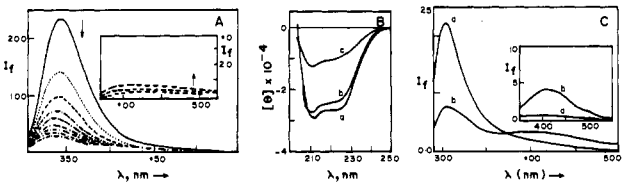

Figure 1. Panel A: Progressive change in the fluorescence spectra of BSA upon irradiation of the protein solution in aqueous buffer (2 h, monitored every 15 min, as the arrow indicates). Irradiation was done at 220 nm as per conditions given in ref 2. Excitation was at 295 nm for the main curves and 320 nm for the inset. I_f indicates fluorescence intensity in arbitrary units. Panel B: CD spectra of BSA before (a) and after irradiation in anaerobic (b) and aerobic (c) conditions, at 220 nm for 2 h. Ellipticity in the y axis is as deg·cm²·deciresidue/mol. CD measurements were done on a JASCO J 20 spectropolarimeter. Panel C: Fluorescence spectra of calmodulin solutions in aqueous buffer before (a) and after (b) irradiation at 220 nm for 2 h. Excitation was at 275 nm for the main curves and 320 nm for the inset.

chain moieties. The fluorescence due to the tryptophan (Trp) residues is progressively reduced, and a new weak emission band is generated in the 415-nm region. Such changes have been observed previously with several peptides and proteins,[3-5] generally upon irradiation of the ^1L_a,^1L_b band of Trp in the 280–300-nm (UVB) region, and the 415-nm emission has been attributed to *N*-formylkynurenine (NFK) and related products that were formed by the oxidation of the indole ring of Trp. Figure 1A shows that irradiation near 220 nm (the ^1B_a band in the UVC region) also produces the same results.

The conformation of the protein is altered in the process. CD spectra (Figure 1B) reveal substantial reduction in the secondary structure of BSA upon photolysis. Similar changes have been noted earlier[5] with a few other proteins. We find that photolysis of BSA denatured in 6 M guanidinium chloride does occur, but less efficiently than in the native form (as monitored by fluorescence, but with no accompanying CD changes, presumably since the unordered conformation of BSA in the denaturant solution is altered no further upon irradiation).

The photoreaction is sensitive to air. CD changes are far less pronounced when BSA is irradiated under anaerobic (N_2 flushed) conditions rather than in ambient aerobic conditions[3,6,7] (Figure 1B). It also depends on the amount of incident light; the losses in CD and fluorescence signals after 2 h of irradiation at 220 nm with spectral band widths of 20, 10, and 5 nm were about 35%, 15%, and 8%, respectively.

Tyr residues are also photolyzed. UVC irradiation of ribonuclease A reduces its Tyr fluorescence but not its CD. Irradiation of another Trp-free protein, calmodulin (CaM), at 220 nm for 2 h led to the loss of Tyr and the formation of a photoproduct that emits around 400 nm, which is very likely to be bityrosine.[8,9] Concimitantly, the secondary structure also changes from an initial 50% helix to 33% after the photoreaction. (The 400-nm emission was not seen with ribonuclease A photolysis. Bityrosine is apparently not formed here, probably because precursor Tyr residues are not appropriately disposed in this molecule as they are in CaM.[9])

Turning to the Phe residue, we irradiated (210 nm, 2 h) a synthetic peptide (comprising the first 26 residues of the sequence of the toxin pardaxin),[10] which contains three Phe residues and no Tyr or Trp, and found a small drop in both the emission and CD spectra, suggesting that Phe residues might also be affected by light.[3]

Photodamage depends on the aromatic residue content; Trp is known to be more photoreactive than Tyr, which in turn is more so than Phe.[3,7] This might explain the larger CD change seen in myoglobin,[1] which has 1.33 mol % Trp, while BSA has only 0.35 mol % Trp and ribonuclease A has no Trp at all.[11]

Interestingly, irradiation of a solution of α-helical poly(L-glutamic acid) with 220-nm light for 2 h brought about a 16% loss in the ellipticity value; somewhat similar results were also obtained with helical poly(L-lysine). This raises the possibility of the peptide chromophore itself being photosensitive, an issue that needs further study.

These results draw attention to the possibility of artifacts arising during spectral studies of protein kinetics (e.g., renaturation assays), which involve continuous spectral measurements in the UV region, though in most routine CD measurements (short-term illumination, 2-nm slits, and nitrogen flushing) these photoreactions may be insignificant and escape detection. Finally, protein photolytic damage can generate oxidation products and covalent aggregates,[12] a striking clinical manifestation of which occurs during light-induced forms of cataract on the eye lens.[13-15]

Registry No. Trp, 73-22-3; Tyr, 60-18-4; ribonuclease, 9001-99-4.

(3) For comprehensive reviews of the photochemistry of aromatic chromophores in proteins, see: Creed, D. *Photochem. Photobiol.* **1984**, *39*, 537, 563, 577.

(4) Dillon, J.; Chiesa, R.; Spector, A. *Photochem. Photobiol.* **1987**, *45*, 147.

(5) Rao, S. C.; Rao, Ch. M.; Balasubramanian, D. *Photochem. Photobiol.* **1990**, *51*, 357. Also, Figure 1A reveals a blue shift of the Trp emission band as irradiation proceeds, suggesting a change in the microenvironment of the fluorophore.

(6) Anaerobic irradiation of Trp at <280 nm yields several photoproducts including kynurenine, while in the presence of air the key product is *N*-formylkynurenine (NFK).[3] Both kynurenine and NFK have very similar spectral features; see: Pileni, M. P.; Walrant, P.; Santus, R. *J. Phys. Chem.* **1976**, *80*, 1804.

(7) Dillon, J.; Spector, A. *Exp. Eye Res.* **1980**, *31*, 591.

(8) Amado, R.; Aesbach, R.; Neukom, H. *Methods Enzymol.* **1984**, *107*, 377.

(9) Anderson, S. R.; Malencik, D. A. In *Fluorescent Biomolecules*; Jameson, D. M., Reinhardt, G. D., Eds.; Plenum Press: New York, 1989; pp 217–245.

(10) The synthetic peptide was 26 amino acids long and had the sequence (in the one letter amino acid code) GFFALIPKIISSPLFKTLLSAVGSAL. We are grateful to G. Sabherwal, M. Renil, M. C. Chandy, and R. Nagaraj for sparing the sample.

(11) *Handbook of Biochemistry*; CRC Press: Cleveland, OH, 1970.

(12) McLaren, A. D.; Shugar, D. *Photochemistry of Proteins and Nucleic Acids*; McMillan: New York, 1964.

(13) Hiller, R.; Giacometti, L.; Yuen, K. *Am. J. Epidemiol.* **1977**, *105*, 450.

(14) Waxler, M.; Hitchins, V. M. *Optical Radiation and Visual Health*; CRC Press: Boca Raton, FL, 1986.

(15) Balasubramanian, D.; Bhat, K. S.; Rao, G. N. *Curr. Sci.* **1990**, *59*, 498.

Direct Observation and Reactivity of Transient Ketenes Generated by Flash Photolysis

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Quantitative studies of ketene reactivities have concentrated on the hydration of a few transient species generated photolytically[1,2a,h-j] and on longer lived species that are usually heavily substituted.[2] Examples of the representative structural types RCH=C=O include ketene itself,[1a,2j] *n*-butylketene,[2a] phenyl-

(1) Bothe, E.; Dessouki, A. M.; Schulte-Frohlinde, D. *J. Phys. Chem.* **1980**, *84*, 3270–3272. (b) Bothe, E.; Meier, H.; Schulte-Frohlinde, D.; von Sonntag, C. *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 380–831.

(2) (a) Allen, A. D.; Kresge, A. J.; Schepp, N. P.; Tidwell, T. T. *Can. J. Chem.* **1987**, *65*, 1719–1723. (b) Allen, A. D.; Tidwell, T. T. *J. Am. Chem. Soc.* **1987**, *109*, 2774–2780. (c) Allen, A. D.; Stevenson, A.; Tidwell, T. T. *J. Org. Chem.* **1989**, *54*, 2843–2848. (d) Allen, A. D.; Gong, L.; Tidwell, T. T. *J. Am. Chem. Soc.* **1990**, *112*, 6396–6397. (e) Allen, A. D.; Baigrie, L. M.; Gong, L.; Tidwell, T. T. *Can. J. Chem.* **1991**, *69*, 138–145. (f) Allen, A. D.; Tidwell, T. T. *Tetrahedron Lett.* **1991**, *32*, 847–850. (g) Tidwell, T. T. *Acc. Chem. Res.* **1990**, *23*, 273–279. (h) Chiang, Y.; Kresge, A. J.; Pruszynski, P.; Schepp, N. P.; Wirz, J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 792–794. (i) Urwyler, B.; Wirz, J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 790–791. (j) Andraos, J.; Kresge, A. J. *J. Photochem. Photobiol. A: Chem.* **1991**, *57*, 165–173.

0002-7863/92/1514-1878$03.00/0

Table I. Hydration Reactivities[a] and Isodesmic Stabilization (Equation 2) of Ketenes

	c-PrCH=C=O (1)	(*E*)-PhCH=CHCH=C=O (5)	PhC≡CCH=C=O (3)	CF_3CH=C=O (4)	*n*-BuCH=C=O[b]	PhCH=C=O[b] (7)	PhCOC(Ph)=C=O (6)
k_{H_2O} (s^{-1})	304	5.76×10^3	7.16×10^4	6×10^2	99	4.77×10^3	6.29×10^3
k_{OH^-} (M^{-1} s^{-1})	2.48×10^5	2.31×10^6			3.29×10^4	1.22×10^6	
k_{H^+} (M^{-1} s^{-1})		2.09×10^4	1.61×10^5		3.98×10^3		
ΔE (kcal/mol)	−1.8	−0.2[c]	0.2[d]	−0.4	0.0[e]	0.7	3.6[f]

[a] Full experimental details are available in the Ph.D. Thesis of J. Andraos, University of Toronto, Toronto, Ontario, Canada, 1991. [b] Reference 2a. [c] CH_2=CH. [d] HC≡C. [e] CH_3. [f] CH=O.

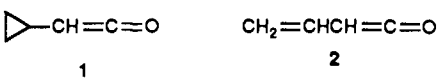
ketene,[1b,2a] other arylketenes,[1b] and (trimethylsilyl)ketene.[2f] We now report studies of ketenes RCH=C=O with cyclopropyl, alkenyl, alkynyl, and CF_3 substituents, studies of PhCOC(Ph)=C=O, and an interpretation of their reactivity in terms of ground-state and transition-state stabilization.

Theoretical calculations[3a-c] on ketenes have been used[3c] to assess the effect of substituent on ketene stability according to the isodesmic reaction of eq 1. The potential energy change, ΔE, for these isodesmic reactions correlates well with the group electronegativities X_{BE}[3d] of the substituents R (eq 2).[3c]

$$RCH{=}C{=}O + CH_3CH{=}CH_2 \rightarrow CH_3CH{=}C{=}O + RCH{=}CH_2 \quad (1)$$

$$\Delta E = -15.6X_{BE} + 42.3 \quad (2)$$

Equation 2 predicts very similar stabilities for ketenes substituted by cyclopropyl, alkenyl, alkynyl, and CF_3 groups, with ΔE values of −1.8, −0.2, 0.2, and −0.4 kcal/mol, respectively.[3c] Of these ketenes, cyclopropylketene (**1**) has been trapped with alcohols[4a] and as the dimer,[4b] and the IR band at 2110 cm^{-1} of (2,2-dimethylcyclopropyl)ketene was observed.[4c] Vinylketene (**2**)



1 **2**

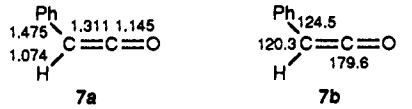
has been observed upon generation at low temperatures,[4d] but on attempted generation of (2-phenylethynyl)ketene (**3**) by photolysis only decarbonylated products were observed (eq 3).[4e] (Trifluoromethyl)ketene (**4**) was not observed on photolysis of the diazo ketone, but was trapped as the ester (eq 4).[4f]

$$PhC{\equiv}CCOCHN_2 \xrightarrow{h\nu} \underset{\mathbf{3}}{[PhC{\equiv}CCH{=}C{=}O]} \xrightarrow{h\nu} PhC{\equiv}CCH_3 \quad (3)$$

$$CF_3COCHN_2 \xrightarrow{h\nu} \underset{\mathbf{4}}{[CF_3CH{=}C{=}O]} \xrightarrow{ROH} CF_3CH_2CO_2R \quad (4)$$

We now report the generation and direct observation by UV spectroscopy of the rates of hydration of **1**, **3**, **4**, (*E*)-(2-phenylethenyl)ketene (**5**), and phenylbenzoylketene (**6**) generated by photochemical Wolff rearrangements of diazo ketones in aqueous solution[2a] as in eqs 3 and 4. The reactivities of the ketenes in H_2O are summarized in Table I, together with data for *n*-BuCH=C=O and PhCH=C=O.[2a]

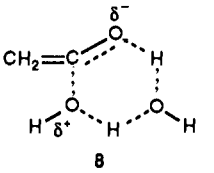
Calculated[2c] 6-31G*//6-31G* bond distances (angstroms) and bond angles (deg) of phenylketene are shown in **7a** and **7b**, respectively. The ΔE value (eqs 1 and 2) is 0.7 kcal/mol, showing that phenylketene has essentially the same ground-state stabilization as **1**–**5** (Table I).



7a **7b**

(μ = 1.50 D)

The calculated (6-31G*//6-31G*) transition-state structure for ketene hydration by water dimer involves in-plane addition to the carbonyl group as shown in **8**.[3a] However, ketene is much more reactive in H_2O at 25 °C ($\Delta H^{\ddagger}$ = 10.3 kcal/mol, $\Delta S^{\ddagger}$ = −15 eu)[1a] than suggested by the calculated gas-phase barrier for formation of **8** (33.4 kcal/mol),[3a] and so the transition state in H_2O solution evidently involves the participation of additional water molecules.



8

Despite the very similar ground-state stabilization of **1**, **3**–**5**, *n*-BuCH=C=O, and **7**, there is a 700-fold variation in reactivity with H_2O (Table I). The higher rates with the better conjugating substituents support the proposal[2] that there is significant enolate character in the transition state. Phenylbenzoylketene (**6**) is also quite reactive as expected because of its two conjugative substituents, even though the ground state of formylketene (O=C-HCH=C=O) is stabilized by 3.6 kcal/mol.[3c] The importance of conjugative interactions by β-substituents in the addition transition state and the direct observation of enediol intermediates[2h,i] argue against persistent proposals[5] that water and alcohols react with ketenes by addition to the C=C linkage. Cyclopropyl and CF_3 groups inductively stabilize adjacent negative charge, but are poor π-acceptors and thus cause modest acceleration of ketene hydration.

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Registry No. 1, 128871-21-6; **3**, 138541-71-6; **4**, 134736-46-2; **5**, 138541-72-7; **6**, 75508-81-5.

Supplementary Material Available: Calculated geometry, energy, and atomic charges of **7** and styrene and kinetic data for **1** and **3**–**6** (20 pages). Ordering information is given on any current masthead page.

(3) (a) Andraos, J.; Kresge, A. J.; Peterson, M. R.; Csizmadia, I. G. *J. Mol. Struct.* **1991**, *232*, 155–177. (b) Nguyen, M. T.; Hegarty, A. F. *J. Am. Chem. Soc.* **1984**, *106*, 1552–1557. (c) Gong, L.; McAllister, M. A.; Tidwell, T. T. *J. Am. Chem. Soc.* **1991**, *113*, 6021–6028. (d) Boyd, R. J.; Edgecombe, K. E. *J. Am. Chem. Soc.* **1988**, *110*, 4182–4186.

(4) (a) Basnak, I.; Farkas, J. *Collect. Czech. Chem. Commun.* **1976**, *41*, 311–316. (b) Berkowitz, W. F.; Ozorio, A. A. *J. Org. Chem.* **1975**, *40*, 527–528. (c) Agosta, W. C.; Smith, A. B., III; Kende, A. S.; Eilerman, R. G.; Benham, J. *Tetrahedron Lett.* **1969**, 4517–4520. (d) Trahanovsky, W. S.; Surber, B. W.; Wilkes, M. C.; Preckel, M. M. *J. Am. Chem. Soc.* **1982**, *104*, 6779–6781. (e) Selvarajan, R.; Boyer, J. H. *J. Org. Chem.* **1971**, *36*, 1679–1682. (f) Brown, F.; Musgrave, W. K. R. *J. Chem. Soc.* **1953**, 2087–2089.

(5) (a) Donohoe, G.; Satchell, D. P. N.; Satchell, R. S. *J. Chem. Soc., Perkin Trans. 2* **1990**, 1671–1674. (b) Poon, N. L.; Satchell, D. P. N. *J. Chem. Soc., Perkin Trans. 2* **1986**, 1485–1490.

(6) Danheiser, R. L.; Miller, R. F.; Brisbois, R. G.; Park, S. Z. *J. Org. Chem.* **1990**, *55*, 1959–1964.