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Ribosomal Peptide Biosynthesis

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Unveiling the biosynthetic pathway of the representative ribosomal peptide ustiloxin B in filamentous fungi

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Abstract: The biosynthetic machinery of the first fungal ribosomal peptide ustiloxin B was elucidated through a series of gene inactivation and heterologous expression studies. The results confirmed the essential requirement of novel oxidases possessing the DUF3328 motif for macrocyclization and highly unique side chain modifications by three oxidases (UstCF1F2) and a PLP-dependent enzyme (UstD). These findings provide a new insight for biosynthesis of ribosomal peptide gene clusters found in various fungi.

Ustiloxins represented by B (**1**) were isolated as phytotoxins from rice false smut caused by the pathogenic fungus *Ustilaginoidea virens* (Figure 1).[1-3] These family members, including a structurally related phomopsin (Figure 1), exhibit potent antimitotic activity and inhibit microtubule assembly.[2] Recently, a biosynthetic gene cluster (*ust*) of **1** was identified in the genome of *Aspergillus flavus* 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 the filamentous fungi, while the precursor peptide gene for α-amanitin was identified in mushrooms (Figure 1).[7] The cluster contained 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 the *Aspergilli* genome sequences.[8] Very recently, gene clusters of new ribosomal peptides, epichloëcyclin and asperipin-2a, have been reported.[8,9] In this communication, we report the complete characterization of the core peptide formation with three oxidation enzymes uniquely found in the gene clusters of the filamentous fungal RiPPs and the subsequent modifications specific for ustiloxin family biosynthesis.

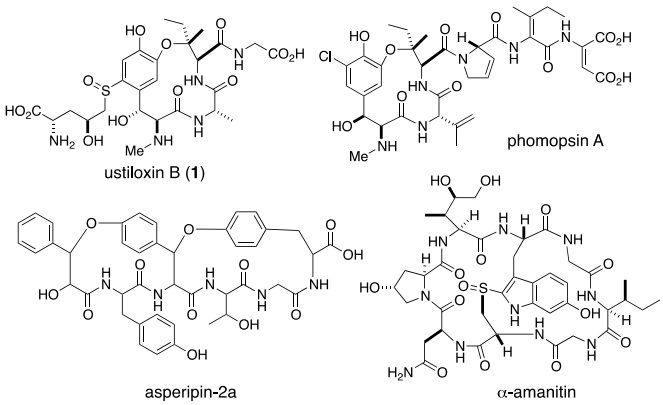

Figure 1. Fungal ribosomal peptides.

On the basis of previous gene knockout experiments, we proposed that 11 genes were responsible for ustiloxin biosynthesis.[8] Detailed LC-MS analysis of the extracts from five mutants, *ΔustM*, *ΔustC*, *ΔustF1*, *ΔustF2*, and *ΔustD*, showed the accumulation of ustiloxin derivatives (**2**-**6**) with characteristic UV spectra (Figure 2, Figure S1). Based on HR-MS data, the molecular formulae of the compounds were deduced as follows: **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 as ustiloxin F and ustiloxin C, respectively, from the ^{1}H-NMR data.[2,3] Comparison of the ^{1}H-NMR spectra of **2** and **3** indicated that these were closely related and that the *N*-methyl signal (δ_H 2.74) in the spectrum of **3** was not present in that of **2**, suggesting that **2** is a desmethyl derivative of **3**. The higher molecular weights of **4**/**5** compared to **3** indicated 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 (**3**: δ_H 7.04) present in **3** indicated the 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**: δ_H 3.43, 3.61 (CH_2); **5**: δ_H 3.15, 3.71 (CH_2)) and the aromatic portion (**4**: δ_H 7.14, 7.27; **5**: δ_H 7.63, 7.42), suggesting that **5** is a sulfoxide derivative of **4**, which we named as ustiloxin H. The proposed structures of **2**, **4**, and **5**

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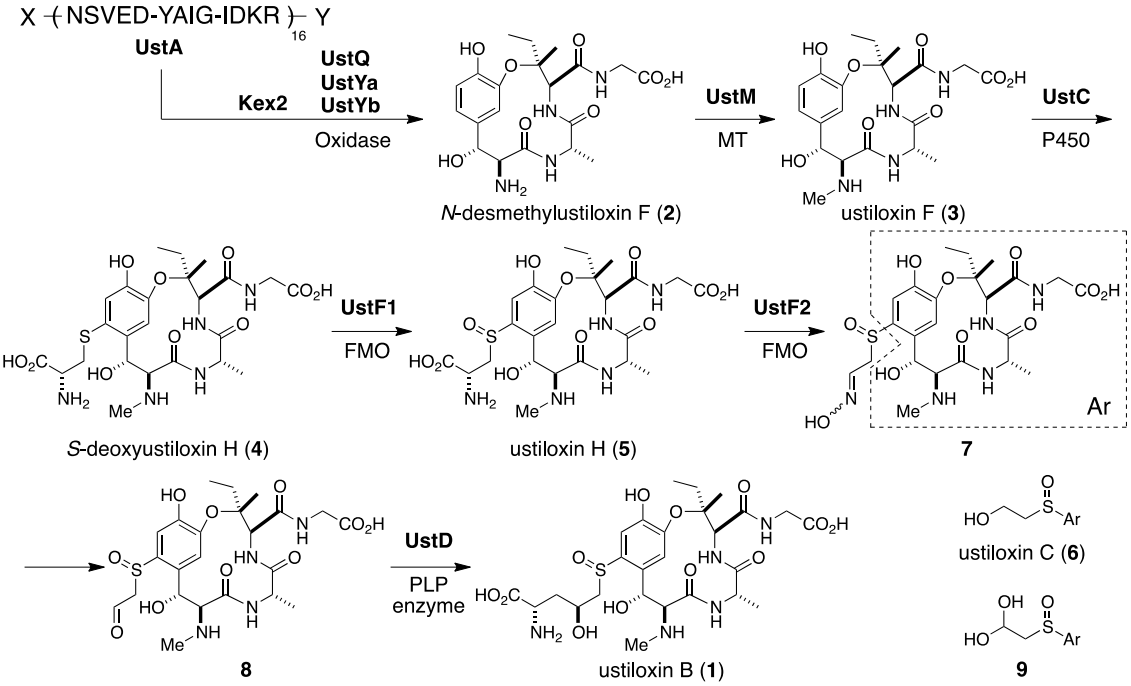
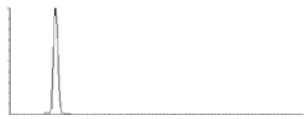


Scheme 1. Proposed biosynthetic pathway of ustiloxin.



) *ΔustD*

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*, (E) **6** produced by *ΔustD*.

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 an aldehyde form **8** of **6** is most likely to be involved. Taking into account these data, we proposed a biosynthetic pathway for **1** as shown in Scheme 1.

For the macrocyclization of the core peptide derived from UstA, the results from the gene inactivation studies on *ustQYaYb* indicated that these genes are essential to give the first cyclization product **2**, although these disruptants did not give any detectable intermediate unlike with the 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 a heterologous expression study in *Aspergillus oryzae*.[10] When we transformed the wild-type NSAR1 strain using different combinations of plasmids (pUSA2-*ustAQ* and pUARA2-*ustYa*/ pUARA2-*ustYb*/pUARA2-*ustYaYb*), three transformants, AO-*ustAQYa*, AO-*ustAQYb*, and AO-*ustAQYaYb*, were obtained. While AO-*ustAQYa* and AO-*ustAQYb* produced no cyclic or linear peptides, AO-*ustAQYaYb* gave **2** as a sole product (Figures 3A-3D). Additionally, incorporation of *ustYb* into AO-*ustAQYa* resulted in the production of **2** (Figure 3E). These results further confirmed the importance of the three oxidation enzymes. Specifically, the homologous oxidases UstYa and UstYb were not redundant, and both enzymes are required to afford the stable intermediate. Taken together with the results of the gene inactivation studies, we speculated that before cyclization, the UstA protein is digested into 16 pieces of trideca-/tetradeca-peptides 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.

The introduction of the *ustM* gene into AO-*ustAQYaYb* generated AO-*ustAQYaYbM*, which produced **3** in a small amount. 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 the ustiloxins accumulated in the mutants have a ustiloxin F core that is essential for the ability to inhibit microtubule polymerization.[2] Although ustiloxins do not inhibit the growth of

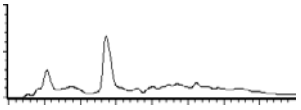


Figure 3. LC-MS profiles of extracts from transformants: (A) WT strain, (B) AO-*ustAQYa*, (C) AO-*ustAQYb*, (D) AO-*ustAQYaYb* (co-transformation), and (E) AO-*ustAQYaYb* (stepwise incorporation).

fungi, they may cause damage to the host. Our findings may aid in the heterologous production of various ribosomal peptides in a versatile host such as *A. oryzae*, which presents a wide range of tolerance against various antibiotics and metabolites that are toxic to eukaryotic cells. [10,12]

We then turned our attention to the functional analysis of Class B bifunctional flavoprotein monooxygenases,[13] UstF1 and UstF2, which participate in the side chain modifications 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), indicating a tight binding of a FAD as is the case for other Class B flavoproteins. Oxidation of **4** with UstF1 was examined in the presence of NADPH. LC-MS analysis of the reaction mixtures showed a new peak corresponding to **5** (Figure 4A). Incubation of **5** with UstF2 and NADPH yielded two isomeric mixtures of oximes **7** (Figure 4B), whose molecular formula 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); δ_H 7.36, *Z* isomer (minor); δ_H 6.89). Extensive NMR analyses confirmed the structure of **7**. Treatment of **7** with 0.1% 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 due 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 formation mechanism of oxime **7** catalyzed by UstF2, a time course analysis was then performed (Figure S5). At the beginning of the reaction, a new peak of **10** was observed along with **7** in the LC-MS analysis. Its molecular weight (*m/z* 618 $[M+H]^+$) strongly suggested 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 2A). Similar oxime formation was reported in caerulomycin A biosynthesis, which involves sequential *N*-hydroxylations with monooxygenase CrmH followed by dehydration instead of decarboxylative dehydration in the case of **7** (Scheme S1).[14]

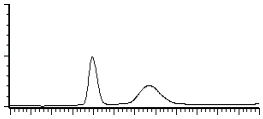
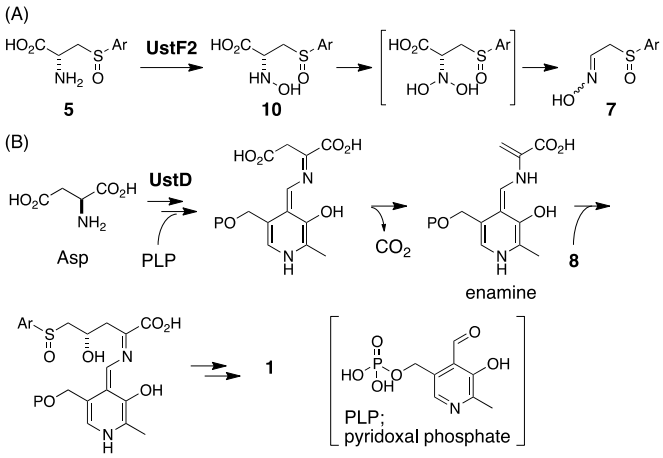


Figure 4. LC-MS profiles of the enzymatic reaction products of (A) UstF1 reaction, (B) UstF2 reaction, (C) UstD reaction with alanine, and (D) UstD reaction with aspartic acid.

Considering that the last transformation is catalyzed by the PLP-dependent enzyme UstD, we speculated that a C3 nucleophile, such as an enamine derived from alanine or aspartate, likely reacts with a putative aldehyde intermediate **8** to give **1**. Therefore, we incubated **9** with a MBP-tagged UstD in the presence of PLP and amino acids. In the case of aspartic acid, formation of **1** was observed (Figures 4C-4D), while in the absence of **9,** we detected a peak of a dansylated alanine in the HPLC analysis after treatment of the reaction mixture with dansyl chloride (Figure S6). These results indicated that UstD catalyzed a decarboxylation of aspartate to yield an enamine followed by condensation with aldehyde **8** to give **1**, as shown in Scheme 2B. Although the mechanism of aspartate β-decarboxylase has been established in various studies including X-ray crystal analysis (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: i) hydroxylation at the benzylic position, ii) hydroxylation at either the aromatic ring of Tyr or β-position of Ile, and iii) 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 ribosomal peptide, asperipin-2a, possessing two macrocyclic ether rings was reported.[8] In its biosynthetic gene cluster consisting of four genes, the UstY homolog was found to be the sole oxidation enzyme. To date, only three gene clusters for the ribosomal peptides have been characterized.[5,8,9] However, a set of biosynthetic genes encoding a precursor peptide and UstY homolog 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 homolog.



Scheme 2. Proposed mechanism of (A) UstF2 catalyzing decarboxylative dehydration and (B) UstD catalyzing C-C bond formation.

In summary, we unveiled the entire biosynthetic pathway of ustiloxin involving nine enzymes utilizing 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, suggesting that this method can be applied for the functional analysis of fungal ribosomal peptides. In addition, functional analysis of the side chain modification enzymes revealed the unique oxidations of sulfur and nitrogen atoms (two homologous-FAD dependent enzymes, UstF1 and UstF2) and decarboxylative C-C bond formation (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; 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 Derkel, 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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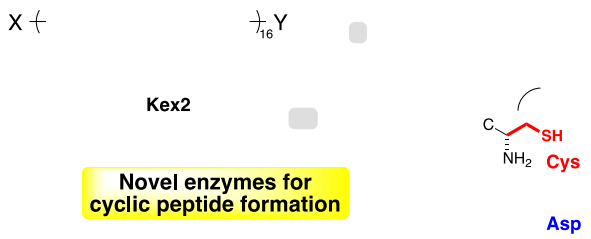

The biosynthetic machinery of fungal ribosomal peptide ustiloxin has been elucidated by gene inactivation, heterologous expression, and in vitro studies. These included the cyclic peptide formation catalysed by novel oxidases harbouring DUF3328 motif and unique modification reactions for construction of the side chain on the aromatic ring. These findings set a new stage for elucidating the fungal ribosomal peptide biosynthesis.