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Enzymatic *cis*-Decalin Formation in Natural Product Biosynthesis

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Abstract

Stereoselective synthesis of *cis*-decalin structures using [4+2] cycloaddition is challenging. We explored the biosynthetic pathway of the fungal natural product fischerin (**1**) to identify a new pericyclase FinI that can catalyze such a reaction. Cocrystal structure of FinI, a predicted *O*-methyltransferase, with the product and SAM provides insight into *cis*-decalin formation in Nature.

The decalin moiety is found prevalently in natural products, exemplified by the cholesterol-lowering lovastatin.[1,2] Decalin can be derived from either a terpene or a polyketide precursor.[1,3] In polyketide-derived decalins, a stereoselective intramolecular Diels-Alder (IMDA) cycloaddition takes place between a dienophile and a diene separated by a four methylene spacer to afford either a *cis*- or *trans*-decalin (Figure 1A).[4,5] The skipped polyene precursor is generated through the programming rules of polyketide synthase (PKS) and associated reductase partners including enoylreductase (ER).[6,7] Biomimetic syntheses of decalin natural products using [4+2] cycloaddition are well-documented.[1,8–14] However, chemical synthesis of a single stereoisomer from the four accessible *endo* and *exo*

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Author Contributions

All authors have given approval to the final version of the manuscript.

Supporting Information

Experimental details, spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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transitions states is challenging, especially for *cis*-decalin compounds.[1,9,13,15–17] Motivated by such challenge to generate *cis*-decalin from skipped polyene precursors, we aimed to identify pericyclases (enzymes that catalyze pericyclic reactions)[18] that can catalyze such transformations stereoselectively.

In recent years, a number of lipocalin-like Diels-Alderases (DAses) have been reported to partner with fungal PKSs to generate decalin structures, including CghA[19], Fsa2[20], MycB[21], Phm7[22], and UcsH[23] (Figure 1A). All of these enzymes, however, stereoselectively form *trans*-decalin scaffolds.[5] Although structural-guided mutations of the active sites in these enzymes have led to formation of *cis*-decalin, the resulting stereoselectivity is not high and mixed *cis* and *trans* products are formed.[24,25] One reported fungal DAse that forms *cis*-decalin is PvhB involved in the biosynthesis of varicidin A (Figure 1B).[26] In this case, however, the *exo*-transition state is kinetically favored due to substitution of the methyl group on the diene with an electron-withdrawing carboxylate that effectively led to an inverse-electron demand Diels-Alder reaction. To date, there is no report of a DAses that can exclusively catalyze *cis*-decalin formation via a normal-electron demand IMDA reaction.

To understand how natural *cis*-decalins are formed, we investigated the biosynthesis of a recently "de-orphaned" natural product, fischerin (**1**), which is a cytotoxic compound with *cis*-decalin and epoxycyclohexane-diol fragments connected through a 2-pyridone (Figure 1B).[27] A close structural analog to **1** is *N*-hydroxyapiosporamide (**2**),[28] which contains a *trans*-decalin. The *fin* biosynthetic gene cluster (BGC, Figure 1C) mined from *Aspergillus carbonarius* was reconstituted to produce **1** (Figure 2A, ii), the structure of which was verified by microcrystal electron microscopy (MicroED).[27] A polyketide-nonribosomal peptide synthetase (PKS-NRPS, FinD), a *trans*-acting ER (FinC) and a ring-expansion P450 ($P450_{RE}$, FinE) collectively biosynthesize a 2-pyridone ketone (Figures 2C and S5). The *fin* BGC also encodes a P450 (*finJ*), an ene-reductase (OYE, *finH*) and a short-chain reductase/dehydrogenase (SDR, *finA*), which are involved in dearomatizing the phenyl group into the epoxycyclohexane-diol (Figures 2C and S5).[27] The *fin* cluster does not encode a lipocalin-like DAse found in other fungal decalin natural product BGCs.

Genes in the *fin* BGC are homologous to those in the putative BGCs of **2** from the genomes of *Apiospora montagnei* (*api*) and *Alternalia solani* (*asol*) (Figure 1C and Table S1).[30] Oikawa et. al. showed that coexpression of *the asol* PKS-NRPS, ER and $P450_{RE}$ in *A. oryzae* led to the production of the 2-pyridone didymellamide B that has the same *trans*-decalin stereochemistry as in **2** (Figure 1B).[30] The authors proposed the IMDA reaction can take place nonenzymatically. Interestingly, these clusters all encode a putative OMT (FinI, ApiI and AsolM) (Figure 1C and Table S1) with a well-conserved *S*-adenosylmethionine (SAM) binding motif (GXGXG) (Figure S9),[29] although no *O*-methylation is required to biosynthesize either **1** or **2.** Given that formation of the kinetically disfavored *cis*-decalin in **1** most certainly requires a dedicated DAse, and OMTs have been repurposed as pericyclases in other biosynthetic pathways,[31–34] we hypothesized that FinI are ApiI could be involved in catalyzing the formation of *cis*-decalin and *trans*-decalin in the respective pathways.

To test this hypothesis, we expressed *finACDEHJ* without *finI* in *A. nidulans*. LCMS analysis revealed production of **1** was abolished in the absence of FinI (Figure 2A, i).

Instead, compound **3** (0.5 mg/L) with the same MWT of **1** emerged (Figure 2A, i). NMR characterization showed **3** has the same epoxycyclohexane-diol moiety as in **1**, but the dienophilic olefin has undergone a Michael addition at C9 and cyclization with pyridone 4-OH to form a fused pyran (Table S5 and Figures S25-S30). The ^{13}C NMR signals suggest that **3** contains a diastereomer **3'** in a 1:1 ratio. We propose the inseparable mixtures of **3** and **3'** forms nonenzymatically from **4** through the reversible Michael reaction (Figure 2C).[35] To investigate the timing of FinI function, we coexpressed *finCDE*, *finCDEJ*, or *finCDEHJ* with or without *finI*. None of the *A. nidulans* transformants showed formation of *cis*-decalin products (Figure S5). Hence, FinI likely functions subsequent to the formation of the epoxycyclohexane-diol. In the absence of FinI, cycloaddition from acyclic precursor to form *cis*-decalin is abolished and instead pyran formation occurs.

To determine if **4**, the putative retro-Michael product of **3/3'**, is as substrate for FinI, we expressed and purified FinI from *Escherichia coli* BL21(DE3) (Figure S6). As similar to LepI, FinI is copurified with SAM.[31] In the absence of FinI, no conversion of **3/3'** to **1** was detected (Figure 2B, i). In contrast, conversion from **3/3'** to **1** was observed in the presence of FinI (Figure 2B, ii), albeit with low efficiency. This led us to propose that the *bona fide* substrate of FinI may be **6,** the *N*-deoxy version of **4,** which can form the pyran **7/7'** nonenzymatically. Pyridone *N*-hydroxylation in **1** is likely catalyzed by the dual function of FinJ, which shows 48% identity to the *N*-hydroxylation P450 TenB from tenellin biosynthesis.[36] This dual function of FinJ as both an aromatic hydroxylase and a *N*-oxidase, however, results in near complete *N*-hydroxylation of all products (**6** to **4**, or **7/7'** to **3/3'**) in *A. nidulans*. In the transformant expressing *finACDEHJ*, only trace amounts of a compound with MWT of **7/7'** (*m*/*z* 416 [M+H$^+$]) with similar retention time to **3/3'** can be detected (Figure 2A, i). Isolation of **7/7'** at quantity and purity required for NMR was not successful, but sufficient amount for in vitro assays was obtained. In the presence of FinI, **7/7'** was more efficiently consumed compared to **3/3'**, and led to the emergence of a new compound **8** with the same MWT as **7/7'** (Figure 2B, iv-vi). Compound **8** was also detected in the *A. nidulans* transformant expressing the full *fin* BGC (Figure 2A, ii) using selected-ion monitoring. More than 100 L of CDST agar cultivation of the transformant allowed us to isolate **8** (<5 mg) and to confirm the compound is *N*-deoxyfischerin with a *cis*-decalin (Table S7 and Figures S37-S42). Collectively, these experiments showed that FinI is the DAse responsible for *cis*-decalin formation, and most likely catalyzes cycloaddition of **6** to form **8**.

The cocrystal structure of FinI with SAM was solved and refined to 2.35 Å (Figures 3A and S10) (Supplementary methods). The dimeric structure resembles closely both canonical SAM-dependent OMTs and repurposed pericyclases such as LepI (Figure S12)[37,38]: an interlocking *N*-terminal dimerization domain and an α/β Rossmann-fold *C*-terminal domain. To understand how FinI catalyzes the *exo*-Diels-Alder reaction, we attempted to cocrystallize FinI with compounds obtained from the reconstitution studies. Whereas attempts with other compounds did not lead to a cocrystal complex, we were able to obtain a crystal structure of FinI-SAM-**8** ternary complex refined to 1.84 Å (Figures 3B and S10). The overall FinI-SAM-**8** structure is very similar to that of FinI-SAM (r.m.s.d. of 0.407 Å for 686 Cα atoms) (Figure S14).

The electron density map revealed that **8** is accommodated in a cavity adjacent to SAM (Figure 3B). The ternary complex enabled us to unambiguously assign the absolute stereochemistry of **8** (Figures 2D and 3B), and by inference that of **1** (the structure solved by MicroED was with relative stereochemistry)[27]. The 2-pyridone ring of **8** is anchored via hydrogen bond interactions between amide nitrogen and D314, and between 2-amide carbonyl and R313. R313 in FinI replaces the general base histidine that is universally conserved in canonical OMTs (Figure S15).[37,38] The electropositive SAM sulfonium methyl carbon forms a potential electrostatic interaction (3.4 Å) with the 2-pyridone nitrogen in **8** (Figure 3B). These interactions involving the amide nitrogen rationalize our observation that *N*-deoxy **6** (from **7/7'**) is a better substrate than the *N*-hydroxy **4** (from **3/3'**) in the in vitro assays. The phenolic oxygen in Y151 forms a direct hydrogen bond with (*R*)-C1'-OH of the epoxycyclohexane-diol moiety in **8**, while the 2'-OH group in SAM and (*S*)-4'-OH in **8** both hydrogen bond to the same water molecule. These interactions support our biochemical observations that the epoxycyclohexane-diol moiety is formed prior to and is required for FinI catalysis, and directly implicate an important role for SAM in aiding substrate recognition.

With respect to catalysis, the C7 carbonyl group of **8** conjugated to the dienophilic olefin forms a direct hydrogen bond with the guanidinium group of R310 (Figure 3B), as well as a water molecule that is hydrogen bonded to Y214 and W143. This strategy to lower the LUMO energy of the dienophile and stabilize the transition state has been noted in other pericyclases.[24,38] The pair R309/R310 is conserved in many canonical OMTs including OxaC, and plays an important role in SAM binding through hydrogen bonding to α-carboxyl group in SAM (Figure S15).[37] In the FinI structure, only R309 is interacting with the SAM carboxylate, while R310 is apparently repurposed to aid the cycloaddition reaction through hydrogen-bonding. The active site pocket of FinI locks the *cis*-decalin part of **8** into a small hydrophobic pocket through interactions with the side chains of W143, L355, F358 and Y151. These interactions suggest that the stereoselectivity of the IMDA reaction is sterically controlled via shape complementarity to prevent the acyclic precursor such as **6** to access other transition states. When the *trans*-decalin version of **8** formed by the same facial selectivity of IMDA reaction was computationally docked into the binding cavity of FinI, prohibitive steric clashes with the residues lining the pocket were clearly evident (Figure S16).

After demonstrating FinI is a *cis*-decalin forming DAse, we examined the role of ApiI in the biosynthesis of the *trans*-decalin **2.** Upon expression of the *api* cluster without ApiI (*apiACDEHJ*) in *A. nidulans,* we observed the accumulation of a putative pyran congener pair **12/12'** (**3/3'** with C12 methyl) as the major product (Figure 2A, iv, and 2C). A small amount of **2** was detected by LCMS, which is in agreement with Oikawa's finding that *trans*-decalin formation can be nonenzymatic.[30] In contrast, upon coexpression of ApiI, the amount of **2** significantly increased while the pyran congener pair can no longer be detected (Figure 2A, v). Purification of **2** confirmed the *trans*-decalin stereochemistry (Table S4 and Figure S19-S24). This is agreement with a role of ApiI in accelerating the *endo*-DA reaction to form *trans*-decalin. To further confirm the function of ApiI, we heterologously expressed *finACDEHJ* with *apiI* in *A. nidulans* to produce a *trans*-decalin analog of **1**.

LCMS analysis revealed that a new compound **5** (0.5 mg/L) with the same MWT as **1** was produced (Figure 2A, iii). NMR analysis confirmed the decalin moiety in **5** is indeed in the *trans* configuration (Table S6 and Figures S31-S36). This is the only stereoisomer that can be detected. Identification of **5** led us to reexamine the metabolite profile of *A. nidulans* expression *finACDEHJ* (Figure 2A, i). Based on retention time and mass, low levels of **5** and likely its *trans*-diastereomer formed via nonenzymatic cyclization can be detected.

In summary, we have identified new DAses FinI and ApiI from BGCs of **1** and **2**, respectively. Molecular insights gained from our structural studies of FinI further showcase how Nature can repurpose a widely occurring OMT structure for catalysis of a synthetically challenging pericyclic reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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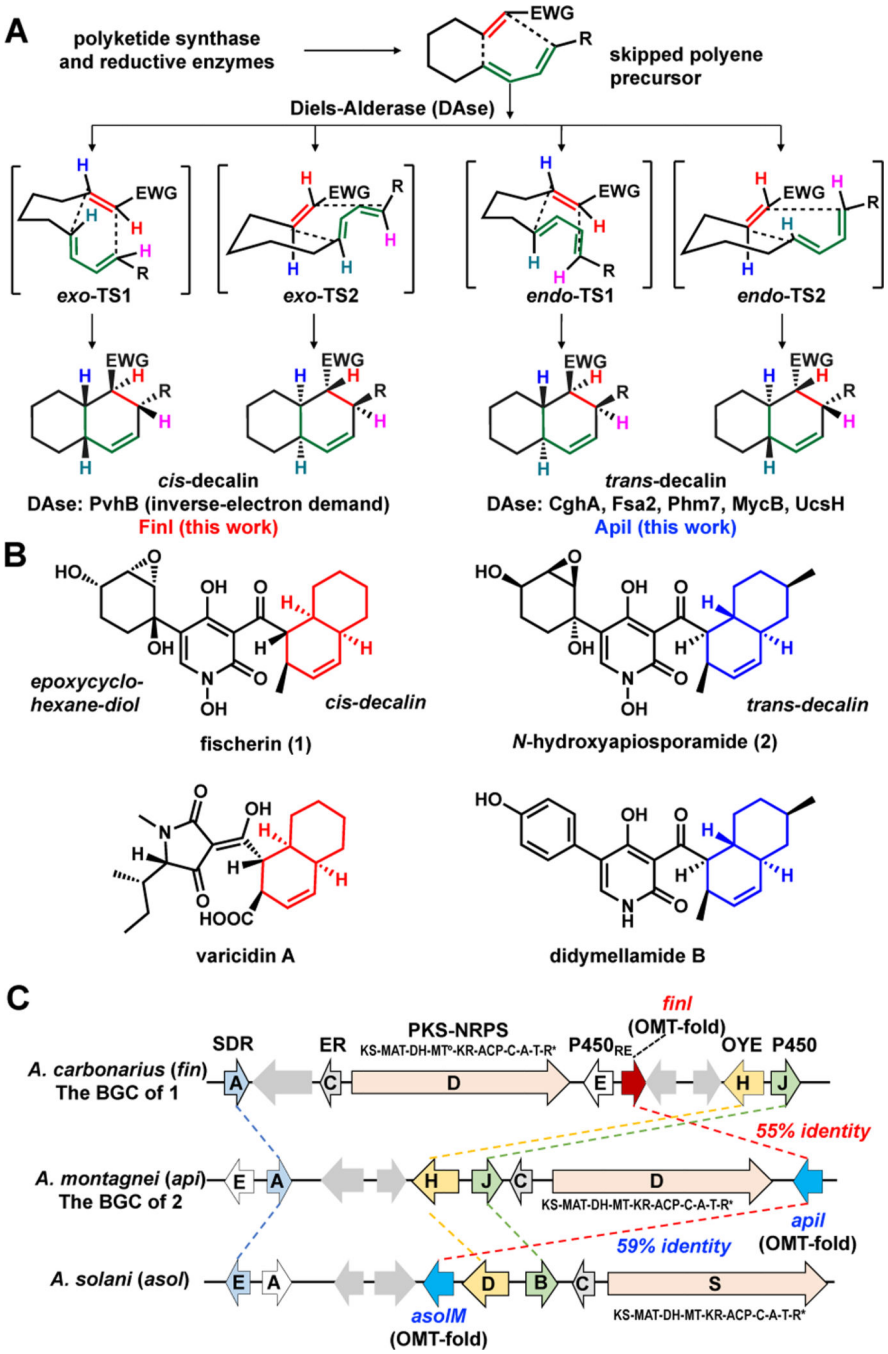

Figure 1.

Decalin-forming Diels-Alder reactions in fungal natural product biosynthesis and BGCs of interest. (**A**) Formation of four possible stereoisomers through IMDA reaction. (**B**) Structures of decalin-containing compounds. (**C**) The BGCs of **1** and **2**; Abbreviations: KS, ketosynthase; MAT, malonyl-CoA:ACP transacylase; DH, dehydratase; MT, methyltransferase; KR, ketoreductase; ACP, acyl carrier protein; C, condensation; A, adenylation; T, thiolation; R, reductase. ER, enoylreductase; OYE, old-yellow enzyme (ene-

reductase); SDR, short-chain dehydrogenase/reductase; OMT, *O*-methyltransferase; CMT, C-methyltransferase.

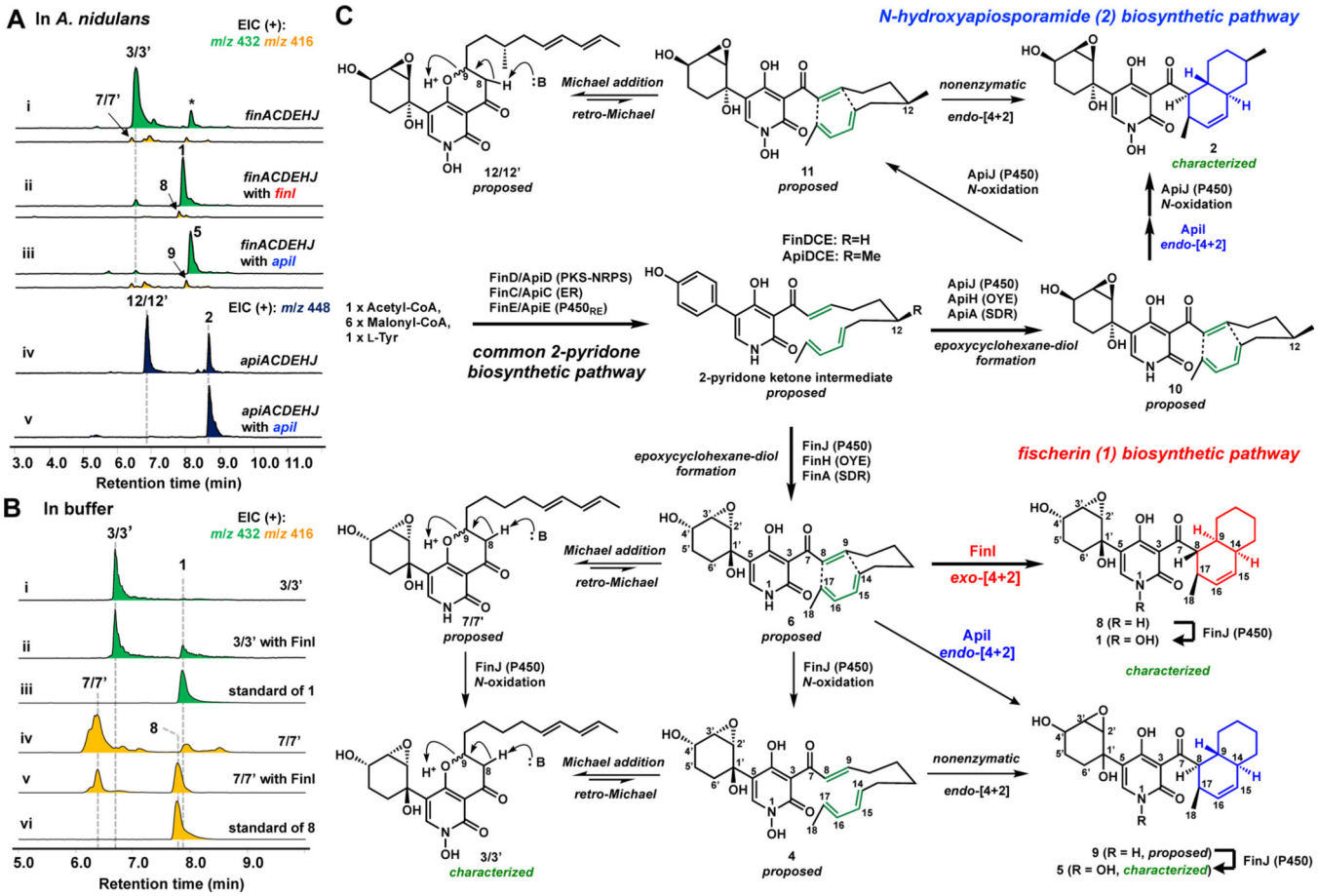

Figure 2.

Discovery of *cis*-decalin forming FinI and *trans*-decalin forming ApiI. (**A**) Products of *A. nidulans* expressing *finACDEHJ* with or without a DAse. *The peak is expected to be a mixture of **5** and the *trans*-diastereomer. (**B**) In vitro reaction of FinI with **3**/**3'** and **7**/**7'**. (**C**) Proposed biosynthetic pathways of **1**. The structures of **5** and **9** are shown with relative stereochemistry.

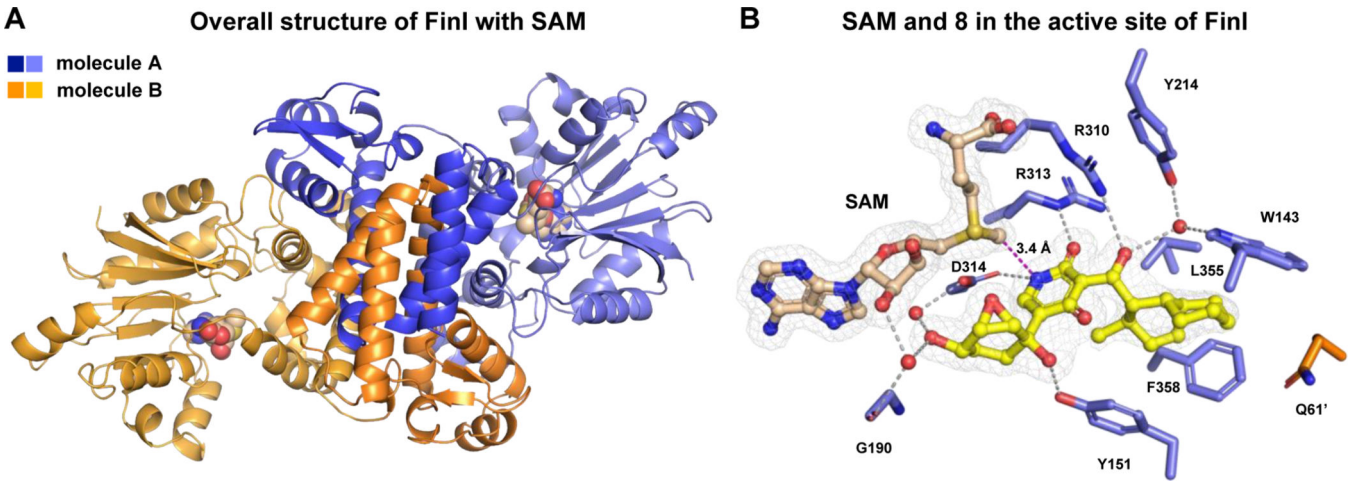

Figure 3.

Structural basis of FinI specificity and stereoselectivity. (**A**) X-ray crystal structure of the FinI-SAM-complex (molecule A in blue and molecule B in orange, *N*-deoxyfischerin (**8**) and SAM (cofactor) shown as spheres); (**B**) The interactions between *N*-deoxyfischerin (**8**), SAM and the active site residues (residues from molecule A, light blue; residues from molecule B, bright orange; SAM, wheat; **8**, yellow). Simulated annealing omit maps are shown in grey mesh and contoured at 1.0 σ. The absolute stereochemistry of **8** was determined from the crystal structure of FinI-SAM-**8**.