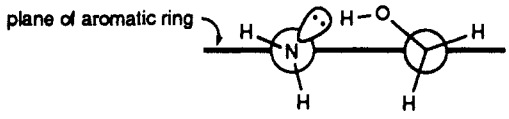
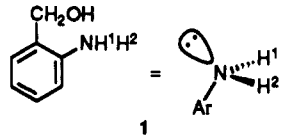
Chart I

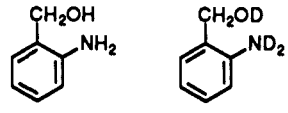


stituted aromatic compounds have been observed and their conformations determined by laser spectroscopy.[7-9] We herein report the first observation of spectra of individual isotopomers of a monosubstituted amine $ArNH^1H^2$, **1**, in which H^1 and H^2 represent diastereotopic hydrogen atoms under slow exchange conditions.[10]



1

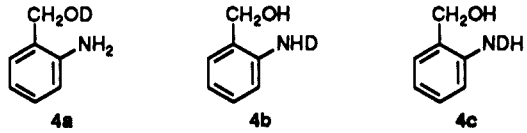
When compared to its all-protiated parent, a deuteriated analogue's first 0_0^0 transition will typically shift to the blue (to higher energy) while all vibronic transitions will shift to the red (to lower energy) relative to that origin.[8d] These isotope effects allow one to distinguish between origin and vibronic transitions.[8d] The mass-resolved excitation spectra[11] of 2-aminobenzyl alcohol (**2**) and its trideuteriated derivative **3** are shown in Figure 1a and



2 **3**

1d, respectively. Comparison of parts a and d of Figure 1 demonstrates that only a single origin transition is present in each spectrum, since each of the five transitions to the blue of the 0_0^0 transition in Figure 1d has a small but definite red shift relative to those in Figure 1a. In addition, the size of these isotope shifts (ca. 3-5%) demonstrates that the observed progressions are not due to motion involving either the NH_2 group or the OH group, but rather due to motion of the entire CH_2OH group in the excited electronic state.

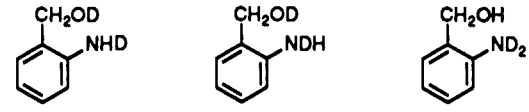
We now distinguish between 2-aminobenzyl alcohol species in which the amino hydrogen atoms H^1 and H^2 are nonequivalent.[10] The $S_1 \leftarrow S_0$ spectrum observed in mass channel m/z 124 (corresponding to 2-aminobenzyl alcohol-d_1 **4** is shown in Figure 1b.



4a **4b** **4c**

The spectrum of **4** is readily interpretable based on the spectra of **2** and **3**: each single transition of **2** and **3** has become a "triplet" in the spectrum of **4**. Each triplet can be further resolved into a single feature and a doublet to higher energy (cm^{-1}) (resolved by ca. 1 cm^{-1}). We interpret these three peaks as arising from the superposition of three spectra, one each for **4a-4c**. The lowest energy feature of the triplet is suggested to arise from **4a**, while the doublet feature is suggested to be associated with structures **4b** and **4c**, in which the two amino hydrogen atoms are diastereotopic.[10] Based on the perpendicular conformation of benzyl alcohol,[7] and on the expected intramolecular hydrogen bonding in **2** in the expansion gas, Chart I illustrates one possible geometry for 2-aminobenzyl alcohol.[10]

The mass-resolved excitation spectrum observed when monitoring mass channel m/z 125 for 2-aminobenzyl alcohol-d_2 (**5**)



5a **5b** **5c**

is shown in Figure 1c. This spectrum is remarkably similar to the spectrum of **4** (Figure 1b), with the exception that the relative positions of the singlet and the doublet are interchanged. Based on the above considerations, Figure 1c arises from the superposition of features from the spectra of **5a-5c**.

The individual diastereomers[10] of **4** and **5** are thus each stable over the time scale of the experiment and can be observed uniquely. The spectra of **4a-4c** and **5a-5c** are consistent with the number of isomers for each compound and the observation that deuterium substitution on the nitrogen atom produces a larger $S_1 \leftarrow S_0$ isotope shift than deuterium substitution on the oxygen atom. The switch in singlet-doublet positions going from Figure 1b to Figure 1c is also consistent with the location of the deuterium atoms in **4a-4c** compared to **5a-5c**: the lower 0_0^0 transitions obtain for the species having a CH_2OD moiety. Upon optical excitation ($S_1 \leftarrow S_0$), the force constants for the amino moiety must change more than those of the hydroxyl moiety.

To our knowledge, this work represents a unique experimental observation of spectra of the individual isotopomers of a monosubstituted amine. Future publications will disclose recent results on chemical reactions in intramolecularly hydrogen bonded systems observed by using these laser jet techniques.

(10) Under conditions in which rotation about $\tau(C_2-C_1-C_\alpha-O)$ is frozen and $\tau \neq 0°$ or $\tau \neq 180°$, H^1 and H^2 are diastereotopic.

(11) The experiment is performed as follows. A sample is irradiated with a laser of energy ν_1, resulting in the generation of the first excited singlet state ($S_0 \rightarrow S_1$). A second photon ν_2 subsequently ionizes those molecules in S_1 ($S_1 \rightarrow I^+$). The ions are detected in given mass channels by time of flight mass spectroscopy, such that only ion current representing a chosen m/z is recorded. The energy of the ν_1 laser is changed, and absorption spectra of mass-selected species are obtained.

Phospholipase A_2 Engineering. 4. Can the Active-Site Aspartate-99 Function Alone?[1]

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The Asp···His···Ser "catalytic triad" is the key catalytic vehicle for serine proteases.[3] Recently this class of enzymes has been extended to include lipases.[4] While the catalytic roles of His and Ser have been relatively well established,[3] that of Asp has been a subject of debate. At least three possible roles have been suggested for the Asp: (a) orienting the conformation of His;[5] (b) stabilizing the appropriate tautomer of His;[5] and (c) neutralizing the positive charge of His during the reaction.[6] Strong evidence for role b has been provided in a recent study which

(1) For paper 3 in this series, see ref 2. This work was supported by Research Grant GM41788 from the NIH. J.P.N. was the recipient of a Monsanto Biotechnology Fellowship. We thank K. J. Hamilton for purification of Y73S and Y73A and J. K. Myers for purification of Y52F/Y73F. Abbreviations: Y, F, V, S, A, D, and N are one-letter designations of Tyr, Phe, Val, Ser, Ala, Asp, and Asn, respectively.

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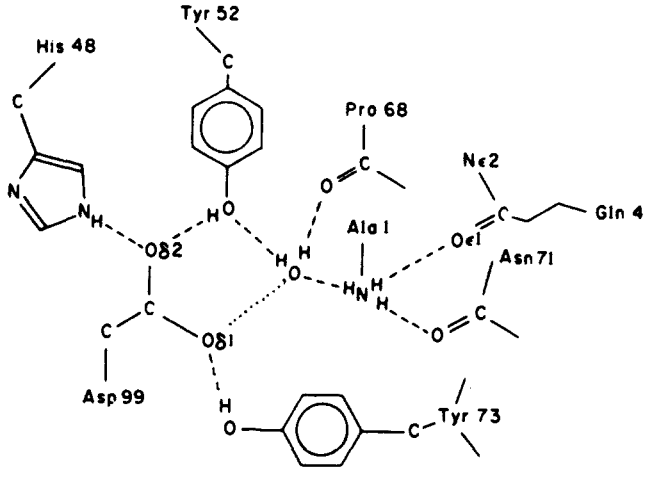

Figure 1. Schematic drawing of the hydrogen-bonding network observed in the 1.7-Å crystal structure of bovine pancreatic PLA2,[12b] as seen previously in ref 12a.

showed that substitution of the Asp-102 of trypsin by Asn caused His-57 to exist in the incorrect tautomer[7] and resulted in a 10^4-fold decrease in k_{cat}/K_m.[8] Some initial evidence for role c has also been presented in a recent meeting.[9] Role a could be inseparable from roles b and c since the latter two may require a proper conformation of His.

Less attention has been given to the observation that, in serine proteases, the carboxylate of the Asp in the catalytic triad is H-bonded to a few other residues. In trypsin, for example, the carboxylate of Asp-102 accepts H bonds from Ser-214 and the backbone amide protons of residues 56 and 57.[10] It is an interesting and important question as to whether these H bonds are required to stabilize the Asp in a proper conformation or the carboxylate of the Asp can perform its catalytic functions without further assistance from other groups.

We examined a similar situation in phospholipase A_2 (PLA2) which uses Asp-99···His-48 as a "catalytic diad", with the role of Ser substituted by water.[11] As shown in Figure 1, the carboxylate oxygens of Asp-99 are H-bonded to Tyr-52 and Tyr-73, which in turn are part of an extensive H-bonding network.[12] The residues His-48, Asp-99, Tyr-52, and Tyr-73, which are invariant among all groups I and II PLA2s (which include all known sequences except bee venom PLA2)[13] and occupy virtually identical positions in all crystal structures,[14] have been collectively termed the "catalytic network" by Sigler.[15] This catalytic network also exists in the crystal structure of a PLA2–inhibitor complex.[16] As depicted in Figure 1, the catalytic network is connected via a water molecule to Ala-1 and Gln-4, which have been suggested to be involved in the "interfacial recognition site" by de Haas.[17]

Table I. Summary of Kinetic Data[a]

enzyme	DC_8PC micelles k_{cat}, s^{-1}	K_m, mM	k_{cat}/K_m, M^{-1} s^{-1}	DC_6PC monomers: sp act., μmol^{-1} min^{-1} mg^{-1}
WT	675	1.4	4.8×10^5	5.3
Y52F	265	1.4	1.9×10^5	4.0
Y73F	580	2.0	2.9×10^5	2.1
Y52F/Y73F	205	2.8	0.7×10^5	0.6
Y52V	1.0	0.7	1.4×10^3	0.3
Y73S	1.7	0.9	1.9×10^3	0.4
Y73A	4.0	1.8	2.2×10^3	0.3
D99N	0.6	1.4	0.4×10^3	

[a] Bovine pancreatic PLA2 was isolated from an *E. coli* expression host BL21(DE3)plysS carrying a plasmid pTO-propla2[19] which contained a synthetic gene coding for bovine proPLA2.[18] The procedures for construction and purification of mutants will be described in detail in the future. The assays for both micellar and monomeric substrates were performed at pH 8.0 (1 mM sodium borate, 25 mM $CaCl_2$, 100 mM NaCl) and 45 °C using a pH stat method. The specific activity of monomers was measured by using 5 mM DC_6PC.

Blocking the amino terminus, modifying Gln-4, or changing their immediate environments have been shown to result in total loss of activity toward micellar but not monomeric substrates.[17]

We used site-directed mutagenesis to probe the roles of Tyr-52 and Tyr-73, particularly in relation to the function of Asp-99. Our system is bovine pancreatic PLA2 overproduced in *Escherichia coli*.[18,19] Table I lists the kinetic data for wild-type (WT) PLA2 and mutated enzymes Y52F, Y73F, Y52F/Y73F (double mutation), Y52V, Y73S, Y73A, and D99N.[1] For the micellar substrate DC_8PC (1,2-dioctanoyl-*sn*-glycero-3-phosphocholine), both k_{cat} and K_m have been obtained, whereas for the monomeric substrate DC_6PC, only specific activities were determined because the activities of the mutated enzymes toward the monomeric substrate approach the limit of detection of our assay system. Surprisingly, the kinetic constants for the mutated enzymes Y52F, Y73F, and Y52F/Y73F are perturbed by <10-fold relative to those of WT for both micellar and monomeric substrates. This suggests that Asp-99 is able to perform its catalytic function without being H-bonded to Tyr-52 and Tyr-73. It is possible that other groups or water molecules could move in to H bond to the carboxylate of Asp-99. However, the fact that both Tyr-52 and Tyr-73 can be changed to Phe suggests that such H bonds are most likely unimportant catalytically.

However, the aromaticity of both Tyr residues appears to be required; mutations at positions 52 and 73 involving the loss of aromatic side chain(s) (Y52V, Y73S, and Y73A) resulted in decreases in the catalytic activity by factors of 200–350 for micellar substrates and 10–20 for monomeric substrates. Such changes are likely to be due to structural perturbations since Tyr-52 and Tyr-73 are involved in perpendicular aromatic–aromatic interactions with Tyr-69 and Tyr-75, respectively; such interactions may serve to stabilize the tertiary structure of proteins.[20] Alternatively, the aromatic rings could be involved in carrying water molecules via H bonding.[21] Structural characterization of these mutated enzymes will be required to test these interpretations.

The mutated enzyme D99N showed a 10^3-fold decrease in k_{cat}/K_m, which is comparable to the result reported for the analogous mutation (D102N) in trypsin.[8] Future structural characterization is needed to determine whether this is due to the incorrect tautomeric form of His-48, as in the D102N mutated enzyme of trypsin.[7]

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The above results suggest the following conclusions: (a) The carboxylate of Asp-99 is able to carry out its function without forming H bonds to Tyr-52 and Tyr-73. (b) The phenolic hydroxyl of neither Tyr-52 nor Tyr-73 is catalytically essential even though they are absolutely conserved in groups I and II PLA2 sequences. (c) The aromatic rings of both residues are required, possibly for structural reasons. (d) If the H-bonding network shown in Figure 1 is really important in interfacial catalysis,[13a,17d] it should not involve Tyr-52 or Tyr-73.

Mechanism of Rhodium-Promoted Conversion of 3-Vinyl-1-cyclopropenes to 1,3-Cyclopentadienes. Stereochemistry of the Carbon–Carbon Bond-Forming Step

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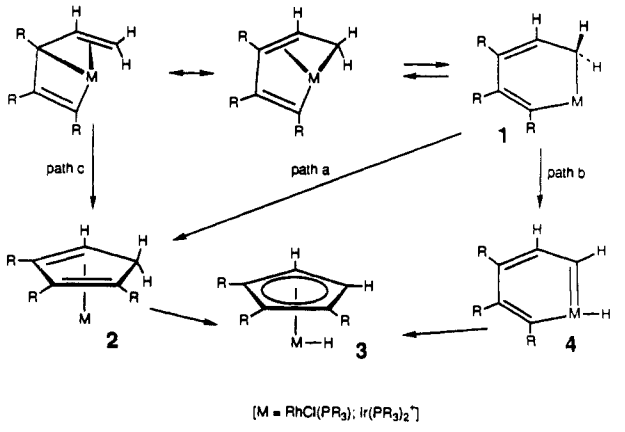
The transition-metal-promoted reactions of cyclopropenes are of great synthetic utility and mechanistic interest.[1] We have demonstrated that 1,2,3-triphenyl-3-vinyl-1-cyclopropene undergoes a facile C–C cleavage reaction promoted by Pt(0), Rh(I), and Ir(I) complexes to give 1,2,3,5-η- or 1,5-η-pentadienediyl (metallacyclohexadiene) complexes of Pt(II),[2] Rh(III),[3] and Ir(III).[3,4] Subsequent ring closure occurs under variable conditions to afford free 1,2,3-triphenyl-1,3-cyclopentadiene,[2] η^4-complexes of this diene,[2] or η^5-cyclopentadienyl(hydrido) complexes presumably derived from the latter.[3,5] It has been suggested that formation of the cyclopentadiene or (cyclopentadienyl)hydrido complexes may occur by either of two mechanisms, in both of which a metallacyclohexadiene intermediate **1** plays a key role (Scheme I).[3,5,6] Reductive elimination and C–C bond formation from **1** (path a) would give a coordinated cyclopentadiene complex **2**; subsequent addition of the *endo*-C–H bond to the metal would yield the (cyclopentadienyl)hydrido complex **3**. Alternatively, α-H elimination from **1** (path b) would afford (metallabenzene)hydrido intermediate **4**, which could then undergo C–C coupling to afford **3**. Here we report that closure to give an η^4-cyclopentadiene ligand can occur stereospecifically from the 1,2,3,5-η-ligand (path c) under conditions where the absence of any metallacyclic intermediates can be demonstrated unambiguously.

Syntheses of the sterically crowded complex **5a** and its D-labeled isotopomer **5b** have been reported; the half-life for scrambling of D between the syn and anti positions in **5b** is ca. 45 days at 110 °C,[7] indicating a high activation barrier for formation of metallacyclohexadiene **6** from **5**. No ring closures of **5a,b** to give a cyclopentadiene ligand were observed under these conditions.[7]

Scheme I



Scheme II

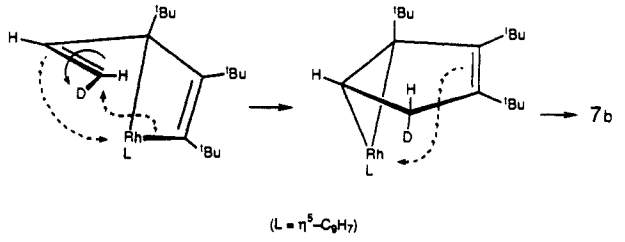
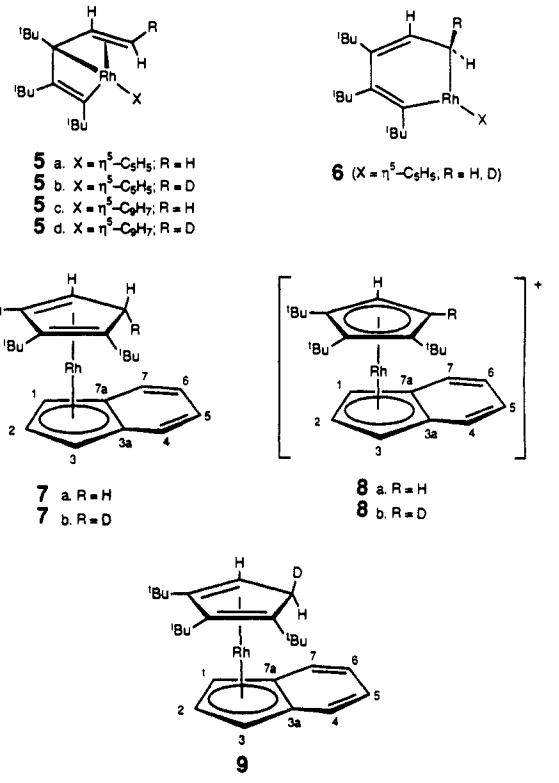


Chart I



In contrast, the indenyl analogues **5c,d**[8] undergo ring closure in refluxing benzene to give (at <50% conversion) their cyclopentadiene isomers **7a,b**; **7b** is formed as a single *endo*-D isoto-

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(8) Prepared from the chloride-bridged dimer[7] and indenylpotassium. **5c**: orange crystals, mp 58–60 °C; ^{1}H NMR ($CDCl_3$) δ 7.17 (m, 2 H, $H_{4,7}$), 6.88 (m, 2 H, $H_{5,6}$), 6.09 (br, 1 H, $H_{1/3}$), 5.96 (br, 1 H, $H_{1/3}$), 5.72 (br, 1 H, H_2), 4.01 (ddd, 1 H, J_{HH} = 10.6, 6.3, J_{RhH} = 2.0, $H_{central}$), 2.77 (dd, 1 H, J_{HH} = 6.6, J_{RhH} = 1.5, H_{syn}), 1.35 (s, 9 H, ^{t}Bu), 1.34 (dd, 1 H, J_{HH} = 10.6, J_{RhH} = 2.2, H_{anti}), 0.94 (s, 9 H, ^{t}Bu), 0.88 (s, 9 H, ^{t}Bu). **5d**: ^{1}H NMR ($CDCl_3$) identical except for δ 4.01 (dd, 1 H, J_{HH} = 10.6, J_{RhH} = 2.0, $H_{central}$), 1.34 (dd, 1 H, J_{HH} = 10.6, J_{RhH} = 2.2, H_{anti}); ^{2}H{^{1}H} NMR ($CHCl_3$) δ 2.77 (s, 1 D, D_{syn}). Satisfactory microanalysis data (C, H) were obtained for all compounds reported here.

0002-7863/90/1512-7076$02.50/0