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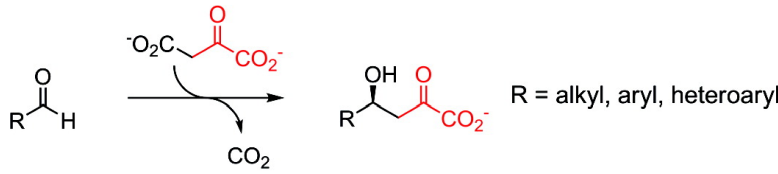

macrophomate synthase

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The Putative Diels−Alderase Macrophomate Synthase is an Efficient Aldolase

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Macrophomate synthase (MPS) from *Macrophoma commelinae* catalyzes an unusual multistep reaction cascade involving (1) decarboxylation of oxaloacetate to give pyruvate enolate, (2) formation of two C−C bonds between the enolate and 2-pyrone **1**, and (3) decarboxylation of the resulting bicyclic intermediate to give **2**, which spontaneously dehydrates in aqueous buffer to afford macrophomate **3** (Scheme 1).[1,2] The suggestion that the C−C bonds form via a concerted Diels−Alder mechanism has elicited considerable interest.[3] However, recent QM/MM calculations indicate that a two-step Michael-aldol sequence is energetically preferred over a [4 + 2] pericyclic reaction.[4] Moreover, MPS is closely related in sequence and structure to 2-dehydro-3-deoxygalactarate (DDG) aldolase, an enzyme that catalyzes the reversible aldol addition of pyruvate to tartronic semialdehyde.[5,6] Given the striking similarity of the MPS and DDG active sites and their shared dependence on Mg^{2+} as a cofactor, we wondered whether MPS might exhibit promiscuous aldolase activity.

To test this hypothesis, we screened a panel of aliphatic, aromatic, and heterocyclic aldehydes as potential reaction partners for oxaloacetate. In test reactions, MPS (0.8 μM) was incubated with the aldehyde (1 mM) for 5 min at room temperature in 50 mM phosphate buffer containing 5 mM $MgCl_2$ at pH 7.0. The reaction was initiated by adding oxaloacetate (2 mM) in four aliquots over 2 h. The samples were then frozen, lyophilized, and analyzed by analytical reverse phase HPLC. In each case, a parallel reaction without enzyme was performed as a control. For reaction mixtures that could not be separated by HPLC, ^{1}H NMR spectroscopy was used to judge the extent of product formation. As summarized in Table 1, the expected aldol product **5** and/or the subsequent elimination product **6** were detected in all cases, as judged by comparisons with authentic samples, in yields ranging from 35 to >90% and with large rate accelerations over background (vide infra). Reactions were also run on a preparative scale (typically 0.5 mmol aldehyde and 0.5 mmol oxaloacetate) to determine accurate yields. The examples in Table 1 indicate that electron-rich aldehydes tend to give more of the elimination product than neutral or electron-poor aldehydes (compare **4a** and **4b** with **4c** or **4d**). Monitoring the rate of elimination product **6a** at 340 nm relative to a control in the absence of enzyme shows that the elimination is not, however, enzyme-catalyzed. This finding is consonant with the observation that MPS does not accelerate the dehydration of **2** to give macrophomate.[2]

The direct addition of pyruvate enolates to electrophiles is difficult in the absence of enzymes,[7] and most reliable methods to introduce this moiety require indirect syntheses.[8] The protocol described here with MPS is operationally simple and uses an inexpensive, commercially available starting material, oxaloacetate, to generate a variety of aromatic and aliphatic γ-hydroxy-α-ketoacids of potential synthetic use. The two limiting factors in substrate scope seem to be the size of the electrophiles and their solubility in aqueous buffer. Although MPS exhibits relatively low enantioselectivity with the few aldehydes from Table 1 that were tested (ca. 40% ee), the diastereoselectivity with glyceraldehyde derivatives **7a** and **7b** (Scheme 2) demonstrates that, at least in certain cases, the enzyme is capable of directing the stereochemical course of the addition reaction. Oxaloacetate preferentially attacks the *Si* face of both glyceraldehyde enantiomers to give 2-keto-3-deoxygluconate derivatives **8a** (8:1 dr) and **8b** (3.5:1 dr), which are intermediates in the Entner−Doudoroff pathway of bacterial sugar acid metabolism.[9]

Since benzaldehyde (**4a**) is one of the most efficiently converted substrates, its reaction kinetics were examined in greater detail. The MPS-catalyzed reactions were carried out at 300 K in 50 mM phosphate buffer (pH 7.0) with 5 mM $MgCl_2$, and the appearance of product was monitored by ^{1}H NMR spectroscopy. The experiments were performed at three different benzaldehyde concentrations (0.3, 1.7, and 6.8 mM), bracketing the known K_m for pyrone **1** (1.7 mM) in the intermolecular reaction with oxaloacetate;[1b] each

Scheme 1. Native Reaction of MPS with 2-Pyrones (**1**→ **3**) and Analogous Reactions with Aldehydes (**4** → **6**)

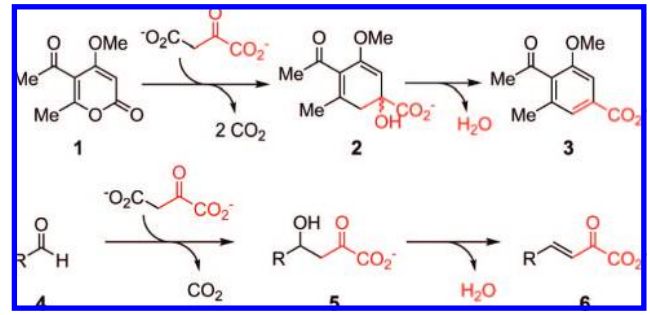
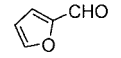
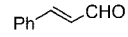
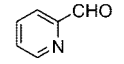


Table 1. Promiscuous Aldolase Activity of MPS[a]

Substrate	Aldehyde	Yield (%)	5:6	ee (%)
4a	PhCHO	70	>20:1	44[b]
4b	4-F-C_6H_4CHO	52	9:1	nd
4c	4-MeO-C_6H_4CHO	30	1:3	nd
4d		49	3:1	nd
4e		35	>20:1	nd
4f		95	>20:1	36[c]
4g	$C_7H_{15}CHO$	96	9:1	nd
4h	Et_2CHCHO	45	>20:1	nd

[a] Reactions were performed at room temperature in 50 mM postassium phosphate buffer (pH 7.0, pH 7.5 for preparative scale) containing 5 mM $MgCl_2$. [**4**] = 1 mM; [oxaloacetate] = 2 mM (added in two portions), [MPS] = 0.8 μM. [b] Determined by ^{1}H NMR of the corresponding SAMP hydrazone. [c] Determined by chiral HPLC after dithioacetal formation and cyclization; the reaction is *S* selective as determined by optical rotation. See Supporting Information for details; nd, not determined.

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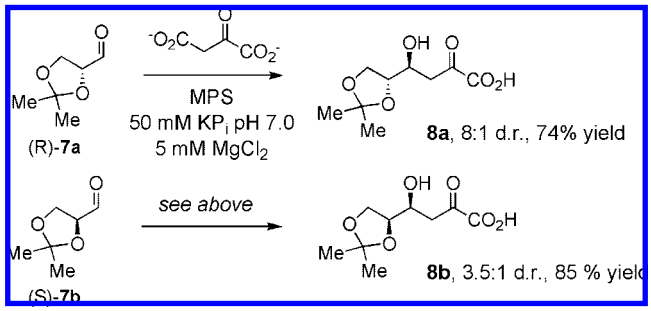
Scheme 2. Synthesis of 2-Deoxysugar Derivatives



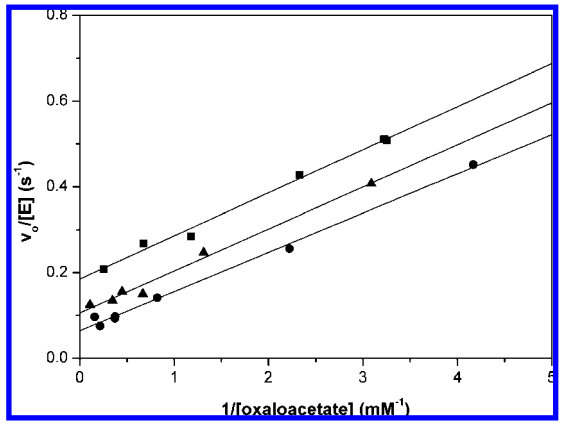


Figure 1. Double reciprocal plots at various benzaldehyde (**4a**) concentrations: ■ 0.3 mM **4a**; ▲ 1.7 mM **4a**; ● 6.8 mM **4a**.

line in Figure 1 was generated by collecting at least five initial velocity points for oxaloacetate concentrations in the range of 50 μM to 10 mM. Saturation kinetics were observed in all cases, and the slopes of the resulting double reciprocal plots were nearly parallel, indicating very little interaction between the substrates. This behavior is consistent with a rate-determining enzyme-catalyzed preactivation step. In this case, the preactivation is the decarboxylation of oxaloacetate. The limiting k_{cat} for the overall reaction was determined by replotting the three apparent k_{cat} values obtained from each data set in Figure 1 and extrapolating to saturating benzaldehyde concentrations. The resulting value is identical within error to that obtained for the MPS-catalyzed decarboxylation of oxaloacetate ($k_{aldol} = 12 \pm 2\ s^{-1}$ vs $k_{decarboxylation} = 12.0 \pm 0.4\ s^{-1}$), which suggests that the C−C bond-forming step is fast relative to decarboxylation.

The MPS-catalyzed retro-aldol reaction of **5a** was also examined. Formation of pyruvate was monitored at 303 K with a coupled NADH/lactate dehydrogenase assay[10] in 50 mM PIPES buffer with 5 mM $MgCl_2$ at pH 7.0. The reaction exhibits Michaelis−Menten behavior with $k_{cat} = 0.23 \pm 0.01\ s^{-1}$ and $k_{cat}/K_m = 3.9 \pm 0.6\ mM^{-1}\ s^{-1}$. Comparison with the corresponding rate constant obtained in the absence of enzyme ($1.0 \times 10^{-6}\ s^{-1}$) shows that MPS accelerates this reaction >10^5-fold. Thus, MPS is roughly 10^2-fold less active than typical pyruvate-specific type II aldolases acting on their native substrates, such as DDG aldolase ($k_{cat} = 27\ s^{-1}$; $k_{cat}/K_m = 420\ mM^{-1}\ s^{-1}$)[5] and *Hpa*I ($k_{cat} = 350\ s^{-1}$; $k_{cat}/K_m = 940\ mM^{-1}\ s^{-1}$),[10] and comparable to or more active than other aldolases that have been analyzed for promiscuous retro-aldol activity, including evolved variants of KDPG aldolase[11] and catalytic antibody 38C2.[12]

Promiscuity has been hypothesized to promote the evolution of new enzyme function, taking advantage of specific mechanistic attributes to facilitate the process of divergence.[13] Like the structurally related DDG aldolase,[5] *Hpa*I,[10] and the tetralin degradation enzyme ThnF,[14] MPS generates a highly reactive pyruvate enolate. Because its binding pocket is relatively capacious, this intermediate can be efficiently trapped by a structurally diverse set of electrophilic aldehydes in addition to its native 2-pyrone substrate, whereas the active sites of the natural aldolases appear to be tailored to specific electrophilic substrates. The modest *S*-selectivity we observe for the promiscuous aldol reactions is consistent with a relatively open binding pocket and further underscores the evolutionary link between MPS and type II pyruvate aldolases, which also preferentially cleave *S*-configured carbinols.[10] The higher selectivity observed with glyceraldehyde derivative **7a**, and the tolerance of the MPS active site to mutation,[15] suggests that relatively minor modification of the substrate or the binding pocket might suffice to reengineer the specificity of this enzyme.

Although MPS promiscuity does not directly address the concerted nature of the addition of pyruvate enolate to 2-pyrones, the fact that the enzyme is capable of efficiently promoting aldol-type chemistry lends plausibility to the proposed two-step Michael-aldol sequence. It is incumbent on proponents of a [4 + 2] Diels−Alder mechanism to provide evidence in favor of this mechanistic alternative.

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Supporting Information Available: Experimental procedures, and spectral and analytical data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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