

Open-chain Tetrapyrroles in Light Sensor Proteins: Phytochromes

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Introduction

Open-chain tetrapyrroles serve as chromophores in accessory light-harvesting antennae of certain algae (phycobilisomes) and in light sensor proteins of higher and lower plants, algae, mosses, and cyanobacteria (phytochromes). In both functional systems the tetrapyrroles, *e.g.*, phycocyanobilin (*PCB*) in the C-phycocyanin of phycobilisomes and phytochromobilin (*PΦB*) in phytochromes,^{1,2} are covalently bound to a cysteine residue of the protein (Figure 1). In either case, primary prerequisite for the function of these biliproteins is that they selectively absorb in wavelength regions (about 550-660 and above 700 nm), in which light is not entirely filtered out by competing absorbers such as, *e.g.*, the chlorophyll-protein antennae of green plants. Nature exploits the considerable conformational flexibility of these tetrapyrroles and the polarizability of the extended π system (both properties being controlled by intimate interaction with the surrounding protein pocket) in order to judiciously fine-tune the vis absorption wavelengths of a single given chromophore according to the particular needs.

The tetrapyrroles serve either of two distinctly different purposes: the rigid containment within antenna protein matrices favors an efficient transfer of the absorbed light energy to a neighboring like chromophore and, ultimately, to the photosynthetic reaction center,³ rather than wasting the energy by any photoreactions. In contrast, the numerous photomorphogenic control functions exerted by the phytochromes are triggered by just such a process. $Z \rightarrow E$ and $E \rightarrow Z$ double-bond photoisomerizations, followed by a cascade of conformational chromophore-protein adaptations, convert the physiologically inactive P_r form ($\lambda_{\max} \sim 665$ nm) into the active P_{fr} form ($\lambda_{\max} \sim 730$ nm; Figure 1), and *vice versa*.⁴ The system $P_r \rightleftharpoons P_{fr}$ thus is photochromic and functions as a light-driven biological switch, a classic example of a dynamic conformational protein change photoinitiated by an internal chromophore.

Phytochromes in higher plants comprise ensembles of chromoproteins which are selectively responsible for the sensing of the various incident light wavelengths and intensities, and for the initiation of the transduction of photomorphogenic signals.⁵ For example, in *Arabidopsis* five different phytochromes, *phyA-E*, have been identified,⁶ with *phyA* predominating in the etiolated (dark grown) stage of the plant and *phyB* being the most abundant form in the green stage. Furthermore, gene sequences encoding for phytochrome-like apoproteins have been found recently also in lower plants and cyanobacteria.^{2,7,8}

Our phytochrome work primarily focuses on the spectroscopic investigation of the complex structural changes and dynamics associated with the $P_r \rightleftharpoons P_{fr}$ photocycle (Figure 2). It thereby has to cope with two major difficulties, *viz.*, the lack of information on the protein structure (other than complete sequence and quaternary shape; *cf.* Figure 1 top), and the extensive spectral overlap of P_r , P_{fr} and the many intermediates. Combinatory studies of native and recombinant phytochromes by many different spectroscopies have been designed to master these challenges (Figure 3).^{2,7,9-25} The native phytochromes consist of a protein of typically 125 kDa (about 1100 amino acid residues) and a single chromophore (*PΦB*; Figure 1 top). N-Terminal phytochrome fragments (*e.g.*, *phyA65*) occur as monomers in solution, whereas the C-terminal domains cause full-length phytochromes to dimerize.

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From the Executive Director

D. C. Neckers, Executive Director, Center for Photochemical Sciences, Bowling Green State University

I ran into a member of the University Board of Trustees at a restaurant recently. He's an attorney, and he introduced me to his colleague as they were going in for lunch. After a couple of minutes he said to his friend, "Neckers is the Director of the Center for Photochemical Sciences. He sends me his newsletter from time to time. After the first two sentences or so I find I can't understand a word." I gulped a bit, looked embarrassed and demurred by saying I hoped at least he would read the editorials. I believed he could understand them.

I'm sure we've all heard comments like this from friends. But I wonder how that attorney would react if I introduced him to the principal bassoonist in the New York Philharmonic and said;

"Here's Judy LaClair. She plays the bassoon. I'd tell you what she does, but I don't understand a thing about it."

I know what most people in cultured society would think. Anyone who told a professional musician that what he/she did for a living was completely incomprehensible would be immediately labeled a cultureless hack by Ms. Manners' gentler society. Yet, I bet many people, even professional people, know as little about playing a bassoon as they know about chemistry. Even people that can't tell major from minor, soprano from bass, or Bach from Scriabin, will go to a concert, suffer through it, and tell all their friends how wonderful it was. It would be uncultured to do otherwise.

So why do non-scientists react in this way to their scientists friends? In my younger days I used to think scientists were at fault by not translating our message so non-scientists could understand it. If we were able to do this, they'd respect what we do, try to understand it at some level, and CP Snow's two cultures would melt into one big happy family. But I'm old and a lot less patient with the world than I used to be. As one who probably errs by trying too hard to simplify scientific things so that they can be understood, I submit our non-scientific colleagues are insulting the dickens out of us everytime they react as my friend did. They think otherwise, but the reality is something quite different. For some reason, the pain of the piano lessons taken as a child becomes enriching and good when the person becomes an adult. But pain suffered in math and in science in school remains vivid to the adult to the point that some percentage of the citizenry isn't willing to listen or to read even when scientists attempt to tell their story in terms they can understand.

I suspect a fundamental disrespect for the quantitative is inculcated in children before they get out of grade school. It's a given every child needs to know how to read. But there's less emphasis on mathematics in American society than elsewhere in the world, so if a child can't do algebra it's ok. Plenty of Americans are handicapped likewise, and both their teachers and parents are willing to excuse it.

I'm sure my friend thought he was offering me a compliment. But at another level, and this was the level at which I took it, I believe what he was really saying was that what scientists do is so unimportant in contemporary America a significant percentage of the citizenry can't be bothered to learn a thing about it.

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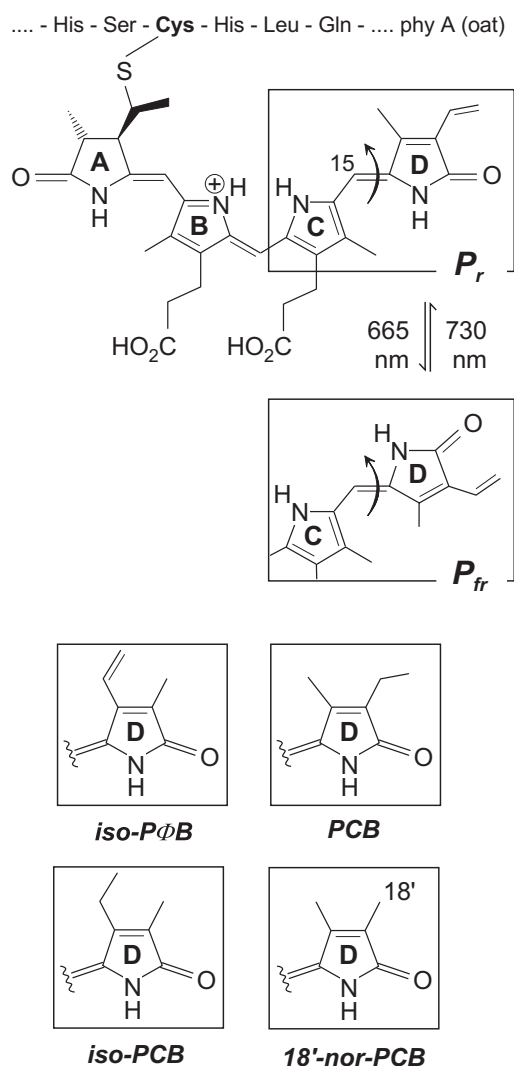
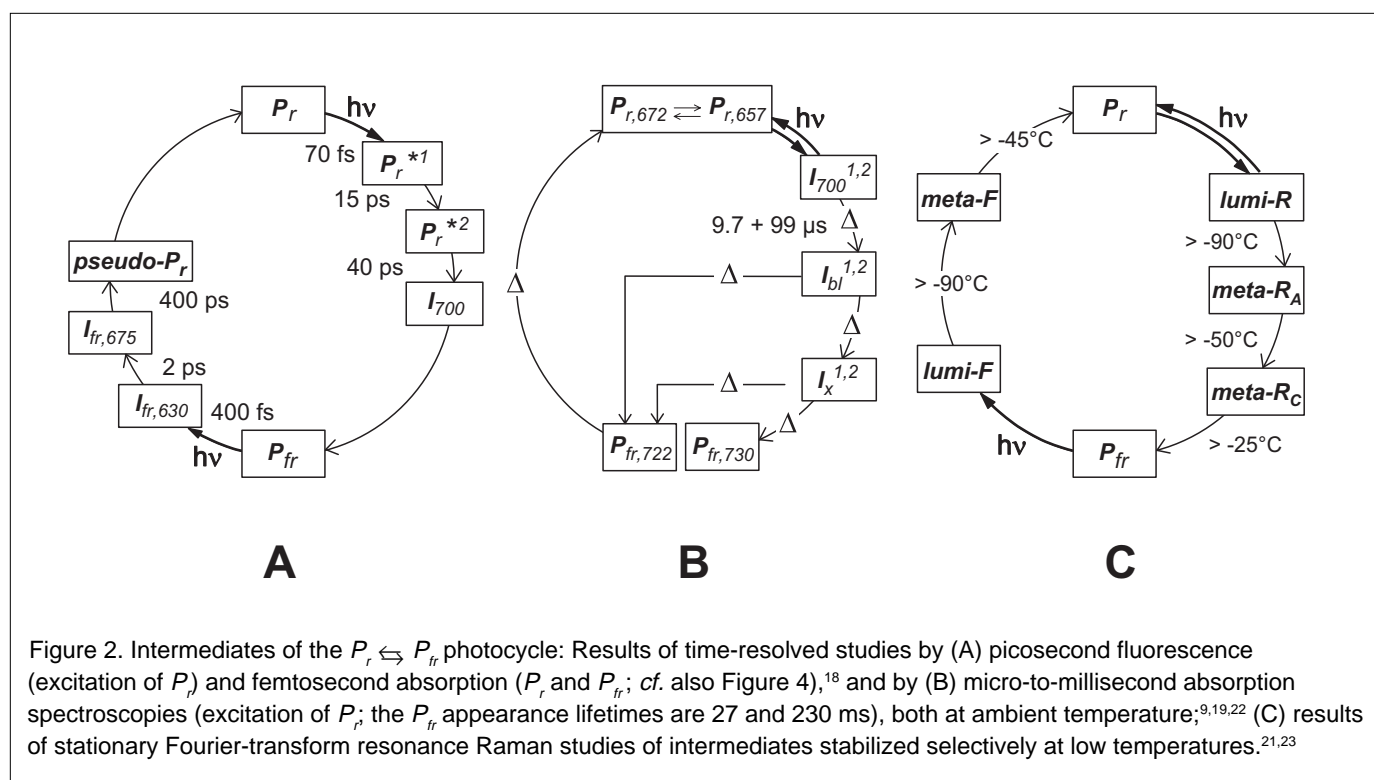


Figure 1. Top: Initiation of the $P_r \rightarrow P_{fr}$ transformation by primary $Z \rightarrow E$ photoisomerization of the 15,16-double bond of phytylchromobilin ($P\Phi B$), the chromophore of phytochrome. Note that only the complete amino acid sequence (but neither secondary nor tertiary protein structures), the constitution of $P\Phi B$ (the conformation shown is judged a likely one by experimental evidence), and the position of the covalent $P\Phi B$ attachment to the protein (Cys321 in *phyA* of oat) are known. Bottom: Chromophores which have been synthesized and assembled with recombinant truncated apophytochrome of oat (*phyA65*) in order to probe structural constraints during the $P_r \leftrightarrow P_{fr}$ transformation.¹⁶

Some results of ultrafast fluorescence and absorption kinetics are summarized in Figure 4.¹⁸ The photoisomerization of P_r is photochemically reversible. It affords either two primary products, $I_{700}^{1,2}$, formed in parallel, or one such product which is in thermal equilibrium with a species of similar absorption properties.¹⁹ The photoequilibrium $P_r \leftrightarrow I_{700}$ is established at essentially all absorption wavelengths of either of the components. Laser-induced optoacoustic spectroscopy at very low laser fluences gave quantum yields of $\Phi_{P_r \rightarrow I_{700}} > 0.14$ and $\Phi_{I_{700} \rightarrow P_r} = 0.4 \pm 0.1$.²⁰ Since the quantum yields of $\Phi_{P_r \rightarrow I_{700}}$ and $\Phi_{P_r \rightarrow P_{fr}} (= 0.16^{26})$ are practically the same, none of the intermediates thermally returns to the parent P_r state to any significant extent. Rather, >80% of the absorbed light energy are wasted as heat and, in accord with low-temperature resonance Raman data²¹, the protein conformation in I_{700} is still similar to that of P_r . This is probably the reason why the $I_{700} \rightarrow P_r$ photoreversion is more efficient than the forward reaction. The complexity of events revealed by the ultrafast kinetics evolving from photoexcited P_r and P_{fr} is juxtaposed by similarly intricate sequences of parallel and consecutive thermal steps, and by the photochemical equilibrium $P_r \leftrightarrow I_{700}$ on the microsecond-to-second time-scale (Figure 2A, B).¹⁹ Extensive comparative studies with native and recombinant *phyAs* showed that the complexity, as much as it has been recognized so far, is an intrinsic property of phytochrome.²² Furthermore, resonance Raman data of intermediates selectively stabilized at low temperatures have afforded insights into the structural changes of the chromophore during the photocycle in terms of the E/Z double-bond isomerization and the torsions around the single bonds of the methine bridges (Figure 2C).^{14,15,21,23} Of particular interest are distinct changes in hydrogen bonding interactions between the protein and the N-H groups in rings B and C of the intermediate *meta-R_C* as well as far-reaching spectral similarities between the chromophores of *meta-R_C* and P_{fr} , which suggest that in the *meta-R_C* $\rightarrow P_{fr}$ step mostly the ultimate conformational changes of the protein are occurring. Marker bands, derived from recent normal mode analyses of tetrapyrroles,²⁴ have been sought out as the diagnostic indicators for the protonated state of the pyrrolic nitrogen to persist throughout the $P_r \leftrightarrow P_{fr}$ photocycle.²³

Our most recent efforts have been directed towards probing the domains of chromophore and protein which control the $P_r \leftrightarrow P_{fr}$ photocycle under *in-vitro* conditions, exploiting the considerable diversity of recombinant *phys* which are now available by heterologous expression in yeasts of full-length and fragmented apoproteins, and their assembly with $P\Phi B$, PCB , and various substitutional isomers and homologs thereof (Figure 3). Four topics are being addressed: kinetic differences in the $I_{700} \rightarrow P_{fr}$ transformations between



phyA–*Cs* upon changing the chromophore structure, the protein sequence (by site-directed mutagenesis) of the binding pocket, and upon polypeptide truncation.

Results of the currently still ongoing studies include, *inter alia*, the following: The decay of I_{700} and formation of P_{fr} and its dark reversion to P_r clearly differ in *phyA* (of oat), *phyB* (of potato and *Arabidopsis*), and *phyC* (of *Arabidopsis*). It remains to be established, however, whether the kinetic differences reflect differences between the phytochromes of green and etiolated plants, or are due to the origin from either di- and monocotyledons.¹³

The ring D substituents of the chromophore (Figure 1 bottom) are adjacent to the site where the primary photoisomerization step occurs and thus are judged to be particularly sensitive to spatial boundary conditions for the reactivity imposed by the protein pocket. It is not surprising, therefore, that seemingly small structural changes in ring D¹⁶ result in substantial kinetic modifications, concerning number and efficiency of reaction channels between the intermediates of the photocycle, and the thermal P_{fr} to P_r back reaction, while the basic aspects of the multistep $P_r \rightarrow P_{fr}$ transformation remain unchanged. For example, the P_r to P_{fr} kinetics of the N-terminal *phyA65* and *phyB66* can be quite different. The decay of the primary photoproduct I_{700} of *phyA65-PCB* is monoexponential and that of the *P Φ B* analogue biexponential, whereas I_{700} of *phyB66* decays monoexponentially irrespective of the chromophore incorporated. Furthermore, the formation of P_{fr} from P_r , on the one hand, is faster with the N-terminal halves than with the full-length phytochromes, indicating an involvement of the C-terminal domain in the relatively slow protein conformational changes. On the other hand, the thermal stabilities of the P_{fr} forms of the N-terminal halves are similar to those of the corresponding full-length phytochromes, the oat *phyA P_{fr}s* being highly stable, whereas the potato *phyB P_{fr}s* are rapidly converted back into the P_r s. Thus, the C-terminus does not regulate this property. Rather, it reflects some structural differences in the N-terminal halves between the phytochromes originating from mono- and dicotyledones.^{13,15} Similar variations have since been observed for the light-induced and thermal transformations of all other recombinant phytochromes listed in Figure 3. However, the results are still too fragmentary to permit conclusions based on any coherent structure-dynamics relationship.

Interestingly, changes in the ring D substituents can also cause appreciable shifts of the absorption maximum. Thus, exchanges of *P Φ B* with *iso-P Φ B* and of *PCB* with *iso-PCB* (Figure 1) in oat *phyA65* result in hypsochromic shifts of 10–14 nm in the P_{fr} forms (contrary to the P_r absorptions which remain unaffected), whereas the absorption maximum of *phyA65-18'-nor-PCB* is quite similar to that of *phyA65-PCB*. The hypsochromic shifts exhibited are therefore attributable to the increase in size of the substituent at position C-17, rather than to an alternate release of steric compression around C-18.¹⁶

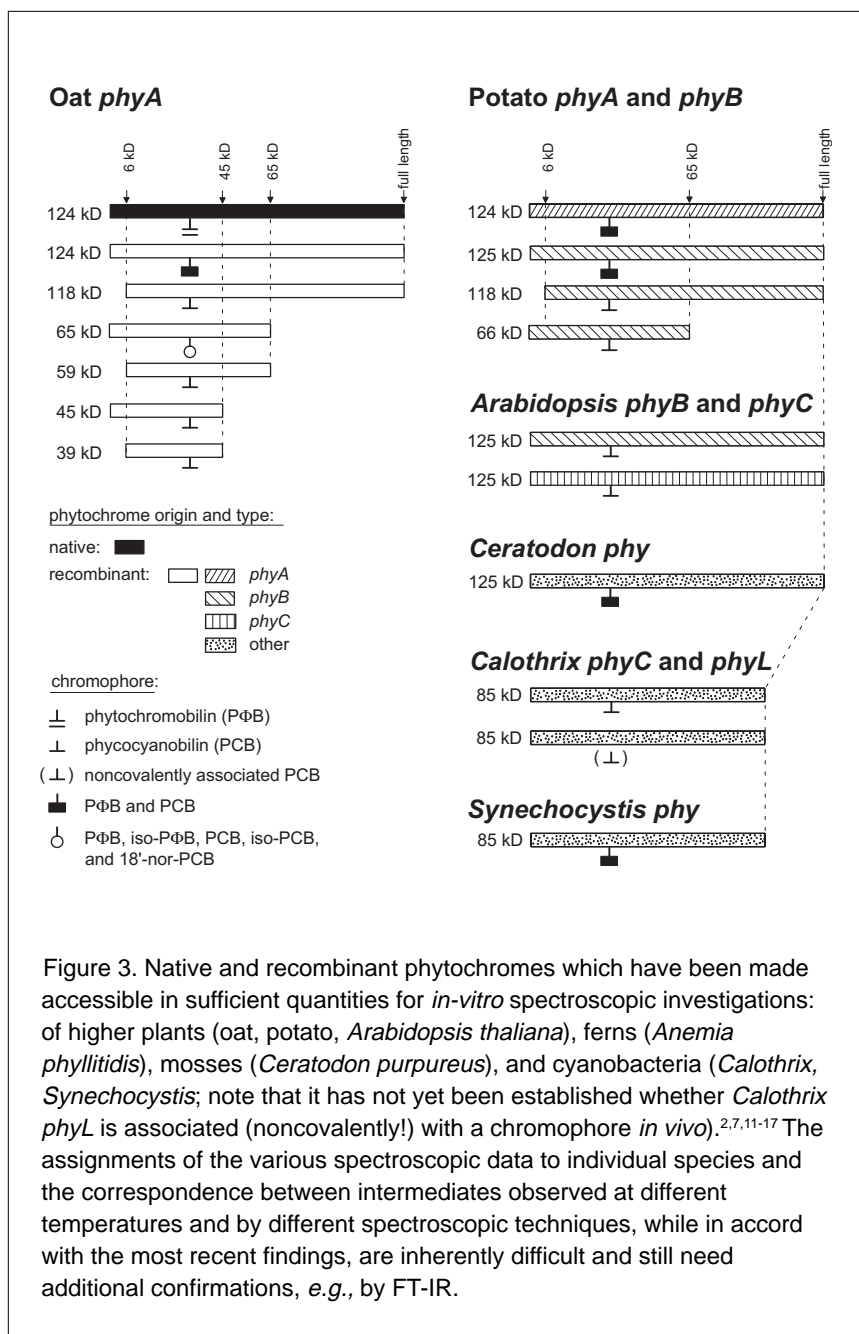


Figure 3. Native and recombinant phytochromes which have been made accessible in sufficient quantities for *in-vitro* spectroscopic investigations: of higher plants (oat, potato, *Arabidopsis thaliana*), ferns (*Anemia phyllitidis*), mosses (*Ceratodon purpureus*), and cyanobacteria (*Calothrix*, *Synechocystis*; note that it has not yet been established whether *Calothrix phyL* is associated (noncovalently!) with a chromophore *in vivo*).^{2,7,11-17} The assignments of the various spectroscopic data to individual species and the correspondence between intermediates observed at different temperatures and by different spectroscopic techniques, while in accord with the most recent findings, are inherently difficult and still need additional confirmations, *e.g.*, by FT-IR.

teins with PΦB and PCB. Thus, *Ceratodon* phytochrome was shown to correspond to *phyA*-PΦB rather than *phyA*-PCB.² Moreover, expression of the apoprotein from open reading frames in *Calothrix* and *Synechocystis*, and assembly with PΦB and PCB furnished recombinant procaryotic phytochromes which are functional *in vitro* in photophysical and photochemical terms but have not been isolated as yet from a natural source.^{7,8}

We should note in this context that such phytochromes appear in fact to be widely spread among cyanobacteria. This suggests that the principle of light sensing with open-chain tetrapyrrole chromophores has been acquired very early in evolution.²⁷ Evidence is accumulating also that phytochrome-like proteins may serve other functions, either with a chromophore or without. As the first example *Calothrix phyL* may be mentioned, in which the cysteine that covalently binds the chromophore in the other phytochromes is replaced by a leucine. Nevertheless, incubation of the recombinant *phyL* apoprotein with PCB afforded a photochromic phytochrome, in which the chromophore evidently is incorporated in a *noncovalent* fashion.²⁵ Not surprisingly, this *phyL* chromoprotein upon photocycling is less stable than the "normal" phytochromes and releases PCB to an appreciable extent.

Our experiments employing site-directed mutagenesis, as another means to explore the interactions between chromophore and protein, are presently still restricted to exchanges of amino acid residues in the domains adjacent to the chromophore-binding cysteine, in view of the missing informations about the three-dimensional structure. In this part of the protein remarkable effects on the kinetics of the $P_r \rightarrow P_{fr}$ conversion have been achieved. The I_{700} decay of the histidine mutant *H319A* is more than twice slower than that of the wild-type chromoprotein (177 *vs.* 75 μ s), whereas an increase in polarity by insertion of charged amino acids accelerates this process (50 μ s for the *L323R/Q324D* double mutant). The mutation studies also gave some insight into the role of the protein during the P_{fr} formation: Since prolines provide conformational flexibility to the protein backbone, proline318 was mutated into alanine. This exchange significantly slowed down the millisecond processes leading to P_{fr} formation (the time constants are 4 and 22 ms for the mutant, and 1.2 and 14 ms for the wild-type protein).¹⁷

The assembly of recombinant phytochromes (Figure 3) has greater potential than has been exploited so far. Clearly, the ongoing work outlined above wants still farther reaching systematic efforts. For example, absorption shifts in the P_r / P_{fr} difference spectra and the $P_r \leftrightarrow I_{700}$ kinetics have been used to identify the chromophore in phytochromes which are accessible only by assembling heterologously expressed recombinant apopro-

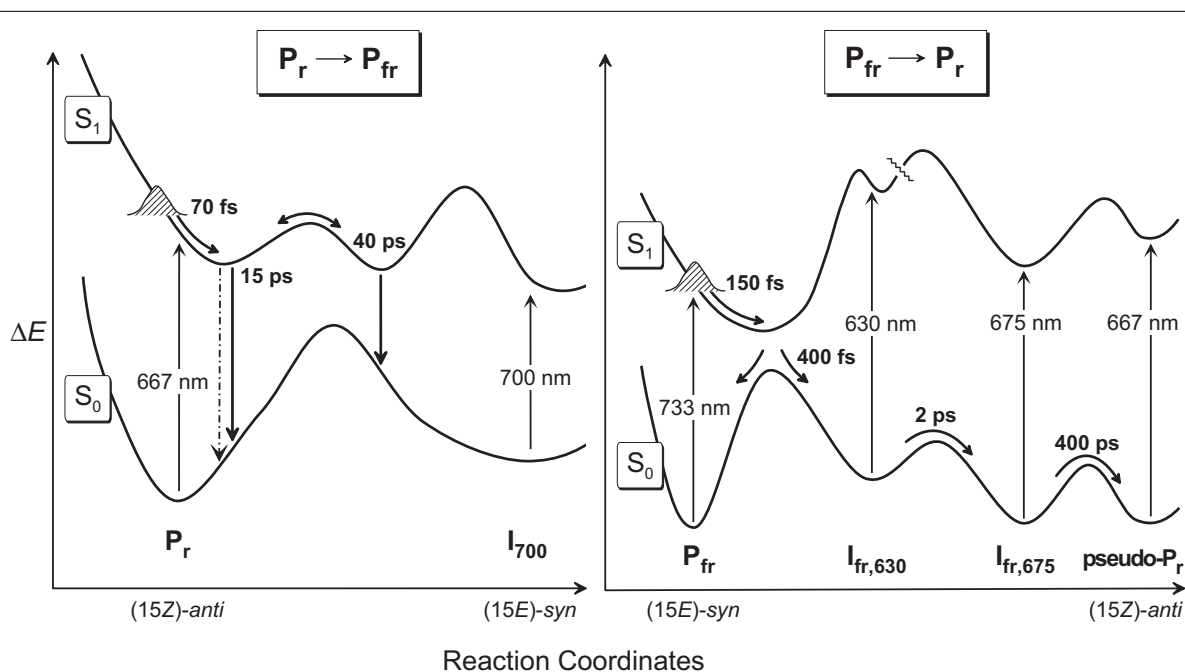


Figure 4. State and potential energy diagrams determined by picosecond fluorescence (P_r and P_{fr}) and femtosecond absorption (P_r and P_{fr}) spectroscopies for parts of the $P_r \leftarrow P_{fr}$ photocycle of native oat *phyA* at ambient temperature.

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Structure and Reactivity of Organic Intermediates as Revealed by Time-Resolved IR Spectroscopy

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Introduction

Time-resolved spectroscopic techniques are important and effective tools for mechanistic photochemical studies. The most popular of these tools, time-resolved UV-Vis absorption spectroscopy, has been applied to a wide variety of chemical problems for approximately 30 years.¹ Although a great deal of information about the reactivity of organic photochemical intermediates (e.g., excited states, radicals, carbenes, and nitrenes) in solution at ambient temperatures has been amassed with this technique, only limited structural information can be extracted from such investigations since absorption bands are usually quite broad and featureless. Questions of bonding, charge distribution, and solvation (in addition to those of dynamics) are more readily addressed with time-resolved vibrational spectroscopy.

Indeed, time-resolved resonance Raman spectroscopy has been successfully employed to study the structure and dynamics of many short-lived molecular species.² This technique, while certainly very powerful, does however have some limitations. It requires a non-fluorescent intermediate that has an absorption band at the wavelength of the scattering light. Scattering must be done at those wavelengths in order to take advantage of resonance enhancement of the Raman signal. Time-resolved infrared (TRIR) spectroscopy, on the other hand, is potentially a more general tool, since it gives one the ability to monitor directly both the structure and dynamics of the reactants, intermediates, and products of most photochemical reactions without the above restrictions. Unfortunately, experimental limitations have severely limited the types of molecular systems that could be studied by TRIR spectroscopy in the past. Most previous work in this field has concentrated on metal carbonyl complexes owing to their strong IR chromophores and experimentally convenient monitoring frequencies.³ In contrast, until recently, relatively few organic systems have been examined by TRIR spectroscopy.

Experimental Approaches to TRIR Spectroscopy

Recent technical advances have greatly expanded the applicability of TRIR spectroscopy, making measurements over a wide temporal and spectral range now feasible. The relative merits of different experimental techniques have been discussed previously by Ford^{3a} and also by Turner.⁴

Although sub-picosecond TRIR experiments are possible,⁵ wide spectral coverage in these cases is difficult since the generation and/or detection of the IR light typically involves nonlinear frequency mixing in materials such as crystalline LiIO₃ under the proper phase-matching conditions. A narrow range of attainable IR probe frequencies can limit the types of molecular systems amenable to investigation.

At the present time, greater spectral coverage is accomplished more readily with TRIR experiments on the nanosecond and slower timescales. Here, mainly two alternatives exist: time-resolved step-scan FTIR spectroscopy and conventional pump-probe experiments. The step-scan FTIR experiment has previously been described in detail.⁶ Briefly, the moving mirror of an FTIR spectrometer is translated in discrete steps rather than continuously as is normally done in a static FTIR experiment. At each mirror position, the photochemical process of interest is triggered (e.g., by a laser pulse) and the temporal changes in detector response are recorded. Data from each mirror position are transposed into a series of interferograms that correspond to different time delays following the trigger. These interferograms are then Fourier transformed to provide a series of time-resolved IR spectra. In conventional nanosecond pump-probe TRIR experiments, spectra are measured one frequency at a time. The pump source is typically a nanosecond laser; the probe source can be broadband IR light from a globar or tunable IR light from a CO laser or a semiconductor diode laser.

Although CO and diode lasers can produce high intensity IR light, these probe sources are limited in their spectral coverage. The CO laser is restricted to the range 2000 - 1500 cm⁻¹ and a typical IR diode laser has a maximum scanning range of 100 - 150 cm⁻¹.^{3a} Of course, a series of diode lasers will cover a broader range of frequencies, but such a setup

can quickly become prohibitively expensive. Normal globar sources are not limited in their spectral range, but unfortunately produce relatively low intensity IR light that typically leads to signal-to-noise problems. Thus, in conventional TRIR pump-probe experiments one is usually faced with a choice between probe sources with high intensity or broad spectral coverage.

One solution to this dilemma has recently been advanced by Hamaguchi⁷ who has made use of a MoSi₂ IR source newly developed by JASCO that provides approximately twice the emissive intensity of conventional globar sources. This probe source was incorporated into a dispersive TRIR spectrometer that allows access to the entire mid-IR spectrum with high sensitivity ($\Delta A < 10^{-5}$) and sufficient time (50 ns) and frequency (4 - 16 cm⁻¹) resolution to probe a wide range of transient intermediates in solution. The block diagram of Figure 1 shows the TRIR spectrometer that we have constructed at Hopkins following this design.

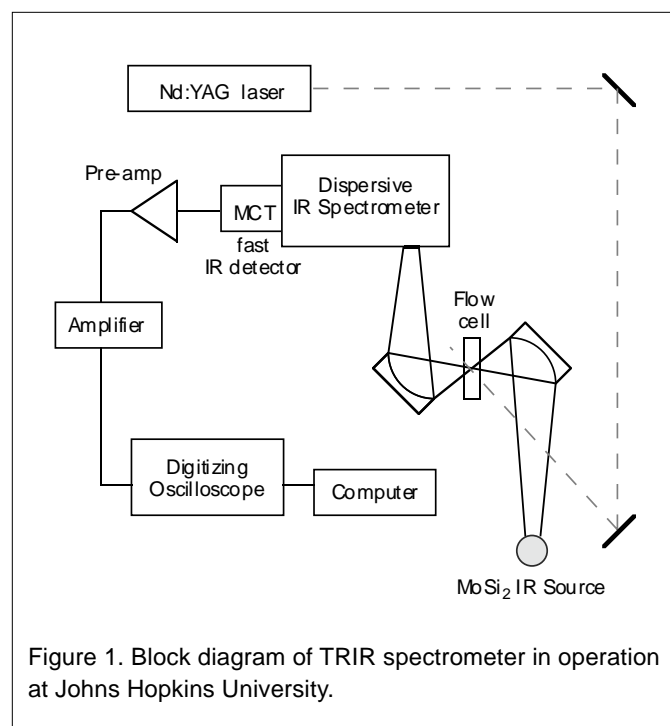


Figure 1. Block diagram of TRIR spectrometer in operation at Johns Hopkins University.

As indicated in Figure 1, the broadband output of the MoSi₂ IR source is crossed with excitation pulses (266 nm, 355 nm, or 532 nm) from a Nd:YAG laser. Changes in IR intensity are monitored as a function of time by an ac-coupled mercury/cadmium/tellurium (MCT) photovoltaic IR detector (Kolmar Technologies, KMPV11-1-J1), amplified by an NF Electronic Instruments 5305 low noise amplifier, and digitized with a Tektronix TDS520A oscilloscope. Data are collected at repetition rates up to 200 Hz, the maximum data handling speed of our digitizing oscilloscope, and acquisition is synchronized with the stepwise scan of a JASCO TRIR-1000 dispersive spectrometer.

TRIR measurements are obtained from three different recordings of the emission spectrum of the IR source. These include a spectrum of the IR source without the sample in the beam path (I_0), a spectrum of the IR source with the sample in the beam path (I), and the infrared intensity changes induced by photoexcitation (ΔI). The absorbance spectrum of the unexcited sample is derived from I_0 and I , $A = \log(I_0/I)$. Since the IR detector is ac-coupled measurement of I_0 and I are performed with an optical chopper (Stanford SR540) to modulate the IR light. The TRIR ab-

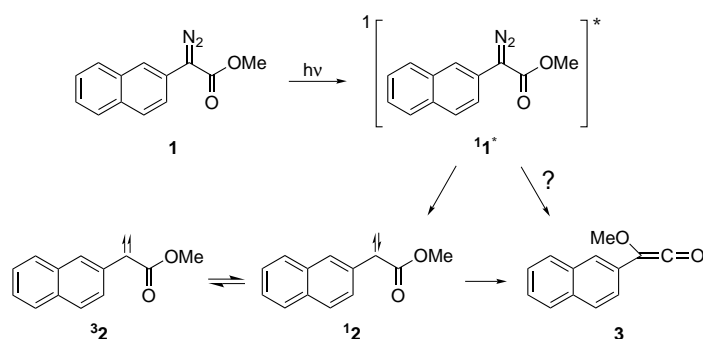
sorbance spectrum is obtained in the form of a difference spectrum, $\Delta A = -\log(1 + \Delta I/I)$. Thus, depletion of reactant gives rise to negative signals, and formation of transient intermediates or products leads to positive bands. In ideal cases, the depletion of reactants, the growth and decay of intermediates, and the growth of products of a photoinitiated reaction can be monitored.

In order to obtain TRIR spectra with sufficient sensitivity, we typically signal average several thousand laser shots at each IR frequency of interest. Thus, a flow cell is necessary to prevent excessive sample decomposition. A reservoir of solution (typically 10 - 20 mL) is continually circulated between two calcium or barium fluoride salt plates. Since the detection of transient species is more problematic in regions with strong solvent bands as a result of low transmission of IR light,⁷ available spectral windows are limited by solvent absorbance and path length.

Although the dispersive TRIR experiment described above does not have the spectral multiplexing advantage of the step-scan FTIR experiment, both experiments require that the transient process being probed is reliably repeatable. (The dispersive experiment demands substantial signal averaging; the step-scan experiment requires data collection at a series of mirror positions.) The step-scan FTIR method, however, can be more sensitive to any variation in measuring conditions compared with the dispersive TRIR experiment. For example, a noise spike in an interferogram will affect the entire spectrum in the step-scan experiment, whereas it will distort only a single point in the dispersive method. Indeed, early step-scan FTIR experiments were plagued by spectral artifacts caused by fluctuating measuring conditions.⁸ An additional difference between the two experiments is that the dispersive method allows the accumulation of accurate kinetic data at a single frequency of interest, whereas step-scan experiment requires collection of the entire TRIR spectrum.

Representative TRIR Studies of Carbene Reactivity

With the dispersive TRIR spectrometer shown in Figure 1 we have begun to examine the structure and reactivity of a variety of organic reactive intermediates.⁹ As a demonstration of the utility of TRIR spectroscopy for the study of such intermediates, representative data from our experiments with carbonyl carbenes are presented below.



Our current time-resolution restricts this approach to the study of carbenes with lifetimes greater than 50 ns. We,^{9d} along with the groups of Bally,¹¹ McMahon,¹¹ and Platz¹² recently examined in detail the chemistry and spectroscopy of 2-naphthyl(carbomethoxy)carbene (2).

Observation of 2-Naphthyl(carbomethoxy)carbene by TRIR Spectroscopy

Typical TRIR data observed following 266 nm laser excitation of diazoester **1** in argon-saturated Freon-113 (1,1,2-trichlorotrifluoroethane) are shown in Figure 2 for the spectral region 1800 - 1550 cm^{-1} . We attribute the positive bands at 1650, 1620, and 1584 cm^{-1} observed over the first 1.0 μs after photolysis to 2-naphthylcarbomethoxycarbene (**2**) based on a comparison with the low-temperature matrix IR data of Bally and McMahon (carbene bands detected at 1660, 1640, 1625, and 1590 cm^{-1});¹¹ the negative band at 1715 cm^{-1} in Figure 2 is due to the depletion of diazoester **1**. (The reported 1640 cm^{-1} signal is not resolved in our TRIR data due to its low intensity and the relative broadness of solution IR bands.) Bally and McMahon demonstrated that the matrix IR band they observed at 1660 cm^{-1} is due to the triplet carbene (**32**) and those observed at 1640, 1625, and 1590 cm^{-1} arise from the singlet carbene (**12**).¹¹ Analogously, we have assigned our TRIR band detected at 1650 cm^{-1} to **32** and those detected at 1620 and 1584 cm^{-1} to **12**.

We further confirmed the identities of the 1650, 1620, and 1584 cm^{-1} bands of Figure 1 by kinetic studies. Platz and co-workers showed that singlet/triplet spin equilibration of carbene **2** is much faster than reaction from either spin state.¹² Consistent with that observation, we find that signals at 1650, 1620, and 1584 cm^{-1} decay at equal rates. Quenching studies performed at 1650 and 1584 cm^{-1} provided rate constants in agreement with previous work.¹³ The quenching rate constants observed at 1650 cm^{-1} (**32**) and 1584 cm^{-1} (**12**) with reagents that could potentially react in a multiplicity-dependent fashion (e.g., methanol and oxygen) are the same within experimental error, again consistent with fast singlet/triplet spin equilibration of carbene **2**.

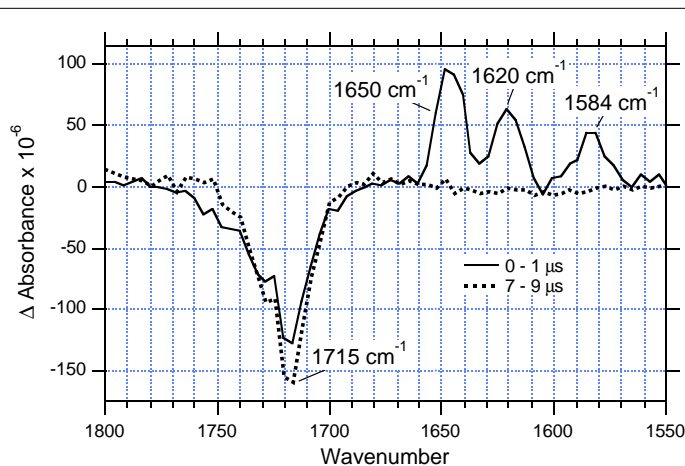


Figure 2. TRIR difference spectra observed from 0 to 1 μs and 7 to 9 μs following 266 nm photolysis (10 ns, 0.4 mJ) of diazoester **1** (1.1 mM) in argon-saturated Freon-113.

Singlet/Triplet Energy Gap of 2-Naphthylcarbomethoxycarbene

One of the most fundamental properties that determines a carbene's reactivity is its spin state. However, even in those cases where the

triplet is of lower energy, chemistry often arises from the higher lying, but more reactive, singlet carbene. Two mechanistic schemes have emerged to explain such observations. The classical mechanism, first advanced by Bethell and co-workers,¹⁴ predicts that the observed barrier (E_a) for a singlet carbene reaction is given by the actual activation barrier of the reaction (ΔH^\ddagger) plus the energy required to populate the singlet from the lower energy triplet carbene (ΔH_{ST}).

$$E_a = \Delta H^\ddagger + \Delta H_{ST}$$

The alternative, proposed by Griller, Nazran, and Scaiano,¹⁵ is an avoided surface-crossing mechanism in which the triplet carbene surface crosses the singlet carbene \rightarrow product surface at a point below the energy of the singlet carbene leading to an observed E_a that is lower than the sum of ΔH^\ddagger and ΔH_{ST} . In this case, if the activation energy for the singlet carbene reaction is very small, one might experimentally observe an activation barrier less than the singlet/triplet energy gap. Indeed, the avoided surface-crossing mechanism was originally formulated to explain such an observation.¹⁵

Thus, accurate measurements of carbene singlet/triplet energy gaps are both fundamentally and mechanistically very important. However, to our knowledge a carbene singlet/triplet gap has never been *directly* measured in solution.¹⁶ Previous experimental estimates of these splittings usually employ a combination of product studies and kinetic measurements and have been forced to rely on assumptions concerning the spin-selectivity of carbene trapping reagents.¹⁷ For example, alcohols are thought to be selective traps of singlet carbene; oxygen and isoprene are believed to be selective triplet carbene traps. Of course, any inaccuracy in these suppositions could lead to critical errors in the estimated energy difference between the two carbene spin states.

Our observation of IR signals of both singlet and triplet 2-naphthylcarbomethoxycarbene (**2**) allows a direct experimental estimate of a carbene singlet/triplet energy gap in solution. Since the relative intensities of these signals are directly related to the concentrations of the singlet and triplet carbene, an equilibrium constant and subsequently a standard free energy difference can be easily derived. To determine the relative concentrations, however, we need the ratio of extinction coefficients for a singlet and a triplet IR band. Fortunately, an estimate of this ratio is available

from the matrix studies of Bally and McMahon who observed that at 12K **2** thermally relaxes only to **32**.¹¹ Thus, the intensity ratio of final **32** band to initial **2** band reflects the ratio of their extinction coefficients. We chose the well-separated signals observed at 1650 cm^{-1} (**2**) and 1584 cm^{-1} (**2**) for this analysis. The low-temperature matrix-determined ratio (**32**/**2**) for these signals is approximately 1.5; the Freon-113 solution TRIR-determined ratio at 21°C is 2.1. These values lead to an equilibrium constant of 1.4 ± 0.2 at 21°C and a free energy difference of 0.2 ± 0.1 kcal/mol, with the triplet carbene lower in energy.

Carbene **2** was assigned a triplet ground state by Bally and McMahon on the basis of low-temperature ESR spectroscopy and theoretical calculations as well of their observation that **2** relaxes thermally to **32** at 12K.¹¹ Thus, our determination that **32** has lower energy than **2** agrees well with their work; however, our experimental value of the singlet/triplet splitting is significantly lower than that calculated by density functional theory ($\Delta H_{ST} = 4.52$ kcal/mol).¹¹ Matrix site effects on observed IR band intensities could certainly lead to errors in our estimated extinction coefficient ratio, but even a fairly considerable error in equilibrium constant translates to a relatively small absolute error in free energy difference.

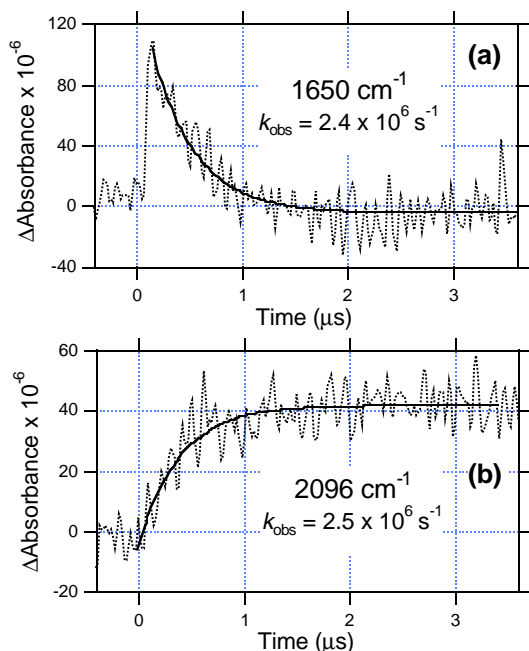
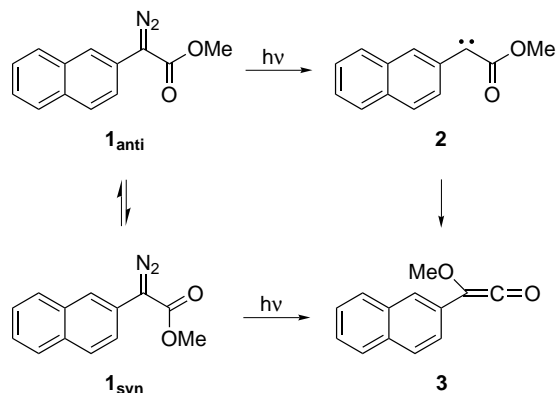


Figure 3. Kinetic traces observed at (a) 1650 cm^{-1} and (b) 2096 cm^{-1} following 266 nm photolysis (10 ns, 0.4 mJ) of ^{15}N -labeled **1** (3.1 mM) in argon-saturated Freon-113. The dotted curves are experimental data; the solid curves are the calculated best fit to a single exponential function.

Moreover, the fact that we can observe singlet carbene signals at ambient temperature indicates that the singlet/triplet energy separation must be on the order of 1 kcal/mol or less.

Previous work by Eisenthal and co-workers¹⁸ has shown that singlet diphenylcarbene is stabilized relative to triplet diphenylcarbene in polar solvent, presumably due to the zwitterionic nature of singlet carbenes.¹⁹ In addition, the groups of Goodman²⁰ and of Moss²¹ have recently provided evidence for the stabilization of singlet carbenes through the formation of energetically favorable carbene – benzene complexes. Thus, a possible explanation for the difference between the calculated (gas phase) and experimental (Freon-113 solution) value of the singlet/triplet splitting for carbene **2** is that the singlet is preferentially stabilized in solution. We are currently pursuing this hypothesis by attempting to measure the singlet/triplet gap for **2** as a function of solvent.



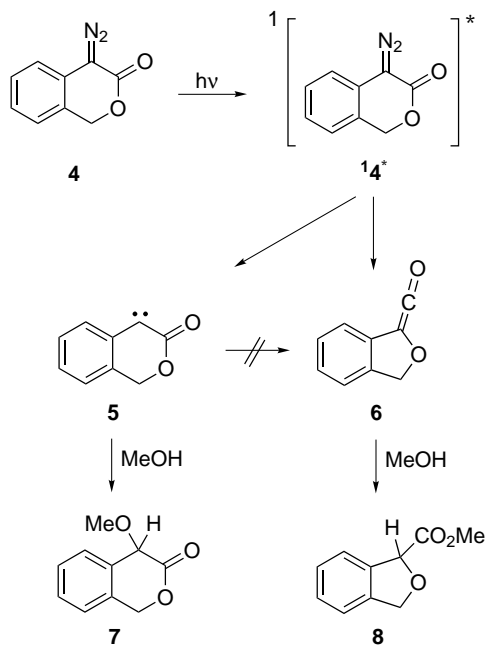
Mechanism of Ketene Formation

As discussed above, the original impetus for our TRIR investigation of diazoester **1** was to monitor directly the kinetics of rearrangement to ketene **3**. Specifically, we were interested in determining the portion of ketene **3** produced directly from the singlet excited state of **1** and that formed from spin-equilibrated carbene **2** (Scheme 1). Since the lifetime of ¹**1*** is likely much less than 1 ns

and certainly well below the present time resolution (50 ns) of our TRIR spectrometer, ketene production only from ¹**1*** would be indicated by a fast, unresolvable increase in ketene IR absorbance following laser photolysis. Production only from carbene **2** would be revealed by a rate of ketene growth equal to that of carbene decay.

We were initially disappointed to find that the rate of ketene production was impossible to monitor for diazoester **1** due to overlap between the positive ketene IR band (at 2096 cm⁻¹) and the strong negative depletion signal of the diazoester at 2090 cm⁻¹. Thus, we prepared and studied doubly ¹⁵N-labeled **1** in order to shift the diazo IR band away from the ketene signal of interest. Here, the diazo depletion band is shifted to 2020 cm⁻¹ and the positive ketene band is now observed at 2096 cm⁻¹. Figure 3 shows that the rate of ketene growth is equal to the rate of carbene decay, clearly demonstrating that ketene **3** arises entirely from carbene **2**.

This result is in excellent agreement with the alcohol trapping experiments of Platz and co-workers who isolated essentially only carbene derived adducts¹² and the calculations of Bally and McMahon.¹¹ Those calculations indicate



that diazoester **1** is planar and exists almost entirely (99%) in conformations in which the diazo and carbonyl groups are *anti*. Previously, Kaplan and co-workers²² demonstrated that diazocarbonyl compounds can exist as equilibrium mixtures of *syn* and *anti* forms (e.g., **1**_{syn} and **1**_{anti}). Kaplan's group, as well as those of Tomioka²³ and of Platz,²⁴ have shown that concerted rearrangement to ketene from an excited state is facile from the *syn* conformation, but carbene production is more likely from the *anti* form (Scheme 2).²⁵ Thus, the preferred conformation of **1** leads to efficient carbene production upon photolysis with very little if any direct rearrangement to ketene **3**.

The lack of excited state involvement for **1** is in contrast to behavior in systems that have substantial equilibrium concentrations of the *syn* conformer. In order to examine the effect that conformation has on ketene growth kinetics, we very recently have prepared and studied 4-diazo-3-isochromanone (**4**),²⁶ a cyclic analogue (phenyl version) of **1** which is locked in the *syn* conformation. In this case, as shown in Figure 4, we observe a carbene IR band at 1686 cm⁻¹ that decays with a lifetime of 525 ns in Freon-113. The ketene IR band at 2106 cm⁻¹, however, in dramatic contrast to the data observed with acyclic diazocarbonyl **1**, is produced faster than the current time resolution (50 ns) of our spectrometer. We, therefore, hypothesize that ketene **6** is

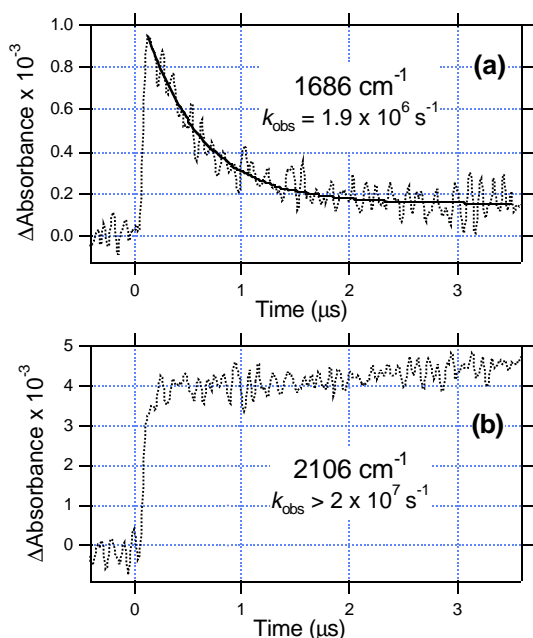


Figure 4. Kinetic traces observed at (a) 1686 cm^{-1} and (b) 2106 cm^{-1} following 266 nm photolysis (5 ns, 4 mJ) of ^{15}N -labeled **4** (2 mM) in argon-saturated Freon-113. The dotted curves are experimental data; the solid curve is the calculated best fit to a single exponential function.

formed entirely from the excited state of **4** (Scheme 3). Consistent with this hypothesis, methanol and oxygen quench carbene **5**, but leave the initial intensity of the ketene IR band unaffected. In addition, photolysis of **4** in neat methanol leads to both carbene-derived (i.e., **7** in 75% absolute yield) and ketene-derived adducts (i.e., **8** in 18% absolute yield), but thermolysis of **4** in neat methanol provides only **7** (91% absolute yield).

Conclusions

The wealth of structural and kinetic information available from TRIR spectroscopy makes it a powerful tool for establishing important structure/reactivity relationships for short-lived reactive intermediates. As demonstrated by our studies on carbonyl carbenes, recent technical advances have greatly expanded the applicability of this technique. As the necessary instrumentation becomes even more refined and easier to implement, application of TRIR spectroscopy to a range of photochemical and photobiological problems promises to become more common. Given its obvious potential, future TRIR work will likely advance our fundamental understanding of reactive intermediates just as the application of time-resolved UV-Vis absorption spectroscopy began to do 30 years ago.

Acknowledgment

I am extremely grateful to my co-workers, especially Ms. Yuhong Wang, Mr. Brett Showalter, Dr. Naod Kebede, and Dr. Alexander Nikolaitchik, for their contributions. Our research is generously supported by the Camille and Henry Dreyfus New Faculty Awards Program, a National Science Foundation Faculty Early Career Development Award, the National Institutes of Health, and the Donors of the Petroleum Research Fund administered by the American Chemical Society.

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1999 Ralph and Helen Oesper Banquet and Symposium Honoring**Professor George S. Hammond**

The Department of Chemistry of the University of Cincinnati and the Cincinnati Section of the American Chemical Society present the 1999 Ralph and Helen Oesper Banquet and Symposium honoring Professor George S. Hammond, Bowling Green State University. A banquet will be held October 28, 1999, at the Faculty Club, University of Cincinnati. Cocktails will begin at 5:30 p.m. followed by a banquet at 7:00 p.m. Professor Richard A. Caldwell, University of Texas at Dallas, will speak at 8:00 p.m. on "*George Hammond's Scientific Legacies*". A symposium will be held October 29 at The Regency Ballroom, The Vernon Manor Hotel, 400 Oak Street, Cincinnati. The schedule follows.

- 8:30 a.m. Professor Douglas C. Neckers, Bowling Green State University
New Chemistry in 3 Dimensions
- 9:15 Professor Richard A. Caldwell, University of Texas at Dallas
Time-resolved Photoacoustic Calorimetry: Techniques, Results, and Prospects
- 10:00 Coffee Break
- 10:15 Professor and Chancellor Mark S. Wrighton, Washington University
Molecular Photochemistry at Interfaces
- 11:00 Dr. Angelo Lamola, Rohm and Haas
Streams from George's River (Assorted Research Topics Through the Years)
- 11:45 Dr. Thomas Dougherty, Roswell Park Cancer Institute
Photodynamic Therapy for Cancer
- 12:30 p.m. Lunch
- 2:00 Professor Michael A. J. Rodgers, Bowling Green State University
Ultrafast Photodynamics of Metallophthalocyanines
- 2:45 Dr. David F. Eaton, DuPont Photopolymer and Electronic Materials
Industrial Aspects of Photochemistry, with Useful Examples
- 3:30 Coffee Break
- 3:45 Professor Nicholas J. Turro, Columbia University
The "Cage Effect": From the Gas Phase to the Molecular Solvent Cage to the Supramolecular Cage to the Superdupermolecular Cage
- 4:40 Introduction of the Guest of Honor, Professor R. Marshall Wilson

Oesper Award Lecture**Professor George S. Hammond, Bowling Green State University***Chemical Dynamics, the Heartland of the Science*

For details, see the web site at www.che.uc.edu/grad_program/oesper_award.html. For information on the symposium, contact Kim Carey, (513) 556-0293, careykr@email.uc.edu, or R. Marshall Wilson, (513) 556-9261, wilsonrm@uc.edu. The mailing address is Department of Chemistry, University of Cincinnati, Cincinnati, Ohio 45221-0172.

From *The Spectrum* Editor

Pat Green

When I was a freshman in college, I was placed in the wrong chemistry course. I was supposed to be in chemistry for non-majors (my degree is secondary education with majors in English and history), but I ended up in freshmen chemistry for majors. I was naïve and did not know I could drop the course, so I ended up with a whopping four credit hour “D”. I threw away my slide rule, got an “A” in chemistry for non-majors, and swore off science forever. Why would I ever need it anyway! I was planning to teach high school English. (Besides wearing those slide rules on belts was pretty nerdy.)

I am sure my chemistry professor would have laughed hilariously if he had known I was going to spend almost 13 years in the Center for Photochemical Sciences and become friends and colleagues with not only chemists, but scientists in many different disciplines all over the world. If anyone had told me that I would ever again absorb any scientific knowledge, let alone hang around with scientists, I would have laughed hilariously!

Yet, this is exactly what has happened. I have spent a major portion of my adult career in the Center of Photochemical Sciences as the administrative director and also directing the graduate programs. And I have loved every minute!

This brings me to the point! I have recently left Bowling Green State University. As of June 30, 1999, I am no longer employed there—or anywhere else for that matter. My spouse has taken a position in Denver, Colorado, and we moved to Denver several weeks ago. It is a wonderful part of the country and a great move.

Thanks to technology, I am still very much a part of the Center. I will remain the editor of *The Spectrum* and also continue other administrative duties. Telecommuting is great and the transition from sitting in my office in Bowling Green to my home in Denver is virtually seamless.

BUT

I miss my scientist colleagues, daily interaction with staff and of course, the students. Since I arrived here, I have had time for some reflection and realize how important the 12 1/2 years in the Center has been to my personal and professional development. Coming into the Center in its infancy and being an active participant in its growth and development has been one of the highlights of my career. More particularly, working with, around and for Doug Neckers has been a great adventure. Doug empowered me with this imprimatur when he hired me. In the interview he said, “I don’t know what we need, but I know I need help with this fledgling Center.”

The rest, as they say, is history. I want to take this opportunity to thank Doug for giving me the freedom to learn, develop, and grow personally as the Center grew. I want to thank the entire Center faculty, who, for the most part, I have watched grow from junior faculty to mature scientists. I am very grateful to have the opportunity to interact with all of the wonderful members of the Center’s Scientific Advisory Board. I also want to thank Alita Frater, my incredible secretary all these past years, who is the real glue of the Center. I want to thank all of the students I have encountered all these years.

I especially want to thank all of you—readers and contributors of *The Spectrum*. *The Spectrum* has been an important element of the Center and has become an established and respected publication. This is directly a result of the high quality of the articles and the feedback of our readers.

I would be greatly remiss if I didn’t mention how honored I am to count among my friends, George Hammond and his spouse Eve. I don’t understand George’s science, but I do understand how important his science has been to the development of the photochemical sciences.

I look forward to my continuing role as editor of *The Spectrum* and wherever the other adventures of the Center for Photochemical Sciences takes me. No matter where I am, the Center for Photochemical Sciences at BGSU will be an important part of my life.

Thanks and continue to enjoy *The Spectrum*.