Enzyme Structure/Mechanism

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Insights into the Mechanistic Pathway of the *Pantoea agglomerans* Phenylalanine Aminomutase**

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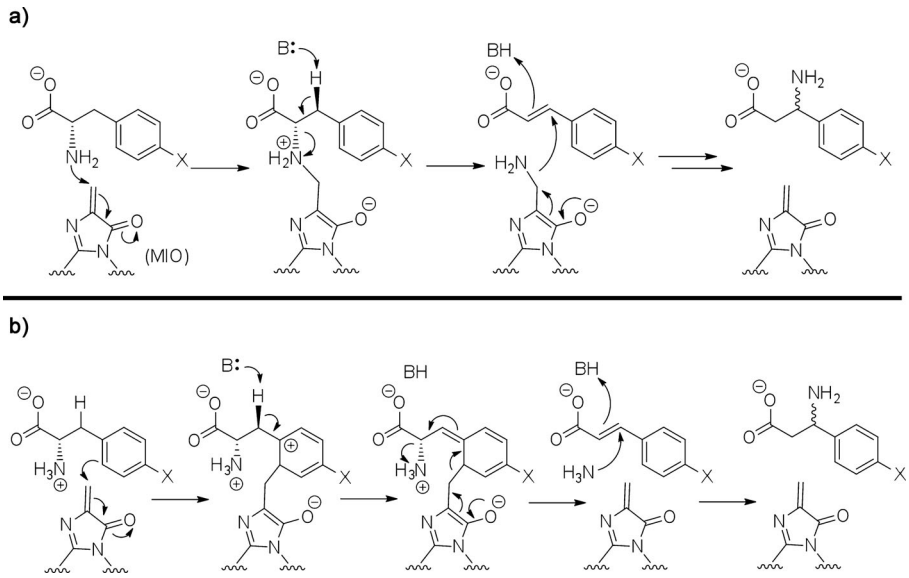
The biosynthesis of the hybrid peptide-polyketide antibiotic andrimid in *Pantoea agglomerans* requires the function of a phenylalanine aminomutase, AdmH (designated *Pa*PAM), which converts (*S*)-α-phenylalanine to (*S*)-β-phenylalanine.[1,2] *Pa*PAM is a member of the class I lyase-like family that includes phenylalanine and tyrosine aminomutases (PAMs[3] and TAMs[4,5], respectively), phenylalanine ammonia lyases (PALs),[6] tyrosine ammonia lyases (TALs),[7] and histidine ammonia lyases (HALs).[8] PALs, TALs, and HALs produce aryl acrylates from the corresponding amino-acid substrate by the elimination of ammonia.

The transformations that are performed by this family of enzymes are, in part, catalyzed by a 4-methylidene-1*H*-imidazol-5(4*H*)-one (MIO) prosthetic group. This cofactor is formed post-translationally from a tandem of active site residues, typically Ala-Ser-Gly, and is believed to function as an electrophile through its α/β-unsaturated keto functional group (Scheme 1).[9] Two mechanisms for these transformations have been proposed; in the first, the amino group of the amino acid substrate acts as a nucleophile and attacks the methylidene of MIO through conjugate addition (earlier reports suggested that MIO was a dehydroalanyl moiety).[10] Ammonia is subsequently expelled from the *N*-alkylated substrate through an α/β-elimination process, which results in the formation of an acrylate reaction intermediate that is released as such in the ammonia lyase reaction.[11] Alternatively, the acrylate remains in the active site for amino group rebound to form the β-amino acid product in the aminomutase reaction.[3,12] A second proposed mechanism suggests that π-electrons at the *ortho*-carbon atom of the phenyl ring of the substrate attack MIO, which acts as a Lewis acid, by Friedel–Crafts-like activation.[6,13,14] The second process has been principally assigned to ammonia lyase reactions that yield unsaturated products by α/β-elimination,[6,8,13] but has also been implicated in the aminomutase reactions.[15]



Scheme 1. Two proposed mechanisms for the conversion of substrate to product in a generic aminomutase: a) amino-group alkylation pathway and b) Friedel–Crafts aryl-alkylation pathway. An ammonia lyase reaction terminates at *trans*-cinnamate or *trans*-coumarate (X = H (phenylalanine) or OH (tyrosine), respectively).

The structures of several enzymes of the MIO-dependent family were characterized in earlier reports.[16] The structure of *Rhodobacter sphaeroides* tyrosine ammonia lyase (*Rs*TAL) in complex with the competitive inhibitor 2-aminoindan-2-phosphonic acid, which is covalently bound by its amino group to MIO in the active site (Protein Data Bank (PDB) 2O7E) was determined.[17] Yet, based on this structure, one of the MIO-based mechanisms was not suggested for the

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*Rs*TAL reaction.[17] Also available are the structures of the *Streptomyces globisporus* L-tyrosine 2,3-aminomutase (*Sg*TAM) in complex with inhibitors 2,2-difluoro-β-tyrosine (PDB 2QVE) and alternatively with (3*R*)-3-amino-2,2-difluoro-3-(4-methoxyphenyl)propanoic acid (PDB 2RJS).[18] These β-amino acids were alkylated at their amine groups by the MIO, which is consistent with the aminoalkyl mechanism that involves the elimination of an alkylamine.

The crystal structure of the Y63F mutant of *Sg*TAM in complex with the tyrosine residue from the substrate (PDB 3KDZ) shows the important catalytic function of the highly conserved Tyr63 in the abstraction of the C_β proton that is coupled to the elimination of the transient alkylammonium group.[9] The structure of the *Taxus canadensis* phenylalanine aminomutase (*Tc*PAM, PDB 3NZ4) was solved as an active site complex with a cinnamate ion, which was bound in a trajectory that is reminiscent of the ligands that are co-crystallized in the *Sg*TAM structures, and suggests a similar mechanism for both enzymes.[3,4,9] The opposite stereochemistry of the product from the reaction catalyzed by *Tc*PAM was postulated to result from simultaneous rotation of the cinnamate intermediate about the single bonds that flank the propene double bond attached between the carboxylate and phenyl groups.[3] This rotamer presents the opposite face of the alkene nearer to the aminated MIO before reattachment of the amino group (see Scheme 1a for the amination sequence). However, automated docking and molecular dynamics simulation studies of *Petroselinum crispum* phenylalanine ammonia lyase (*Pc*PAL, PDB 1W27)[19] imply that the Friedel–Crafts mechanism is used by both PAL and PAM enzymes.[15]

Apparently, there is still debate about the mechanism that is used by these enzymes, and the nature of the stereochemical control is still not well understood. *Pa*PAM catalyzes the production of the β-amino acid that is the enantiomer of the product made by *Tc*PAM, but has the same stereochemistry as the product made by *Sg*TAM. To provide further information on the stereochemistry of this class of catalysts, the X-ray crystal structure of *Pa*PAM was determined at 1.7 Å resolution with cinnamate included in the crystallization buffer.

*Pa*PAM has similar architecture to the class of MIO-dependent aminomutases and ammonia lyases.[3,4,16,17,19] The monomer consists of mostly α helices that run parallel to one another and form a four helix bundle at the center (Figure 1a). The catalytically relevant species is a dimer of dimers, in which the two monomers in the asymmetric unit are related by a crystallographic twofold axis to the other two monomers that comprise the catalytically functional *Pa*PAM tetramer, and each subunit contains an active site (Figure 1b). At the end of this bundle is the active site, which resides at the interfaces between three of the monomers in the tetramer, and includes residues from all three protomers. *Pa*PAM, similar to other bacterially derived members of this family whose structures are known, lacks the C-terminal capping domain that is present in the related plant enzymes, such as *Tc*PAM.[3] The inner-loop region, which rests just above the active site, is packed tighter towards MIO than that of *Tc*PAM, and is well ordered. However, the *Pa*PAM MIO is made autocatalytically from a Thr-Ser-Gly tandem instead of from the common Ala-Ser-Gly sequence.

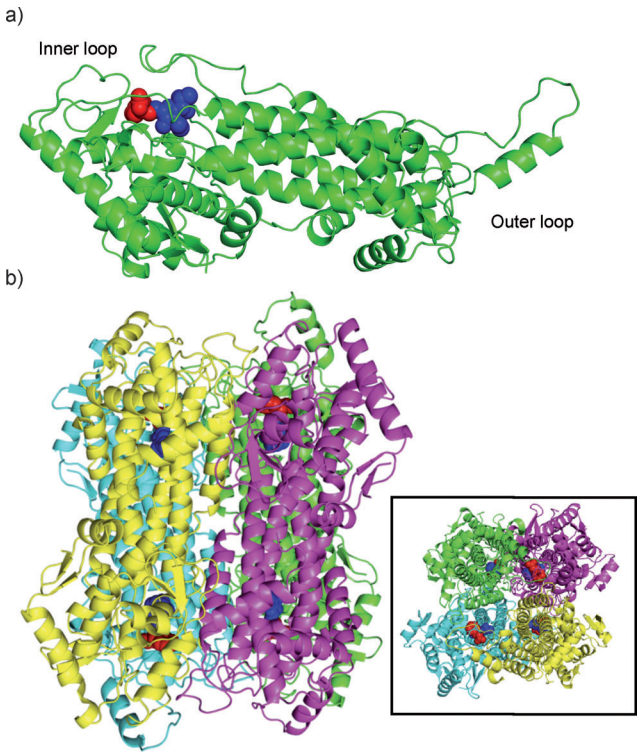

Figure 1. a) *Pa*PAM monomer showing the inner and outer loop regions. b) Side view of *Pa*PAM tetramer (Inset: Top view). MIO is highlighted with blue spheres. The (*S*)-β-phenylalanine is highlighted with red spheres.

The active sites of the two monomeric structures in the asymmetric unit differ in what is bound to them. In monomer "A", a molecule that is indistinguishable (by electron density) from β-phenylalanine is found covalently attached by its amino group to the methylidene carbon atom of MIO. This complex is consistent with an enzyme-bound β-phenylalanine-type intermediate along the conjugate amino-addition pathway (Scheme 1a, see also Figure S1 in the Supporting Information). In contrast, the electron density of monomer "B" suggests partial occupancy of two ligand types. The electron density for a β-phenylalanine-type complex is evident, as seen in monomer "A", but there is also electron density that is consistent with a molecule that is indistinguishable from α-phenylalanine, which is covalently attached by its amino group to the methylidene carbon atom of MIO (Figure 2a). These complexes are, to our knowledge, the first structural identification of naturally occurring pathway intermediates from an unmodified MIO-dependent enzyme, and provide additional evidence that reactions catalyzed by *Pa*PAM do not proceed through a Friedel–Crafts pathway, as recently proposed.[15]

Presumably, the α- and β-phenylalanines were formed by the reaction of a cinnamate ion with the covalent amino–MIO adduct, which was made when the recombinant protein was expressed in *E. coli* and retained during protein purification (see Experimental in the Supporting Information). Notably, the Luria-Bertani (LB) media (pH 7.3) that was used to grow

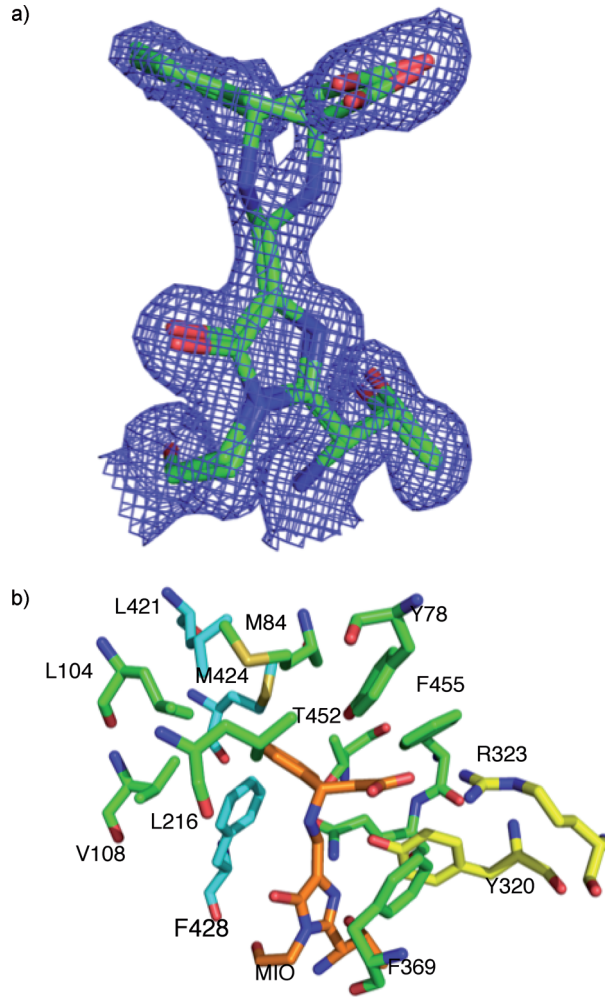

Figure 2. a) Electron density ($2F_o-F_c$ map, blue mesh) calculated at 1.0 σ around the α- and β-phenylpropanoid that is covalently bound to the MIO found in monomer "B" (C_α and C_β are indicated). Atoms are color-coded as carbon: green; oxygen: red; nitrogen: blue. b) Active site of *Pa*PAM in complex with a MIO-bound (3*S*)-β-phenylalanine-type ligand (orange carbon atoms). Active site residues contributed by three monomers are colored accordingly (carbons: cyan, yellow, or green for each monomer; oxygen: red; nitrogen: blue).

the bacteria for this crystallography study was estimated to contain ammonia at a concentration of 2.4 mM, as assessed in an earlier study.[20] Therefore, LB media was a practical source of ammonia that could bind to the MIO of *Pa*PAM that was overexpressed in *E. coli*. To assess whether *Pa*PAM could transfer the amino group to the cinnamate ions that were added to the crystallization buffer, in a manner similar to that of other MIO-dependent enzymes,[21] *Pa*PAM (7 mg in 1 mL assay), cinnamic acid (1.25 mM), and ammonia (60 μM) were incubated together for 48 h at 31 °C. The assay buffer was treated with derivatizing reagents to convert the putative, biosynthetically derived amino acids to their *N*-carbonyl methyl esters. Analysis of the derivatized products showed that α-phenylalanine (6.6 ± 0.9 nmol) and β-phenylalanine (8.7 ± 0.9 nmol) were produced. These results indicate that a concentration as low as 60 μM of ammonia in the bulk media was sufficient to aminate the MIO of *Pa*PAM and to catalyze the reverse reaction. Similarly, MIO adducts of either amino[11,17] or hydroxy[16] groups of MIO were detected in previous crystallographic studies of enzymes in this class.

Furthermore, evaluation of the α- and β-phenylalanine complexes of monomer "B" (Figure 2a) suggests that during the course of the amino group isomerization catalyzed by *Pa*PAM, the phenylpropanoid carbon backbone remains mostly stationary above the amino group that is attached to the MIO moiety. These configurations are consistent with the mechanism of stereoselectivity for this enzyme that proceeds with inversion of configuration at each migration terminus.[22]

The *Pa*PAM active site contains key catalytic residues that are found in other structurally characterized enzymes in the class I lyase-like family (Figure 2b). Tyr 78 (Tyr 80 in *Tc*PAM and Tyr 63 in *Sg*TAM) is positioned above and within 3.5 Å of both the α- and β-carbon atoms of the phenylalanine complexes, and is poised to de- and reprotonate the intermediate phenylalanine complexes at both the α and β positions. Tyr 320 (Tyr 322 in *Tc*PAM and Tyr 308 in *Sg*TAM) is only 2.6 Å from the amino group of the α-phenylalanine intermediate, and is thought to facilitate proton transfers when the amino group is removed from the α-phenylalanine substrate and added to MIO.

In contrast, differences in the residues near the active site of *Pa*PAM are notable when compared with those of other MIO-dependent catalysts (Figure S2 in the Supporting Information). For example, Phe 455 (Asn 458 in *Tc*PAM) is a glutamine or asparagine in almost all other enzymes in the family. When the *Tc*PAM–cinnamate structure[3] is overlaid onto the *Pa*PAM–phenylalanine structure, the *Tc*PAM–cinnamate ligand clashes with the sterically bulkier Phe 455. In the *Pa*PAM structure, however, the covalently-bound α- and β-phenylalanine ligands avoid the steric clash with Phe 455 by altering their trajectory through the active site, which avoids the collision (Figure 3a). The new orientation results in a weaker monodentate salt bridge with Arg 323 (versus the bidentate salt bridge that is present in *Tc*PAM) and is almost identical to the trajectory of tyrosine in the *Sg*TAM structure. This similar alignment of ligands is reinforced by the superimposition of the bridging atoms (likely nitrogen) of the α- and β-phenylpropanoid–MIO adducts in *Pa*PAM and the nitrogen and oxygen atoms, respectively, of the α-difluoro-β-tyrosine and 2,3-dihydroxycoumarate inhibitor–MIO adducts in the two *Sg*TAM structures (Figure 3b).[12,18] *Sg*TAM, however, contains a nonconserved His 93 (Val 108 in *Pa*PAM) that forms a hydrogen bond with the hydroxy group of the tyrosine substrate.[9] This hydrogen-bonding interaction, which is absent in *Pa*PAM, enforces the trajectory of tyrosine in the *Sg*TAM active site. Thus, *Sg*TAM and *Pa*PAM catalyze equivalent stereochemistries, presumably by orienting their substrates identically in their active sites, but use a distinct set of enzyme–substrate interactions to accomplish this.

To assess the role of Phe 455 in the reaction, a study of Phe 455 mutants was conducted. At steady state, *Pa*PAM converts (*S*)-α-phenylalanine to (*S*)-β-phenylalanine/*trans*-cinnamate in a 90:10 ratio. In contrast, *Pa*PAM mutants F455A and F455N (F455N is analogous to N458 of *Tc*PAM) each form the same products, but at approximately 2% of the

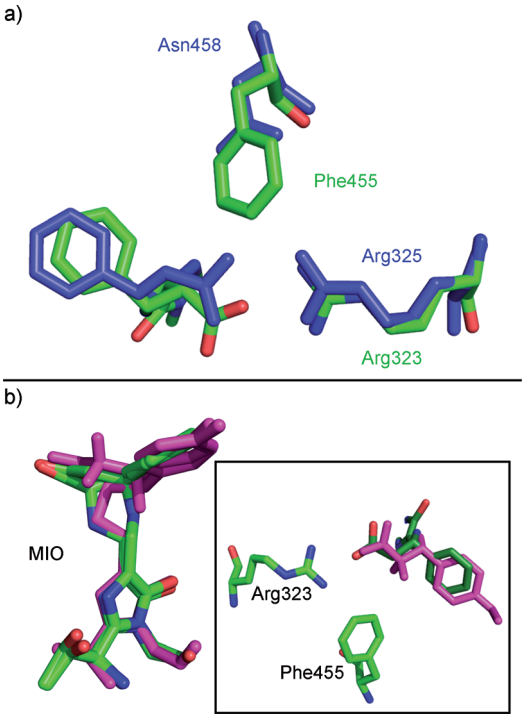

Figure 3. Comparison of *Pa*PAM with *Sg*TAM and *Tc*PAM. a) Overlay of the *Tc*PAM active site (showing the cinnamate, Arg 325, and Asn 458 (all blue)) and the *Pa*PAM active site (showing the (*S*)-β-phenylalanine/MIO adduct, Arg 323, and Phe 455 color-coded as carbon: green: oxygen: red; nitrogen: blue). b) Side view (Inset: top view) of the overlay of two *Sg*TAM active site structures containing inhibitor-MIO complexes (magenta) (inhibitors are (2*S*,3*S*)-3-(4-fluorophenyl)-2,3-dihydroxy)propanoic acid (PDB 2RJR) and α-difluoro-β-tyrosine (PDB 2QVE)) and the active site of *Pa*PAM molecule B (containing both α- and β-phenylalanine-MIO complexes).

rate of *Pa*PAM, in a 40:60 ratio, where *trans*-cinnamate prevails. These data are consistent with the hypothesis that Phe 455 is important for the proper orientation of the substrate in the active site for transfer of the amino group from the α to the β position.

Intrigued by the possibility that other class I lyase-like enzymes that catalyze the formation of the same (*S*)-β-phenylalanine produced by *Pa*PAM may exist, a BLAST[23] search was performed, and five other MIO-dependent enzymes, each from a distinct organism, were found to have a Phe residue that is equivalent to Phe 455 (Figure S2 in the Supporting Information). In each case, all of the other residues in the vicinity of the active site were also identical to those of *Pa*PAM. Of these enzymes, EncP from *Streptomyces maritimus*, was characterized as a slow phenylalanine ammonia lyase ($k_{cat} = 0.0061\ s^{-1}$) that is required for the biosynthesis of the antibiotic enterocin in this organism.[24–26] Subsequent studies, however, showed that this enzyme has much higher phenylalanine aminomutase activity (with (*S*)-β-phenylalanine as the product) than lyase activity below 50 °C.[27] Furthermore, another organism (*Vibrionales bacterium SWAT-3*) identified by the BLAST search produces andrimid, which contains an amino acid moiety that is derived from (*S*)-β-phenylalanine.[28] Taken together, this leads to the reasonable conclusion that perhaps all of these enzymes produce (*S*)-β-phenylalanine. There is no report of a metabolite that contains β-phenylalanine that has been identified thus far in the other four organisms.

In conclusion, the structure of *Pa*PAM was solved as α- and β-phenylpropanoid adducts, presumably with (*S*)-α- and (*S*)-β-phenylalanine. These intermediates provide strong evidence that *Pa*PAM reacts by an alkylamine elimination pathway (such as a Hoffmann-type or E2 elimination process), which involves covalent attachment between the amino group of the substrate and the product as well as the MIO cofactor, as demonstrated previously for *Sg*TAM.[18] The results indicate that the carbon skeleton of the (*S*)-phenylalanine substrate remains in one rotameric conformation while the exocyclic C–N bond of the amine–MIO adduct rotates into position below the α- and β-carbon atoms to complete the isomerization reaction (Figure 2 a). Thus, the structure also confirms the inversion of configuration at each migration terminus during the isomerization of the α-amino acid substrate into its β-isomer.[22]

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[1] M. Jin, M. A. Fischbach, J. Clardy, *J. Am. Chem. Soc.* **2006**, *128*, 10660.
[2] P. D. Fortin, C. T. Walsh, N. A. Magarvey, *Nature* **2007**, *448*, 824.
[3] L. Feng, U. Wanninayake, S. Strom, J. Geiger, K. D. Walker, *Biochemistry* **2011**, *50*, 2919.
[4] C. V. Christianson, T. J. Montavon, S. G. Van Lanen, B. Shen, S. D. Bruner, *Biochemistry* **2007**, *46*, 7205.
[5] D. Krug, R. Mueller, *ChemBioChem* **2009**, *10*, 741.
[6] M. J. MacDonald, G. B. D'Cunha, *Biochem. Cell Biol.* **2007**, *85*, 273.
[7] J. A. Kyndt, T. E. Meyer, M. A. Cusanovich, J. J. Van Beeumen, *FEBS Lett.* **2002**, *512*, 240.
[8] M. Baedeker, G. E. Schulz, *Eur. J. Biochem.* **2002**, *269*, 1790.
[9] H. A. Cooke, S. D. Bruner, *Biopolymers* **2010**, *93*, 802.
[10] K. R. Hanson, E. A. Havir, *Arch. Biochem. Biophys.* **1970**, *141*, 1.
[11] J. C. Calabrese, D. B. Jordan, A. Boodhoo, S. Sariaslani, T. Vannelli, *Biochemistry* **2004**, *43*, 11403.
[12] C. V. Christianson, T. J. Montavon, G. M. Festin, H. A. Cooke, B. Shen, S. D. Bruner, *J. Am. Chem. Soc.* **2007**, *129*, 15744.
[13] B. Schuster, J. Retey, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 8433.
[14] L. Poppe, J. Retey, *Angew. Chem.* **2005**, *117*, 3734; *Angew. Chem. Int. Ed.* **2005**, *44*, 3668.
[15] S. Bartsch, U. T. Bornscheuer, *Angew. Chem.* **2009**, *121*, 3412; *Angew. Chem. Int. Ed.* **2009**, *48*, 3362.
[16] T. F. Schwede, J. Rétey, G. E. Schulz, *Biochemistry* **1999**, *38*, 5355.
[17] G. V. Louie, M. E. Bowman, M. C. Moffitt, T. J. Baiga, B. S. Moore, J. P. Noel, *Chem. Biol.* **2006**, *13*, 1327.
[18] T. J. Montavon, C. V. Christianson, G. M. Festin, B. Shen, S. D. Bruner, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3099.
[19] H. Ritter, G. E. Schulz, *Plant Cell* **2004**, *16*, 3426.
[20] K. D. Dunkley, T. R. Callaway, V. I. Chalova, R. C. Anderson, M. M. Kundinger, C. S. Dunkley, D. J. Nisbet, S. C. Ricke, *Anaerobe* **2008**, *14*, 35.

[21] N. J. Turner, *Curr. Opin. Chem. Biol.* **2011**, *15*, 234.
[22] N. D. Ratnayake, U. Wanninayake, J. H. Geiger, K. D. Walker, *J. Am. Chem. Soc.* **2011**, *133*, 8531.
[23] E. W. Sayers, T. Barrett, D. A. Benson, S. H. Bryant, K. Canese, V. Chetvernin, D. M. Church, M. DiCuccio, R. Edgar, S. M. Federhen, *Nucleic Acids Res.* **2009**, *37*, D5.
[24] M. C. Moffitt, G. V. Louie, M. E. Bowman, J. Pence, J. P. Noel, B. S. Moore, *Biochemistry* **2007**, *46*, 1004.
[25] L. K. Xiang, B. S. Moore, *J. Biol. Chem.* **2002**, *277*, 32505.
[26] L. K. Xiang, B. S. Moore, *J. Bacteriol.* **2005**, *187*, 4286.
[27] T. Grecu, Masters of Philosophy Thesis, University of Manchester (UK), **2010**.
[28] J. M. Oclarit, H. Okada, S. Ohta, K. Kaminura, Y. Yamaoka, T. Iizuka, S. Miyashiro, S. Ikegami, *Microbios* **1994**, *78*, 7.

