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Design and characterization of mechanism-based inhibitors for the tyrosine aminomutase *Sg*TAM

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Abstract—The synthesis and evaluation of two classes of inhibitors for *Sg*TAM, a 4-methylideneimidazole-5-one (MIO) containing tyrosine aminomutase, are described. A mechanism-based strategy was used to design analogs that mimic the substrate or product of the reaction and form covalent interactions with the enzyme through the MIO prosthetic group. The analogs were characterized by measuring inhibition constants and X-ray crystallographic structural analysis of the co-complexes bound to the aminomutase, *Sg*TAM.

Biosynthetic building blocks based on cinnamates and β-amino acids are key components of many therapeutically important natural products.[1] These biosynthetic precursors can be derived from α-amino acids by the action of ammonia lyases and aminomutases. The prosthetic group 4-methylideneimidazole-5-one (MIO, Fig. 1) is unique to these classes of enzymes.[2] MIO is a potent electrophile that is formed by the self-condensation of the tripeptide sequence alanine–serine–glycine in the protein backbone.[3] Ammonia lyases catalyze the elimination of ammonia from aromatic amino acids to form α,β-unsaturated carboxylic acids, while 2,3-aminomutases promote additional chemical steps resulting in the net 1,2-amine migration to generate β-amino acids.[4]

We have recently solved the first X-ray crystal structure of an MIO-based aminomutase, *Sg*TAM, demonstrating that all structurally characterized MIO-containing enzymes have the same overall protein fold and likely chemical mechanism.[5] *Sg*TAM catalyzes the conversion

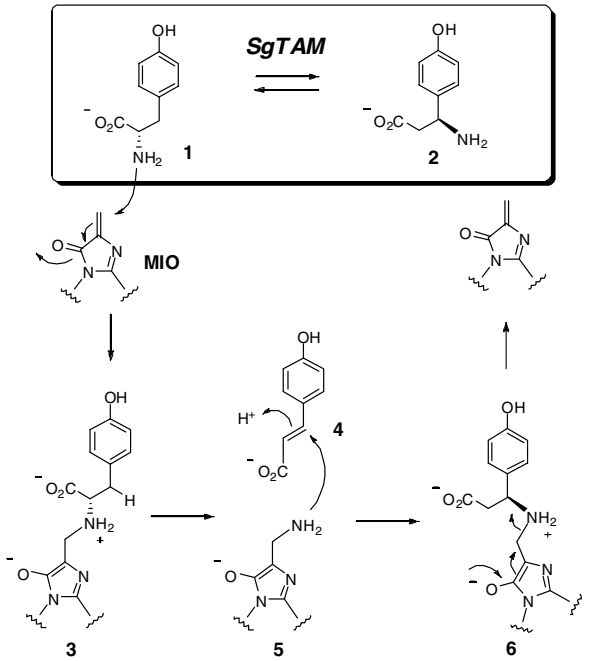

Figure 1. The mechanism of the MIO-based aminomutase *Sg*TAM. The overall reaction [L-tyrosine to (*S*)-β-tyrosine] is boxed. The MIO prosthetic group is derived from the protein backbone and shown abbreviated.

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of L-tyrosine to (*S*)-β-tyrosine, the first step in the biosynthesis of the β-amino acid moiety of the enediyne antitumor–antibiotic C-1027.[4a,b] The proposed mechanism for *Sg*TAM, illustrating the role of the MIO prosthetic group, is diagrammed in Figure 1. The α-amine of L-tyrosine adds into the electrophilic moiety via a conjugate addition. Formation of this covalent adduct facilitates deprotonation of the β-hydrogen and elimination of ammonia to produce 4-hydroxycinnamate (**4**). In the reaction of ammonia lyases, the olefin intermediate dissociates from the active site and the MIO-adduct releases ammonia.[5] Aminomutases retain these intermediates in the active site, allowing readdition of the MIO-bound amine nucleophile at the β-position of the substrate. The product (*S*)-β-tyrosine is ultimately released, regenerating the prosthetic group.

The exploitation of aminomutases with altered specificity is a potential route to produce novel aromatic β-amino acids or natural product derivatives. This can be accomplished through either the use of aminomutases as chemoenzymatic tools in vitro or the application of engineered biosynthesis in vivo.[6] Determination of the substrate scope and enzymatic chemistry is a key step toward this goal. Evaluation of inhibitors through biochemical studies and X-ray crystallography provides a structural basis for analog binding and recognition. Described here is the synthesis and characterization of two classes of mechanism-based inhibitors for the tyrosine aminomutase *Sg*TAM. The substrate or product analogs were designed to form covalent adducts with the MIO, mimicking intermediates along the reaction pathway. We have previously described the synthesis and initial characterization of a fluorine-substituted analog (α,α-difluoro-β-tyrosine, **8a**) that mimics the product of this reaction.[7] The X-ray crystal structure of **8a** bound to *Sg*TAM provided a structural basis for recognition of the natural substrate L-tyrosine and for the overall reaction mechanism. Synthesis, biochemical and structural characterization of additional analogs based on the structure of **8a** are presented along with a novel class of inhibitors based on cinnamate epoxides.

Figure 2 illustrates two classes of inhibitors for MIO-containing enzymes. Analogs based on cinnamate epoxides (**7a**–**c**) were designed to mimic the *para*-hydroxycinnamate intermediate in the reaction while presenting a reactive functionality in the active site. The design rationale is based on the similar geometry of the epoxide to the double bond in *para*-hydroxycinnamate. In addition, the stereochemistry of the epoxide mimics that of L-tyrosine and (*S*)-β-tyrosine. The synthesis of epoxide-containing analogs (**7a**–**c**) begins with various para-substituted ethyl cinnamates (**12a**–**c**, Scheme 1). Enantioselective epoxidation with Shi's dioxirane catalyst efficiently produced the desired epoxides.[8] Saponification of the ethyl ester protecting group gave the desired analogs as potassium salts.[9] As opposed to the free acids, the epoxides were stored as stable potassium carboxylates. The methoxy- and fluoro-substituents of **7a** and **7b** were incorporated to be mimics of the phenol on the natural substrate L-tyrosine. The synthesis of **7**

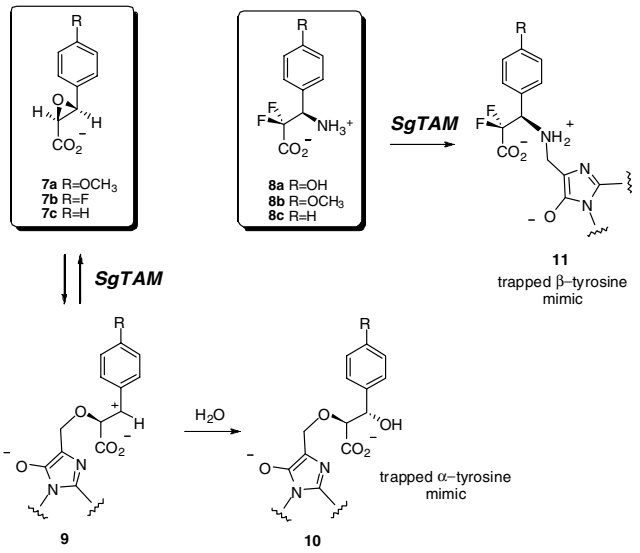
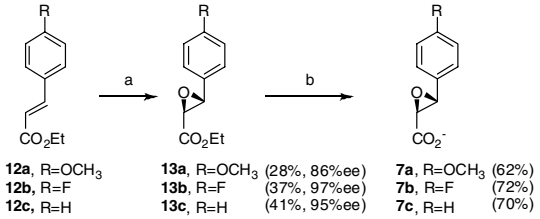


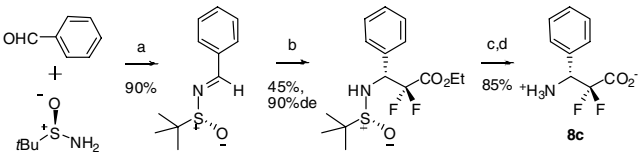
Figure 2. Structures of tyrosine aminomutase inhibitors based on cinnamate epoxides (**7a**–**c**) and α,α-difluoro-β-tyrosine (**8a**–**c**). The proposed binding mode for each inhibitor class is illustrated.



Scheme 1. Synthesis of epoxide-based inhibitors of *Sg*TAM. Reagents: (a) 30 mol% Shi's epoxidation catalyst, oxone, $NaHCO_3$, Na_2EDTA, cat. TBAHS, H_2O–CH_3CN; (b) KOH, MeOH. Isolated yields and percent enantiomeric excess are indicated.

where R = OH was not feasible as the final product is not stable under neutral pH conditions.

Analogs based on the product, (*S*)-β-tyrosine (**8a**, Fig. 2), also form adducts with the MIO.[7] The inclusion of α-fluorines in **8a** prevents the reverse reaction [(*S*)-β-tyrosine to L-tyrosine] and generates a product-like intermediate co-complex (Fig. 2, **11**). The synthesis of the α,α-difluoro analogs (**8a**–**c**) follows published work using Ellman's sulfinamide chemistry to prepare α,α-difluoro-β-phenylalanines.[10] The synthesis of **8c** is summarized in Scheme 2 as an example. Briefly, the route starts with benzaldehyde (or 4-substituted benzaldehydes), which is condensed with (*R*)-*tert*-butylsulfinimine.[11] A Reformatsky-type addition of $BrZnCF_2CO_2Et$ followed

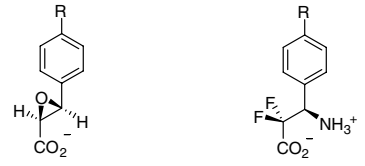


Scheme 2. General synthesis of α,α-difluoro-based inhibitors of *Sg*TAM. Reagents: (a) $CsCO_3$, DCM; (b) Zn^0, ethyl bromodifluoroacetate, THF; (c) 6 N HCl; (d) *i*-PrOH, propylene oxide.

by global deprotection yields the desired β-tyrosine analogs.

Binding of the designed inhibitors (**7a**–**c** and **8a**–**c**) was evaluated by determination of their inhibition constants (IC_{50}) with *Sg*TAM. The natural substrate L-tyrosine was incubated with the enzyme and various concentrations of analogs. The relative binding efficiency was measured using an HPLC-based assay and OPA derivatization of the starting L-tyrosine and product (*S*)-β-tyrosine.[4b] The results (Fig. 3) show all analogs inhibit the reaction in a concentration-dependent manner and the designed substitutions are tolerated by the enzyme. To observe adequate turnover in the HPLC assay, a high concentration (0.5 mg/mL) of enzyme and substrate L-tyrosine (75 μM) was necessary, resulting in IC_{50} values in the mM range.

To determine the structural basis of inhibition, the co-complex structures of each of the inhibitors were determined through X-ray crystallography. The structures from each of the two inhibitor classes gave very similar binding modes, therefore the structures of **7b** and **8b** are illustrated to represent their respective classes (Fig. 4). The structure of the epoxide analog **7b** confirms that the analog forms a covalent interaction with the electrophilic MIO through the epoxide oxygen. Electron density for a β-hydroxyl is clear in the maps, corresponding to the addition of water as illustrated in Figure 2. The binding mode of **7b** is analogous to that



Analog	R	IC_{50} (mM)
7a	OMe	3.0
7b	F	11.6
7c	H	5.8

Analog	R	IC_{50} (mM)
8a	OH	4.8
8b	OMe	5.3
8c	H	7.3

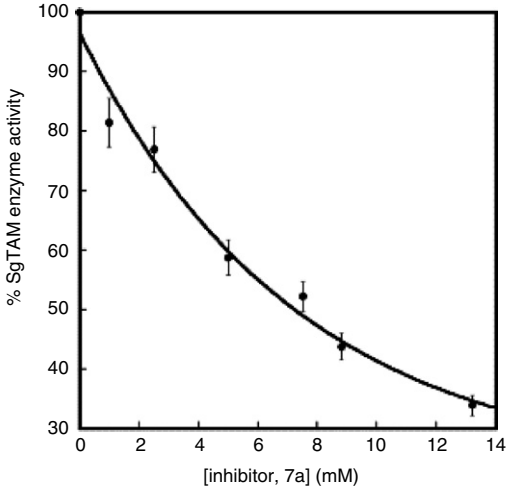


Figure 3. IC_{50} measurements for synthetic inhibitors of *Sg*TAM. Experiments were done in triplicate and a representative graph is illustrated.

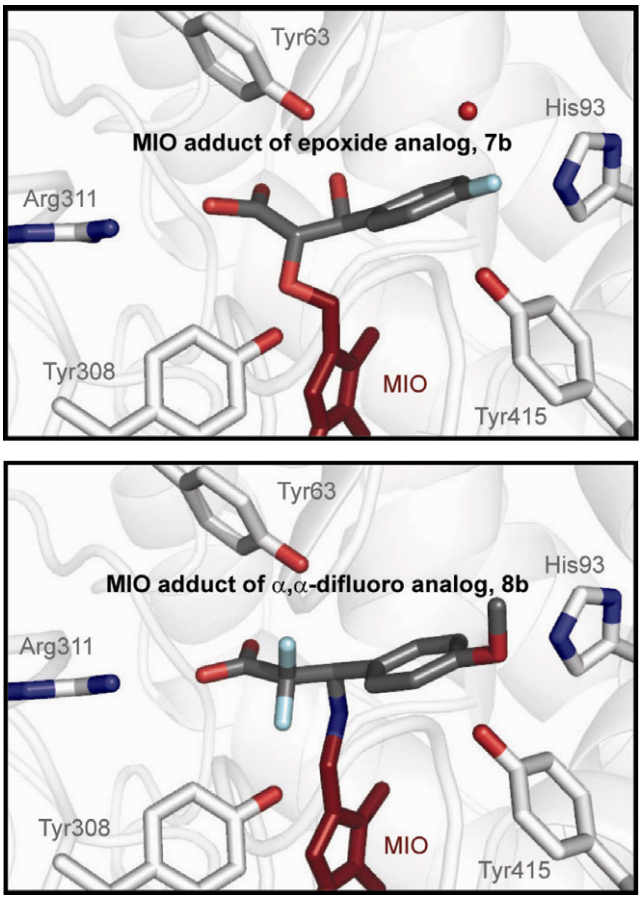


Figure 4. X-ray crystal structures of representative inhibitors bound in the active site of the aminomutase *Sg*TAM. The inhibitors (dark gray), active site amino acid residues (light gray), and MIO prosthetic group (dark red) are illustrated as sticks.

predicted for amino acids bound to MIO-based enzymes. The presence of the 4-fluoro group in **7b** pushes the aryl ring slightly away from the recognition elements His93 and Tyr415. These residues are predicted to form hydrogen bonds with the substrate 4-hydroxyl.[12] The 2,3-diol observed in the crystal structure is a single diastereomer (2*R*,3*S*) and results from attack and inversion at the 3-position of **7**. To provide evidence that the ring opening is enzyme-catalyzed, **7c** was incubated under standard assay conditions with and without *Sg*TAM. Substrate analog **7c** was converted to the corresponding diol when incubated in the presence of *Sg*TAM, but was recovered intact after a 24-incubation in buffer without enzyme.[13] Therefore, the hydrolysis of the epoxide ring was considered to be enzyme-catalyzed as shown in Figure 2. The observed diol can be generated either through an S_N1 (as shown) or S_N2-type mechanism. The result is a structure that is a close mimic of the starting adduct between L-tyrosine and MIO.

In addition to the previously reported structure of **8a** bound to *Sg*TAM, the biochemical evaluation of the analogs bearing substitutions at the 4-position suggests these will also form a covalent co-complex. The structures of **8b** and **8c** bound to *Sg*TAM were solved (see Fig. 4) and both displayed a similar binding mode. The 4-methoxy analog interacts with the two residues

(Tyr415 and His93) important in tyrosine recognition. Hydrogen bonding interactions are maintained, and the methyl group of the methoxy substrate displaces a bound water molecule that is present in the enzyme structure when the 4-hydroxy or the 4-fluoro inhibitor is covalently bound.

Overall, the results provide a structural basis for covalent catalysis and substrate recognition by MIO-based enzymes. Of particular note is the novel use of epoxide analogs as mechanism-based trapping reagents to provide structural insights into L-tyrosine binding and recognition. The structures of the bound inhibitors support a mechanism of catalysis via an amino/MIO adduct and the utilization of Tyr63 as a catalytic base to promote the observed 1,2 amino shift catalyzed by *Sg*TAM.[7] The L-tyrosine analog (**7b**) interacts with one face of the MIO while the product-like analog (**8b**) forms a bond with the opposite face. This finding supports a catalytic mechanism in which the amine bound to the MIO (**5**, Fig. 1) rotates while the substrate remains relatively fixed, resulting in the observed 1,2-amine shift and inversion of configuration at C3.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.046.

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