

Award Accounts

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Involvement of the Diels–Alderases in the Biosynthesis of Natural Products

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Recent studies on a novel C–C bond formation enzyme, Diels–Alderase, show that this unusual enzyme is involved in the biosynthesis of secondary metabolites. In this account, I describe studies related to Diels–Alderases including biomimetic synthesis, the biological utilization of the Diels–Alder reaction, and natural Diels–Alderases. The function and catalytic mechanism of natural Diels–Alderases, such as solanapyrone synthase, lovastatin nonaketide synthase, and macrophomate synthase, are of great interest due to the diversity of molecular skeletons in natural Diels–Alder adducts. The first structure analysis of macrophomate synthase provided information on detailed mechanisms regarding active site organization among the substrates, metal and amino acid residues, and regarding how to avoid product inhibition.

Nature creates a huge diversity of molecular skeletons found in the natural products. Besides common major biosynthetic pathways (mevalonate pathway, deoxyxylulose phosphate pathway, shikimate pathway, polyketide pathway, non-ribosomal polypeptide pathway, or mixed pathway of two of these), further modifications increase structural diversity in the secondary metabolites. Such modifications include polyether formations, phenolic oxidative couplings, spiroketalizations, and the Diels–Alder reactions that are described in this article.

During structure elucidations of phytotoxic metabolites produced by phytopathogenic fungi in our laboratory, we frequently encountered Diels–Alder type adducts, such as betaenone B (**1**),[1] solanapyrones A (**2**), and D (**3**),[2] pyrrenocine A (**4**, precursor of **5**),[3] pyrenochaetic acid A (**5**),[4] and chaetoglobosin O (**6**)[5] (Fig. 1). These observations strongly indicated involvements of the enzyme responsible for biochemically unusual Diels–Alder reactions, "often named Diels–Alderases". After these findings, we recognized that there are a number of natural [4 + 2] adducts including polyketides, terpenoids, phenylpropanoids, alkaloids, and natural products formed via mixed biosynthetic pathways in the literature.[6] Extensive examples of natural [4 + 2] adducts have been reviewed,[6] and representative examples are shown in Fig. 2. These include intramolecular adducts keramaphidin B (**9**) and ilicicolin H (**10**), a simple intermolecular adduct plagiospiroside A (**12**). Dimer torreyanic acid (**8**), tetramer quatromicin A_3 (**13**), and hetero-Diels–Alder adduct brevianamide A (**11**) are also found. In addition, there are unique metabolites: intra- and intermolecular [4 + 2] adduct longithorone A (**7**), and tandem intramolecular adduct kijanimicin (**14**). Although considerable efforts have been made to identify the enzymatic Diels–Alder reaction,

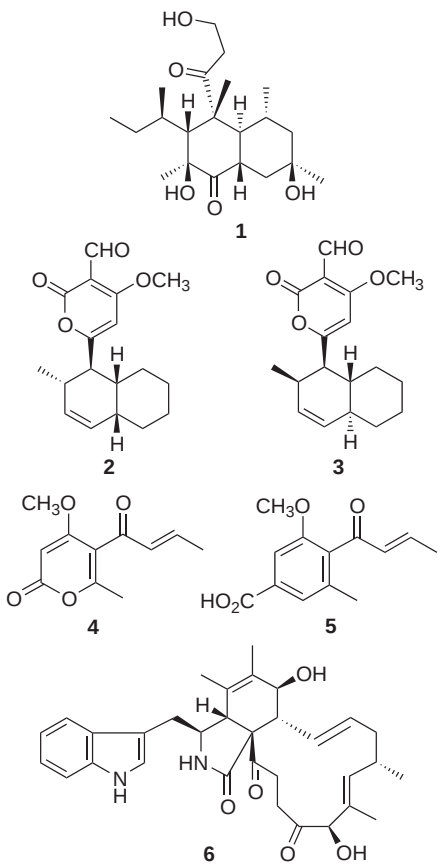

Fig. 1. Phytotoxins isolated from phytopathogenic fungi in our research group.

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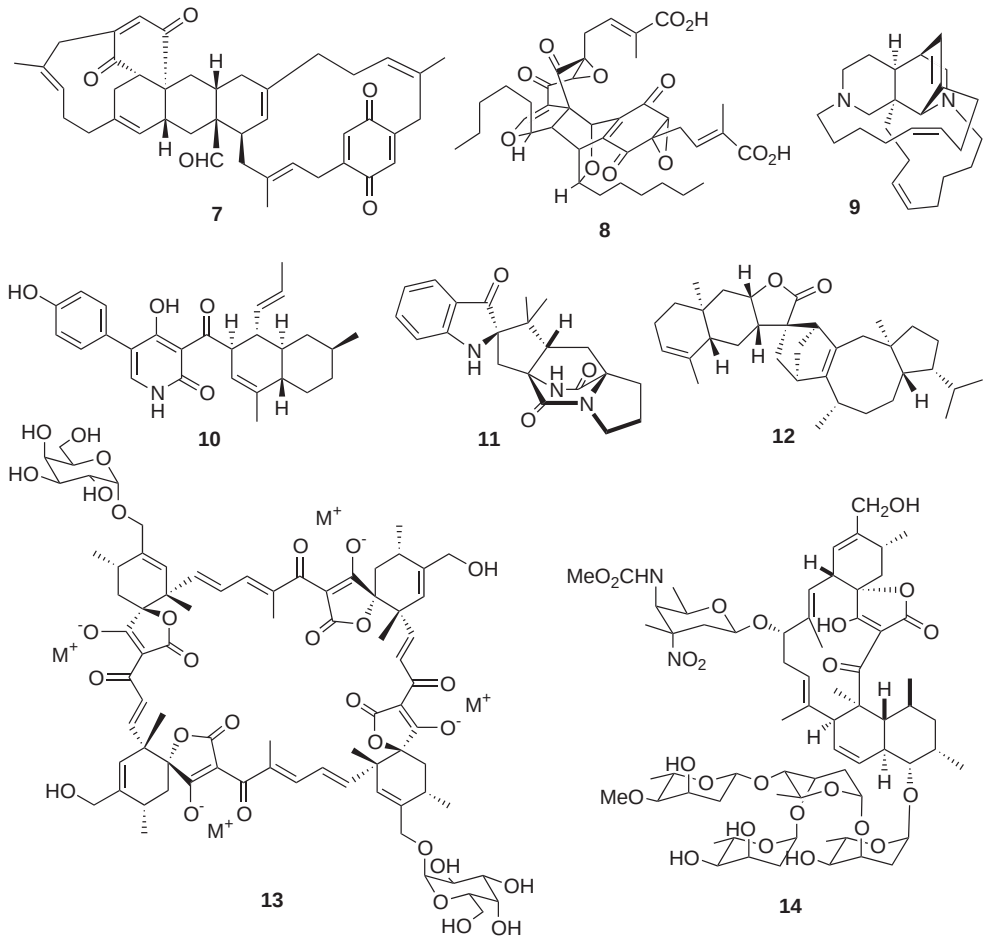


Fig. 2. Representative examples of natural [4 + 2]-adducts.

there was no report on the enzyme catalysing the Diels–Alder reaction until our first report in 1995.[7]

The Diels–Alder reaction is synthetically very useful because it forms a carbocyclic system with high regio- and stereoselectivity under mild conditions in a concerted manner.[8,9] Since the Diels–Alder reaction is a powerful tool in organic synthesis for forming four chiral centres or quaternary stereogenic centres, it has been applied to the synthesis of complex pharmaceutical and biologically active compounds.[10] The importance of the Diels–Alder reaction prompted us to explore the Diels–Alderases, in the hope of applying them to organic synthesis as the general catalyst of Diels–Alder reactions.

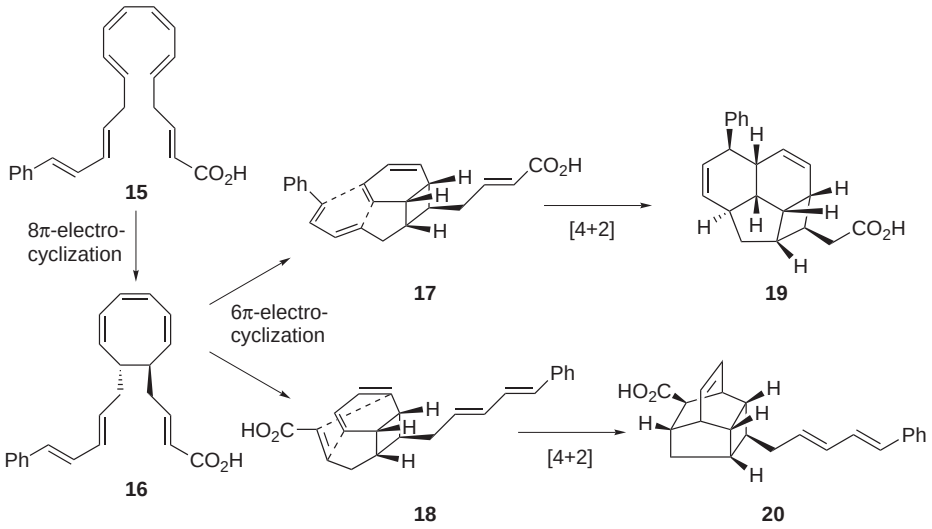
In this account, I describe studies related to Diels–Alderases including biomimetic synthesis, the biological utilization of the Diels–Alder reaction, and natural Diels–Alderases. The function and catalytic mechanism of natural Diels–Alderases are of great interest due to the diversity of molecular skeletons in natural Diels–Alder adducts. This topic is also discussed based on the stereostructure of macrophomate synthase.

1. Biomimetic Syntheses to Examine Feasibility of Biosynthetic Diels–Alder Reaction

To prove a biogenetic proposal is a challenging study in organic synthesis. Elegant total syntheses based on biogenesis have been achieved in an efficient way. We can find representative examples in efficient syntheses of tropinone (Robinson)[11] and *proto*-daphiniphylline (Piettre and Heathcock).[12] Similar approach is found in the total synthesis of natural [4 + 2] adducts. An indication of possible involvement of a Diels–Alder reaction in the biosynthesis of natural products provides information that their total synthesis may be achieved via a biomimetic approach. Thus, a number of total syntheses using Diels–Alder reaction have been reported.

Differentiation between non-enzymatic and enzymatic reaction in the biosynthesis of natural [4 + 2] adducts is important to study Diels–Alderases. For rigorous proof of Diels–Alderase-catalyzed reaction, isolation and characterization of the corresponding enzyme is essential. However, such study requires enormous efforts as described in the section 3. Instead of direct proof, biomimetic syntheses using Diels–Alder reaction sometimes provide useful information on the involvement of Diels–Alderases. In this section, I would like to introduce selected examples of total syntheses along this line.

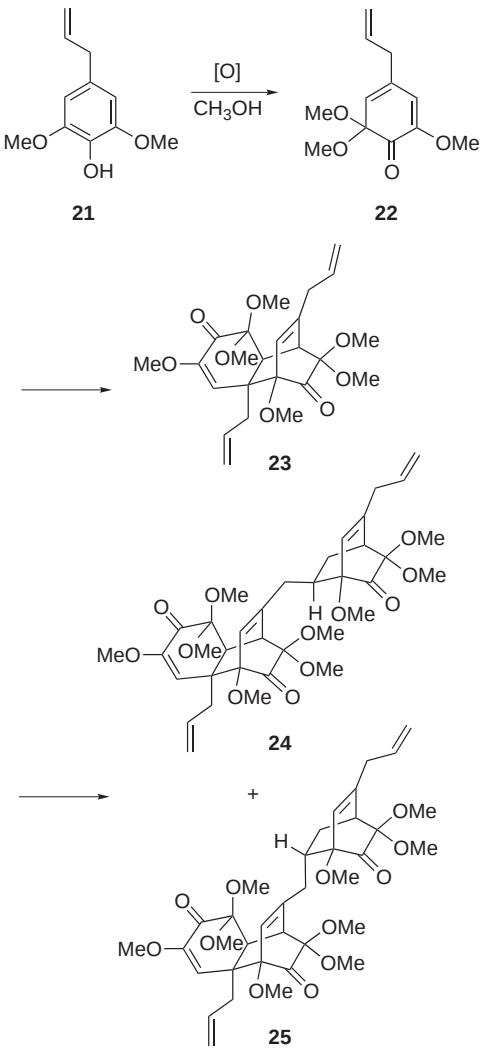
1.1 Natural [4 + 2] Adducts Which Are Plausibly Formed in a Non-enzymatic Manner. 1.1.1 Endiandric Acids: Careful examination of structures of natural products sometimes provides useful information on biosynthesis. Endiandric acids B (**19**) and C (**20**) were isolated from the plant *Endiandra introsa* as a mixture of structurally related compounds.[13] Based on logical retrosynthetic disconnection of **19** and **20**, elegant biogenesis as shown in Scheme 1 was proposed by Black and co-workers.[14] Starting from a common



Scheme 1.

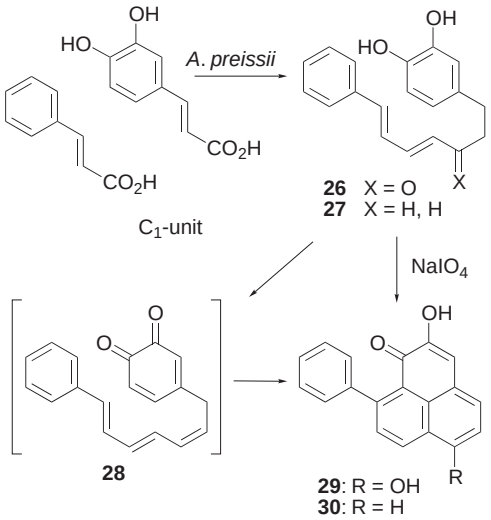
precursor **15**, the first electrocyclization affords cyclooctatriene **16**. Conformational isomers of **16** provided 6π-electrocyclization products **17** and **18** that are finally converted to **19** and **20**, respectively by Diels–Alder cycloaddition. Nicolaou and co-workers proved that this route is chemically feasible.[15] Since all endiandric acids were isolated as racemates, their formations would proceed in a non-enzymatic manner. In the biosynthesis of endiandric acids, the reactive polyene precursor is released from the active site of the corresponding polyene formation enzyme (possibly dehydrogenase), and is spontaneously cyclized without assistance of an enzyme to give a series of structurally related cycloadducts.

1.1.2 Asatone and Related Metabolites: Dimeric [4 + 2] adducts are frequently found in natural products. Retrosynthetic analysis of such a compound provides information on the dimerization reaction. The dimer of neolignan, asatone (**23**) was isolated from the plant *Asarum teitonense* (Scheme 2).[16] Later, two closely related novel trimers, heterotropatrione (**24**) and isoheterotropatrione (**25**), were also isolated.[17] Based on the oligomeric structure of neolignans, biosynthetic pathways of these metabolites were proposed as shown in Scheme 2. Oxidation of a phenol **21**, and the subsequent addition of methanol produced a dienone **22** that dimerizes to give **23** at room temperature through a Diels–Alder reaction. Further cycloadditions of **23** with **22** yield **24** and **25**, respectively. Having no optical activity, all these lignans would be formed by spontaneous cycloadditions of the dienone **22** in the absence of an enzyme after enzymatic oxidation of **21**. This proposal was supported by the result that the anodic oxidation of phenol **21** produced reactive quinone methide which underwent cycloaddition to give **23** quantitatively.[18] Later, I will introduce an example where an oxidase catalyzes not only oxidation but also a Dies–Alder reaction in the same active site of solanapyrone synthase (section 3.1).



Scheme 2.

1.1.3 Diarylheptanoid Anigorufone: Bazan et al. proposed the involvement of a Diels–Alder reaction in the biosynthesis of phenylphenalenone lachanthocarpone (**29**) based on the conversion of diarylheptanoid **26** to **29**, with $NaIO_4$ at room temperature, via the orthoquinone **28**[19] (Scheme 3). To examine the intermediacy of the diarylheptanoid in the biosynthesis of anigorufone (**30**), Steiner and co-workers fed the ^{13}C-labelled diarylheptanoid **27** to the cultured root of *Anigozanthos preissii*.[20] The anigorufone (**30**) isolated showed signifi-



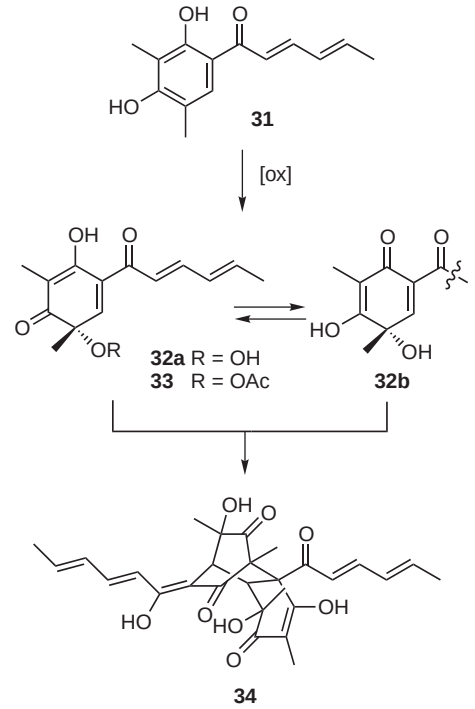
Scheme 3.

cant incorporation establishing involvement of oxidation of **27** to **28**, followed by a Diels–Alder reaction as proposed by Bazan et al. As in the case of asatone (**23**), an oxidase would provide the reactive precursor **28** for a cycloaddition. Then, the cycloaddition proceeded after releasing it from the active site of the oxidase to give racemic products. Again involvement of a Diels–Alderase is not essential in this case.

1.1.4 Bisorbicillinol and Related Metabolites: Next, I introduce an example in which a chirality of a product is not conclusive evidence for distinguishing non-enzymatic and enzymatic reactions. The bisorbicillinoids are a family of structurally diverse fungal metabolites represented by bisorbicillinol (**34**), which shows DPPH radical scavenging activity (Scheme 4).[21] Based on extensive studies of the structure elucidation and the biosynthesis of the bisorbicillinoids, Abe et al. proposed that the stable monomer sorbicillin (**31**) enantioselectively oxidized to the reactive sorbicillinol (**32a**), which dimerizes via two different [4 + 2] cycloadditions to provide **34** and sorbiquinol.[22] During the purification of sorbicillinol **32a**, it was found that the concentration of a solution of **32a** caused a [4 + 2] cycloaddition to give **34**, indicating that **32a** is highly reactive and that the conversion is non-enzymatic and occurs under mild conditions with complete regio- and stereoselectivity.[22] In the synthetic studies of the bisorbicillinoids,[23] basic hydrolysis of acetate **33** gave two discrete quinolates (bis-deprotonated forms of **32a** and **32b**) that underwent cycloaddition after subsequent acidification. Involvement of a Diels–Alderase is not necessary in this case because a non-enzymatic reaction provided a single product and because enantioselective oxidation of **31** introduced the chirality in **32a**, thus determining the stereochemistry in **34**.

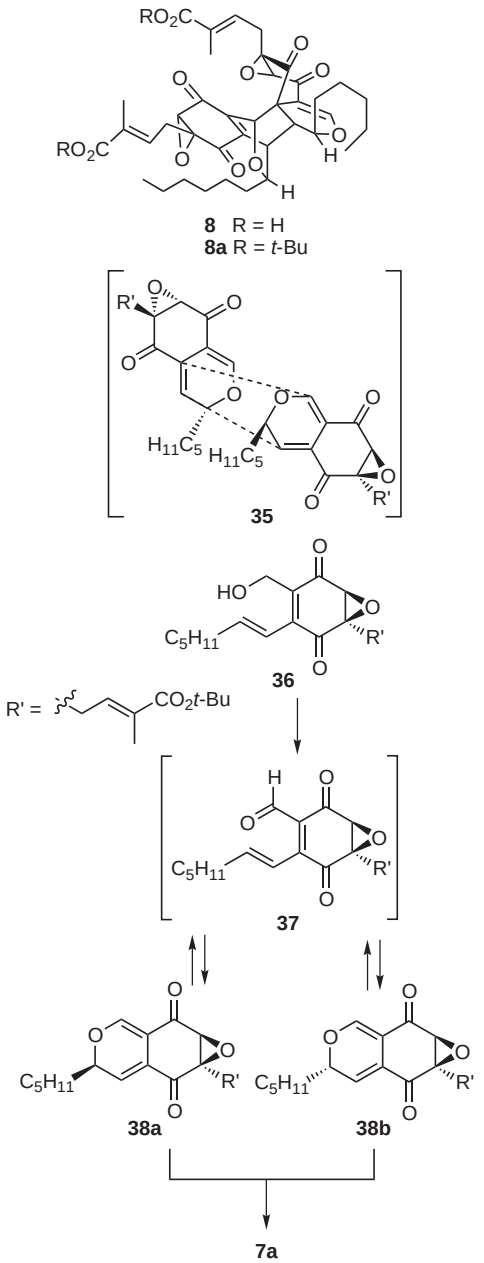
Detection of a significant amount of monomer **32a** in the bisorbicillinol producing fungus indicated that an oxidase provided the chiral reactive substrate **32a** and that the Diels–Alder reaction of **32a** was promoted in aqueous medium without a Diels–Alderase.

1.1.5 Torreyanic Acid: This is one excellent example where biomimetic synthesis explains an unusual event in the biosynthesis. Porco and co-workers extensively studied the synthesis of the fungal metabolite torreyanic acid (**8**).[24] Isolation of the corresponding monomer (+)-ambuic acid[25] suggested involvement of a Diels–Alder reaction in the biosynthesis of **8**. In the biomimetic synthesis of **8**, oxidation of alcohol **36** with Dess–Martin periodinane provided aldehyde **37** at room temperature, which rapidly converted via oxaelectrocyclization to *syn*- and *anti*-pyrans **38a** and **38b**, respectively (Scheme 5). Though aldehyde **37**, 2*H*-pyrans **38a** and **38b** existed as an equilibrium mixture, both steric and substituent effects shifted the equilibrium to the formation of the 2*H*-pyrans over aldehyde **37**. Spontaneous Diels–Alder dimerization of pyrans **38a** and **38b** proceeded with complete regio- and diastereoselectivity to give *endo*-adduct **8a**. In the retro-Diels–Alder reaction of **8a** at 60 °C, signals originating from pyrans **38a** and **38b** were detected in the ^{1}H NMR spectrum, but no aldehyde signal was observed. Theoretical calculations indicate that dienal formation is a disfavored process (11–12 kcal/mol) in the electrocyclization and that a remarkable difference exists between the energies of the transition states in the Diels–Alder reaction: the most favored one, **35**, is 9.4 kcal/mol more stable than the alternative. These calculations and the facile reaction to reverse the reaction explain the exclusive formation of a single diastereomer of **8a**. The high reactivity valves of the substrates **38a** and **38b**, which may be produced by an corresponding dehydrogenase, and the excellent diastereoselectivity in the cycloaddition indicate that the corresponding Diels–Alder reaction proceeds in non-enzymatic manner.



Scheme 4.

1.1.6 Plagiospirolide A: Synthetic studies on intermolecular adducts of terpenes have also been made by several groups. To examine the chemical feasibility of a [4 + 2] cycloaddition, Kato et al. synthesized the terpenoid adduct, plagiospirolide A (**12**)[26] (Scheme 6). Diels–Alder reaction of diplophyllin (**39**) and a mixture of unidentified diterpenes **40a** and **40b** proceeded under nearly physiological conditions to afford

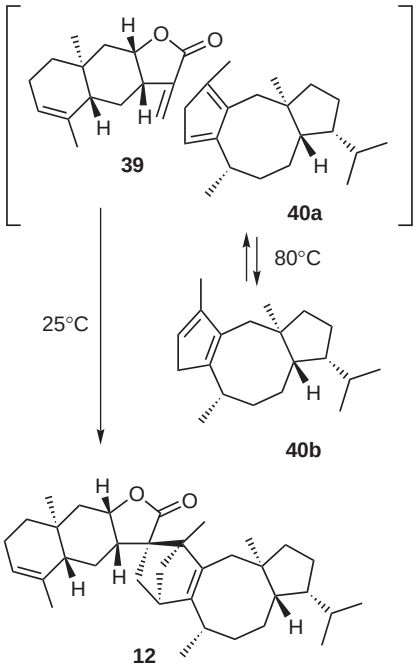


Scheme 5.

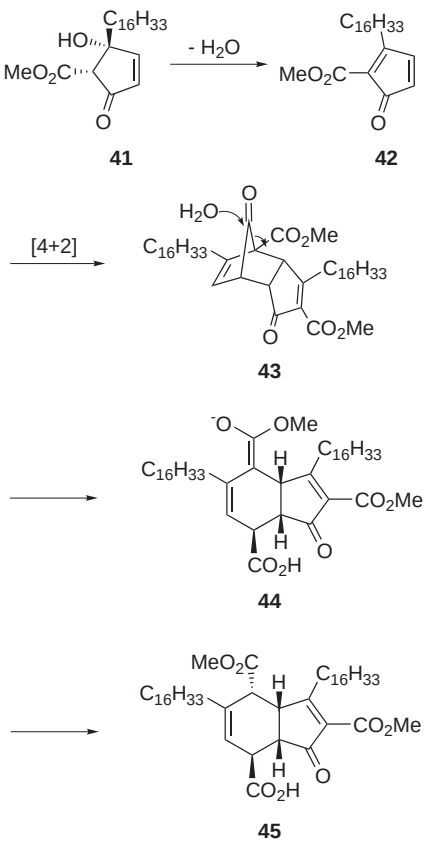
a single product, plagiospirolide A (**12**). This observation indicates that the natural adduct is most likely the result of a non-enzymatic transformation in the oil body of the liverwort.

Examples shown in this section indicate that a significant number of natural [4 + 2] adducts are produced in a non-enzymatic manner and that chirality of the adducts is not conclusive evidence for the involvement of Diels–Alderases.

1.2 Natural [4 + 2] Adducts Which Are Plausibly Formed by Diels–Alderase. 1.2.1 Manzamenones: Previously, Kobayashi and co-workers proposed a biosynthetic pathway for manzamenone A (**45**) via a [4 + 2] cycloaddition based on occurrence of monomer equivalents such as untenone A (**41**).[27] Consideration of the reactivity of diene led Whitehead and co-workers to re-examine this biosynthetic route.[28] They proposed dimerization of cyclopentadienone **42** possibly



Scheme 6.



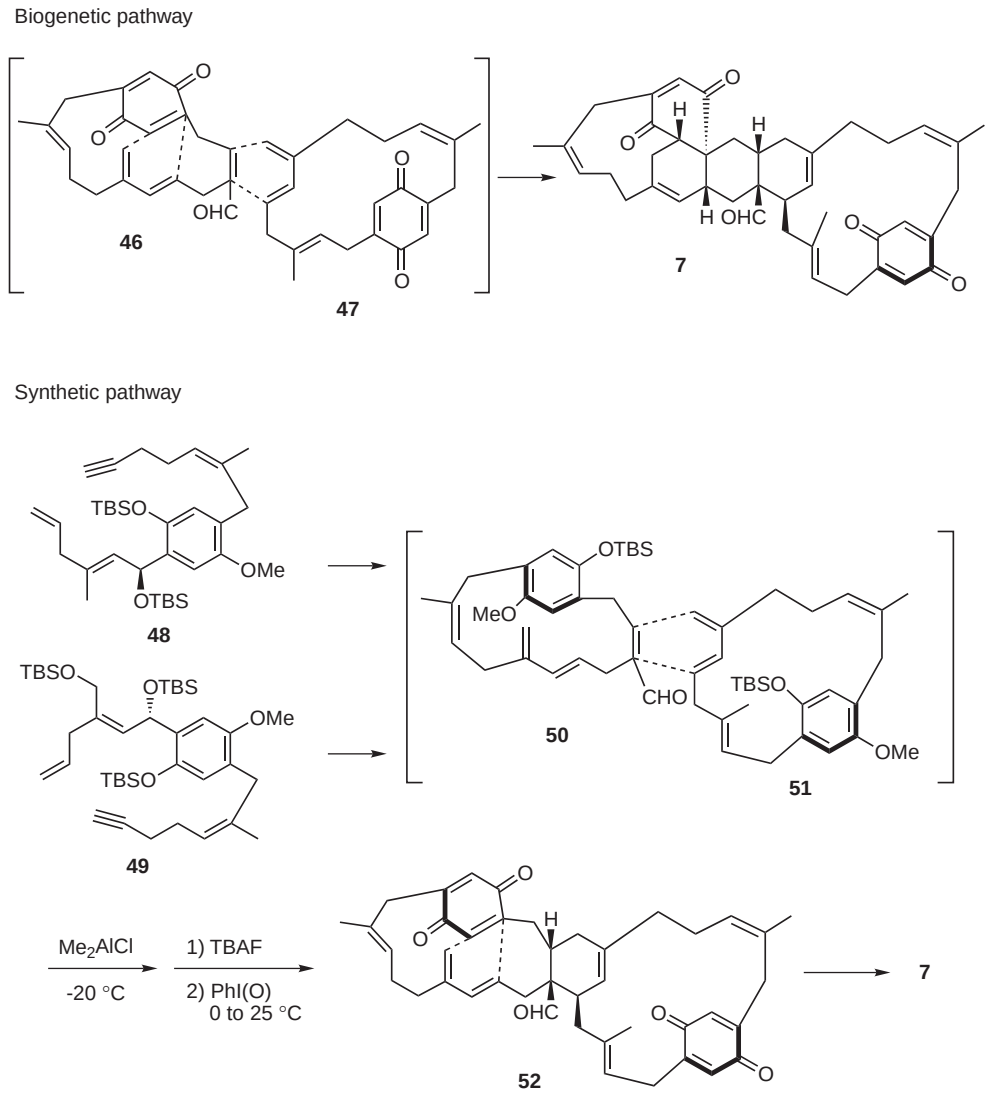
Scheme 7.

derived from untenone A (**41**) to afford an adduct **43** that undergoes a retro-Dieckmann condensation via **44** to yield **45** (Scheme 7). This route was supported by the biomimetic synthesis of manzamenone A (**45**). Dehydration of **41** and the sub-

sequent cycloaddition proceeded under mild conditions (neat, 40 °C) with high *endo*-selectivity to give **43** which is further converted to **45** under the same conditions. Involvement of Diels–Alderase is obvious, since dimerization of the achiral precursor afforded the chiral adduct. In this case, a dehydratase which converts **41** to achiral dienone **42** may catalyze the Diels–Alder reaction.

1.2.2 Longithorones: The unique prenylated [12]-paracyclophane quinone dimer longithorone A (**7**) was isolated from the tunicate *Aplidium longithorax* as a cytotoxic agent, and it plausibly originates from a cycloaddition reactions between precursors **46** and **47**.[29] Later, a series of monomers and dimers were isolated from the same tunicates, indicating the possible involvement of a Diels–Alderase.[29] The monomers are novel farnesylated benzoquinones possessing either para- or meta-bridged structures. These compounds have restricted rotations in their macrocyclic rings, resulting in atropisomerism. Total synthesis of **7** was achieved by Shair and co-workers[30] (Scheme 8). Macrocyclizations of **48** and **49** with ene-yne methathesis formed a conjugated diene system that is ready for a Diels–Alder reaction. In this cyclization, chirality transfer from the removable stereogenic center to the atropisomer was achieved to give **50** and **51**. The cycloaddition between **50** and **51** proceeded in the presence of Me_2AlCl with complete *endo*-selectivity to give two possible diastereomers. The adduct was converted into quinone **52**, which is reactive enough to undergo a second Diels–Alder reaction at room temperature, yielding **7**. The first Diels–Alder reaction required a Lewis acid, and this inherent low reactivity and facial selectivity strongly suggest the involvement of a Diels–Alderase in the first intermolecular reaction.

1.2.3 1,2-Dialkyldecalin Polyketides: In our previous review,[6] we described extensive examples of 1,2-dialkyldecalin polyketides that are presumably biosynthesized via intramolecular Diels–Alder reaction of a linear polyolefin chain, and that presumably have a decalin moiety with two neighboring alkyl groups at the same ring (Chart 1). Many synthetic chemists, including our group, choose biomimetic Diels–Alder reactions to construct the molecular skeletons of 1,2-dialkyldecalin polyketides and to install multiple chiral centers in a single step. This approach is attractive because the synthesis of a linear functionalized chain is relatively easy. Later, representative exam-



Scheme 8.

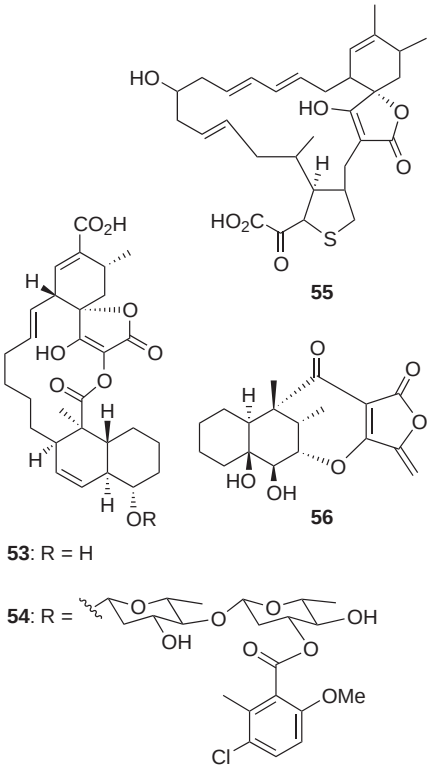
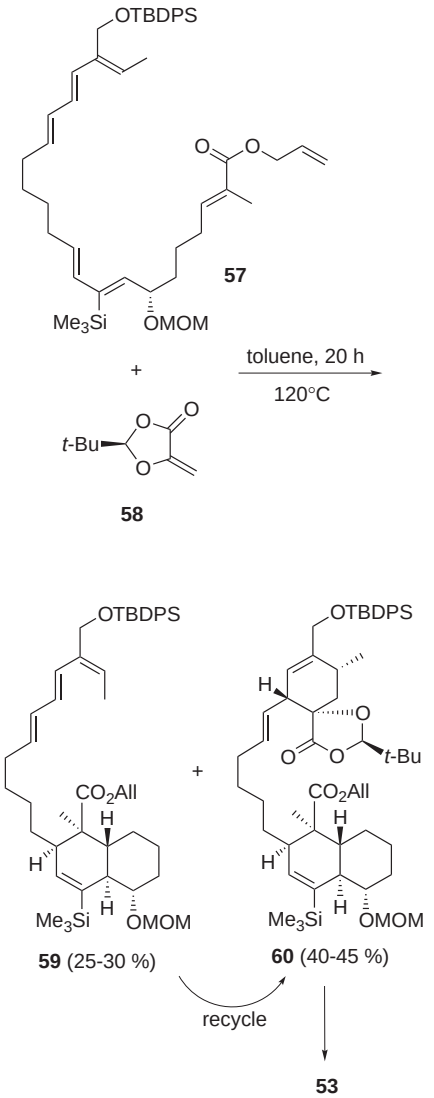


Chart 1.

ples are introduced in the sections of betaenone, solanapyrone, and lovastatin. In this section, I describe a synthesis of a spirotetronate polyketide.

Chlorothricolide (**53**) is the aglycon of an antibiotic, chlorothricin (**54**),[31] which is a member of the 1,2-dialkyldecalin polyketides (Chart 1). Isolation of tetronothiodin (**55**)[32] and tetrodecamycin (**56**)[33] strongly suggested that **54** is biosynthesized via unusual intra- and intermolecular Diels–Alder reactions.[6c] Synthesis of **53** incorporating tandem intra- and intermolecular Diels–Alder reactions has been reported by Roush and co-workers (Scheme 9).[34] Based on the knowledge of the relative reactivity of two pairs of internal and terminal components (dienophiles and dienes), they chose hexaene **57** and dienophile segment **58** as key synthetic intermediates. The Diels–Alder reaction of **57** and **58** in toluene at 120 °C proceeded with reasonably high diastereoselectivity (**60**:other diastereomers, 67:23) which is nearly the same ratio as that predicted by the cycloadditions using individual pairs of diene and dienophile models. Isomerization of the double bond under cycloaddition conditions allowed the recycling of recovered **59** to **60**. Conversion of **60** to **53** was achieved in nine steps. Since controlling diastereoselectivity in both intra- and intermolecular Diels–Alder reactions is difficult to achieve, a Diels–Alderase most likely involves in the biosynthesis of spirotetronate polyketides such as chlorothricin (**54**).

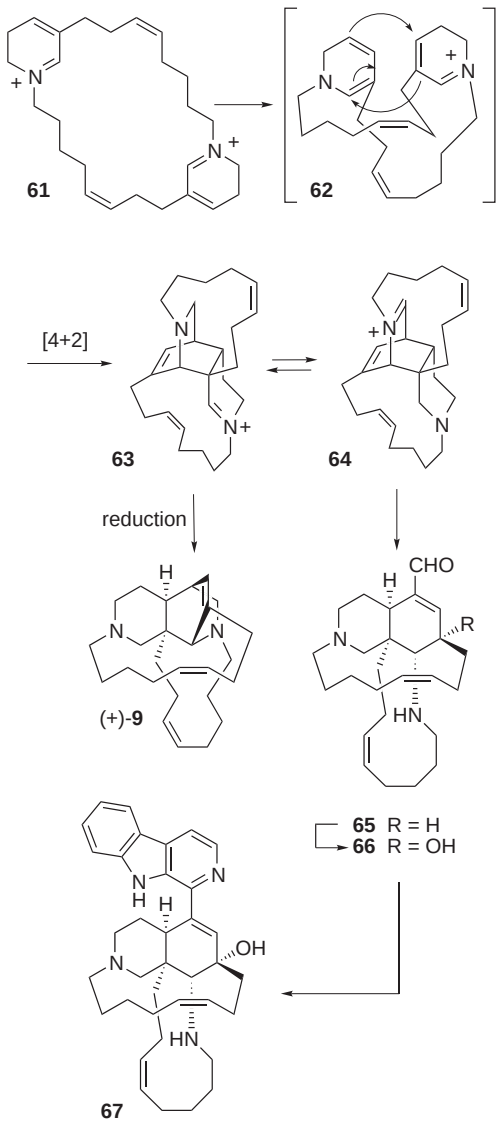
1.2.4 Manzamines: The manzamines are a family of unique heterocyclic alkaloids isolated from marine sponges. Manzamine B (**67**) were isolated from *Haliclona* sp.[35] Later, many manzamine-related alkaloids were isolated from other sponges. In 1992, plausible biogenetic precursors ircinal B (**66**) from *Ircinia* sp. were reported.[36] At the same time, Baldwin and Whitehead proposed an elegant biogenetic pathway involving a Diels–Alder reaction.[37]



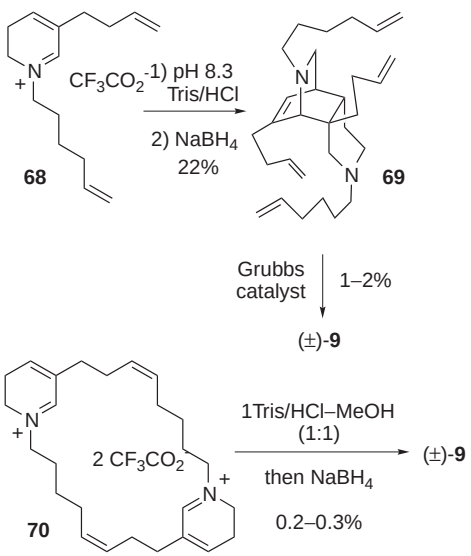
Scheme 9.

In Baldwin and Whitehead's pathway (Scheme 10), intramolecular Diels–Alder cyclization of the bis-dihydropyridine intermediate **61** via isomerization to **62** gives iminium salt **63**. Redox exchange and hydrolysis of the resultant **64** affords aldehyde **65**. Isolations of **66** and keramaphidin B (**9**) from *Amphimedon* sp.[38] strongly support this biogenetic pathway.

Recently, Baldwin et al. have completed the biomimetic synthesis of keramaphidin B (**9**) (Scheme 11).[39] The intermolecular cycloaddition of dihydropyridinium salt **68** provided **69** in 22% yield under the conditions of the model synthesis. The ring-closing metathesis of **69** gave **9** (1–2%) along with monocyclized products (10–20%). Alternatively, they achieved the keramaphidin synthesis via an intramolecular Diels–Alder reaction of the proposed precursor, macrocyclic dihydropyridinium salt **70**. Cycloaddition of **70** in a MeOH-buffer followed by $NaBH_4$ reduction produced keramaphidin B (**9**) in 0.2–0.3% yield. These results established the feasibility of the construction of both keramaphidine and the halicyclamine cores via a biomimetic Diels–Alder reaction (Scheme 10). This result is the first piece of chemical evidence for Baldwin and



Scheme 10.



Scheme 11.

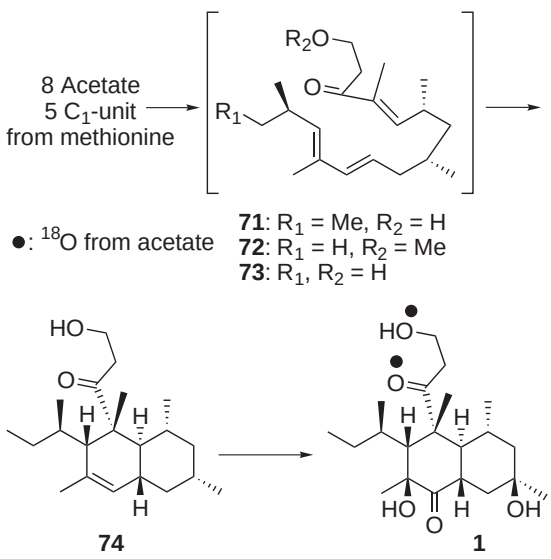
Whitehead's hypothesis. The low yield of cycloadduct **9** was mainly attributable to a limited population of disproportionation-product **62** from **61**. Thus, the putative Diels–Alderase involved in the biosynthesis of manzamines would avoid the problem of disproportionation. Furthermore, the enzyme would limit conformation of the substrate and would stabilize the transition state.

2. Biosynthetic Studies to Confirm Involvement of Diels–Alderases

The search for a Diels–Alderase attracts many chemists, including our group. To prove the existence of a Diels–Alderase, the identification of a substrate in the Diels–Alderase reaction concerned is essential. Since, in most cases, the final products are modified by oxidation, acylation, and alkylation, the oxidation level of plausible precursors is examined to reduce the number of possible substrates. When a precursor has been proposed, isotopically labeled precursors are synthesized and incorporated. I will give examples which provide significant information on a biological Diels–Alder reaction.

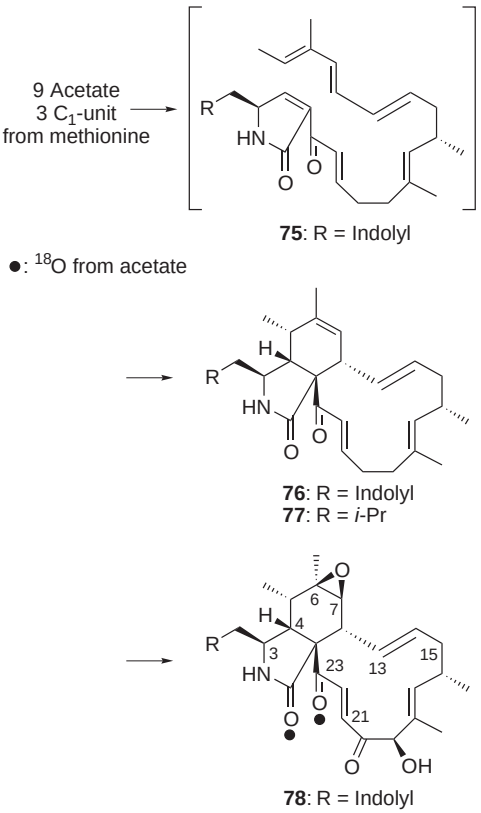
2.1 Betaenones. Although the structures of the phytotoxins betaenone B (**1**) and its derivatives[1,40] from *Phoma betae* contain an oxidatively modified 1,2-dialkyldecalin system, the relative stereochemistry of **1** suggested that these are *endo*-adducts of a Diels–Alder reaction (Scheme 12). To determine the actual substrate in this Diels–Alder reaction, a feeding experiment with ^{18}O-labelled acetate was conducted, indicating that oxygen atoms on the decalin ring were introduced after elongation of the polyketide chain.[41] This was confirmed by the following observations: 1) isolation of probetaenone I (**74**)[41] in the incubation of the fungus with cytochrome P-450 inhibitors; 2) successful conversion of ^{13}C-labelled **74** into **1**.[42] Both betaenone B (**1**)[43] and probetaenone I (**74**)[44] were synthesized to examine the feasibility of a biomimetic synthesis. Unfortunately, no conclusive evidence was obtained from incorporation experiments with linear precursor analogs **71**, **72**, and **73** with whole cell or cell-free system.[45]

2.2 Chaetoglobosins. Using a similar strategy to the case of the betaenones, we investigated a biosynthetic pathway of



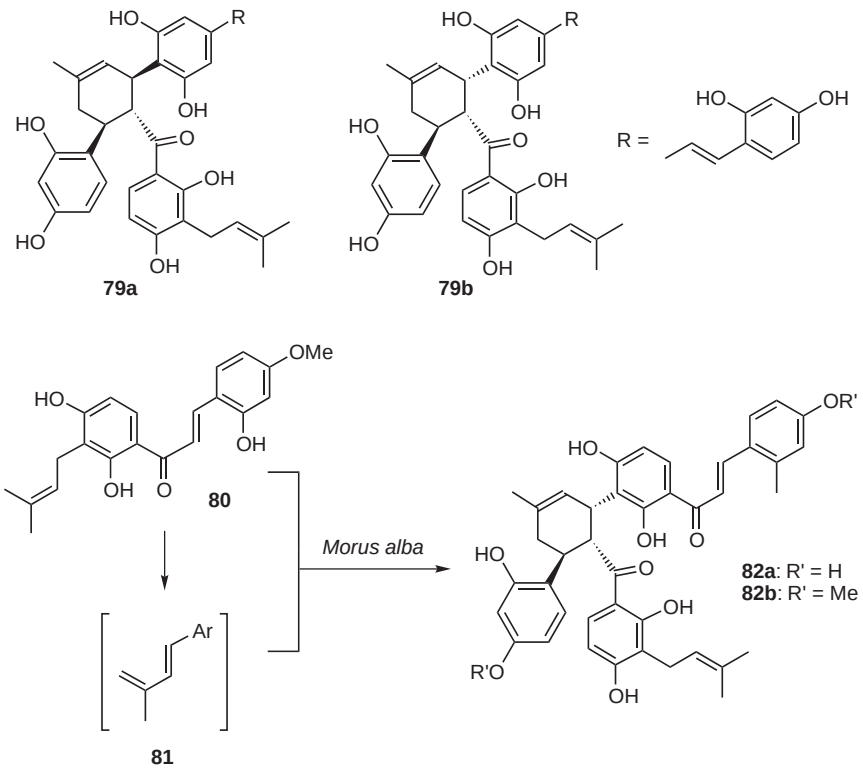
Scheme 12.

the fungal metabolite chaetoglobosin A (**78**)[46] from *Chaetomium subbafine*. Origins of the oxygen atoms in **78** were examined by incorporation of ^{18}O-labelled acetate and oxygen gas, indicating that the oxygen atoms of the carbonyl groups are derived from acetate and others are introduced by hydroxylations.[47] Treatment of the fungus with cytochrome P-450 inhibitors caused accumulation of various less-oxidized intermediates such as the non-oxidized polyketide intermediate prochaetoglobosin I (**76**).[48] Occurrence of proxiphomin (**77**)[49] in the cytochalasin metabolites suggests that both **76** and **77** are adducts of intramolecular [4 + 2] cycloadditions (Scheme 13). Low diastereoselectivity in the relevant Diels–Alder reaction in the synthetic study[50] indicates that transformation from **75** to **76** must involve enzyme-catalysis.



Scheme 13.

2.3 Kuwanons. Kuwanons, constituents of moraceous plants, are phytoalexins.[51] Based on their structures, kuwanons X (**79a**) and Y (**79b**) are regarded as adducts of a precursor chalcone and stilbene with the diene derived from the prenyl side chain. Isolation of these diastereomers as optically active forms strongly indicated that the achiral precursors afford *endo*- and *exo*-adducts via an enzymatic Diels–Alder reaction.[51] Nomura and co-workers reported a series of incorporation experiments to examine this hypothesis.[52] In a feeding experiment with the non-natural methoxychalcone **80** into callus tissue of *Morus alba*, the dimeric adducts **82a** and **82b** were obtained, indicating that dehydrogenation of the prenyl group followed by [4 + 2] cycloaddition between **80** and **81** yielded the non-natural adduct **82b** (Scheme 14).[52] In the case of metabolites described in the section 1.1, an oxidative enzyme may convert a precursor to reactive diene or dienophile and release it from the active site. The subsequent Diels–Alder reaction gave racemic products. On the other hand, a plausible dehydrogenase involving kuwanon biosynthesis may convert **80** to the reactive diene **81** but in this case the same enzyme may provide an active site for the intermolecular Diels–Alder



Scheme 14.

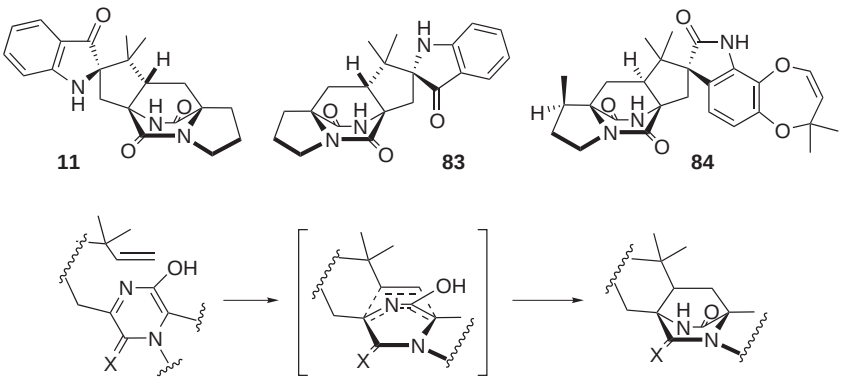
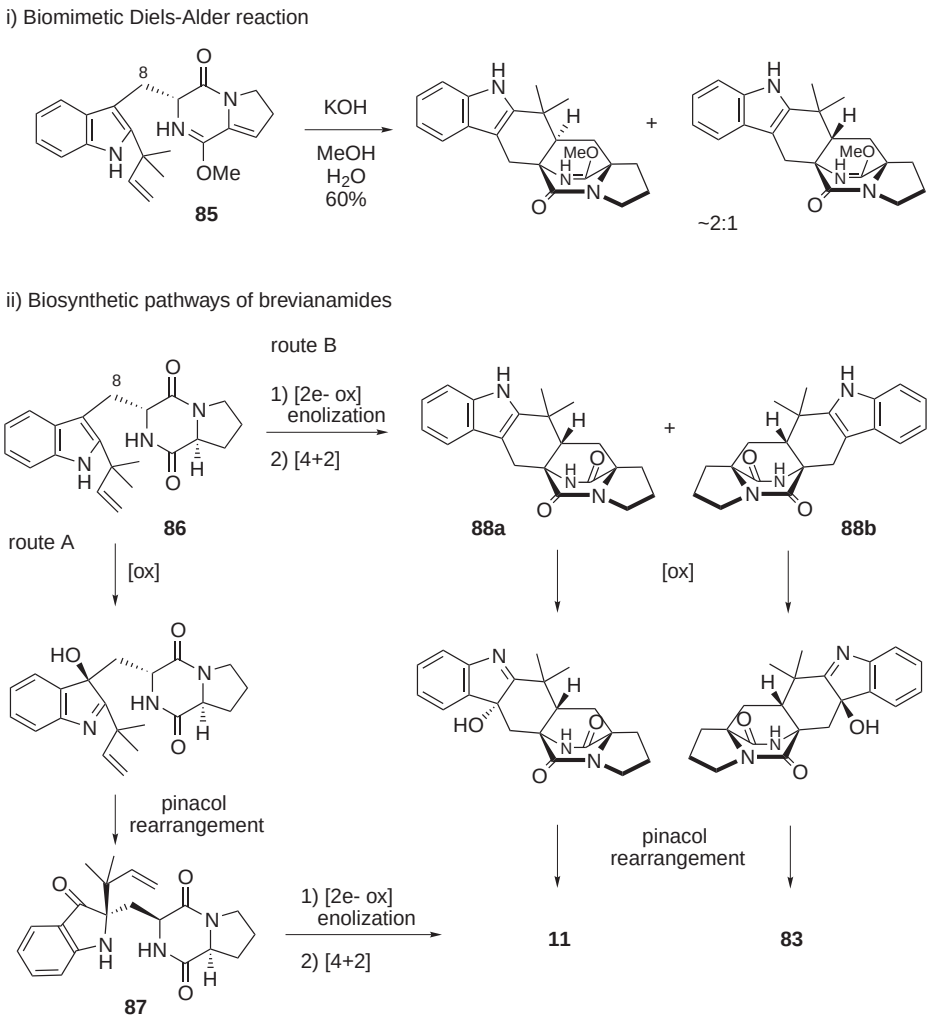


Fig. 3.



Scheme 15.

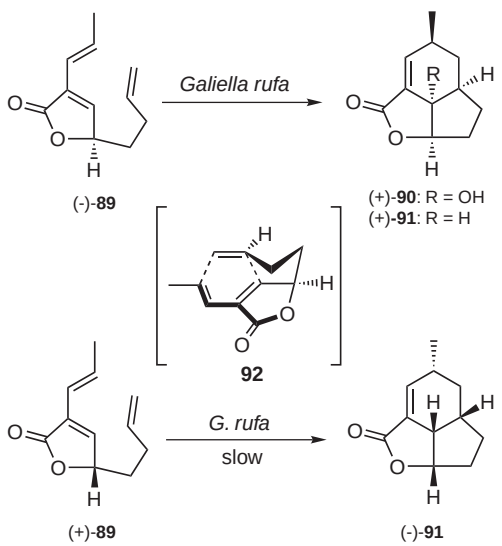
reaction between **80** and **81** to afford chiral **82b**. Similar example is introduced in the biomimetic synthesis of manzamenone (section 1.2.1). Unfortunately, no characterization of the corresponding enzyme has been reported.

2.4 Brevianamides and Paraherquamides. Brevianamides A (**11**), B (**83**), and paraherquamide (**84**) constitute a structurally unique family of fungal metabolites containing the bicyclo[2.2.2]diazaoctane skeleton, which is proposed to be constructed via an intramolecular Diels–Alder reaction between an azadiene and an isoprene unit (Fig. 3). To date, more than 30 compounds belonging to this family have been reported in the literature.[53] Extensive synthetic and biosynthetic studies of this family have been carried out by Williams group, and have recently been reviewed.[53]

It was found that isotopically labeled deoxybrevianamide E (**86**) was efficiently incorporated into **11** and **83** when fed to the culture of brevianamide-producing fungus (Scheme 15). This established that **86** is an actual intermediate of breviana-

mides, and that prenylation take place after the formation of the bicyclo[2.2.2]diazaoctane skeleton.[54] There are two alternative routes A and B, as shown in Scheme 15, concerning the timings of azadiene-formation/cycloaddition and oxidative rearrangement on indole ring. Efforts on synthesizing the plausible intermediate **87** have been unsuccessful due to the instability of **87**. Although isotopically labeled racemic adducts **88a** and **88b** were not converted into brevianamides in the feeding experiment, Williams and co-workers preferred route B since recent incorporation experiments on paraherquamide (**84**) showed that hetero-Diels–Alder reaction proceeded prior to the oxidative rearrangement. Total synthesis of **11** and **83** was achieved via a biomimetic hetero-Diels–Alder reaction of **85**[55] (Scheme 15). This biomimetic synthesis provides a strong support for the assertion that the plausible Diels–Alder reaction in Scheme 15 route A is chemically feasible. The oxidative enzyme as shown in the section 2.3 (kuwanons) could be responsible for formation of the azadiene and the subsequent Diels–Alder reaction. Significant progress has been made in the biosynthesis of the paraherquamides and brevianamides, but identification of the real substrates for the Diels–Alder reaction and elucidation of the mechanism forming the reactive azadiene system still remain to be solved.

2.5 Galiellalactone. The fungal metabolite galiellalactone (**90**), obtained from *Galiella rufa*, is a potent inhibitor of interleukin-6 signalling in HepG2 cells.[56] Sterner and co-workers established that pregaliellalactone (**89**) is converted into desoxygaliellalactone (**91**) in intact cells via an inverse-electron demand Diels–Alder reaction (Scheme 16).[57] Non-enzymatic reaction of **89** to **91** via transition state **92** proceeded slowly (half life 65 h) in aqueous media at room temperature, while the reaction in toluene required heating to 140 °C and was completed in 5 h. In this reaction, *endo*-isomer **91** was obtained as a single product. There it was found that the resting cell can convert (−)-**89** to (+)-**91** at an 8 times higher rate than the conversion of the corresponding enantiomer (+)-**89**, while acceleration was not observed using autoclaved mycelia. In this case, a plausible protein as an auxiliary protein in the case



Scheme 16.

of lignan biosynthesis[58] may simply catalyze the Diels–Alder reaction by an entropy trapping mechanism.

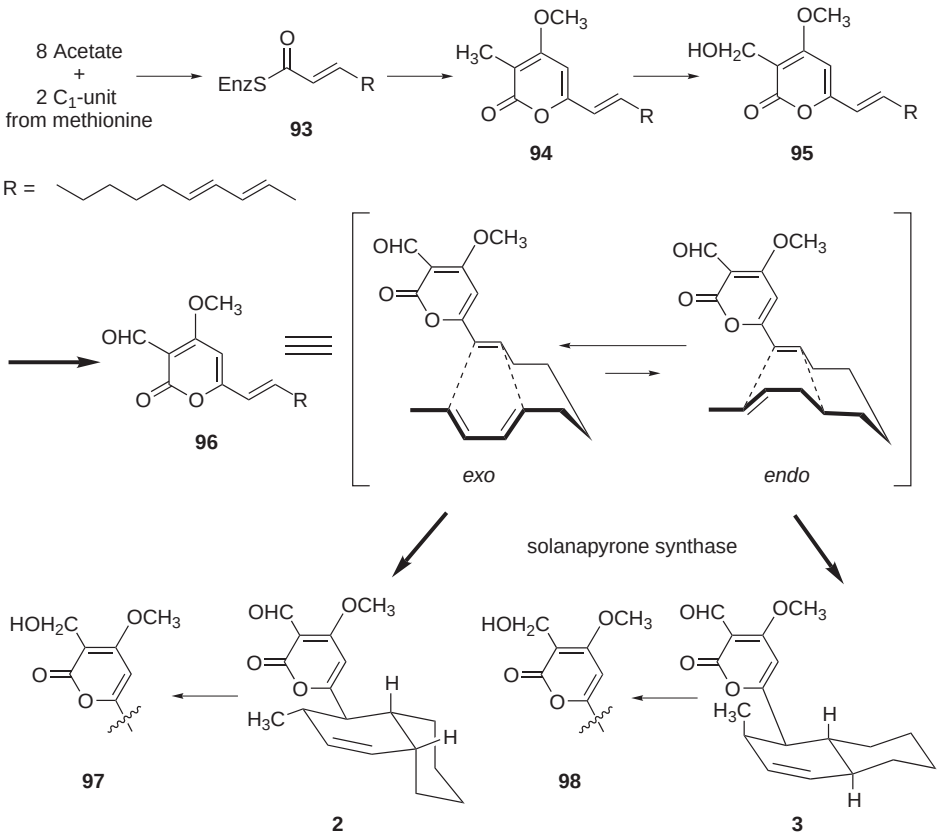
3. Natural Diels–Alderase

Since we reported the enzymatic activity of solanapyrone synthase in 1995 as the first Diels–Alderase,[7] two additional Diels–Alderases: lovastatin nonaketide synthase[59] and macrophomate synthase,[60] have been purified and characterized. Two of them catalyze intramolecular Diels–Alder reactions, while the last one catalyzes an intermolecular Diels–Alder reaction. We have recently reported the detailed reaction pathway[61,62] of macrophomate synthase and its catalytic mechanism based on the crystal structure.[63] In this section, I describe three natural Diels–Alderases and discuss the mechanisms of their catalysis.

3.1 Solanapyrone Synthase. Solanapyrones were isolated as phytotoxic substances from phytopathogenic fungi *Alternaria solani*.[2] The solanapyrone family consists of diastereomers A (**2**), D (**3**), and their reduced forms B (**97**) and E (**98**). Isolation of these substances as optically active forms strongly indicates that solanapyrones are biosynthesized from an achiral linear triene precursor such as **93** via an enzyme-catalyzed Diels–Alder reaction. Biomimetic synthesis of **2** and **3** via a [4 + 2] cycloaddition proved the feasibility of the biosynthetic Diels–Alder reaction.[64] A series of feeding experiments of simple precursors and plausible synthetic precursors excluded the intermediacy of **93**.[66b] Incorporation of isotopically labeled biosynthetic precursors, prosolanapyrones I (**94**) and II (**95**) into (−)-solanapyrones unambiguously confirmed the biosynthetic pathway of solanapyrones, as shown in Scheme 17.[65,66]

To establish the involvement of Diels–Alderase in this reaction, we next examined the enzymatic conversion of **95** and **96**. In cell-free extracts of *A. solani*, we found enzymatic activity catalyzing the Diels–Alder reaction[7] from **95** to (−)-**2** with excellent enantioselectivity (99% ee) and relatively high *exo*-selectivity (6:1). Subsequently, we reported the partial purification and properties of the enzyme, solanapyrone synthase (SPS),[67] which is the first example of a Diels–Alderase. In addition, we showed that, in the presence of molecular oxygen, the partially purified enzyme converted **95** to **2** and **3** with accompanying formation of hydrogen peroxide. Based on the chromatographic behavior of the enzyme,[67] we proposed that the single enzyme catalyzes the oxidation from the alcohol **95** to the reactive aldehyde **96**, which is further converted to the adducts **2** and **3** by the Diels–Alder reaction. Because of scarcity and instability, SPS has not been purified as a single band on SDS-PAGE.

To assess the intrinsic reactivity of prosolanapyrones and the diastereoselectivity of the corresponding reaction, we have examined non-enzymatic Diels–Alder reactions under various conditions.[64b] In less polar solvents, heating was required for the effective cycloaddition of **94**, **95**, and **96**. Increase in the oxidation level of the 3-substituent in the prosolanapyrones enhances the reaction rate. This can be explained by reduction of the LUMO energy of the dienophile moiety in the pyrone precursors. *endo*/*exo*-Selectivities with **94**, **95**, and **96** were essentially the same in various organic solvents. The slight preference for *endo*-selectivity in less polar solvents suggests that there is little steric congestion in both *endo*- and *exo*-transition



Scheme 17.

states.

When the non-enzymatic Diels–Alder reaction of prosolanapyrone III (**96**) was carried out in organic solvents at 30 °C, no reaction occurred. In aqueous medium, however, the reaction was accelerated and gave *endo*-adducts with high selectivity (**2**:**3** = 3:97).[64b] These effects were observed in the reaction of **96** but not in that of **95**. This observation indicated that the oxidation of prosolanapyrone II (**95**) enhanced the reactivity of the substrate significantly for the Diels–Alder reaction. For Diels–Alder reactions in aqueous media, similar rate accelerations and the predominant formation of *endo*-adducts have been reported.[68] Breslow explained this phenomenon by the hydrophobic effect:[68] water forces the substrate to form the more compact *endo*-transition state, reducing its molecular surface exposed to the aqueous medium. On the other hand, Ruiz-López et al. emphasized the importance of hydrogen bonding between the water and dienophile carbonyl group to reduce the LUMO energy of the dienophile and to enhance the reactivity of the substrate.[69] Due to the effects described above, the background reaction could not be ignored under standard enzymatic reaction conditions. Contrary to the non-enzymatic reaction, the enzymatic conversion of **95** provided preferentially *exo*-adduct **2**. In general, the *exo*-selective cycloaddition can not be achieved by simple heating or by use of Lewis acid catalysts. These observations indicate that the major roles of solanapyrone synthase are the oxidation of prosolanapyrone II (**95**) to the more reactive III (**96**) and the stabilization of the *exo*-transition state.

3.2 Lovastatin Nonaketide Synthase. The biosynthesis of the cholesterol-lowering drug lovastatin (**102**) isolated from *Aspergillus terreus* has been extensively investigated by Vederas and co-workers. Incorporation experiments with multiple labeled acetate and ^{18}O-oxygen suggested that oxygen atoms of the side chain are derived from acetate and that the oxygen atom on the decalin ring is obtained from molecular oxygen.[70] Later, a blocked mutant of *A. terreus* converted 4a,5-dihydromonacolin L (**101**) to **102**, confirming that these are intermediates (Scheme 18).[71] Because lovastatin (**102**) does not have an electron-withdrawing group in the dienophile moiety, it was proposed that the requisite Diels–Alder reaction of **99a** occurred at the hexaketide stage to give **100**.

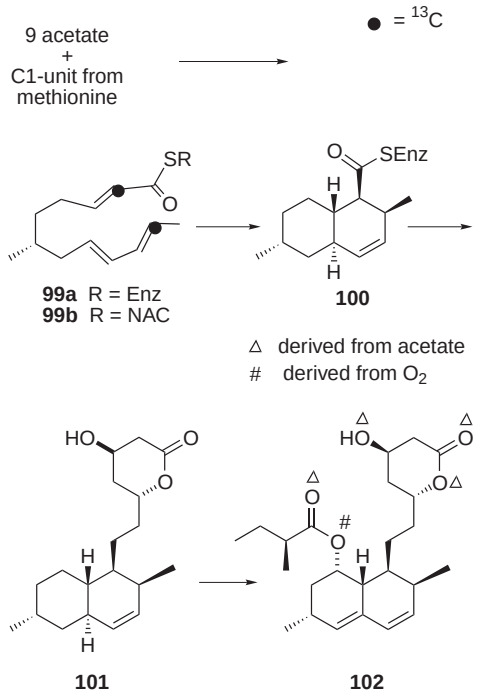
In 1999, the biosynthetic gene cluster of lovastatin (**102**) was cloned by Hutchinson's group.[72] They succeeded in achieving heterologous expression of whole genes in *A. nidulans* to produce dihydromonacolin L (**101**). Using the purified lovastatin nonaketide synthase (LNKS), hexaketide triene precursor **99b** was incubated without co-factors and substrates to give three adducts: **103a**, **103b**, and **103c** in a ratio (1:15:15), as shown in Scheme 19. The minor *endo*-product **103a** is confirmed to be the one with the same stereochemistry as natural **102**.[59] Due to the inability of the denatured enzyme to form the adduct **103a**, LNKS catalyses this cycloaddition with significant rate acceleration. In the lovastatin biosynthesis, hexaketide precursor **99b** should load on the corresponding ketosynthase domain of LNKS; then it would be processed downstream to yield **102**. Since the adducts **103a**–**103c** were obtained as NAC thioesters, the obligatory thioester exchange did not occur in the Diels–Alder reaction. To understand the

detailed mechanism of LNKS, one must convert the adduct **103a** to dihydromonacolin L (**101**) by LNKS. It is interesting that SPS catalyzes the Diels–Alder reaction after chain elongation while LNKS catalyses it during polyketide chain construction. Since most of the modification reactions in polyketide biosynthesis take place after chain elongation, LNKS is the first reported enzyme capable of chain modification prior to termination of polyketide chain extension.

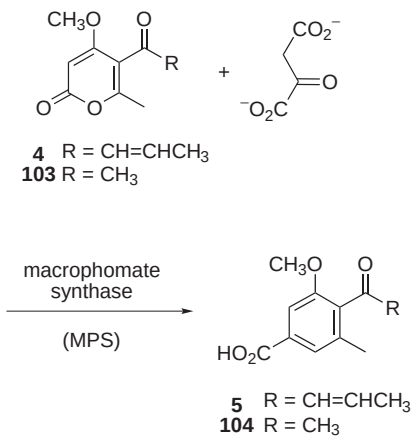
3.3 Macrophomate Synthase. The phytopathogenic fungus, *Macrophoma commelinae* has the ability to transform 2-pyrone **104** into the corresponding benzoate analogue macrophomate (**105**) (Scheme 20).[73] This conversion is very similar to that of pyrrenocine A (**4**)[3] to pyrenochaetic acid A (**5**),[4] and was effectively inhibited by bicyclic intermediate analogs.[74] This complex aromatic conversion is catalyzed by a single enzyme, macrophomate synthase (MPS),[60] with oxalacetate as a substrate for the C3-unit precursor. MPS is a Mg^{2+}-dependent enzyme with 339 amino acid residues (MW = 36244 Da). The catalytic mechanism of the whole pathway was investigated extensively, and it was shown that it proceeds through three separate steps: decarboxylation, two carbon–carbon bond formations, and decarboxylation with concomitant dehydration.[61,62] In the absence of 2-pyrone **104**, MPS simply acts as a decarboxylase with high catalytic efficiency (Scheme 21).

The crystal structure of the MPS complexed with pyruvate and Mg^{2+} was determined with a resolution of 1.70 Å (Fig. 4).[63] In the crystal structure, the C-terminal 40 residues (residues 300–339) that are not important in catalysis were deleted. The molecule is hexameric and the protomer core region consists of 8 stranded β-barrel surrounded by $8 + 3$ α-helices with a $(\beta/\alpha)_8$ barrel fold.

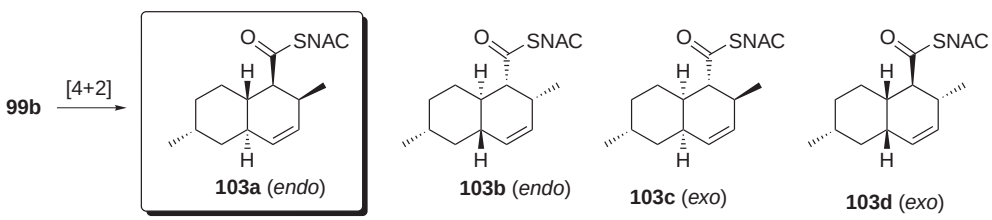
At the catalytic cavity, Mg^{2+} is located in an octahedral coordination site (Fig. 5A). Two of the ligands of Mg^{2+} are the side-chain carboxyl oxygens of Glu185 and Asp211. Two other coordination sites are filled with two water molecules which are in turn hydrogen bonded to the protein. The last two coordination sites are occupied by the C2-carbonyl and C1-carboxyl oxygen atoms of pyruvate enolate. This firmly bound structure clearly defines the precise orientation of the pyruvate enolate (Fig. 5A) of which two carboxyl oxygen atoms are also hydrogen-bonded to the main-chain amide protons of Gly210 and Asp211. This complex is further stabilized by interaction between the carbonyl oxygen of pyruvate and the side-chain of Arg101. With these bonds, the pyruvate enolate is tightly placed in this position. The active site is at the C-terminal end of the β-barrel, which is covered by the loop from the 3-fold-related chain.



Scheme 18.

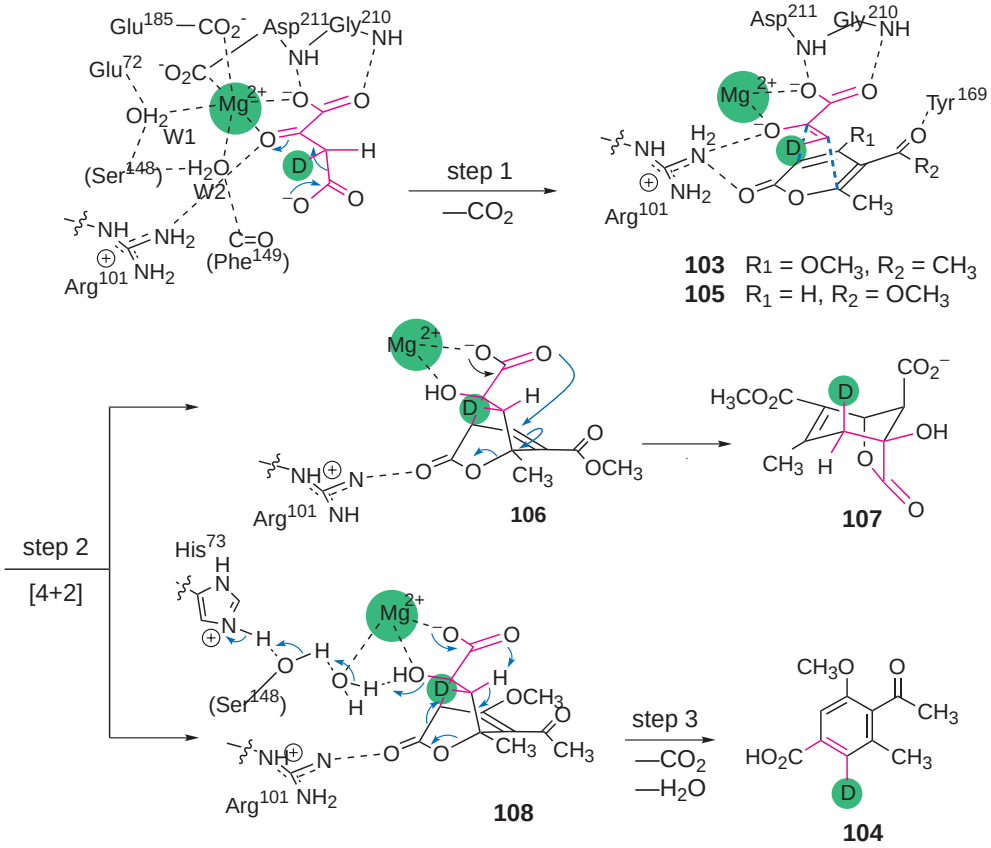


Scheme 20.



	103a (*endo*)	103b (*endo*)	103c (*exo*)	103d (*exo*)
toluene, 160 °C, 4 d	0	1	1	0
20 mM Tris, pH 7.7				
without LNKS	0	1	1	0
with LNKS	1	15	15	0

Scheme 19.



Scheme 21.

On the basis of this structural information, the pathway of the MPS reaction can be outlined as follows (Scheme 21): oxalacetate is incorporated into the active site of MPS in a similar way to that of pyruvate. Lewis acidity of the magnesium promotes decarboxylation to form the enolate anion, which is stabilized by an electron sink provided by the divalent cation. Steric congestion of the peptide backbone allows the 2-pyrone **104** access only from one side of the enolate plane, where the catalytic pocket is open. As shown in Fig. 5B, the 2-pyrone molecule is fixed in place through two hydrogen bonds between the carbonyl oxygen of 2-pyrone, Arg101, the C5-acyl oxygen, and Tyr169. The flexible loop (residues 139–170) with hydrophobic side-chains (Phe149, Pro151, and Trp152) from the 3-fold-related protomer shields this transition state from the solvent. The stacking direction of 2-pyrone and pyruvate enolate is exactly as expected from the product. Importance of the hydrogen bonds in the present model is confirmed by the experiment with two mutants, R101S and Y169F. Both mutations dramatically disturbed MPS activity whilst retaining the decarboxylase activity. These hydrogen bonds act not only in substrate recognition but also enhance reactivity in the inverse electron demand Diels–Alder reaction by reducing the LUMO energy of the diene. The binding model explains the substrate specificity[75] and the stereochemical course of whole reaction pathway.[61,62]

Based on formation of the aberrant adduct **108** with pyrone **106**[61] and the observation that dehydration proceeds formally in an *anti*-sense,[62] it was proposed that the higher energy [4 + 2] adducts **107** and **109** are transformed to either the rearranged product **108** or the benzoate analogue **105**, as shown in Scheme 21. The binding structure indicates that the free carboxylate oxygen is located close to the C4 position in the electron-deficient olefin. Thus, the carboxylate in the higher energy bicyclo[2.2.2]octane **107** attacks the β-position of the α,β-unsaturated ester moiety, followed by rearrangement and ring opening to afford stable bicyclo[3.2.1]octane **108**. In the reaction with the normal adduct **109**, substitution of the electron-donating methoxy group reduces the reactivity of the α,β-unsaturated ester, resulting in the fact that the nucleophile, C1-carboxylate can not attack this moiety. This alters the reaction path to form the aromatic compound **105**.

The stereochemical course of conversion from the adduct **109** to macrophomate (**105**)[62,74] is shown in Scheme 21. Since inspection of the active site does not identify any basic residue proximal to the *pro-R* proton at C6, the C1-carboxylate of **109** may abstract the proton accepting the developing charge on the lactone oxygen with Arg101. The concomitant elimination of the hydroxy group would facilitate the protonation from water, which is further relayed by Ser148 and His73. Kinetic analyses of MPS for the decarboxylation (k_{cat} 16.3 s^{-1}), the aberrant adduct formation (k_{cat} 5.9 s^{-1}), and the overall reaction (k_{cat} 0.6 s^{-1}) reveal that the last degradation step is the rate-determining step.[62] This might be reflected by the inefficiency of intramolecular deprotonation of the *pro-R* hydrogen in intermediate **109**.

Diels–Alder reaction usually does not require any catalytic process, and thus rate acceleration could be achieved by simply stabilizing the transition state. In fact, it is reported that

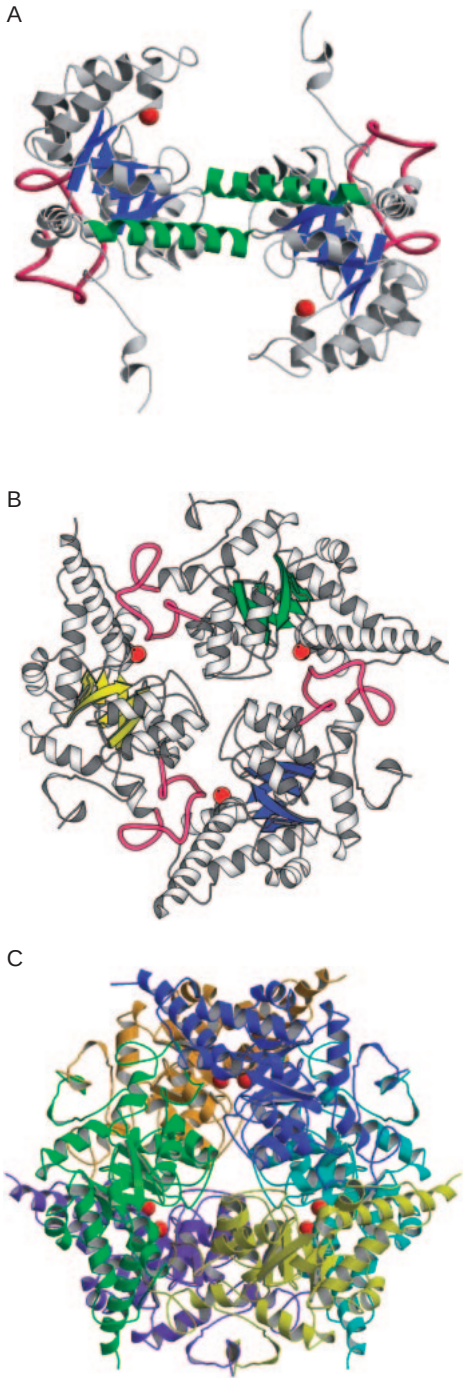


Fig. 4. Overall structures of macrophomate synthase. A: dimer; B: trimer; C: hexamer (functional unit).

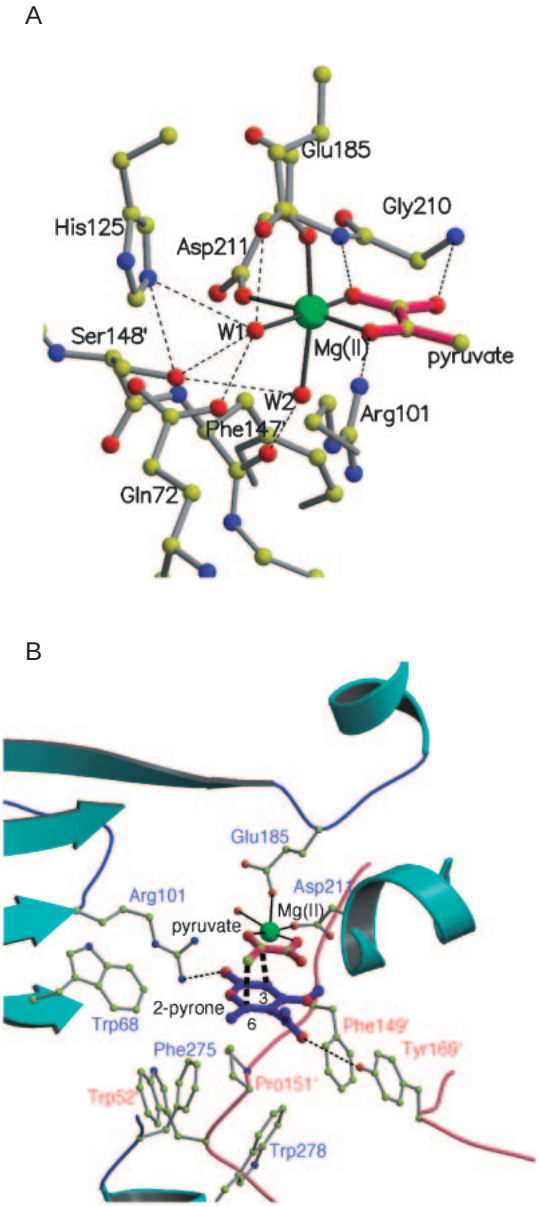


Fig. 5. Active site view of macrophomate synthase. A: Schematic representation depicting the detailed interactions of Mg^{2+} and its ligands; B: The residues of the active site and proposed binding model of pyruvate enolate and pyrone **104**.

the antibody that is elicited by transition state analogue is able to catalyze Diels–Alder reaction.[76] In this case, Diels–Alderase antibody provides a pocket acting as an entropy trap. As mentioned above, using this strategy, several Diels–Alderase antibodies have been created to date. Product inhibition is an inherent problem in the reaction with Diels–Alderase antibody because of the resemblance between the product and the transition state. MPS in which uncatalyzed CO_2 elimination from the adduct **109** is programmed provides one of solution for the product inhibition.

In summary, the general strategy of enzyme catalysis of the Diels–Alder reaction is entropy trapping with activation of the substrate by hydrogen bonding. Catalytic antibodies provide good examples of this strategy. Auxiliary proteins found in lignan biosynthesis[58] could be an example of natural proteins, although the corresponding Diels–Alderase has not been found yet. The plausible protein catalyzing the formation of galiellalactone might belong to this class. Three examples of natural Diels–Alderases: solanapyrone synthase, lovastatin nonaketide synthase, and macrophomate synthase catalyse not only the Diels–Alder reaction but also oxidation, polyketide chain formation, and decarboxylation, respectively. These enzymes convert the corresponding substrates into reactive Diels–Alder

substrates, which are not released from the active sites and readily undergo cycloaddition in the active sites by forcing them into reactive conformations. Thus, among many natural plausible Diels–Alderases described in this review, at least the three examples shown above can at least be classified as producers of reactive substrates with an entropy trap for [4 + 2] cycloaddition. Compared with artificial biomolecular catalysts such as catalytic antibodies[76] and RNA Diels–Alderase,[77] these types of natural Diels–Alderases have obvious advantages when one utilizes highly reactive substrates which are not stable in reaction media.

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