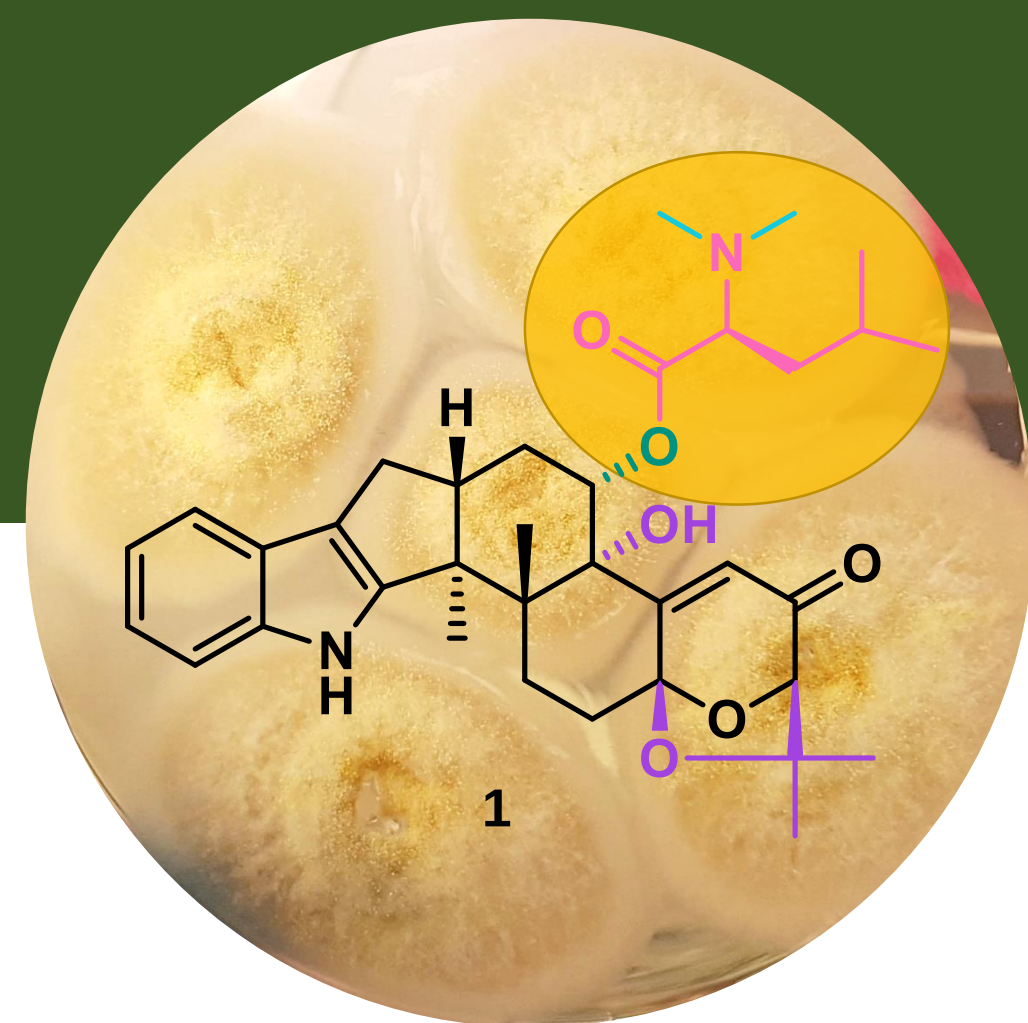


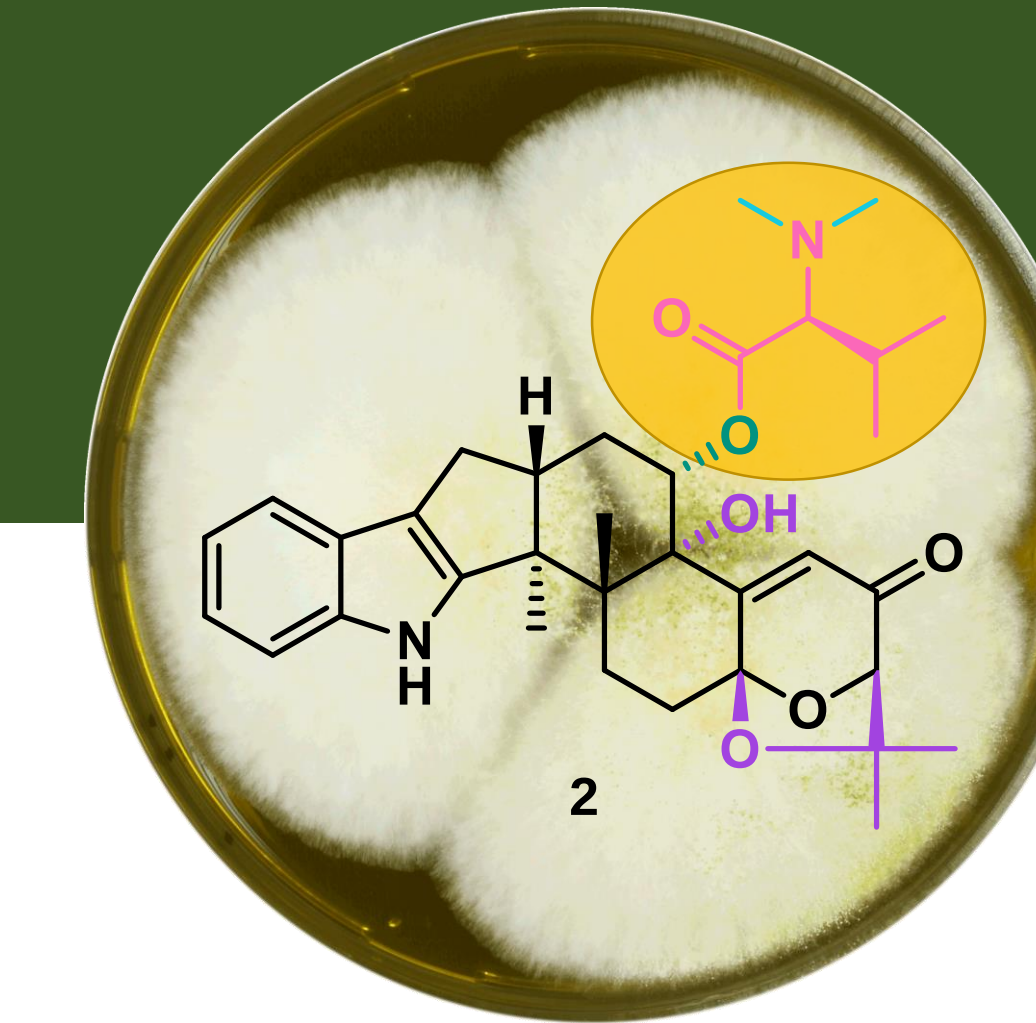
ACEing aminoacylation in indole diterpene pathways

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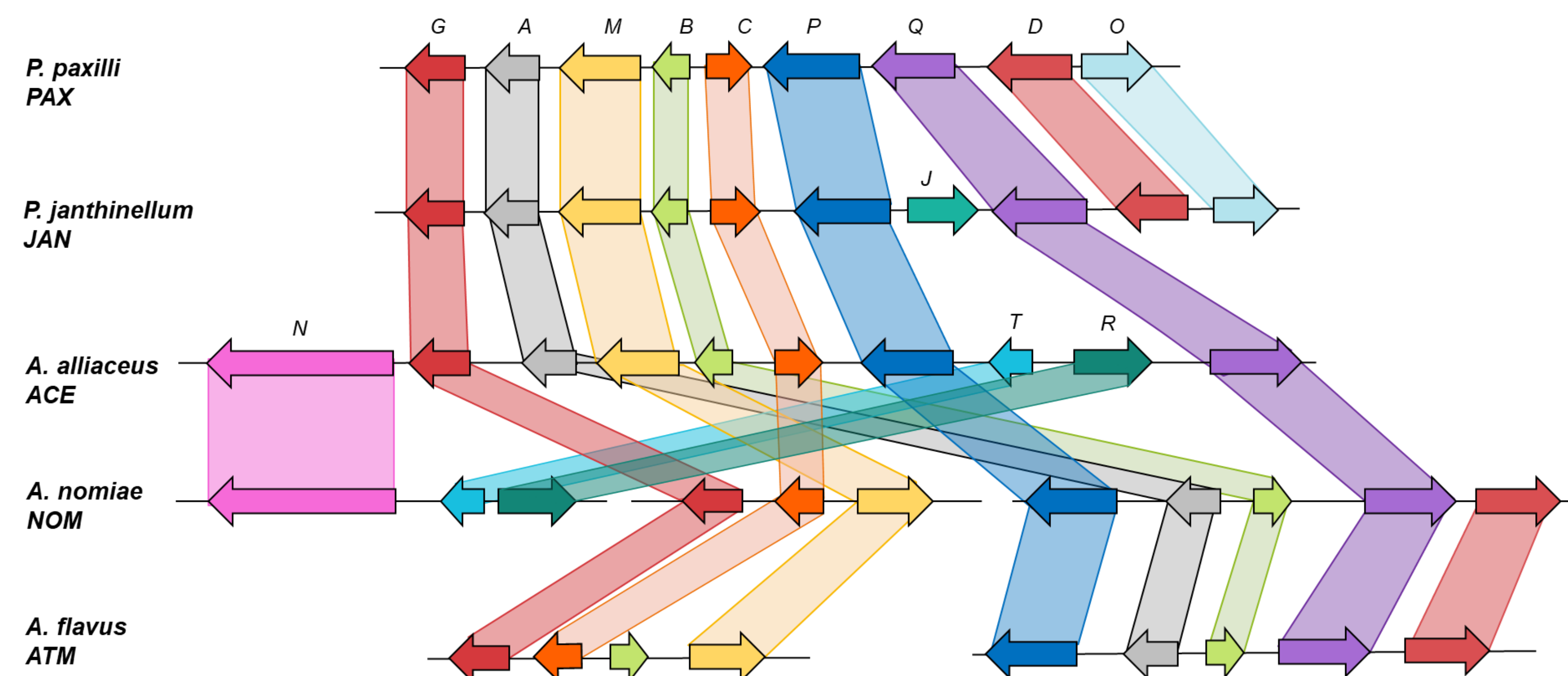
Indole diterpenes (IDT) are a structurally diverse and bioactive class of secondary metabolites produced by filamentous fungi. Decoration of core IDT structures is key to their bioactivity diversity. Very rarely is aminoacylation linked to terpene biosynthesis, and there are only two examples reported for IDTs: 14-(*N,N*-dimethylleucyloxy)paspalinine (**1**) from *Aspergillus alliaceus*¹, that exhibits potassium channel blocker activity², and 14-(*N,N*-dimethylvalyloxy)paspalinine (**2**) from *Aspergillus nomiae* that exhibits potent anti-insecticidal activity³. By understanding the biosynthetic pathways to these IDTs through heterologous expression in *Penicillium paxilli*, we can access these bioactive metabolites more efficiently.



The first IDT biosynthetic gene clusters containing single module NRPS-like enzymes

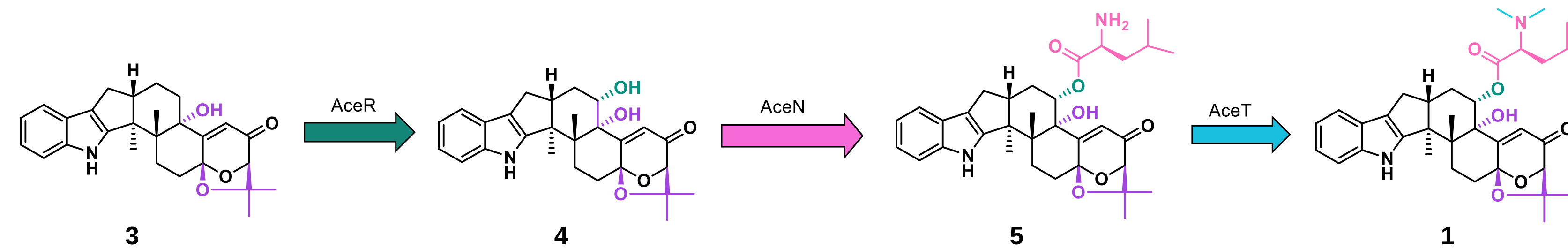
Whole genome sequencing was carried out for a *Aspergillus alliaceus* ATCC® 20738™ and the sequence was interrogated for IDT genes. A putative IDT biosynthetic gene cluster (the *ACE* cluster) containing ten open reading frames was identified. Seven of these were homologous to genes in the *JAN* cluster of *Penicillium janthinellum*⁴ and the *PAX* cluster of *Penicillium paxilli*⁵, respectively.

Using BLASTp analysis, we predicted the other three genes to encode a cytochrome P450 monooxygenase (AceR), a monomodular NRPS-like enzyme (AceN) and a SAM-dependent methyltransferase (AceT). A biosynthetic pathway for production of **1** was proposed based on chemical structure and homology to known IDT pathways, where functions were assigned for all *ACE* cluster genes. Most notably the three new genes are predicted to be involved in derivatisation of paspalinine.



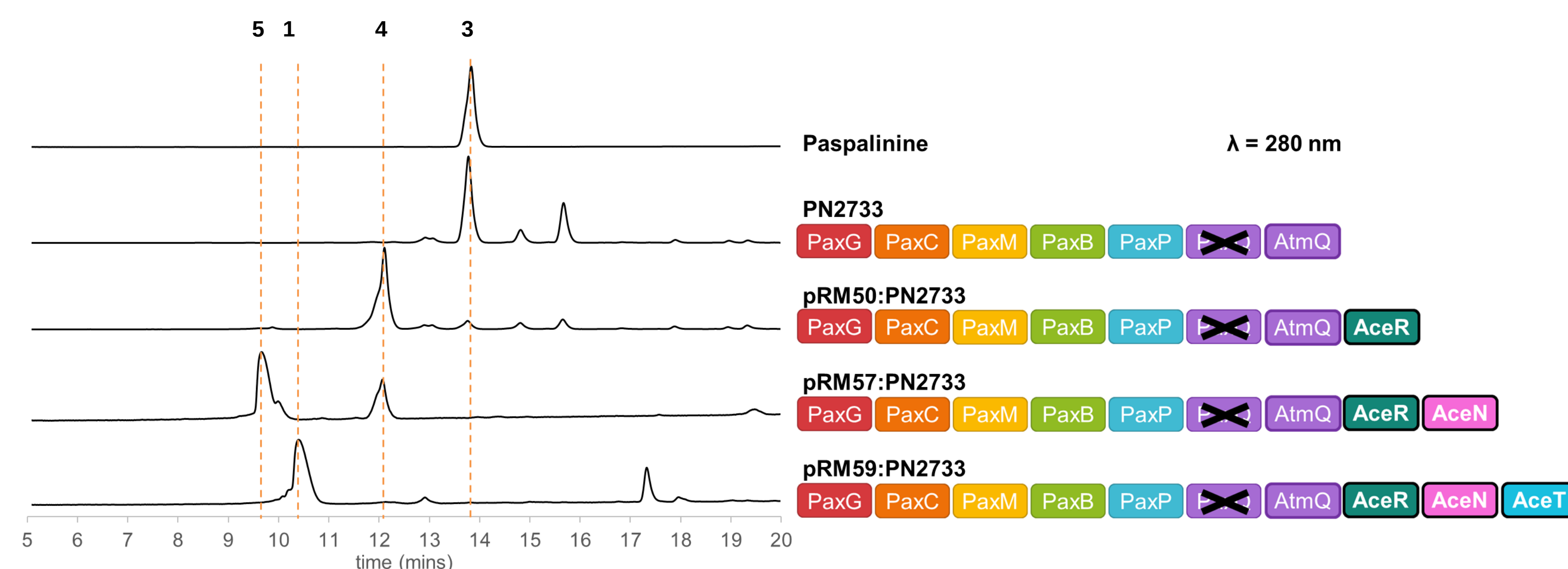
A top BLASTp hit for AceN was a hypothetical protein from *Aspergillus nomiae* NRRL 13137, the only other organism known to produce an aminoacylated IDT, so we also interrogated this genome for IDT genes that are potentially responsible for the production of **2**. Three putative IDT clusters with a total of 11 open reading frames were identified (The *NOM* clusters). Unlike the *ACE* cluster, two of the *NOM* clusters mirrored the synteny of the *ATM* cluster of *Aspergillus flavus*⁶. The *NOM* cluster has homologues to the three new *ACE* genes, so we also predict their role will be in derivatisation of a paspalinine core. The *ACE* cluster and *NOM* clusters are the first IDT biosynthetic gene clusters identified with genes encoding single module NRPS-like enzymes.

Reassembly of 14-(*N,N*-dimethylleucyloxy)paspalinine biosynthesis in *Penicillium paxilli*



To determine the function of the three new clustered *ACE* genes and to establish their respective roles in biosynthesis of paspalinine (**3**) derivatives, we constructed various multi-gene plasmids for transformation into a *P. paxilli* (PN2733) strain that produces **3**. First, a plasmid harbouring was transformed into PN2733. LC-MS results showed that **3** was converted to 14-hydroxypaspalinine (**4**), confirming that AceR is a cytochrome P450 that hydroxylates the C-14 of paspalinine. AceR is the first enzyme to afford a functionality at C-14 of the paspalinine/penitrem class of indole diterpenes.

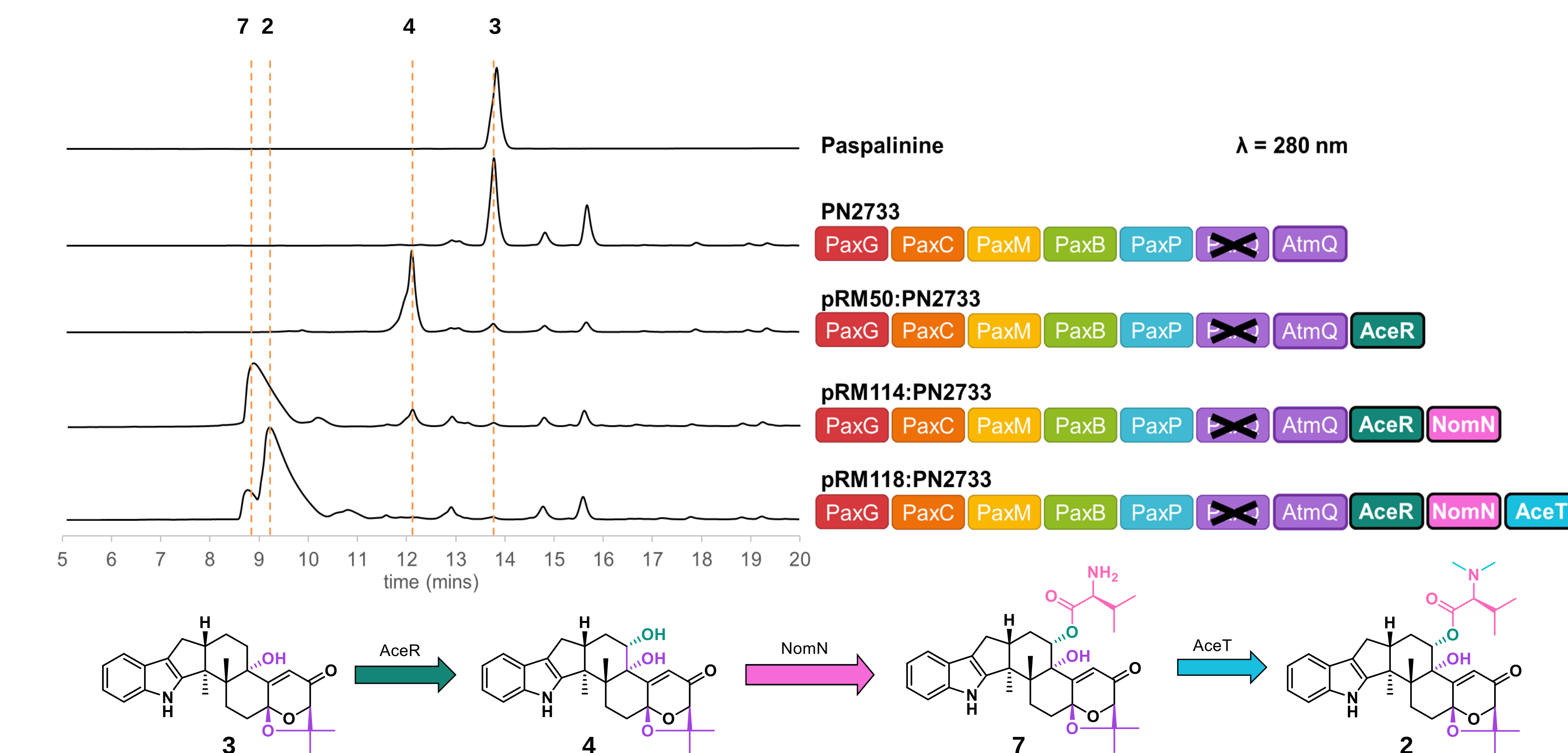
When PN2733 was transformed with a plasmid harbouring both *aceR* and *aceN* the novel intermediate 14-(leucyloxy)paspalinine (**5**) was produced. Therefore, AceN is a single-module NRPS-like enzyme that catalyses the aminoacylation of L-leucine with **4**. This is the first use of an NRPS-like enzyme in IDT biosynthesis, and an extremely rare use of an NRPS in decoration of a terpene core.



When all three genes (*aceR*, *aceN* and *aceT*) were introduced into PN2733, the resulting organism was able to successfully convert **5** to **1**, confirming AceT as an *N*-methyltransferase (that dimethylates the free amine of the L-leucine moiety of **5**); the first occurrence of a characterised methyltransferase in IDT biosynthesis. Thus, we have completely delineated the biosynthetic pathway to IDTs produced by *A. alliaceus*.

Reassembly of 14-(*N,N*-dimethylvalyloxy)paspalinine biosynthesis in *Penicillium paxilli*

To reconstruct 14-(*N,N*-dimethylvalyloxy)paspalinine biosynthesis in *P. paxilli*, we hypothesised that the only gene required from *A. nomiae* was *nomN*. Given AceR leads to biosynthesis of **4**, a plasmid harbouring *aceR* and *nomN* was transformed into PN2733. LC-MS analysis revealed the production of a new biosynthetic intermediate, 14-(valyloxy)paspalinine (**7**). Therefore, NomN is a single-module NRPS-like enzyme that catalyses the aminoacylation of L-valine with **4**. A plasmid with *aceR*, *nomN* and *aceT* was then transformed into PN2733. LC-MS confirmed the conversion of **7** to **2**, demonstrating that AceT can exhibit promiscuity in the *N*-methylation of aminoacylated IDTs: it can accept both **5** and **7** as substrates. Overall, biosynthesis of **2** has been reassembled in *P. paxilli*.



Summary

We identified IDT biosynthetic gene clusters in two *Aspergilli*, which are the first to contain genes encoding NRPS-like enzymes. Heterologous expression of *ACE* and *NOM* cluster genes in *P. paxilli* allowed us to confirm the function of four genes that have novel functions. Overall, we have delineated the biosynthetic pathways to both 14-(*N,N*-dimethylleucyloxy)paspalinine and 14-(*N,N*-dimethylvalyloxy)paspalinine, and characterised the first uses of NRPS-like enzymes and a methyltransferase in IDT biosynthesis.⁸ This study could lead to greater understanding of amino acid specificity of fungal NRPSs and reveal more NRPS-like enzymes with decorative capabilities.

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