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Macrophomate synthase: unusual enzyme catalyzing multiple reactions from pyrones to benzoates

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Abstract

Macrophomate synthase which catalyzes unusual multiple reactions from 2-pyrone (**3**) to macrophomic acid (**1**) has been purified in a homogenous state. The macrophomate synthase is a dimeric enzyme which requires Mg^{2+} as a co-factor and whose molecular mass is 40 kDa. Study on substrate specificity showed that the enzyme is capable of converting various 2-pyrones to the corresponding benzoates. © 1999 Elsevier Science Ltd. All rights reserved.

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To date, a few enzymes catalyzing more than four different conversions, such as dehydroquinate synthase[1] and 2-deoxy-*scyllo*-inosose synthase[2] are known. An investigation on reaction mechanisms of the enzymes is of great importance to understand how they can catalyze the multiple reactions for application of the enzymes to organic synthesis. These would provide information to modify an existing enzyme for adding another function which is useful for organic synthesis. Herein, we report purification and characterization of macrophomate synthase which catalyze the multistep conversion via formation of two C–C bonds concomitant with two decarboxylations and dehydration.

Previous studies[3] with whole cells of the fungus *Macrophoma commelinae* showed that macrophomic acid (**1**)[4] and pyrenochaetic acid (**2**)[5] were derived from the corresponding 2-pyrones **3** or pyrenocine A (**4**)[6] and a substrate for C_3-unit as shown in Scheme 1. In these cases, the condensations occur at both C2 and C3 positions of the precursor acid and this type of the transformation from 2-pyrone to benzoate analog is totally different from common routes via shikimate and polyketide pathways. In a biosynthetic study of **1**,[7] α,β-hetero atom-substituted C_3-acid or its equivalent such as phospho*enol*pyruvate and 2-phosphoglycerate is proposed as a substrate for C_3-unit due to intact incorporation of [*U*-^{13}C]-glycerol and stereoselective retention of deuterium labeled glycerols. In re-examination of the C_3-unit precursor, we found oxalacetate to be a more efficient and a direct precursor when we used a crude enzyme obtained from chromatography on DEAE Sepharose FF.[8] This conversion does not require any co-factor except an

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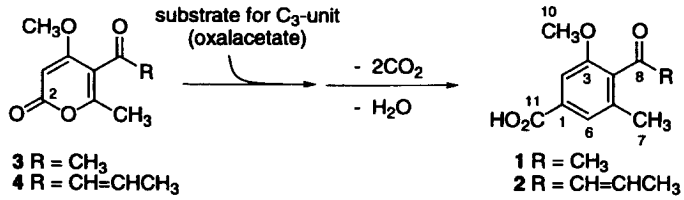
Mg^{2+} ion. The purified enzyme utilized oxalacatate as a sole substrate for C_3-unit. We named the enzyme as macrophomate synthase that catalyzes the unusual condensation of C_3-unit precursor with 2-pyrone.



Scheme 1. Unusual enzymatic transformations from 2-pyrones to benzoates

With oxalacetate as a substrate, macrophomate synthase was purified to homogeneity by chromatography on DEAE Sepharose FF, Q Sepharose FF (HiLoad 26/10) and butyl Sepharose 4 FF. The purified enzyme migrated on SDS-PAGE as a single band with a molecular mass of 40 kDa (Fig. 1). The native molecular mass was determined by gel filtration using Diol-200 was 80 kDa indicating that macrophomate synthase is a dimeric enzyme. The isoelectric point for this enzyme was determined as 5.3 by isoelectric electrophoresis. The macrophomic acid produced by the enzymatic reaction was found to be identical with synthetic **1**[3,9] by comparison of their ^1H NMR and FD-MS spectra. The observed relatively higher K_m values (1.7 mM for **3** and 1.2 mM for oxalacetate) agree with previous observation that various 2-pyrones were converted into the corresponding benzoates.[3]

Substrate specificity of the macrophomate synthase was next examined (Scheme 2).[10] The phytotoxins pyrenocines A (**4**) and B (**5**) were efficiently converted to the pyrenocheatic acids A (**2**) and B (**8**), respectively, as previously reported with the whole cell.[3] In these cases, **4** was not converted to **8** and **5** was not to **2** although each pyrone produced both benzoates in the whole cell experiment.[3] Whereas, 2-pyrones **6**[3] and **7**[3] which was not transformed with the whole cells, were converted to the corresponding benzoates **9**[3] and **10**.[11] This indicated that the pyrone **7** was degraded rapidly in the whole cell before macrophomate synthase converted it to the benzoate. It should be noted that the simple pyrone **7** was completely consumed within 10 min while 47% of **3** was converted at the same reaction time.

Macrophomate synthase catalyzes fairly complex reactions involving formally two C–C bond formations, two decarboxylations and dehydration. This reaction is closely related to our synthesis of pyreno-

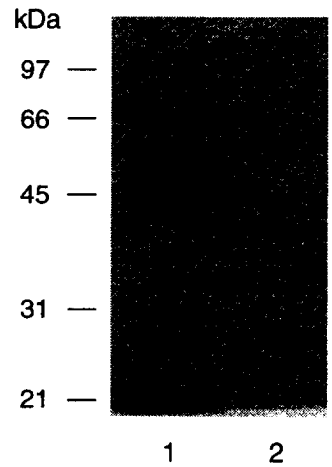
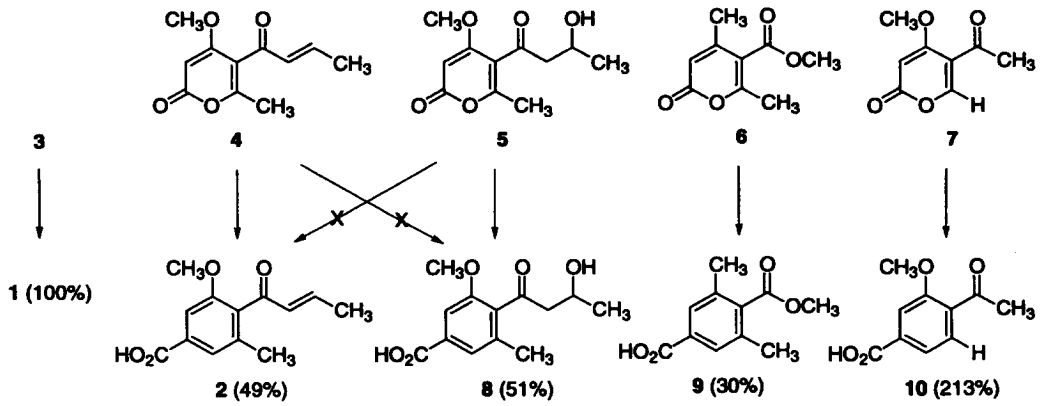
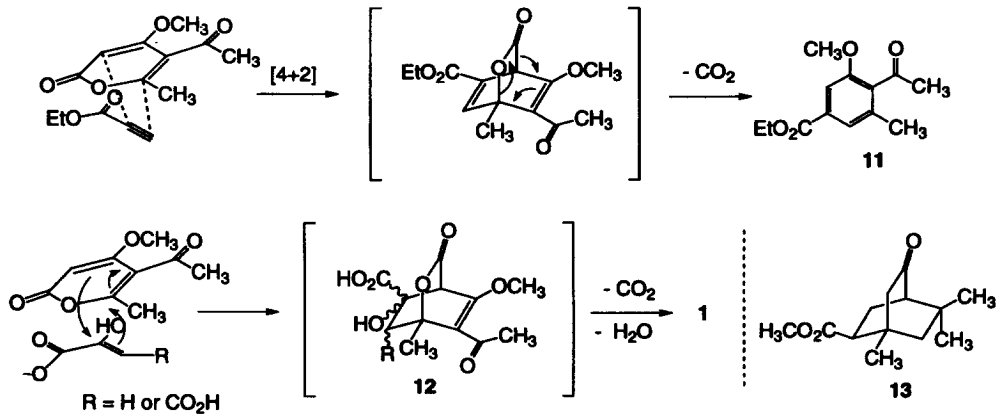


Figure 1. SDS-PAGE (10% gel) of purified macrophomate synthase. The gel was stained with Coomassie Blue. Lane 1: molecular mass standard. Lane 2: purified macrophomate synthase. Numbers at left indicate positions of molecular mass standard in kilodaltons



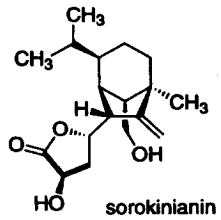
Scheme 2. The enzymatic conversions of various 2-pyrones. The values in parentheses are relative product formation against that of **1**

cheatic acid (**2**) starting from 2-pyrone **3** and ethyl propiolate to yield **11** via Diels–Alder reaction.[9] On the basis of this similarity and stereoselective incorporations of deuterium labeled glycerols,[7] we initially proposed[7] that the unusual transformation proceeds via inverse-electron-demand Diels–Alder reaction[12] between 2-pyrone and phospho*enol*pyruvate. Since it is difficult to produce a suitable dienophile from oxal-acetate, we tentatively proposed an alternative route as shown in Scheme 3. In this route, oxalacetate acts as a nucleophile and the 2-pyrone **3** as a Michael acceptor. A plausible bicyclic intermediate **12** might facilitate the decarboxylation and the dehydration due to its rigid conformation. This pathway is partly supported by the result that a bicyclic compound **13**[7] caused a 23% inhibition (**13**: 5 mM, pyrone **3**: 5 mM, oxalacetate: 5 mM). Currently, detail of the catalytic mechanism is not available but the data shown above indicate that the macrophomate synthase is a new type of enzyme.

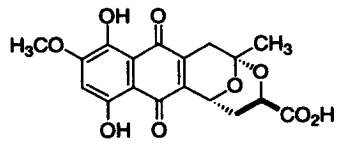


Scheme 3. Proposed reaction mechanism of the macrophomate synthase

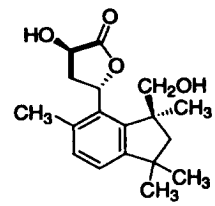
In literature, condensation of C_3-unit precursor to polyketides (marticin)[13], terpenoids (sorokinianin,[14] botryslactone)[15] and fatty acid derivatives[16] are often found in the biosynthesis of secondary metabolites. Although there are several proposals for the formation of such metabolites, none of them is established at enzyme level. Our study on the macrophomate synthase could provide a representative example for the condensation of C_3-unit precursor to various electrophiles shown above.



sorokinianin



marticin



botryslactone

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References

1. Widlanski, T. S.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 1873–1875; Widlanski, T. S.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 2299–2300.
2. Iwase, N.; Kudo, F.; Yamauchi, N.; Kakinuma, K. *Bioscience, Biotechnology & Biochemistry* **1998**, *62*, 2396–2407.
3. Sakurai, I.; Suzuki, H.; Shimizu, S.; Yamamoto, Y. *Chem. Pharm. Bull.* **1985**, *33*, 5141–5143; Sakurai, I.; Miyajima, H.; Akiyama, K.; Shimizu, S.; Yamamoto, Y. *Chem. Pharm. Bull.* **1988**, *36*, 2003–2011.
4. Sakurai, I.; Shimizu, S.; Yamamoto, Y. *Chem. Pharm. Bull.* **1988**, *36*, 1328–1335.
5. Sato, H.; Konoma, K.; Sakamura, S. *Agric. Biol. Chem.* **1981**, *45*, 1675–1679.
6. Sato, H.; Konoma, K.; Sakamura, S. *Agric. Biol. Chem.* **1979**, *43*, 2409–2411; Sato, H.; Konoma, K.; Sakamura, S.; Furusaki, A.; Matsumoto, T.; Matsuzaki, T. *Agric. Biol. Chem.* **1981**, *45*, 795–797.
7. Oikawa, H.; Yagi, K.; Watanabe, K.; Honma, M.; Ichihara, A. *J. Chem. Soc., Chem. Commun.* **1997**, 97–98.
8. The mycelium of *M. commelinae* IFO 9570 was cultured under shaking as described by Yamamoto et al.[3] From the mycelia (1 kg), the macrophomate synthase (0.62 mg) was obtained by a three-step purification. The overall yield of macrophomate synthase from this purification (161 times) is 0.15%.
9. Ichihara, A.; Murakami, K.; Sakamura, S. *Tetrahedron* **1987**, *43*, 5245–5250.
10. Reactions were carried out at 30°C for 10 min in enzyme solution (50 mM PIPES containing 5 mM $MgCl_2$, 5 mM of oxalacetic acid and 5 mM of pyrones, pH 7.2, 100 μl). Capillary electrophoretic analysis (Beckman, P/ACE 5000) of reaction products was performed [ϕ75 μm×57 cm, free 20 nl electrophoresis, 100 mM borate buffer (pH 8.1) and 50 mM SDS, constant current 90 μA, temp. 15°C, UV 200 nm, 5 nl injection, t_R (min) pyrones: **3** 3.6, **4** 6.8, **5** 5.4, **6** 5.1, **7** 6.6; benzoates: **1** 4.3, **2** 5.9, **8** 5.7, **9** 4.4, **10** 7.2].
11. Selected data for the benzoate, **9**: ^{1}H NMR (270 MHz, $CDCl_3$): δ 7.75 (d, *J*=8.6 Hz, 1H), 7.69 (dd, *J*=8.6, 1.3 Hz, 1H), 7.67 (br.s, 1H), 3.98 (s, 3H), 2.63 (s, 3H).
12. Afarinkia, K.; Vinader, V.; Nielson, T. D.; Posner, G. H. *Tetrahedron* **1992**, *48*, 9111–9171.
13. Holenstein, J. E.; Kern, H.; Stoessl, A.; Stothers, J. B. *Tetrahedron Lett.* **1983**, *24*, 4059–4062.
14. Nakajima, H.; Toratsu, Y.; Fujii, Y.; Ichinoe, M.; Hamasaki, T. *Tetrahedron Lett.* **1998**, *39*, 1013–1016.
15. Kimura, Y.; Fujioka, H.; Hamasaki, T.; Furihata, K.; Fujioka, S. *Tetrahedron Lett.* **1995**, *36*, 7673–7676.
16. Chesters, N. C. J. E.; O'Hagan, D. *J. Chem. Soc., Perkin Trans. 1* **1997**, 827–834.