Biosynthesis of macrophomic acid: plausible involvement of intermolecular Diels–Alder reaction

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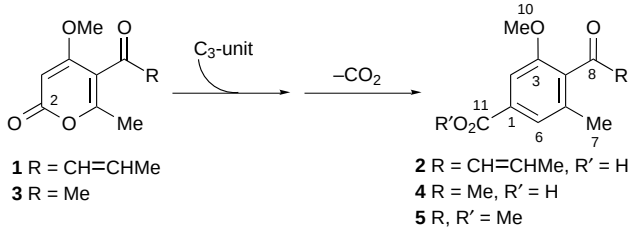
The biosynthetic precursor of the fungal metabolite macrophomic acid 4 from *Macrophoma commelinae* has been studied by feeding experiments and enzymatic conversion from the pyrone 3 to 4 in a cell-free system. Based on the experimental data, a biosynthetic pathway for 4 involving an unprecedented intermolecular Diels–Alder reaction is proposed.

In 1979 phytotoxin pyrenocine A **1**[1,2] was isolated from *Pyrenochaeta terrestris* by our group, and soon afterwards the structurally related pyrenochaetic acid A **2**[3] was also found from the same fungus. In spite of their striking structural similarity, we were unable to examine their biosynthetic origins due to their scarcity in the culture extracts. Later, Sakurai *et al*. reported that the fungus *Macrophoma commelinae* can convert pyrone **3** to macrophomic acid **4** (Scheme 1).[4,5] To our surprise, this fungus also converted **1** to **2** and its related analogues found in *P. terrestris*. Our continued interest in the biosyntheses of pyrenocines and pyrenochaetic acids prompted us to examine the unusual aromatic ring formation in *M. commelinae*.

Although Sakurai *et al*. established that the whole pyrone skeleton of **3** was directly transformed into benzoic acids with the loss of the C-2 carbon as CO_2, the origin of the C_3-unit of **4** remained to be established.[4,5] In order to answer this question, we employed several incorporation experiments. Administration of ^{13}C- or ^{2}H-labelled precursors to *M. commelinae*, followed by diazomethane treatment of the extracts, afforded methyl ester **5** which was examined by either ^{13}C or ^{2}H NMR analysis.

When [1-^{13}C]-l-serine was administered, an enhancement of the C-11 signal in the ^{13}C NMR spectrum of **5** formed in this experiment was observed. Normalization of the enhanced peak indicates an enrichment of 3.4 atom%. Similarly, after administration of [1-^{13}C]-l-alanine, high enrichment (6.3 atom%) at C-1 was observed. Since pyruvate is also incorporated without the loss of the C-1 carbon,[5] these data indicate that serine, alanine and pyruvate are converted to a common intermediate which is directly incorporated into **4**.

Next, a feeding experiment with [U-^{13}C]glycerol was conducted. In the ^{13}C NMR spectrum of the methyl ester **5** from the feeding experiment, two doublets at δ 166.5 (C-11, *J* 74.5 Hz) and 124.3 (C-6, *J* 60.3 Hz), and a doublet of doublets at δ 131.3 (C-1, *J* 74.5, 60.3 Hz) were observed. Both the splitting and the enrichment of the resonance show that incorporation of the labels, after the cycling of the C_3-unit through the tricarboxylic acid (TCA) and pentose-phosphate (PP) cycles, occurs at significant levels (Table 1). The observed coupling of the C-1 signal establishes the intact incorporation of glycerol.



1 R = CH=CHMe
3 R = Me

2 R = CH=CHMe, R′ = H
4 R = Me, R′ = H
5 R, R′ = Me

Scheme 1

Administration of (1*RS*, 2*S*)- and (1*RS*, 2*R*)-[1-^{2}H]glycerols prepared from 1,2;5,6-di-*O*-isopropylidene-d-mannitol[6,7] to *M. commelinae* was then investigated. As expected, only one signal, assigned to 6-H, was observed at δ 7.36 in the spectra of **5** from both experiments. Enrichment of (2*R*)-[^{2}H]-glycerol was estimated to be 3.7%. On the other hand, negligible enrichment of ^{2}H (0.23%) was found in the case of (2*S*)-[^{2}H]glycerol. The results are explained by the intermediacy of the C_3-unit as a (2*R*)-3-phosphoglycerate (3-PG) derivative.

In order to obtain information on the origin of the hydrogen atom at C-6, feeding experiments with specifically ^{2}H-labelled (1*R*, 2*R*)-[1-^{2}H]glycerol (58% ee) and (1*S*, 2*R*)-[1-^{2}H]glycerol (59% ee)† were conducted. Analysis of **5** formed in these experiments showed that the ^{2}H-label at C-6 was retained at a 5.5 times higher level in the case of (1*R*, 2*R*)-glycerol compared with (1*S*, 2*R*)-glycerol. The observed selective labelling indicates that the C–C bond formation between the pyrone **3** and the C_3-unit is stereoselective, and that the pathway involves an intermediate which has diastereotopic methylene protons.

Intact incorporation of [U-^{13}C]glycerol and retention of the 1-^{13}C label in the feeding experiments of l-serine and l-alanine exclude the possibility that TCA intermediates, *i.e.* oxaloacetate, directly convert into **4**. Among the other possible sources of the C_3-precursor, the observed results in the feeding experiment with specifically ^{2}H-labelled glycerols exclude the possibility of the involvement of pyruvate and alanine as the C_3-unit. Experimental evidence in this study leaves the most obvious candidate, PEP, as an intact substrate in the novel aromatic ring formation. Since the previous study suggested that PEP was not incorporated into **4**,[5] we initiated a cell-free study. Using **3** and PEP as substrates, enzymatic formation of **4** in the cell-free system was found.‡§ This finding established that macrophomic acid **4** is formed from the pyrone **3** and PEP by the specific enzyme(s).

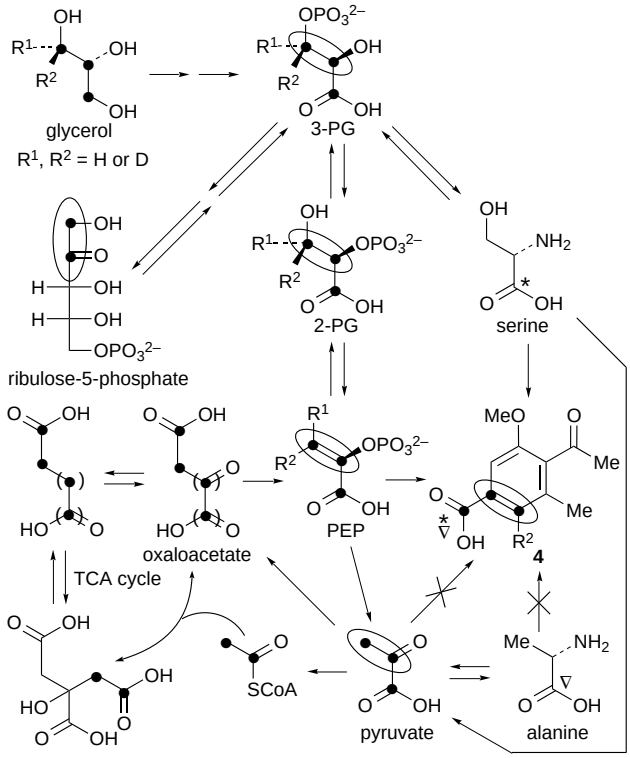
For the biosynthesis of macrophomic acid, Sakurai *et al*. proposed an anion-based condensation of **3** and PEP.[5] On the basis of our experimental data, we propose an alternative pathway for the conversion of **3** to **4**, as shown in Scheme 3. In this pathway, an intermolecular inverse-electron-demand Diels–Alder reaction of the pyrone **3** and the dienophile

Table 1 Incorporation of ^{13}C- or ^{2}H-labelled precursors into **5** in *M. commelinae* in the presence of the pyrone **3**

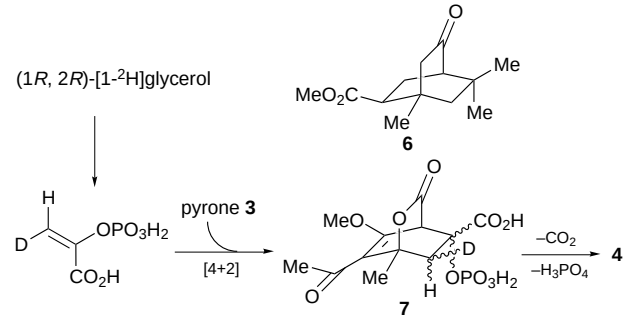
		Enrichment		
Entry	Precursor	C-6	C-1	C-11
1	[1-^{13}C]-l-alanine			3.4
2	[1-^{13}C]-l-serine			6.3
3	[U-^{13}C]glycerol	1.9	1.6[a] (1.0, 1.9)[b]	2.9
4	(1*RS*, 2*R*)-[1-^{2}H]glycerol	3.7		
5	(1*RS*, 2*S*)-[1-^{2}H]glycerol	0.23		
6	(1*R*, 2*R*)-[1-^{2}H]glycerol	34		
7	(1*S*, 2*R*)-[1-^{2}H]glycerol	7.1		

[a] Enrichment for doublet of doublets. [b] Enrichments for two doublets.

intermediate PEP affords a plausible adduct **7** which is transformed into **4** by successive retro-Diels–Alder reaction and *syn*-elimination of phosphoric acid. Compared with the hypothesis of Sakurai *et al*., our pathway significantly simplifies this unusual conversion. The results in the feeding experiments with (1*R*, 2*R*)- and (1*S*, 2*R*)-[1-^{2}H]glycerols are consistent with the selective retention of ^{2}H at C-6 in **5** *via* [3-^{2}H]-PEP. Interestingly, pyrenochaetic acid A **2** was chemically synthesized from pyrenocine A **1** *via* the plausible biomimetic route.[8]



Scheme 2



Scheme 3

To test this mechanism, the bicyclic compound **6**[9] which mimicked the plausible intermediate **7**, was synthesized. As expected, the intermediate analogue **6** inhibited the enzymatic conversion of **3** (10 μm) and PEP (100 μm) to **4**. The IC_{50} of **6** was determined to be 200 μm. This observation lends further support to our hypothesis, although the anion-based condensation is not rigorously excluded.

Currently, we are working on purifying the enzyme responsible for this unusual biological transformation.

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Footnotes

† The stereospecifically labelled glycerols were synthesized by Sharpless asymmetric dihydroxylation of deuterium-labelled allyl benzyl ethers. Details will be reported elsewhere.

‡ The crude enzyme was prepared as follows. The mycelium of *M. commelinae* IFO 9570 was cultured under shaking as described by Sakurai *et al*. During preincubation for induction of the desired enzyme, mycelia were collected by filtration and suspended in 50 mm potassium phosphate (0.45% NaCl, pH 7.2) containing a 100 mm DMF solution of pyrone **3** (final concentration 1 mm). This was further incubated under shaking for 3 h. The mycelia were collected by filtration and suspended in the same buffer. The mixture was homogenized with a mortar and pestle. Cell debris was removed by centrifugation (1.207 g, 15 min) and the supernatant was used as a crude enzyme.

§ Reactions were carried out at 30 °C for 4 h in mixtures containing the crude enzyme (1 ml), the pyrone **3** in DMF (10 μl, final concentration 100 μm) and phosphoenolpyruvate in H_2O (10 μl, final concentration 100 μm) in a total volume of 1.02 ml. Denatured enzyme (100 °C, 10 min) was used as a control. HPLC analysis of reaction products was performed with an ODS column (Wakosil-II 5C18, MeCN–H_2O, 3 : 2, 1 ml min^{-1}, UV 250 nm). Product formation was quantified *via* calibration with a standard. After 4 h, **3** and PEP were completely converted into **4**.

References

1 H. Sato, K. Konoma and S. Sakamura, *Agric. Biol. Chem.*, 1979, **43**, 2409.
2 H. Sato, K. Konoma, S. Sakamura, A. Furusaki, T. Matsumoto and T. Matsuzaki, *Agric. Biol. Chem.*, 1981, **45**, 795.
3 H. Sato, K. Konoma and S. Sakamura, *Agric. Biol. Chem.*, 1981, **45**, 1675.
4 I. Sakurai, H. Suzuki, S. Shimizu and Y. Yamamoto, *Chem. Pharm. Bull.*, 1985, **33**, 5141.
5 I. Sakurai, H. Miyajima, K. Akiyama, S. Shimizu and Y. Yamamoto, *Chem. Pharm. Bull.*, 1988, **36**, 2003.
6 S. Takano, E. Goto, M. Hirama and K. Ogasawara, *Heterocycles*, 1981, **16**, 381.
7 S. Takano, K. Seya, E. Goto, M. Hirama and K. Ogasawara, *Synthesis*, 1983, 116.
8 A. Ichihara, K. Murakami and S. Sakamura, *Tetrahedron*, 1987, **43**, 5245.
9 R. A. Lee, *Tetrahedron Lett.*, 1973, 3333.

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