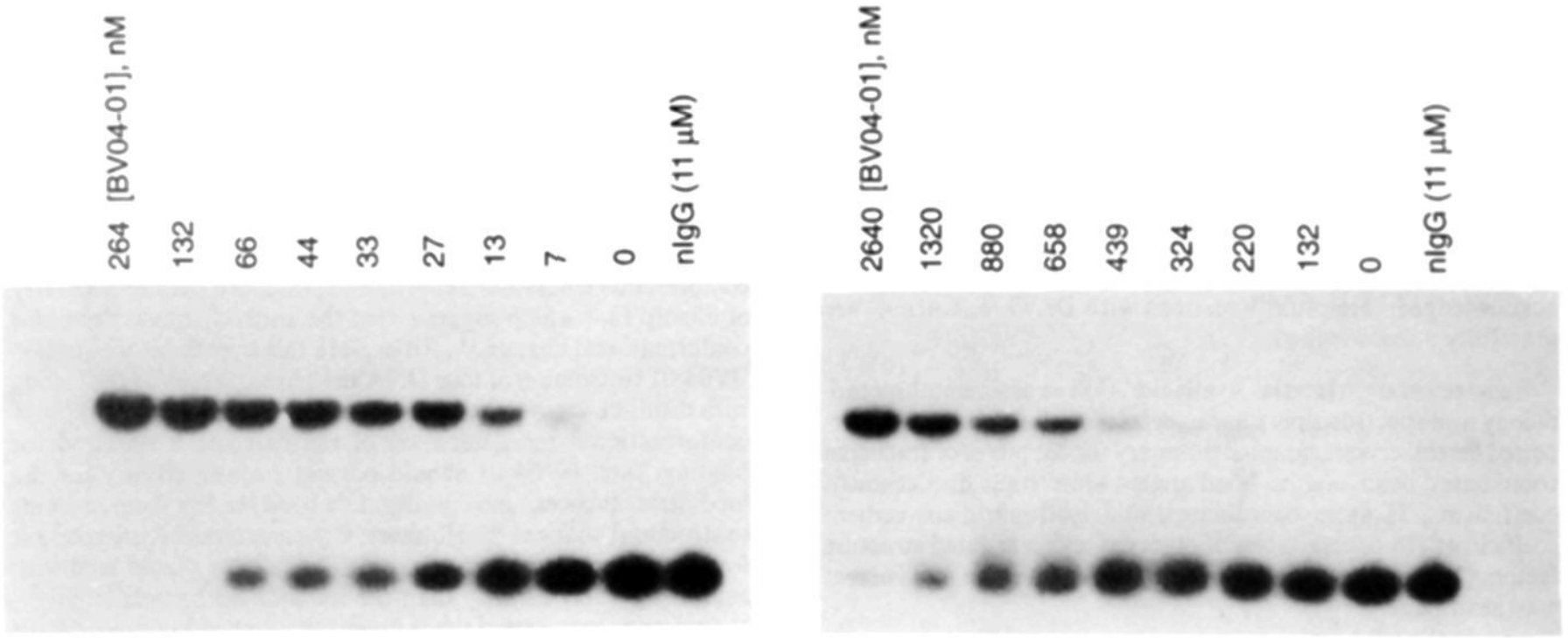
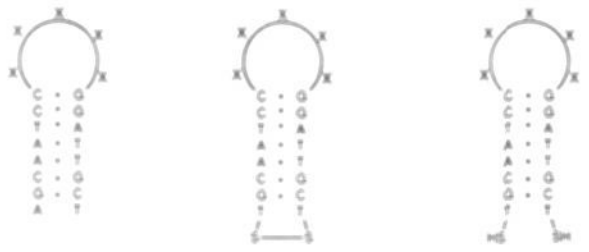


Figure 1. Representative gel shift data. (Left) Hairpin **1**. (Right) Hairpin **5**. [^{32}P] end-labeled hairpin (0.1 nM, ~2300 cpm) was titrated with antibody in TBE at pH 8.3. After equilibration for 1 h at 25 °C the mixtures were loaded onto a 6% polyacrylamide gel (9:1 acrylamide:bisacrylamide) at 300 V for 90 s and then electrophoresed at 106 V for 30 min at 4 °C. The high cross-linking ratio affords a nonrestrictive gel that permits the complex to enter the matrix.[20] The gels were then autoradiographed at −70 °C for 18 h. To determine K_d values, DNA concentrations were quantified by densitometry and the data were fit to the binding isotherm for the equilibrium Ab + DNA ⇌ Ab-DNA by nonlinear least-squares regression. The protein concentration at which half the DNA is bound corresponds to the apparent K_d.



	unmodified		crosslinked		reduced	
X= T	34 ± 2	(1)	523 ± 37	(5)	31 ± 5	(9)
C	48 ± 3	(2)	> 3000	(6)	49 ± 7	(10)
A	53 ± 4	(3)	> 3000	(7)	48 ± 4	(11)
G	104 ± 8	(4)	788 ± 34	(8)	108 ± 9	(12)

Figure 2. Apparent dissociation constants for BV04-01·hairpin complexes (K_d per IgG binding site, nM). Each K_d is the average of at least three separate measurements with the associated error representing error to the fit.

tinguishable from binding to **1**–**4**.[21] To provide further support that BV04-01 alters the structure of DNA upon binding, we synthesized **13** and **14**, which are less stable than **1**.[22] If induced

13 **14**

fit is important for complexation, then less binding energy will be needed for structural reorganization of these new hairpins, resulting in tighter binding to the antibody. Gel shift measurements showed that both **13** and **14** form tighter complexes than **1** with BV04-01 (K_d(**13**) = 17 ± 3 nM and K_d(**14**) = 22 ± 4 nM). However, the differences in stability between **1** and **13** and **1** and **14** are not fully expressed as added binding energy, which suggests that the hairpins may not be completely unfolded when bound to the antibody.

To illuminate the specificity of anti-DNA, future studies must focus on examining both free- *and* bound-state DNA geometries. Such data will hopefully guide the synthesis of molecules that specifically block autoantibody recognition of DNA and form the basis for new and effective therapies to combat SLE.

Acknowledgment. This work was supported by an Arthritis Investigator Award from the National Arthritis Foundation and by a grant from the NIH (GM 46831) to G.D.G. Funding from the University of Michigan Multipurpose Arthritis Center (NIH Grant AR 20557) is also acknowledged. P.C.S. is an NIH Molecular Biophysics Predoctoral Fellow.

(21) NOESY spectra indicate that the cross-link does not alter the conformation of native DNA structure. Cain, R.; Glick, G. D., unpublished results.

(22) T_m(**1**) = 55.8 °C, T_m(**13**) = 47.7 °C, T_m(**14**) = 49.5 °C. ΔG(**1**) = 5.3 kcal/mol, ΔG(**13**) = 4.2 kcal/mol, ΔG(**14**) = 4.4 kcal/mol (from van't Hoff analysis of UV thermal denaturation curves).

Formation of a Stable Metallacyclobutene Complex from α-Diazocarbonyl and Alkyne Substrates

Joseph M. O'Connor,*,† Hongli Ji, and Mahzad Iranpour

Department of Chemistry (216), University of Nevada Reno, Nevada 89557

Arnold L. Rheingold*

Department of Chemistry, University of Delaware Newark, Delaware 19716

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The metal-catalyzed reactions of α-diazocarbonyl compounds with alkenes[1] and alkynes[2] are widely employed in the synthesis of hetero- and carbocyclic organic molecules. A central mechanistic consideration in these reactions is the nature of the inter-

† After July 1, 1993, address correspondence to this author at: Department of Chemistry (0506), University of California at San Diego, La Jolla, CA 92093-0506.

(1) For recent leading references and a review, see: (a) Doyle, M. P.; Pieters, R. J.; Martin, S. F.; Austin, R. E.; Oalmann, C. J.; Müller, P. *J. Am. Chem. Soc.* **1991**, *113*, 1423. (b) Doyle, M. P. *Chem. Rev.* **1986**, *86*, 919. (c) Maxwell, J. L.; Brown, K. C.; Bartley, D. W.; Kodadek, T. *Science* **1992**, *256*, 1544. (d) Taber, D. F.; Hoerrner, R. S. *J. Org. Chem.* **1992**, *57*, 441.

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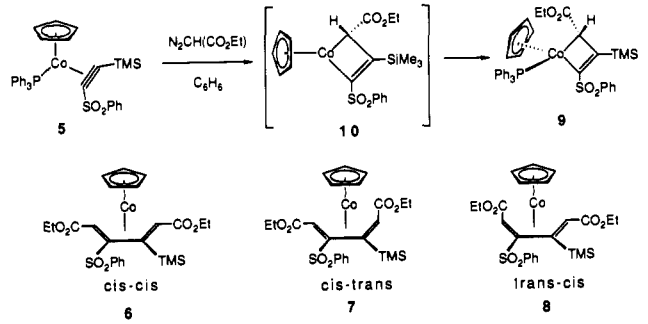
Scheme I



5 10 9

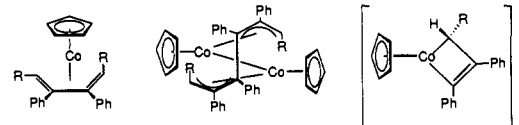
6 7 8

mediate in which the alkyne (or alkene) and carbene are assembled at the metal center. In the case of alkyne substrates, three related yet fundamentally distinct species have been proposed: metallacyclobutene **I**, η^1- or η^3-vinylcarbene II, and zwiterion III.[3]

I II III

Product distribution studies indicate that the intermediate may vary depending on reaction conditions and the specific substrate.[2a,b] We now present the first direct proof that diazocarbonyl and alkyne substrates are indeed viable precursors to metallacyclobutene complexes.[4,5]

Hong previously reported the reaction of $(\eta^5\text{-}C_5H_5)Co(PPh_3)(\eta^2\text{-}PhC{\equiv}CPh)$ (**1**) with alkyl diazoacetates to give a mixture of two diene complexes (e.g., **2**) and two dinuclear complexes (e.g., **3**), presumably via a common unobserved metallacyclobutene intermediate (**4**).[6] We have found that by simple



2 (two isomers) 3 (plus isomer) 4

manipulation of the alkyne substituents and reaction conditions, the outcome of this reaction can be dramatically altered to provide a stable metallacyclobutene product as a single regio- and stereoisomer.

The η^2-alkyne complex $(\eta^5\text{-}C_5H_5)Co(PPh_3)(\eta^2\text{-}Me_3SiC{\equiv}CSO_2Ph)$ (**5**)[7] was prepared by a procedure similar to that reported for **1**.[8] When a benzene solution of **5** (250 mg, 0.4 mmol, 0.02 M) and ethyl diazoacetate (0.6 mmol) is allowed to stand at room temperature for ~18 h and the reaction mixture is then subjected to a chromatographic workup (alumina, 3% ethylacetate/benzene), four distinct bands are observed. The first three bands correspond to the η^4-diene products **6**, **7**, and **8**.[9,10] The last band to elute

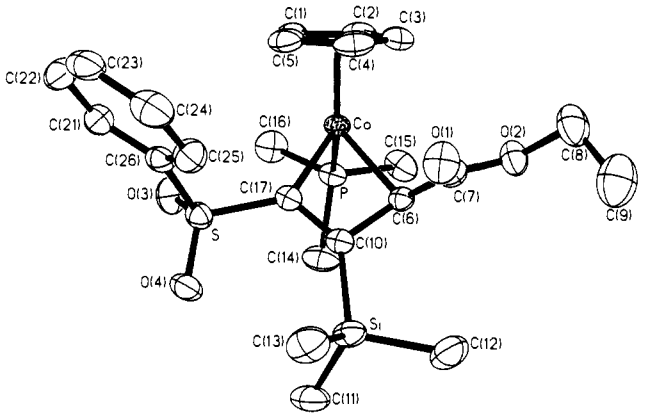


Figure 1. ORTEP drawing of **11** showing selected atom labeling: Co–P 2.167(1) Å, Co–C(6) 2.054(4) Å, Co–C(10) 2.574 Å, Co–C(17) 1.918(4) Å, C(6)–C(10) 1.523(5) Å, and C(10)–C(17) 1.340(6) Å, C(6)–Co–C(17) 66.7(2)°, Co–C(6)–C(10) 90.8(2)°, C(6)–C(10)–C(17) 99.5(3)°, Co–C(17)–C(10) 103.0(3)°.

gives a red crystalline solid, which is isolated in 43% yield as a single regio- and stereoisomeric cobaltacyclobutene complex **9** (Scheme I). From the ^1H NMR spectrum of **9**, it is clear that starting complex **5** has incorporated a $CH(CO_2Et)$ fragment from the ethyl diazoacetate. The ^1H NMR spectrum ($CDCl_3$) of **9** exhibits singlets at δ −0.23 (9 H, $SiMe_3$) and 4.21 (5 H, C_5H_5), a triplet at 1.43 (J = 7.2 Hz, 3 H, CH_2CH_3), and a quartet at 4.26 (J = 7.2 Hz, 2 H, CH_2CH_3). In addition, a doublet at δ 1.48 (J_{PH} = 7.2 Hz, 1 H) is assigned to a hydrogen atom on the α-carbon of the metallacycle. Irradiation of the methine doublet at δ 1.48 results in observation of a small NOE for the PPh_3 resonance but not for the cyclopentadienyl hydrogen resonance. This result supports an (SS, RR) relative stereochemistry for **9**, as expected on the basis of a simple steric argument: the ester substituent is located anti to the bulky PPh_3 ligand. In the $^{13}C\{^1H\}$ NMR spectrum ($CDCl_3$) the absence of a downfield shift in the carbene region of the ^{13}C NMR spectrum is inconsistent with a vinylcarbene structure (II).

The indicated regiochemistry for formation of **9** is supported by a single crystal X-ray diffraction study of the PMe_3 analogue **11** (Figure 1),[11] formed as a 1:1 mixture of diastereomers upon reaction of **9** with PMe_3 at 80 °C.[7] The crystallographic data for **11** indicate a planar[12] localized metallacyclobutene ring [C(6)–C(10), 1.523(5); C(10–C(17), 1.340(6) Å], with the C(6)–Co–C(17) angle constrained to 66.7°.[13] For comparison, the metallacyclobutene ring in *fac*-Ir[$CH_2C(p\text{-tol}){=}C(p\text{-tol})(PMe_3)_3$] has a localized double bond [C—C, 1.525(7); C=C, 1.344(8) Å] and a C–Ir–C angle of 64.5(2)°.[4d]

The formation of **6–8** and **9** presumably involves initial formation of a common unsaturated metallacyclobutene intermediate (**10**) (Scheme I), which is then trapped with either additional carbene to give diene products **6–8** or PPh_3 to give **9**. When the reaction of **5** and ethyl diazoacetate is carried out in the presence of a 9-fold excess of PPh_3, the rate of reaction is significantly diminished and the yield of **9** is only 27% after 4 days of reaction

(3) In certain cases, the transition state for cyclopropanation may involve reaction of the alkyne substrate directly at the metal–carbene carbon via a concerted process with no charge buildup: Brown, K. C.; Kodadek, T. *J. Am. Chem. Soc.* **1992**, *114*, 8336.

(4) Stable metallacyclobutenes of the later transition metals have been prepared by alternate routes; for leading references, see: (a) Casey, C. P.; Chae, S. Y. *J. Am. Chem. Soc.* **1992**, *114*, 6597. (b) O'Connor, J. M.; Pu, L.; Woolard, S.; Chadha, R. K. *J. Am. Chem. Soc.* **1990**, *112*, 6731. (c) Hemond, R. C.; Hughes, R. P.; Robinson, D. J.; Rheingold, A. L. *Organometallics* **1988**, *7*, 2239. (d) Calabrese, J. C.; Roe, D. C.; Thorn, D. L.; Tulip, T. H. *Organometallics* **1984**, *3*, 1223.

(5) For conversion of metal–carbene complexes and alkynes to stable metallacyclobutene complexes, see: (a) Tebbe, F. N.; Harlow, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 6149. (b) McKinney, R. J.; Tulip, T. H.; Thorn, D. L.; Coolbaugh, T. S.; Tebbe, F. N. *J. Am. Chem. Soc.* **1981**, *103*, 5584. (c) Grubbs, R. H.; Tumas, W. *Science* **1989**, *243*, 907.

(6) Hong, P.; Aoki, K.; Yamazaki, H. *J. Organomet. Chem.* **1978**, *150*, 279.

(7) See supplementary material for full characterization.

(8) Alkyne complex **5** was prepared from $(\eta^5\text{-}C_5H_5)Co(PPh_3)_2$ and $TMSC{\equiv}CSO_2Ph$ at room temperature (1 h) in C_6H_6: Yamazaki, H.; Hagihara, N. *J. Organomet. Chem.* **1970**, *21*, 431.

(9) A related NMR scale reaction was run, and the four new cyclopentadienyl compounds (**6**, **7**, **8**, and **9**) were observed by ^1H NMR spectroscopy in ~6:3:1:12 ratio, respectively (80% combined yield).

(10) (a) For η^4-diene complexes the anti hydrogens resonate at higher field than the syn hydrogens in the ^1H NMR spectrum: Emerson, G. F.; Mahler, J. E.; Kochhar, R.; Pettit, R. *J. Org. Chem.* **1964**, *29*, 3620. (b) In the ^1H NMR spectra the vinyl hydrogen chemical shifts for the diene ligands in **6–8** are observed as follows: for **6**, δ 0.06 (s, 1 H) and 1.10 (s, 1 H); for **7**, 1.09 (s, 1 H), 3.73 (s, 1 H); and for **8**, 1.13 (s, 1 H), 4.55 (s, 1 H), respectively.

(11) Crystallographic data for $C_{23}H_{34}CoO_4PSSi$: M = 524.6, triclinic, $P\bar{1}$, a = 8.762(2) Å, b = 9.332(2) Å, c = 16.783(3) Å, α = 87.43(3)°, β = 85.06(3)°, γ = 73.13°, V = 1308.1(7) Å^3, Z = 2, D_{calcd} = 1.332 g cm^{-3}, m(Mo Kα) = 8.60 cm^{-1}, Mo Kα radiation (λ = 0.710 73 Å), T = 296 K, $R(F)$ = 4.19%, $R_w(F)$ = 5.46%.

(12) Deviations from the mean plane defined by Co, C(6), C(10), and C(17) are as follows: Co, −0.002; C(6), +0.003; and C(10), −0.004; C(17), +0.004; S, −0.110; Si −0.069; C(7), +1.208.

(13) To our knowledge, **11** is the first observation and structural characterization of a first-row metallacyclobutene complex of the late transition metals.

(balance of cobalt material is **5**). However, when 1 equiv of PPh_3 (0.016 mmol) is added to a benzene solution of ethyl diazoacetate (0.027 M) and **5** (0.027 M), the diazoacetate is slowly consumed over the course of 4 days and an 84% yield of cobaltacyclobutene **9** is observed by ^1H NMR spectroscopy.[14] The isolated metallacyclobutene **9** is stable to ethyl diazoacetate in benzene at room temperature. However, when a benzene-d_6 solution of **9** and ethyl diazoacetate (1.5 equiv) is heated at 50 °C, diene complexes **6–8** are formed in the same ratio as that observed from reaction of **5** with ethyl diazoacetate.[10]

The dramatic difference in product distribution for **5** vs **1** is presumably related to the stability of the unsaturated cobaltacyclobutene intermediate. The bulky trimethylsilyl group inhibits the formation of bimetallic products, and the electron-withdrawing sulfone substituent stabilizes the intermediate toward subsequent reaction with additional carbene. When the reaction of **1** and ethyl diazoacetate is repeated in the presence of PPh_3, no metallacyclobutene product is observed by ^1H NMR spectroscopy. Detailed mechanistic studies are currently underway to determine the specifics of metallacyclobutene ring formation.

Acknowledgment. Support from the National Science Foundation is gratefully acknowledged. Acknowledgment is made to the donors of The Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. We thank the American Cancer Society Junior Faculty Fellowship Program for support of our programs.

Supplementary Material Available: Characterization data for **5–9** and **11**; listings of fractional coordinates, bond distances, bond angles, hydrogen atom coordinates, and thermal parameters (8 pages); table of observed and calculated structure factors (10 pages). Ordering information is given on any current masthead page.

(14) In a related NMR experiment, a 93% yield of **9** was achieved by further addition of ethyl diazoacetate after ~80% conversion.

Detection of an Enzyme–Intermediate Complex by Time-Resolved Solid-State NMR Spectroscopy

Jeremy N. S. Evans,*,†,‡ Richard J. Appleyard,† and Wendy A. Shuttleworth†

Departments of Biochemistry/Biophysics and Chemistry
Washington State University
Pullman, Washington 99164-4660

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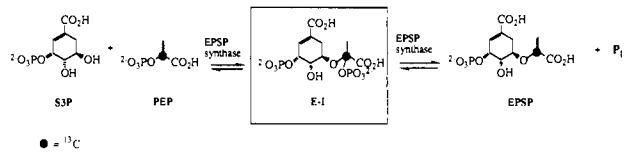
The elucidation of the structure of an enzyme–substrate complex at various points along the reaction coordinate is one of the most sought after goals of enzyme chemistry. The two techniques that generate the most structural information, X-ray crystallography and NMR spectroscopy, also are among the slowest and most insensitive methods for the data collection.[1] In this communication, we report the first application of a new NMR method, which we call time-resolved solid-state NMR spectroscopy, to the direct detection of the transient enzyme–intermediate complex of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, a key enzyme in the aromatic amino acid biosynthetic pathway. Unlike X-ray diffraction of enzyme crystals, which has difficulty defining

Scheme I



intermediate structures due their intrinsic motions, time-resolved solid-state NMR spectroscopy focuses primarily on the intermediate. This provides a generally applicable technique for "mapping out" intermediate structures as a function of time.

Time-resolved solid-state NMR spectroscopy is complementary to X-ray crystallography, which to date has achieved time resolution of the order of 0.5 h with cryoenzymological methods[2] and 1–3 s with Laue diffraction methods,[3,4] although in theory Laue methods can achieve time-resolutions in the millisecond regime.[5] However, the limitations of time-resolved Laue diffraction methods that arise from substrate diffusion and transient lattice disorder give rise to diffuse electron density maps, and this has been improved only to an extent by using caged substrate molecules.[6] Disordered substrates are not too surprising, since during catalysis the enzyme-bound substrate is dynamic and undergoing significant molecular motion, which is where NMR spectroscopy is useful. Although NMR spectroscopy has been used to study rapid reactions in solution, in particular by continuous-flow and stopped-flow methods,[7] the time-resolution to date is at very best ca. 20 ms and in practice of the order of 200 ms to >10 s. The method of time-resolved solid-state NMR spectroscopy which we introduce here has a time-resolution achievable on the order of ca. 2 ms.

We have examined a well-characterized enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19), which catalyzes the penultimate step in the aromatic amino acid biosynthetic pathway in higher plants and bacteria. EPSP is formed from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) (see Scheme I). The enzyme is a monomer with molecular weight M_r = 46 000, and the cloned *E. coli* gene has been used to generate a hyperexpressing strain,[8] so the bacterial enzyme is available in gram quantities. Furthermore, EPSP synthase is the primary site of action of the herbicide glyphosate[9] or *N*-phosphonomethylglycine.

This enzyme has been extensively studied by kinetic and biophysical methods in the last five years and is one of a limited number of enzymes for which the full kinetic and thermodynamic profile has been determined.[10] The direct observation of the enzyme–intermediate E·I complex was first reported by our laboratory[11] and later confirmed by another laboratory.[12] The E·I

* To whom correspondence should be addressed at the Department of Biochemistry/Biophysics. Telephone: (509) 335-3343. FAX: (509) 335-9688. EMAIL: EVANSJ@WSUVMS1.CSC.WSU.EDU.
† Department of Biochemistry/Biophysics.
‡ Department of Chemistry.

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