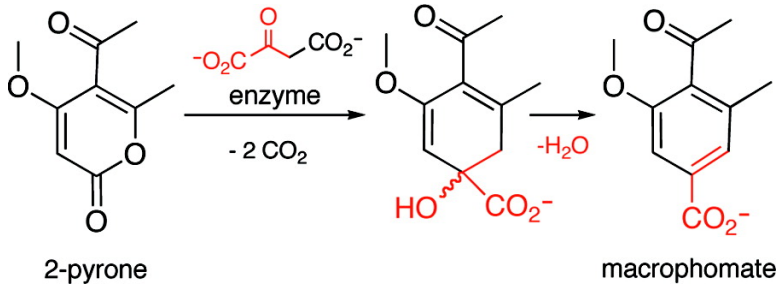
Communication

Trapping and Structural Elucidation of an Intermediate in the Macrophomate Synthase Reaction Pathway

Jrg M. Serafimov, Thomas Westfeld, Beat H. Meier, and Donald Hilvert

J. Am. Chem. Soc., **2007**, 129 (31), 9580-9581• DOI: 10.1021/ja073087y • Publication Date (Web): 18 July 2007



2-pyrone macrophomate

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 3 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 07/18/2007

Trapping and Structural Elucidation of an Intermediate in the Macrophomate Synthase Reaction Pathway

Jörg M. Serafimov,[§] Thomas Westfeld,[‡] Beat H. Meier,[‡] and Donald Hilvert*,[§]

Laboratory of Organic Chemistry, ETH Zurich, CH-8093 Zurich, Switzerland, and Laboratory of Physical Chemistry, ETH Zurich, CH-8093 Zurich, Switzerland

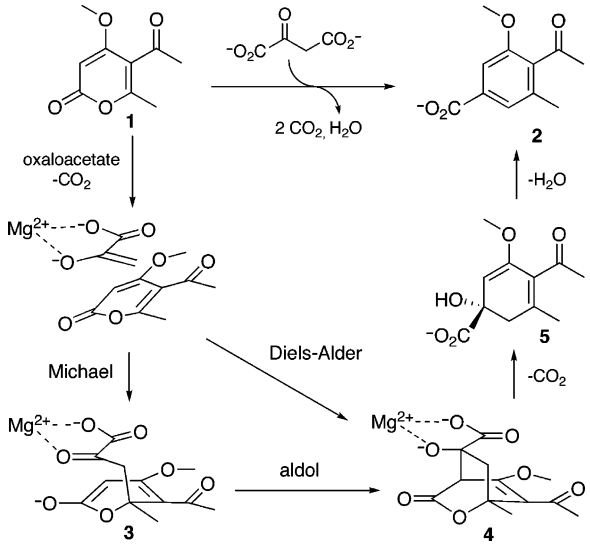
Received May 2, 2007; E-mail: hilvert@org.chem.ethz.ch

Macrophomate synthase (MPS), which promotes the conversion of 2-pyrones to benzoates (Scheme 1),[1−3] has garnered considerable attention as a possible natural Diels−Alderase.[4,5] The multistep reaction cascade is initiated by Mg^{2+}-assisted decarboxylation of the co-substrate oxaloacetate. Addition of the resulting pyruvate enolate to 2-pyrone **1** is then thought to afford a bicyclic intermediate **4** that subsequently undergoes decarboxylation and dehydration to give macrophomate **2**. Although the initial C−C bond-forming step has been put forward as a rare example of an enzyme-catalyzed [4 + 2] cycloaddition,[2,3] this conclusion is at odds with recent QM/MM calculations, which suggest that a two-step Michael-aldol sequence is energetically preferred over a concerted pericyclic pathway.[6]

Isolation of the Michael adduct **3**, which has been calculated to be more stable than **4**,[6] would resolve the mechanistic controversy. Although intermediates other than the pyruvate enolate have not been previously detected,[2,3] the feasibility of trapping such a species is supported by indications from work with a substrate analogue that a step after oxaloacetate decarboxylation probably limits macrophomate production.[2] We have now obtained direct spectroscopic evidence for the buildup of an intermediate during MPS catalysis. The reaction between oxaloacetate and 2-pyrone **1** to give macrophomate **2** can be conveniently monitored at 305 nm. When both substrates are mixed together in aqueous buffer in the presence of MPS, the absorbance initially decreases rapidly to a minimum value and then increases again as the reaction goes to completion (Figure 1). This type of biphasic reaction profile is diagnostic for the accumulation of a transient species.[7] We were able to confirm the formation of the intermediate by quenching the reaction with acetonitrile when the absorbance had reached its minimum and analyzing the sample by reverse phase HPLC (on a Nucleosil 300-5 C18 column eluted with a linear gradient from 90:10 to 45:55 water/acetonitrile over 55 min). A new peak was observed at 46 min that does not correspond to **1** (34 min) or to macrophomate **2** (50 min) (see Supporting Information). Unfortunately, more detailed characterization of this compound, for example by LC-MS, was hindered by its facile conversion to macrophomate.

Although attempts to isolate the MPS reaction intermediate failed, it can also be detected by ^{1}H NMR spectroscopy (Figure 2). In the course of the transformation, new resonances that do not correspond to the final product appear, concomitant with the disappearance of the substrates. With time, these signals decay and are replaced by those of macrophomate. The transient species was trapped by quenching the reaction with deuterated acetonitrile and cooling the sample to 260 K. Its structure was established unambiguously as **5** by a complete 2D NMR spectroscopic analysis (see Supporting Information for details). Although it was not possible to determine the absolute configuration of this compound, based on a docking model of the cycloaddition transition state at the active site,[3] it is likely that the single stereocenter has the *S* configuration, as shown in Scheme 1.

Scheme 1. Possible Mechanisms for the MPS-Catalyzed Formation of Macrophomate (**2**) from 2-Pyrone **1** and Oxaloacetate



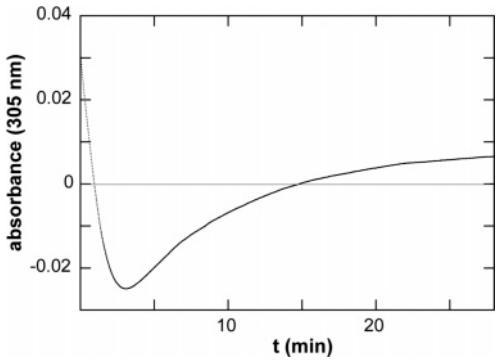


Figure 1. Spectroscopic detection of an MPS reaction intermediate. The time course of the MPS-catalyzed reaction between oxaloacetate (150 μM) and 2-pyrone **1** (200 μM) was monitored at 305 nm. The reaction was carried out in 50 mM PIPES buffer containing 5 mM $MgCl_2$ at pH 7.0 and 30 °C with [MPS] = 0.3 μM.

The kinetics of the MPS-catalyzed transformation were determined by ^{1}H NMR spectroscopy at 271 K as a function of oxaloacetate concentration, holding 2-pyrone constant at 8 mM. The formation of **5** follows Michaelis−Menten kinetics with an apparent k_{cat} value of 0.80 ± 0.06 s^{-1} and a K_m for oxaloacetate of 2.4 ± 0.5 mM. Because the rates of oxaloacetate consumption in

[§] Laboratory of Organic Chemistry.
[‡] Laboratory of Physical Chemistry.

10.1021/ja073087y CCC: $37.00

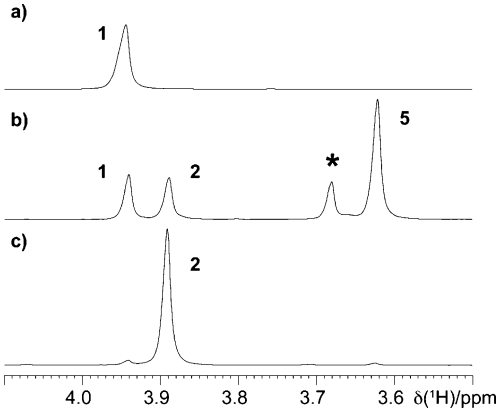


Figure 2. MPS reaction monitored by 400 MHz ^{1}H NMR. The reaction was carried out in 50 mM phosphate, 5 mM $MgCl_2$ at pH 7.0 and 271 K with [oxaloacetate] = 12 mM, [**1**] = 2.5 mM, and [MPS] = 1 μM. Spectra of the starting 2-pyrone (**1**) (a) and the reaction mixture after 140 min (b) and 1000 min (c) are shown. For the purposes of illustration, only the spectral region with the $-OCH_3$ resonances is presented (complete spectra are available in the Supporting Information). The signal marked with an asterisk (∗) corresponds to oxaloacetate.

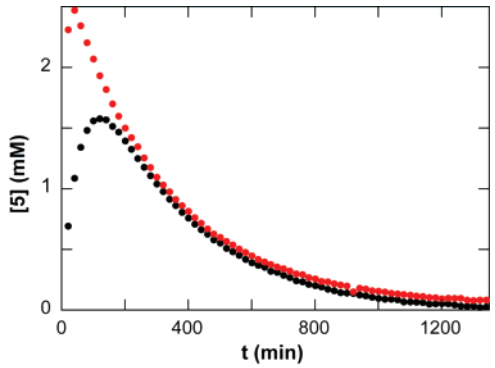


Figure 3. Total time course for the formation and decay of compound **5** in the presence of different MPS concentrations. The reaction was carried out in 50 mM phosphate buffer containing 5 mM $MgCl_2$ at pH 7.0 and 271 K with [oxaloacetate] = 12 mM, [**1**] = 2.5 mM, [MPS] = 1 μM (black) or 5 μM (red). The data were obtained by ^{1}H NMR spectroscopy using TMSP as an internal standard.

the presence and absence of pyrone coincide within experimental error with the rate at which **5** appears (and **1** disappears), all steps up to and including the formation of **5** must be at least as fast as the decarboxylation of oxaloacetate. These findings are consistent with mutagenesis experiments showing that decarboxylase activity can be reduced by as much as a factor of 20 without impacting the formation of macrophomate.[8]

The relatively slow decomposition of compound **5** limits the overall conversion of the 2-pyrone to macrophomate. However, this reaction is not catalyzed by MPS. As shown in Figure 3, raising the enzyme concentration 5-fold increases the rate of formation of the intermediate, as expected, but does not affect its breakdown. Analysis of the progress curves gives a rate constant of ca. 5×10^{-5} s^{-1} for the formation of **2** from **5** at pH 7.0 and 271 K.[9] Given that the final step in the reaction sequence is uncatalyzed and presumably does not occur at the MPS active site, the enzyme's designation as a macrophomate synthase is an apparent misnomer.

Identification of **5** as the true product of MPS catalysis clarifies the final stages of the reaction pathway leading from 2-pyrone **1** to macrophomate **2**. The bicyclic compound **4**, which is presumed to be a key intermediate in all previously published mechanisms, apparently decomposes by CO_2 extrusion to yield **5**, rather than by elimination of water or concerted loss of water and CO_2 as previously proposed.[2] This reaction could conceivably occur either in a stepwise or a concerted fashion. Aromatization then provides a strong driving force for the subsequent (uncatalyzed) dehydration of **5** to give macrophomate.

Although our results shorten the MPS reaction cascade by one step, they do not shed light on the nature of the early C−C bond-forming events. Indeed, the fact that **5** is formed at the same rate that oxaloacetate undergoes decarboxylation makes it extremely unlikely that either the Michael adduct **3** or the bicyclic intermediate **4** can be directly observed with currently available methods. As a consequence, other approaches will be required to resolve whether MPS truly functions as a natural Diels−Alderase.[4]

Acknowledgment. This work was generously supported by the ETH Zürich and a Kekulé fellowship from the Fonds der deutschen chemischen Industrie (J.M.S.). We are grateful to Prof. H. Oikawa (Hokkaido University) for the gift of the MPS-encoding plasmid pETMPS.

Supporting Information Available: Experimental details and structural determination of **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Oikawa, H.; Yagi, K.; Watanabe, K.; Honma, M.; Ichihara, A. *Chem. Commun.* **1997**, 97−98. (b) Watanabe, K.; Mie, T.; Ichihara, A.; Oikawa, H.; Honma, M. *Tetrahedron Lett.* **2000**, *41*, 1443−1446.
(2) Watanabe, K.; Mie, T.; Ichihara, A.; Oikawa, H.; Honma, M. *J. Biol. Chem.* **2000**, *275*, 38393−38401.
(3) Ose, T.; Watanabe, K.; Mie, T.; Honma, M.; Watanabe, H.; Yao, M.; Oikawa, H.; Tanaka, I. *Nature* **2003**, *422*, 185−189.
(4) For natural Diels−Alderases, see: (a) Auclair, K.; Sutherland, A.; Kennedy, J.; Witter, D. J.; Van den Heever, J. P.; Hutchinson, C. R.; Vederas, J. C. *J. Am. Chem. Soc.* **2000**, *122*, 11519−11520. (b) Stocking, E. M.; Williams, R. M. *Angew. Chem., Int. Ed.* **2003**, *42*, 3078−3115.
(5) For representative examples of catalytic antibodies and ribozymes that accelerate Diels−Alder reactions, see: (a) Xu, J.; Deng, Q.; Chen, J.; Houk, K. N.; Bartek, J.; Hilvert, D.; Wilson, I. A. *Science* **1999**, *286*, 2345−2348. (b) Romesberg, F. E.; Spiller, B.; Schultz, P. G.; Stevens, R. C. *Science* **1998**, *279*, 1929−1933. (c) Serganov, A.; Keiper, S.; Malinina, L.; Tereshko, V.; Skripkin, E.; Höbartner, C.; Polonskaia, A.; Phan, A. T.; Wombacher, R.; Micura, R.; Dauter, Z.; Jäschke, A.; Patel, D. J. *Nat. Struct. Mol. Biol.* **2005**, *12*, 218−224. (d) Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *Nature* **1997**, *389*, 54−57.
(6) Guimarães, C. R. W.; Udier-Blagović, M.; Jorgensen, W. L. *J. Am. Chem. Soc.* **2005**, *127*, 3577−3588.
(7) Because macrophomate is fluorescent, whereas the starting materials are not, its formation can be monitored by the continuous increase in fluorescence at 420 nm using an excitation wavelength of 310 nm. A significant lag phase is consistently observed for the appearance of product in the total time courses of the MPS-catalyzed reaction, providing additional evidence for the occurrence of a relatively stable intermediate.
(8) Serafimov, J. M.; Lehmann, H. C.; Oikawa, H.; Hilvert, D. *Chem. Commun.* **2007**, 1701−1703.
(9) It is not possible to deduce the rate at which macrophomate was formed in previous studies (e.g., ref 2) because the published value of k_{cat} (0.6 s^{-1}) was determined at an unspecified enzyme concentration. Nonetheless, based on the increase in fluorescence observed at 420 nm for reactions in 50 mM PIPES, 5 mM $MgCl_2$ (pH 7.0), we estimate the rate constant for the conversion of **5** to **2** to be approximately 1.5×10^{-3} s^{-1} at 303 K. If we assume 5% conversion for an MPS reaction with 15 mM **1** (10 times K_m), then the resulting 0.75 mM **5** would yield macrophomate at an initial rate of (0.75 mM)$(1.5 \times 10^{-3}$ s$^{-1}) \approx 1$ μM s^{-1}. If we further assume an MPS concentration of 1 μM, this "V_{max}" would correspond to an apparent "k_{cat}" of 1 s^{-1}, which is similar in magnitude to the literature value.

JA073087Y