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-pyrene to 4-acetyl-3-methoxy-5-methyl-benzoic acid (macrophomic acid). To investigate the substrate diversity of the enzyme, 40 analogs of 2-pyrene 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-pyrene; oxalacetate

Only a few enzymes catalyzing more than four different conversions such as dehydroquininate synthase1,2) and 2-deoxy-scyllato-inosose synthase3) 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 stereoregulated manner.

Yamamoto’s group4,5) 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 A8,9), and a C3-acyl precursor by the fungus, Macrophoma commelinae, as shown in Scheme 1. We have recently determined the C3-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-pyrene 3 to afford benzoate 1 via the formation of two C-C bonds concomitant with two decarboxylation and dehydration reactions.

The addition of the C3-acyl to polyketides,13) terpenoids14,15) and fatty acid derivatives16) 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-216) of the C3-acyl and formation at C-3,13,15) In some cases, an oxalacetate has been proposed to be the C3-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.19) To test the ability of macrophomate synthase to accept various pyrones as

\[
\begin{align*}
3 & \text{ R = CH}_3 \\
4 & \text{ R = CH} = \text{CHCH}_3
\end{align*}
\]

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 macrophamate synthase which catalyzes the multistep conversion.

Materials and Methods

General procedures. IR spectra were measured with a System 2000 FT-IR, 1H- and 13C-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 F254. Anhydrous reactions were carried out under an argon atmosphere.

Enzyme. The partially purified enzyme(2) of macrophamate 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(2) 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 MgCl2.

Substrates. Pyrenocines A (4) and B (13a), and pyrenocarboxylic acids A (2) and B (14a) were kindly presented by Prof. H. Sato. Compounds 7c, 7d, 8a, 8c, 8d, 8e, 8g, 8h, 9a, 9b, 11a, 11b, 11c, 11e, 11g, 11h, 13b, 13c, 13d, 13e, 13f and 13k were synthesized according to the literature, and compounds 5a, 7a, 7b and 13j were purchased from Aldrich.

Authentic reagents. All authentic samples, except for 2, 10a and 14a, were prepared by biotransformation, using whole cells of Macrophama commelinae as described previously.5

5-Carboxy-4,6-dimethyl-2H-pyrane-2-one (5e). A solution of 5a (500 mg, 2.7 mmol) in H2SO4 (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 Na2SO4. The mixture was evaporated in vacuo, and the black oily residue was purified by flash chromatography (CHCl3/Methanol/H2O, 95/5) to give 5e(20) (417 mg, 92%) as colorless needles, mp 152-155°C (lit. mp 155°C).

5-Allyloxybenzoxycarbonyl-4,6-dimethyl-2H-pyrane-2-one (5b). To a mixture of 5e (40 mg, 0.24 mmol) and CH2Cl2 (1 ml) was added a suspension of DCC (50 mg, 0.24 mmol) and DMAP (3 mg, 0.025 mmol) in CH2Cl2 (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 (CHCl3) to give 5b (6.6 mg, 14%) as a yellow oil. IR νmax (NaCl) cm⁻¹: 3627, 3085, 2933, 1634, 1557, 1378, 1083, 860, 778, 1H-NMR (270 MHz, CDCl3) δ: 6.02 (1H, s, Ar-H), 5.98-5.85 (1H, m, -CH2-CH2=CH2), 5.33 (2H, dd, J = 8.9, 1.3 Hz, -CH=CH2), 4.77 (2H, d, J = 6.6 Hz, -O-CH2CH=CH2), 2.39 (3H, s), 2.22 (3H, s). MS (EI) m/z: 208 (M⁺). HRMS (EI) m/z (M⁺): calcd. for C11H12O4, 208.0735; found, 208.0771.

5-Benzoxycarbonyl-4,6-dimethyl-2H-pyrane-2-one (5e). Compound 5e was synthesized as described previously, except for using benzylalcohol, as an amorphous solid, mp 109-112°C. IR νmax (KBr) cm⁻¹: 2936, 1723, 1547, 2370, 1247, 1082, 888, 756, 636, 565. 1H-NMR (270 MHz, CDCl3) δ: 7.32-7.27 (5H, m, Ar-H), 5.95 (1H, s, Ar-H), 4.61 (2H, -O-CH2-CH2-), 2.41 (3H, s), 2.21 (3H, s). MS (EI) m/z: 258 (M⁺). HRMS (EI) m/z (M⁺): calcd. for C12H14O3, 258.0892; found, 258.0927.

4,6-Dimethyl-5-[(N-hexyl)aminocarbonyl]-2H-pyrane-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 Et3N (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. NH4Cl and brine. The organic layer was dried over anhydrous Na2SO4 and evaporated in vacuo to afford a residue which was purified by PTLC (CHCl3/Methanol, 9/1) to give 5d (2.3 mg, 17%) as a yellow oil. IR νmax (NaCl) cm⁻¹: 2930, 2859, 1728, 1645, 1601, 1413, 1315, 1036, 814, 587. 1H-NMR (270 MHz, CDCl3) δ: 12.1 (br. s, 1H, -CO-NH-CH2-), 5.52 (1H, s, Ar-H), 2.43 (3H, s), 2.23 (3H, s), 1.86-0.96 (10H, m, -NHNH2-CH2-CH2-), 0.91 (3H, t, J = 4.0 Hz, -CH2-CH3). MS (EI) m/z: 251 (M⁺). HRMS (EI) m/z (M⁺): calcd. for C18H24N4O2, 251.1522; found, 251.1516.
(CHCl₃/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. ¹H-NMR (270 MHz, CDCl₃): δ: 7.51–7.15 (5H, m, Ar-H), 3.99 (1H, s, Ar-H), 3.97 (2H, s, -S-C₆H₅-Ar), 3.73 (3H, s, Ar–OC₂H₅), 2.29 (3H, s, Ar–CH₃). MS (EI) m/z: 262 (M⁺). HRMS (EI) m/z (M⁺): calcld. for C₁₆H₁₄O₃S, 262.0663; found, 262.0642.

4-Methoxy-6-methyl-3-[(phenylsulfonyl)oxy]methyl]-2H-pyran-2-one (8). To a solution of 8b (109 mg, 0.42 mmol) in CH₂Cl₂ (14 ml) was added a solution of mCPBA (80%, 98.7 mg, 0.46 mmol). After stirring at -40°C for 1 h, the mixture was successively treated with sat. NaHSO₃, sat. NaHCO₃ and brine, dried over anhydrous Na₂SO₄, filtered and evaporated in vacuo. Purification by PTLC (CHCl₃/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. ¹H-NMR (270 MHz, CDCl₃): 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–OC₂H₅), 2.29 (3H, s, Ar–CH₃). MS (FD) m/z: 278 (M⁺). HRMS (FD) m/z (M⁺): calcld. for C₁₆H₁₄O₃S, 278.0613; found, 278.0583.

5-Acetyl-6-methyl-4-pentoxyl-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% NaHCO₃ and brine. The organic layer was dried over anhydrous Na₂SO₄ and evaporated in vacuo to afford a residue which was purified by PTLC (CHCl₃/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. ¹H-NMR (270 MHz, CDCl₃): δ: 5.41 (1H, s, Ar-H), 3.97 (2H, t, J = 6.6 Hz, -CH₂-C₆H₄-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, -CH₃-CH₂-). MS (EI) m/z: 238 (M⁺). HRMS (EI) m/z (M⁺): calcld. for C₁₃H₁₂O₃S, 238.1205; found, 238.1195.

5-Acetyl-4-[(tert-butyl)diphenylsiloxy]-6-methyl-2H-pyran-2-one (11f). To a mixture of imidazole (82 mg, 1.2 mmol) and TBDPSiCl (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 Na₂SO₄ and evaporated in vacuo. The residue was purified by PTLC (CHCl₃/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. ¹H-NMR (270 MHz, CDCl₃): 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, -S-C₆H₅). MS (EI) m/z: 406 (M⁺). HRMS (EI) m/z (M⁺): calcld. for C₁₈H₁₃O₃Si, 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 (43.5 mg, 80%) was synthesized by using 4-hydroxy-5-hydroxymethyl-6-methyl-2H-pyran-2-one (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 CH₂Cl₂ was added Dess-Martin periodinane (51 mg, 0.12 mmol). After stirring at room temperature for 2 h, the mixture was quenched with 5% NaHCO₃ and then extracted with AcOEt. The organic extract was washed with brine, dried over anhydrous Na₂SO₄ and evaporated in vacuo. Purification by PTLC (CHCl₃/MeOH, 95/5) gave 13e (17 mg, 84%) as colorless needles, mp 132–135°C (lit. mp 130–133°C).

4-Methoxy-5-[(methoxymethyl)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 Na₂SO₄, and evaporated in vacuo. Purification by PTLC (CHCl₃/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. ¹H-NMR (270 MHz, CDCl₃): δ: 5.47 (1H, s, Ar-H), 4.12 (2H, s, Ar–CH₂–OCH₃), 3.84 (3H, s, Ar–OC₂H₅), 3.37 (3H, s, -CH₃–OCH₃), 2.32 (3H, s, Ar–CH₃). MS (EI) m/z: 184 (M⁺). HRMS (EI) m/z (M⁺): calcld. for C₁₉H₁₈O₄S, 184.0736; found, 184.0729.

4-Methoxy-5-[[methoxymethoxymethyl]-6-methyl-2H-pyran-2-one (13h). To a solution of alcohol 13f (2.0 mg, 0.01 mmol) in CH₂Cl₂ (0.5 ml) were added MOMCl (0.04 ml, 0.5 mmol) and i-Pr₂NEt (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. NaHCO₃, and brine, dried over anhydrous Na₂SO₄, filtered and evaporated in vacuo. Purification by PTLC (CHCl₃/MeOH, 92:8) gave 13b (1.7 mg, 79%) as white crystals, mp 81–83°C. IR νmax (KBr) cm⁻¹: 2956, 2897, 1708, 1558, 1460, 1389, 1260, 1092, 1030, 910, 840. ¹H-NMR (270 MHz, CDCl₃) δ: 5.46 (1H, s, Ar-H), 4.64 (2H, s), 4.38 (2H, s), 3.85 (3H, s, Ar-OC₃H₃), 3.39 (3H, s, -CH₂-OC₃H₃), 2.33 (3H, s, Ar-C₂H₅). MS (EI) m/z: 214 (M⁺). HRMS (EI) m/z (M⁺): calcd. for C₁₀H₁₄O₄S, 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 CeCl₃·7H₂O (700 mg, 1.88 mmol) in MeOH (30 ml) was added NaBH₄ (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 Na₂SO₄ and evaporated in vacuo to afford a residue which was purified by flash chromatography (CHCl₃/acetone, 8/2) to give 13i (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 macrophage 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 oxalacetaldehyde, 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. 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 (6.4×250 mm). The mobile phase was solution A [10% CH₃CN in a 10 mM phosphate buffer (pH 2.6)] and solution B [40% CH₃CN 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. ⁴

4-Acetyl-3-methoxybenzoic acid (10a). To identify product 10a in the enzymatic reaction of 2-pyrene 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 Na₂SO₄ and evaporated in vacuo. The residue was purified by PTLC (CHCl₃/MeOH, 91/1) to give benzoate 10a (0.9 mg, 90%) as a yellow oil. IR νmax (NaCl) cm⁻¹: 3751, 2861, 2842, 1019, 669, 466. ¹H-NMR (270 MHz, CDCl₃) 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-OC₃H₃), 2.63 (3H, s, -CO-CH₃). MS (EI) m/z: 194 (M⁺). HRMS (EI) m/z (M⁺): calcd. for C₁₆H₁₀O₄, 194.0579; found, 194.0569.

Results and Discussion

Most of the enzyme reactions of the 2-pyrones with the partially purified enzyme of macrophage 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 isodehydroacetate 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 hydroxide. In this case, we used the purified enzyme to ensure an accurate.

Oxalacetate analogs

Initially, C₇-precurors other than the oxalacetate were explored as alternative substrates. Various analogs were tested as the C₇-precuror such as diaacids (succinate, malonate, fumarate, itaconate, malate and malonate), keto acids (pyruvate, 3-bromopyruvate, acetooacetate, α-ketoglutarate and β-ketoglutarate) and amino acids (aspartate, asparagine and glutamate). As previously reported, pyruvate was the only compound converted to 1 in the presence of 3. Although pyruvate acted as a less efficient substrate (oxalacetate, Kₐ 1.2 mm; pyruvate, Kₐ 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-pyrene 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
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-pyrones 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, benzoxyl, 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, pentoxylo and tert-butyldiphenylsiloxy derivatives 11d and 11f were tested. Pyrones with the larger substituents than the benzoxyl group were not substrates. No conversion was apparent in the reactions with 2-pyrones 11e and 11g containing an acetoxyl 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 4–13e were all converted to corresponding benzoates 2–14e 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 13l, did not act as substrates, but corresponding esters 13b and 13e 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 allyloxy carbonyl were not allowed as substrates in the enzymatic reaction. In addition to the data already shown, the inability to convert 2-pyrene 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.9 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

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<th>Conversion (%)</th>
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<tr>
<td>11a&lt;sup&gt;17&lt;/sup&gt;</td>
<td>R = OEt</td>
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<tr>
<td>11b&lt;sup&gt;21&lt;/sup&gt;</td>
<td>R = OBn</td>
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<tr>
<td>11c&lt;sup&gt;5, 21&lt;/sup&gt;</td>
<td>R = Cl</td>
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<tr>
<td>11d</td>
<td>R = OC&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;11&lt;/sub&gt;</td>
</tr>
<tr>
<td>11e&lt;sup&gt;5, 17&lt;/sup&gt;</td>
<td>R = OAc</td>
</tr>
<tr>
<td>11f</td>
<td>R = OTBDPS</td>
</tr>
<tr>
<td>11g&lt;sup&gt;17&lt;/sup&gt;</td>
<td>R = OH</td>
</tr>
<tr>
<td>11h&lt;sup&gt;22&lt;/sup&gt;</td>
<td>R = CH&lt;sub&gt;3&lt;/sub&gt;</td>
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<tr>
<td>11i</td>
<td>R = H</td>
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* 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

<table>
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<th>Conversion (%)</th>
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<td>4</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; = OCH&lt;sub&gt;3&lt;/sub&gt;, R&lt;sup&gt;2&lt;/sup&gt; = COCH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>13a</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; = OCH&lt;sub&gt;3&lt;/sub&gt;, R&lt;sup&gt;2&lt;/sup&gt; = COCH&lt;sub&gt;2&lt;/sub&gt;CH(OH)CH&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>13b&lt;sup&gt;6&lt;/sup&gt;</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; = OCH&lt;sub&gt;3&lt;/sub&gt;, R&lt;sup&gt;2&lt;/sup&gt; = CO&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
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<td>13c&lt;sup&gt;6&lt;/sup&gt;</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; = OCH&lt;sub&gt;3&lt;/sub&gt;, R&lt;sup&gt;2&lt;/sup&gt; = CO&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
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<td>13d&lt;sup&gt;23, 24&lt;/sup&gt;</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; = OCH&lt;sub&gt;3&lt;/sub&gt;, R&lt;sup&gt;2&lt;/sup&gt; = Br</td>
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<tr>
<td>5a</td>
<td>R&lt;sup&gt;1&lt;/sup&gt; = CH&lt;sub&gt;3&lt;/sub&gt;, R&lt;sup&gt;2&lt;/sup&gt; = CO&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
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13<sup>e, l</sup> | R<sup>1</sup> = OCH<sub>3</sub>, R<sup>2</sup> = CHO. 13<sup>f, l</sup> | R<sup>1</sup> = OCH<sub>3</sub>, R<sup>2</sup> = CH<sub>2</sub>OH. 13<sup>g, l</sup> | R<sup>1</sup> = OCH<sub>3</sub>, R<sup>2</sup> = CO<sub>2</sub>H.<sup>l</sup> | 13<sup>h, l</sup> | R<sup>1</sup> = OCH<sub>3</sub>, R<sup>2</sup> = CH<sub>2</sub>OCH<sub>2</sub>OCH<sub>3</sub> | 13l | R<sup>1</sup> = OCH<sub>3</sub>, R<sup>2</sup> = CH<sub>2</sub>OH<sub>2</sub>CH. 13j | R<sup>1</sup> = OCH<sub>3</sub>, R<sup>2</sup> = H. 13k<sup>6</sup> | R<sup>1</sup> = OCH<sub>3</sub>, R<sup>2</sup> = CO<sub>2</sub>H. 5b | R<sup>1</sup> = CH<sub>3</sub>, R<sup>2</sup> = CO<sub>2</sub>Alkyl. 5c | R<sup>1</sup> = CH<sub>3</sub>, R<sup>2</sup> = CO<sub>2</sub>Bn. 5d | R<sup>1</sup> = CH<sub>3</sub>, R<sup>2</sup> = CONHC<sub>2</sub>H<sub>4</sub>. 5e | R<sup>1</sup> = CH<sub>3</sub>, R<sup>2</sup> = CO<sub>2</sub>H. |

show low cell permeability than the less polar ones. The use of the partially purified enzyme of macrophate 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-
Substrate Diversity of Macrophomate Synthase

hyde were not hydrolyzed or reduced in the partially purified enzyme. For example, pyrenocine A (4) was converted only to pyrenocaetic acid A (2) in the enzymatic reaction, but 4 afforded pyrenoecaetic acids B (14a) and C along with 2 in the whole-cell experiments. 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-pyrene 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 dehydorination 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.

Acknowledgments

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