 keto carbonyl available for exo bonding interactions[4g] of the type proposed by Boxer and Closs,[6] Fong et al.,[4f,g] and Katz et al.[7] This interpretation[4g] has recently been confirmed by independent experiments in the laboratories of Clarke,[9] Schaafsma,[48] and their co-workers, except that Schaafsma et al.[48] had incorrectly interpreted their data on the basis of the dimeric Chl a monohydrate stoichiometry, (Chl a·H_2O)$_2$, even though their solutions were prepared in the presence of a large excess of water.

In summary, we have verified the stoichiometries of structures A and B which have, respectively, been proposed to be the photosystems I and II reaction center aggregates in plant photosynthesis.[4i,47] In both A and B, the Mg atom of one Chl a molecule and the ring V cyclopentanone group of a second Chl a molecule play reciprocal roles in providing (i) the bonding interactions C=O···H(H)O···Mg and (ii) the charge-transfer mechanism via photoactivated proton shifts $^+$C–O–H···(H)O–Mg$^-$. These roles are manifested in the observed photochemical activities[4i,j,k,11] and the long-wavelength shifts[4h] in the optical absorption of A and B. The present work has focused on the determination of the stoichiometry of the two lowest states of Chl a hydration. We assume that the structural properties of (Chl a·$2H_2O$)$_n$ are closely similar to those given by Strouse et al.[5] for the corresponding dihydrate aggregate of the ethyl chlorophyllide. The optical spectrum of the monohydrate film was given[11b] earlier in the form of the action spectrum of the photogalvanic response of Chl a·H_2O on Pt, which was analyzed in terms of a distribution consisting mostly of monomeric and dimeric complexes of Chl a·H_2O (see Figure 1B of ref 11b). Based on the stoichiometric assignments of Chl a·H_2O aggregation, it has been possible to systematize the multifarious physical and photochemical properties of the chlorophyll.[4e-k]

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Communications to the Editor

Total Synthesis of (±)-Seychellene.
Use of Silyloxydienes in Synthesis[1]

Sir:

Seychellene (**1**), a plant sesquiterpene found in commercial patchouli oil and used in perfumery,[2] has been a common synthetic target due to its novel tricyclic structure. Several fine syntheses of seychellene have been published[3–6] which range in yield from extremely low to ≃7%. We wish to report now a very direct and efficient total synthesis of seychellene (**1**) in only 10 steps, which demonstrates the very high synthetic utility of 2-silyloxydienes[7,8] in Diels–Alder approaches to polycyclic natural products.

In our approach we chose to build the necessary bicyclo[2.2.2]octane system in the first step of the synthesis with appropriate functionality so positioned that the final ring closure could be simply accomplished. To this end the 2-trimethylsilyloxycyclohexadiene (**3**), which was prepared in 95% yield (LDA, THF, −78 °C/Me$_3$SiCl) from the readily available[9] 2,3-dimethylcyclohexenone (**2**), was reacted with methyl vinyl ketone **4** (neat, sealed tube, 110 °C, 18 h) to afford a 79% yield of the Diels–Alder adduct **5** (oil; bp 80 °C (0.2 mm); NMR (CCl$_4$) δ 1.90 (s, 3 H), 1.50 (s, 3 H), 1.07 (s, 3 H), 0.13 (s, 9 H); IR (liquid film) 1700, 1670 cm^{-1}) (Scheme I). We assigned the structure **5**, namely with the acetyl group endo

and 1,4 to the trimethylsilyloxy function, to this Diels–Alder product on the basis of analogy to our earlier work[7] and to that of others[8] which had shown that the trimethylsilyloxy group was a very good director in such cycloaddition reactions. Indirect evidence for this assignment was obtained when the silyl enol ether of **5** was hydrolyzed in acid whereupon only dione **6a** (NMR (CCl$_4$) δ 2.12 (s, 3 H), 1.11 (s, 3 H), 1.00 (d, 3 H, J = 7 Hz); IR (liquid film) 1710 cm^{-1}) was formed, and in base where a mixture of **6a** and a more stable isomer **6b** (NMR

Scheme I