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Functional (Mis)Assignment in the Tomaymycin Biosynthetic Pathway

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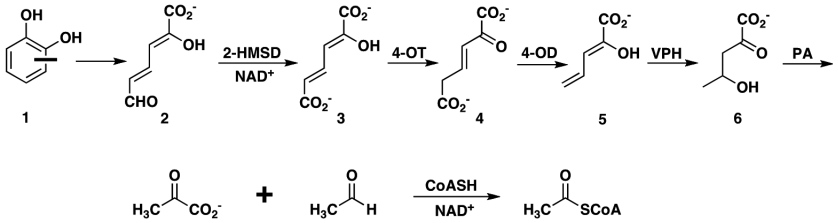
Abstract

4-Oxalocrotonate tautomerase (4-OT) catalyses the conversion of 2-hydroxymuconate to 2-oxo-3-hexenedioate in microbial pathways for the degradation of aromatic hydrocarbons. Pro-1 functions as a general base and shuttles the 2-hydroxy proton to C-5 of the product. Two arginine residues, Arg-11 and Arg-39, facilitate the reaction by participating in binding and catalysis. The same reaction is carried out by a heterohexamer 4-OT (hh4-OT) in thermophilic bacteria. The α-subunit of the hh4-OT identified the 4-OT homologue TomN in the biosynthetic cluster for the C ring of the antitumor antibiotic tomaymycin. TomN shares 58% pairwise sequence similarity with 4-OT including the three key catalytic residues. Kinetic and mutagenesis studies show that TomN catalyses the canonical 4-OT reaction with comparable efficiency using the same mechanism. However, the proposed function for TomN involves a very different reaction from that carried out by 4-OT. These results suggest that the assignment for TomN and the sequence of events leading to the C ring of tomaymycin might not be correct.

Introduction

Many bacterial species use aromatic compounds as their sole sources of carbon and energy because they have pathways to convert these compounds into substrates for the Krebs cycle (e.g., pyruvate and acetyl CoA) [1]. Initially, the aromatic compound is converted to

catechol or a derivative. Subsequently, these catechols are processed by one of the many so-called meta-fission pathways. Meta-fission refers to a mode of catechol ring fission, as shown in Scheme 1. In the catechol meta-fission pathway, catechol 2,3-dioxygenase is responsible for ring-cleavage of catechol (**1**) to yield 2-hydroxymuconate semialdehyde (**2**). The aldehyde is oxidized by the NAD^+-dependent 2-hydroxymuconate semialdehyde dehydrogenase (2-HMSD), to yield 2-hydroxymuconate (**3**). Ketonisation of **3** to 2-oxo-3-hexenedioate (**4**) is catalysed by 4-oxalocrotonate tautomerase (4-OT). Decarboxylation of **4** by the metal-dependent 4-oxalocrotonate decarboxylase (4-OD) generates 2-hydroxy-2,4-pentadienoate (**5**). The metal-dependent vinylpyruvate hydratase (VPH) converts **5** to 4*S*-hydroxy-2-keto-pentanoate (**6**) by the addition of water. Cleavage of **6** by a pyruvate aldolase (PA) yields pyruvate and acetaldehyde. PA is tightly associated with acetaldehyde dehydrogenase, and this complex uses NAD^+ and CoASH to produce acetyl CoA from acetaldehyde [2].



Scheme 1

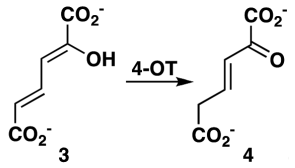
The focus of this article will be the reactions catalysed by 4-oxalocrotonate tautomerase (4-OT) [3], the heterohexamer 4-oxalocrotonate tautomerase (hh4-OT) [4], and a 4-OT homologue known as TomN [5]. 4-OT was initially cloned from the TOL plasmid pWW0 in *Pseudomonas putida* mt-2. Organisms harbouring this plasmid can process simple aromatic hydrocarbons such as benzene, toluene, *m*- and *p*-xylene, 3-ethyltoluene, and 1,2,4-trimethylbenzene. The hh4-OT is found in the thermophilic organism *Chloroflexus aurantiacus* J-10-fl. The genomic context suggests that the hh4-OT is also part of a pathway for the degradation of aromatic hydrocarbons. TomN is found in the biosynthetic pathway for tomaymycin, a potent antitumor antibiotic agent.

The three enzymes are in the 4-OT family, which is one family in the tautomerase superfamily [6]. This superfamily is a group of structurally homologous proteins characterized by a β-α-β building block (see Figure 1) and a catalytic amino-terminal proline (Pro-1). Superfamily members are made up of short monomers (61 – 84 amino acids) or longer monomers, which are about twice as long. A short monomer codes for a single β-α-β unit, whereas a longer monomer codes for two β-α-β units that are connected by a short linker. Tautomerase superfamily enzymes carry out tautomerisation, dehalogenation, hydration, and decarboxylation reactions [7]. Thus far, Pro-1 is critical for all of these activities. As tautomerase superfamily members, the three enzymes share mechanistic and structural

similarities. They also show subtle, but telling differences. The similarities and differences have implications for the assignment of function and the evolution of new activities in the tautomerase superfamily.

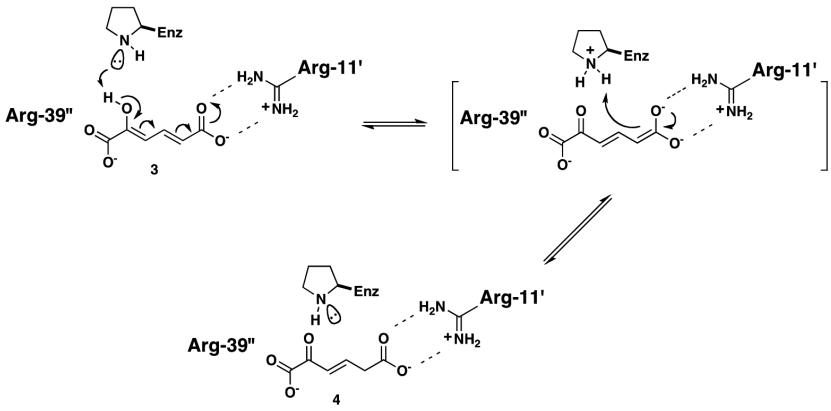
4-Oxalocrotonate Tautomerase

The mechanism and structure of the canonical 4-OT-catalyzed reaction (**3** to **4**, Scheme 2) have been studied for more than 20 years. The enzyme carries out a simple proton transfer reaction without the assistance of co-factors. A catalytic basein 4-OT abstracts the proton from the 2-hydroxy group of 2-hydroxymuconate (**3**) and places it at C-5 of 2-oxo-3-hexenedioate (**4**), in a highly stereoselective manner [3]. 4-OT is a hexamer, where each subunit is made up of 62 amino acids. It is also a founding member of the tautomerase superfamily.



Scheme 2

Kinetic, mechanistic, and structural studies identified Pro-1, Arg-11, Arg-39, and Phe-50 (from different monomers) as key players in the 4-OT-catalyzed conversion of **3** to **4.** The proposed mechanism for 4-OT is shown in Scheme 3, where the primed residues refer to the different monomers. Pro-1, which has a pK_a of ~6.4 (determined by ^{15}N NMR titration), is the catalytic base responsible for the proton transfer from the 2-hydroxy group of **3** to C-5 of **4** [8]. The interaction between Arg-11 and the C-6 carboxylate group (of **3**) binds substrate and draws electron density to C-5 to facilitate protonation at C-5 [9]. Arg-39 interacts with the 2-hydroxy group and a carboxylate oxygen of C-1. Mutagenesis shows that the role of Arg-39 is primarily catalytic, where the positively charged guanidinium moiety stabilizes the developing carbanionic character after deprotonation of the 2-hydroxy group. Phe-50 (not shown) is a major contributor to a hydrophobic pocket near the prolyl nitrogen of Pro-1 [10]. The proximity of the pocket is largely responsible for the low pK_a of Pro-1.



Scheme 3

The proposed mechanism for 4-OT is supported in part by the results of mutagenesis studies. The steady state kinetic parameters for the wild type enzyme and the P1A, R11A, and R39A mutants are summarized in Table 1. The first notable observation is that the wild type-catalysed reaction is near the diffusion-controlled limit. The second notable observation is the change in these kinetic parameters upon mutagenesis of the key residues. Changing Pro-1 to an alanine reduces k_{cat} (58-fold), but does not affect K_m [11]. The P1A mutant retains a catalytic base in the form of a primary amine so activity is not unexpected. Changing Arg-11 to an alanine has a major effect on K_m (9-fold increase) as well as a major effect on k_{cat} (87.5-fold reduction). These observations are consistent with the proposed binding and catalytic role. Finally, changing Arg-39 to an alanine has little effect on K_m, but a major effect on k_{cat} (125-fold reduction). These observations are consistent with a catalytic role for Arg-39.

Table 1. Kinetic parameters for 4-OT and mutants.

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
4-OT[a]	180 ± 30	3500 ± 500	1.9×10^7
P1A[b]	100 ± 12	60 ± 3	6.0×10^5
R11A[a]	1600 ± 300	40 ± 6	2.5×10^4
R39A[a]	290 ± 40	28 ± 2	9.7×10^4

[a]The steady state kinetic parameters were determined as described [9]. [b]The steady state kinetic parameters were determined as described elsewhere [11].

As noted above, 4-OT is a homohexamer. It is made up by the oligomerisation of a single β-α-β-building block (Figure 1) [12]. Two monomers form a dimer, and the three dimers assemble to form the hexamer (Figure 1). The GIGG motif (Gly-51, Ile-52, Gly-53, and

Glu-54) is critical for oligomer assembly and is a tell-tale sign of a hexamer in a short sequence. This motif is responsible for a β-hairpin at the end of each monomer, which interacts with the adjoining dimer and stabilizes the structure.

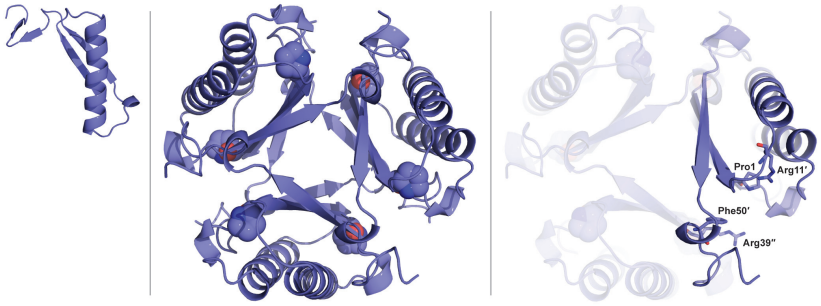
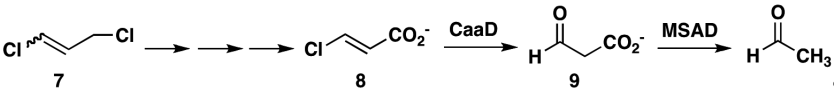


Figure 1. The 4-OT monomer (left), the 4-OT homohexamer (center), and a close-up of one of six active sites with the key residues labeled (right) (PDB code 4OTA). The 4-OT monomer shows signature tautomerase superfamily β–α–β building block. The catalytic amino-terminal proline is shown in space-filling form. The primed residues refer to different monomers.

There are six active sites per hexamer, which are located at the dimer interfaces (two per interface). A close-up of one active site shows the positions of the critical residues, Pro-1, Arg-11, Arg-39, and Phe-50 (Figure 1). These residues are contributed from three different monomers. Hence, activity is only observed for the hexamer, and not the individual dimers.

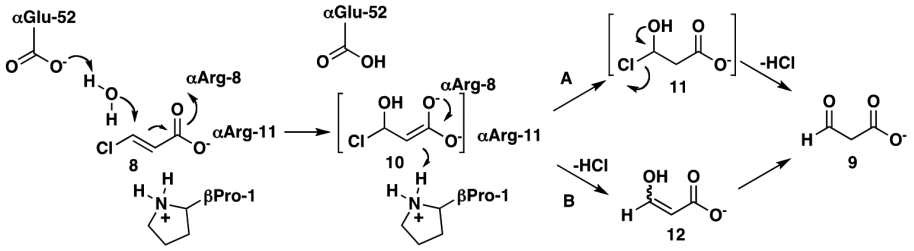
In the tautomerase superfamily, Pro-1 can function as a catalytic base or acid. In *trans*-3-chloroacrylic acid dehalogenase (CaaD), another 4-OT family member, Pro-1 functions as a catalytic acid with a pK_a of ~9.2 (determined by ^{15}N NMR titration) [13]. CaaD is found in a catabolic pathway for the nematacide, 1,3-dichloropropene (**7**, Scheme 4). In three enzyme-catalysed steps, **7** is converted to *trans*-3-chloroacrylate (**8**). Hydrolytic dehalogenation of **8** by CaaD produces malonate semialdehyde (**9**). Decarboxylation of **9** produces acetaldehyde, which is presumably channelled into the Krebs cycle. CaaD is a heterohexamer consisting of three α-subunits (with 75 amino acids) and three β-subunits (with 70 amino acids) [14]. The enzyme carries out the hydrolytic dehalogenation without the assistance of co-factors.



Scheme 4

The key players in the CaaD-catalysed conversion of **8** to **9** are βPro-1, αArg-8, αArg-11, and αGlu-52 (Scheme 5). αGlu-52 activates water for attack at C-3 of **8** (Scheme 5). The arginine residues, αArg-8 and αArg-11, interact with the C-1 carboxylate group, thereby binding and polarizing the substrate. The combined actions result in the enediolate inter-

mediate **10.** The enediolate can undergo two fates. In path A, the enediolate picks up a proton from βPro-1 to generate the chlorohydrin intermediate **11.** Direct expulsion of the chloride produces **9.** In path B, the enediolate undergoes an α,β-elimination of HCl to yield **12.** Tautomerisation and protonation at C-2 by βPro-1 completes the reaction.



Scheme 5

The structure shows that an α- and β-subunit form a heterodimer (Figure 2) [14]. Three heterodimers form the CaaD heterohexamer. Although both subunits have an amino-terminal proline, only the βPro-1 is involved in catalysis. Hence, there are three active sites per heterohexamer. A close-up of one active site shows the positions of the critical residues (Figure 2). Notably, three key residues in CaaD (βPro-1, αArg-11, and αPhe-50) superimpose with those of 4-OT. A fourth residue (αArg-8) superimposes with Leu-8 in 4-OT.

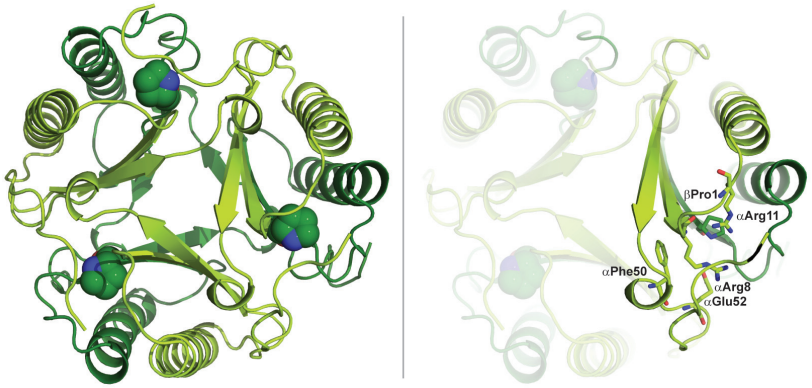
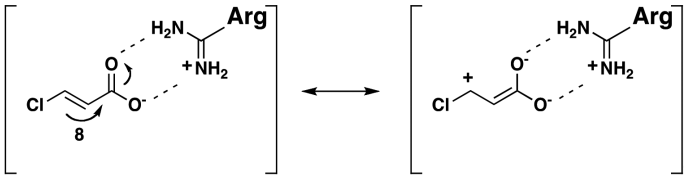


Figure 2. The CaaD heterodimer in the heterohexamer (left) and a close-up of one of three active sites with the key residues labeled (right) (PDB code 3EJ3). The catalytic Pro-1 is shown in space-filling form.

These structural similarities coupled with the observation that both substrates (i.e., **3** and **8**) have an acrylate moiety suggested that 4-OT might have a low level CaaD activity. Subsequent experimentation confirmed that 4-OT does indeed have a low level CaaD activity, supporting the evolutionary link between CaaD and 4-OT [15]. The low level activity is dependent on Pro-1 and one of the two arginines (Arg-11 or Arg-39). One arginine might interact with the C-1 carboxylate group of **8** and create a partial positive charge at C-3

(Scheme 6). It is less clear what Pro-1 does in the reaction. The low level CaaD activity of 4-OT is a very clear example of catalytic promiscuity in the tautomerase superfamily and lends support to the idea that 4-OT and 4-OT homologues can serve as templates for the creation of new enzymatic activities (such as CaaD).



Scheme 6

The CaaD activity of 4-OT is enhanced in the L8R-4-OT [16]. This mutation increases the CaaD activity 50-fold (as assessed by the k_{cat}/K_m values), due mostly to a ~9-fold increase in k_{cat}. The mutation does not change the pK_a of Pro-1 or have structural consequences. The additional arginine might narrow the binding modes such that **8** is more likely to bind in catalytically productive mode. An attempt to further enhance the CaaD activity in 4-OT by adding an αGlu-52 equivalent was not successful. Ile-52 in 4-OT occupies the same position as αGlu-52 in CaaD. However, changing this isoleucine to a glutamate only reduced the 4-OT activity with no increase in CaaD activity.

A comparison of the k_{cat} value for the low level CaaD activity of 4-OT to that of CaaD does not suggest a robust reaction (Table 2) [16]. However, a comparison of this rate with that of the uncatalysed reaction shows that 4-OT enhances the reaction 10^8-fold (compared with the 10^{12}-fold rate enhancement exhibited by CaaD) [17]. The presence of the second arginine residue in the L8R-4-OT enhances the reaction 10^9-fold – a 10-fold increase. The L8R-4-OT only requires an additional 1000-fold rate enhancement to be a fully active CaaD. Pro-1, Arg-11 or Arg-39, and Arg-8 are determinants of the low level CaaD activity. One additional determinant was identified in the hh4-OT studies. However, mutations that might provide the 1000-fold rate enhancement have not been determined.

Table 2. The low-level CaaD activity of 4-OT.

Enzyme	k_{cat} (s^{-1})	Rate Enhancement
4-OT[a]	8.3×10^{-4}	~10^8
CaaD	3.8	~10^{12}
L8R-4-OT	8.8×10^{-3}	~10^9
No Enzyme[b]	2.2×10^{-12}	-

[a]The steady state kinetic parameters were determined as described elsewhere [15,16].
[b]The non-enzymatic rate was determined by Horvat and Wolfenden [17].

The Heterohexamer 4-Oxalocrotonate Tautomerase

In the course of ongoing sequence analysis, two 4-OTs were identified in the thermophile *Chloroflexus aurantiacus* J-10-fl. in a putative meta-fission pathway (based on sequence similarities with other meta-fission pathway enzymes) [4]. One 4-OT, with 72 amino acids, triggers the tautomerase annotation (in PSI-BLAST), but lacks Pro-1. A nearby 4-OT, also made up of 72 amino acids, has Pro-1, but does not trigger the tautomerase annotation. Both sequences have the two potentially catalytic arginines. A tryptophan residue is found in place of Phe-50 in the sequence that triggers the tautomerase annotation. At first, it was puzzling to find two 4-OTs in a meta-fission pathway because there is no apparent reason for two tautomerases. It also had potential implications for the tautomerase superfamily because it appeared that a member without a Pro-1 might have been discovered. Unfortunately, all attempts to express the two genes separately resulted in insoluble protein.

The mystery surrounding these two sequences was solved when co-expression of the two genes produced a stable heterohexamer where each dimer consists of an α- and β-subunit (Figure 3). The two subunits are arbitrarily designated the α- and β-subunits by their positions in the genome – the α-subunit appears first. The α-subunit sequence lacks Pro-1, but triggers the tautomerase annotation.

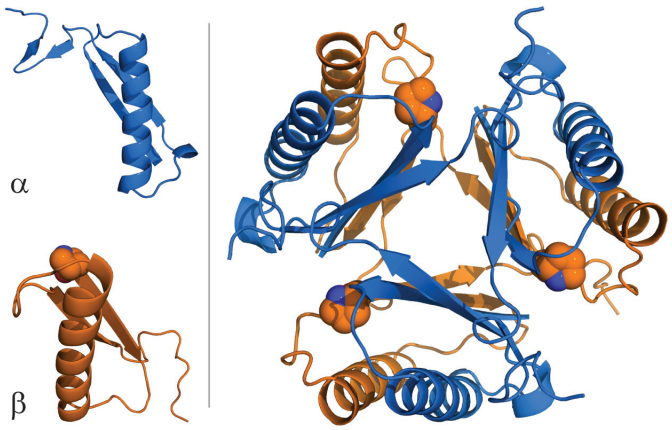


Figure 3. The α- and β-subunits of the hh4-OT (left) and the hh4-OT heterohexamer (right) (PDB code 3MB2). The three active sites are located at the interfaces. The catalytic amino-terminal proline is shown in space-filling form.

Both subunits have two arginine residues that could function in the mechanism. The α-subunit has Arg-12 and Arg-40 and the β-subunit has Arg-11 and Arg-39. Replacing the arginine residues in the β-subunit (with alanines) has only a minimal effect on the activity. However, changing βPro-1, αArg-12, or αArg-40 to an alanine has a major impact on the kinetic parameters for the reactions using **3** (Table 3) [4]. Moreover, the effects on the kinetic parameters parallel those seen with 4-OT. Changing βPro-1 to an alanine reduces k_{cat}

(83-fold) and K_m (4-fold). As noted for 4-OT, the P1A mutant has a catalytic base in the form of a primary amine so activity is expected. Changing αArg-12 to an alanine has significant effects on K_m (15-fold increase) and k_{cat} (70-fold decrease). These observations are consistent with the proposed binding and catalytic role of αArg-12. Finally, changing αArg-40 to an alanine increases K_m (5-fold) and reduces k_{cat} (46-fold). These observations are consistent with a catalytic role for αArg-40. These results identified βPro-1, αArg-12, and αArg-40 as critical residues, and indicate that the hh4-OT uses a mechanism like that proposed for 4-OT (see Scheme 3).

Table 3. Kinetic parameters for the hh4-OT and mutants[a].

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
4-OT	70 ± 8	3000 ± 100	4.3×10^7
βP1A	17 ± 2	36 ± 1	2.1×10^6
αR12A	1033 ± 510	43 ± 19	4.2×10^4
αR40A	345 ± 100	65 ± 14	1.9×10^5

[a]The steady state kinetic parameters were determined as described [4].

There is one notable difference between the hh4-OT and 4-OT – the hh4-OT lacks a low level CaaD activity. Incubation of **8** with a large quantity of the hh4-OT did not result in any detectable turnover after 7 weeks. This observation is somewhat surprising because an overlay of the two active sites shows little difference other than the replacement of Phe-50 (in 4-OT) with αTrp-51 (in hh4-OT) (Figure 4) [4].

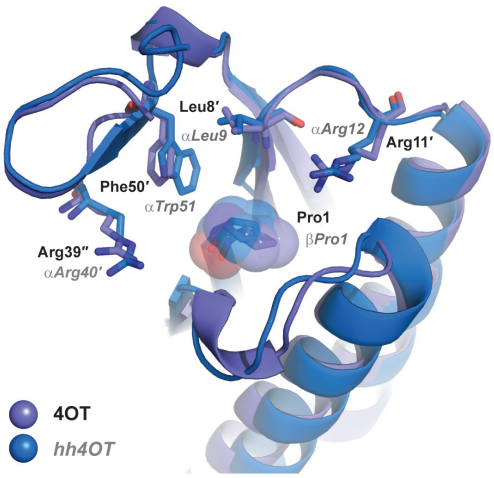


Figure 4. An overlay of the active sites of the hh4-OT and 4-OT. The active sites are similar except αTrp-51 in the hh4-OT replaces Phe-50 in 4-OT. The primed residues refer to different monomers.

However, the additional bulk on tryptophan might crowd Pro-1 and prevent alignment of **8** in the active site in such a way that it can undergo dehalogenation (Figure 5). This result identified Phe-50 as another determinant of the low level CaaD activity in 4-OT.

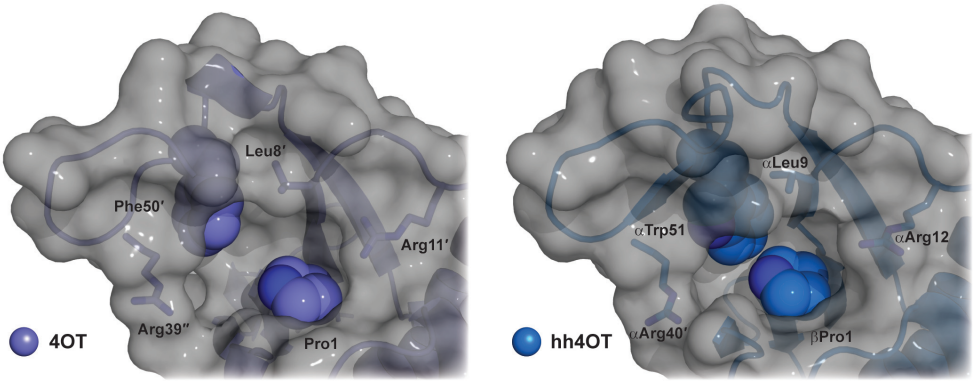
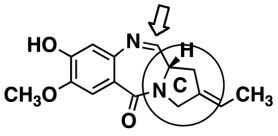


Figure 5. Space filling models showing the active sites of 4-OT (left) and the hh4-OT (right). αTrp-51 in the hh4-OT appears to crowd βPro-1 and might prevent alignment of **8** in the active site. The primed residues refer to different monomers.

TomN, a 4-Oxalocrotonate Tautomerase

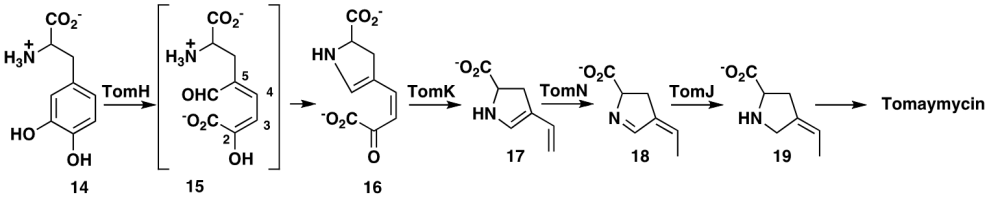
The α-subunit of the hh4-OT led to the discovery of TomN [5]. TomN catalyses one step in the proposed biosynthetic pathway for the C ring of tomaymycin (**13**, Scheme 7) [18]. Tomaymycin and related pyrrolo[1,4]benzodiazepines (PBDs) such as sibiromycin are antibiotic antitumor agents [19]. The antitumor activity of these compounds results from sequence-specific DNA alkylation. Interest in this activity spawned the design, synthesis, and characterization of many derivatives. The chemical lability of the imine bond (see arrow in **13**, Scheme 7) complicates synthetic efforts and prompted the recent cloning of the sibiromycin and tomaymycin biosynthetic clusters. Biochemical characterization of the individual enzymes making up these pathways and manipulation of the corresponding genes might expand the repertoire of PBD analogues to include synthetically inaccessible ones and make feasible semi-synthetic approaches.



Tomaymycin (13)

Scheme 7

In the currently proposed pathway, hydroxylation of tyrosine produces L-dopa (**14**) (Scheme 8) [18]. TomH, a 2,3-dioxygenase, converts **14** to **16**, presumably through **15** (as the *s-cis* or *s-trans* isomer) [20]. TomK processes **16** to **17** and oxalate. TomN catalyses the tautomerisation of **17** to **18.** TomJ, an F420-dependent enzyme, carries out the reduction of the imine bond in **18** to yield **19**, which is subsequently incorporated into **13.** Although this scheme accounts for the construction of the C ring, the functions assigned to the individual enzymes are tentative and there is little biochemical evidence to support them.



Scheme 8

The proposed TomN-catalysed reaction (**17** to **18** in Scheme 8) is very different from the canonical 4-OT-catalyzed reaction (**3** to **4** in Scheme 2). This observation is particularly striking because TomN shows 58% pairwise sequence similarity with 4-OT and has Pro-1, Arg-11, and Arg-39. In view of these similarities, it seemed odd that the reactions were so different and that TomN processes a monacid (i.e., **17**), whereas 4-OT processes a diacid (i.e., **3**). These observations prompted a kinetic and mutagenic analysis of TomN using **3** because the proposed TomN substrate is not available [5].

This analysis indicates that TomN carries out the canonical 4-OT reaction using the same residues (Pro-1, Arg-11, and Arg-39) with slightly less efficiency (5-fold, as judged by k_{cat}/K_m values) (Table 4). Changing Pro-1 to an alanine severely reduces k_{cat} (1233-fold), but has less of an effect on K_m (2.8-fold decrease). The change in k_{cat} is much more significant than that seen for the P1A mutant of 4-OT. Changing Arg-11 to an alanine results in a 2-fold increase in K_m and a 132-fold decrease in k_{cat}. The effect on K_m is not as significant as that measured for the R11A-4-OT, but is still consistent with a binding and catalytic role for Arg-11. Finally, changing Arg-39 to an alanine has little effect on K_m, but has a dramatic effect on k_{cat} (9250-fold reduction). These observations are consistent with a catalytic role for Arg-39, but the reduction in k_{cat} is much more severe than that measured for the R39A-4-OT.

Table 4. Kinetic parameters for TomN and mutants[a].

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
TomN	512 ± 225	1850 ± 630	3.6×10^6
P1A	180 ± 10	1.5 ± 0.1	8.3×10^3
R11A	1050 ± 240	14 ± 3	1.3×10^4
R39A	440 ± 60	0.2 ± 0.02	4.5×10^2

[a]The steady state kinetic parameters were determined as described [5].

The crystal structure for TomN shows that the enzyme is a hexamer constructed from the signature β-α-β building block (Figure 6).

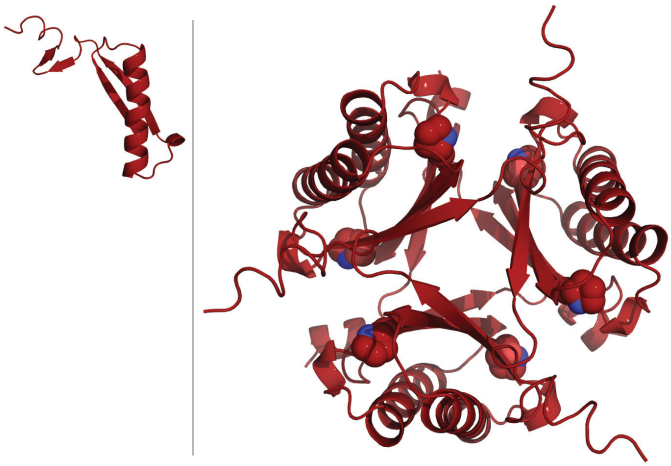


Figure 6. The TomN monomer (left) and the TomN homohexamer (right) (PDB code 3ry0). The catalytic amino-terminal proline is shown in space-filling form in the homohexamer.

The active site of TomN is very similar to those of 4-OT and the hh4-OT (not shown) (Figure 7) [5]. The key catalytic residues (Pro-1, Arg-11, and Arg-39) are positionally conserved. Trp-50 (in TomN) is found in place of Phe-50. These structural observations are consistent with the results of the kinetic and mutagenesis studies, indicating that TomN functions much like the canonical 4-OT.

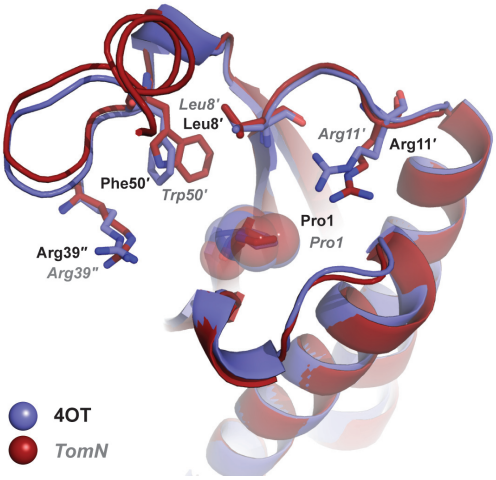
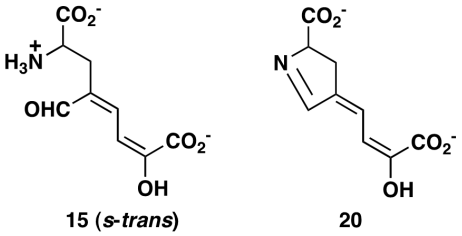


Figure 7. An overlay of the active sites of TomN and 4-OT. The active sites are similar except Trp-50 in the TomN replaces Phe-50 in 4-OT. The primed residues refer to different monomers.

The conversion of **3** to **4** is not likely the biological reaction for TomN, as suggested by the differences in K_m and k_{cat} for the wild type enzymes and the mutants. Nonetheless, the fact that TomN is an efficient 4-OT raises questions about the currently proposed function for TomN in the tomaymycin pathway. If TomN does not convert **17** to **18**, it might process a diacid substrate such as **15** (shown as the *s-trans* isomer in Scheme 9) or **20**, which is the cyclic counterpart of **15.** These possibilities are being examined. The outcome might also require a re-evaluation of the proposed sequence of events leading to the formation of the C ring and the functions of the enzymes.



Scheme 9

TomN is the first 4-OT (and the first tautomerase superfamily member) found in a biosynthetic pathway instead of a catabolic pathway. The group of biosynthetic 4-OTs is growing. 4-OT homologues have recently been identified in the pyridomycin and pristinamycin biosynthetic pathways (Pyr5 and SnbT, respectively). Pyridomycin is an anti-mycobacterial antibiotic with some unusual structural features [21]. Semi-synthetic water-soluble

derivatives of pristinamycin I and II are the active constituents of Synercid, which is used for the treatment of various infections caused by Gram-positive bacteria and vancomycin-resistant *Enterococcus faecium* [22]. The roles of Pyr5 and SnbT in these pathways are not yet known, but elucidation of TomN's role in the tomaymycin pathway will provide insight.

Conclusions

Three 4-OTs (defined by their ability to convert **3** to **4**) have been characterized. They carry out the canonical 4-OT reaction with similar catalytic efficiencies and mechanisms, underscoring the difficulty of functional annotation for closely related family members in a superfamily. The assignment (or mis-assignment) of function for TomN best exemplifies the problem. Characterization of the enzymes has resulted in the discovery of a burgeoning group of biosynthetic 4-OTs.

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