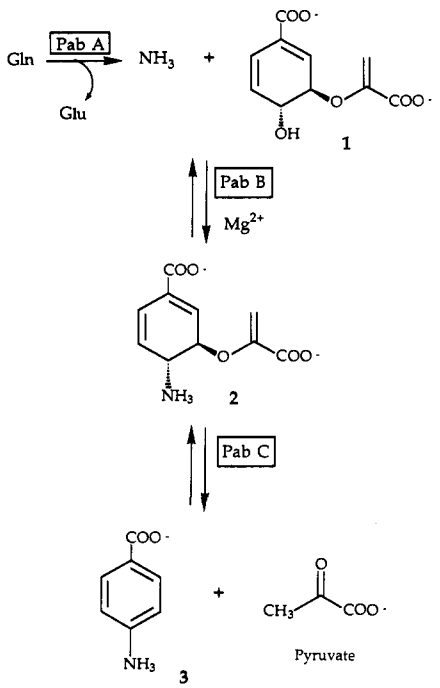
Scheme I



to chorismate. As shown in the spectra in the top panel of Figure 1, a duplicate set of resonances appeared after addition of enzyme. The ratio of peak heights of the original and new resonances reached a constant value which reflected the equilibrium constant for the reaction measured by chemical-quench methods.

The proton chemical shifts are in agreement with those previously reported by Teng et al. for the chemically synthesized 4-amino-4-deoxychorismate.[1] The resonances were shifted downfield (relative to chorismate) for all of the protons except those at the 4-position. The 4-H proton was shifted *upfield* by approximately 0.5 ppm to 4.25 ppm, as anticipated in replacing the C-4 oxygen with nitrogen. The coupling constant measured between H-3 and H-4 is −14 Hz, establishing a trans stereochemical relationship analogous to chorismic acid.

Further structural information was provided by enzymatically synthesizing **2** using ^{15}N-labeled ammonium bicarbonate (see legend to Figure 1). A 2D proton–carbon correlated spectrum is shown in the middle panel of Figure 1. The spectrum shows the anticipated chemical shifts for carbons and protons, with C-4, the allylic carbon, bearing the amino group and H-4 located at 53.7 ppm. A proton-decoupled natural abundance ^{13}C NMR spectrum (53 ppm region) is shown in the inset to the middle panel. The resonance having a chemical shift of 53.7 ppm was split into a doublet with a coupling constant, $^1J_{CN} = -8$ Hz, demonstrating that the [^{15}N]amino group is attached to C-4. The final confirmation of the structure for **2** was provided by the ^{15}N NMR spectrum shown in the bottom panel. The resonance observed upfield at −98.3 ppm is attributable to residual NH_4^+ while the resonance downfield at −77.2 ppm is due to the intermediate.

The enzymatic and structural data in this report provide definitive identification of **2** as a true intermediate which is formed by the action of PabB on chorismate. As discussed previously,[7-9] isomeric hydroxy- or aminochorismate compounds have been isolated or proposed also in the biosynthesis of isochorismate and anthranilate, respectively. It is interesting to note that the absolute stereochemistry of chorismate is retained in the corresponding isomeric intermediates, implying that a double inversion of configuration must have taken place. In each of these cases, the enzymatic transformation requires magnesium. It is reasonable to suggest that all three pathways utilize a common intermediate which can be attacked by the appropriate nucleophile to give the desired product. We are currently exploring this possibility in studies underway to establish the complete kinetic pathway for the conversion of chorismate to PABA.

Acknowledgment. We thank J. Lecomte for helpful discussions.

(6) The value of the equilibrium constant was measured from reaction mixtures containing 50 μM [^{14}C]chorismate, 1 μM PabB, 50 mM $(NH_4)_2SO_4$, and 10 mM $MgCl_2$ in 50 mM TRIS pH 8.6 buffer at 37 °C. The reactions were quenched at 35 and 75 min with $CHCl_3$ and analyzed by RP-HPLC. The results showed that the equilibrium was established within 35 min and that the value for the equilibrium constant was 6.1 M^{-1}.

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Design of a Novel Type of Zinc-Containing Protease Inhibitor

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Carboxypeptidase A (CPA, EC 3.4.17.1)[1,2] serves as the prototypic enzyme for zinc-containing proteases.[3] The key catalytic groups of CPA are Zn^{2+}, Glu-270, and Arg-145. The carbonyl group of the scissile peptide bond is polarized by the Zn^{2+}, and the activated carbon is attacked by the carboxylate of Glu-270 (anhydride pathway[4]) or by a water molecule with the assistance

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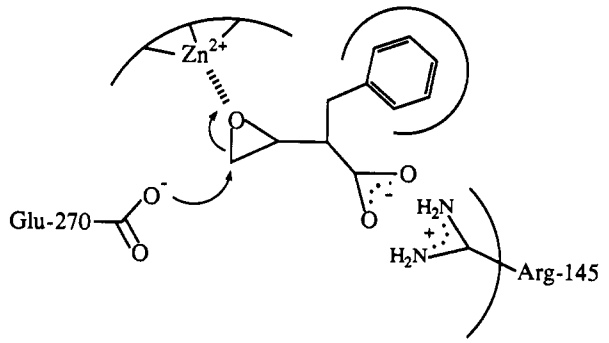

Figure 1. Schematic representation of the hypothetical Michaelis complex formed upon the interaction of BEBA with CPA.

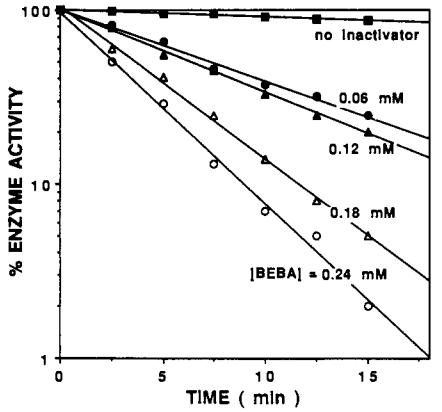

Figure 2. Plot that demonstrates time-dependent inactivation of CPA by BEBA. The buffer solution of CPA and BEBA (about 1.5 μM CPA for any given [BEBA]) was incubated at room temperature for 0.5 h, and then at 2.5-min intervals 50-μL samples were removed and added to 950 μL of the assay mixture (400 μM). Absorbances at 254 nm were measured immediately.

of Glu-270 (promoted water pathway[5]).[6]

Oxiranes are stable compounds, but in the presence of a Lewis acid such as $AlCl_3$ or $ZnCl_2$ they are susceptible to nucleophilic ring opening. Accordingly the oxiranes having structural features for complexing with Zn^{2+} at the active site are expected to interact with Glu-270 with resultant covalent modification (Figure 1). 2-Benzyl-3,4-epoxybutanoic acid (BEBA) is the compound designed to serve such a purpose.

BEBA[7] was readily prepared from 2-benzyl-2-vinylacetic acid[8] by epoxidation with *m*-chloroperoxybenzoic acid (MCPBA). In kinetic studies,[9] it showed a time-dependent loss of CPA activity (Figure 2), and the rate of inactivation was directly proportional to the concentrations of BEBA up to 1.88 μM and then independent at higher concentrations (saturation kinetics).

K_m and k_{inact} were determined[2a] to be 6.25 mM and 0.05 min^{-1}, respectively, by using the following simplied kinetic scheme:

$$E + I \underset{}{\overset{K_m}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_{inact}} E - I$$

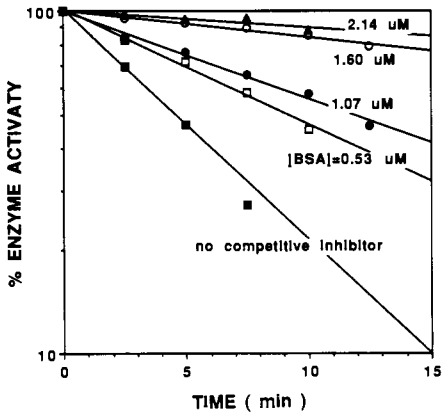

Figure 3. The competitive inhibitor experiment carried out at various concentrations of benzylsuccinic acid (BSA). Typically, a 1000-μL solution of 1.0 mM BSA, 1.5 μM CPA, and 0.12 mM BEBA in the Tris buffer was stirred gently at room temperature, and a sample of the mixture (100 μL) was removed and added to 900 μL of the assay mixture (400 μM) at various time intervals. The activity was monitored at 254 nm.

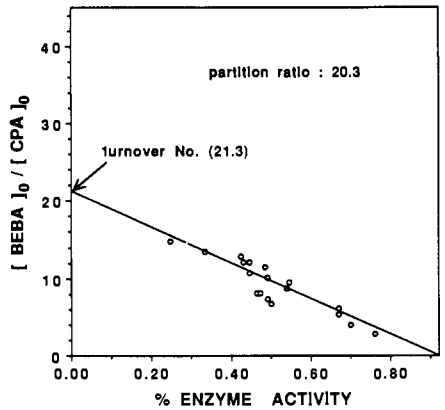

Figure 4. The determination of the partition ratio from plotting of $[BEBA]_0/[CPA]_0$ vs the fraction of the activity remaining after dialysis for 24 h at 5 °C.

The K_i (reversible inhibitor constant) value of 300 ± 14 μM was obtained from the plot of K_m' against concentrations of BEBA.[10] Plotting of the logarithm of the observed inactivation rate constants of CPA against the logarithm of concentrations of BEBA according to the method of Levy et al.[11] gave a slope of 1.2, which strongly suggests that the irreversible interaction occurs with 1:1 stoichiometry. That the irreversibility is due to covalent modification was supported by the dialysis experiment of the incubation (5 °C, 15 h) mixture of CPA and BEBA in excess. The enzyme activity did not return after 24 h.

As shown by Figure 3, the rate of the inactivation became slower in the presence of benzylsuccinic acid, a known reversible competitive inhibitor[2a] of CPA, which indicates that the inactivation is active-site directed. Finally, the partition ratio,[12,13] the efficiency measure of an inactivator, was determined[13] to be 20.3 (Figure 4), which reveals that BEBA is a highly effective inactivator of CPA.[14]

The above kinetic data, taken together, strongly suggest that BEBA is a novel type of CPA inactivator (pseudomechanism-based inactivator[15]) of CPA[16] and provide a new avenue to the design

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(6) In spite of extensive studies over the past several decades, the mechanistic details of CPA are still in question.

(7) The epoxidation reaction was carried out by a standard method and purified by silica gel column chromatography eluting with chloroform and methanol to obtain BEBA as a thick oil: ^1H NMR (300 MHz, $CDCl_3$/TMS) δ 2.04–2.30 (m, 1 H), 2.47–2.52 (m, 1 H), 2.60–2.73 (m, 1 H), 2.85–2.96 (m, 1 H), 3.10–3.22 (m, 2 H), 7.18–7.32 (m, 5 H); high-resolution mass spectrum (EI^+) *m/z* 192.076 (M^+, calcd 192.079); IR (neat) 1730, 1270 cm^{-1}.

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(14) The partition ratio would be further improved if an enantiomerically pure BEBA were used.

of active-site-directed irreversible inhibitors of zinc-containing proteases. The stereochemical effect of BEBA in the inactivation action and applications of this novel approach to the design of therapeutically useful inactivators are in progress.

Acknowledgment. We thank the Korea Science and Engineering Foundation and the Ministry of Education, Republic of Korea, for financial support.

(15) We feel that pseudomechanism-based inactivator is a preferred terminology that properly reflects the mode of the inhibitory action: The oxirane ring of BEBA is *activated not by a chemical change but through coordination with the active-site* Zn^{2+} to form a covalent linkage with the carboxylate of Glu-270 (Figure 1). This inactivator is not an affinity label because the latter would not require such activation.

(16) The feature of the inhibitory mode of BEBA is consistent with the anhydride pathway. An alternative mechanism by which promoted water functions as a nucleophile may also be operational, but in this case no covalent modification of CPA is expected.

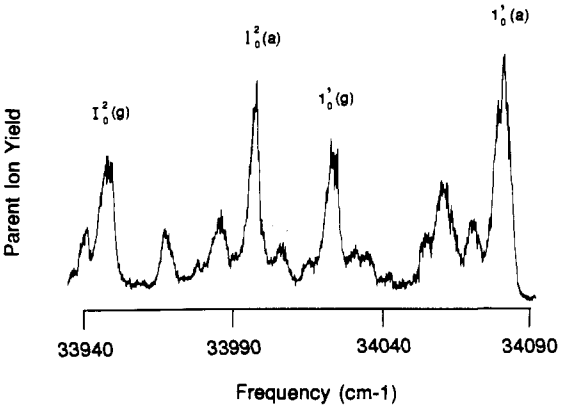

Figure 1. Wavelength dependence of the two-step ionization yield of *p-n*-propylaniline as recorded with a time-of-flight mass spectrometer. Peaks correspond to transitions between the vibrationless ground electronic state and various vibronic levels of the $^1B_2$ first excited singlet state of this molecule.

Dependence of *p-n*-Propylaniline Ionization Potential on Molecular Conformation: Comparison of Experiment with Theory

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Cooling of molecules in supersonic jets often reduces the complexity of electronic spectra sufficiently that contributions from different molecular conformations can be clearly resolved. Some of the earliest cases demonstrating this involved the alkylbenzene and alkylaniline compounds studied by Smalley and co-workers.[1-3] They showed that when the alkyl substituent contains three or more carbon atoms, a splitting of sharp vibronic features occurs. Their generally accepted interpretation was that at least two molecular configurations exist: one with the alkyl chain folded back over the benzene ring and the other with it extended away from the ring. For substituent chains with four or more carbon atoms, further spectral splittings may imply the existence of additional conformations. The electronic spectrum associated with the folded (gauche) configuration is red-shifted relative to that of the extended (anti) configuration.[1-4] Although conventional photoelectron spectra of room temperature molecules exhibit such broadened and unresolved line shapes that conformational variations are barely discernible,[5-8] high resolution laser photoelectron spectroscopy makes it possible to directly measure the slight differences between ionization potentials of molecules in these two conformations. The present study involves gauche and anti

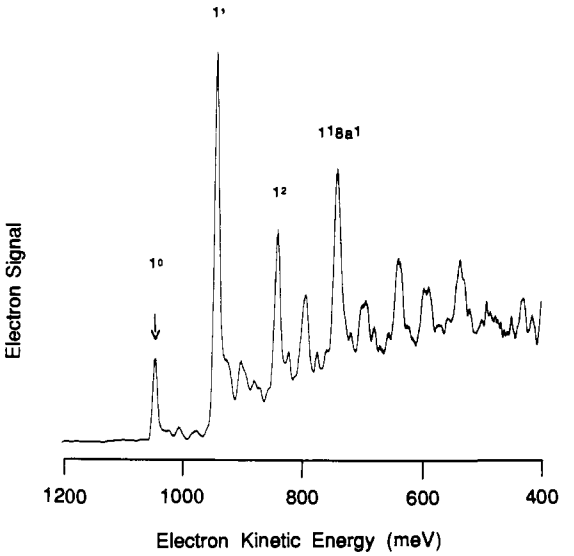
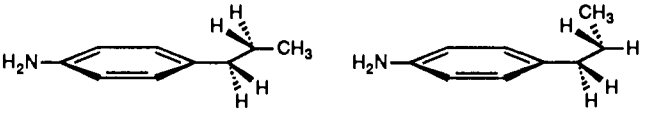


Figure 2. Photoelectron spectrum obtained by exciting the 1^1_0 transition of *anti-n*-propylaniline and photoionizing the electronically excited molecules with the same light pulse. Only *anti-n*-propylaniline ions are generated. The vertical scale corresponds to electron counts, in arbitrary units. A few ion vibrational assignments are indicated.

conformations of *p-n*-propylaniline, which have the following structures:



anti-p-n-propylaniline *gauche-p-n*-propylaniline

Our pulsed supersonic beam photoelectron spectrometer, which cools aromatic molecules to approximately 8 K, has been thoroughly described.[9] Laser photoelectron spectra are recorded by exciting a particular vibronic level of a molecule with a pulse of tunable laser light and photoionizing the molecule from that initially selected level. To tune our laser to any desired spectral feature, we record the wavelength dependence of the two-step ionization yield, detecting the positive ions formed using a time-of-flight mass spectrometer. A typical wavelength dependence is displayed in Figure 1. At the low light intensities we used, little ion fragmentation was produced. A few spectral assignments that match those of Powers et al. are indicated in Figure 1. In this notation, X^n_0 refers to a transition between the vibrationless ground electronic state and the $v = n$ level of mode

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