

# ENZYMATIC HALOGENATION IN NATURAL PRODUCT BIOSYNTHESIS

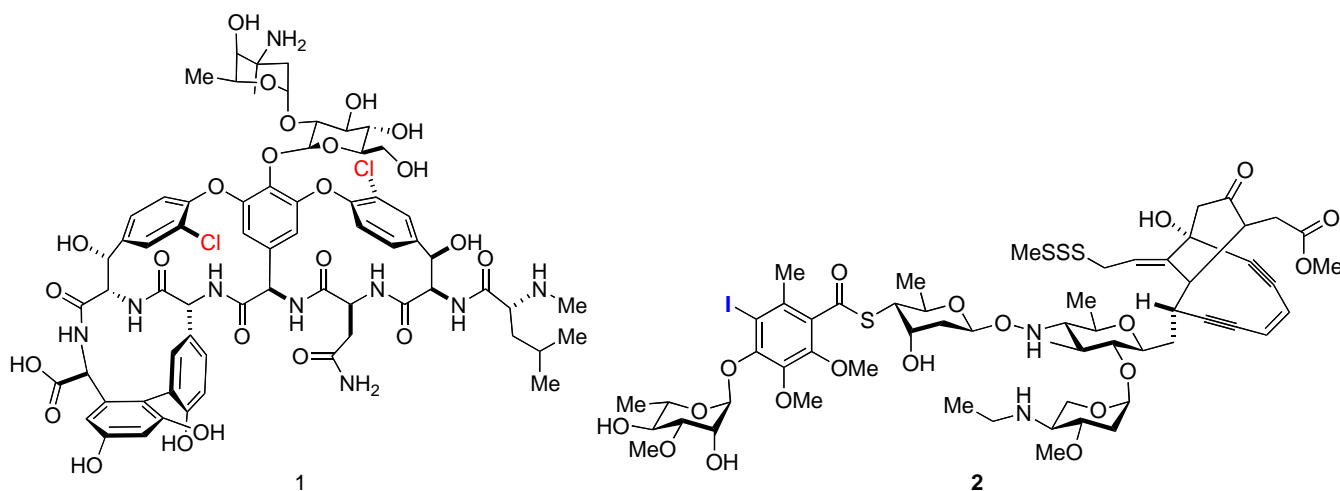
Reported by John T. Witteck

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## INTRODUCTION

Halogenation reactions are an integral part of organic chemistry. One of the first reactions learned by an organic chemistry student is the  $S_N1$  displacement of an alcohol with a hydrohalic acid to form an alkyl halide. The student also learns that halides are good leaving groups for both elimination and substitution reactions.<sup>1</sup> The theme of halides as nucleophiles or leaving groups (nucleofuges) pervades even complex molecule total synthesis. Nature, as well, has integrated halides into many natural products.

To date, over 4500 halogenated natural products have been discovered<sup>2</sup> representing a broad spectrum of biological activity. Well known examples such as vancomycin (**1**) and calicheamycin (**2**) are used clinically to treat bacterial infections and myelogenous leukemia, respectively. In many cases, biological activity is thought to arise at least in part from the unique structural and electronic characteristics imparted by the halogen functional group.<sup>3</sup> Unlike synthetic organic chemistry, nature uses few carbon centers as electrophiles and has therefore evolved unique mechanisms for the introduction of halogen atoms in natural product biosynthesis.



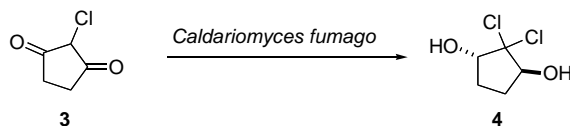
**Figure 1.** Structures of bioactive halogenated natural products. Halogens are highlighted in blue and red.

## HEME DEPENDENT HALOGENATION

Halogenated natural products have been studied for many decades.<sup>3</sup> While studying the biosynthesis of caldariomycin (**4**) by the fungus *Caldariomycin fumago*, an enzyme with structural similarities and homology to horseradish peroxidase was found.<sup>4</sup> This enzyme was shown to depend on hydrogen peroxide and a ferriprotoporphyrin for activity.<sup>4,5</sup> Moreover, partially purified enzyme can

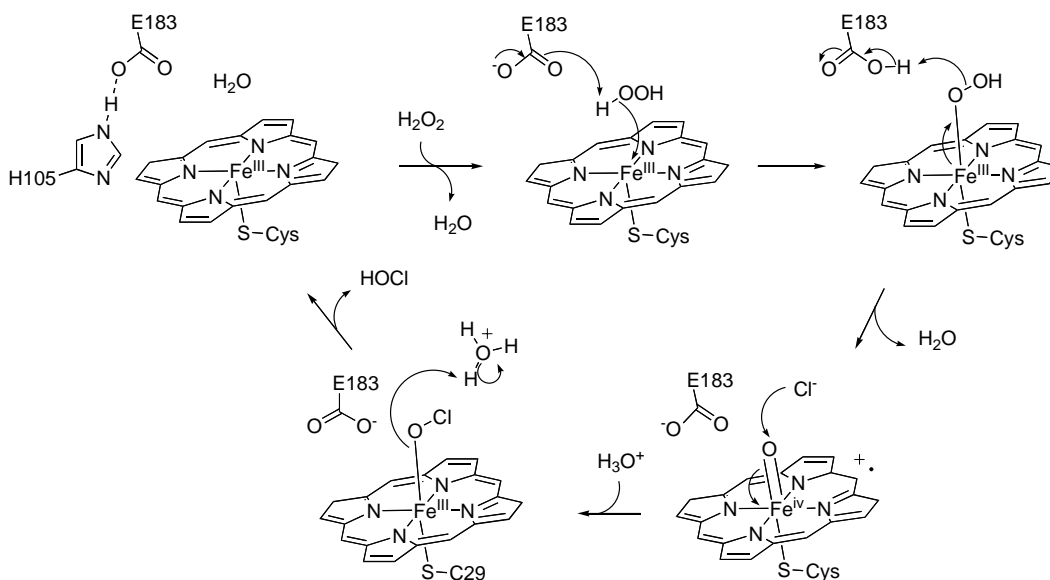
catalyze the chlorination of 2-chlorocyclopentane-1,3-dione (**3**) to 2,2-dichlorocyclopentane-1,3-dione (**4**), a precursor to caldariomycin.<sup>5</sup>

**Scheme 1. Halogenation of Cyclopentane-1,3-dione by *Caldariomyces fumago***



Further mechanistic insights that: (1) the chlorinating species is electrophilic, and (2) formation of this electrophilic species is rate determining were subsequently revealed.<sup>6</sup> On the basis of these observations it was proposed that hydrogen peroxide activates the heme to an iron(IV)-oxo porphyrin radical cation. This radical is attacked by free halide to form of an iron-bound hypohalite. This intermediate is then protonated to release HOCl which is the electrophilic halogenating agent<sup>7</sup> (Scheme 2). This proposal is supported by the observation that Cl<sub>2</sub> and HOCl are released from the enzyme in absence of substrate.<sup>8</sup> In addition, the enzyme functions optimally at low pH, which is conducive to the formation of HOCl. The X-ray crystal structure analysis of the chlorinating enzyme has allowed identification of the specific residues important in catalysis, such a catalytic diad of His105 and Glu183. It also shows the porphyrin is anchored to the active site by Cys29 which is analogous to P450 oxidases rather than the expected His for peroxidases. The generally accepted mechanism for heme dependent halogenating enzymes is shown (Scheme 2).

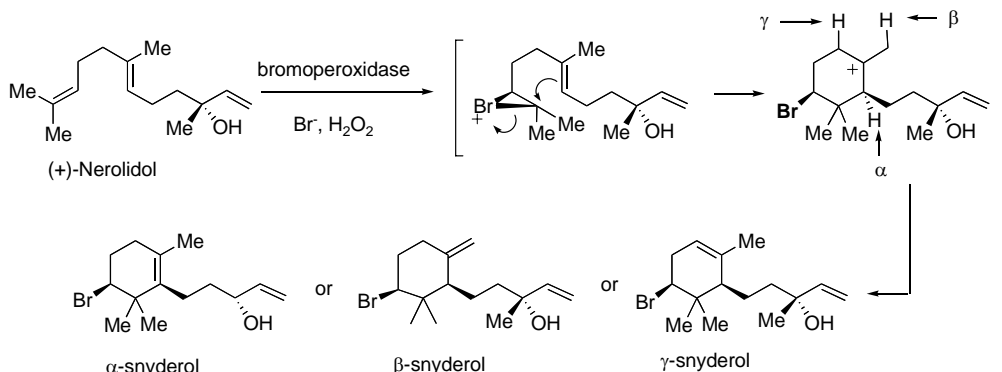
**Scheme 2. Mechanism of Heme-Dependent Halogenation**



## VANADIUM DEPENDENT HALOGENATION

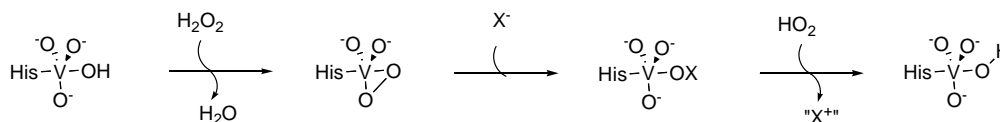
The discovery of heme-dependent halogenating enzymes prompted an extensive search for related enzymes involved in the biosynthesis of other natural products. Interestingly, enzymes from red sea algae responsible for the conversion of nerolidol to  $\alpha$ -, $\beta$ -, or  $\gamma$ -snyderol contain stoichiometric amounts of vanadate instead of the expected heme cofactor.<sup>9</sup> However, like heme-dependent chloroperoxidases the enzyme is also dependent on hydrogen peroxide for activity.<sup>10</sup>

**Scheme 3. Synthesis of  $\alpha$ -, $\beta$ -, or  $\gamma$ -Snyderol from Nerolidol by Vanadium Bromoperoxidase.**



The X-ray crystal structure analysis of the vanadium dependent enzymes shows a trigonal bipyramidal coordination geometry at vanadium with hydrogen bonding from active site residues to the vanadate oxygens and an apically coordinated histidine anchoring it to the active site.<sup>11</sup> An X-ray crystal structure of the activated form of the enzyme shows a distorted trigonal bipyramidal geometry at vanadium with one peroxo atom pseudoapically bound to vanadium and the other occupying an equatorial position.<sup>11</sup> In contrast to the redox role of iron, vanadium acts as a strong Lewis acid activator of H<sub>2</sub>O<sub>2</sub> (Scheme 4).<sup>12</sup> However, as with heme-dependent enzymes HOX is thought to be the halogenating agent. Although, the exact halogenating species has not yet been experimentally confirmed. On the other hand, algae that produce the enzyme use vanadium-dependent halogenation to produce HOX as a defense mechanism.<sup>13</sup>

**Scheme 4: Intermediates on vanadium dependent haloperoxidation.**



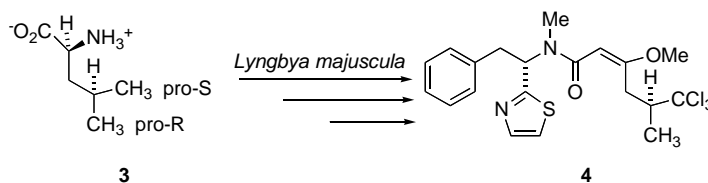
In the case of  $\alpha$ -, $\beta$ -, and  $\gamma$ -snyderol synthesis, the electrophilic halogen source adds to a polyene bound in the active site.<sup>3</sup> This process generates a bromonium ion that is attacked diastereoselectively by a nearby double bond to produce the six membered ring and a tertiary carbocation. Depending on which

hydrogen atom is removed to afford a new double bond, one of the three snyderols can be synthesized from a common intermediate (Scheme 4).

## A NEW CLASS OF HALOGENATING ENZYMES

For years, all biohalogenation was thought to be catalyzed by either of these classes of enzymes, which are grouped as a family and labeled haloperoxidases due to their dependence on hydrogen peroxide.<sup>13</sup> However, feeding experiments in the study of barbamide biosynthesis shows that chlorination of L-leucine (**3**), the precursor to barbamide (**4**) is diastereoselective.<sup>14</sup> The pro-*R* and pro-*S* methyl groups of L-leucine were individually <sup>13</sup>C-labeled and fed in parallel experiments to the producing organism (Scheme 5). Carbon-13 NMR analysis of isolated barbamide showed that only the pro-*S* carbon was chlorinated. Because enzymatic halogenation by hypohalous acid was historically thought to be non-specific, this result implies the existence of another type of halogenating enzyme. These types of enzymes would be found in the biosynthesis of natural products, such as barbamide, that require stereoselective or site-selective halogenation reactions.

**Scheme 5: Stereoselective Chlorination and Incorporation of L-Leucine into Barbamide.**



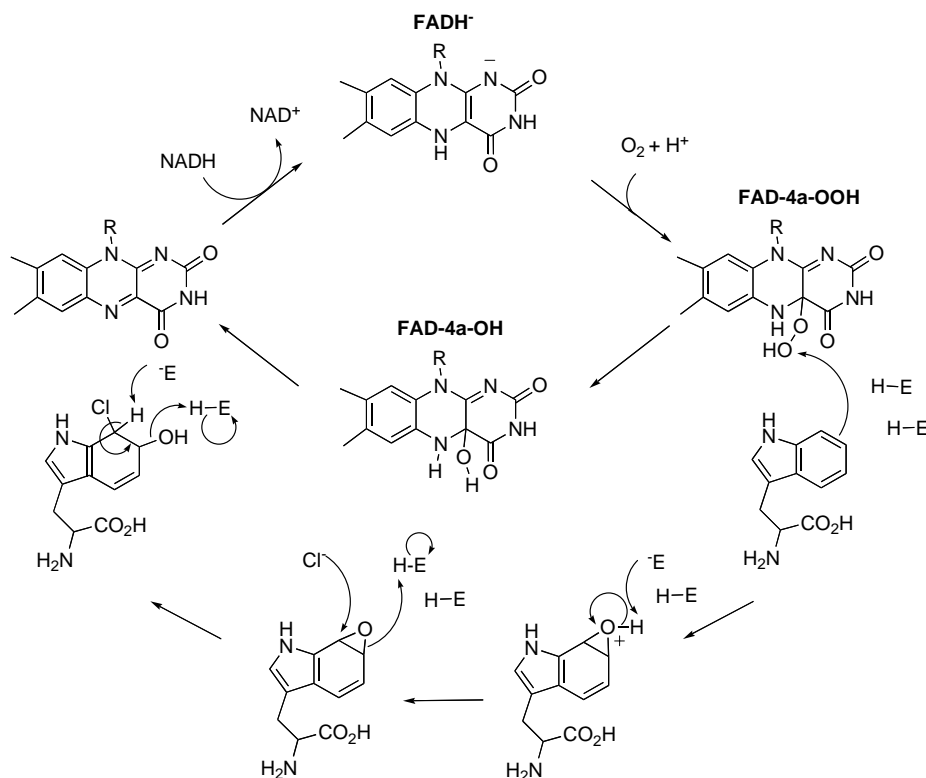
## FAD DEPENDENT HALOGENASES

A good candidate for a new class of enzymatic halogenation is PrnA. Sequence homology suggested that PrnA, the enzyme that catalyzes the site-selective chlorination of tryptophan to 7-chlorotryptophan (7-Cl-Trp) also did not use heme or vanadium as a cofactor.<sup>15</sup> The purified enzyme is competent for catalysis only in the presence of a second protein, a non-specific flavin reductase much like a flavin dependent oxygenase (Scheme 6). Subsequent experiments confirmed dependence on FAD, NADH and O<sub>2</sub>, much like the analogous systems

The initial mechanism was formulated by analogy to two-component FAD dependent oxygenases.<sup>15</sup> This mechanism involves formation of a peroxide adduct at the C4a position (FAD-4a-OOH) of the reduced flavin. Oxygen transfer from FAD-4a-OOH forms a benzene oxide. A chloride ion bound in the active site opens the epoxide. Elimination of water restores aromaticity and provides the chlorinated natural product (Scheme 5).

Spectroscopic evidence indeed suggests that FAD-4a-OOH is formed.<sup>15</sup> However, by analogy to the mechanism of haloperoxidases, a second mechanism is proposed in which a 4a-FAD-hypochlorite (FAD-4a-OCl) species is formed by attack of chloride ion on 4a-FAD-OOH. This intermediate would directly interact with the substrate as an electrophilic or radical chlorinating species.<sup>15</sup> However, neither mechanism has been confirmed experimentally.

**Scheme 6: First proposed mechanism of FAD dependent halogenation**



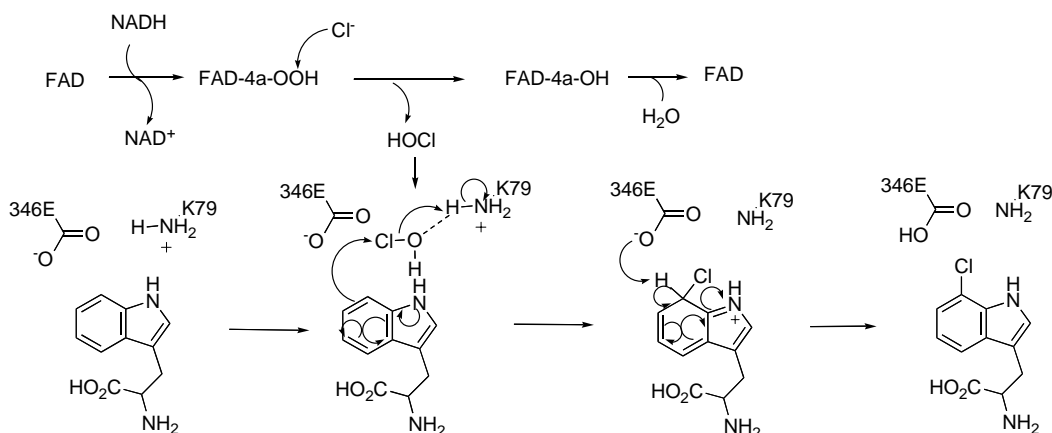
The X-ray crystal structure analysis of PrnA suggests a new mechanism in which hypochlorous acid is produced. This proposal is based on the fact that the flavin and substrate are 10 Å apart and separated by a lysine residue.<sup>17</sup> However, some enzymes can shift the substrate 7 – 8 Å after binding, and thus the crystallographic evidence is not conclusive.<sup>19</sup>

Further investigation supports hypochlorous acid as the electrophile.<sup>18</sup> Kinetic studies using UV-visible spectroscopy and quenched-flow experiments with radioactive labeled [<sup>14</sup>C]-Trp show that 7-Cl-Trp is formed after formation and disappearance of FAD adducts. Also, it was found that the chlorination of Trp is rate limiting.<sup>17</sup> These data support a mechanism in which HOCl formed from a FAD-4a-OCl adduct and is the electrophilic halogenating species (Scheme 7).

A conserved Lys79 between the flavin and substrate is thought to act as a shuttle for the HOCl.<sup>7</sup> Lys79 could either serve as a general acid to facilitate the initial electrophilic attack or it may undergo

chlorination to form a chloramine. The chloramine or HOCl would then be the ultimate halogenating species. At this time the exact role Lys79 plays in halogenation is unclear. In either case, site directed mutagenesis demonstrates the important role of Lys79 in both the regioselectivity and activity of halogenation.<sup>20</sup>

**Scheme 7. Mechanism of FAD Dependent Halogenation**



## NON-HEME IRON DEPENDENT HALOGENASES

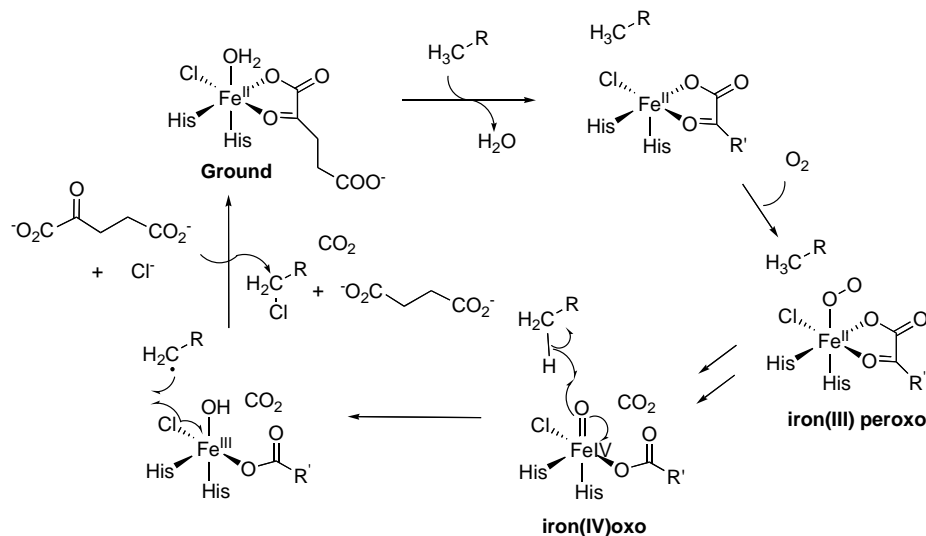
No obvious homologs to flavin-dependent halogenases have been found in the sequenced gene clusters for natural products containing halogenated aliphatic carbons.<sup>21</sup> In the case of purified SyrB2, which halogenates the  $\gamma$ -position of L-Thr, high homology with Fe(II)/2-ketoglutarate (2-KG) oxygenases has been established.<sup>22</sup> The purified enzyme is indeed dependent on Fe(II)/2-KG and O<sub>2</sub> for activity.<sup>22</sup> This dependence has also been demonstrated for BarB1 and BarB2, the enzymes responsible for halogenation in barbamide biosynthesis.

At first, the mechanism was assumed to mimic the characteristic 2-KG dependent oxygenases.<sup>26</sup> In this mechanism, Fe(II) is bound by two histidine residues, a carboxylate (Asp/Glu) residue, water and 2-KG. Binding of the substrate displaces water and allows for molecular oxygen to bind. 2-KG then decarboxylates to yield a high energy Fe(IV) complex. Hydrogen atom extraction from the substrate generates the methylene radical and Fe(III). Carbon dioxide is released and chloride displaces the succinate and then combines with the methylene radical to give the chlorinated product. Product is then released and binding of 2-KG and water resets the enzyme.

However, the X-ray crystal structure analysis of SyrB2 suggests another mechanism.<sup>18</sup> As predicted, two histidine ligands and 2-KG are coordinated to an iron center. However, alanine occupies the position at which an Asp/Glu ligand is found in 2-KG dependent oxygenases and chloride is directly coordinated to the iron in the ground state. Substrate binding displaces the water molecule and allows O<sub>2</sub>

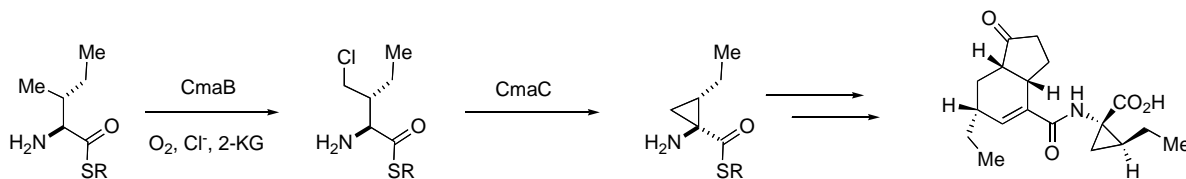
to bind. Decarboxylation affords the iron(IV)-oxo species which abstracts a proton from the carbon center. The methylene radical then combines with chlorine. The product, succinate and CO<sub>2</sub> are released and then binding of 2-KG and chloride resets the catalytic cycle (Scheme 8).

**Scheme 8. Catalytic Cycle of Non-Heme Iron Halogenation**



An understanding of the function of halogenating enzymes has already offered insight into the biosynthesis of coronatine (**10**). A previously uncharacterized enzyme, CmaB, has high homology with Fe(II)/2-KG dependent halogenases.<sup>24</sup> On this basis, CmaB was purified and reconstituted with Fe(II) and 2-KG. This reconstituted enzyme converts *L*-allo-isoleucine (**7**) to  $\gamma$ -chloro-*allo*-isoleucine (**8**). A second enzyme, CmaC, then uses this substrate to create the cyclopropanated amino acid **9** which is incorporated into coronatine (Scheme 9).

**Scheme 9. Cryptic Chlorination in Coronatine Biosynthesis**



## CONCLUSIONS AND OUTLOOK

The predominantly electrophilic and oxidative mechanisms of H<sub>2</sub>O<sub>2</sub>-dependent haloperoxogenases and O<sub>2</sub>-dependent halogenases have been substantially clarified. Though some work still remains to elucidate the halogenating species or intermediates for some of these classes of enzymes. Future research may eventually design enzyme variants or biomimetic catalysts that could effect stereoselective reactions in synthetic organic chemistry.

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