ORGANIC LETTERS

2007
Vol. 9, No. 7
1195−1198

Model Studies for Heme Oxygenase-Catalyzed Porphyrin Meso Hydroxylation

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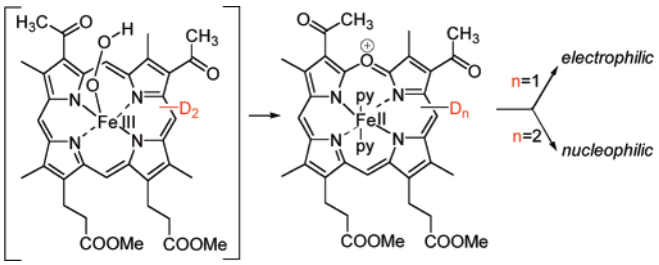
Received December 20, 2006

ABSTRACT



Nonenzymatic model studies based on a porphyrin analogue (2,4-diacetyldeuteroporphyrin) that avoid the steric effect complications of the heme oxygenase active site were carried out to determine the polarity of the ferric hydroperoxide attacking species. Mass spectral and deuterium-labeling experiments indicate that the porphyrin meso positions that are at higher π-electron densities in ferric 2,4-diacetyldeuteroporphyrin are selectively attacked. This supports an electrophilic aromatic substitution mechanism for the heme oxygenase-catalyzed porphyrin meso hydroxylation.

The physiological oxidation of heme (**1**) to biliverdin (**3**) is catalyzed by heme oxygenase in a reaction that consumes O_2 and reducing equivalents provided by NADPH-cytochrome P450 reductase (Scheme 1).[1] The first step of this catalytic sequence involves regiospecific hydroxylation of the porphyrin α-*meso*-carbon, which leads to the well-established heme degradation intermediate, α-*meso*-hydroxyheme (**2**).[2] The regiospecificity of this meso-hydroxylation step accounts for the regiospecificity of the ultimate product biliverdin IXα (**3**).[3] The meso-hydroxylation reaction proceeds via ferric hydroperoxyheme (**4**),[4] which reacts with the aromatic porphyrin *meso*-carbon, leading to substitution of the hydrogen atom by the hydroxyl group (Scheme 2, top). Regardless of whether it is a stepwise or a concerted mechanism, the ferric hydroperoxyl group could undergo either a homolytic or a heterolytic cleavage, generating one of three different species, OH^+, OH^-, or $OH^\bullet$, which reacts with the porphyrin *meso*-carbon.

Porphyrin is a macrocyclic aromatic molecule known to be versatile in accommodating different attacking species at its meso positions.[5] In the porphyrin cavity, the iron-bound peroxide is also capable of catalyzing very different reactions.[6] The electronic properties of the reaction between the iron-bound peroxide and the porphyrin meso positions, leading to porphyrin meso hydroxylation, are unknown. This

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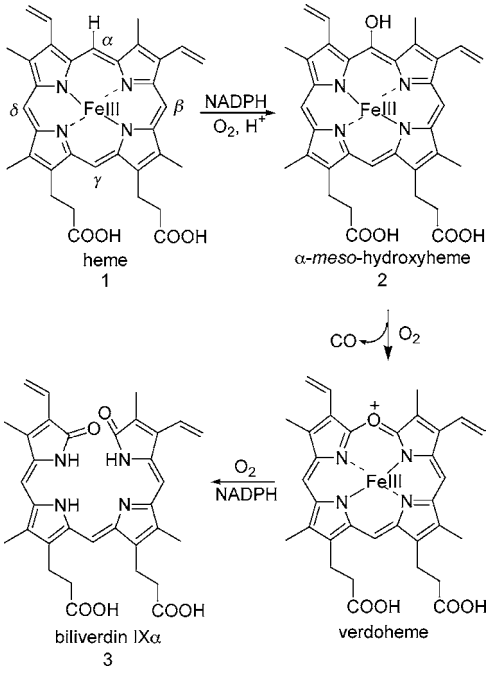
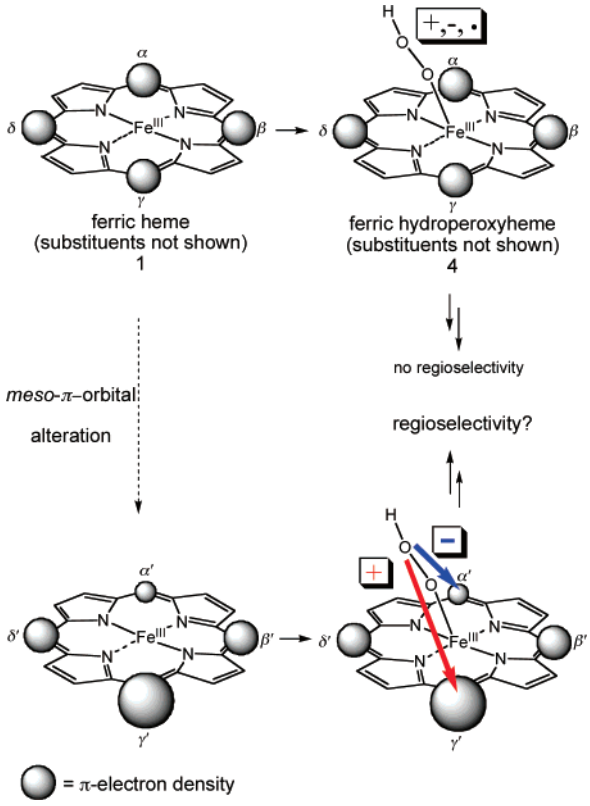
10.1021/ol063088f CCC: $37.00
Published on Web 03/08/2007

Scheme 1. Heme Oxygenase-Catalyzed Heme Degradation



Scheme 2. Hypothetical Example of Reactivities of π-Electrons at Porphyrin Meso Positions[a]



[a] The π-electron densities are arbitrarily assigned to show an expected outcome.

reaction takes place in the active site of heme oxygenase, where, as crystallographic studies show, steric hindrance at the β-, γ-, and δ-meso positions of the natural substrate, ferric protoporphyrin IX (heme, **1**), leads to α-regiospecificity.[7] This biased regioselectivity indicates that the enzymatic reaction is not a good model to study electronic properties of the heme meso-hydroxylation reaction.

A nonenzymatic model of the heme oxygenase reaction that involves a coupled oxidation with no steric hindrance has demonstrated that the four meso positions are nonselectively cleaved to generate four biliverdin isomers.[8] Much effort has been expended to elucidate the electronic properties of the ferric hydroperoxyl attacking species involved in the oxidation. On the basis of enzymatic reactions of meso-substituted mesohemes, Ortiz de Montellano and co-workers concluded that an electrophilic mechanism was important.[4] Crusats and co-workers carried out a nonenzymatic coupled oxidation reaction of a heme analogue, 3-demethyl-3-(trifluoromethyl)mesoheme IX (CF_3-heme).[9] The regioselectivity of the biliverdin products obtained from the nonenzymatic oxidation of CF_3-heme was supported by porphyrin meso atomic charge data obtained from theoretical calculations on an analogue of CF_3-heme, which also led to the conclusion of an electrophilic mechanism for heme oxygenase. This nonenzymatic coupled oxidation model is superior to the enzymatic reaction for the study of electronic properties of the heme hydroxylation reaction without steric complications of the enzyme active site. However, the mechanistic conclusions were based on data obtained from theoretical calculations of the electronic effects of each of the meso positions, which were not validated by experiment. Here we describe experiments to determine the relative electronic effects of the meso positions of a more electron-deficient and much more regioselective heme model than that described above.

Recently, we reported that the replacement of the vinyl substituents of protoporphyrin IX with acetyl substituents not only leads to lower meso position π-electron densities but also results in pronounced meso position selectivities (the β- and γ-meso positions are much more nucleophilic (basic) than the α- and δ-meso positions).[10] In light of this study, we carried out the coupled oxidation reaction of ferric 2,4-diacetyldeuteroporphyrin (**6**) to provide experimental support regarding the controversy about the heme oxygenase-

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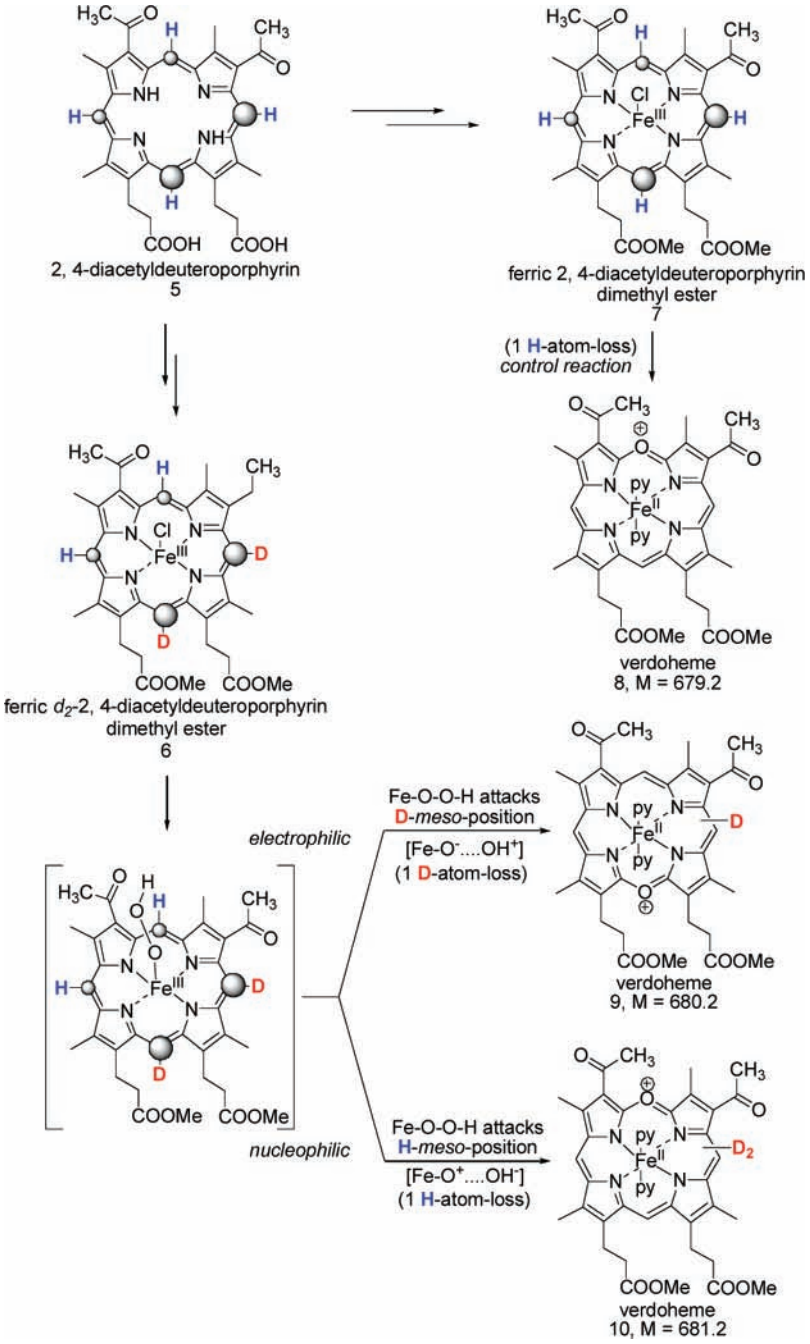
Scheme 3. Model Studies of Porphyrin Meso Hydroxylation: Electrophilic Mechanism or Nucleophilic Mechanism?[a]



catalyzed porphyrin meso-hydroxylation mechanism, whether it is a radical mechanism, an electrophilic mechanism, or a nucleophilic mechanism.

Our initial plan was to identify the regioselectivity of porphyrin meso hydroxylation from the ratio of the biliverdin products; the acetyl substituents of the biliverdin products after the ferric 2,4-diacetyldeuteroporphyrin reaction could be converted to vinyl groups[11] to set up a convenient HPLC- or TLC-based regioselectivity identification using the biliverdin reference compounds from the nonselective ferric protoporphyrin (heme) reaction.[12] However, following meso hydroxylation of ferric 2,4-diacetyldeuteroporphyrin, the verdoheme products could not be hydrolyzed to biliverdins. Although the verdohemes were very stable in aqueous pyridine solution, under many different hydrolytic conditions,[8,13] decomposition of verdohemechrome was observed, which may have been induced by the effects of the acetyl substituents. To circumvent this problem, we designed a mass spectral approach based on deuterium-labeled 2,4-diacetyldeuteroporphyrin (**5**, Scheme 3).

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As reported recently,[10] the β- and γ-meso positions of 2,4-diacetyldeuteroporphyrin (**5**) can be fully deuterated by concentrated D_2SO_4 (87 wt %). The α- and δ-meso positions are inert to these deuteration conditions because of their low π-electron densities (as a result of the strong electron-withdrawing and polarizing effects of the two acetyl groups). Thus, in this diacetyl porphyrin analogue, the two nucleophilic meso positions (β and γ) became labeled with deuterium atoms, and the two electrophilic meso positions (α and δ) retained their hydrogen atoms. Iron was inserted into the central cavity, and the modified ferric heme (**6**) was kept in its dimethyl ester form for the nonenzymatic model study (the methyl ester form of heme is known to generate the same results in this reaction as the free acid form[14]).

The coupled oxidation reaction of heme in pyridine−H_2O solution with oxygen and sodium ascorbate as the reducing agent is known to be a good nonenzymatic model for the heme oxygenase reaction.[8,9] In this model reaction of modified ferric heme (**6**), the electronic properties of the four meso positions will determine the regioselectivity (Scheme 3). There are three possible outcomes: (1) If the ferric hydroperoxide selectively attacks either of the deuterium-labeled meso positions (β- and γ-positions), which are at a higher π-electron density, then it would support a [Fe^{III}−$O^{-}\cdots OH^{+}$] hydroxylation species, indicating an electrophilic mechanism, and one of the deuterium atoms would be removed in the heme−verdoheme conversion. (2) If the ferric hydroperoxide selectively attacks either of the hydrogen-labeled meso positions (α- or δ-positions), which are at a lower π-electron density, then it would support a [Fe^{III}−$O^{+}\cdots OH^{-}$] hydroxylation species, indicating a nucleophilic mechanism, and no loss of deuterium would occur during the heme−verdoheme conversion. (3) If the ferric hydroperoxide shows no selectivity, then a radical mechanism would be favored. Two reasonable assumptions were made in the establishment of this methodology: (1) substitutions of the vinyl groups by acetyl groups and of hydrogen by deuterium do not cause significant steric changes in the coupled oxidation reaction, and (2) the slight energy difference between a C−D bond and a C−H bond does affect the hydroxylation reaction significantly.

Conversion of the modified ferric heme (**6**) to verdoheme can be easily monitored by mass spectrometry.[14] The nondeuterated modified ferric heme (**7**) was first tested to provide a control reaction. The *m*/*z* value for verdoheme is 679.6 (**8**). After reaction of d_2-heme **6**, the *m*/*z* value of the verdoheme product (**9**) was found to be 680.7, which indicates that one deuterium was lost from **6** in this step, suggesting that the porphyrin meso positions that have higher π-electron densities (containing a deuterium atom) are selectively attacked by the ferric hydroperoxide. This steric-effect-free nonenzymatic model, therefore, supports an electrophilic aromatic substitution mechanism, attack of the porphyrin π-electrons on the ferric hydroperoxide, a mechanism previously proposed by Ortiz de Montellano and coworkers[4] and Crusats et al.[9]

The model study reported here experimentally tests the heme oxygenase-catalyzed porphyrin meso-hydroxylation mechanism. The regioselectivity observed in the coupled oxidation reaction of ferric 2,4-diacetyldeuteroporphyrin is attributed to the strong electron-polarizing resonance effects of the acetyl groups on the porphyrin meso positions (unlike in many other iron-containing porphyrins, including CF_3-heme,[9] the small regioselectivity differences observed among the four meso positions result from induction effects). By introduction of a pronounced meso π-electron density difference, 2,4-diacetyldeuteroporphyrin acts as both a model compound for theoretical calculations of porphyrin aromaticity and a probe compound for enzyme mechanism studies.

Acknowledgment. The authors are grateful to the National Institutes of Health (GM 49725) for financial support of this research. We thank Graham R. Lawton (Department of Chemistry, Northwestern University) for suggesting the use of the deuterated modified heme for direct determination of the site of attack.

Supporting Information Available: Experimental details about preparations of **6** and **7** and mass spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

OL063088F

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