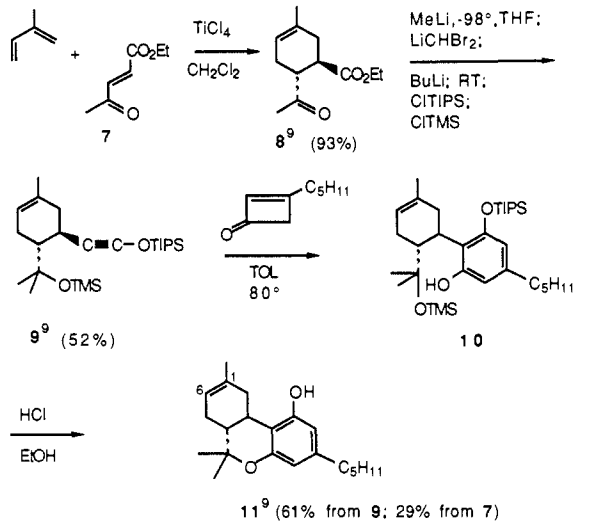
Scheme II



perature, 0.1 equiv of titanium tetrachloride, in methylene chloride) afforded the Diels–Alder product **8** in 93% yield, as a 20:1 mixture of regioisomers (major isomer shown).[18] This ester was converted into silyloxyacetylene **9** in a second step,[4] via successive treatment in tetrahydrofuran with methyllithium (1 equiv, −90 °C), dibromomethyllithium (2.2 equiv, −78 °C), and *n*-butyllithium (7 equiv, −78 °C), warming to room temperature, cooling, and then silylating first the ynolate anion oxygen with triisopropylsilyl chloride (7 equiv, −78 °C → room temperature) and then the more hindered tertiary alkoxide with trimethylsilyl chloride (10 equiv, −78 °C → room temperature). The homologation/rearrangement reaction central to this step was expected to proceed with retention of stereochemistry,[19] and indeed the trans substituted product **9** was obtained (52% yield after flash chromatography). Heating a 1:1 mixture of silyloxyacetylene **9** and 3-pentylcyclobutenone[15] in toluene at 80 °C for 1 h smoothly afforded the desired resorcinol product **10**. Without purification, this tertiary silyl ether was treated with refluxing acidic ethanol to afford Δ-6-tetrahydrocannabinol (**11**)[20,22] in 61% yield for two steps from **9**. This novel four-step synthesis, proceeding in 29% overall yield from ester **7**, nicely illustrates the potential utility of ester-derived silyloxyacetylenes in 2 + 2 cycloadditions with vinylketenes.

Acknowledgment. We thank Dr. Steven Carr and Walter Johnson of the Physical and Structural Chemistry Department for determination of exact mass spectra as well as Dr. Charles DeBrosse and Pricilla Offen of the Analytical Chemistry Department for NOE and COSY NMR experiments reported herein.

(15) Cyclobutenone **5-H** was prepared in 70% yield from 3-ethoxycyclobutenone by addition of *n*-pentylmagnesium bromide in ether, followed by treatment with aqueous hydrochloric acid; see ref 8 and 11.

(16) Danheiser, R. L.; Savariar, S. *Tetrahedron Lett.* **1987**, *28*, 3299.

(17) McMurry, J. E.; Blaszczak, L. C. *J. Org. Chem.* **1974**, *39*, 2217.

(18) When the reaction was performed in toluene at 60 °C, with no catalyst, a nearly 1:1 mixture was obtained. The high degree of regioselectivity observed in the Lewis acid catalyzed reaction with isoprene surprised us, although some enhancement of the desired product was expected. See: (a) Lee, I.; Rhyu, K. B.; Jeon, Y. K. *J. Korean Chem. Soc.* **1979**, *23*, 286. (b) Kakushima, M.; Scott, D. G. *Can. J. Chem.* **1979**, *57*, 1399. (c) Kahn, S. D.; Pan, C. F.; Overman, L. E.; Hehre, W. J. *J. Am. Chem. Soc.* **1986**, *108*, 7381.

(19) Kowalski, C. J.; Haque, M. S.; Fields, K. W. *J. Am. Chem. Soc.* **1985**, *107*, 1429.

(20) Spectral data for product **11** was identical with that reported in the literature,[21] and COSY NMR clearly showed that the olefinic ring hydrogen at C-6 was separated from the benzylic hydrogen by two intervening carbon centers (not one, as would be expected if the methyl were at C-6 and the hydrogen at C-1).

(21) (a) Binder, M.; Franke, I.; Schmidt, B.; Dietrich, W. *Helv. Chem. Acta* **1982**, *65*, 807. (b) Taylor, E. C.; Lenard, K.; Shvo, Y. *J. Am. Chem. Soc.* **1966**, *88*, 367. (c) Fahrenholtz, K. E.; Lurie, M.; Kierstead, R. W. *J. Am. Chem. Soc.* **1967**, *89*, 5934.

(22) For a review of cannabinoid total synthesis, see: Razdan, R. K. In *The Total Synthesis of Natural Products*; ApSimon, J., Ed.; John Wiley: New York, 1981; Vol. 4, p 185.

Hydride Transfer Catalyzed by Lactate Dehydrogenase Displays Absolute Stereospecificity at C_4 of the Nicotinamide Ring

V. E. Anderson* and R. D. LaReau

Department of Chemistry, Brown University
Providence, Rhode Island 02912

Received January 12, 1988

The stereospecificity of hydride transfer to and from C_4 of the nicotinamide ring catalyzed by dehydrogenases has been intensively studied,[1] and recent controversy surrounding the mechanistic importance of the stereospecificity has renewed interest in this topic.[2] This communication reports the results of an experiment designed to detect rare nonstereospecific hydride transfer to or from the nicotinamide ring. In determining the energy difference between the two diastereomeric transition states each 1.35 Kcal/mol of stabilization energy results in a factor of 10 increase in the stereospecificity of the reaction. Thus, the over 10^4 increase in sensitivity afforded by this measurement expands the accessible energy range by over 5 Kcal/mol. The absolute stereospecificity of enzyme catalyzed reactions has been discussed by Cornforth who suggested experiments similar to the one described in this communication.[3]

The inability of ^{1}H NMR to detect trace (<1%) contaminants[4] and the uncertainty of the purity of chiral [4-^{3}H]NADH[5] limits the sensitivity of the methods commonly used to determine NADH stereochemistry. The initial work of Westheimer and co-workers using deuterium isotope ratio mass spectrometry was only accurate to ±2% at best.[6] In careful radioactive studies there has been evidence of nonstereospecific hydride transfers.[7] ^{1}H NMR studies utilizing nuclear Overhauser effects have shown that the nicotinamide ring of NAD(P) can bind in either a syn or anti fashion to glucose-6-phosphate dehydrogenases.[8] The ability of dehydrogenases to utilize both the α and arabino configurations at the C_1' and C_2' positions of the ribose ring, respectively,[2d] suggests the active site can accommodate altered nucleotide structures.

If 10 μM [4-^{3}H]NAD is added to a solution containing 50 mM (*S*)-lactate and 5 mM pyruvate in the presence of lactate dehydrogenase from pig heart (Sigma), any nonstereospecific hydride transfer either to or from the nicotinamide ring results in the appearance of ^{3}H in the (*S*)-lactate. The rate of hydride transfer in the dynamic equilibrium established by the presence of the enzyme can be monitored by the increase in the intensity of the pyruvate signal in the ^{1}H NMR if [3-$^2H_3$]pyruvate is present initially. After a fixed incubation time, when each NAD molecule has undergone an average of 10^5 turnovers, carrier lactate is added, the tritiated nucleotides removed by filtration through charcoal, and the lactate recrystallized to constant specific activity as the Zn^{2+} salt. The radioactivity derived from [4-^{3}H]NAD that ap-

(1) (a) For reviews, see: You, K. *Meth. Enzymol.* **1982**, *87*, 101. (b) You, K. *CRC Crit. Rev. Biochem.* **1985**, *17*, 313.

(2) (a) Nambiar, K. P.; Stauffer, D. M.; Kolodziej, P. A.; Benner, S. A. *J. Am. Chem. Soc.* **1983**, *105*, 5886. (b) Oppenheimer, N. J. *J. Am. Chem. Soc.* **1984**, *106*, 3032. (c) Benner, S. A.; Nambiar, K. P.; Chambers, G. K. *J. Am. Chem. Soc.* **1985**, *107*, 5513. (d) Oppenheimer, N. J.; Marshner, T. M.; Malver, O.; Kam, B. L. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1986; p 15. (e) Benner, S. A.; Nambiar, K. P. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1986; p 29.

(3) Cornforth, J. In *Structures of Complexes between Biopolymers and Low Molecular Weight Molecules*; Bartmann, W., Snatzke, G., Eds.; John Wiley and Sons: New York, **1982**; pp 1–16.

(4) Arnold, L. J.; You, K.; Allison, W. S.; Kaplan, N. O. *Biochemistry* **1976**, *15*, 4844.

(5) Jarabak, J.; Talalay, P. *J. Biol. Chem.* **1960**, *235*, 2147.

(6) (a) Fisher, H. F.; Conn, E. E.; Vennesland, B.; Westheimer, F. H. *J. Biol. Chem.* **1953**, *202*, 687. (b) Fisher, H. F.; Lowes, F. A.; Ofner, P.; Westheimer, F. H.; Vennesland, B. *J. Biol. Chem.* **1953**, *202*, 699.

(7) (a) Walton, D. J. *Biochemistry* **1973**, *12*, 3472. (b) Chan, P. C.; Bielski, B. H. J. *J. Biol. Chem.* **1975**, *250*, 7266.

(8) (a) Levy, H. R.; Ejchart, A.; Levy, G. C. *Biochemistry* **1983**, *22*, 2792. (b) Groneborn, A. M.; Clore, G. M.; Hobbs, L.; Jeffrey, J. *Eur. J. Biochem.* **1984**, *145*, 365.

0002-7863/88/1510-3695$01.50/0

Table I

conditions[a]	no. of turnovers	total counts (cpm) ppt	1st recrystllztn	2nd recrystllztn	stereospcfcty (%)
10 μM NAD^+ 654080 cpm ^{3}H-NAD^+ 8.5 μM LDH[b]	1.8×10^5	5.52×10^3	2.30 ± 0.15[c] $\times 10^3$	$2.31 \pm 0.15 \times 10^3$	99.9999980
10 μM NAD^+ 435200 cpm ^{3}H-NAD^+ 8.5 μM LDH[b]	1.3×10^5	2.00×10^3	$5.64 \pm 0.16 \times 10^2$	$5.05 \pm 0.16 \times 10^2$	99.9999991
10 μM NAD^+ 2640000 cpm ^{3}H-NAD^+ 8.5 μM LDH[b]	$>2.0 \times 10^5$	6.46×10^3	$3.78 \pm 0.19 \times 10^3$	$3.85 \pm 0.19 \times 10^3$	99.9999990
10 μM NAD^+ 988800 cpm ^{3}H-NAD^+ no enzyme	1–100[d]	3.99×10^3	$<1.5 \times 10^2$		
10 μM NAD^+ 988800 cpm ^{3}H-NAD no enzyme	1–100[d]	5.17×10^3	0		
8 μM NAD^+ 212480 cpm NAD^3H no enzyme	0	9.39×10^2	$1.29 \pm 0.14 \times 10^2$	$9.39 \pm 0.12 \times 10^1$	

[a] All incubations additionally included 50 mM lactate, 5 mM pyruvate in phosphate buffer, pD 8.0. [b] Lactate dehydrogenase from pig heart. [c] Standard deviation of three samples. [d] Enzyme (0.1 μM) was added for the final 10 s.

pears in the isolated lactate of three separate determinations and controls is shown in Table I. The data show that the hydride added to C_4 of the oxidized nicotinamide ring is removed in the subsequent oxidation by pyruvate over 99.999998% of the time.

The sensitivity of this protocol is derived from four advantages: the final analysis does not rely on a diastereomeric separation, the chiral purity of all the materials is not critical, the amplification obtained by using a dynamic equilibrium allows low concentrations of labeled NAD to be employed, and a minimal amount (<1 nM) of free [4-^{3}H]NADH is produced. The low NADH concentration limits nonenzymatic anomerization[9] and reduction of the pyruvate and/or NAD by free [4-^{3}H]NADH.[10] The small amount of apparent nonstereospecific transfer reproducibly detected may only tentatively be assigned to a lactate dehydrogenase catalyzed reaction. A contaminating *pro*-4S dehydrogenase in conjunction with its cosubstrate could have produced the small amount of nonstereospecific transfer observed.

Nonstereospecific hydride transfer will occur when C_2 of pyruvate and lactate are bound on opposite faces of the nicotinamide ring during a reduction oxidation cycle. This happens either if the glycosidic bond is fixed and the carboxylic acids bind on opposite faces of the ring or if the carboxylic acids bind at the same site and the nicotinamide ring rotates from the anti to syn configuration. For the first possibility, the transfer of the *pro*-4R hydrogen[9] is favored by at least 10 Kcal/mol over the transfer of the *pro*-4S hydrogen.

To determine the relative energies of the transition states for the syn and anti configurations of the nicotinamide ring, free kinetic access between the two conformations has to be possible between the reduction and subsequent oxidation of the nicotinamide. This access is guaranteed if the NADH dissociates from the enzyme before reducing pyruvate. The partitioning of the binary enzyme–NADH complex is estimated from the ratio of equilibrium exchange rates of NAD to NADH and lactate to pyruvate to be 0.05[12] and from steady-state kinetic data to be 0.013.[13] If the nicotinamide ring could dissociate and rotate independently of the ADP-ribose it would result in much greater kinetic access to the syn configuration. If the transition state for dissociation of the ring and entire molecule is characterized by the difference in free energies for binding of ADP-ribose and NADH, 4.8 and 7.2 Kcal/mol, respectively,[14] the reduced nicotinamide ring would dissociate 20 times faster than the entire NADH molecule. Consequently, the ordered release of pyruvate and NADH *may* cause the measured stereospecificity to overstate the energy difference between the transition states for the transfer of the *pro*-4R and *pro*-4S hydrogens from the anti and syn configurations, respectively, by *at most* a factor of 75 which would reduce the minimum energy difference to 8.0 Kcal/mol.

The over 8–10 Kcal/mol difference in transition-state energies measured would be interesting to compare to energy minimizations of NADH·pyruvate ternary complexes with the nicotinamide ring constrained to be in either a syn or anti configuration. Steric effects certainly contribute to this energy difference; however, lactate dehydrogenase utilizes NAD analogues that are derivatized at C_5 and C_6 of the nicotinamide ring,[16] and the boundary on the C_5 and C_6 side of the nicotinamide ring is formed by the flexible αDβD loop[16b] suggesting the syn conformation could be accommodated. Specific favorable interactions between the protein and carboxamide in the preferred anti conformation must contribute the energy not accounted for by steric exclusion. The nature of the interactions leading to the large energy difference measured is currently being investigated by determining the stereospecificity of hydride transfer as the substituent at C_3 of the pyridine ring is varied.

Additionally, by establishing the absolute stereospecificity of the pig heart lactate dehydrogenase reaction, it may be used analytically to determine if other dehydrogenases exhibit the same

(9) Oppenheimer, N. J. In *The Pyridine Nucleotide Coenzymes*; Everse, Anderson, V., You, K., Eds.; Academic Press: New York, 1982; p 53.
(10) Ludowieg, J.; Levy, A. *Biochemistry* **1964**, *3*, 373.
(11) The work of Fischer et al., ref 4b, and Cornforth and Ryback (Cornforth, J. W.; Ryback, G. *Biochem. Biophys. Res. Commun.* **1962**, *9*, 371) demonstrated the *pro*-4R hydrogen was routinely transferred.
(12) Silverstein, E.; Boyer, P. D. *J. Biol. Chem.* **1964**, *239*, 3901.
(13) The partitioning of the E-NADH complex is obtained from the ratio of V_{max} for lactate oxidation to $V/K_{pyruvate}$ [pyruvates]. The data is from Schwert et al. (Schwert, G. W.; Miller, B. R.; Peanasky, R. J. *J. Biol. Chem.* **1967**, *242*, 3295.
(14) Hinz, H. J.; Steininger, G.; Schmid, F.; Jaenicke, R. *FEBS Lett.* **1978**, *87*, 83.
(15) (a) Grau, U.; Kapmeyer, H.; Trommer, W. E. *Biochemistry* **1978**, *17*, 4621. (b) Holbrook, J. J.; Liljas, A.; Steindel, S. J.; Rossmann, M. G. In *The Enzymes* Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. XI, p 191.
(16) (a) White, J. L.; Hackert, M. L.; Buehner, M.; Adams, M. J.; Ford, G. C.; Lentz, P. J., Jr.; Smiley, I. E.; Steindel, S. J.; Rossmann, M. G. *J. Mol. Biol.* **1976**, *131*, 55. (b) Grau, U. M.; Trommer, W. E.; Rossmann, M. G. *J. Mol. Biol.* **1981**, *151*, 289.

absolute stereospecificity when suitable dynamic equilibria cannot be established.

Acknowledgment. We acknowledge the referees who brought Cornforth's discussion of enzyme stereospecificity to our attention and who prompted a more accurate discussion of the consequences of the ordered mechanism. Financial support from the NIGMS (GM-36562) is gratefully acknowledged.

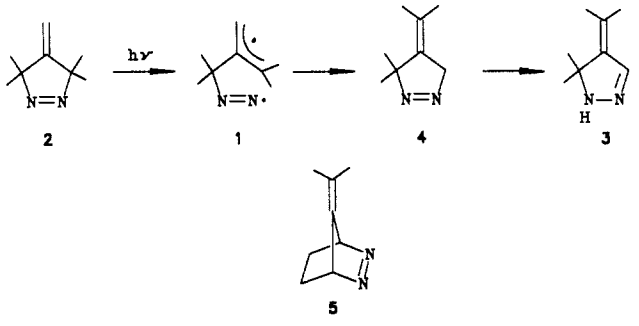
Direct Evidence for Both Thermal and Photochemical Stepwise Cleavage in a Pair of Isomeric Azo Compounds

D. E. Reedich and R. S. Sheridan*[1]

Departments of Chemistry, University of Nevada Reno, Nevada 89557
University of Wisconsin, Madison, Wisconsin 53706

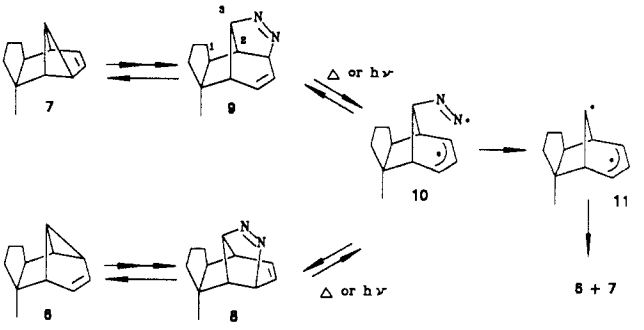
Received February 16, 1988
Revised Manuscript Received April 4, 1988

Despite numerous investigations of the formation of biradicals from azo compounds, the question of one-bond versus two-bond cleavage is still poorly resolved.[2] Considerable evidence in favor of initial thermal cleavage of the weakest C-N bond has accumulated.[3-7] Direct support for one-bond cleavage in photolyses of azo compounds is, in contrast, less abundant.[2,8] Recently, Adam and Dörr[9] reported evidence for diazenyl biradical **1** for-



mation in the photochemical denitrogenation of **2**, where 0.5% of rearranged **3** was observed by GC-MS. This product was presumed to be formed from "turnaround" isomer **4**. It was noted that previous efforts by others[10] had failed to detect similar rearrangement in thermolyses of **2**. These results are particularly enigmatic, since similar azo compound **5** is reported to give turnaround thermally but not photochemically.[4b-d] We now wish to report evidence for both thermal and photochemical stepwise cleavage in a pair of isomeric pyrazolines. A theoretical model consistent with known results is proposed.

Azo compounds **8** and **9** were synthesized from meta photoadducts of 1-methylcyclopentene and benzene, **6** and **7**, by using procedures detailed by us previously.[11-13] Thermal decomposition of **8** and **9** at 100 °C cleanly gave mixtures of the vinylcyclo-



propane isomers **6** and **7** in ratios of 2.5:1 and 1:4.9, respectively. We,[13] and Askani and co-workers,[14] have reported that similar azo compounds exhibit preferential ring closure away from nitrogen and have attributed this selectivity to concerted six-electron cycloreversion. As in our previous studies,[13] the regiospecificity is not complete, and some closure on the same side as nitrogen (e.g., **6** from **9**) is observed. These results suggest that stepwise cleavage of both azo compounds to **10**, and hence to approximately regiorandom product formation (via **11**), occurs in competition with concerted cycloreversion. This scenario raises the possibility of turnaround of the azo isomers, which was confirmed by careful monitoring of the reactions.

HPLC analysis during the thermolysis of **8** showed the formation of a small amount of **9**, which reached a maximum level of 10% of starting **8** and then decreased along with **8**. Similarly, thermolysis of **9** under the same conditions gave production of **8** up to a steady-state concentration of 4% of starting material. Simplex fitting[15] of the concentrations to first-order kinetics indicates that 28% of **8** produces **9** and 13% of **9** gives **8**. High field ^{1}H NMR taken at short thermolysis times confirmed the rearrangement of the isomers. Strikingly, irradiation also caused turnaround of the azo isomers, although to a lesser extent. Photolysis of **8** or **9** at 366 nm produced a maximum level of ca. 2% (based on the amount of starting azo compound) of the alternate azo isomer, along with **6** and **7**. The azo compounds have comparable absorptions at this wavelength.[11]

These results suggest at least some stepwise cleavage in both of these thermal and photochemical denitrogenations. Adam and

(1) Fellow of the Alfred P. Sloan Foundation, 1986-1988. Address correspondence to this author at the University of Nevada.

(2) (a) Dervan, P. B.; Dougherty, D. A. In *Diradicals*; Borden, W. R., Ed.; Wiley: New York, 1982. (b) Engel, P. S. *Chem. Rev.* **1980**, *80*, 99.

(3) (a) Roth, W. R.; Martin, M. *Liebigs Ann. Chem.* **1967**, *702*, 1. (b) Roth, W. R.; Martin, M. *Tetrahedron Lett.* **1967**, 4695. (c) Allred, E. L.; Smith, R. L. *J. Am. Chem. Soc.* **1969**, *91*, 6766. (d) Adam, W.; Hannemann, K.; Peters, E.-M.; Peters, K.; von Schnering, H. G.; Wilson, R. M. *J. Am. Chem. Soc.* **1987**, *109*, 5250. (e) Chang, M. H.; Dougherty, D. A. *J. Am. Chem. Soc.* **1982**, *104*, 2333. (f) Chang, M. H.; Jain, R.; Dougherty, D. A. *J. Am. Chem. Soc.* **1984**, *106*, 4211.

(4) (a) Engel, P. S.; Gerth, D. B. *J. Am. Chem. Soc.* **1983**, *105*, 6849. (b) Cichra, D. A.; Duncan, C. D.; Berson, J. A. *J. Am. Chem. Soc.* **1980**, *102*, 6527. (c) Berson, J. A. *Acc. Chem. Res.* **1978**, *11*, 446. (d) Cichra, D. A.; Platz, M. S.; Berson, J. A. *J. Am. Chem. Soc.* **1977**, *99*, 8507.

(5) Neuman, R. C., Jr.; Lockyer, G. D., Jr. *J. Am. Chem. Soc.* **1983**, *105*, 3982.

(6) (a) Dannenberg, J. J.; Rocklin, D. *J. Org. Chem.* **1982**, *47*, 4529. (b) Dannenberg, J. J. *J. Org. Chem.* **1985**, *50*, 4963. (c) Hiberty, P. C.; Jean, Y. *J. Am. Chem. Soc.* **1979**, *101*, 2538.

(7) See, also: Schmittel, M.; Rüchardt, C. *J. Am. Chem. Soc.* **1987**, *109*, 2750, and references therein.

(8) (a) Holt, P. L.; McCurdy, K. E.; Adams, J. S.; Burton, K. A.; Weisman, R. B.; Engel, P. S. *J. Am. Chem. Soc.* **1985**, *107*, 2180. (b) Adams, J. S.; Burton, K. A.; Andrews, B. K.; Weisman, R. B.; Engel, P. S. *J. Am. Chem. Soc.* **1986**, *108*, 7935.

(9) Adam, W.; Dörr, M. *J. Am. Chem. Soc.* **1987**, *109*, 1240.

(10) (a) Crawford, R. J.; Tokunaga, H. *Can. J. Chem.* **1974**, *52*, 4033. (b) Crawford, R. J.; Tokunaga, H.; Schrijver, L. M. H. C.; Godard, J. C. *Can. J. Chem.* **1978**, *56*, 998.

(11) The stereo- and regiochemistry of all compounds was confirmed by extensive ^{1}H NMR decoupling experiments at 270 MHz. Spectral and synthetic details are given in the Supplementary Material.

(12) For a recent review of aryl meta photoadditions, see: Mattay, J. *J. Photochem.* **1987**, *37*, 167, and references therein.

(13) (a) Sheridan, R. S. *J. Am. Chem. Soc.* **1983**, *105*, 5140. (b) Reedich, D. E.; Sheridan, R. S. *J. Am. Chem. Soc.* **1985**, *107*, 3360.

(14) (a) Askani, R.; Hornykiewytoch, T.; Schwertfeger, W.; Jansen, M. *Chem. Ber.* **1980**, *113*, 2154. (b) Askani, R.; Hornykiewytoch, T.; Muller, K. M. *Tetrahedron Lett.* **1983**, *24*, 513.

(15) The concentrations of azo compounds relative to an internal standard were fit to an interconverting unimolecular scheme with the SMPLXKS program, from Serena Software, Bloomington, IN. The best-fit rate constants are $k(8,9) = 5.3 \times 10^{-4}$, $k(8,6+7) = 1.36 \times 10^{-3}$, $k(9,8) = 2.3 \times 10^{-4}$, and $k(9,6+7) = 1.69 \times 10^{-3}$ s^{-1} at 99.4 °C. It should be noted that the regiochemistry of product formation is too random to be accounted for by rapid equilibration of the azo isomers with selective formation of only **6** from **8** and **7** from **9**. Previous azo compounds of this general structure reported from these laboratories[13] exhibit no turnaround. These compounds were methyl substituted at the 3-position (numbered as in **9**), and the corresponding tertiary diazenyl radicals are likely too unstable to rearrange.[4a,6a,b]

0002-7863/88/1510-3697$01.50/0