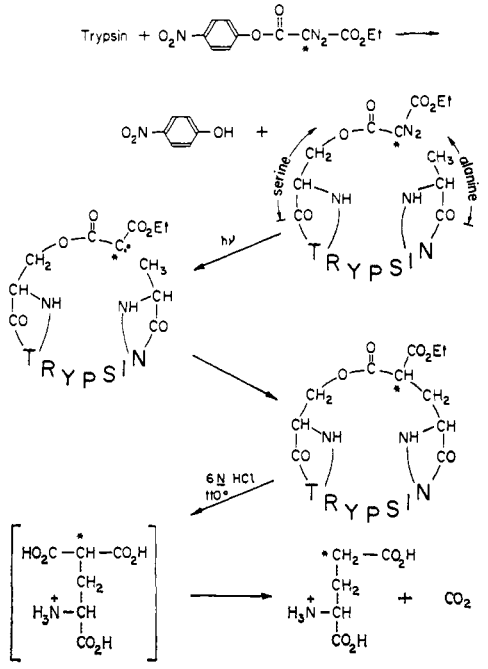
A Method for Marking the Hydrophobic Binding Sites of Enzymes. An Insertion into the Methyl Group of an Alanine Residue of Trypsin[1]

Sir:

Many reagents have been developed that combine with reactive amino acids in enzymes.[2] However, marking the hydrophobic side chains of the aliphatic amino acids (*e.g.*, the methyl group of alanine, the isopropyl group of valine, etc.) constitutes a much greater challenge. We have previously described[3-5] studies on diazoacyl reagents that were designed to resolve this problem; these reagents can be attached at the active sites of some enzymes and on subsequent photolysis yield carbenes that may be expected[6] to insert into the carbon–hydrogen bonds of alkyl groups. Although the products so far identified from the photolysis of diazoacetylchymotrypsin[3,4] are restricted to those formed from reactive amino acids, the present communication describes the successful insertion of a carbene into an alkyl group in trypsin. Photolysis of a labeled diazomalonyl derivative of trypsin followed by hydrolysis has yielded radioactive glutamic acid; the product has been identified chromatographically, by carrier techniques, and enzymatically. Glutamic acid is presumed to have been formed by the attack of a carbene, generated photochemically, on the methyl side chain of an alanine residue, in accordance with the diagrams below.



In order to prepare the reagent (ethyl *p*-nitrophenyldiazomalonate[7]) needed to acylate trypsin, ethyl diazoacetate was prepared[8] from glycine-2-^{14}C (0.48 mCi/mmol) and allowed to react successively with phosgene and sodium *p*-nitrophenoxide.[7] The reagent acylates trypsin, presumably at the active serine.[9]

Photolysis at 254 nm of the diazoacyl derivative of 660 mg of trypsin followed by reduction and aminoethylation of the disulfide bridges[10] yielded a modified protein which was digested twice with Worthington TPCK trypsin. The insoluble "core" material (67 mg) from this digestion was hydrolyzed (6 *N* HCl at 110°) and the hydrolysate was chromatographed on a 0.9 × 150 cm column of Dowex 50 WX8 (eluted at 55° with pH 3.28 buffer); the major radioactive component appeared with glutamic acid at 192–207 ml of eluent. The radioactive material was rechromatographed on a 0.9 × 50 cm column of Dowex 2X8 and eluted with a linear gradient from pH 6.72 to pH 3.25; the radioactivity (solution V, 140,000 dpm) appeared with glutamic acid.

The identification of this radioactive material as glutamic acid was confirmed as follows. (1) A portion of solution V was applied to the 150-cm column of a Beckman-Spinco amino acid analyzer along with other amino acids (including 1-carboxymethylhistidine); the radioactivity appeared with glutamic acid and was cleanly separated from other amino acids. (2) A portion of solution V was added to carrier L-glutamic acid and cocrystallized from ethanol–water. The counts per minute per milligram, for five successive recrystallizations, were 39.7, 40.2, 38.3, 38.6, and 37.8 (10-mg samples were counted for 100 min; counts in the glutamic acid were 20 times background). (3) A third portion of solution V (6950 dpm) was treated at pH 5 with 0.5 unit of L-glutamic decarboxylase[11] to convert the glutamic acid to γ-aminobutyric acid. The residue, after acid hydrolysis and evaporation, was applied to the 50-cm column of the amino acid analyzer and eluted (60 ml/hr) with pH 3.28 buffer and (after 110 min) a 0.35 *N* pH 4.25 citrate buffer. The residual glutamic acid appeared at 75 min and the γ-aminobutyric acid at 283 min. The ratio of glutamic acid to γ-aminobutyric acid from colorimetric measurements was 19:81; radiochemical measurements gave a ratio of 23:77.

^{14}C-Glutamic acid was also identified from a sample of photolyzed diazoacyl-trypsin that was directly hydrolyzed with hydrochloric acid.

Apparently the carbene from the diazomalonate residue attacks the methyl group of an alanine residue; the resulting substituted malonic acid undergoes decarboxylation during the acid hydrolysis of the protein to yield glutamic acid. No alanine in the sequence[12,13] for trypsinogen is near the "active serine," but two are adjacent to the histidine that is attacked by the chloromethyl ketone analog of lysine,[14] and, in chymotrypsin,[15] the corresponding residues are close to the active site. Furthermore, these alanine residues are present in the "core" of trypsin that is resistant to tryptic hydrolysis.[16]

(1) This work was supported by Grant GM-04712 from the Institute of General Medical Sciences of the National Institutes of Health.

(2) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," John Wiley & Sons, Inc., New York, N. Y., 1967.

(3) A. Singh, E. R. Thornton, and F. H. Westheimer, *J. Biol. Chem.*, 237, PC3006 (1962).

(4) J. Shafer, P. Baronowsky, R. Laursen, F. Finn, and F. H. Westheimer, *ibid.*, 241, 421 (1966).

(5) H. Chaimovich, R. J. Vaughan, and F. H. Westheimer, *J. Am. Chem. Soc.*, 90, 4088 (1968).

(6) W. von E. Doering and L. H. Knox, *ibid.*, 83, 1989 (1961).

(7) R. J. Vaughan and F. H. Westheimer, *Anal. Biochem.*, in press.

(8) E. B. Womack and A. B. Nelson, "Organic Syntheses," Coll. Vol. III, John Wiley & Sons, Inc., New York, N. Y., 1955, p 392.

(9) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 34, 49 (1965).

(10) M. A. Raftery and R. D. Cole, *J. Biol. Chem.*, 241, 3457 (1966).

(11) Prepared by Dr. M. O'Leary according to R. Shukuya and G. W. Schwert, *ibid.*, 235, 1649 (1960).

(12) F. Šorm, V. Holeyšovský, O. Mikeš, and V. Tomášek, *Collection Czech. Chem. Commun.*, 30, 2103 (1965).

(13) K. A. Walsh and H. Neurath, *Proc. Nat. Acad. Sci. U. S.*, 52, 884 (1964).

(14) E. Shaw and S. Springhorn, *Biochem. Biophys. Res. Commun.*, 27, 391 (1967).

(15) B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, *Nature*, 214, 652 (1967).

(16) V. Tomášek, O. Mikeš, V. Holeyšovský, and F. Šorm, *Collection Czech. Chem. Commun.*, 29, 3122 (1964).

Although this research opens the way to the chemical determination of the hydrophobic binding sites of proteins, some caution in interpretation is required. The yield of radioactive glutamic acid is small (1–3%) and although the identification of the glutamic acid is reasonably secure, its detailed mode of formation must still be proved. At most, the equations shown in this communication must be read as diagrams pertaining only to that portion of the photolyzed product that yields glutamic acid.

(17) National Science Foundation Fellow, 1965–1969.

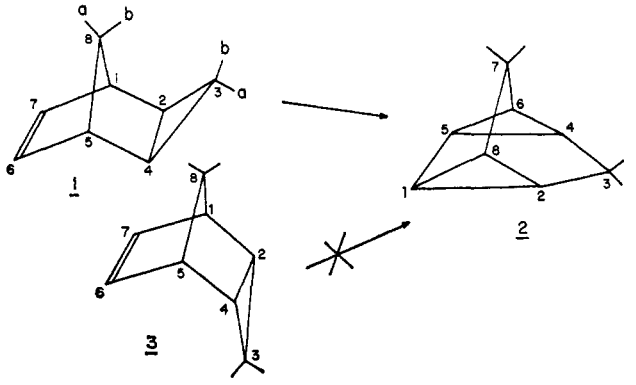
Ronald J. Vaughan,[17] F. H. Westheimer
James B. Conant Laboratory, Harvard University
Cambridge, Massachusetts 02138
Received November 15, 1968

Rhodium(I)-Catalyzed Valence Isomerization of *exo*-Tricyclo[3.2.1.$0^{2,4}$]oct-6-ene

Sir:

Valence isomerizations are receiving continuing interest. Recently several examples have been reported of these reactions in the presence of catalytic amounts of transition metal complexes.[1,2] It was concluded that reactions which are "forbidden" (energetically unfavorable) according to the Woodward–Hoffmann postulate under thermal conditions may occur under mild conditions in the presence of a suitable transition metal complex.[2,3]

In this communication we report on an example of a transition metal complex catalyzed valence isomerization in which a cyclopropane ring and a double bond are involved. *exo*-Tricyclo[3.2.1.$0^{2,4}$]oct-6-ene (**1**),[4,5] either pure or dissolved in $CDCl_3$, was converted quantitatively into tetracyclo[3.3.0.$0^{2,8}$.$0^{4,6}$]octane (**2**) in the presence of 10 mole % of $Rh_2(CO)_4Cl_2$ at room temperature.

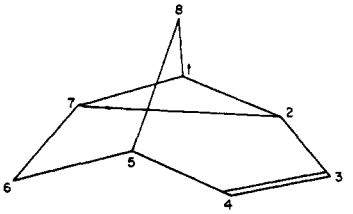


Product **2** had been reported earlier by Freeman[6] and LeBel.[7] Our spectral data (complex peaks centered at 1.61 and 1.26 ppm in the ratio 6:4; parent peak at *m/e* 106 and C–H stretching frequency at 3020 cm^{-1}, characteristic of cyclopropane rings) are about identical with those reported by Freeman and LeBel.

During the reaction, the red catalyst $Rh_2(CO)_4Cl_2$ was converted into a yellow, catalytically less active, complex. The elemental analysis and the infrared data (a terminal carbonyl group (2040 cm^{-1}), an acyl group (1700 cm^{-1}), and a bridged chloride (283 cm^{-1})) suggest a structure similar to that reported for the complex obtained from cyclopropane and $Rh_2(CO)_4Cl_2$, which contains a 1-rhodiacylopent-2-one ring.[8]

The *exo* arrangement of the fused cyclopropane ring in **1** is required for the occurrence of valence isomerization. This is demonstrated by the failure of the *endo* isomer **3**[9] to undergo a similar reaction. Even at 100° **3** did not react. The results can be explained in terms of the geometry of the tricyclic systems. In the *exo* isomer **1**, the π orbitals of the double bond, together with the orbitals forming the cyclopropyl bent bond between C_2 and C_4, are ideally situated for interaction with the orbitals of the rhodium atom, considering the reported edgewise coordination of cyclopropane toward tetravalent platinum.[10]

It is of interest to note that valence isomerization of **1** under thermal (200°) and photochemical conditions has been reported to yield tricyclo[3.2.1.$0^{2,7}$]oct-3-ene (**4**)[11] and **2** (20%)[6], respectively. Irradiation of the



4

endo isomer **3** leads also to **2** (19%).[6] Apparently, the spatial arrangement of the fused cyclopropane is not critical under these conditions. The reaction pathways of the conversion of **1** into **2** under catalyzed as well as under photochemical conditions have in common that occupied antibonding orbitals—either by back-bonding from the metal or by excitation—are involved

The conversion of **1** into **2** suggests a possible route for the preparation of tetracyclo[3.3.1.$0^{2,8}$.$0^{4,6}$]nonane (**6**) (triasterane) from *exo,exo*-tetracyclo[3.3.1.$0^{2,4}$.$0^{6,8}$]-nonane (**5**).[12]

All attempts to effect the conversion failed; the starting material was quantitatively recovered. Only the complex $Rh_2(CO)_4Cl_2$ was converted into a yellow complex, with presumably a structure similar to that of the complex obtained from **1**.

(1) H. C. Volger and H. Hogeveen, *J. Am. Chem. Soc.*, **89**, 2486 (1967); *Rec. Trav. Chim.*, **86**, 830 (1967); *Chem. Commun.* 1133 (1967).
(2) W. Merk and R. Pettit, *J. Am. Chem. Soc.*, **89**, 4788 (1967).
(3) F. D. Mango and J. H. Schachtschneider, *ibid.*, **89**, 2484 (1967).
(4) S. D. Koch, R. M. Kliss, D. V. Lopiekes, and R. J. Wineman, *J. Org. Chem.*, **26**, 3122 (1961); H. E. Simmons and R. D. Smith, *J. Am. Chem. Soc.*, **81**, 4256 (1959).
(5) Nmr data of **1**: δ (ppm from TMS) H(1,5) 2.71, H(2,4) 1.03, H(3a) 0.79, H(3b) 1.45, H(6,7) 6.35, H(8a) 1.06, H(8b) 0.96; J(1,5–6,7) 1.72 cps (triplet), J(3a, 3b) = 6.3 cps; J(2,3b) = 3.0 cps J(2,3a) = 7.5 cps, J(8a,8b) $\approx$ 8 cps (AB pattern).
(6) P. K. Freeman, D. G. Kuper, and V. N. Mallikarjuna Rao, *Tetrahedron Letters*, 3303 (1965).
(7) N. A. LeBel and R. N. Liesemer, *J. Am. Chem. Soc.*, **87**, 4301 (1965).
(8) R. M. Roundhill, D. N. Lawson, and G. Wilkinson, *J. Chem. Soc.*, *A*, 845 (1968).
(9) K. Wiberg and W. J. Bartley, *J. Am. Chem. Soc.*, **82**, 6375 (1960); K. Tori and M. Ohtsuru, *Chem. Commun.* 886 (1966).
(10) N. A. Bailey, R. D. Gillard, M. Keeton, R. Mason, and D. R. Russell, *ibid.*, 396 (1966).
(11) H. E. Simmons quoted in H. Prinzbach, W. Eberbach, M. Klaus, G. v. Veh, and U. Scheidegger, *Tetrahedron Letters*, 1681 (1966).
(12) Nmr data of **5**′ (for preparation see ref 4): δ (ppm from TMS) H(1,5) 2.35, H(2,4,6,8) 1.07, H(3a,7a) 0.31, H(3b,7b) 0.82, H(9) 0.51, J(1,9) = 1.70 cps, J(1,2) < 0.30 cps, J(2,3a) = 7.0 cps, J(2,3b) = 3.20 cps, and J(3a,3b) = 6.40 cps. The assignment of the *exo,exo* configuration was based on comparison of the nmr data with those of cyclopropane derivatives with established *exo* and *endo* configurations.[9,13]
(13) R. E. Pincock and J. Haywood-Farmer, *Tetrahedron Letters*, 4759 (1967).