Biosynthesis

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Unveiling the Biosynthetic Pathway of the Ribosomally Synthesized and Post-translationally Modified Peptide Ustiloxin B in Filamentous Fungi

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In memory of Miho Izumikawa

Abstract: *The biosynthetic machinery of the first fungal ribosomally synthesized and post-translationally modified peptide (RiPP) ustiloxin B was elucidated through a series of gene inactivation and heterologous expression studies. The results confirmed an essential requirement for novel oxidases possessing the DUF3328 motif for macrocyclization, and highly unique side-chain modifications by three oxidases (UstCF1F2) and a pyridoxal 5′-phosphate (PLP)-dependent enzyme (UstD). These findings provide new insight into the expression of the RiPP gene clusters found in various fungi.*

The ustiloxins, for example ustiloxin B (**1**), were isolated as phytotoxins from rice false smut caused by the pathogenic fungus *Ustilaginoidea virens* (Figure 1).[1–3] Members of this family, which includes a structurally related phomopsin (Figure 1), exhibit potent antimitotic activity and inhibit microtubule assembly.[2] Recently, a biosynthetic gene cluster (*ust*) for **1** was identified in the genome of *Aspergillus flavus* by using a sequence-motif-independent de novo detection algorithm (MIDDAS-M) for secondary-metabolite gene clusters.[4,5] Ribosomally synthesized and post-translationally modified peptides (RiPPs) are often found in the genomes of various species of bacteria.[6] In contrast, the ustiloxin gene cluster was the first example of an RiPP from filamentous fungi, while the gene for the precursor peptide of α-amanitin was identified in mushrooms (Figure 1).[7] The cluster contains a unique precursor protein (UstA) that has an unprecedented 16 repeated core peptide regions. After identification of the *ust* cluster, we found more than 94 homologous clusters in *Aspergillus* genome sequences.[8] Very recently, gene clusters for two RiPPs, epichloëcyclin and asperipin-2a, have been reported.[8,9] Herein, we report complete characterization of the formation of the core peptide by three oxidation enzymes uniquely found in the gene clusters of the filamentous fungal RiPPs, and subsequent modifications specific for ustiloxin biosynthesis.

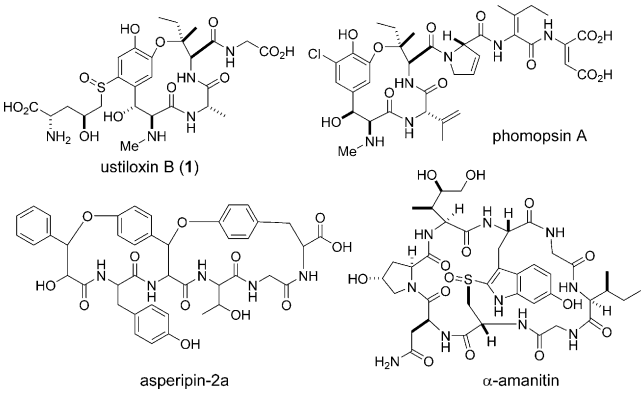

Figure 1. Representative fungal RiPPs.

On the basis of previous gene-knockout experiments, we proposed that 11 genes are responsible for ustiloxin biosynthesis.[8] Detailed LC–MS analysis of extracts from the mutants *ΔustM*, *ΔustC*, *ΔustF1*, *ΔustF2*, and *ΔustD* showed the accumulation of ustiloxin derivatives **2**–**6** with characteristic UV spectra (Figure 2, Figure S1 in the Supporting Information). Based on HR-MS data, the molecular formulae of the compounds were deduced to be **2**: $C_{20}H_{28}N_4O_8$, **3**: $C_{21}H_{30}N_4O_8$, **4**: $C_{24}H_{35}N_5O_{10}S$, **5**: $C_{24}H_{35}N_5O_{11}S$, and **6**: $C_{23}H_{34}N_4O_{10}S$. Among these metabolites, **3** and **6** were readily identified from the ^{1}H-NMR data as ustiloxin F and ustiloxin C, respectively.[2,3] Comparison of the ^{1}H-NMR spectra of **2** and **3** indicated that these are closely related, and the N-methyl signal ($\delta_H = 2.74$) in the spectrum of **3** was not present in that of **2**, thus suggesting that **2** is a desmethyl derivative of **3**. The higher molecular weights of **4**/**5** compared to **3** indicate the addition of a cysteine residue ($C_3H_5NSO_2$/$C_3H_5NSO_3$). In the NMR spectra of **4** and **5**, the lack of one of the aromatic proton present in **3** ($\delta_H = 7.04$) indicates

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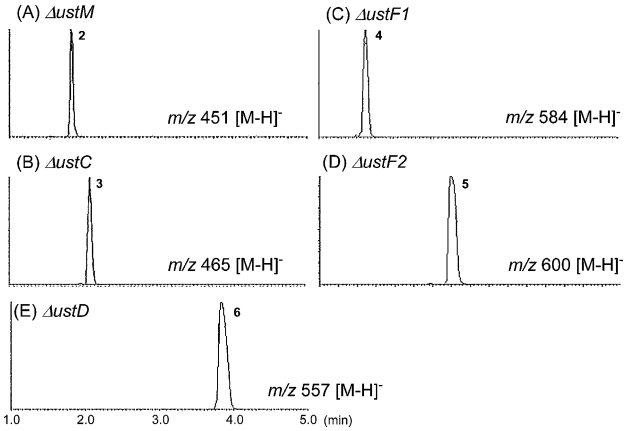


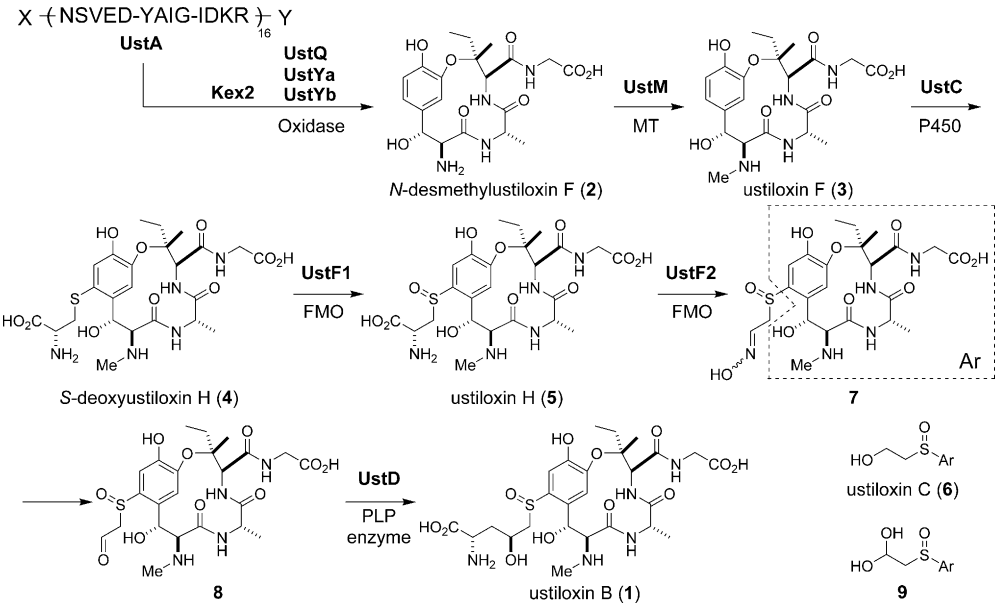
Figure 2. LC–MS profiles of the ustiloxin derivatives. A) **2** produced by *ΔustM*, B) **3** produced by *ΔustC*, C) **4** produced by *ΔustF1*, D) **5** produced by *ΔustF2*, and E) **6** produced by *ΔustD*.

substitution by a cysteine residue. Additionally, the signal patterns were nearly identical, and the shifted signals were observed only at the Tyr side chain (**4**: $\delta_H = 3.43, 3.61$ (CH_2); **5**: $\delta_H = 3.15, 3.71$ (CH_2)) and the aromatic portion (**4**: $\delta_H = 7.14, 7.27$; **5**: $\delta_H = 7.63, 7.42$), thus suggesting that **5**, which we named ustiloxin H, is a sulfoxide derivative of **4**. The proposed structures of **2**, **4**, and **5** were confirmed by extensive NMR analyses, including COSY, HSQC, and HMBC. These data enabled us to speculate on the functions of the five *ustMCF1F2D* genes. For the last transformation with UstD, condensation of a C3 nucleophile with **8**, the aldehyde form of **6**, is most likely to be involved. Taking into account these data, we proposed a biosynthetic pathway for **1** (Scheme 1).

For macrocyclization of the core peptide derived from UstA, the results from gene inactivation studies with *ustQYaYb* indicate that these genes are essential to give the first cyclization product **2**, although the disruptants did not give any detectable intermediate, unlike with modification of the genes for the reactions of the aromatic side chain. The *ustQ* gene displays homology with a tyrosinase. The *ustYa* and *ustYb* genes exhibit no homology with functionally characterized enzymes but have a common DUF3328 motif. To characterize the function of those genes, we conducted heterologous expression in *Aspergillus oryzae*.[10] We transformed the wild-type NSAR1 strain with different combinations of plasmids (pUSA2-*ustAQ* with pUARA2-*ustYa*, pUARA2-*ustYb*, or pUARA2-*ustYaYb*) to obtain the transformants AO-*ustAQYa*, AO-*ustAQYb*, and AO-*ustAQYaYb*. While AO-*ustAQYa* and AO-*ustAQYb* produced no cyclic or linear peptides, AO-*ustAQYaYb* gave **2** as the sole product (Figure 3 A–D). Additionally, incorporation of *ustYb* into AO-*ustAQYa* resulted in the production of **2** (Figure 3 E). These results further confirm the importance of the three oxidation enzymes. Specifically, the homologous oxidases UstYa and UstYb are not redundant, and both enzymes are required to afford the stable intermediate. Taken together with the results of the gene inactivation studies, we speculate that before cyclization, the UstA protein is digested into 16 trideca-/tetradecapeptides by Kex2 proteinases.[11] To generate mature cyclic peptide **2**, both the N- and C-terminal sequences of the tridecapeptide must be cleaved. Currently, however, there is no information on the protease and the timing for these cleavages.

Introduction of the *ustM* gene into AO-*ustAQYaYb* generated AO-*ustAQYaYbM*, which produced **3** in small amounts. Based on the unusual observation that the transporter deletion mutant *ΔustT* accumulated **2**, we postulated that the introduction of *ustT* may improve the yield of **3**. Indeed, the generated AO-*ustAQYaYbMT* resulted in a three-fold increased production of **3** (Figure S2). All of the ustiloxins that accumulated in the mutants have a ustiloxin F core that is essential for their ability to inhibit microtubule polymerization.[2] Although ustiloxins do not inhibit the growth of fungi, they may cause damage to the host. Our findings may aid the heterologous production of various RiPPs in a versatile host such as *A. oryzae*, which presents broad tolerance against various antibiotics and metabolites that are toxic to eukaryotic cells.[10,12]

We then turned our attention to functional analysis of the Class B bifunctional flavoprotein monooxygenases[13] UstF1 and UstF2, which participate in modification of the side chain



Scheme 1. Proposed biosynthetic pathway of ustiloxins. MT = methyltransferase, P450 = cytochrome P450 monooxygenase, FMO = flavin-containing monooxygenase.

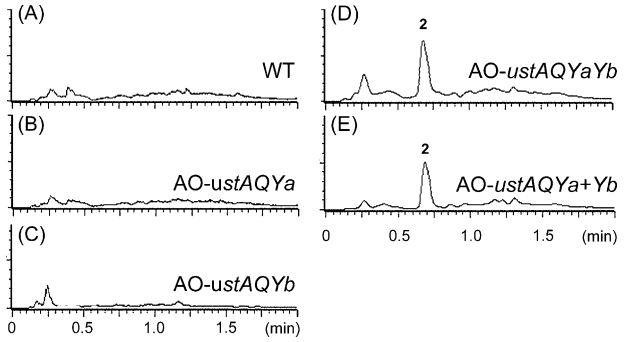
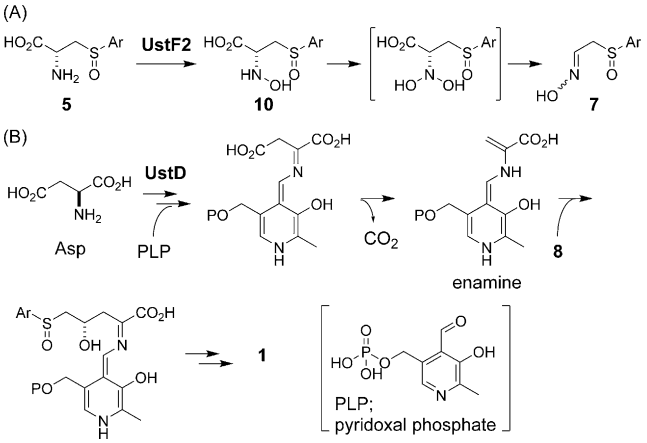


Figure 3. LC–MS profiles of extracts from *Aspergillus oryzae* transformants: A) WT strain, B) AO-*ustAQYa*, C) AO-*ustAQYb*, D) AO-*ustAQYaYb* (co-transformation), and E) AO-*ustAQYaYb* (stepwise incorporation).



Scheme 2. Proposed mechanism of A) decarboxylative dehydration catalyzed by UstF2 and B) C–C bond formation catalyzed by UstD.

of **4**. Both purified maltose binding protein (MBP)-tagged enzymes showed a yellow color and strong absorption at 450 nm in the UV/visible spectra (Figure S3), thus indicating tight binding of flavin adenine dinucleotide (FAD), as is the case for other Class B flavoproteins. Oxidation of **4** by UstF1 was examined in the presence of NADPH. LC–MS analysis of the reaction mixtures showed a new peak corresponding to **5** (Figure 4 A). Incubation of **5** with UstF2 and NADPH yielded

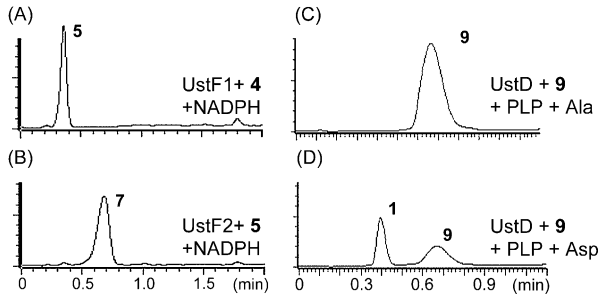


Figure 4. LC–MS profiles of the enzymatic products of the reactions of A) UstF1, B) UstF2, and C) UstD with alanine, and D) UstD with aspartic acid.

an *E*/*Z* mixture of oxime **7** (Figure 4 B), the molecular formula of which was determined to be $C_{23}H_{34}N_5O_{10}S$ by HR-MS analysis. In the ^{1}H-NMR spectrum of **7**, the α-proton of a Cys residue in **5** was missing (NMR spectra in the Supporting information), and new oxime methine signals appeared (*E* isomer (major): $\delta_H = 7.36$, *Z* isomer (minor): $\delta_H = 6.89$). Extensive NMR analyses confirmed the structure of **7**. Treatment of **7** with 0.1% trifluoroacetic acid (TFA) readily afforded the corresponding geminal diol **9**, a hydrate form of **8**. During NMR measurement of **7** and **9** in D_2O, we observed relatively rapid H/D exchange of C2′-CH_2 in the side chain owing to their acidity (Figure S4, NMR spectra in the Supporting Information). Reduction of **9** with $NaBH_4$ yielded **6**, which was identical to ustiloxin C from the *ustD* deletion mutant in all respects. To elucidate the mechanism of the formation of oxime **7** as catalyzed by UstF2, a time-course analysis was performed (Figure S5). At the beginning of the reaction, a new peak for **10** was observed along with **7** in the LC–MS sprectra. Its molecular weight (*m*/*z* 618 $[M+H]^+$) strongly suggests that **10** is a monohydroxylated intermediate of **5**. Based on these observations, we proposed a reaction mechanism involving two rounds of N-hydroxylation followed by decarboxylative dehydration to give **7** (Scheme 2 A). Similar oxime formation was reported in the biosynthesis of caerulomycin A, which involves sequential N-hydroxylations with monooxygenase CrmH followed by dehydration instead of decarboxylative dehydration as in the case of **7** (Scheme S1 in the Supporting Information).[14]

Considering that the last transformation is catalyzed by the pyridoxal 5′-phosphate (PLP)-dependent enzyme UstD, we speculated that a C3 nucleophile, such as an enamine derived from alanine or aspartate, likely reacts with the putative aldehyde intermediate **8** to give **1**. Therefore, we incubated **9** with MBP-tagged UstD in the presence of PLP and amino acids. In the case of aspartic acid, formation of **1** was observed (Figure 4 C–D), while in the absence of **9**, we detected a peak for dansylated alanine by HPLC after treatment of the reaction mixture with dansyl chloride (Figure S6). These results indicate that UstD catalyzes decarboxylation of aspartate to yield an enamine followed by condensation with aldehyde **8** to give **1** (Scheme 2 B). Although the mechanism of aspartate β-decarboxylase has been established in various studies, including by X-ray crystallography (Scheme S2),[15] to our knowledge, condensation of the resultant enamine with an aldehyde in the same active site has not been reported before.

For the formation of the first cyclization product **2**, three oxidation steps are required: 1) hydroxylation at the benzylic position, 2) hydroxylation at either the aromatic ring of Tyr or β-position of Ile, and 3) oxidative cyclization. Based on the putative function of tyrosinase, UstQ may catalyze the oxidation of a phenol moiety, whereas the uncharacterized DUF3328 proteins UstYa and UstYb are most likely responsible for the remaining two-step oxidations. Very recently, a new RiPP, asperipin-2a, which possesses two macrocyclic ether rings, was reported.[8] In its biosynthetic gene cluster, which consists of four genes, the UstY homologue was found to be the sole oxidation enzyme. To date, only three gene clusters for the ribosomal peptides RiPPs have been charac-

terized.[5,8,9] However, a set of biosynthetic genes encoding a precursor peptide and UstY homologue have been identified in many fungal genomes.[8,9] This circumstantial evidence suggests that the most intriguing macrocyclization of the core peptide is catalyzed by a UstY homologue.

In summary, we have unveiled the entire biosynthetic pathway of ustiloxin, which involves nine enzymes, through gene inactivation, heterologous expression, and in vitro functional analyses. In particular, the heterologous expression data were very useful for elucidating the key role of UstA/Q/Ya/Yb in the macrocyclic formation of ustiloxins, thus suggesting that this method can be applied to the functional analysis of fungal ribosomal peptides RiPPs. In addition, functional analysis of the enzymes responsible for the side-chain modifications revealed unique oxidations of sulfur and nitrogen atoms (by two homologous FAD-dependent enzymes, UstF1 and UstF2) and decarboxylative C–C bond formation (by the PLP-dependent enzyme UstD). Currently, the unprecedented oxidative cyclization of the core peptide catalyzed by UstQYaYb is under investigation.

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[1] Y. Koiso, M. Natori, S. Iwasaki, S. Sato, R. Sonoda, Y. Fujita, H. Yaegashi, Z. Sato, *Tetrahedron Lett.* **1992**, *33*, 4157–4160.
[2] Y. Koiso, Y. Li, S. Iwasaki, K. Hanaoka, T. Kobayashi, R. Sonoda, Y. Fujita, H. Yaegashi, Z. Sato, *J. Antibiot.* **1994**, *47*, 765–773.
[3] Y. Koiso, N. Morisaki, Y. Yamashita, Y. Mitsui, R. Shirai, Y. Hashimoto, S. Iwasaki, *J. Antibiot.* **1998**, *51*, 418–422.
[4] M. Umemura, H. Koike, N. Nagano, T. Ishii, J. Kawano, N. Yamane, I. Kozone, K. Horimoto, K. Shin-ya, K. Asai, J. J. Yu, J. W. Bennett, M. Machida, *PLoS One* **2013**, *8*, e84028.
[5] M. Umemura, N. Nagano, H. Koike, J. Kawano, T. Ishii, Y. Miyamura, M. Kikuchi, K. Tamano, J. J. Yu, K. Shin-ya, M. Machida, *Fungal Genet. Biol.* **2014**, *68*, 23–30.
[6] P. G. Arnison, M. J. Bibb, G. Bierbaum, A. A. Bowers, T. S. Bugni, G. Bulaj, J. A. Camarero, D. J. Campopiano, G. L. Challis, J. Clardy, P. D. Cotter, D. J. Craik, M. Dawson, E. Dittmann, S. Donadio, P. C. Dorrestein, K. D. Entian, M. A. Fischbach, J. S. Garavelli, U. Goransson, C. W. Gruber, D. H. Haft, T. K. Hemscheidt, C. Hertweck, C. Hill, A. R. Horswill, M. Jaspars, W. L. Kelly, J. P. Klinman, O. P. Kuipers, A. J. Link, W. Liu, M. A. Marahiel, D. A. Mitchell, G. N. Moll, B. S. Moore, R. Muller, S. K. Nair, I. F. Nes, G. E. Norris, B. M. Olivera, H. Onaka, M. L. Patchett, J. Piel, M. J. T. Reaney, S. Rebuffat, R. P. Ross, H. G. Sahl, E. W. Schmidt, M. E. Selsted, K. Severinov, B. Shen, K. Sivonen, L. Smith, T. Stein, R. D. Sussmuth, J. R. Tagg, G. L. Tang, A. W. Truman, J. C. Vederas, C. T. Walsh, J. D. Walton, S. C. Wenzel, J. M. Willey, W. A. van der Donk, *Nat. Prod. Rep.* **2013**, *30*, 108–160.
[7] a) H. E. Hallen, H. Luo, J. S. Scott-Craig, J. D. Walton, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 19097–19101; b) H. Luo, H. E. Hallen-Adams, J. S. Scott-Craig, J. D. Walton, *Fungal Genet. Biol.* **2012**, *49*, 123–129.
[8] N. Nagano, M. Umemura, M. Izumikawa, J. Kawano, T. Ishii, M. Kikuchi, K. Tomii, T. Kumagai, A. Yoshimi, M. Machida, K. Abe, K. Shin-ya, K. Asai, *Fungal Genet. Biol.* **2016**, *86*, 58–70.
[9] R. D. Johnson, G. A. Lane, A. Koulman, M. Cao, K. Fraser, D. J. Fleetwood, C. R. Voisey, J. M. Dyer, J. Pratt, M. Christensen, W. R. Simpson, G. T. Bryan, L. J. Johnson, *Fungal Genet. Biol.* **2015**, *85*, 14–24.
[10] a) R. Fujii, A. Minami, T. Tsukagoshi, N. Sato, T. Sahara, S. Ohgiya, K. Gomi, H. Oikawa, *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1813–1817; b) Y. Ye, A. Minami, A. Mandi, C. Liu, T. Taniguchi, T. Kuzuyama, K. Monde, K. Gomi, H. Oikawa, *J. Am. Chem. Soc.* **2015**, *137*, 11846–11853; c) C. Liu, K. Tagami, A. Minami, T. Matsumoto, J. C. Frisvad, H. Suzuki, J. Ishikawa, K. Gomi, H. Oikawa, *Angew. Chem. Int. Ed.* **2015**, *54*, 5748–5752; *Angew. Chem.* **2015**, *127*, 5840–5844; d) A. Minami, C. Liu, H. Oikawa, *Heterocycles* **2016**, *92*, 397–421; e) T. Ugai, A. Minami, R. Fujii, M. Tanaka, K. Gomi, H. Oikawa, *Chem. Commun.* **2015**, *51*, 1878–1881.
[11] a) N. C. Rockwell, D. J. Krysan, T. Komiyama, R. S. Fuller, *Chem. Rev.* **2002**, *102*, 4525–4548; b) A. Yoshimi, M. Umemura, N. Nagano, H. Koike, M. Machida, K. Abe, *AMB Express* **2016**, *6*, 9.
[12] a) A. Kovalchuk, A. J. M. Driessen, *BMC Genomics* **2010**, *11*, 177; b) M. Machida, K. Asai, M. Sano, T. Tanaka, T. Kumagai, G. Terai, K. Kusumoto, T. Arima, O. Akita, Y. Kashiwagi, K. Abe, K. Gomi, H. Horiuchi, K. Kitamoto, T. Kobayashi, M. Takeuchi, D. W. Denning, J. E. Galagan, W. C. Nierman, J. Yu, D. B. Archer, J. W. Bennett, D. Bhatnagar, T. E. Cleveland, N. D. Fedorova, O. Gotoh, H. Horikawa, A. Hosoyama, M. Ichinomiya, R. Igarashi, K. Iwashita, P. R. Juvvadi, M. Kato, Y. Kato, T. Kin, A. Kokubun, H. Maeda, N. Maeyama, J. Maruyama, H. Nagasaki, T. Nakajima, K. Oda, K. Okada, I. Paulsen, K. Sakamoto, T. Sawano, M. Takahashi, K. Takase, Y. Terabayashi, J. R. Wortman, O. Yamada, Y. Yamagata, H. Anazawa, Y. Hata, Y. Koide, T. Komori, Y. Koyama, T. Minetoki, S. Suharnan, A. Tanaka, K. Isono, S. Kuhara, N. Ogasawara, H. Kikuchi, *Nature* **2005**, *438*, 1157–1161.
[13] a) W. J. H. van Berkel, N. M. Kamerbeek, M. W. Fraaije, *J. Biotechnol.* **2006**, *124*, 670–689; b) K. Crozier-Reabe, G. R. Moran, *Int. J. Mol. Sci.* **2012**, *13*, 15601–15639.
[14] Y. Zhu, Q. Zhang, S. Li, Q. Lin, P. Fu, G. Zhang, H. Zhang, R. Shi, W. Zhu, C. Zhang, *J. Am. Chem. Soc.* **2013**, *135*, 18750–18753.
[15] a) S. S. Tate, A. Meister, *Adv. Enzymol. Relat. Areas Mol. Biol.* **1971**, *35*, 503–543; b) H.-J. Chen, T.-P. Ko, C.-Y. Lee, N.-C. Wang, A. H.-J. Wang, *Structure* **2009**, *17*, 517–529.

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