Elucidation of ustilaginoidin biosynthesis reveals a previously unrecognised class of ene-reductases†

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Ustilaginoids are a type of mycotoxin featuring a dimeric naphtho-γ-pyrene skeleton, produced by the rice false smut pathogen *Ustilaginoidea virens*. Here we used gene disruption, heterologous expression in *Aspergillus oryzae*, feeding experiments, and *in vitro* experiments to fully elucidate the biosynthesis of ustilaginoids. A new route to dimeric 2,3-unsaturated naphtho-γ-pyrones via dimerization of YWA1 (and 3-methyl YWA1) followed by dehydration was discovered. Intriguingly, the reduction of the 2,3-double bond of the pyrene ring was catalyzed by a phospholipid methyltransferase-like enzyme (UsgR). The reductase was specific for reduction of monomeric, linear naphtho-γ-pyrenones, but not for the dimers. Atroposelective coupling of various monomers by the laccase (UsgL) led to diverse ustilaginoids. Moreover, 3-epimerism of the 3-methyl-2,3-dihydro-naphtho-γ-pyrones adds additional complexity to the biosynthesis.

Intriguingly, the stereoselective reduction of the 2,3-double bond of 4H-benzo[g]chromen-4-one was catalyzed by a distinct class of ene-reductases not found previously. The biosynthesis of ustilaginoids was reconstructed in *A. oryzae* and *in vitro* by using various monomers with the laccase.

Results

Deletion of various genes in the *usg* BGC

The *usg* BGC comprises genes encoding a non-reducing polyketide synthase (nrPKS, *wpks1*), a putative dehydrase (*usgD*), a C-methyltransferase (C-MeT, *usgM*), a laccase (*usgL*), a putative flavin-dependent oxidoreductase (*usgO*), and several proteins of unknown function (Fig. 1A). Interestingly, *UsgM* has a ψ-ACP-MT didomain, as multiple sequence alignment revealed that the phosphopantetheine modification site in this ACP domain is DPL, which differs from the classic DSL sequence (Fig. 1C and ESI Fig. S1†). Such a didomain was also found in the trans-acting C-MeT (TlnC) that was involved in the biosynthesis of tricholignan A,13 in which ψ-ACP was crucial for methylation.

Using the CRISPR/Cas9-mediated gene knock-out method,18 we successively deleted various genes in the *usg* BGC. Deletion of *wpks1* completely abolished the production of ustilaginoids (Fig. 2(i)), while deletion of *usgM*, encoding the trans-acting C-methyltransferase, led to the accumulation of *usg* F (18), G (11), and A (10) (Fig. 2(ii)), all without a 3-methyl substitution. Deletion of the laccase-encoding gene (*usgL*) resulted in the production of semi-ustilaginoids, i.e., hemiustilaginoidin F (5), ephemiustilaginoidin D (6), and hemiustilaginoidin D (7) (Fig. 2(iii)). The chemical profiles of these deleted mutants were in good agreement with those in previous reports.10,11

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suggesting the role of these genes in the biosynthesis of ustilaginoids. Heterologous expression of the nrPKS in A. aculeatus revealed that YWA1 (1) was the first metabolite in ustilaginoidins biosynthesis; however, the dehydration from YWA1 or its derivative to form the Δ₂-derivatives, and subsequent reduction to yield the 2,3-dihydrogenated derivatives were unclear yet. Thus, we set forth to delete other putative genes in the BGC.

Deletion of the putative dehydratase gene (usgD) led to the accumulation of usg K (21), L (15), and M (17), in addition to the minor congeners including usg N (13) and D (16), and an unknown metabolite (22, Rt 12.2 min, MW 560, vide infra) (Fig. 2(vii)). In contrast, usg D (16) and E (12), the 2,3-reduced congeners of 21 and 15, respectively, are present as the two dominant metabolites in the wild type (WT) (Fig. 2(xii)). This was unexpected, and it seemed that usgD played a role in Δ₂-reduction.

Meanwhile, deletion of usgR, which encodes a protein of unknown function (belonging to the phospholipid methyltransferase superfamily, Fig. S2†), resulted in the accumulation of the Δ₂-type of ustilaginoids (i.e., 10, 15, and 21) (Fig. 2(viii)). Interestingly, when both usgR and usgL were deleted, the ΔusgR/ΔusgL mutant accumulated only 2,3-unsaturated, monomeric naphtho-γ-pyrones (2 and 4) (Fig. 2(iv)). Their structures were elucidated as norrubrofusarin (2) and 3-methyl-norrubrofusarin (4) by UV, MS, and NMR analysis (Fig. S30–S33 and S41–S44; Tables S11 and S13†). Thus, UsgR was deduced to be the Δ₂-reductase. Consistent with this, further deletion of usgR on the ΔusgM background led to the production of 10 as the sole ustilaginoidin metabolite (Fig. 2(xi)). In addition, the reduction
was quite efficient, as only the reduced monomers can be seen in the ΔusgL (Fig. 2(iii)) or ΔusgM/ΔusgL mutant (Fig. 2(vi)), and the reduced dimers were dominant in the WT (Fig. 2(xii)).

As an apparent reductive role for usgD could not be ruled out, several double deletion experiments involving this gene were conducted (Fig. 2(v), (ix) and (x)). Interestingly, when the laccase encoding gene was deleted, the double-deletion mutant (ΔusgD/ΔusgL) further produced 3-methyl-YWA1 (3) as a major compound, and three minor peaks of YWA1 (1) and 2 (m/z 531.08 [M−H]− and 533.09 [M + H]+), respectively. Compound 9 was purified and characterized by NMR as a 1/2-heterodimer (Fig. S33–S35 and Table S14†). However, an attempt to isolate 8 for NMR analysis failed, as it was quickly converted to the dehydrated products (9 and 10) after workup. Based on the HRMS and UV data, and its relationship to 10, the structure of 8 was proposed (Scheme 1). Similarly, we found that the minor metabolite (22 in ΔusgD (Fig. 2(vii)) was quickly converted to usg K (21) upon isolation. Based on this relationship, together with the HRMS data, the structure of 22 was characterized as a 3/4-heterodimer. This suggested that the laccase can dimerize YWA1 and its 3-methyl congener, and dehydration could happen in the dimeric YWA1 or its analogue without usgD (Scheme 1). The third double mutant (ΔusgR/ΔusgD) produced four monomeric naphtho-γ-pyrones (1–4) (Fig. 2(ix)), albeit in minor amounts, and dimer 22, compared to that of ΔusgR (Fig. 2(viii)). It seems that the dimerization was not 100% completed without both usgD and usgR, as there were no monomeric congeners found in either the ΔusgD or ΔusgR strain (Fig. 2(vii) and (viii)). Similarly, monomers were uncovered in the ΔusgM/ΔusgD mutant (Fig. 2(x)), but not in the ΔusgM/ΔusgR mutant (Fig. 2(xi)).

When comparing the profiles of ΔusgD/ΔusgL and ΔusgD (Fig. 2(v) and (vii)), one can see that the abundant peaks in the

Scheme 1  Biosynthesis of the dimeric naphtho-γ-pyrones revealed by gene-deletion experiments and heterologous expression in A. oryzae.
former, except 3, were for the reduced monomer (7), while in the latter, the abundant one was a dimer of $\Delta^2$-7 (i.e., usg K (21)). In contrast, in the WT (Fig. 2[xii]), the 2,3-reduced dimers, i.e., usg E (12) and usg D (16), are dominant. This suggested that the reduction for $\Delta^2$-monomers was quite efficient (Fig. 2[v]), but not for the $\Delta^2$-dimers (Fig. 2[vii]). The formation of 2,3-unsaturated metabolites indicates that dehydration happens without usg D, albeit slower. The accumulation of $\Delta^2$-dimers in $\Delta$usgD (Fig. 2[vii]) indicates an alternative route to the $\Delta^2$-dimers starting from dimeric YWA1 and its 3-methyl derivatives via sequential dehydration (Scheme 1, top), as otherwise, this mutant should enrich reduced dimers if it follows the route shown in the left side of Scheme 1.

Interestingly, deletion of the putative FAD dependent oxidoreductase encoding gene usgO did not change the metabolite profile compared to the WT (Fig. S14†). This was in contrast to the previous report, in which the $\Delta$usgO mutant still produced all the ustilaginoidins compared to the WT, but the ratio of semi-reduced usg vs. the fully reduced counterparts (i.e., usg N vs. E; usg M vs. D) increased, so a reductive role of usgO was proposed. The discrepancy might result from the different medium used (rice and PSB media in this study vs. PSA in the literature†). The heterologous expression in A. oryzae and feeding experiments (vide infra) has unambiguously excluded the catalytic role of usgO in ustilaginoidin biosynthesis.

**Heterologous reconstruction of the ustilaginoidin biosynthesis in A. oryzae NSAR1**

To verify the function of the usg genes, we carried out a heterologous expression of combinations of the usg genes in A. oryzae NSAR1 (Table 1 and Fig. 3). All gene fragments were amplified from cDNA and cloned into fungal expression vectors (see ESI Fig. S8†). Expression of the uvpks1 alone (Table 1, entry 1) resulted in the production of YWA1 (1) as the major compound, as well as its dehydrated derivative norrubrofusarin (2) in a smaller amount (Fig. 3, EXP1). It is unknown whether the dehydration was catalyzed by the dehydratase of the host, or non-enzymatically. Further expression of the dehydratase (usgD) in A. oryzae (Table 1, entry 2) led to an almost complete turnover to norrubrofusarin (2) (Fig. 3, EXP2), revealing that this DH was highly efficient. Co-expressing uvpks1 and C-Met usgM in A. oryzae (Table 1, entry 4) resulted in the observation of two new metabolites (3 and 4) compared to that of EXP1. LC-MS analysis revealed that they were 3-methyl derivatives of 1 and 2, respectively (Fig. 3, EXP4). Likewise, adding the DH to the previous set of genes in A. oryzae (Table 1, entry 8) converted the hemiketals (1 and 3) to the dehydrated congeners (2 and 4) almost quantitatively (Fig. 3, EXP8).

![HPLC-MS analysis of the expression of usg genes in A. oryzae. EXP1, 16: UV (λ = 280 nm) traces of extracts of A. oryzae transformants. EXP refers to the gene combinations in Table 1. * = unrelated peaks. For EXP10, EXP12 and EXP15, 4 and 7 were resolved by using a different column (Fig. S18†). For EXP13, peaks of 18, 11, and 10 were resolved likewise (Fig. S19†).](image)

**Table 1** Combinations of usg genes expressed in A. oryzae

| Uvpks1 | UsgD | UsgR | UsgM | UsgL | UsgO |
|--------|-----|------|------|------|------|
| EXP | PKS | DH | Red | MeT | Laccase | Redox | Product |
| 1 | ✓ | — | — | — | — | — | —
| 2 | ✓ | ✓ | — | — | — | — | —
| 3 | ✓ | — | ✓ | — | — | — | —
| 4 | ✓ | — | — | ✓ | — | — | —
| 5 | ✓ | — | — | — | ✓ | — | —
| 6 | ✓ | — | — | — | — | ✓ | —
| 7 | ✓ | ✓ | — | — | — | — | —
| 8 | ✓ | ✓ | ✓ | — | — | — | —
| 9 | ✓ | ✓ | — | — | — | — | —
| 10 | ✓ | — | — | ✓ | — | — | —
| 11 | ✓ | — | — | — | ✓ | — | —
| 12 | ✓ | ✓ | ✓ | — | — | — | —
| 13 | ✓ | ✓ | — | — | — | — | —
| 14 | ✓ | — | ✓ | — | — | — | —
| 15 | ✓ | — | — | ✓ | — | — | —
| 16 | ✓ | ✓ | ✓ | — | — | — | —

* Trace amount.
a reductase. Intriguingly, the reduction was highly stereoselective. The absolute configurations of 5–7 were determined previously, and all have a 2R configuration. Regarding the 3-methyl substrate, this enzyme predominantly gave a 2,3-trans-dimethyl product (7), in addition to a minor amount of 2,3-cis product (6).

Although there was neither a YWA1 (1) nor 3-methyl-YWA1 (3) moiety found in the reported ustilaginoids, we hypothesized that they could be dimerized by the laccase. Indeed, when both wuks1 and usgL were co-expressed in A. oryzae (Table 1, entry 5), we observed three dimers including the 1/1-homodimer (8), 1/2-heterodimer (9), and 2/2-homodimer (10), in addition to the monomers (1 and 2) (Fig. 3, EXP5). Again, adding the DH to the above combination (EXP9, Fig. 3) led to the enrichment of the dehydrated monomer (2) and dimer (10), as expected.

In EXP13, combining the PKS, DH, Red, and laccase resulted in the production of the monomers (2 and 5), and the dimeric metabolites (9–11 and 18) building from 2, 5, and 1 (Fig. 3). Meanwhile, expressing all the usg genes except the reductase usgR (EXP14) led to the almost exclusive production of Δ2-metabolites, including the monomers (2 and 4), and the 2/2-homodimer (10), 2/4-heterodimer (15), and 4/4-homodimer (21) (Fig. 3).

Finally, heterologous expression of all five genes (Table 1, entry 16) led to the observation of a variety of ustilaginoids (10–18), as well as the monomers 2, 4, 5, and 7. The identity of the ustilaginoids was confirmed by co-chromatography with the standards isolated previously by our group.

The putative FAD-dependent oxidoreductase, usgO, was also co-expressed with key usg genes (Table 1, EXP6, 11, and 15) in A. oryzae to confirm our deduction from the knockout experiments. Indeed, UsgO does not function as a tailoring enzyme, and as can be seen from Fig. 3 (EXP6, 11, and 15), the metabolite profiles did not change compared to those of EXP1, EXP5, and EXP12, respectively. However, when usgO was present, the ratio of the dehydrated product (2) to its precursor (1) was significantly decreased, compared to those without usgO (Fig. 3, EXP6 vs. EXP1, and EXP11 vs. EXP5, all lacking a DH in the combinations), especially in EXP11, where the 1/1-homodimer (8) increased, but the 2/2-homodimer (10) decreased, probably resulting from the difference in availability of 1 and 2. In EXP15, however, the metabolite profile was the same as that of EXP12. It seems that UsgO might have inhibited side dehydration when the DH was absent.

Meanwhile, we fed 2 and 4 to A. oryzae expressing usgO, respectively, and no conversion was found (Fig. S12†). This again indicated that UsgO was not a reductase. Furthermore, the semi-reduced ustilaginoids, i.e., usg M (17) and O (14), were also fed to this host; however, no turnover was detected by LC-MS (Fig. S12†). This excludes a possible role of usgO in adjusting the equilibrium of fully reduced and semi-reduced ustilaginoids proposed previously.

**In vitro biosynthesis of ustilaginoids with the laccase**

Since the laccase was shown to dimerize norrubrofasarin (2) *in vitro*, we further tested all the possible monomers found in the reported ustilaginoids, together with their precursors (YWA1 (1) and 3-methyl-YWA1 (3)) in various combinations. The results are shown in Fig. 4, S9,† and Table 2.

The cell-free extract (CFE) of A. oryzae expressing the laccase (usgL) in citrate buffer (pH 6.0) was able to dimerize norrubrofasarin (2) (entry L7, Table 2) to produce usg A (10) (Fig. 4, L7), which was consistent with a previous report, while no dimerization was found in Tris buffer (pH 8.0), indicating that the protonation of the phenolic hydroxyl group is essential for the activity. The CFE was constructed thereafter in citrate buffer for all the experiments (Table 2). The results are shown in Fig. 4 (for full data, see Fig. S9†).

When using YWA1 (1) as the substrate, the expected dimer (8) could not be detected. Instead, a partial dehydration product (9) and the fully dehydrated congener (10) were found to be the major products, together with the dehydrated monomer 2 (entry L1, Table 2, and Fig. 4). In the control experiment, 1 was treated with boiled CFE, and ca. 2/3 of 1 was found to be converted to 2 (Fig. S9,† trace EXP L1). It is possible that the dimerization surpassed the dehydration in the case of 1, which first gave rise to 8, and subsequent dehydration to 9, and finally to 10, as the yield of 10 in EXP L1 was significantly higher than that in EXP
The dimer usg K (with 3-methyl-YWA1 (norrubrofusarin) was completely used up (EXP L2). The above data indicated that the yield of 3-methyl-YWA1 (CA2) was not tested due to its limited amount. As mentioned previously, 3-methyl-YWA1 (3) was quickly dehydrated under the tested conditions, leading to a trace product of 4/4-homodimer (21) (EXP L12 and L13). However, adding a 2,3-dihydrogenated monomer (3) to the reaction mixture (EXP L14) significantly increased the yield of the dimerized product, including a 3/3-homodimer (usg O, 14), though 21 was still obtained in a trace amount. In EXP L15, upon mixing 3 with one other 2,3-reduced monomer (7), the dimeric products were minor; however, they included the 7/7-homodimer (16) and 4/7-heterodimer (usg M, 17), respectively, in a descending order of yield.

Interestingly, using 2 instead of 1 in various combinations (entries L7–L11, Table 2) led to a similar profile of the dimerized products (except in the case of 9) to that found with 1 (entries L1 and L3–L6, respectively); however, the yield was significantly lower. This also revealed that the laccase preferred 1 over 2.

As the second major product, and a 6/7-heterodimer (iso-chaetocromin B2, 20), 2/2-homodimer (10), and 1/2-heterodimer (9) as minor metabolites. These dimers arose from the combination of four available monomers: 1 and its dehydrated product 2, and 7 and its 3-epimer 6, as all of them were detected in the control experiment (Fig. S9). Similarly, when 7 was fed alone to the CFE (EXP L21), the dimeric products 16/20 were produced, as well as 6.

When comparing EXP L5 vs. L2, and L6 vs. L4, one can see that the 2,3-reduced monomer was more efficiently converted by the laccase than its cis-analogue. And judging from the peak area of the residual starting material, the 3-methyl substitution has a negative impact on the dimerization (Fig. 4, L6 vs. L5; L3 vs. L1; L7 vs. L16).

Interestingly, using 2 instead of 1 in various combinations (entries L7–L11, Table 2) led to a similar profile of the dimerized products (except in the case of 9) to that found with 1 (entries L1 and L3–L6, respectively); however, the yield was significantly lower. This also revealed that the laccase preferred 1 over 2.

As mentioned previously, 3-methyl-YWA1 (3) was quickly dehydrated under the tested conditions, leading to a trace product of 4/4-homodimer (21) (EXP L12 and L13). However, adding a 2,3-dihydrogenated monomer (3) to the reaction mixture (EXP L14) significantly increased the yield of the dimerized product, including a 5/3-homodimer (18) and 5/4-heterodimer (usg O, 14), though 21 was still obtained in a trace amount. In EXP L15, upon mixing 3 with one other 2,3-reduced monomer (7), the dimeric products were minor; however, they included the 7/7-homodimer (16) and 4/7-heterodimer (usg M, 17), respectively, in a descending order of yield.

Meanwhile, using 3-methyl-norrubrofusarin (4) instead of 3 in the combination (EXP L16–L18, Table 2) resulted in a noticeable increase of the dimeric products; however, a similar profile was found (Fig. 4, L16–L18 vs. L13–L15). This suggested that 4 was preferred by UsgL over 3, which was in contrast to 1 vs. 2.

In EXP L19, the 5/5-homodimer (usg F, 18) was generated in a good yield using 5 as the substrate. When mixing 5 and 7 in EXP L20, the 5/7-heterodimer (usg E, 12) was the major product, which was followed by the 7/7-homodimer (usg D, 16) and 5/5-homodimer (usg F, 18) as the 2nd and 3rd major products, respectively, together with a minor amount of 20. When comparing with the control (Fig. S9†), one can find that 5 was consumed more than 7, suggesting that 5 might be a better substrate for the laccase.

Obermaier et al. reported that the atrop selectivity of the laccase was dependent on the protein concentration. In the current study, the atropselectivity of UsgL was investigated at different concentrations using 5 as the substrate, which was chosen due to its better reactivity; diastereomeric products were formed if the atrop selectivity varied, and thus there was no need to use a chiral column. Indeed, the P/M-selectivity varied with the change of the protein concentration, with a higher concentration causing a higher M-selectivity (Fig. S10†). These results were consistent with a previous study using 2 as the substrate.12

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| Table 2 | Construction of ustilaginidins using various combinations of monomers with a cell-free extract of A. oryzae-usgL. |
|---------|----------------------------------------------------------------------------------------------------------|
| Monomer | EXP 1 | 2 | 3 | 4 | 5 | 7 | Product |
|---------|-------|---|---|---|---|---|---------|
| L1      | ✓     |   |   |   |   |   | 2, 9, 10 |
| L2      | ✓ ✓   |   |   |   |   |   | 9, 10   |
| L3      | ✓ ✓ ✓ | ✓ |   | ✓ |   |   | 2, 4, 9, 10, 15, 21 |
| L4      | ✓ ✓ ✓ | ✓ |   | ✓ |   |   | 2, 9, 10, 15, 21 |
| L5      | ✓ ✓ ✓ | ✓ |   | ✓ |   | ✓ | 2, 9–11, 18 |
| L6      | ✓ ✓ ✓ | ✓ |   | ✓ | ✓ | ✓ | 2, 6, 9, 10, 13, 16, 20 |
| L7      | ✓ ✓ ✓ | ✓ |   | ✓ | ✓ | ✓ | 10 |
| L8      | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 4, 10, 15, 21 |
| L9      | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 10, 15, 21 |
| L10     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 10, 11, 18 |
| L11     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 6, 13, 16 |
| L12     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 4, 21 |
| L13     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 21 |
| L14     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 4, 14, 18, 21 |
| L15     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 4, 6, 16, 17 |
| L16     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 21 |
| L17     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 14, 18, 21 |
| L18     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 6, 16, 17 |
| L19     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 18 |
| L20     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 6, 12, 16, 18, 20 |
| L21     | ✓ ✓ ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | 6, 16, 20 |

a 6 was not tested due to its limited amount. b Trace amount.
UsgR is a distinct class of ene-reductases

Bioinformatics analysis indicated that UsgR contained a conserved domain belonging to the phospholipid methyltransferase superfamily, similar to DUF1295 (a family of unknown function), and the C-terminal domain of the steroid 5α-reductase family (Fig. S21†). UsgR was predicted to be a membrane-bound protein (Fig. S3†). An attempt to express it in *Escherichia coli* failed despite various trials. Then, two truncated versions of UsgR were designed and tested: UsgR76-273 and UsgR160-264. The former was designed based on the alignment to Δ14-sterol reductase in SWISS-MODEL, while the latter contained only the predicted conserved domain of UsgR (Fig. S21†). We found that UsgR76-273 was not expressed in *E. coli* despite testing various expression vectors and strains, while UsgR160-264 was expressed in three vectors (pETM10, pMBP_1a, and pGEX6p-1) upon addition of IPTG (Table S9†). However, further analysis of the expression pattern revealed that UsgR160-264 was insoluble and found only in the cell debris (Fig. S22†).

We expressed usgR in *A. oryzae*, and the CFE showed no reducing ability towards the ene-substrates (2 and 4), not surprisingly. Then, we fed the substrates to the *A. oryzae* strain expressing usgR, in DPY medium and citrate buffer, respectively (Fig. 5 and S11†). The results showed that norrubrofasarin (2) was fully converted to its dihydrogenated product 5 (Fig. 5(i) and (ii)). Meanwhile, feeding 3-methyl-norrubrofasarin (4) led to the production of two reduced epimers (6 and 7), with the 2,3-trans-methylated one (7) as the predominant product (approx. ratio of 6 to 7, 9 : 91 and 7 : 93, Fig. 5(iii) and (iv)), respectively. On the other hand, we fed 7 and 5 to the same strain, respectively, and no conversion back to 4 and 2 was found after 48 h. This was in contrast to the steroid 5α-reductase (EC 1.3.1.22), which catalyzed the conversion of 3-oxo-Δ4 steroids into their corresponding Δα form, and *vice versa*. By the same token, we fed the ene-substrates to the *E. coli* and *A. oryzae* recombinant strains expressing usgR160-264, and no products were found in neither strain (Fig. S23 and S24†). This indicated that this truncated protein was nonfunctional.

The heterologous expression of usgR in yeasts *Pichia pastoris* GS115 and *Saccharomyces cerevisiae* BY4741 succeeded as well, as confirmed by feeding experiments (Fig. S25A and B†), albeit less efficient than in *A. oryzae* (Fig. S25C†). However, the microsomal fractions from these recombinant strains failed to reduce 2 despite various trials (Fig. S25†).

Interestingly, when we fed usg A (10), L (15), and K (21), three dimeric Δ2-naphtho-γ-pyrones, to the *A. oryzae-usgR*, no product was found after 48 h (Fig. 5(v) and S11†).

For the 2,3-dimethyl precursor, the reduction gave a predominant 2,3-trans product (7), with a minor amount of 3-epimer (6). It is probable that the 3-epimerization is non-enzymatic, as the keto–ene tautomerism could destroy the stereocenter at C-3 (Fig. 6). The formation of 7 could be thermodynamically favored because it is more stable when both methyl groups are oriented towards a different face. In order to test these hypotheses, 6 or 7 was incubated with various tested buffers, media or solvent (Table 3). The results showed that 7 was stable in the citrate buffer, DPY and PSB media, and in

**Fig. 6** Keto–ene tautomerism led to epimerism at C-3.

**Table 3** The ratio of 6 to 7 under various tested conditions

| Tested conditions | Substrate | Ratio of 6/7<sup>a</sup> |
|-------------------|-----------|--------------------------|
| Citrate buffer    | 7         | <1 : 99 (2 d, 28°C)      |
|                   | 6         | 2 : 98 (30 d, 4°C)       |
| DPY               | 7         | <1 : 99 (2 d, 28°C)      |
|                   | 6         | 2 : 98 (30 d, 4°C)       |
| PSB               | 7         | <1 : 99 (2 d, 28°C)      |
|                   | 6         | 2 : 98 (30 d, 4°C)       |
| MeOH              | 7         | <1 : 99 (2 d, 28°C)      |
|                   | 6         | 2 : 98 (30 d, 4°C)       |
| DMSO              | 7         | <1 : 99 (2 d, 28°C)      |
|                   | 6         | 2 : 98 (30 d, 4°C)       |
| Boiled CFE of *A. oryzae-usgL* | 7     | 8 : 92 (1 d, 28°C)       |
| *A. oryzae-usgL*, citrate buffer | 4     | 7 : 93 (2 d, 28°C)       |
| *A. oryzae-usgL*, DPY | 4     | 9 : 91 (2 d, 28°C)       |
| ΔusgL             | —         | 8 : 92–7 : 93            |

<sup>a</sup>The ratio was estimated from the peak area at 280 nm. The feeding experiments and CFE tests were performed at 28 °C for 2 days and 1 day, respectively. CFE: cell-free extract constructed in citrate buffer.
MeOH, for 2 d (the same as the feeding experiment), while the cis-isomer 6 was partially (≈20%) converted to the trans-isomer (7) in the citrate buffer [pH = 6]; however, it was very stable in the tested media and in MeOH. This indicated that 7 was more stable than 6. However, ≈2% of 7 was converted to 6, after about 1 month at 4 °C, while a ~6% conversion rate was found in DMSO even when it was stored at −20 °C. Interestingly, when 7 was incubated with the boiled CFE of A. oryzae-usgL, 8% of 6 was formed. This ratio is close to that found in the feeding experiment (4 + A. oryzae-usgR) and is also similar to that in the knockout mutant (ΔusgL). These data suggested that the cellular components of the host promoted the epimerism, but non-enzymatically.

Although a large number of ene-reductases have been discovered for asymmetric reduction of activated C=C bonds,14 no examples have been reported for the reduction of naphtho-γ-pyrenones, to the best of our knowledge. Moreover, only a few enzymatic reductions of chromen-4-one-containing substrates, including flavones15 and isoflavones,16 have been reported. In this study, we also tested some representatives containing such a moiety, including one flavone (23), one isoflavone (24), four chromen-4-ones (25–28), and one benzo[h]chromen-4-one (29) (Fig. 7); however, no reduction was found after feeding them to A. oryzae-usgR for 2 d (data not shown). This suggested that the reductase was specific for reduction of linear naphtho-γ-pyrenones (i.e., 4H-benzo[g]chromen-4-one) (e.g. 2 and 4).

### Discussion

Bis-naphtho-γ-pyrones are widely distributed in filamentous fungi, and displayed a broad range of biological activities.17 Among them, ustilaginoidins and chaetochromins are mycotoxins, sharing a 9/9′-linked bis-naphtho-γ-pyrene skeleton but differing in the axial configuration (M and P, respectively).

As can be seen from the structures of ustilaginoidins, they vary at the constituted monomers due to having different saturations at C-2/C-3, and methylation at C-3 (Fig. 1). Different biosynthetic schemes have been proposed for these monomers,11,12 but the key steps (methylation, dehydrogenation, and reduction) have not been validated by biochemical or genetic studies. Moreover, it is unclear whether some modifications on the dimers could happen or not during the biosynthesis.

In this study, the complete biosynthetic pathway of ustilaginoidins was revealed, as shown in Fig. 1B and Scheme 1. UVPKS1 incorporated one acetyl CoA and six malonyl CoA to form an ACP-bound heptaketide, and was further processed by the product template (PT) and thioesterase (TE) domain before releasing the phenolic ketone, which was spontaneously ketalized to produce YWA1 (1). UsgL contains a ψ-ACP-MT didomain, similar to TlnC,18 in which ψ-ACP was essential for the C-methylation of tricholignan A. The ψ-ACP of TlnC was found to inhibit the KS (β-ketoacyl synthase) catalyzed chain elongation to facilitate methylation. It is likely that UsgL can work in a similar manner. It should work on the ACP-bound growing ketides before chain release to form the 3-methyl derivative (3). This can explain why feeding YWA1 (1) to A. oryzae-usgM or its CFE did not result in any 3-methylated product (Fig. S13†). Moreover, the methylation was so efficient that the 3-methyl derivatives are predominant (Fig. 2, xii; especially iii and iv), and judging from ΔusgL/UsgL (Fig. 2[iiv]), the ratio of non-methylation vs. methylation is ca. 1 to 7.

The hemi-ketals, YWA1 (1) and its derivative (3), were dehydrated by UsgD to form the Δ3-derivatives (2 and 4). These metabolites were prone to dehydration without UsgD, albeit it was slower (Fig. 3, EXP1 and EXP4; Fig. 2[v]). The reduction of the 2,3-double bond by UsgR afforded the 2,3-dihydro-naphtho-γ-pyrones (5–7). The reduction was stereoselective, yielding the 2R substrates. With regard to the 2,3-dimethyl precursor, the major product was the trans-isomer (7), in addition to a minor amount of cis-isomer (6). The production of the 3-epimer is probably non-enzymatic under the culture conditions.

Atroposelective dimerization of these monomers by the laccase UsgL gives rise to the diverse ustilaginoidins. In vitro experiments revealed that the reactivity of the monomers followed the order 3-nomethyl > 3-methyl; 2,3-saturated > 2,3-unsaturated; surprisingly, YWA1 (1) > its dehydrated counterpart (2); however, the trend is reversed for 3-methyl YWA1 (3). To compensate for the relatively low reactivity, the fungus produced a significant excess of 3-methyl metabolites to non-methylated ones as mentioned above. All these together contribute to the observed diversity of ustilaginoidins (Scheme 1 and Fig. 2[xii]).

Meanwhile, the gene-deletions and heterologous expression experiments revealed complex interactions between the DH (usgD), C-MeT (usgM), reductase (usgR), and laccase (usgL). For instance, gene-deletion of usgD did not result in the enrichment of 2-hydroxyl derivatives (Fig. 2[vii]), while further deletion of either usgL, usgR, or usgM did (Fig. 2[v,i], (ix) and (x)). Moreover, disrupting usgD alone did not lead to the accumulation of monomers (Fig. 2[vii]), but upon further deletion of either the upstream usgM or the downstream usgR, the monomers appeared (Fig. 2[ix] and (x)). This indicated two different ways to biosynthesize the Δ3-dimers (i.e., 10, 15, and 21), either through dimerization of the Δ3-monomers, or dimerization of the 2-hydroxyl intermediates followed by dehydration (Scheme 1). Further reduction of these Δ3-dimers by usgR or the putative

![Fig. 7 Tested compounds (23–29) containing a chromen-4-one moiety.](image-url)
oxidoreductase (usgO) was not observed (Scheme 1). Thus, the fully reduced dimers (12, 16, and 18–20) and the semi-reduced ustilaginoidins (11, 13, 14, and 17) had to be formed by dimerization of two corresponding monomers by the laccase.

The non-enzymatic epimerism at C-3 was found between the monomers (6 and 7) as stated previously (Table 3). Likewise, the epimerism could be possible in the dimers containing either 6 or 7. Indeed, we observed a partial conversion (6%) from usg D (16) to its 3-epimer 20, from usg M (17) to its 3’-epimer M1, and from usg N (13) to its 3’-epimer usg P, in the frozen samples of 16, 17, and 13, respectively, which had been stored at –20 °C for 4 years. This suggested one alternative route to generate the 6-containing dimers (such as 19 and 20) via 3-epimerization of the corresponding dimers (e.g. 12 → 19, 16 → 20), in addition to the laccase-catalyzed dimerization of the respective monomers (Scheme 1).

UsgR is a previously unknown reductase that specifically catalyzed the reduction of 4H-benzo[g]chromen-4-one. A BLAST search using the usgR sequence as a query identified 97 putative enzymes (amino acid sequence identity ≥30%, coverage ≥80%, e-value ≤1e–10) from over 73 different species of ascomycetes (Table S1†). Phylogenetic analysis revealed that it is closely related to Metarhizium spp. and Thermothelomyces sp. (Fig. S4†). Further searching for similar PKSs and DHs in the genome of these fungi resulted in 12 species that could be possible producers of 2,3-reduced naphtho-γ-pyrones (Table S2†), including the species in the genera of Metarhizium (three species), Thermothelomyces, Rhizodiscina, Lindomyces, Melanomma, Corynespora, Glonium, Cenococcum, Pseudogyrospora, and Chaetomium. Interestingly, the DH was found to cluster with the PKSs in all these species, among which nine species contained the reductase in the same cluster (Fig. S5†). As shown in Fig. S5, high synteny was found for U. virens, C. olivicolor, T. thermophiles, M. robertsii, M. brunneum, and M. anisopliae, indicating that they could produce similar metabolites. Among them, Chaetomium spp. are well-known producers of chaetochromins, though not reported from C. olivicolor. M. anisopliae is reported to produce monomeric (indigotides G, H, and B) and dimeric 2,3-reduced naphtho-γ-pyrones (i.e., 16 and 20), in which 2,3-trans-dimethylated products were found together with the 2,3-cis products; however, the stereochemistry (C-2, C-3) of the pyrone ring was assigned reversely between the monomers and the dimers. Comparing the CD profiles of these monomers with those of 5–7 (ref. 10) revealed that the stereochemistry was probably incorrectly assigned, and should be revised to 2R. Hence, the reductases in these species showed the same stereoelectivity.

In summary, the biosynthesis of ustilaginoidins was revealed by gene-deletions and reconstructed by heterologous expression in A. oryzae, and in vitro experiments. The dehydration step was cryptic at first, but was unveiled by further deletion of one other gene in the ΔusgD background, and confirmed by heterologous expression. A new route to 2,3-unsaturated dimeric naphtho-γ-pyrones was discovered via dimerization of YWA1 (and 3-methyl YWA1) followed by dehydration. The reduction of C2=C3 was catalysed by UsgR, which was distinct from any characterized ene-reductases. The reduction was stereo-controlled, and worked only on monomeric, linear naphtho-γ-pyrenones. In addition, the epimerism at C-3 was non-enzymatic. Finally, the laccase atroposelectively coupled the various building blocks to generate ustilaginoidins, in which different substrate preferences were found. It is interesting to note that ustilaginoidins with a 2-hydroxymethyl substituent were found in the RFS balls, but not in the culture of U. virens; hence, the oxygenase-encoding gene must locate outside the usg BGC.

Data availability

The datasets supporting this article have been uploaded as part of the ESL†

Author contributions

Conceptualisation, supervision and writing – L. Z., and D. L.; investigation and methodology – D. X., R. Y., Z. Z., G. G., S. Z., J.-R. X., J. L., Y.-L. P., D. L.; all authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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