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Substrate Diversity of Macrophomate Synthase Catalyzing an Unusual Multistep Transformation from 2-Pyrones to Benzoates

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Macrophomate synthase, which we have recently purified, catalyzes an unusual multistep transformation from 5-acetyl-4-methoxy-6-methyl-2-pyrone to 4-acetyl-3-methoxy-5-methyl-benzoic acid (macrophomic acid). To investigate the substrate diversity of the enzyme, 40 analogs of 2-pyrone were prepared and their relative efficiency was examined in the enzymatic conversions. The experimental results reveal the structural requirements of the substrates and the rough size of the enzyme active site, and eliminate the ambiguity caused by contamination by other enzymes in the whole-cell experiments.

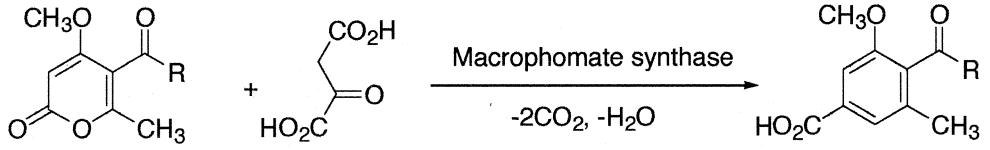
Key words: macrophomate synthase; *Macrophoma commelinae*; 2-pyrone; oxalacetate

Only a few enzymes catalyzing more than four different conversions such as dehydroquinate synthase[1,2] and 2-deoxy-*scyllo*-inosose synthase[3] are known. An investigation on the reaction mechanisms for such enzymes is of great importance to understand how they can catalyze multiple reactions for the application of organic synthesis to reduce a long series of reaction steps. This would provide information to modify an existing enzyme for adding another function or to create a new enzyme that can transform molecules in a stereocontrolled manner.

Yamamoto's group[4,5] have previously shown that macrophomic acid (**1**)[6] and the phytotoxin, pyrenochaetic acid (**2**),[7] were biotransformed from the corresponding 2-pyrones, **3** and **4** (pyrenocine A[8,9]), and a C_3-acid precursor by the fungus, *Macrophoma commelinae*, as shown in Scheme 1. We have recently determined the C_3-precursor to be an oxalacetate, and have purified and characterized the enzyme named as macrophomate synthase.[10–12] This single enzyme catalyzes multiple reactions of oxalacetate with 2-pyrone **3** to afford benzoate **1** *via* the formation of two C–C bonds concomitant with two decarboxylation and dehydration reactions.

The addition of the C_3-acid to polyketides,[13] terpenoids[14,15] and fatty acid derivatives[16] is often to be found in the biosynthesis of a secondary metabolite. There are two modes of condensation pattern, C–C bond formation at C-2[16] of the C_3-acid and formation at C-3.[13–15] In some cases, an oxalacetate has been proposed to be the C_3-unit precursor.[14,16] Although there are several proposals for the formation of such metabolites, none of them has been established at an enzyme level. In the case of the conversion catalyzed by macrophomate synthase, condensation occurs at both the C-2 and C-3 positions, and this type of aromatic ring formation is totally different from the common routes *via* the shikimate and polyketide pathways.

Previous studies on biotransformation with the whole cell of *M. commelinae* have shown that various 2-pyrones were converted into the corresponding benzoates.[5] To test the ability of macrophomate synthase to accept various pyrones as



3 R = CH_3
4 R = CH=$CHCH_3$

1 R = CH_3
2 R = CH=$CHCH_3$

Scheme 1. Enzymatic Conversions of Macrophomate Synthase.

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substrates, and to explore the synthetic utility of the enzyme, this biotransformation was extensively re-examined for the enzyme. We report have an investigation of the substrate diversity of macrophomate synthase which catalyzes the multistep conversion.

Materials and Methods

General procedures. IR spectra were measured with a System 2000 FT-IR, ^{1}H- and ^{13}C-NMR spectra with a JEOL EX-270 spectrometer, and mass spectra with JEOL JMS-AX500 and JMS-SX102A spectrometers. Column chromatography used Merck Kieselgel 60 (0.04–0.063), and TLC was performed on Merck Kieselgel 60 F_{254}. Anhydrous reactions were carried out under an argon atmosphere.

Enzyme. The partially purified enzyme[12] of macrophomate synthase used in this study was obtained after HiLoad Q Sepharose HP column chromatography of disrupted cells of *M. commelinae* (specific activity of 44 nmol/min/mg). The purified enzyme[12] was obtained as described previously (specific activity of 130 nmol/min/mg). The enzyme solution used in this study consisted of 50 mM piperazine-1,4′-bis(2-ethanesulfonate) at pH 7.2 containing 5 mM $MgCl_2$.

Substrates. Pyrenocines A (**4**) and B (**13a**), and pyrenochaetic acids A (**2**) and B (**14a**) were kindly presented by Prof. H. Sato. Compounds **7c**,[17] **7d**,[18] **8a**,[19] **8c**,[19] **8d**,[19] **8e**,[19] **8g**,[19] **8h**,[19] **3**,[20] **9a**,[20] **11a**,[17] **11b**,[5] **11c**,[5,21] **11e**,[5,17] **11g**,[17] **11h**,[22] **13b**,[5] **13c**,[5] **13d**,[23,24] **13e**,[5] **13f**[5] and **13k**[5] were synthesized according to the literature, and compounds **5a**, **7a**, **7b** and **13j** were purchased from Aldrich.

Authentic reaction products. All authentic samples, except for **2**, **10a** and **14a**, were prepared by biotransformation, using whole cells of *Macrophoma commelinae* as described previously.[5]

5-Carboxy-4,6-dimethyl-2H-pyran-2-one (5e). A solution of **5a** (500 mg, 2.7 mmol) in H_2SO_4 (95.0%, 10 ml) was stirred for 5 days at 80°C. The mixture was poured into ice-cooled water and extracted with AcOEt. The combined organic extract was washed with brine and dried over anhydrous Na_2SO_4. The mixture was evaporated *in vacuo*, and the black oily residue was purified by flash chromatography ($CHCl_3$/MeOH, 95/5) to give **5e**[25] (417 mg, 92%) as colorless needles, mp 152–155°C (lit. mp 155°C).

5-Allyloxycarbonyl-4,6-dimethyl-2H-pyran-2-one (5b). To a mixture of **5e** (40 mg, 0.24 mmol) and CH_2Cl_2 (1 ml) was added a suspension of DCC (50 mg, 0.24 mmol) and DMAP (3 mg, 0.025 mmol) in CH_2Cl_2 (1 ml). Allyl alcohol (0.1 ml, 1.7 mmol) was added, and the resulting mixture was stirred at room temperature for 8 h. The reaction mixture was evaporated *in vacuo* to afford an oily residue which was purified by PTLC ($CHCl_3$) to give **5b** (6.6 mg, 14%) as a yellow oil. IR ν_{max} (NaCl) cm^{-1}: 3627, 3085, 2933, 1634, 1557, 1378, 1083, 860, 778; ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 6.02 (1H, s, Ar-*H*), 5.98–5.85 (1H, m, -CH_2-C*H*=CH_2), 5.33 (2H, dd, J=8.9, 1.3 Hz, -CH=CH_2), 4.77 (2H, d, J=6.3 Hz, -O-CH_2-CH=), 2.39 (3H, s), 2.22 (3H, s). MS (EI) m/z: 208 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{11}H_{12}O_4$, 208.0735; found, 208.0771.

5-Benzyloxycarbonyl-4,6-dimethyl-2H-pyran-2-one (5c). Compound **5c** was synthesized as described previously, except for using benzylalcohol, as an amorphous solid, mp 109–112°C. IR ν_{max} (KBr) cm^{-1}: 2936, 1723, 1547, 2370, 1247, 1082, 888, 756, 636, 565. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.32–7.27 (5H, m, Ar-*H*), 5.95 (1H, s, Ar-*H*), 4.61 (2H, s, -O-CH_2-Ar), 2.41 (3H, s), 2.21 (3H, s). MS (EI) m/z: 258 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{15}H_{14}O_4$, 258.0892; found, 258.0927.

4,6-Dimethyl-5-[(N-hexyl)aminocarbonyl]-2H-pyran-2-one (5d). To a suspension of **5e** (9.3 mg, 0.06 mmol) in AcOEt (0.75 ml) was added hexylamine (16.7 ml, 0.17 mmol), diethylphosphoryl cyanide (DEPC, 0.02 ml, 0.12 mmol) and Et_3N (18 ml, 0.17 mmol). After stirring at room temperature for 18 h, the mixture was poured into ice-cooled water and extracted with AcOEt. The organic extract was successively washed with sat. NH_4Cl and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a residue which was purified by PTLC ($CHCl_3$/MeOH, 9/1) to give **5d** (2.3 mg, 17%) as a yellow oil. IR ν_{max} (NaCl) cm^{-1}: 2930, 2859, 1728, 1645, 1601, 1413, 1315, 1036, 814, 587. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 12.1 (br. s, 1H, -CO-N*H*-CH_2-), 5.52 (1H, s, Ar-*H*), 2.43 (3H, s), 2.32 (3H, s), 1.86–0.96 (10H, m, -NH-(CH_2)$_5$-CH_3), 0.91 (3H, t, J=4.0 Hz, -CH_2-CH_3). MS (EI) m/z: 251 (M^+). HRMS (EI) m/z (M^+): calcd. for $C_{14}H_{21}NO_3$, 251.1522; found, 251.1516.

4-Methoxy-6-methyl-3-[(thiophenyl)methyl]-2H-pyran-2-one (8b). To a suspension of K_2CO_3 (804 mg, 5.82 mmol) and 4-hydroxy-6-methyl-3-[(thiophenyl)methyl]-2*H*-pyran-2-one[19] (490 mg, 1.97 mmol) in DMF (2 ml) was added methyl *p*-toluenesulfonate (542 mg, 2.91 mmol) in DMF (1 ml) at 0°C, and the mixture stirred at room temperature for 12 h.[23] The reaction mixture was poured into water and extracted with CH_2Cl_2. The organic extract was successively washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a residue which was purified by silica gel column chromatography

($CHCl_3$/acetone, 98:2) to give **8b** (549.3 mg, 100%) as a colorless oil. IR ν_{max} (NaCl) cm^{-1}: 2951, 1695, 1645, 1598, 1464, 1353, 1260, 1177, 1037, 804, 661, 555. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.51–7.15 (5H, m, Ar-*H*), 5.99 (1H, s, Ar-*H*), 3.97 (2H, s, -S-C*H*$_2$-Ar), 3.73 (3H, s, Ar-OC*H*$_3$), 2.29 (3H, s, Ar-C*H*$_3$). MS (EI) *m/z*: 262 (M^+). HRMS (EI) *m/z* (M^+): calcd. for $C_{14}H_{14}O_3S$, 262.0663; found, 262.0642.

4-Methoxy-6-methyl-3-[(phenylsulfoxy)methyl]-2H-pyran-2-one (8f). To a solution of **8b** (109 mg, 0.42 mmol) in CH_2Cl_2 (14 ml) was added a solution of *m*CPBA (80%, 98.7 mg, 0.46 mmol). After stirring at −40°C for 1 h, the mixture was successively treated with sat. $NaHSO_3$, sat. $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. Purification by PTLC ($CHCl_3$/MeOH, 9:1) gave **8f** (82.9 mg, 75%) as yellow crystals, mp 119–122°C. IR ν_{max} (KBr) cm^{-1}: 3067, 2847, 1703, 1642, 1565, 1476, 1383, 1227, 1034, 1019, 748, 690. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.78–7.46 (5H, m, Ar-*H*), 5.99 (1H, s, Ar-*H*), 4.08 (1H, d, J=12.2 Hz), 3.88 (1H, d, J=12.2 Hz), 3.73 (3H, s, Ar-OC*H*$_3$), 2.29 (3H, s, Ar-C*H*$_3$). MS (FD) *m/z*: 278 (M^+). HRMS (FD) *m/z* (M^+): calcd. for $C_{14}H_{14}O_4S$, 278.0613; found, 278.0583.

5-Acetyl-6-methyl-4-pentyloxy-2H-pyran-2-one (11d). To a mixture of NaH (60% in mineral oil, 50 mg, 1.25 mmol) in DMF (1 ml) was added **11g** (147 mg, 0.88 mmol) in DMF (2 ml) at 0°C. The mixture was stirred at room temperature for 15 min. 1-Bromopentane (0.5 ml, 3.3 mmol) was added to the mixture, and stirring was continued for 12 h at room temperature. After being quenched with 10% HCl, the reaction mixture was diluted with AcOEt and then successively washed with 10% $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a residue which was purified by PTLC ($CHCl_3$/MeOH, 95/5) to give **11d** (42.7 mg) as a yellow oil. IR ν_{max} (NaCl) cm^{-1}: 2958, 1739, 1622, 1564, 1382, 1267, 1088, 956, 808, 460. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 5.41 (1H, s, Ar-*H*), 3.97 (2H, t, J=6.6 Hz, -CH_2-C*H*$_2$-O-), 2.39 (3H, s), 2.23 (3H, s), 1.76 (2H, m), 1.35 (4H, m), 0.87 (3H, t, J=6.9 Hz, -CH_2-C*H*$_3$). MS (EI) *m/z*: 238 (M^+). HRMS (EI) *m/z* (M^+): calcd. for $C_{13}H_{18}O_4$, 238.1205; found, 238.1195.

5-Acetyl-4-(tert-butyldiphenylsiloxy)-6-methyl-2H-pyran-2-one (11f). To a mixture of imidazole (82 mg, 1.2 mmol) and TBDPSCl (0.16 ml, 0.6 mmol) in DMF (3 ml) was added a solution of **11g** (100 mg, 0.6 mmol) in DMF (5 ml), and the resulting mixture was stirred at room temperature for 12 h. The reaction mixture was poured into water. After diluting with AcOEt, the reaction mixture was washed with brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was purified by PTLC ($CHCl_3$/MeOH, 9/1) to give **11f** (25 mg, 10%) as a yellow oil. IR ν_{max} (NaCl) cm^{-1}: 2929, 1716, 1428, 1113, 821, 702, 506. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.72–7.70 (4H, m, Ar-*H*), 7.40–7.37 (6H, m, Ar-*H*), 5.57 (1H, s, Ar-*H*), 2.25 (3H, s), 2.40 (3H, s), 1.07 (9H, s, -C(C*H*$_3$)$_3$). MS (FD) *m/z*: 406 (M^+). HRMS (FD) *m/z* (M^+): calcd. for $C_{24}H_{26}O_4Si$, 406.1600; found, 406.1631.

5-Formyl-4-methoxy-6-methyl-2H-pyran-2-one (13e). Compound **13e** was synthesized by using essentially the same procedure as that just described. Compound **13f**[5] (43.5 mg, 80%) was synthesized by using 4-hydroxy-5-hydroxymethyl-6-methyl-2*H*-pyran-2-one[5] (50 mg, 0.32 mmol) and yielded as colorless crystals, mp 165–167°C (lit. mp 163–166°C).

To a solution of **13f** (20 mg, 0.12 mmol) in 5 ml of CH_2Cl_2 was added Dess-Martin periodinane[26,27] (51 mg, 0.12 mmol). After stirring at room temperature for 2 h, the mixture was quenched with 5% $NaHCO_3$ and then extracted with AcOEt. The organic extract was washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. Purification by PTLC ($CHCl_3$/MeOH, 95/5) gave **13e**[20] (17 mg, 84%) as colorless needles, mp 132–135°C (lit. mp 130–133°C).

4-Methoxy-5-[(methoxy)methyl]-6-methyl-2H-pyran-2-one (13g). To a suspension of NaH (60% in mineral oil, 7 mg, 0.18 mmol) in THF (1 ml) was added alcohol **13f** (10 mg, 0.06 mmol), and the mixture was stirred at room temperature for 30 min. A solution of methyl *p*-toluenesulfonate (0.05 ml, 0.27 mmol) was then added. After stirring at room temperature for 2 h, the mixture was quenched with ice-cooled water and extracted with AcOEt, and the combined organic extract was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. Purification by PTLC ($CHCl_3$/MeOH, 9:1) gave **13g** (1.0 mg, 9.2%) as white crystals, mp 58–61°C. IR ν_{max} (KBr) cm^{-1}: 2931, 1724, 1650, 1566, 1412, 1256, 1091, 842, 735, 533. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 5.47 (1H, s, Ar-*H*), 4.12 (2H, s, Ar-C*H*$_2$-OCH$_3$), 3.84 (3H, s, Ar-OC*H*$_3$), 3.37 (3H, s, -CH_2-OC*H*$_3$), 2.32 (3H, s, Ar-C*H*$_3$). MS (EI) *m/z*: 184 (M^+). HRMS (EI) *m/z* (M^+): calcd. for $C_9H_{12}O_4$, 184.0736; found, 184.0729.

4-Methoxy-5-[[(methoxy)methoxy]methyl]-6-methyl-2H-pyran-2-one (13h). To a solution of alcohol **13f** (2.0 mg, 0.01 mmol) in CH_2Cl_2 (0.5 ml) were added MOMCl (0.04 ml, 0.5 mmol) and *i*-Pr_2NEt (0.06 ml, 0.3 mmol). After stirring at room temperature for 1 h, the mixture was poured into

ice-cooled water and extracted with AcOEt. The organic extract was successively treated with 2 M HCl, sat. $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. Purification by PTLC ($CHCl_3$/MeOH, 92:8) gave **13h** (1.7 mg, 79%) as white crystals, mp 81–83°C. IR ν_{max} (KBr) cm^{-1}: 2956, 2897, 1708, 1558, 1460, 1389, 1260, 1092, 1030, 910, 840. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 5.46 (1H, s, Ar-*H*), 4.64 (2H, s), 4.38 (2H, s), 3.85 (3H, s, Ar-OC*H*$_3$), 3.39 (3H, s, -CH_2-OC*H*$_3$), 2.33 (3H, s, Ar-C*H*$_3$). MS (EI) *m/z*: 214 (M^+). HRMS (EI) *m/z* (M^+): calcd. for $C_{10}H_{14}O_5$, 214.0841; found, 214.0860.

5-Hydroxymethyl-4-methoxy-6-methyl-2H-pyran-2-one (13i). To a solution of **3** (100 mg, 0.55 mmol) and $CeCl_3 \cdot 7H_2O$ (700 mg, 1.88 mmol) in MeOH (30 ml) was added $NaBH_4$ (100 mg, 2.64 mmol) in MeOH (20 ml) and the mixture was stirred at 0°C for 20 min. The reaction was quenched with acetone, and the mixture was evaporated *in vacuo*. Water was added to the reaction mixture, and the resulting mixture was extracted with AcOEt. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to afford a residue which was purified by flash chromatography ($CHCl_3$/acetone, 8/2) to give **13i**[20] (107 mg, 95%) as white crystals, mp 133–136°C (lit. mp 127–130°C).

Assay conditions. All the compounds, except acetate **11e** and aldehyde **13e**, were assayed by using the partially purified enzyme of macrophomate synthase. In the cases of **11e** and **13e**, the purified enzyme was used under the same conditions. The prepared enzyme solution was preincubated with 5 μl of a 0.1 M DMF solution of the various analogues at 30°C for 5 min. The reaction was initiated by adding 5 μl of 0.1 M oxalacetate, and stopped by adding 100 μl of 2-propanol after a 60-min incubation at 30°C. The enzyme solution boiled at 100°C for 10 min was used as a control.[12] The reaction mixture was filtered with EB-DISK 13 (Cica), and an aliquot was analyzed by reversed-phase HPLC. The apparatus was a Waters™ 996 photodiode array detector, Waters™ 600 controller, Waters™ 717 plus autosampler, and a column of Wakosil 5C18 (ϕ4.6×250 mm). The mobile phase was solution A [10% CH_3CN in a 10 mM phosphate buffer (pH 2.6)] and solution B [40% CH_3CN in a 10 mM phosphate buffer (pH 2.6)] with a linear gradient of solutions A and B (0–45% B, 0–15 min; 45–70% B, 15–20 min; 70% B, 20–25 min) at a flow rate of 1 ml/min and temperature of 50°C. Retention times **3**, 17.7 min; and **1**, 29.3 min. Detection was UV at 250 nm.[5]

4-Acetyl-3-methoxybenzoic acid (10a). To identify product **10a** in the enzymatic reaction of 2-pyrone **9a**, the reaction was employed on a 10-times larger scale for 3 h. To the reaction mixture was added 100 μl of 2 M HCl, and the resulting mixture was extracted with AcOEt. The AcOEt layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was purified by PTLC ($CHCl_3$/MeOH, 9/1) to give benzoate **10a** (0.9 mg, 90%) as a yellow oil. IR ν_{max} (NaCl) cm^{-1}: 3751, 2861, 2842, 1019, 669, 466. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.75 (1H, d, J=8.6 Hz, Ar-*H*), 7.69 (1H, dd, J=8.6, 1.3 Hz, Ar-*H*), 7.67 (1H, br. s, Ar-*H*), 3.98 (3H, s, Ar-OC*H*$_3$), 2.63 (3H, s, -CO-C*H*$_3$). MS (FD) *m/z*: 194 (M^+). HRMS (FD) *m/z* (M^+): calcd. for $C_{10}H_{10}O_4$, 194.0579; found, 194.0569.

Results and Discussion

Most of the enzyme reactions of the 2-pyrones with the partially purified enzyme of macrophomate synthase proceeded cleanly to provide essentially single products as shown in Fig. 1. Under standard conditions, natural substrate **3** was completely converted to **1** in 60 min. Typical examples of the enzymatic reaction are shown in Fig. 1. Methyl isodehydracetate **5a** was converted to corresponding benzoate **6a**. The formation of **6a** was confirmed by its online UV spectrum (Fig. 1-f) which is similar to that of **1** (Fig. 1-e). The benzoates produced were easily characterized by their retention times and UV profiles in comparison with those of authentic samples. The crude enzyme was sufficiently pure for each transformation, except acetate **11e** which was hydrolyzed to alcohol **11g** by contaminated hydrolase. In this case, we used the purified enzyme to ensure an accurate.

Oxalacetate analogs

Initially, C_3-precursors other than the oxalacetate were explored as alternative substrates. Various analogs were tested as the C_3-precursor such as diacids (succinate, malonate, fumarate, itaconate, malate and malonate), keto acids (pyruvate, 3-bromopyruvate, acetoacetate, α-ketoglutarate and β-ketoglutarate) and amino acids (aspartate, asparagine and glutamate). As previously reported,[12] pyruvate was the only compound converted to **1** in the presence of **3**. Although pyruvate acted as a less efficient substrate[12] (oxalacetate, K_m 1.2 mM; pyruvate, K_m 35.2 mM), this observation strongly indicates that the oxalacetate was converted to an enolpyruvate *via* decarboxylation. Until now, all attempts to detect the enzymatic conversion from an oxalacetate to a pyruvate in the absence of 2-pyrone **3** have failed.

Simple pyrones and related analogs

When structurally simple pyrones **7a** and **7b** were used as substrates, none of them was transformed. In addition, neither of the derivatives closely related

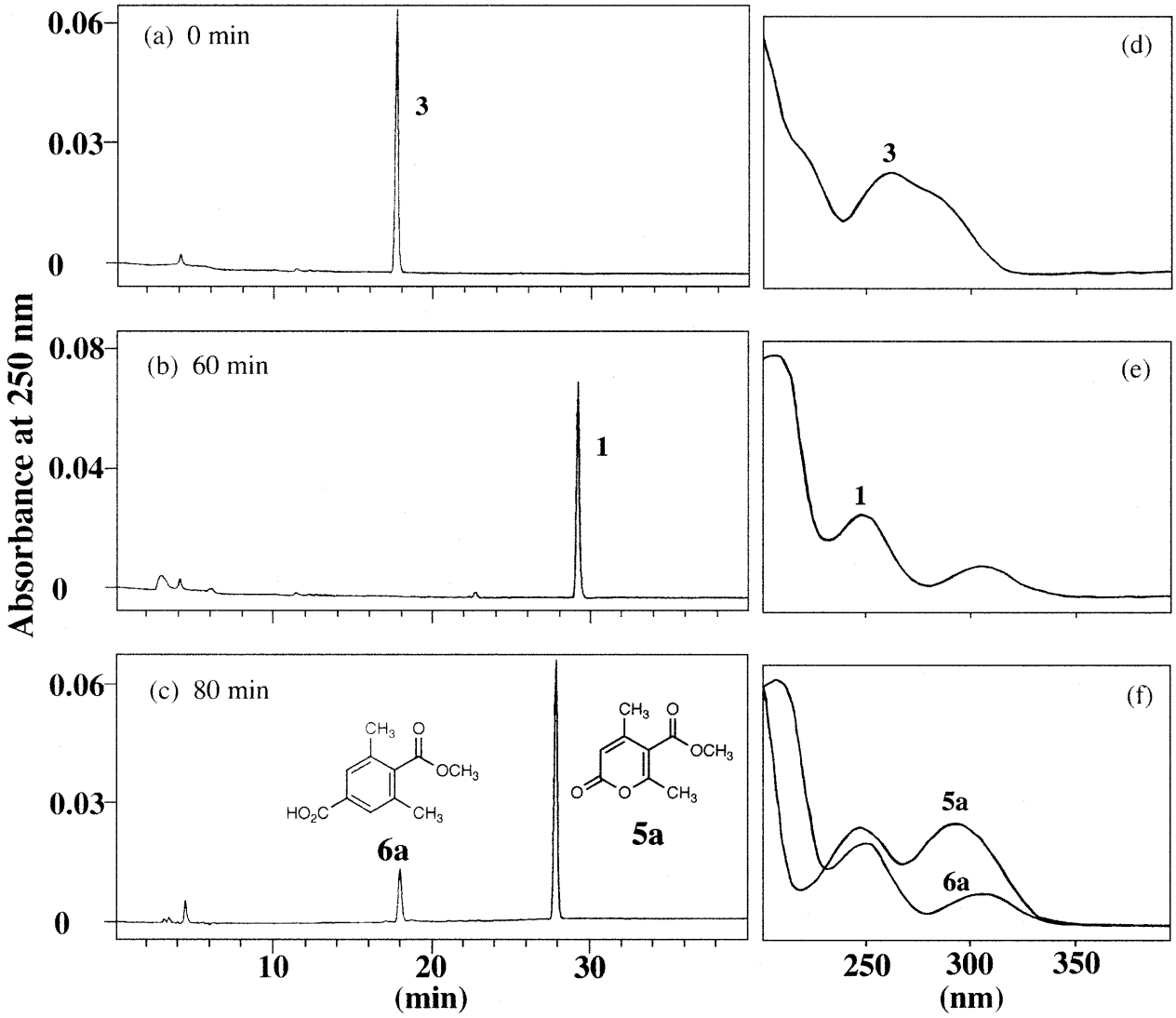


Fig. 1. HPLC Profiles and UV Spectra for **1**, **3**, **5a** and **6a**.

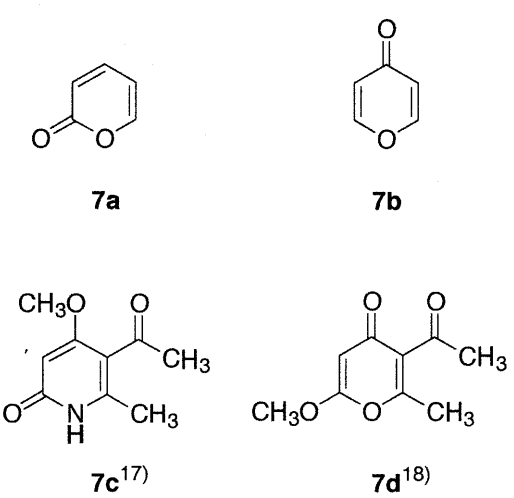


Fig. 2. Pyrone Analogs Tested in the Enzymatic Reactions.

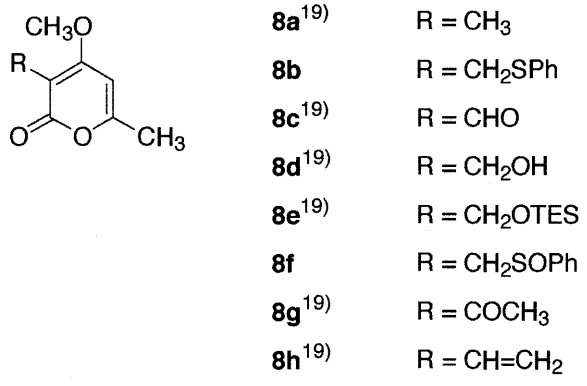
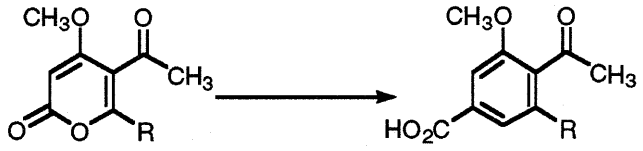


Fig. 3. Pyrone Analogs Bearing Various Substituents at C-3.

to 2-pyrone **3**, 2-pyridone **7c** and 4-pyrone **7d**, was consumed (Fig. 2). These results indicate that a simple 2-pyrone skeleton itself was not a sufficient structural requirement for the substrate, although it

Table 1. Conversion of Pyrone Analogs Bearing Various Substituents at C-6



				Conversion (%)
3[20]	R = CH_3	**1**	R = CH_3	100
9a[20]	R = H	**10a**	R = H	100
9b	R = Ph	**10b**	R = Ph	24

was essential for recognition of the enzyme.

Pyrone analogs bearing various substituents at C-3 or C-6

We initially expected that an electron-withdrawing group at C-3 would equally affect the reactivity of the 2-pyrone such as the pyrone bearing one at C-5. To investigate this, 2-pyrones **8a–8h** possessing various substituents at C-3 were synthesized and tested (Fig. 3). Regardless of the size of the substituents, nature of the attached groups (electron-withdrawing or electron-donating), none of them was transformed into a corresponding benzoate. This indicates that the active site of the corresponding position in macrophomate synthase was fairly restricted.

On the other hand, pyrones with different substituents at C-6 were transformed into the corresponding benzoates at different rates (Table 1). 6-Normethylpyrone **9a** was a better substrate (100%) than the natural one, and 6-phenylpyrone **9b** showed poor conversion (24%). In these cases, steric factors seemed to have a predominant effect, although electronic factors might have contributed in the reaction of **9b**.

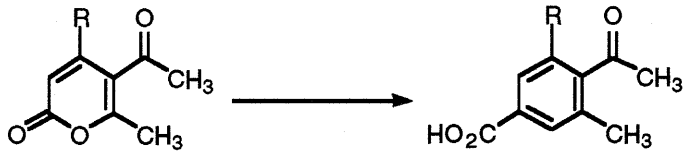
Pyrone analogs bearing various substituents at C-4 or C-5

The effects of 4 and 5-substituents on the 2-pyrones are next examined. Macrophomate synthase accepted a variety of 4-substituted analogs as shown in Table 2. The ethoxy, benzyloxy, chloro and methyl analogs (**11a**, **11b** and **11c**) were converted to benzoates **12a**, **12b** and **12c** at moderate rates (15–45%). To investigate the size limit of the C-4 substituent, pentyloxy and *tert*-butyldiphenylsiloxy derivatives **11d** and **11f** were tested. Pyrones with the larger substituents than the benzyloxy group were not substrates. No conversion was apparent in the reactions with 2-pyrones **11e** and **11g** containing an acetoxy or polar hydroxy group. It is noteworthy that simple pyrones **11h** and **11i** without the 4-substituent were rapidly consumed (within 60 and 10 min, respectively) but none of the products could be detected in an HPLC analysis under standard conditions. These results clearly indicate that the macrophomate synthase exhibited relaxed substrate specificity on the 4-substitution.

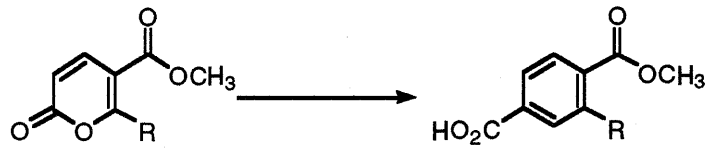
Remarkable tolerance of the macrophomate synthase was also observed in the reaction of 2-pyrones bearing various groups at C-5 as shown in Table 3. Crotonyl, 3-hydroxybutyroyl, methoxycarbonyl and ethoxycarbonylpyrones **4–13c** were all converted to corresponding benzoates **2–14c** at reasonable rates (27–39%), while bromo analog **13d** exhibited only poor conversion (5%). In the HPLC-UV analysis, the reaction products of 5-formylpyrone **13e** gave multiple peaks which displayed marked differences in their UV pattern from those of the other benzoate products. Use of the purified enzyme gave essentially the same result. The reason for this is obscure, although **13e** is regarded as a reactive substrate due to the presence of a carbonyl group at C-5. Pyrones **13f**, **13g**, **13h**, **13i**, **13j** and **13k** with polar groups at C-5, except natural product **13i**, did not act as substrates, but corresponding esters **13b** and **13c** did (similar to the relationship between 4-hydroxy **11g** and 4-methoxy **3**). We therefore synthesized methoxymethyl and methoxymethoxymethyl derivatives **13g** and **13h** to evaluate the effect of a hydroxy group on the C-5 side chain. These are not recognized as substrates for the macrophomate synthase. To assess the size limit of the 5-substituents, three pyrones **5a**, **5b**, **5c** with larger groups were synthesized (Table 3). Substituents larger than allyloxycarbonyl were not allowed as substrates in the enzymatic reaction. In addition to the data already shown, the inability to convert 2-pyrone **13j** bearing no 5-substituent indicates the electron-withdrawing group at C-5 to be essential for the substrate. These data clearly show the limitation of size for the 5-substituents.

Side reactions were serious problems in the case of the whole cell experiments, since hydrolysis, reduction, methylation and hydrogenation occurred.[5] In addition, relatively higher recovery of the polar substrate indicates that these compounds assumed to

Table 2. Conversion of Pyrone Analogs Bearing Various Substituents at C-4



				Conversion (%)
11a[17]	R = OEt	**12a**	R = OEt	45
11b[5]	R = OBn	**12b**	R = OBn	42
11c[5, 21]	R = Cl	**12c**	R = Cl	15
11d	$R = OC_5H_{11}$	**12d**	$R = OC_5H_{11}$	0
11e[5, 17]	R = OAc	**12e**	R = OAc	0
11f	R = OTBDPS	**12f**	R = OTBDPS	0
11g[17]	R = OH	**12g**	R = OH	0

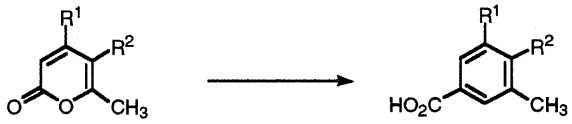


11h[22]	$R = CH_3$	**12h**	$R = CH_3$	0*
11i	R = H	**12i**	R = H	0**

* The substrate was consumed within 60 min.

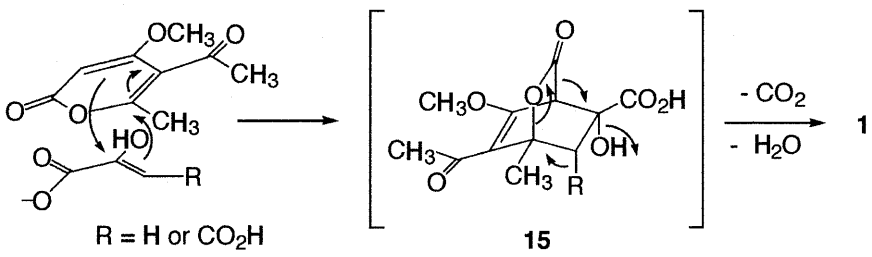
** The substrate was consumed within 10 min.

Table 3. Conversion of Pyrone Analogs Bearing Various Substituents at C-5



			Conversion (%)
4	$R^1 = OCH_3$ $R^2 = COCH{=}CHCH_3$	**2** $R^1 = OCH_3$ $R^2 = COCH{=}CHCH_3$	39
13a	$R^1 = OCH_3$ $R^2 = COCH_2CH(OH)CH_3$	**14a** $R^1 = OCH_3$ $R^2 = COCH_2CH(OH)CH_3$	31
13b[5]	$R^1 = OCH_3$ $R^2 = CO_2CH_3$	**14b** $R^1 = OCH_3$ $R^2 = CO_2CH_3$	31
13c[5]	$R^1 = OCH_3$ $R^2 = CO_2C_2H_5$	**14c** $R^1 = OCH_3$ $R^2 = CO_2C_2H_5$	27
13d[23,24]	$R^1 = OCH_3$ $R^2 = Br$	**14d** $R^1 = OCH_3$ $R^2 = Br$	5
5a	$R^1 = CH_3$ $R^2 = CO_2CH_3$	**6a** $R^1 = CH_3$ $R^2 = CO_2CH_3$	26
13e[5] $R^1 = OCH_3$ $R^2 = CHO$, **13f**[5] $R^1 = OCH_3$ $R^2 = CH_2OH$, **13g** $R^1 = OCH_3$ $R^2 = CH_2OCH_3$, **13h** $R^1 = OCH_3$ $R^2 = CH_2OCH_2OCH_3$, **13i** $R^1 = OCH_3$ $R^2 = CH(OH)CH_3$, **13j** $R^1 = OCH_3$ $R^2 = H$, **13k**[5] $R^1 = OCH_3$ $R^2 = CO_2H$ **5b** $R^1 = CH_3$ $R^2 = CO_2Allyl$, **5c** $R^1 = CH_3$ $R^2 = CO_2Bn$, **5d** $R^1 = CH_3$ $R^2 = CONHC_6H_{13}$, **5e** $R^1 = CH_3$ $R^2 = CO_2H$		0

show low cell permeability than the less polar ones.[5] The use of the partially purified enzyme of macrophomate synthase clarified the ambiguity about transformation of the tested compounds caused by such other enzymes as esterase, reductase, methyl transferase and oxidase. Esters, ketones and alde-



Scheme 2. Proposed Reaction Mechanism for Macrophomate Synthase.

hyde were not hydrolyzed or reduced in the partially purified enzyme. For example, pyrenocine A (**4**) was converted only to pyrenochaetic acid A (**2**) in the enzymatic reaction, but **4** afforded pyrenochaetic acids B (**14a**) and C[7] along with **2** in the whole-cell experiments.[5] Problems with the cell permeability of polar compounds **13f**, **13g**, **13h**, **13i**, **13j**, **13k** were also eliminated.

Based on the result for the oxalacetate analogs, we speculate that the first reaction of this unusual enzymatic transformation was decarboxylation, in which the oxalacetate produced an enol form of pyruvate. One might rationally assume that a Michael reaction of the enolate with 2-pyrone **3** would successively proceed to C–C bond formation first and then to C–C bond formation between C-5 and the carbonyl group which has been formed from the enolate (Scheme 2). Resulting bicyclic intermediate **15** would then undergo spontaneous decomposition *via* dehydration and decarboxylation to yield corresponding benzoate **1**. This assumption explains the reactivity of 2-pyrones **3**, **9a** and **9b** concerning 6-substitution.

In summary, our study on the substrate diversity of the macrophomate synthase has revealed the structural requirements of the substrates and the rough size of the enzyme active site, and has eliminated the ambiguity caused by contamination by the other enzymes. We are currently working on cloning the gene coding the macrophomate synthase and its expression. Tolerance could be modified and this may enable us to prepare various benzoate derivatives.

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