# L-[2,5-$H_2$]Phenylalanine, an Alternate Substrate for Rat Liver Phenylalanine Hydroxylase†

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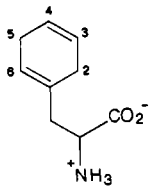
ABSTRACT: The phenylalanine analogue L-[2,5-$H_2$]phenylalanine (**1**) was found to be a viable substrate ($K_M$ = 0.45 mM, $k_{cat}$ = 8 $s^{-1}$) for L-phenylalanine-activated, rat liver phenylalanine hydroxylase (EC 1.14.16.1) (PAH). The PAH-catalyzed oxidation of **1** was stoichiometric with the oxidation of cofactor, 6-methyltetrahydropterin. Spectral and chromatographic data of the product from the oxidation of **1** by PAH were found to be in accord with a 3,4-epoxide. The enzymatic epoxidation of **1** is consistent with the hypothesis of an intermediate arene oxide on the reaction coordinate for PAH hydroxylation of L-phenylalanine.

The non-heme iron containing enzyme, (Fisher et al., 1972) phenylalanine hydroxylase (EC 1.14.16.1) (PAH), catalyzes the insertion of atomic oxygen into the carbon–hydrogen bond of the 4-ring position of L-phenylalanine to yield L-tyrosine. The enzyme requires a tetrahydropterin cofactor [i.e., biopterin or 6-methyltetrahydropterin (6-$MPH_4$)], which is oxidized ultimately to the quinonoid pterin (6-$MPH_2$) during PAH turnover (Kaufman, 1964).

Recent work on the mechanism of the pterin oxidation by PAH during substrate hydroxylation has revealed that 6-$MPH_4$ is first converted to 6-methyl-4a(*S*)-hydroxypterin [6-M-(4a-OH)$PH_2$] (Dix et al., 1985) (see Scheme I). This intermediate then undergoes a pH-dependent dehydration to yield 6-$MPH_2$ (Lazarus et al., 1981). This dehydration has been found to be also catalyzed by a rat liver carbinolamine dehydratase (CAD) Lazarus et al., 1983).

In contrast to the above-described pterin oxidation, the mechanism that PAH employs to regioselectively insert oxygen into the aromatic amino acid is not fully understood. The discovery of the PAH-induced "NIH shift", with phenylalanines labeled in the 4-ring position, has resulted in the postulation of an intermediate arene oxide (Guroff et al., 1967) (see Scheme II). This arene oxide is then thought to ring open to yield a stable carbocation, which undergoes a 1,2-sigmatropic rearrangement to give a cyclohexadienone. In the case of a hydrogen isotope label in the 4-ring position of the parent phenylalanine, an isotope effect selects for the protium atom as the cyclohexadienone aromatizes to tyrosine (see Scheme II). This proposed mechanism, then, is consistent with the NIH shift of the label from the 4-ring position to the 3-ring position in the aromatic. It is uncertain if the post arene oxide transformations depicted in Scheme II would require PAH catalysis.

The existence of this arene oxide intermediate has never been verified. Therefore, we decided to probe for this intermediate by employing an alternate substrate that could be oxidized by PAH yet lacks the ability to undergo ring opening and subsequent rearrangement. One compound that fits these criteria is L-[2,5-$H_2$]phenylalanine (**1**) (Snow et al., 1968). In analogy with Scheme II, this compound might yield an




**1**

epoxide upon oxidation by PAH. This epoxide if ring opened would generate a less stable secondary carbocation instead of the resonance-stabilized carbonium ion shown in Scheme II, thus increasing the probability of isolating the epoxide as the final product. Isolation and characterization of an epoxide derived from the reaction of **1** with PAH would clearly suggest an intermediary arene oxide in the enzymatic oxidation of phenylalanine. This paper, then, describes the studies of the reaction of **1** with PAH, as well as furnishing proof for the formation of an epoxide from **1** upon enzymatic oxidation.

## Experimental Procedures

Commercially available reagents were used without further purification. Nuclear magnetic resonance spectroscopy was done on a Bruker WB-360 (360 MHz) with chemical shifts being referenced versus the transmitter offset for $D_2O$ or the position of the phenyl protons in phenylalanine ($\delta$ = 7.26 ppm). Direct-injection probe mass spectroscopy was performed on a Finnigan Model 3200 spectrometer employing chemical ionization (methane) as the excitation method. Optical rotation measurements were done on a Perkin-Elmer Model 241 polarimeter. Fluorescence emission spectra were taken on either a Perkin-Elmer MPF 44A or a SLM 8000C spectrofluorometer. The sorbent for thin-layer chromatography was cellulose (Eastman-Kodak, 13254) with the mobile phases specified below. Melting points were determined on a Fisher-Johns apparatus and were not stem corrected. The pH of the various reaction buffers was adjusted to the desired value by using a Radiometer Model 22 pH meter with a standard internal referenced glass electrode (Model GK2402C). 6-Methyltetrahydropterin (6-$MPH_4$) was prepared as previously described (Storm et al., 1971). Dihydropterin reductase (DHPR) was purified and assayed by the method of Craine et al. (1972).

*Preparation of* L-[*2,5-$H_2$*]*Phenylalanine* (**1**). The preparation of compound **1** was done in accord with the published procedure of Snow et al. (1968) and gave product yields ranging between 40% to 68% on reaction scales of 0.91–9.1 mmol. Compound **1** was obtained as a white to slightly off-

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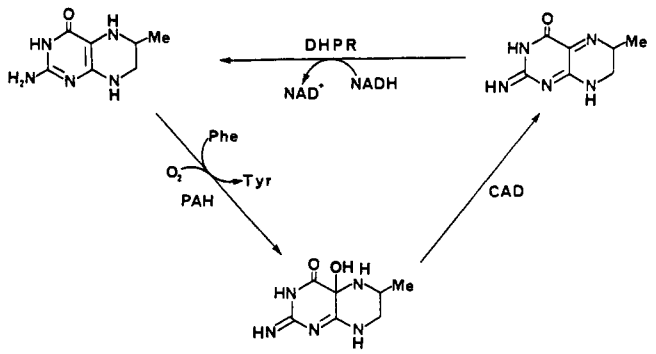
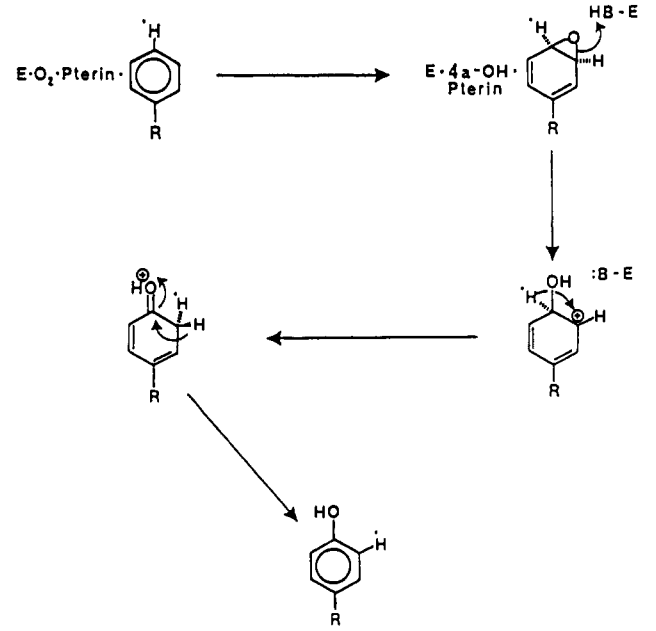
Scheme I




Scheme II




white solid that melted with decomposition between 230 and 240 °C [lit. 235–236 °C (Snow et al., 1968)]. The product gave one spot on thin-layer chromatography (TLC) ($R_f$ = 0.75, cellulose, 3:2:0.6:2.4 butanol/pyridine/acetic acid/water), which yielded the previously observed brick-red color upon treatment with ninhydrin (Snow et al., 1968). Optical rotation (Na D line, concentration of sample = 0.04 g/mL, cell path = 1 dm) gave the following specific rotation: $[\alpha]^{27°C}$ −29.25 (L-phenylalanine, $[\alpha]^{27°C}$ −26.37). MS (chemical ionization, methane) (relative intensity of base peak) $M^+ - 2$, 165 (0.05); $M^+$, 167 (100); $M^+ + 28$, 195 (0.11); impurity peaks, 205 (0.02) and 223 (0.03). The compound can be stored, as a solid or in aqueous solution, for several months at <4 °C. At >25 °C oxidation to phenylalanine takes place.

*Purification of Rat Liver Phenylalanine Hydroxylase.* Phenylalanine hydroxylase was purified according to the modified Shiman procedure (Gottschall et al., 1982) and gave enzyme that possessed specific activities ranging between 9.3 and 11.2 μmol $min^{-1}$ (mg of protein)$^{-1}$. Total protein concentration was determined by absorbance at 280 nm (extinction of 1 absorbance unit/mg of protein) and confirmed by the bicinchoninic acid (BCA) colorimetric assay (Pierce).

*Phenylalanine Hydroxylase Substrate Activation Study.* The ability of **1** to serve as an allosteric activator (Shiman & Gray, 1980) of PAH was tested in the following manner. PAH, at a concentration of 0.45 μM, was preincubated for 15 min with various concentrations of either **1** or phenylalanine (0–20 mM) at 25.0 °C at pH 6.8 (50 mM potassium phosphate, total volume 50 μL). From these solutions 10 μL was removed and added to 990 μL of a 50 mM potassium phosphate solution, pH 6.8, containing 6 mM 1,4-dithiothreitol (DTT), 60 μg/mL catalase, and 60 μM 6-$MPH_4$. The production of tyrosine was then monitored by the Shiman assay (Gottschall et al., 1982) which involved observing the change in absorbance at 275 nm. Tyrosine production was then calculated from these data by using a molar extinction of 0.17 $\mu M^{-1}$ $cm^{-1}$.

*PAH Fluorescence Change upon Binding Substrates.* The change in PAH fluorescence upon binding the activator substrate (Shiman, 1985) was performed in the following manner. An emission spectrum from 300 to 420 nm of a solution of 50 mM potassium phosphate, pH 6.8, at 25.0 °C, containing 0.25 μM PAH was taken at an excitation wavelength of 275 nm. This experiment was repeated, except 0.1 mM phenylalanine or 0.1–0.2 mM **1** were present in the solution. Comparison of these spectra with the spectrum in the absence of substrate revealed the difference in the emission characteristics of PAH upon binding substrate activators.

*Determination of Steady-State Kinetic Parameter Determination for* **1** *with Phenylalanine Hydroxylase.* Steady-state parameters for **1** with PAH were determined from phenylalanine-activated PAH and entailed preincubating a 4.0 μM solution of PAH in 1 mM phenylalanine at pH 6.8 for 15 min at 25.0 °C. From this solution, 10 μL was removed and added to 990 μL of 50 mM potassium phosphate, pH 6.8, containing 120 μM NADPH, 60 μg/mL catalase, 1.43 μM dihydropterin reductase (Sigma), 60 μM 6-$MPH_4$, and various concentrations of **1** (0.05–2.0 mM). The rate of PAH turnover was determined by the NADPH/DHPR assay (Kaufman & Mason, 1982), a procedure that observes the decrease in absorbance at 340 nm due to the consumption of NADPH as dihydropterin is reduced by DHPR, or by the method of Shiman (Gottschall et al., 1982). The reaction velocities for **1** with PAH were then calculated from these data by using the molar extinction for NADPH of 6.4 $\mu M^{-1}$ $cm^{-1}$ at 340 nm. The velocities were corrected for the contribution to turnover due to the phenylalanine used in the activation of PAH. The resulting double-reciprocal plot (1/[S] versus 1/$v$) was computer fit to the Michaelis–Menten parameters (Cleland, 1970; Cornish-Bowden & Endrenyi, 1981).

*Stoichiometry of* **1** *versus NADPH with PAH.* The reaction stoichiometry between **1** and NADPH was determined by observing the turnover of PAH in the presence of a limiting amount of **1**. This experiment entailed taking 4.0 μM PAH that had been preincubated in a solution of 1.0 mM phenylalanine at 25.0 °C for 15 min at pH 6.8 and removing 10 μL and adding it to 990 μL of 50 mM potassium phosphate, pH 6.8, containing 120 μM NADPH, 60 μg/mL catalase, 60 μM 6-$MPH_4$, and either 0.05 or 0.10 μM compound **1**. The amount of NADPH consumed was monitored by the decrease in absorbance at 340 nm. Since a significant amount of phenylalanine was added from the activation solution, this amount (10 μM) was subtracted from the total amount of NADPH consumed to yield the change due only to **1**. At the apparent end point additional PAH (4 nmol) and NADPH (100 nmol) were added to ensure completion of the reaction.

*Detection of 6-M-(4a-OH)$PH_2$ with* **1** *and PAH.* The detection of 6-M-(4a-OH)$PH_2$ with **1** and PAH was accomplished by the method of Lazarus et al. (1981). This method exploits the slow rate of 6-M-(4a-OH)$PH_2$ dehydration at basic pHs coupled to the large change in molar extinction between 6-$MPH_4$, 6-M-(4a-OH)$PH_2$, and 6-$MPH_2$ species. This procedure involved taking 10 μL of a 4.0 μM PAH solution in 1.0 mM phenylalanine (PHE) at 25.0 °C and adding

it to 990 μL of a 20 mM potassium borate solution, pH 8.4, containing 1 mM **1**, 60 μg/mL catalase, and 20 μM 6-$MPH_4$. The production of 6-M-(4a-OH)$PH_2$ and its subsequent dehydration were observed by monitoring the overall change in absorbance at 244 nm.

*Preparation of Products from PAH Reaction for Mass Spectral Analysis.* The reaction solution for mass spectral product analysis of the reaction of **1** with PAH contained the following: 1 mM **1**, 6 mM DTT, 60 μg/mL catalase, 3.18 μM PAH, and 60 μM 6-$MPH_4$. This solution was kept at 25.0 °C for 12 h, followed by heating to 50.0 °C for an additional 2 h. The solution was filtered through an Amicon ultrafiltration system (YM-10 membrane, $M_r$ 10000 cutoff) followed by lyophilization of the filtrate. The resultant residue was then triturated with methylene chloride (3 × 100 mL), dried under reduced pressure to give a white powder, and stored at −20 °C. Approximately 1–2 mg of this material was subjected to direct-injection probe/mass spectral (DIP/MS) analysis. A control experiment was run, employing the same reaction conditions described above, except that phenylalanine was the substrate instead of **1**.

*Treatment of* **1** *with m-Chloroperbenzoic Acid.* For product analysis via TLC, treatment of **1** with *m*-chloroperbenzoic acid (MCPBA) was done in water. This involved adding 20 μL of a 100 mM ethanolic solution of MCPBA to a 19 mM solution of **1** in 50 mM potassium phosphate, pH 6.8, followed by reaction at ambient temperature for 30 min. Reaction aliquots were removed at 0, 5, 15, and 30 min, respectively, and analyzed for products via the TLC method.

For product analysis with $^1H$ NMR spectroscopy, **1** (25 mg, 0.15 mmol) was dissolved in deuterium oxide (0.5 mL). Added to this was an aliquot (50 μL) of a 0.1 M ethanolic solution of MCPBA. The reaction solution was then subjected to $^1H$ NMR analysis with time points taken at 0, 2, 10, 180, 300, and 2880 min.

*Treatment of the Reaction Product from* **1** *and PAH,* **1** *and MCPBA, and Phenylalanine and PAH with Reduced Glutathione and Glutathione S-Transferase.* The product sample from the reaction of **1** and PAH was prepared in accord with the procedure used for the above-described DIP/MS analysis, except that the reaction solution was not heated to 50.0 °C. The resulting lyophilized solid was dissolved in water (10 mL) that contained 1 mM reduced glutathione and 80 μg/mL rat liver glutathione *S*-transferase (Jerina, 1976), followed by standing at 25.0 °C for 18 h. The reaction solution was filtered through an Amicon ultrafiltration apparatus (YM-10 membrane, $M_r$ 10000 cutoff) and lyophilized to yield a white powder. This material was redissolved in a minimal amount of water (2.0 mL) and subjected to TLC product analysis.

Conditions identical with those described above were used for the reaction of the MCPBA-treated **1** with reduced glutathione and glutathione *S*-transferase, except that aliquots were removed and analyzed via TLC at 0, 5, 15, and 30 min.

In the reaction involving phenylalanine and PAH, the reduced glutathione and glutathione *S*-transferase were present during PAH turnover. The reaction solution contained the following: 6 mM DTT, 1 mM phenylalanine, 30 μg/mL catalase, 1 mM glutathione, 80 μg/mL glutathione *S*-transferase, 0.2 μM PAH, and 60 μM 6-$MPH_4$ in 50 mM potassium phosphate, pH 6.8 (total volume 9.75 mL). The reaction solution was allowed to stand at 25.0 °C for 18 h in air. The solution was ultrafiltered ($M_r$ 10000 cutoff) and lyophilized to give a white filamentous powder. This material was taken up in a minimum amount of water (0.5 mL), and products were analyzed by TLC.

*Product Analysis of Various Reactions by Thin-Layer Chromatography.* The products and product standards for the above-described reactions were spotted onto a 3 × 10 cm cellulose plate (100-μm thickness) and eluted with a solution of 1-butanol/pyridine/acetic acid/water (3:2:0.6:2.4) at ambient temperature. After development, the eluent was allowed to completely evaporate and the plate was sprayed with a ninhydrin reagent [0.3 g of ninhydrin (Aldrich) and 0.4 mL of acetic acid in 100 mL of 1-butanol] followed by heating at 140 °C until the desired color had been obtained.

*$^1H$ NMR Spectroscopy of Enzyme-Derived Products.* $^1H$ NMR analysis was performed on the remaining solid material from the above-described DIP/MS analysis experiment. This solid was taken up in water (15 mL), filtered, and lyophilized to give an off-white solid, which was then lyophilized twice from deuterium oxide. This slightly off-white solid was triturated with deuterium oxide (0.5 mL), followed by dimethyl sulfoxide-$d_6$ (0.5 mL) and finally by additional deuterium oxide (0.5 mL). The majority of the product was found in the dimethyl sulfoxide extract, while the first deuterium oxide extraction contained a great deal of potassium phosphate. The dimethyl sulfoxide sample was then subjected to $^1H$ NMR analysis employing an inversion–recovery pulse sequence (WEFT) (1.8-s delay time between the 90° and 180° pulses) and two-dimensional correlated (COSY) (Aue et al., 1976; Nagayama et al., 1980) NMR spectroscopy (Bruker Aspect 3000 pulse sequence). These spectra were referenced to the phenyl ring protons of phenylalanine ($\delta$ = 7.26 ppm).

A second experiment was done by utilizing the same reaction procedure as outlined in the DIP/MS experiment except the solution was not heated to 50.0 °C. The resulting off-white solid was triturated with deuterium oxide (2.0 mL). The triturate was filtered through glass wool, and an aliquot (0.5 mL) was analyzed by $^1H$ NMR spectroscopy (reference $H_2O$).

Authentic **1** (10 mg, 0.06 mmol) was dissolved in deuterium oxide (0.5 mL) and subjected to $^1H$ NMR analysis (reference $H_2O$).

## Results and Discussion

In order for rat liver PAH to be fully active, it must first bind substrate to an allosteric site, that is, at a separate locus from the active site (Shiman & Gray, 1980). This requisite preturnover binding of activator is thought to influence organization of the catalytic site on the enzyme and in turn causes a slight, but detectable, "red shift" in the emission spectrum of PAH (Shiman, 1985). In order to gain information for the substrate viability of **1**, we first determined whether it was an effective activator of the enzyme.

The ability of **1** to act as an activator of PAH was tested by preincubating the enzyme in various concentrations of **1** at 25.0 °C and at pH 6.8. An aliquot of each of these solutions was then withdrawn and PAH activity measured by the Shiman assay (Gottschall et al., 1982) using the normal substrate. The initial velocities obtained from these assays would indicate the amount of active PAH present as a function of the concentration of activator. Figure 1 depicts a plot of PAH initial velocity for the production of tyrosine versus the concentration of activator (i.e., phenylalanine or **1**), showing that **1** does not activate the enzyme to the same extent that phenylalanine does. In fact, full activation of PAH was not attained, even after preincubating the enzyme in 20 mM **1**. On the other hand, both the normal substrate and compound **1** caused a red shift in the fluorescence emission spectrum of the enzyme of 14 and 9 nm, respectively. This result suggests that the enzyme was undergoing similar conformational changes with **1** as with phenylalanine; however, the resultant

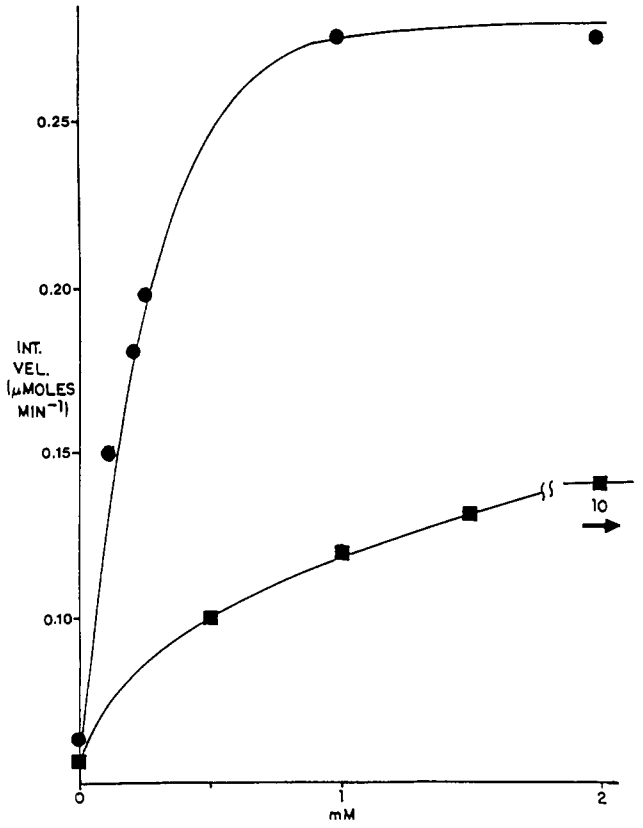



FIGURE 1: Plot of PAH initial velocity ($\mu$mol of Tyr $min^{-1}$) versus the concentration of activator. Conditions listed under Experimental Procedures. Key: (●) PHE; (■) **1**.

PAH was not as kinetically active as phenylalanine-activated PAH. This inability of **1** to activate PAH was unexpected, especially in light of the fluorescence data supporting the binding of **1** to PAH and its substrate properties. One possible explanation may be that the puckered cyclohexadiene ring in **1** does not induce formation of the catalytic site in PAH. Similarly, L-tryptophan is a substrate for PAH (Renson et al., 1961) but at concentrations less than 5 mM does not activate the enzyme (Shiman & Gray, 1980).

Therefore, in order to test convincingly the ability of **1** to act as a substrate for the enzyme, we used phenylalanine-activated PAH (Shiman & Gray, 1980). This was accomplished by preincubating PAH with sufficient aromatic amino acid to induce full activation of the enzyme, then adding portions of this material to solutions containing various concentrations of **1**, and assaying the amount of pterin turnover by using the NADPH/DHPR assay (Kaufman & Mason, 1982). Compound **1** displayed saturation kinetics with PAH; a reciprocal replot of these data yielded the steady-state parameters (Cleland, 1970; Cornish-Bowden & Endrenyi, 1981) $K_M = 0.45 \pm 0.12$ mM and $k_{cat} = 8 \pm 0.88$ $s^{-1}$, for comparison with phenylalanine $K_M = 0.18$ mM and $k_{cat} = 10$ $s^{-1}$. These data clearly show that compound **1** is equivalent to phenylalanine as a substrate for PAH.

PAH can oxidize pterin cofactors without concomitant hydroxylation of substrate. The nonstoichiometric uncoupling of pterin oxidation from substrate hydroxylation can be effected by both substrate analogues (e.g., 4-chlorophenylalanine) and unnatural cofactors (e.g., tetrahydropterin) (Storm & Kaufman, 1968; Fisher & Kaufman, 1973; Dix & Benkovic, 1985). Since the assay system employed compound **1** in excess, one cannot conclude that the substrate was indeed being oxidized in a stoichiometric manner relative to cofactor. This question was addressed by observing the amount of

Table I: List of $R_f$'s for Substrates and Products for the Reaction of PAH with **1**

| compound | $R_f$[a] | color[b] |
|---|---|---|
| phenylalanine | 0.68 ± 0.08 (8) | blue |
| tyrosine | 0.60 ± 0.12 (3) | blue |
| **1** | 0.73 ± 0.05 (9) | orange |
| reduced glutathione | 0.33 ± 0.10 (6) | blue |
| oxidized glutathione | 0 (6) | blue |

[a] $R_f$ values and the standard errors were calculated from multiple runs. The numbers in parentheses are the number of data points in the calculations. [b] Represents the color of the spot on the TLC plate after development with ninhydrin (see Experimental Procedures).

NADPH consumed in the presence of a limiting amount of **1**. By use of the NADPH/DHPR assay the amount of pterin oxidized per added **1** can be accurately determined from the amount of NADPH consumed. The result of this experiment is that 50.0 and 100.0 nmol of **1** resulted in 52.0 ± 3.0 and 102.0 nmol of NADPH consumption, respectively. Thus the reaction of PAH with **1** appears to be fully coupled to cofactor oxidation.

Further evidence for a coupled reaction of **1** with PAH is the formation of an intermediate 6-M-(4a-OH)$PH_2$—a species that has been shown to be on the reaction coordinate for the hydroxylation of phenylalanine by PAH (Lazarus et al., 1981). With compounds that "uncouple" PAH, 6-M-(4a-OH)$PH_2$ does not appear to form when 6-$MPH_4$ is oxidized to 6-$MPH_2$ (Dix & Benkovic, 1985). In order to probe for 6-M-(4a-OH)$PH_2$ formation in the reaction of **1** and PAH, a spectroscopic method was employed to monitor the molar absorptivities of the various anticipated pterin species (Lazarus et al., 1981). Under single-turnover conditions (pH 8.4) there is an initial increase followed by a decrease in absorbance at 244 nm, which is due to the initial formation of 6-M-(4a-OH)$PH_2$ ($\epsilon = 16\,500$ $M^{-1}$ $cm^{-1}$) from 6-$MPH_4$ ($\epsilon = 4000$ $M^{-1}$ $cm^{-1}$) (Lazarus et al., 1981) that then dehydrates to 6-$MPH_2$ ($\epsilon = 7200$ $M^{-1}$ $cm^{-1}$). When these reaction conditions were employed with **1** and PAH at pH 8.4, one observed the expected absorbance change versus time curve for 6-M-(4a-OH)$PH_2$ (data not shown), confirming the coupled nature of the reaction.

The next obvious question was the identity of the product produced from **1** upon exposure to PAH. No increase in absorbance at 275 nm was detected from the reaction of PAH with **1**, therefore suggesting that tyrosine was not produced. The PAH reaction mixture was analyzed for new products by TLC (see Experimental Procedures). The chromatography solutes were made visible with ninhydrin and gave very characteristic colors which could be used to confirm the identification of the reaction products (Table I). The orange ninhydrin color obtained with **1**, although unusual, was consistent with previous observations in the literature (Snow et al., 1968). When a large-scale reaction (see Experimental Procedures) of **1** and PAH was carried out, followed by TLC product analysis, two major spots were observed. One gave the same $R_f$ and ninhydrin color as **1**, while the other ($R_f = 0.51 \pm 0.01$; ninhydrin color, brown) did not comigrate with either **1** or tyrosine, in contrast to the control with phenylalanine where the sole product was tyrosine. For this experiment, PAH was activated with compound **1**, in order to avoid the production of tyrosine obtained from phenylalanine-activated PAH. Treatment of **1** with the epoxidizing agent, MCPBA, gave a product spot identical with that obtained with PAH.

In order to elucidate the structure of this new product, the reaction solution of PAH and **1** was treated with reduced

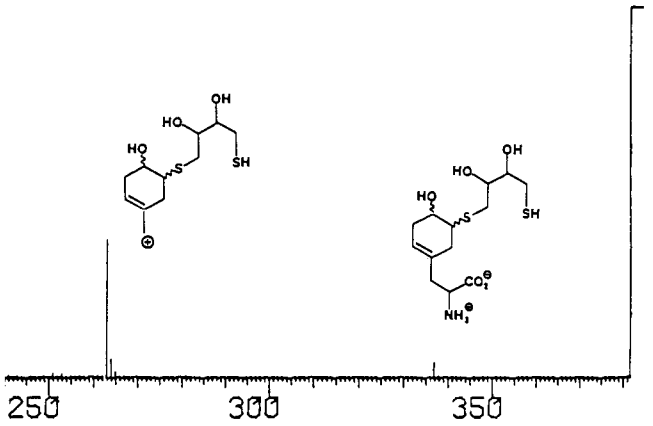


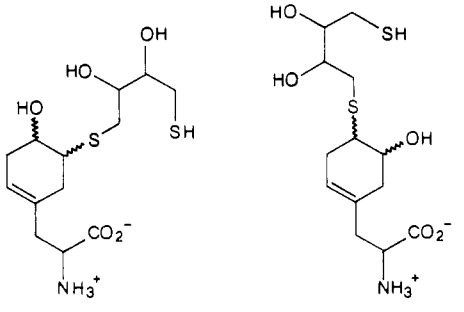

FIGURE 2: Direct-injection probe/chemical ionization mass spectrum from $m/e$ 250–370 of PAH-oxidized **1**.

glutathione and glutathione *S*-transferase, an enzyme that catalyzes the nonspecific attack of reduced glutathione on epoxides (Jerina, 1976) (eq 1). TLC analysis of this reaction


(1)

solution (see Experimental Procedures) yielded, in addition to phenylalanine resulting from the oxidation of **1**, three major spots consistent with **1**, oxidized glutathione (see Table I), and a new product ($R_f$ 0.06). When the hydroxylation of phenylalanine by PAH was done in the presence of reduced glutathione and glutathione *S*-transferase, no detectable glutathione adduct could be observed by TLC analysis. The only spots were those of the expected product, tyrosine; residual substrate; and reduced glutathione. When MCPBA-treated **1** was exposed to reduced glutathione and glutathione *S*-transferase, the initial product ($R_f$ 0.51) disappeared over time with concomitant replacement by a new product with ($R_f$ 0.04). These results collectively imply that the initial product produced in both the enzymatic and chemical oxidation is an epoxide, which can be converted enzymatically to a glutathione conjugate.

Attempts to intercept this epoxide with a thiol reagent, such as DTT, failed to yield an adduct at ambient temperature, a result which is in accord with data in the literature (Jerina, 1976). In order to facilitate the reaction of DTT with the putative epoxide, the reaction solution of compound **1** with PAH was heated to 50 °C with DTT. DIP/MS of this reaction solution, with selective ion monitoring ($m/e$ 200–380), gave the spectrum shown in Figure 2. Three higher molecular weight components at 205, 263, and 337 were detected in this sample. The ion peak at $m/e$ 337 is consistent with an adduct of **1**–DTT–atomic oxygen. Two possible structures that would be in accord with this ion peak are compounds **2** and **3**. Both



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could arise by first a PAH-induced epoxidation of **1** followed

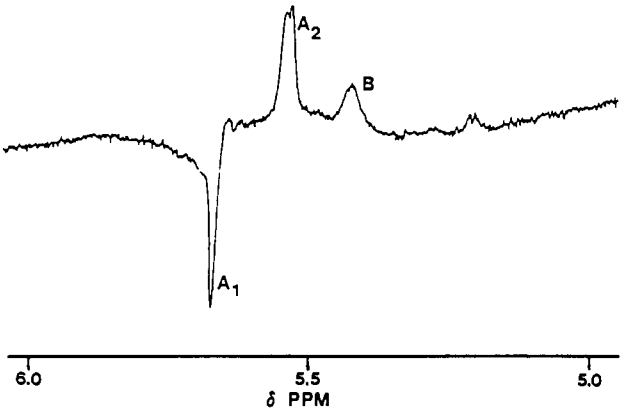
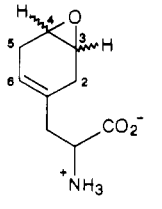



FIGURE 3: Inversion–recovery $^1$H NMR spectrum for the olefinic region of PAH-oxidized **1**.

by sulfhydryl opening of this epoxide to give the putative DTT adduct. Epoxide opening would occur, presumably, in a nonregioselective manner, so that both **2** and **3** would be formed. The ion peak at $m/e$ 263 is 74 mass units smaller than 337 and corresponds to a loss of glycine from either **2** or **3**. This elimination of glycine could occur, from structures such as **2** and **3**, by a McLafferty-like rearrangement (Silverstein et al., 1981). The presence, then, of this McLafferty product indicates that the compound responsible for the ion peak at 337 contains an amino acid moiety. The detection of **2** and **3** supports the formation of an epoxide from the reaction of **1** and PAH, although the molecular ion of this epoxide ($m/e$ 183) could not be detected conclusively because of the overlap with the $M^+ + C_3H_7$ ion peak for DTT ($m/e$ 181).

Comparison of the $^1$H NMR spectroscopic analysis, in deuterium oxide of the product sample from the reaction of PAH with **1** with that of authentic **1**, indicated resonances for a new compound at $\delta$ = 5.43 ppm (broad singlet) and $\delta$ = 3.51 ppm (multiplet). Treatment of **1** with MCPBA, followed by NMR analysis (see Experimental Procedures), at various time periods resulted in a gradual appearance of the resonance at $\delta$ = 5.43 ppm. The location of this NMR resonance is consistent with an olefinic proton, presumably at ring position 6 in **1**. The spectrum also showed three sets of multiplets at $\delta$ = 3.57, 3.52, and 3.34 ppm, with one resonance appearing in the same region as the putative epoxide shift ($\delta$ = 3.52 ppm). The appearance of more than one epoxide methine in the MCPBA-treated **1** is due, presumably, to polyepoxidation of **1** to yield several oxidized products. From these data, one can tentatively assign the NMR resonances for the putative epoxide, **4**, as the following: $\delta$ = 5.43 ppm (H-6 in **4**), 3.52 ppm (H-3 and -4 in **4**).

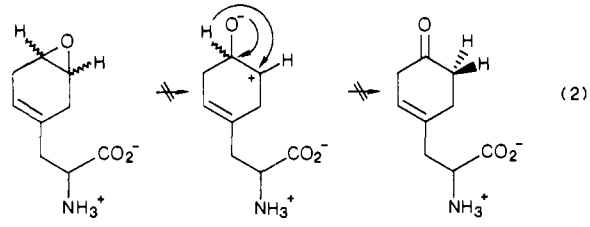


4

Figure 3 shows the $\delta$ = 6.1–4.6 ppm region of an $^1$H NMR inversion–recovery spectrum from a sample of PAH-oxidized **1**. From this figure, there is a sign change in the peaks for residual **1** at $\delta$ = 5.63 (H-3 and -4 in **1**) and 5.53 ppm (H-6 in **1**) (labeled $A_1$ and $A_2$, respectively), which is due to a large difference in the relaxation rates ($\tau$) for the respective protons. The putative olefin proton in the product epoxide $\delta$ = 5.43 ppm

has the same peak sign as the olefin proton in **1** ($A_2$) and suggests that they both possess similar $\tau$ values. A COSY experiment (Aue et al., 1976; Nagayama et al., 1980) on this sample revealed that the resonances at $\delta$ = 5.53 ppm (H-6 in **1**) and 5.43 ppm in **4** are coupled to the same methylene. This result, then, strongly supports the assignment of $\delta$ = 5.43 ppm as the olefin proton (H-6) in **4**.

These product analyses clearly support the formation of **4** from the reaction of **1** with PAH. The detection and trapping (**2** and/or **3**) of **4** from the reaction of PAH with **1** suggest that the epoxide, once formed, is relatively stable and dissociates from the enzyme. The failure to observe enzyme-induced rearrangement (eq 2) may be due to the attenuated




(2)

stability of the secondary carbocation resulting from the ring opening of **4**. Therefore, one cannot rule out a role for PAH in catalyzing a rearrangement of the arene oxide from phenylalanine. This is especially true in light of the inability to detect any glutathione adduct from the reaction of phenylalanine with PAH in the presence of reduced glutathione and glutathione *S*-transferase. On the other hand the lack of detectable inhibition of PAH arising from **4** suggests a low number of active site nucleophilic residues in the epoxide region.

Compound **4** is enantiomeric (i.e., 3*R*,4*S*, or 3*S*,4*R*) with respect to the epoxide ring system; however, this chirality cannot be determined from the data presented here. Recent work on the use of chiral lanthanide shift reagents in the determination of epoxide enantiomers (Yeh et al., 1986) could allow one to establish any stereospecificity in the epoxidation of **1** by PAH. This use of shift reagents can only be accomplished in nonaqueous NMR solvents, therefore necessitating the careful derivatization of **4** in order to render it soluble in organic solvents. By analogy, the PAH-catalyzed formation of **4** from compound **1** is consistent with the mechanism in Scheme II in which an arene oxide is formed in the hydroxylation of the normal substrate.

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