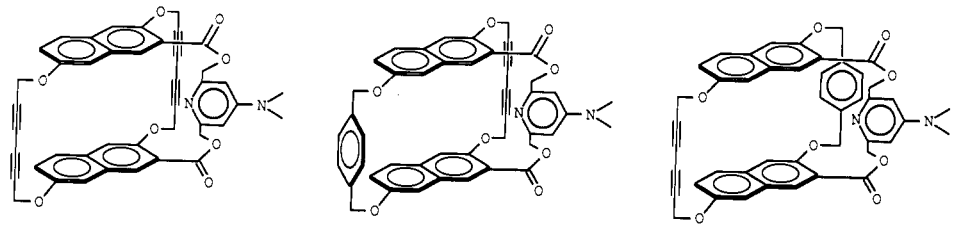

Figure 1.

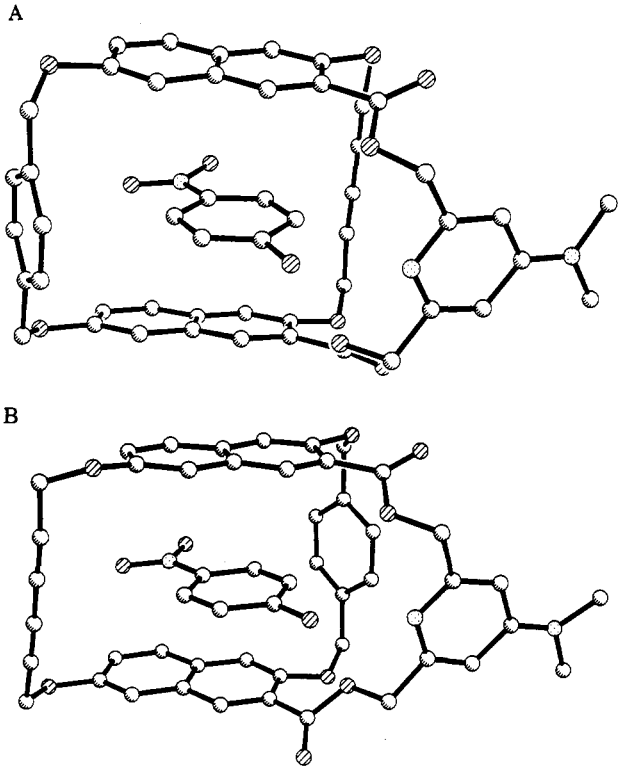

Figure 2. X-ray structure of pyridine–distal *p*-nitrophenol complex **1-D** (A) and structure of pyridine–proximal isomer complex **1-P** (B). Hydrogen atoms are not shown for clarity.

(3) Virtually identical behavior is exhibited by the **1-P** host: at −85 °C the *o*-nitro proton lying over the *p*-xylene spacer appears at δ 3.68, an upfield shift of 4.5 ppm.[4b]

(4) Use of *p*-nitrophenol-*2,6-d*$_2$ shows that it is the aromatic proton ortho to the nitro group of *p*-nitrophenol that is responsible for the high field peaks in the **1-P** complex. A chemical shift change of this magnitude in complexes of **1-D** and **1-P** implies that the *o*-nitro proton is uniquely positioned atop the xylene spacer in both cases.

Observations 1–4 by themselves do not require edge–face stabilization, but the following do.

(5) Single-crystal X-ray structures of these complexes are shown in Figure 1. Examination of them shows the PNP aromatic proton[9] closest to the xylene spacer to be the one ortho to the nitro substituent. It lies 0.1 Å off-center from the mean plane of the xylene in both cases at a distance of 2.72 Å (**1-D**) and 2.56 Å (**1-P**) from the xylene's plane. The corresponding distances from the xylene center to the guests' C3 carbons are 3.51 and 3.44 Å, respectively.

(6) The host naphthalene rings are not parallel: angles of 16° for **1-D** and 4° for **1-P** are found.[8] Nevertheless the guest–xylene angle is 90° within experimental accuracy of the X-ray structure refinement.

Of the series of cyclophane-based hosts possessing concave functionality studied by us, hosts **1-P** and **1-D** enjoy a uniquely high affinity for phenol guests. Both possess a briding *p*-xylene spacer, which is approximately 0.9 Å shorter than a 2,4-hexadiyne unit. This encourages (but does not require) a snugger fit and hence greater degree of immobilization of the phenolic guest. Further, introduction of a *p*-xylene spacer also permits interaction between the edge of the aromatic guest and the face of the spacer. We suggest that the remarkable positioning of the guest in both of the above complexes arises because the detailed geometry of these complexes is dominated by formation of a π hydrogen bond involving the electron-rich *p*-xylene spacer and the acidic *o*-nitro proton of the guest.[10]

Supplementary Material Available: Complete X-ray refinement details and labeled ORTEP drawings of *p*-nitrophenol complexes of **1-P** and **1-D** (24 pages); listings of observed and calculated structure factors (43 pages). Ordering information is given on any current masthead page.

(10) Partial support by the N.S.F and the Department of Navy is acknowledged. We also thank Douglas R. Powell and John M. Desper for the data acquisition and structural refinement of the X-ray structures of **1-P** and **1-D**, respectively.

Mechanism-Based Inactivation of Peptidylglycine α-Hydroxylating Monooxygenase (PHM) by a Substrate Analogue, D-Phenylalanyl-L-Phenylalanyl-D-Vinylglycine: Inhibition of Formation of Peptide C-Terminal Amides

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The biosynthesis of many peptide hormones proceeds by post-translational cleavage of precursor proteins to generate a primary amide functionality at the carboxyl terminus.[1] The amidation results from two-step processing of precursors bearing a C-terminal glycine residue (e.g., **1**, Scheme I).[2] A bifunctional enzyme, peptidylglycine α-amidating monooxygenase (PAM),[2d-f] can catalyze the full transformation or it may be cleaved into two monofunctional proteins. The first, peptidylglycine α-hydroxylating monooxygenase (PHM), catalyzes a stereospecific hydroxylation in a process dependent on oxygen, copper, and ascorbate. The second enzyme, peptidyl-α-hydroxyglycine α-amidating lyase (PAL), then promotes the decomposition of the carbinol amide to a peptide amide and glyoxylate. This final step

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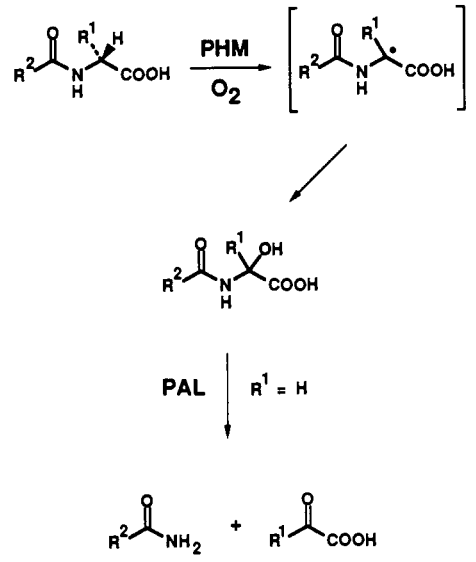
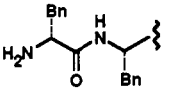
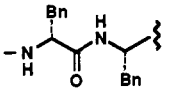
Scheme I

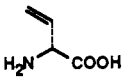


1 R^1 = H R^2 = X = 

2 R^1 = $HC{=}CH_2$ R^2 = X

3 R^1 =  R^2 = X

5 R^1 = $HC{=}CH_2$ R^2 = DANSYL– 

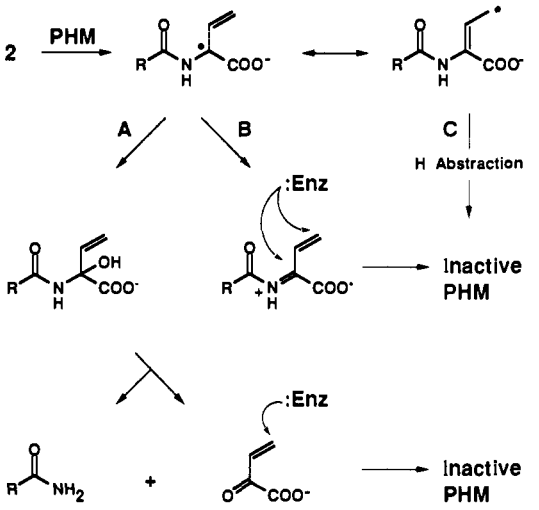


4

occurs spontaneously under alkaline conditions, but at physiological pH requires the action of the enzyme and is stereospecific.[3] It appears probable that a family of closely related PAM, PHM, and PAL proteins with broad, but varying, substrate specificity occur in all animals and generate a host of amidated hormones.[4] Hence, selective inhibitors of particular PHMs would be useful tools in endocrinology and may have medicinal applications. PHM shares cofactor and kinetic similarities to dopamine β-hydroxylase (DBH)[5] and is irreversibly inhibited by *trans*-4-phenyl-3-butenoic acid,[6] a mechanism-based DBH inhibitor. In the present report, we describe the mechanism-based inactivation of PHM by a substrate analogue, D-phenylalanyl-L-phenylalanyl-D-vinylglycine (**2**), and interaction of PHM with D-phenylalanyl-L-phenylalanyl-D-cyclopropylglycine (**3**).

The design of these potential inhibitors is based on the stereospecific abstraction of the glycine *pro-S* hydrogen during the

Scheme II



oxidative process,[7] the ability of the enzyme to convert peptides terminating in D-alanine instead of glycine,[8] and the possibility that a transient radical may form at the glycine α-carbon during direct hydroxylation[9] in analogy to DBH oxidations.[5,10] It seemed that such radical character could lead to abortive reactions if it could be stabilized or relocated at another site. Alternatively, the hydroxylation of the α-carbon could generate a reactive intermediate after further transformation. The key amino acid in **2**, D-vinylglycine (**4**), is available in four steps from *N*-Cbz-D-glutamic acid by modification of Hanessian's procedure for generation of L-vinylglycine[11-13] and is readily transformed to the tripeptide by solution-phase methods (supplementary material).[14] Since the ring-opening reaction of cyclopropyl-substituted radicals provides another means of relocating radical character and a clock for estimating lifetimes of radicals in biochemical processes,[15] the cyclopropyl-bearing tripeptide **3** was also an attractive target. Palladium-catalyzed addition of diazomethane[16] to benzyl *N*-Cbz-D-vinylglycinate, an intermediate in the synthesis of **4**, produces a D-cyclopropylglycine derivative which is readily elaborated to tripeptide **3**.

Interaction of cyclopropyl peptide **3** with PHM from porcine pituitary showed that it acts as a poor competitive inhibitor (K_I > 5 mM). Apparently, substitution of the glycine *pro-R* hydrogen with a group which is much more sterically demanding than methyl (as in a D-alanine terminus) cannot be tolerated. However, preliminary tests with **2** and PHM suggested a time-dependent inhibition process; subsequent preincubation studies confirmed

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(14) In some preparations, small amounts of peptide containing an L-vinylglycine moiety were obtained due to isomerization either during the preparation of D-vinylglycine[11b] or during subsequent deprotection. These are easily separated by HPLC. The D-Phe-L-Phe-L-vinylglycine was prepared using commercially available L-vinylglycine (Sigma) and found to be a weak, competitive inhibitor of PHM (50% inhibition at 0.35 mM).

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this and established an apparent K_I of 20 μM.[17] Incubation of the enzyme with 250 μM **2** for 45 min, followed by removal of the inhibitor, gives no recovery of activity.[18] Furthermore, treatment with 4 M urea and inhibitor removal also fails to restore activity,[18] thereby demonstrating that the inactivation is irreversible and probably not due to a slow-binding inhibitor with a high dissociation constant. Preincubation of PHM with substrate (D-Phe-L-Phe-Gly) (**1**)) and **2** shows protection of the enzyme in proportion to the amount of added substrate, which indicates that **2** acts at the active site of the enzyme. Treatment of PHM with **2** in the absence of Cu^{II} and ascorbate for 1 h followed by removal of the inhibitor results in no significant loss of activity. The requirement that all cofactors be present for inactivation to occur illustrates that **2** is a mechanism-based inhibitor.

Possible mechanisms of action for **2** are depicted in Scheme II. Abstraction of the α-hydrogen from the D-vinylglycine residue produces a conjugated radical at the α-carbon. Direct hydroxylation (path A) would generate a vinylglycine carbinol amide that can cleave to give the peptide amide and the α-keto acid, a potential alkylating species. Oxidative electron transfer (path B) could form a very reactive *N*-acyl iminium species that could result in the covalent attachment of the inhibitor to the enzyme. Path C involves interaction of the resonance-stabilized radical at the distal primary carbon with amino acid residues in the enzyme active site (e.g., hydrogen abstraction). To gain further insight, the inhibitor was resynthesized with a fluorescent 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl) group attached to the amino terminus. Compound **5** also irreversibly inhibits PHM. A large sample of the enzyme was inactivated with this fluorescent tripeptide, a small amount of active PHM was then added to aid in isolation, and the inactivated enzyme was separated from **5** by gel filtration chromatography. Fractions containing amidating activity were pooled and concentrated as were those which had a UV absorption at 250 nm (λ_{max} of **5**). Spectrofluorimetric analysis of these samples showed no fluorescence associated with the PHM fractions above the minimum level of detection (ca. 5 pmol). A single late-eluting fraction which did fluoresce was further analyzed by HPLC and found to contain the tripeptide **5**.

These results indicate that the substrate analogues **2** and **5** inactivate PHM by a process that results in cleavage of the terminal portion of the peptide substrate (e.g., path A, Scheme II).[19] Current work on the detailed mechanism of this inactivation, along with studies on a variety of other inhibitors of PHM, will be described later.

Acknowledgment. We wish to thank Mr. Darrell Belke for assistance in acquiring the scanned fluorescence spectra and Professor Larry Wang for use of the SLM/Aminco 8000C spectrofluorimeter. Stimulating discussions with Dr. Arlindo Castelhano (Syntex Canada) are gratefully acknowledged. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada, the Alberta Heritage Foundation for Medical Research, and postdoctoral fellowships from NATO (U. S. National Science Foundation) and the Killam Foundation to T.M.Z.

Supplementary Material Available: Schemes for the syntheses of compounds **2-5** (4 pages). Ordering information is given on any current masthead page.

(17) The enzyme was isolated as described previously.[7a] Preincubation mixtures (250 μL) contained 50 μL of enzyme and varying amounts of **2** in a cocktail of 1 mM ascorbate, 5 mM $CuSO_4$, 25 mM KI, 0.125 mg/mL catalase, and 1 mg/mL BSA in 50 mM Na_2HPO_4/NaH_2PO_4 and 200 mM NaCl, pH 6.8 buffer. Aliquots (25 μL) of the preincubated mixtures were diluted into 475-μL solutions containing 1.26 μM D-Phe-L-Phe-[1,2-^{14}C]Gly (specific activity 113 μCi/μmol) and 83 μM unlabeled **1** (10 × K_M) in the same cocktail and incubated for 1 h. The percent of substrate converted was determined as described previously.[9]

(18) The inhibitor was removed by three dilution/concentration steps using an Amicon (Beverly, MA) Centricon-30 ultrafiltration device. Uninhibited PHM remains active during this procedure.

(19) Path B is also possible if cleavage of the *N*-acyl imine bond occurs.

A Novel FeC_{60} Adduct in the Solid State

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The 7-Å diameter cavity in buckminsterfullerene has long been considered as a possible host for various atoms and molecules.[1] There have been several attempts to incorporate metal atoms such as La into this cavity, and these studies[2-6] have mainly employed mass spectrometry for characterization. The question of whether the metal atom is inside or outside the spheroidal hollow cage in these species has not been fully resolved. Freiser and co-workers[4] have generated an FeC_{60} complex in the gas phase by means of a ligand-exchange reaction and have also characterized NiC_{60} and $Ni(C_{60})_2$ in the gas phase. One example where an atom has been entrapped in C_{60} is that where a high-energy beam of C_{60} is made to interact with helium to give HeC_{60} in the gas phase.[5] Chai et al.[6] have recently prepared fullerenes with La trapped in the cages by laser vaporization. Metal complexes of C_{60} where the metal is present outside the cavity have been prepared by solution methods and characterized.[7,8] We investigated an iron adduct of C_{60}, FeC_{60} (I), where iron atoms were introduced during the contact-arc vaporization of graphite, expecting that this would result in the entrapment of Fe in the cavity. In order to understand some of the unique features of this adduct, we have compared its properties with those of solid FeC_{60} (II), where Fe is clearly bound externally to the fullerene.

In our effort to obtain the Fe adduct of C_{60} with the metal inside the cage, we carried out the contact-arc vaporization of graphite in an atmosphere of $Fe(CO)_5$. The arc chamber was pumped to a base vacuum of $\sim 10^{-6}$ Torr, and He gas was admitted to about 50 Torr through a needle valve. $Fe(CO)_5$ was admitted to the system from a reservoir at the same rate as He. The soot[9] was washed with diethyl ether, the residue was Soxhlet-extracted with toluene for about 3 h, and the extract was vacuum-dried at 380 K to remove any volatile iron compounds. The mass spectrum of the product of solvent extraction[10] showed the presence of mass peaks other than those due to C_{60} and C_{70}, which could be ascribed to FeC_{60}. The sample showed an additional spot in the TLC other than those due to C_{60} and C_{70} with an R_f value close to that of C_{70}. After C_{60} was separated out by column chromatography, the new product was eluted along with a small proportion of C_{70}.

The mass spectrum of the new product (in mixture with C_{70}) obtained after column chromatography (Figure 1) showed peaks at 776, 752, and 728, ascribable to FeC_{60}, FeC_{58}, and FeC_{56}, besides peaks at 720 and 840;[11] a peak at 388 was seen due to

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(9) The ^{57}Fe Mössbauer spectrum of the soot showed 6-finger patterns attributable to magnetic Fe_3C and α-Fe along with a singlet due to a new species.
(10) The mass spectrum of the residue left over after the solvent extraction did not show any volatile species up to 400 °C.
(11) An intense peak at m/z 720 is found in the mass spectrum of pure C_{70} as well.

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