An ongoing challenge in chemical research is to design catalysts that select the outcomes of the reactions of complex molecules. Chemists rely on organocatalysts or transition metal catalysts to control stereoselectivity, regioselectivity and periselectivity (selectivity among possible pericyclic reactions). Nature achieves these types of selectivity with a variety of enzymes such as the recently discovered pericyclases—a family of enzymes that catalyse pericyclic reactions. Most characterized enzymatic pericyclic reactions have been cycloadditions, and it has been difficult to rationalize how the observed selectivities are achieved. Here we report the discovery of two homologous groups of pericyclases that catalyse distinct reactions: one group catalyses an Alder-ene reaction that was, to our knowledge, previously unknown in biology; the second catalyses a stereoselective hetero-Diels–Alder reaction. Guided by computational studies, we have rationalized the observed differences in reactivities and designed mutant enzymes that reverse periselectivities from Alder-ene to hetero-Diels–Alder and vice versa. A combination of in vitro biochemical characterizations, computational studies, enzyme co-crystal structures, and mutational studies illustrate how high regioselectivity and periselectivity are achieved in nearly identical active sites.

Pericyclic reactions are concerted chemical transformations in which all bonding changes occur in a cyclic array of atoms. These reactions are classics in synthesis, and form cyclohexenes and pyrans, respectively. These pericyclic reactions are catalysed by enzymes in nature. The Alder-ene reaction, originally named the ‘substituting addition’ reaction by Kurt Alder’s laboratory in 1943, has been difficult to rationalize how the observed selectivities are achieved. Here we report the discovery of two homologous groups of pericyclases that catalyse distinct reactions: one group catalyses an Alder-ene reaction that was, to our knowledge, previously unknown in biology; the second catalyses a stereoselective hetero-Diels–Alder reaction.

To understand the reactivity of the QM in pericyclic reactions, we performed quantum mechanical calculations to determine transition state (TS) geometries and to quantify the barriers to possible pericyclic reactions from the same reactive QM. Because Alder-ene reactions are less exothermic (about −27 kcal mol⁻¹) than cycloadditions (about −38 kcal mol⁻¹), we anticipated that the Alder-ene reaction would be intrinsically more difficult than hetero-Diels–Alder reactions and that periselectivity would strongly favour the latter.

To identify the potential pericyclases, we searched the genomes of the producing strains for the exclusive formation of either 1 or 2 (and 3) from the producing strains. To identify the potential pericyclases, we searched the genomes of the producing strains for the exclusive formation of either 1 or 2 (and 3) from the producing strains.

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Fig. 1 | Pericyclic reactions in natural product biosynthesis. a. Known and unknown enzymatic examples of pericyclic reactions. b. The biosynthesis of leporin B involves the multifunctional O-methyltransferase-like pericyclase LepI. c. Theoretical investigations indicate that hetero-Diels–Alder TS-2 is nonenzymatically favoured by 1.9 kcal mol⁻¹ over Alder-ene TS-1 from a common intermediate. Further transformations lead to the natural products pyridoxatin, cordypyridone, asperpyridone and fusaricide. d. The biosynthetic gene cluster (lep) of leporin B from Aspergillus flavus, the putative biosynthetic gene cluster (adx) of pyridoxatin from Albophoma yamanashiensis, the putative biosynthetic gene cluster (pdx) of pyridoxatin from Aspergillus bombycis, and the putative biosynthetic gene cluster (epi) of fusaricide from Epicoccum sorghinum FT1062. PKS-NRPS, polyketide synthase-nonribosomal peptide synthetase; TF, transcription factor; MCT, monocarboxylate transporter; P450, cytochrome P450; SDR, short-chain dehydrogenase/reductase; ER, enoylreductase; PC, pericyclase. e. The proposed biosynthesis of the Alder-ene product (8) and the hetero-Diels–Alder product (9) from the common intermediate. f. One-pot in vitro tandem assay of 5 with PdxG in the presence or absence of selected pericyclases.

(adx/epiC), a partnering enoylreductase (adxD/epiD), a ring expansion P450 (adxA/epiA), a putative N-hydroxylation P450 (adxB/epiB), a short-chain dehydrogenase/reductase (SDR) (adxG/epiG) and a putative O-methyltransferase (OMT) (adxI/epiI). We searched the National Center for Biotechnology Information (NCBI) database for other homologous clusters, which are conserved in many sequenced fungal strains such as Aspergillus bombycis (pdxA), Monosporascus cannonballus (modx), Uncinocarpus reesii (api) and Hymenoscyphus scutulata (hpi) (Fig. 1d, Extended Data Fig. 2). On the basis of the sequence of the previously discovered pericyclase LepI in leporin biosynthesis⁴ (Fig. 1b, d), we hypothesized that the predicted OMT-fold enzymes in these pathways (AdxI, Epil, PdxI, ModxI, Upil and HpiI) are potential...
pericyclases. We proposed that each enzyme would catalyse the stereoselective dehydration of the alcohol (6) to 7. Adxl from the 1-producing strain would then catalyse the subsequent Alder-ene reaction, whereas Epi from the 3-producing strain would catalyse the hetero-Diels–Alder reaction (Fig. 1e). While there is high sequence identity between these enzymes (59–83%; Extended Data Fig. 2b), they all display very low identity to Lepl (~15%).

We performed the coupled in vitro reactions using enzymatically and chemoenzymatically prepared ketone 5 (Supplementary Information), the SDR PdxG as the putative ketoreductase, and one of the proposed pericyclases. In the presence of PdxG and a cofactor NADPH, 5 is reduced to the alcohol 6 (Fig. 1f, i). (Extended Data Fig. 3a, b). In solution, 6 readily underwent nonenzymatic dehydration to generate both (E)- and (Z)-QM that rehydrated to form 6 and the C7 diastereomer 7 (Extended Data Fig. 4d). After 2 h, small amounts of O4-hetero-Diels–Alder product 9 and atropisomeric Alder-ene product 8 were detected in a ratio of 3 to 1 (Fig. 1f, ii) along with three other unidentified minor products (Extended Data Figs. 1, 4e).

Extended incubation times (10 h) with PdxG generated the over-reduced 10, which is presumably derived from a reduction of the QM (Fig. 1f, iii), (Extended Data Fig. 3c). When Adxl, Pdxl or Modxl was incubated with PdxG, NADPH and 5, it predominately formed 8 (~98:2, 8:9: Fig. 1f, iv, v, vi), (Extended Data Fig. 2c). On the other hand, when we added Epi, Upl or HpiI to the reaction mixtures containing PdxG, NADPH and 5, the periselectivity was switched and 9 was the predominant product (<5:95, 8:9: Fig. 1f, vii, Extended Data Fig. 2c). Because 8 and 9 can be chemically interconverted in harsh acidic conditions, we investigated whether these pericyclases could catalyse such a transformation. However, no enzyme was able to catalyse the interconversion (Fig. 1e, Extended Data Fig. 4c), which indicates that these enzymes catalyse the observed reactions with strong periselectivity. Although these new pericyclases are predicted S-adenosyl-l-methionine (SAM)-dependent OMTs 13, none was copurified with SAM (Extended Data Fig. 4a), and neither SAM nor S-adenosyl-l-homocysteine (SAH) was required for catalysis (Extended Data Figs. 2c, 4b). Overall, our biochemical data show that the group of Adxl, Pdxl and Modxl catalyse the Alder-ene reaction and form a bona fide Alder-ene adduct. The group of Epi, Upl and HpiI were characterized as SAM-independent enzymes that catalyse a hetero-Diels–Alder reaction to form a trans-fused hexahydroisochromene. These discoveries expand the repertoire of reactions catalysed in nature.

To gain mechanistic insight into the enzyme-catalysed Alder-ene reaction, X-ray crystal structures of apo-Pdxl, substrate analogue complex Pdxl–5 and product complex Pdxl–8 were solved and refined to 2.0 Å, 2.0 Å and 2.4 Å resolutions, respectively. The apo-Pdxl structure adopts a classic α,β-Rossmann OMT fold 16 (Fig. 2a) and forms a wren dimer structure by interlocking the N-terminal helices (Fig. 2b, lime and royal blue). The Pdxl–5 and Pdxl–8 structures are overall very similar to that of apo-Pdxl (root mean squared deviation (r.m.s.d.) of 0.162 and 0.202 for 831 Cα atoms, respectively). The chain A active sites of Pdxl–5 and Pdxl–8 are shown in Fig. 2c and Extended Data Fig. 5. The pyridone ring forms hydrogen bonds with the side chains of K337, H161 and Q412, as well as with water molecules via T232, D233 and H336 in the active site. The extended alkylation chain of 5 is pointed towards hydrophobic residue I366 and is not in a near-attack conformation for the pericyclic reaction. The C7 ketone of substrate analogue 5 is positioned syn to the C4-hydroxyl and is indicative that the alcohol substrate undergoes a syn-dehydration facilitated by K337 to generate (Z)-QM 7 (Extended Data Fig. 10). In order to generate an (E)-QM, the C7 alcohol would need to rotate by 180° and be syn to the C2-oxygen. The fact that this (E)-QM geometry differs greatly from that of the substrate analogue indicates that it is most likely that 7 is generated in situ. Substitution of K337 to alanine completely abolished the dehydration activity, but it could be rescued by mutation to arginine (Extended Data Figs. 7, 9a), which supports the role of K337 as a general base for the dehydration step.

To investigate how the active site of Pdxl catalyses the energetically unfavourable Alder-ene reaction and suppresses the hetero-Diels–Alder reaction, we performed multiple 500-ns classical molecular dynamics (MD) simulations of 7 docked into Pdxl. As K337 is expected to be protonated after dehydration of 6 to 7, we modelled the side chain of K337 as an ammonium ion. We analysed the conformation of the alkyl chain of the reactive 7 throughout the simulations and found that the alkyl chain can reorganize from the extended, unreactive conformation seen in Pdxl–5 into a reactive near-attack conformation for 50–100 ns (Extended Data Fig. 6c). In all simulations, H161 and Q412 formed hydrogen bonds with N1 and the C2-carbonyl of the pyridone, respectively, whereas K337 and H336 form hydrogen bonds with the C4-carbonyl (Extended Data Fig. 6a, b). K337 maintains this hydrogen bond to the pyridone C4-carbonyl of 7 for longer durations than H336 in the simulation, which implies that the protonated K337 may facilitate the Alder-ene reaction by hydrogen bond catalysis.

Next, we quantified how the K337 and H161 hydrogen bonds affect the reaction rate with a truncated catalytic-residue ‘theozyme’ model; K337 is modelled as a methyl ammonium and H161 as an imidazole (Fig. 3a). The calculations indicate that the energetic barrier for the Alder-ene transition state in the theozyme (TS-3) is reduced by 11.7 kcal mol⁻¹, a rate acceleration of more than 10⁷, when compared to the nonenzymatic reaction (TS-1) (Extended Data Fig. 1b). Protonation of the carbonyl makes C7 highly electrophilic and decreases the nucleophilicity of the carbonyl oxygen. Both of these factors suppress the hetero-Diels–Alder transition state (TS-4) and favour TS-3 by 3.4 kcal mol⁻¹, whereas the O4-hetero-Diels–Alder via TS-4 has a barrier of 12.6 kcal mol⁻¹. This indicates that the Pdxl active site alters the electronics of the reaction, by protonation of O4, to favour the Alder-ene reaction and achieve the observed periselectivity.

It should be noted that the Alder-ene theozyme model also predicts a distinct, yet favourable hetero-Diels–Alder reaction via TS-5 to form the cis-fused O2-hetero-Diels–Alder 4-pyridone adduct 11 (Fig. 3a). However, 11 is not found in the in vitro Pdxl reaction mixtures (Fig. 1f, Extended Data Figs. 4e, 7). This implies that an additional degree of regio- and periselectivity is exerted by Pdxl to disfavour the formation of TS-5. The Pdxl structure with a docked TS-5 indicates that the side chain of T232 and a water molecule (W) cause disfavourable interactions with the terminal C4 methyl in TS-5 (Extended Data Fig. 5d). The threonine residue acts as a steric barrier to prevent 7 from accessing the TS-5 conformation. To test this hypothesis, we prepared Pdxl mutants in which T232 is replaced with alanine, serine or valine (T232A/S/V mutants) and performed the coupled in vitro reactions. Indeed, the less hindered T232A and T232S mutants generated an appreciable amount of a new compound as compared to the wild-type Pdxl (Fig. 3c, Extended Data Figs. 4f, 7). Large-scale in vitro reaction of the Pdxl(T232S) mutant led to the isolation and determination of structure of the expected 11, which is one of the minor, nonenzymatic cyclized products from 7 (Extended Data Figs. 1, 4e). Mutation to valine (Pdxl(T232V)), the steric isostere of threonine, did not produce any 11 (Fig. 3c, 7). Extended Data Fig. 7) supporting the role of T232 in sterically preventing the formation of TS-5.

Pdxl crystal structures, molecular dynamics and the ‘theozyme’ model indicate that the K337 residue acts as a general acid catalyst to favour the Alder-ene reaction over the O4-hetero-Diels–Alder reaction. Then, we calculated the O4-hetero-Diels–Alder ‘theozyme’ model (Fig. 3b) by removing the lysine from the Alder-ene theozyme model (Fig. 3a), and indeed the O4-hetero-Diels–Alder reaction is preferred by 1.7 kcal mol⁻¹ (TS-6 versus TS-7). This suggests that the group of
enzymes (EpII, HpII and UpII) that catalyse the O4-hetero-Diels–Alder reaction, in contrast to PdxI, must avoid proton transfer to the pyridone C4 carbonyl to achieve the opposite periselectivity. Nevertheless, the corresponding lysine is conserved in these three enzymes as well (Extended Data Fig. 2b). We solved and refined the crystal structures of apo-HpII and HpII–ketone 5 to 1.3 Å and 1.5 Å resolution, respectively (Fig. 2d). These structures are highly similar to that of PdxI (r.m.s.d. of 0.468 for 415 Cα atoms; Fig. 2b–d, Extended Data Fig. 5c). Notably, the binding modes of 5 in PdxI and HpII are essentially identical (Fig. 2c, d, Extended Data Fig. 5c) and nearly all amino acid residues in the active site between PdxI and HpII are conserved except for V413 in PdxI and the corresponding residue M415 in HpII (Fig. 2c, d, Extended Data Figs. 2b, 5c). This key residue sits below the pyridone binding site and neighbours the aforementioned lysine (K337 in PdxI, K338 in EpII and K339 in HpII). The HpII–ketone 5 complex clearly shows that K339, unlike K337 in PdxI, does not form a hydrogen bond to the 4-hydroxy on the pyridone ring; this distance is stretched out from 3.2 Å in PdxI to 4.1 Å in HpII (Fig. 2c, d). To verify that the lysine residue is not catalytic, we prepared the more stable mutant K338A of EpII. In contrast to PdxI(K337A) mutant, EpII(K338A) retained the majority (about 80%) of its enzymatic activity and showed the same periselectivity as wild-type EpII (Fig. 3c, Extended Data Figs. 8, 9b). Thus, the EpII-catalysed reactions do not require hydrogen bonding between the C4-oxygen on the pyridone ring and K338 (Extended Data Figs. 8–10). From the crystal structure of HpII, this loss of a hydrogen bond is caused by the bulkier side chain of M415, which shifts the lysine side chain further away from the substrate (Fig. 2d, Extended Data Fig. 5c). Consistently, homologous pericyclases such as EpII, UpII and HpII all catalyse the hetero-Diels–Alder reaction to form 9, and the methionine residue is conserved (M411, M407 and M415, respectively). By contrast, pericyclases such as PdxI, AbxI and ModxI catalyse the Alder-ene reaction, and the valine residue (V413) is conserved (Extended Data Fig. 2b, c).

We next explored whether the periselectivity of PdxI could be switched to favour the hetero-Diels–Alder reaction by replacement of its V413 residue with alanine, isoleucine or methionine. Mutating valine to alanine or isoleucine, which are smaller than methionine, retained the periselectivity of PdxI for the Alder-ene reaction (Fig. 3c, Extended Data Figs. 7, 9a). By contrast, the V413M mutant showed reversed periselectivity, switching the major reaction type from Alder-ene to hetero-Diels–Alder, forming 8 and 9.
in a ratio of 40:60 compared to the wild-type ratio of more than 98:2 (Fig. 3c, Extended Data Figs. 7, 9a). On the other hand, the mutation of M411 in Epil to the less bulky valine or cysteine altered the product ratio (8:9) from 5:95 to 25:75 (Fig. 3c, Extended data Figs. 8, 9b). The mutation of M411 alone in Epil is not sufficient to reverse the periselectivity, suggesting that other factors such as shape complementarity to restrict the movement of 7 would also contribute to maintaining the observed periselectivity for the hetero-Diels–Alder reaction.

As the conformational flexibility of 7 is expected to be restricted by both M411 and T231 residues in the Epil active site, on the basis of the HpiI–5 complex (Fig. 2d), we mutated the T231 residue to smaller alanine or serine residues. This mutation would increase the conformational flexibility of 7 in the Epil active site to form the key hydrogen bond with K338 for the Alder-ene reaction. Indeed, while the hetero-Diels–Alder product 9 still remained the major product, Epil(T231A) and Epil(T231S) increased the ratio of Alder-ene product 8 along with the O2-hetero-Diels–Alder product 11 as seen in PdxI(T232A/S) mutants.
(Fig. 3c. Extended Data Figs. 8, 9b). Intriguingly, the double mutant Epil(M411V/T231A) showed reversed periselectivity with a ratio (8:9) of 66:33. Other less bulky double mutants, such as M411V/T231S, M411T/T231A, M411C/T231A and M411G/T231A, showed similarly reversed periselectivities. On the basis of these results, we conclude that the replacement of the methionine and threonine with smaller residues enlarges the enzyme active site and allows greater conformational sampling of 7 and protonation of the C4 carbonyl, thus leading to the opposite periselectivity.

Our results show that the group of PdxI, Adxl and Modxl are multifunctional enzymes that catalyse the stereoselective syn-dehydration of 6 to 7 and the subsequent Alder-ene reaction of 7 to 8 in a stereo-, regio- and periselective manner. By contrast, the group of Epil, Upil and Hpil catalyse the same stereoselective syn-dehydration of 6 to 7 but with orthogonal periselectivity and catalyse the hetero-Diels–Alder reaction of 7 to 9. Computational studies, comparative analysis of the enzyme co-crystal structures and site-directed mutagenesis provided a detailed picture of the catalytic mechanism for Pdxl and Epil (Extended Data Fig. 10). Pdxl uses K337 as general acid catalyst to facilitate the otherwise energetically unfavourable Alder-ene reaction, while the methionine substitution in Epil abolishes this interaction to allow only the O4-hetero-Diels–Alder reaction. The steric effect of T232 in Pdxl and T231 in Epil inhibits the formation of the O2-hetero-Diels–Alder product 11 to further control regioselectivity.

In conclusion, we have characterized two homologous groups of enzymes and identified how subtle evolutionary divergence leads to the production of different natural products. The insight gained from our research will serve as a basis for developing new biocatalysts that catalyse various natural and unnatural Alder-ene and hetero-Diels–Alder reactions that are valuable synthetic transformations.

Online content
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**Methods**

**Material, fungal strains and culture condition**

*A. bombycis* NRRRL26010 was obtained from the Agricultural Research Service Culture Collection (NRRL). *A. nidulans* FGSC A1145 was obtained from the Fungal Genetics Stock Center (http://www.fgsc.net/). *A. bombycis* and *E. sorghinum* FT1062 were maintained on PDA (potato dextrose agar, BD) for 3–5 days for sporulation or in liquid PDB medium (PDA medium without agar) for isolation of genomic DNA. *A. nidulans* was maintained on Czapek-Dox (CD) agar for sporulation or in liquid CD–ST medium for gene overexpression, compound production and RNA extraction (http://www.fgsc.net/).

**General DNA manipulation technique**

*Escherichia coli* TOP10 was used for cloning, following standard recombinant DNA techniques. DNA restriction enzymes were used as recommended by the manufacturer (New England Biolabs, NEB). PCR was performed using Phusion High-Fidelity DNA Polymerase (NEB). The gene-specific primers are listed in the Supplementary Information. PCR products were confirmed by DNA sequencing. *E. coli* BL21(DE3) (Novagen) was used as the *E. coli* host for protein expression. GeneArt Seamless Cloning and Assembly kit (Thermo Fisher Scientific) was used for the construction of pET28b-derived protein expression vectors. In vivo homologous recombination using *Saccharomyces cerevisiae* was used for the construction of the *A. nidulans* overexpression plasmids.

**Protein expression and purification of PdxG, PdxI, Epil, their homologues and mutants from *E. coli* BL21 (DE3)**

The open reading frame of *pdxG*, *pdxI*, *epil*, their homologues and mutants from *E. coli* BL21 (DE3) was amplified using cDNA from the transformant of *A. nidulans* as a template by PCR with primers of pMO20017-fl/fl-r1 and ligated with a linear pET28b expression vector, which was amplified by PCR with pMOvec-fl/fl-r1 to generate pMO20017 using GeneArt Seamless Cloning and Assembly kit (Thermo Fisher Scientific). The identity of the resulting vector pMO20017 was confirmed by DNA sequencing. The codon-optimized synthetic genes of *pdxG*, *adxl*, *modxl*, *epil*, *upil* and *hpiI* were synthesized by Integrated DNA Technologies Co (IDT). These synthesized genes were ligated with the linear pET28b expression vector as above. The oligonucleotide primers used to construct plasmids for expression of PdxI and Epil mutants are listed in the Supplementary Information. The plasmids pMO20017 and pMO90027 containing the wild-type *pdxI* gene and *epil* genes, respectively, were used as the template for PCR-based site-directed mutagenesis. The primers of pdx-M-fl/pdx-H161A-r1 and pdx-H161A-fl/pdx-M-r1 were used to amplify the H161A mutant, and the resulting two overlapping fragments and the pET28b expression vector amplified using the primers of pdx-vec-fl/fl-r1 were combined to generate the H161A expression plasmid using GeneArt Seamless Cloning and Assembly kit (Thermo Fisher Scientific). Other mutants were constructed in the same manner using primer pairs (ex. pdx-M-fl/pdx (or epil)-mutation position-r1 and pdx (or epil)-mutation position-fl/pdx-M-r1). DNA sequencing was used to confirm the identities including the mutated positions of the expression plasmids.

**Protein crystallization**

Crystals of the Se-Met PdxI were grown at 16 °C using the sitting drop vapour diffusion method in 2-μl drops containing an 1:1 mixture of the protein solution (20 mg/ml Se-Met PdxI in buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl and 3 mM DTT) and a reservoir solution (0.1 M MOPS/HEPES-Na (pH 7.5), 12.5% PEG 10000, 12.5% PEG 3350, 12.5% MPD, 0.03 M of each NPS (0.3 M sodium nitrate, 0.3 M disodium hydrogen phosphate, 0.3 M ammonium sulfate)). Stick-like crystals appeared after three days at 16 °C. Crystals of native PdxI were grown at 16 °C using the sitting drop vapour diffusion method in 2-μl drops containing an 1:1 mixture of the protein solution (20 mg/ml PdxI in buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl and 3 mM DTT) and a reservoir solution (16% PEG 8000, 40 mM potassium phosphate monobasic, 20% glycerol). Stick-like crystals appeared after two days at 16 °C.

All PdxI-compound complexes were crystallized using the sitting drop vapour diffusion method at 16 °C. Purified PdxI (20 mg/ml) was first incubated with 2.0 mM compound and 2–4% DMSO in buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl and 3 mM DTT on ice for 30 min and followed by centrifuge. One microlitre of protein solution was mixed with 1 μl precipitant solution. Stick-like crystals appeared after two days at 16 °C. The PdxI–S complexes were grown under the same conditions.

The native HpiI crystals were grown in 0.1 M MES (pH 6.5), 15% PEG 6000, 5% MPD. Prism-like crystals appeared after two days at 16 °C. The HpiI–S complexes were crystallized in 16% PEG 8000, 40 mM potassium phosphate monobasic, 20% glycerol. Prism-like crystals appeared after two days at 16 °C.
All crystals were flash-frozen in liquid nitrogen after being transferred to a cryoprotectant solution consisting of mother liquor supplemented with 10–15% (v/v) glycerol.

Data collection and structure determination

All X-ray diffraction data were recorded at the Shanghai Synchrotron Radiation Facility (SSRF). For Se-Met PdxI, native PdxI and the PdxI–5 complex, data were collected at BL18U1 (λ = 0.97930 Å). For the HpiI–5 complex, data were collected at BL19U1 (λ = 0.97855 Å). For the PdxI–5 complex, data were collected at BL17U1 (λ = 0.97915 Å). Data reduction and integration for PdxI–5 and HpiI–5 were achieved using XDS34; for other crystals, we used the HKL3000 package35. The statistics for data collection are listed in the Supplementary Information. All PdxI crystals belonged to space group P21, for data collection are listed in the Supplementary Information. All PdxI XDS34; for other crystals, we used the HKL3000 package35. The statistics for data collection are listed in the Supplementary Information.

Activity assay of PdxG-catalysed reaction using the ketone 5 as the substrate

Assays for PdxG activity with 600 μM 5 in HEPES buffer (100 mM HEPES, 2 mM NADPH, pH 8.0) were performed at 50 μl scale with 3 μM PdxG at 30 °C for 20 min. Then the reaction was quenched with an equal volume of cold acetonitrile. Protein was precipitated and removed by centrifugation and the supernatant analysed by LC–MS on a Shimadzu 2020 EV LC–MS (Kinetex 1.7 μm C18 100 Å, LC Column 100 × 2.1 mm) using positive- and negative-mode electrospray ionization with a linear gradient of 5–95% acetonitrile MeCN–H2O with 0.5% formic acid in 15 min followed by 95% MeCN for 3 min with a flow rate of 0.3 ml min⁻¹. The results are shown in Extended Data Fig. 3a. For kinetic analysis of the PdxG-catalysed reaction, 50-μl reaction mixtures containing 3 μM PdxG, 2 mM NADPH, and the different concentrations of 5 (10, 60, 120, 300, 480, 600, 540, 1,200 μM) in HEPES buffer were incubated at 30 °C for 20 min. Then, the reaction was quenched with an equal volume of cold acetonitrile. Protein was precipitated and removed by centrifugation and the supernatant analysed by LC–MS. The consumption of 5 was estimated by a standard curve of 5 that was generated from peak areas at 330 nm by HPLC. Data fitting was performed using GraphPad Prism 8, and kcat and km values represent mean ± s.d. of three independent replicates. The results are shown in Extended Data Fig. 3b.

In vitro reaction of PdxI and Epil using the alcohol 6 as the substrate

The alcohol 6 was prepared as follows. NaNbH₄ (1.2 mg, 30 μmol) was added to the solution of compound 5 (1.0 mg, 3 μmol) in 0.5 ml EtOH at 0 °C and the mixture was stirred at room temperature for 15 min. The reaction mixture was quenched with 0.5 ml 1M HCl, and was extracted with 0.5 ml ethyl acetate twice. The extract was concentrated, and the residue was purified by HPLC with an analytical C18 column of Kinetics New column, 5 μm, 4.6 × 250 mm (Phenomenex). The fractions were not concentrated owing to instability and were immediately used as the substrate solution.

Activity assays for PdxI and Epil towards 6 in HEPES buffer (100 mM HEPES, pH 8.0) were performed at 50-μl scale with 30 μM PdxI (or 50 mM T2325 mutant) and 20 μM Epil (or 40 μM T2314 mutant), at 30 °C for 2 h. Then the reactions were quenched with 70 μl cold acetonitrile. Protein was precipitated and removed by centrifugation, and the supernatant was analysed by LC–MS as described above. These results are shown in Extended Data Fig. 4d, e.

Coupled in vitro reaction of PdxG and pericyclases used in this study using 5 as the substrates

In assays with PdxI, PdxI homologues and PdxI mutants, 50 μl reaction mixture containing 150 μM 5 in HEPES buffer (pH 8.0) containing 100 mM HEPES, 1 mM NADPH was incubated with 3 μM PdxG and 50 μM pericyclase of interest at 30 °C for 2 h. In assays with Epil and the mutants, 50 μl reaction mixture containing 300 μM 5 in HEPES buffer (pH 8.0) containing 100 mM HEPES. 1 mM NADPH was incubated with 3 μM PdxG and 40 μM pericyclase of interest at 30 °C for 2 h. Reactions were quenched with 70 μl cold acetonitrile. Protein was precipitated and removed by centrifugation and the supernatant was analysed by HPLC using a C18 column (Phenomenex Luna C18 (2) 5 μm, 2.0 × 100 mm) with a linear gradient of 15–35% acetonitrile MeCN–H2O in 4 min followed by 35% MeCN for 1 min followed by 95% MeCN for 5 min with a flow rate of 0.3 ml min⁻¹. The results are shown in Fig. 1f and Extended Data Fig. 2c. The supernatant was also analysed by HPLC using a C18 column (Phenomenex Luna C18 (2) 5 μm, 2.0 × 100 mm) with a linear gradient of 15–35% acetonitrile MeCN–H2O in 4 min followed by 35% MeCN for 1 min followed by 95% MeCN for 5 min with a flow rate of 0.3 ml min⁻¹. The results are shown in Extended Data Figs. 7, 8. To measure the relative product ratio of 8, 9 and 11 and the relative activities of the pericyclases, concentrations of 8, 9 and 11 were estimated using separate standard curves of 8, 9 and 11 that were based on peak areas at 290 nm, 290 nm and 268 nm by HPLC, respectively. The data are shown in Fig. 3c and Extended Data Fig. 9. The error bars represent s.d. of three independent replicates.

Measurement of the presence of SAM in PdxI and Epil, and the effects of SAM and SAH for PdxI and Epil catalysis

To measure whether SAM was copurified with PdxI and Epil, more than 200 μM of PdxI and Epil in 50 μl storage buffer (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 8.0) was denatured by acetonitrile. Then, the solutions were centrifuged and the supernatants analysed by LC–MS. The standards of SAM were also analysed by LC–MS using a C18 column (Kinetex 1.7 μm C18 100 Å, LC Column 100 × 2.1 mm) with a linear gradient of 5–95% acetonitrile MeCN–H2O with 0.5% formic acid in 15 min followed by 95% MeCN for 3 min with a flow rate of 0.3 ml min⁻¹. The results are shown in Extended Data Fig. 4a. To measure the effects of SAM and SAH in the PdxI- and Epil-catalysed reactions, 50 μl reaction mixtures containing 3 μM PdxG, 1 mM NADPH, and 30 μM PdxI or 20 μM Epil, either with or without 1 mM SAM or 1 mM SAH were incubated at room temperature for 10 min. Then, 150 μM of 5 was added to the reaction mixture to initiate the enzymatic reaction and further incubated at 30 °C for 2 h. Then, the reaction mixtures were quenched by 70 μl of acetonitrile. After centrifugation, the supernatant was subjected to HPLC analysis using a C18 column (Phenomenex Luna C18 (2) 5 μm, 2.0 × 100 mm) with a linear gradient of 35% acetonitrile MeCN–H2O with 0.5% formic acid in 15 min followed by 95% MeCN for 5 min with a flow rate of 0.3 ml min⁻¹. The results are shown in Extended Data Fig. 4b.

In vitro reaction of PdxI and Epil using 8 and 9 as the substrates

To rule out the possibility that PdxI and Epil catalyse the retro-hydroalkoxylation of 9 to 8 and the hydroalkoxylation of 8 to 9, respectively, we performed in vitro reactions of PdxI using 9 as the substrate and of Epil using 8 as the substrate. Fifty microlitres of reaction mixture containing 100 μM of the substrate compound in HEPES...
buffer (100 mM HEPES, pH 8.0) was incubated with 50 μM PdxI or 40 μM HpiI at 30 °C for 24 h. The reactions were quenched with 70 μl acetonitrile. Protein was precipitated and removed by centrifugation and the supernatant analysed by HPLC using a C18 column (Kinetex 1.7 μm C18 100 Å, LC Column 100 × 2.1 mm) with a linear gradient of 5–95% acetonitrile MeCN–H2O with 0.5% formic acid in 15 min followed by 95% MeCN for 3 min with a flow rate of 0.3 ml min⁻¹. The results are shown in Extended Data Fig. 4c.

Density functional theory calculations
Initial conformational searches were conducted using Schrödinger’s Maestro 2017-2 version 11.2.014[4]. The geometry of conformers were recalculated with the density functional and basis set ωB97X-D/6-31G(d,p) as implemented in Gaussian 16 Rev. A.03 (suite[42–48]. This functional was chosen for its ability to reproduce CCSD geometry calculations of asynchronous Diels–Alder reactions as well as its general applicability for accurately calculating reaction barriers[49,50]. Structures of interest were further optimized at the reported level of theory, ωB97X-D/6-31+G(d,p)[48,51,52]. Following Head-Gordon’s suggested basis set for energetics[53], we computed single point energies at the ωB97X-D/def2-QZVP level of theory[54,55]. We believe these methods to accurately calculate energetics for the reported systems and recommend them for use.

Molecular dynamic simulations
Classical molecular dynamics (MD) simulations were performed with the GPU code pmemd from the AMBER 16 package[56]. Parameters for ligands were generated within the antechamber module with the genparm method. Further extensions of Gaussian-type basis sets for use in molecular orbital studies of organic molecules. J. Chem. Phys. 86, 2257-2261 (1992).

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Author contributions M.O., C.S.J., K.N.H. and Y.T. developed the hypothesis and conceived the idea for the study. M.O., C.S.J., Y.C., J.Z., K.N.H. and Y.T. designed the experiments. M.O. performed all in vivo and in vitro experiments, as well as compound isolation and characterization. D.T., D.K., S.C. and M.C.T. performed compound isolation and characterization. S.M.A., J.V.C., J.S.B., E.P. and N.K.G. designed and performed synthesis of the cluster. M.O. and Y.C. performed protein purification. Y.C. performed all structural biology. C.S.J. performed all in vivo and in vitro experiments, as well as compound isolation and characterization.

Competing interests The authors declare no competing financial interests.

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Extended Data Fig. 1 | Density functional theory calculations for non-enzymatic Alder-ene and hetero-Diels–Alder reactions from (Z)-QM and (E)-QM and Alder-ene theozyme. a. Transition states, products and energies for eight hetero-Diels–Alder and Alder-ene reactions are shown. In the transition states, the Alder-ene reactions adopt a conformation where the pyridone and forming cyclohexane are perpendicular to each other compared to the hetero-Diels–Alder reactions that are more co-planar in geometry. The Alder-ene reactions are synchronous and the hetero-Diels–Alder reactions are asynchronous, but concerted. TS-1 and TS-2 lead to 8' and 9 with barriers of 25.1 and 23.2 kcal·mol⁻¹, respectively. The structures 8a', b', c', 9b, c and 11 are isomers of natural product scaffolds with barriers greater than TS-1.

b. Alder-ene theozyme of (Z)-quinone methide complex leading to Alder-ene adduct (8'), O4- and O2-hetero-Diels–Alder adducts (9 and 11) with energies reported as enthalpies and Gibbs free energies.
Extended Data Fig. 2 | Homologous biosynthetic gene clusters of pyridoxatin (1) and fusaricide (3) and the functions of PdxI and EpiI homologues. a, Putative biosynthetic gene clusters of 1 and 3 (and 2), and their homologous biosynthetic gene clusters found in NCBI database. b, Key active site residues shown in an alignment with those from PdxI and EpiI homologues. Key residues involved in PdxI and EpiI catalysis are colored. c, In vitro analysis of PdxG and selected pericyclases using 5 as the starting substrate. The periselectivity can be correlated with the identity of the amino acid at position 413 (in PdxI, indicated in red dashed box). If valine occupies this position, the enzyme catalyses the Alder-ene reaction. On the other hand, if methionine occupies the position, the enzyme catalyses the hetero-Diels–Alder reaction.
Extended Data Fig. 3 | Biochemical characterization of the ketoreductase PdxG.

**a**, In vitro reaction of 3 μM PdxG with 2 mM NADPH using 600 μM 5 as the substrate. 60% conversion from 5 to 6 was observed within 20 min. **b**, Kinetic analysis of PdxG-catalysed reduction of 5. Reaction mixtures containing 3 μM PdxG, 2 mM NADPH and different concentrations of 5 (10 μM to 1.2 mM) were incubated at 30 °C for 20 min. **c**, Formation of 10 from 6 in the presence of 2 mM NADPH can be observed both with and without PdxG. Compound 6 was obtained from chemical reduction of 5 with NaBH₄. Since 10 can be formed in the presence of only NADPH, we conclude NADPH can nonenzymatically reduce the QM to 10, which accounts for the result in Fig. 1f.

Replicates.
Extended Data Fig. 4  | Biochemical characterization of PdxI and Epil.  

a, LC/MS analyses of chemically denatured PdxI and Epil show no trace of SAM after purification.  

b, HPLC analyses of in vitro reactions of 150 μM 5 with 3 μM PdxG, 1 mM NADPH and 30 μM PdxI or 20 μM Epil at 30 °C for 2 h in the presence or absence of cofactors. SAM or SAH does not alter the enzymatic activity of PdxI and Epil.  

c, Since the interconversion of 8 and 9 could be envisioned by hydroalkoxylation/retro-hydroalkoxylation, we examined the possibility that PdxI and Epil could catalyse the reaction of 9 to 8 and 8 to 9, respectively. The in vitro reactions of 100 μM 9 or 8 with 50 μM PdxI or 40 μM Epil at 30 °C for 2 h were performed. However, no conversion of 9 to 8 and 8 to 9 was observed.  

d, In vitro reaction of PdxI and Epil using 6 as the substrate. To obtain 6 for in vitro reaction, we chemically reduced 5 by NaBH₄. Since this reduction proceeds non-stereoselectively, 6 and diastereomer 6’ were formed. After isolation of 6 and 6’ by HPLC, fractions containing 6 were not concentrated because of the instability and were immediately used as the substrate for PdxI and Epil.  

e, LC/MS analysis of in vitro reactions catalysed by pericyclases using 6 as the substrate. Shown are compounds detected by selected ion monitoring at (M+H)+ of 248. In this mode, 6 is detected as the fragment ion. In the absence of either enzyme, 6 was converted to several products nonenzymatically, including 11. Minor compounds not isolated are indicated with *. In the presence of PdxI, 6 was nearly all converted to 8. In the presence of Epil, 6 was nearly all converted to 9. Mutation of T232S in PdxI or T231A in Epil changed selectivities of the enzymes to give other products, including 11. For additional mutagenesis data, please see Extended Data Figs. 7, 8.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Overlays of crystal structures with transition state structures for Alder-ene and hetero-Diels–Alder reactions. **a**. Overlay of Alder-ene TS-3 with 5 bound in PdxI. Note the extended conformation of the alkyl chain versus the folded transition state geometry. The pyridone is bound by hydrogen bonds from K337, H161, Q412, and water mediated hydrogen bonds from T232, D233 and H336. **b**. Overlay O4-hetero-Diels–Alder TS-6 with 5 bound in HpiI. The pyridone is bound by hydrogen bonds from H161, Q414, and water mediated hydrogen bonds from T232, D233 and H338. Note that the K339 hydrogen bond to the pyridone O4 is not present in this structure. **c**. Overlay of PdxI-5 and HpiI-5. Omit maps not shown for clarity. Both PdxI and HpiI bind the pyridone such that it is prone to a syn-dehydration assisted by K337 (PdxI) or water molecule W (HpiI) and water molecules surrounding C7. The inset shows how V413 (in PdxI) or M415 (in HpiI) affects the orientation of the lysine residue (K337 in PdxI or K339 in HpiI) and its ability to hydrogen bond to the 4-OH of the pyridone. **d**. Overlay of PdxI-5, HpiI-5, Alder-ene TS-3, O4-hetero-Diels–Alder TS-6 and O2-hetero-Diels–Alder TS-5. Omit maps not shown for clarity. TS-3 and TS-6 bind in the active site sans disfavourable interactions whereas TS-5 clashes with T232. As both the calculated Alder-ene transition structure TS-3 and hetero-Diels–Alder TS-6 are quite similar in geometry and both easily fit into the PdxI active site, PdxI cannot solely rely on shape complementarity to catalyse the reaction with observed periselectivity. **e**. Chain B active site of PdxI-product (8) complex. Note the closer distances between K337 and the pyridone O4, the change in coordination of water mediated hydrogen bond from H336, and H161 shifting from a N1 hydrogen bond (in PdxI-5) to an O2 hydrogen bond. **f**. Overlay of Alder-ene TS-3 with 8 bound in PdxI. Note high similarity in structures of 8 and TS-3. This suggests that the enzyme distorts the product structure towards that of the Alder-ene transition state.
Extended Data Fig. 6 | Molecular dynamic simulation of 7 in the PdxI active site. Distances over time of hydrogen bonds to the various positions of the pyridone are tracked in chain A (a) and chain B (b) of the active site. Left panels show H336 and K337 form hydrogen bonds to the 4-position substituent on the pyridone ring. Right panel shows Q412 and H161 remain hydrogen bonded to 2-position substituent and pyridone nitrogen N1, respectively, for the majority of the simulation. c, Molecular dynamic simulations were initiated from an extended conformation (dihedral = -180°). Over time, we monitored this conformation to see if the alkyl chain could spontaneously fold to a reactive conformation (dihedral = -20°). Indeed, for short durations of the simulations we observe the chain folding into a reactive conformation for a pericyclic reaction.
Extended Data Fig. 7 | HPLC analysis of in vitro reaction of PdxI and mutants. Mutation of the catalytic base K337A abolished the activity, while K337R mutant could retain approximately 10% activity. Individual substitution of H336A, Q412A and H161A all completely abolished the activity. Mutation of D233A or D233N completely abolish enzymatic activity. In contrast, in the D233E mutant 60% of activity and the original periselectivity were retained. This suggests that the carboxylate group of D233 in PdxI is important for enzyme function. A single mutation, V413M is sufficient to change the periselectivity from Alder-ene (>98:2, 8:9) to hetero-Diels–Alder reaction (40:60, 8:9). Further, mutation of T232 to either alanine or serine, but not valine, can lead to the formation of the O2-hetero-Diels–Alder product 11 along with the Alder-ene product 8. The data show one representative experiment from at least three independent replicates. Reaction conditions: 150 μM 5 with 3 μM PdxG, 1 mM NADPH and 50 μM PdxI (wild type or mutant) at 30 °C for 2 h.
In contrast to PdxI, substitution of K338 to alanine did not abolish and retained the activity (83%) (Extended Data Fig. 9b). H336A, H161A and Q410A (corresponding to Q412 in PdxI) mutants were highly insoluble and cannot be assayed. Although D232A and D232N mutations completely abolished the enzymatic activity, the D233E mutation retained 53% of activity and maintained the original periselectivity. This suggests that the carboxylate group of D232 in EpiI is also important for enzyme function. Mutation of Y205F retained 89% activity and maintained the original periselectivity, suggesting the hydroxy group of Y205 is not essential for catalysis. The M411V (corresponding to V413 in PdxI) and M411C mutations increased the Alder-ene product ratio compared to the wild type of EpiI. The T232A and T232S mutations but not T232V mutation, generated the O2-hetero-Diels–Alder product 11 and the Alder-ene product 8 as the minor products, with the hetero-Diels–Alder product 9 as a major product. The double mutation M411V/T231A of EpiI reversed the periselectivity from the native hetero-Diels–Alder reaction (<5:95, 8:9) to the energetically disfavoured Alder-ene reaction (2:1, 8:9), although the enzymatic activity is only moderately decreased (Extended Data Fig. 9). In the double mutant, 11 was formed due to the mutation of T231. Other double mutants such as M411V/T231S, M411T/T231A, M411C/T231A and M411G/T231A also reversed periselectivity. The data shown are that of one representative experiment from at least three independent replicates.

Reaction condition: 300 μM 5 with 3 μM PdxG, 1 mM NADPH and 40 μM EpiI (wild type or mutant) at 30 °C for 2 h.
Extended Data Fig. 9 | Relative activities of PdxI, EpiI and mutants. The activity of each mutant is compared to that of wild-type PdxI or EpiI quantified by the formation of S, 9 and 11. Error bars indicate s.d. of three independent replicates. Asterisks indicate mutants with no measurable activity. a, The relative enzymatic activity of PdxI mutants. Reaction conditions: 150 μM 5 with 3 μM PdxG, 1 mM NADPH and 50 μM PdxI mutants at 30 °C for 2 h. b, The relative activity of EpiI mutants. Reaction condition: 300 μM 5 with 3 μM PdxG, 1 mM NADPH and 40 μM EpiI mutants at 30 °C for 2 h.
Extended Data Fig. 10 | See next page for caption.
Proposed mechanisms of PdxI- and EpiI-catalysed reactions. 

a. The catalytic cycle of PdxI-catalysed reaction is initiated by the deprotonation of the 4-hydroxy group by K337 followed by the syn-dehydration to 7 assisted by the extend water hydrogen bonding network mediated by H336. Subsequently, protonated K337 serves as the general acid catalyst and forms the strong hydrogen bonding with 4-carbonyl oxygen of 7 to set the stage for the periselective Alder-ene reaction. Note that the steric effect of T232 inhibits the formation of the O2-hetero-Diels–Alder product 11 to further control regioselectivity. The alkyl chain folds to a reactive conformation and readily undergoes an Alder-ene reaction. After this, the tautomerization is facilitated by K337 and possibly water mediated by H336 to form and release 8. Then, the next catalytic cycle initiates. 

b. The catalytic cycle of EpiI-catalysed reaction, in contrast to PdxI, is initiated by the deprotonation of the hydroxy group by an alternative general base, possibly water followed by the syn-dehydration to 7. Since the key lysine residue does not form hydrogen bonding with 4-carbonyl oxygen of 7 due to the bulkier side chain of M411 (corresponding to V413 in PdxI), the favoured hetero-Diels–Alder reaction takes place to form and release 9. As same as PdxI, the steric effect of T231 inhibits the formation of the O2-hetero-Diels–Alder product 11 to further control regioselectivity. Then, the next catalytic cycle initiates.