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# Studies on Metabolites of *Macrophoma commelinae*. IV. Substrate Specificity in the Biotransformation of 2-Pyrones to Substituted Benzoic Acids[1)]

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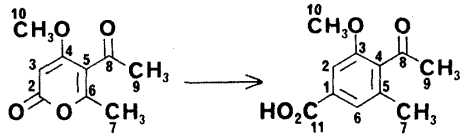
Substrate specificity in the novel biotransformation from 2-pyrone derivatives to substituted benzoic acids by *Macrophoma commelinae* (IFO 9570) was investigated by means of feeding experiments with various compounds. Among them, 2-pyrone derivatives substituted by an electron-donating group at C-4, by an electron-withdrawing group at C-5 and by an alkyl group at C-6 were converted to the corresponding benzoic acid derivatives in fairly good yields. The $C_3$-unit precursors and intermediates were examined in stationary or shaking culture. Based on the experimental results obtained, a mechanism for this unique reaction is proposed.

**Keywords**——*Macrophoma commelinae* IFO 9570; fungi; 2-pyrone; substituted benzoic acid; biotransformation; substrate specificity; aromatic ring formation; macrophomic acid

In a previous paper,[1a)] we reported that *Macrophoma commelinae* (IFO 9570) was able to transform 5-acetyl-4-methoxy-6-methyl-2-pyrone (**1a**) to 4-acetyl-3-methoxy-5-methylbenzoic acid (**2a**, macrophomic acid). In feeding experiments using various compounds labeled with a radioactive or stable isotope, we obtained the following results. 1) Each carbon atom from C-3 to C-10 of pyrone **1a** is retained at the corresponding position of the benzoic acid product (**2a**), but a carbonyl carbon at C-2 is eliminated as $CO_2$. 2) The C-1, C-6 and C-11 carbons of **2a** are derived from another carbon source, probably pyruvate or its equivalent.



**1a** **2a**

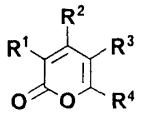
Chart 1

In this paper, we wish to describe the biotransformation of a variety of **1a** analogues by this fungus and also to discuss the mechanism of this unique reaction based on the experimental results.

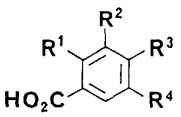
2-Pyrone analogues were administered to the resting cells of *M. commelinae* as previously described.[1a)] After further stationary standing for 10 d, the culture filtrate was extracted with ethyl acetate (AcOEt), and the AcOEt extract was separated by silica gel preparative thin-layer chromatography (PTLC). The isolated benzoic acid derivatives were identified on the basis of the spectral data and elemental analyses. The yields are summarized in Table I.

2-Pyrone derivatives (**1a**—**1l**) were converted in fairly good yields. Among them, pyrenocine A (**1l**),[10)] a metabolite of *Pyrenochaeta terrestris* (IFO 30173), was converted to the benzoic acid derivatives (pyrenochaetic acids A, B and C)[11)] together with a water adduct compound (pyrenocine B (**1k**)). Pyrenocine B was also converted to pyrenochaetic acids (A, B

TABLE I. Transformation of 2-Pyrone Derivatives **1** to Benzoic Acid Derivatives **2** by *M. commelinae*



**1**



**2**

| Compd. **1** | $R^1$ | $R^2$ | $R^3$ | $R^4$ | Yield of isolated **2** (%) | Recovery of **1** (%) |
|---|---|---|---|---|---|---|
| **a**[1] | H | $OCH_3$ | $COCH_3$ | $CH_3$ | 73 | 8 |
| **b** | H | $OCH_3$ | $CO_2CH_3$ | $CH_3$ | 59 | 33 |
| **c** | H | $OC_2H_5$ | $COCH_3$ | $CH_3$ | 56 | 32 |
| **d** | H | $OCH_2C_6H_5$ | $COCH_3$ | $CH_3$ | 56 | 13 |
| **e** | H | $OCH_3$ | $CO_2C_2H_5$ | $CH_3$ | 56 | 36 |
| **f** | H | $OCH_3$ | $C\equiv N$ | $CH_3$ | 50 | 25 |
| **g** | H | $CH_3$ | $CO_2CH_3$ | $CH_3$ | 47 | 25 |
| **h**[2] | H | $CH_3$ | $CO_2C_2H_5$ | $CH_3$ | 45 | 14 |
| **i** | H | $OCH_3$ | $COC_6H_5$ | $CH_3$ | 38 | 28 |
| **j** | H | Cl | $COCH_3$ | $CH_3$ | 24 | 1 |
| **k** | H | $OCH_3$ | $COCH_2CH(OH)CH_3$ | $CH_3$ | 12 | 27 |
| **l** | H | $OCH_3$ | $COCH=CHCH_3$[a] | $CH_3$ | 11 | 9 |
| **m**[2] | H | H | $CO_2CH_3$ | $CH_3$ | 2 | 0 |
| **n** | H | $OCH_3$ | $COCH_3$ | $C_6H_5$ | 2 | 0 |
| **o**[3] | H | $OCH_3$ | Br | $CH_3$ | 1 | 45 |
| **p**[4] | $COCH_3$ | $OCH_3$ | H | $CH_3$ | 0 | 50 |
| **q**[5] | H | OH | $COCH_3$ | $CH_3$ | 0 | 16 |
| **r** | H | $OCOCH_3$ | $COCH_3$ | $CH_3$ | 0 | 0 |
| **s**[6] | H | $OCH_3$ | H | $CH_3$ | 0 | 84 |
| **t** | H | $OCH_3$ | $CH_2OH$ | $CH_3$ | 0 | 52 |
| **u** | H | $OCH_3$ | $CH_2CH_2OH$ | $CH_3$ | 0 | 55 |
| **v** | H | $OCH_3$ | CHO | $CH_3$ | 0 | 0 |
| **w** | H | $OCH_3$ | $CO_2H$ | $CH_3$ | 0 | 55 |
| **x**[7] | H | $CH_3$ | $CO_2H$ | $CH_3$ | 0 | 82 |
| **y** | H | H | $CO_2H$ | H | 0 | 62 |
| **z**[8] | H | $OCH_3$ | $COCH_3$ | H | 0 | 0 |
| **A**[9] | H | $OCH_3$ | $CO_2C(CH_3)=CH$ | | 0 | 17 |
| **B** | H | H | $CH=CHCH=CH$ | | 0 | 35 |
| **C** | H | OH | $CH=CHCH=CH$ | | 0 | 36 |
| **D**[4] | H | $OCH_3$ | $CH=CHCH=CH$ | | 0 | 79 |
| **E** | H | H | $CH=CHC(OH)=CH$ | | 0 | 65 |

*a*) (*E*)-Isomer.

and C) together with pyrenocine A (Chart 2).

The transformations of **1m**, **1n** and **1o** occurred in poor yields, and the other compounds (**1p**—**1z**) were not transformed; in many cases high recoveries of the administered compounds were observed. Side reactions occurred in the following cases. In the esters (**1h** and **1m**), hydrolysis to the corresponding pyrone-5-carboxylic acids was noted (8% and 44%, respectively). In certain carbonyl compounds (**1n** and **1v**), reduction proceeded mainly to give 5-(1-hydroxyethyl)-4-methoxy-6-phenyl-2-pyrone and 5-hydroxymethyl-4-methoxy-6-methyl-2-pyrone (57% or 32%, respectively). Compound **1p**, in which the acetyl group at C-5 in **1a** was moved at C-3, was not converted to a benzoic acid derivative, but the acetyl group was similarly changed to a hydroxyethyl or vinyl group.

Unexpectedly, the 4-hydroxy (**1q**) and 4-acetoxy (**1r**) analogues were not converted to the corresponding benzoic acids, but both afforded the 4-methoxybenzoic acid derivative (**2a**) in

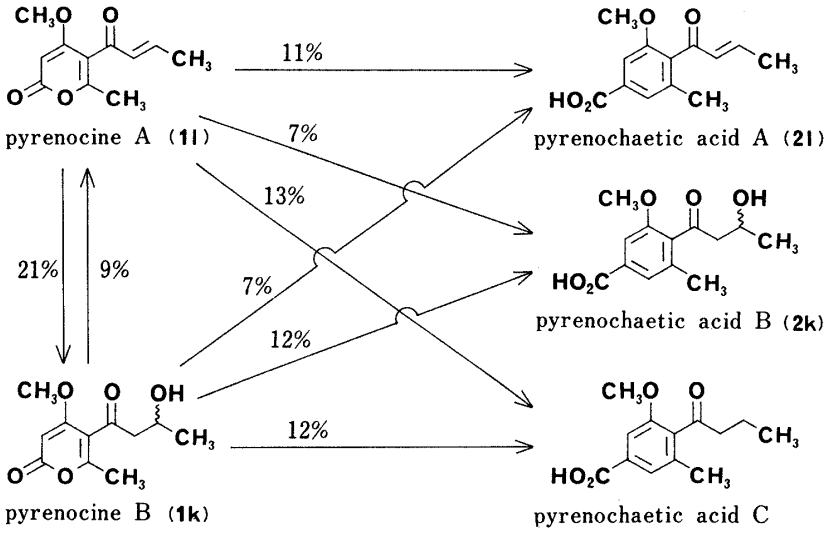



Chart 2

low yields (2% and 4%, respectively). In the case of **1z**, which has no substituent at C-6, hydrogenation to 5-acetyl-5,6-dihydro-4-methoxy-2-pyrone proceeded in poor yield (3%).

As shown in Table I, 2-pyrones having an electron-withdrawing group at C-5, such as ketone (**1a**, **1c**, **1d** and **1i**—**1l**), alkoxycarbonyl (**1b**, **1e**, **1g** and **1h**) and nitrile (**1f**), were converted to corresponding benzoic acids in relatively good yields, but compounds without a substituent (**1p** and **1s**) were not. Further, 2-pyrones having an electron-donating group such as hydroxyalkyl (**1t** and **1u**) at C-5 were not converted. An aldehyde (**1v**) and some carboxylic acids (**1w**—**1y**) were not transformed, unexpectedly. This may be caused by the rapid reduction of the aldehyde to a hydroxymethyl group in the former, and in the latter by the low permeability based on the high polarity of the acid group, since the corresponding esters (**1b**, **1e**, **1g** and **1h**) were converted. Thus, the presence of an electron-withdrawing group at C-5 seemed to be necessary for the progress of this reaction.

Also, the presence of an electron-donating group at C-4 seemed to be necessary to transform the pyrone ring. Namely, 2-pyrones substituted by an alkoxy (**1a**—**1f**, **1i**, **1k** and **1l**), alkyl (**1g** and **1h**) or chloro (**1j**) group at C-4 were converted in fairly good yields, whereas a compound without a substituent (**1m**) was converted in low yield (2%). The failure in the transformation of the 4-hydroxy derivative (**1q**) as described above may be caused by its low permeability or low stability.

Compounds **1y** and **1z** without a substituent at C-6 were not converted. The presence of a methyl group at this position was effective for the transformation, whereas a phenyl group was hardly effective. Coumarin derivatives were not transformed.

The phenyl group at C-6 of **1n** or the benzoyl group at C-5 of **1i** was retained in the corresponding position of the benzoic acid product, so it seems that the condensation of the pyrone ring with a $C_3$ unit proceeds prior to the cleavage of the pyrone ring (*cf.* Chart 3).

In addition to pyruvate, which was reported[1a] as a $C_3$ unit source, radioactive succinate, malate and α-ketoglutarate were found to be incorporated into the benzoic acid (**2a**) at relatively high levels. However, the incorporation ratio of radioactive phosphoenolpyruvate was relatively low and a remarkable amount (77.6%) of the added radioactivity was observed in the culture medium (Table II). This observation may suggest that phosphoenolpyruvate is not able to pass through the cell membrane.

Meanwhile, in shaking culture, it was observed that the transformation activity of the cells reached a maximum in incubation with **1a** for 8 h and the optimal pH value and temperature were 7.3 and 30 °C, respectively. The resting cells pre-incubated with **1a** (see

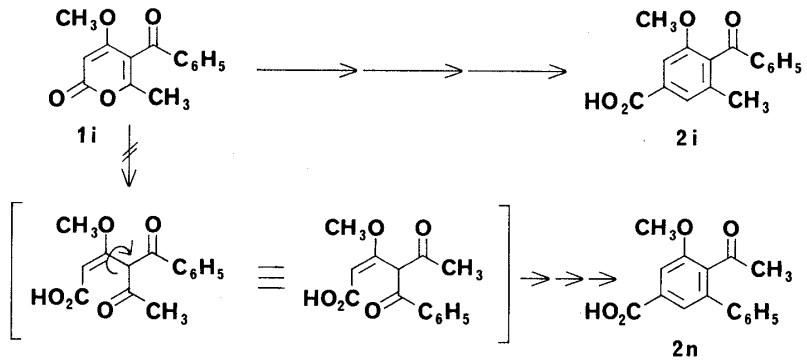


Chart 3

TABLE II. Incorporation Ratio (%) of $^{14}C$-Labeled Precursors into Macrophomic Acid (**2a**) in Stationary Culture

| Precursor | Culture medium (containing **2a**) | Evolved $CO_2$ | **2a** |
|---|---|---|---|
| Sodium[1-$^{14}C$]acetate | 12.5 | 11.7 | 1.2[a] |
| Sodium[1-$^{14}C$]pyruvate | 5.1 | 53.5 | 1.5[a] |
| Sodium[3-$^{14}C$]pyruvate | 22.1 | 14.2 | 8.0 |
| L-[U-$^{14}C$]Malic acid | 9.5 | 25.5 | 5.2 |
| [U-$^{14}C$]Succinic acid | 11.9 | 23.1 | 7.4 |
| [U-$^{14}C$]α-Ketoglutaric acid | 6.9 | 25.4 | 4.2 |
| [1,5-$^{14}C$]Citric acid | 6.7 | 24.2 | 0.8 |
| Cyclohexylammonium phosphoenol-[1-$^{14}C$]pyruvate | 77.6 | 8.5 | 0.5 |

*a*) The radioactivity is specifically distributed in the carboxyl carbon of **2a** (see ref. 1*a*).

Experimental) began to transform the added **1a** to **2a** immediately on shaking. The rate of conversion from **1a** to **2a** was much faster (100% in 4 h) than that in stationary culture, and the yield of the isolated **2a** reached 94%.

To obtain further information on the reaction, we re-examined the conditions of shaking culture with pre-incubated cells. The candidate $C_3$ unit precursors were added together with **1a** to a constant weight of the above pre-incubated cells suspended in a mixture of phosphate buffer and saline and reacted under shaking, and the conversion ratios were checked by high performance liquid chromatography (HPLC) every hour.

The members of the tricarboxylic acid (TCA) cycle, pyruvate and glucose did not show much difference in promoting effect on the rate of reaction, as shown in Fig. 1.

In the cases of phenylpyruvate and acrylate, the transformations did not proceed, though **2a** accumulated. Malonate, which is known as an inhibitor of the TCA cycle, did not inhibit the transformation.

In a shaking experiment with the pre-incubated cells, the sum of added **1a** and product (**2a**) was almost constant throughout the reaction time (0—4 h) (Fig. 2) and no peak suggesting the presence of an intermediate was observed. When a rather large quantity (5 times the usual amount) of **1a** was administered to pre-incubated wet cells in suspension, almost all (93%) of **1a** was consumed in 23 h. From these results, the $C_3$ carbon source must be a material which is abundantly available in fungal cells.

All efforts to obtain a cell-free system were unsuccessful, and we obtained an unexpected result in the feeding experiment with radioactive phosphoenolpyruvate as described above. Nevertheless, phosphoenolpyruvate is considered to be the most likely precursor of the $C_3$

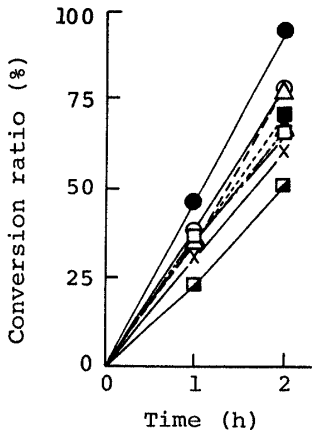



Fig. 1. Effect of Carbon Sources on the Ratio of Transformation

●, glucose; ○, pyruvate; △, succinate; ■, malate; ▲, citrate; □, oxalacetate; ×, control; ◪, acetate.

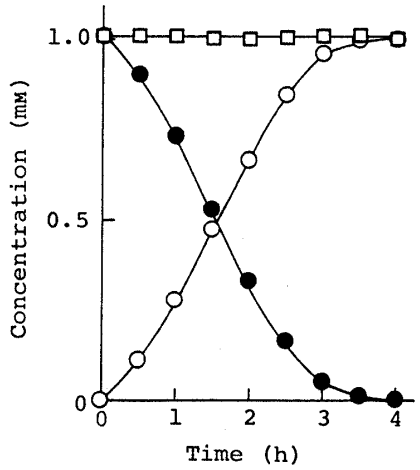



Fig. 2. Time Course of Concentrations of **1a** and **2a** in the Reaction Mixture with Pre-incubated Cells in Shaking Culture

●, **1a**; ○, **2a**; □, sum of **1a** and **2a**.

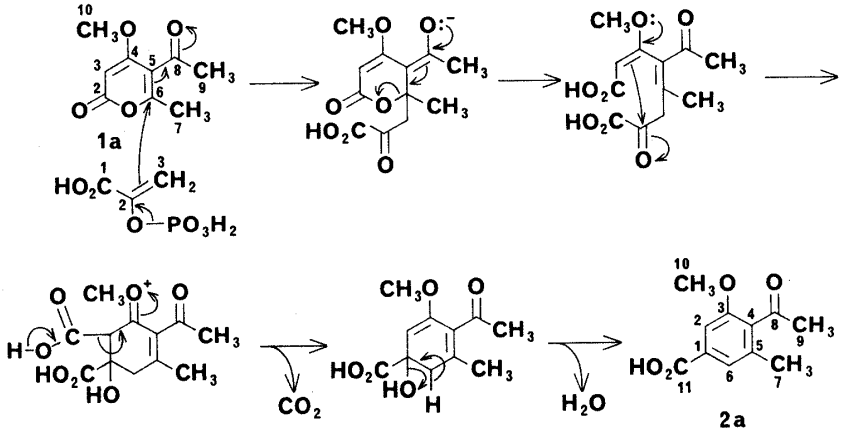



Chart 4

unit. The reaction may proceed by a similar mechanism to shikimate formation, as shown in Chart 4.

Initially, phosphoenolpyruvate attacks C-6 of the pyrone ring activated by an electron-withdrawing substituent at C-5, and then the pyrone ring opens. The C–C bond formation between the C-3 carbon of the pyrone ring and the carbonyl carbon derived from phosphoenolpyruvate was followed. This step seems to be accelerated by an electron-donating substituent at C-4. Finally, decarboxylation of the C-2 carbon of the pyrone and dehydration occur to afford the benzoic acid derivatives (Chart 4).

## Experimental

The general experimental procedures were the same as in Part III.[1b]

**Substrates**——Macommelinol (**1u**) was isolated from culture filtrate of *M. commelinae* (IFO 9570).[8] Pyrenocines A (**1l**) and B (**1k**) were obtained from culture broth of *Pyrenochaeta terrestris* (IFO 30173).[10] New compounds were synthesized as follows and other compounds were synthesized according to the literature cited in Table I. Coumalic acid (**1y**), coumarin (**1B**), 4-hydroxycoumarin (**1C**) and umbelliferone (**1E**) were purchased.

**4-Methoxy-6-methyl-2-oxo-2*H*-pyran-5-carboxylic Acid (1w) and Its Methyl Ester (1b)**——Dipyrone methyl ether (**1A**, 1.62 g)[9] was dissolved in 1 N NaOH (125 ml) and the solution was vigorously shaken at room temperature for 25 min. The solution was acidified with 4 N HCl (50 ml) and kept overnight at 0 °C. The filtrate of the mixture was

extracted with AcOEt (200 ml × 5), and the extract was dried ($Na_2SO_4$) and evaporated *in vacuo*. The residue was separated with PTLC (AcOH : $CHCl_3$ = 1 : 9) into two main bands. Recrystallization of the upper band from acetone–AcOEt yielded **1w** (370 mg) as colorless prisms, mp 183—186 °C. The structure was determined from the following data. MS *m/z*: 184 ($M^+$), 169, 166, 156, 125, 43. UV $\lambda_{max}^{EtOH}$ nm: 287. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3120—2500, 1744, 1712, 1667, 1632, 1554. $^1$H-NMR (DMSO-$d_6$) $\delta$: 2.23 (3H, s), 3.78 (3H, s), 5.56 (1H, s). *Anal.* Calcd for $C_8H_8O_5$: C, 52.18; H, 4.38. Found: C, 52.20; H, 4.36.

**1w** (191 mg) was methylated with ethereal diazomethane ($CH_2N_2$) and the product was recrystallized from cyclohexane to obtain **1b** (84 mg) as colorless needles, mp 114—115 °C. MS *m/z*: 198 ($M^+$), 183, 170, 167, 125, 43. UV $\lambda_{max}^{EtOH}$ nm: 244, 282. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1754, 1710, 1638, 1564. $^1$H-NMR ($CDCl_3$) $\delta$: 2.29 (3H, s), 3.79 (3H, s), 3.82 (3H, s), 5.40 (1H, s). *Anal.* Calcd for $C_9H_{10}O_5$: C, 54.54; H, 5.09. Found: C, 54.32; H, 5.05.

**5-Acetyl-4-ethoxy-6-methyl-2-pyrone (1c)**——The 4-hydroxy compound (**1q**, 1.00 g) synthesized according to the literature[5] was ethylated by the method of Suzuki *et al.*[4] with diethyl sulfate. Recrystallization from cyclohexane yielded **1c** (891 mg) as colorless microcrystals, mp 115—118 °C. MS *m/z*: 196 ($M^+$), 181, 168, 153, 140, 125, 111, 43. UV $\lambda_{max}^{EtOH}$ nm: 219, 257, 282. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1767, 1693, 1622, 1555. $^1$H-NMR ($CDCl_3$) $\delta$: 1.43 (3H, t, $J$ = 6.9 Hz), 2.25 (3H, s), 2.40 (3H, s), 4.05 (2H, q, $J$ = 6.9 Hz), 5.37 (1H, s). *Anal.* Calcd for $C_{10}H_{12}O_4$: C, 61.21; H, 6.17. Found: C, 61.21; H, 6.23.

**5-Acetyl-4-benzyloxy-6-methyl-2-pyrone (1d)**——A mixture of NaH (*ca.* 50% oil suspension; 204 mg) and hexamethylphosphoric triamide (HMPT, 3 ml) was added to a solution of **1q** (589 mg) in HMPT (6 ml) at 0 °C and the whole was stirred at room temperature for 15 min in an atmosphere of nitrogen. Benzyl bromide (0.5 ml) was added and stirring was continued for 3.5 h at room temperature. The reaction mixture was diluted with AcOEt (100 ml) and successively washed with 10% HCl (30 ml × 2), 10% $NaHCO_3$ (30 ml × 2) and 10% brine (30 ml × 2). The organic layer was dried ($Na_2SO_4$) and evaporated to dryness *in vacuo*. The residue was separated with PTLC (MeOH : $CHCl_3$ = 1 : 80) into two main bands. Recrystallization of the lower band from cyclohexane yielded **1d** (199 mg) as colorless needles, mp 104.5—106 °C. MS *m/z*: 258 ($M^+$), 91. UV $\lambda_{max}^{EtOH}$ nm: 258, 282. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1738, 1680, 1616, 1553, 1500. $^1$H-NMR ($CDCl_3$) $\delta$: 2.23 (3H, s), 2.33 (3H, s), 4.98 (2H, s), 5.47 (1H, s), 7.23 (5H, s). *Anal.* Calcd for $C_{15}H_{14}O_4$: C, 69.75; H, 5.46. Found: C, 69.50; H, 5.40.

**Ethyl 4-Hydroxy-6-methyl-2-oxo-2*H*-pyran-5-carboxylate (3)**——According to the literature,[12] a mixture of malonyl chloride (5 ml) and ethyl acetoacetate (5 ml) was heated for 20 min on a water bath, and left to stand for 2 h at room temperature. Then AcOEt (200 ml) was added to the reaction mixture and the whole was extracted with 10% $NaHCO_3$ (30 ml × 3). The aqueous layer was acidified (pH 3.5) and extracted with AcOEt (100 ml × 5). The AcOEt extract was subjected to silica gel column (3 × 16 cm) chromatography with $CHCl_3$. From the early eluted fraction (200 ml), **3** (2.23 g) was obtained as a yellow-orange oil.

**Ethyl 4-Methoxy-6-methyl-2-oxo-2*H*-pyran-5-carboxylate (1e)**——The above ester (**3**, 2.23 g) was methylated with NaH/$Me_2SO_4$.[4] Recrystallization from cyclohexane yielded **1e** (1.59 g) as colorless microneedles, mp 93—94 °C. MS *m/z*: 212 ($M^+$), 197, 184, 167, 156, 125, 43. UV $\lambda_{max}^{EtOH}$ nm: 243, 282. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 1723, 1651, 1569. $^1$H-NMR ($CDCl_3$) $\delta$: 1.32 (3H, t, $J$ = 7.0 Hz), 2.28 (3H, s), 3.77 (3H, s), 4.27 (2H, q, $J$ = 7.0 Hz), 5.36 (1H, s). *Anal.* Calcd for $C_{10}H_{12}O_5$: C, 56.60; H, 5.70. Found: C, 56.32; H, 5.76.

**5-Hydroxymethyl-4-methoxy-6-methyl-2-pyrone (1t)**——This was synthesized by a modification of the literature procedure.[13] Borane-methyl sulfide (BMS) (1.2 ml) was added dropwise to a stirred solution of the above ester **3** (2.31 g) in $C_6H_6$ (12 ml) over 15 min under a nitrogen atmosphere at 0 °C. The reaction mixture was stirred for an additional 22 h at ambient temperature. MeOH (15 ml) was added and evaporated *in vacuo*. This treatment was repeated again. Recrystallization of the residue from MeOH–AcOEt yielded 4-hydroxy-5-hydroxymethyl-6-methyl-2-pyrone (**4**, 786 mg) as colorless microprisms, mp 163—166 °C (dec.). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3254, 3085—2574, 1692 (sh), 1680, 1634, 1560. $^1$H-NMR (DMSO-$d_6$) $\delta$: 2.22 (3H, s), 4.0 (1H, br, exchangeable with $D_2O$), 4.20 (2H, s), 5.24 (1H, s), 11.5 (1H, br, exchangeable with $D_2O$).

A mixture of **4** (500 mg) and anhydrous $K_2CO_3$ (2.21 g) in butanone (13.5 ml) was heated at 100 °C for 30 min. $Me_2SO_4$ (0.37 ml) in butanone (4.2 ml) was added and the mixture was refluxed for 5 h. After cooling, the reaction mixture was filtered and the precipitate was washed with acetone (20 ml). The filtrate and washing were combined and evaporated to dryness *in vacuo*. The residue was subjected to PTLC with acetone–$C_6H_6$ (1 : 4). Recrystallization of the upper main band from $C_6H_6$ yielded **1t** (341 mg) as colorless needles, mp 114—118 °C. MS *m/z*: 170 ($M^+$), 142, 127, 43. UV $\lambda_{max}^{EtOH}$ nm: 282. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3381, 1691, 1636, 1558. $^1$H-NMR ($CDCl_3$) $\delta$: 2.24 (1H, t, $J$ = 6.0 Hz, exchangeable with $D_2O$), 2.29 (3H, s), 3.80 (3H, s), 4.39 (2H, d, $J$ = 6.0 Hz), 5.38 (1H, s). *Anal.* Calcd for $C_8H_{10}O_4$: C, 56.46; H, 5.93. Found: C, 56.43; H, 5.93. Recrystallization of the lower band from $C_6H_6$ yielded the 4-pyrone isomer (40 mg) as colorless plates, mp 124—127.5 °C. MS *m/z*: 170 ($M^+$), 155, 113, 71, 43. UV $\lambda_{max}^{EtOH}$ nm: 243. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3356, 1666, 1609, 1584. $^1$H-NMR ($CDCl_3$) $\delta$: 2.29 (3H, s), 3.7 (1H, br, exchangeable with $D_2O$), 3.82 (3H, s), 4.43 (2H, br s), 5.44 (1H, s). *Anal.* Found: C, 56.38; H, 5.90.

**4-Methoxy-6-methyl-2-oxo-2*H*-pyran-5-aldehyde (1v)**——A solution of **1t** (300 mg) in $CH_2Cl_2$ (6 ml) was added in one batch to a suspension of pyridinium chlorochromate (PCC, 3.9 g) in $CH_2Cl_2$ (60 ml). The dark reaction mixture was stirred for 15 min at 27 °C, placed on a silica gel column (3 × 24 cm), and eluted with AcOEt, then the eluate was evaporated *in vacuo*. Recrystallization of the residue from $CHCl_3$–cyclohexane yielded **1v** (237 mg) as

colorless needles, mp 130—133 °C; spectral data were identical with those of an authentic sample.[8] *Anal.* Calcd for $C_8H_8O_4$: C, 57.14; H, 4.80. Found: C, 56.93; H, 4.74.

**4-Methoxy-6-methyl-2-oxo-2*H*-pyran-5-nitrile (1f)**——The aldehyde **1v** (477 mg) was dissolved in EtOH (25 ml) and a solution of hydroxylamine hydrochloride (220 mg) and anhydrous sodium acetate (260 mg) in water (22 ml) was added. The mixture was allowed to stand for 30 min at room temperature, then the EtOH was removed *in vacuo*. The formed precipitate was collected by filtration, washed with water and dried under vacuum. The residue (507 mg) was recrystallized from $C_6H_6$ to obtain the oxime of **1v** as colorless microneedles, mp 185—187 °C. MS *m/z*: 183 ($M^+$), 168, 153, 137, 125, 43. UV $\lambda_{max}^{EtOH}$ nm: 237, 289. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3282 (br), 1749, 1637, 1571. $^1$H-NMR ($CDCl_3$) $\delta$: 2.39 (3H, s), 3.80 (3H, s), 5.45 (1H, s), 6.8 (1H, br, exchangeable with $D_2O$), 7.95 (1H, s). *Anal.* Calcd for $C_8H_9NO_4$: C, 52.46; H, 4.95; N, 7.65. Found: C, 52.37; H, 4.95; N, 7.53.

According to the literature,[14] the oxime of **1v** (507 mg) suspended in ether (25 ml) was treated with thionyl chloride (1.5 ml) and refluxed for 3.5 h. The mixture was evaporated to dryness and subjected to PTLC with acetone–$C_6H_6$ (1 : 4). Recrystallization of the product from $CHCl_3$–cyclohexane yielded the nitrile (**1f**, 208 mg) as colorless microcrystals, mp 176—184 °C. High-resolution MS *m/z*: 165.0424 (required for $C_8H_7NO_3$: 165.0425). MS *m/z*: 165 ($M^+$), 150, 137, 43. UV $\lambda_{max}^{EtOH}$ nm: 241, 285. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 2232, 1747, 1639, 1568. $^1$H-NMR ($CDCl_3$) $\delta$: 2.49 (3H, s), 3.87 (3H, s), 5.41 (1H, s). *Anal.* Calcd for $C_8H_7NO_3$: C, 58.18; H, 4.27; N, 8.48. Found: C, 57.78; H, 4.16; N, 8.43.

**Methyl 4,6-Dimethyl-2-oxo-2*H*-pyran-5-carboxylate (1g)**——The carboxylic acid (**1x**, 397 mg)[7] was methylated with ethereal $CH_2N_2$ and separated with PTLC ($CHCl_3$). Recrystallization of the product from cyclohexane yielded **1g** (386 mg) as colorless needles, mp 66.5—68.5 °C. MS *m/z*: 182 ($M^+$), 154, 139, 123. UV $\lambda_{max}^{EtOH}$ nm: 248, 295. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1751, 1729, 1633, 1553. $^1$H-NMR ($CDCl_3$) $\delta$: 2.18 (3H, d, $J$ = 1.6 Hz), 2.36 (3H, s), 3.81 (3H, s), 5.91 (1H, br). *Anal.* Calcd for $C_9H_{10}O_4$: C, 59.33; H, 5.53. Found: C, 59.50; H, 5.61.

**5-Benzoyl-4-methoxy-6-methyl-2-pyrone (1i)**——5-Benzoyl-4-hydroxy-6-methyl-2-pyrone (805 mg)[15] was methylated with $NaH/Me_2SO_4$.[4] Recrystallization of the product from cyclohexane yielded **1i** (606 mg) as colorless needles, mp 101—103 °C. MS *m/z*: 244 ($M^+$), 229, 201, 105, 77. UV $\lambda_{max}^{EtOH}$ nm: 256, 277 (sh). IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1731, 1673, 1641, 1593, 1563. $^1$H-NMR ($CDCl_3$) $\delta$: 2.13 (3H, s), 3.63 (3H, s), 5.43 (1H, s), 7.33—7.80 (5H, m). *Anal.* Calcd for $C_{14}H_{12}O_4$: C, 68.84; H, 4.95. Found: C, 68.84; H, 4.88.

**5-Acetyl-4-chloro-6-methyl-2-pyrone (1j)**——According to the literature,[16] a mixture of **1q** (1.00 g), triphenylphosphine (3.12 g), $CHCl_3$ (20 ml) and $CCl_4$ (20 ml) was refluxed for 1 h under a nitrogen atmosphere. After cooling, the mixture was diluted with $C_6H_6$ (100 ml) and filtered. The filtrate was evaporated *in vacuo* and the residue was subjected to silica gel column chromatography (3 × 27 cm, acetone–$C_6H_6$) following PTLC with acetone–$C_6H_6$ (1 : 9). Recrystallization of the product from cyclohexane yielded the 4-chloro derivative (**1j**, 122 mg) as colorless prisms, mp 90—92 °C. MS *m/z*: 188, 186 ($M^+$), 173, 171, 145, 143, 43. UV $\lambda_{max}^{EtOH}$ nm: 258, 304. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1742, 1708, 1614, 1545, 573, 542. $^1$H-NMR ($CDCl_3$) $\delta$: 2.26 (3H, s), 2.49 (3H, s), 6.25 (1H, s). *Anal.* Calcd for $C_8H_7ClO_3$: C, 51.50; H, 3.78. Found: C, 51.38; H, 3.64.

**5-Acetyl-4-methoxy-6-phenyl-2-pyrone (1n)**——5-Acetyl-4-hydroxy-6-phenyl-2-pyrone (254 mg) prepared according to literature[15] was methylated with $NaH/Me_2SO_4$.[4] Recrystallization of the product from cyclohexane yielded **1n** (190 mg) as colorless needles, mp 95.5—98 °C. MS *m/z*: 244 ($M^+$), 229, 201, 105, 77. UV $\lambda_{max}^{EtOH}$ nm: 229, 304. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1724—1701, 1627, 1598, 1549, 1493. $^1$H-NMR ($CDCl_3$) $\delta$: 2.19 (3H, s), 3.81 (3H, s), 5.52 (1H, s), 7.38 (5H, br). *Anal.* Calcd for $C_{14}H_{12}O_4$: C, 68.84; H, 4.95. Found: C, 68.86; H, 4.99.

**4-Acetoxy-5-acetyl-6-methyl-2-pyrone (1r)**——Acetyl chloride (1 ml) was added to **1q** (500 mg)[5] and the mixture was refluxed for 4 h. Excess acetyl chloride was removed by evaporation and the residue was recrystallized from cyclohexane. **1r** (478 mg) was obtained as colorless needles, mp 62—66 °C. MS *m/z*: 210 ($M^+$), 168, 140, 125. UV $\lambda_{max}^{EtOH}$ nm: 258, 295. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1776, 1726, 1683, 1620, 1549, 1255, 1057, 1014. $^1$H-NMR ($CDCl_3$) $\delta$: 2.25 (3H, s), 2.31 (3H, s), 2.38 (3H, s), 6.06 (1H, s). *Anal.* Calcd for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80. Found: C, 57.10; H, 4.74.

**Administration Experiments with Pyrone Derivatives**——A standard procedure was carried out as follows. *M. commelinae* (IFO 9570) was cultivated in 500 ml Roux flasks containing 200 ml of a malt extract medium. After 10 d the culture broth was removed, and the mycelial mat was gently washed once with 200 ml of 10 mM phosphate buffer (pH 6.0, $NaH_2PO_4$–$K_2HPO_4$), then incubated in 10 mM phosphate buffer (200 ml) under sterile conditions. Each pyrone derivative (1.2 mmol) dissolved in EtOH (5 ml) was distributed into five flasks. The culture was stopped on the 20th day, and the obtained culture filtrate was concentrated to about 150 ml *in vacuo*, acidified (pH 2) with concentrated HCl and extracted with AcOEt (150 ml × 4). The AcOEt extract was redissolved in AcOEt (60 ml) and treated with 10% $NaHCO_3$ (20 ml × 2). The organic layer was dried ($Na_2SO_4$) and evaporated *in vacuo* (neutral fraction). The aqueous layer was acidified (pH 2) and extracted with AcOEt (50 ml × 5). The AcOEt layer was dried ($Na_2SO_4$) and evaporated *in vacuo* (acidic fraction). The neutral and acidic fractions were each separated by silica gel PTLC, and the metabolites were purified by recrystallization.

**2b**——Colorless needles from $C_6H_6$, mp 184—188 °C. MS *m/z*: 224 ($M^+$), 192, 149, 134. UV $\lambda_{max}^{EtOH}$ nm: 244, 299. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3070—2490, 1735, 1689, 1582, 1488. $^1$H-NMR ($CDCl_3$) $\delta$: 2.29 (3H, s), 3.82 (3H, s), 3.86 (3H, s), 7.36 (1H, br s), 7.46 (1H, br s), 8.71 (1H, br, exchangeable with $D_2O$). *Anal.* Calcd for $C_{11}H_{12}O_5$: C, 58.92; H, 5.40. Found: C, 58.69; H, 5.33.

**2c**——Colorless microcrystals from $C_6H_6$, mp 182—184.5 °C. MS *m/z*: 222 ($M^+$), 207, 179. UV $\lambda_{max}^{EtOH}$ nm: 251,

304. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 2980—2400, 1688, 1572, 1476. $^1$H-NMR (pyridine-$d_5$) $\delta$: 1.22 (3H, t, $J$=6.7 Hz), 2.25 (3H, s), 2.47 (3H, s), 3.95 (2H, q, $J$=6.7 Hz), 7.62 (1H, br s), 7.70 (1H, br s), 9.35 (1H, br s, exchangeable with $D_2O$). *Anal.* Calcd for $C_{12}H_{14}O_4$: C, 64.85; H, 6.35. Found: C, 64.81; H, 6.21.

**2d**——Colorless needles from $C_6H_6$, mp 151—154 °C. MS *m/z*: 284 ($M^+$), 179, 91. UV $\lambda_{max}^{EtOH}$ nm: 250, 303. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3100—2500, 1694, 1605, 1581, 1503. $^1$H-NMR ($CDCl_3$) $\delta$: 2.25 (3H, s), 2.45 (3H, s), 5.07 (2H, s), 7.27 (5H, br s), 7.48 (2H, br s), 7.6 (1H, br, exchangeable with $D_2O$). *Anal.* Calcd for $C_{17}H_{16}O_4$: C, 71.82; H, 5.67. Found: C, 71.57; H, 5.60.

**2e**——Colorless prisms from $C_6H_6$, mp 165—166 °C. MS *m/z*: 238 ($M^+$), 193, 192. UV $\lambda_{max}^{EtOH}$ nm: 244, 298. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3050—2500, 1728, 1690, 1578, 1486. $^1$H-NMR ($CDCl_3$) $\delta$: 1.39 (3H, t, $J$=6.9 Hz), 2.33 (3H, s), 3.84 (3H, s), 4.37 (2H, q, $J$=6.9 Hz), 7.37 (1H, br s), 7.47 (1H, br s), 8.8 (1H, br, exchangeable with $D_2O$). *Anal.* Calcd for $C_{12}H_{14}O_5$: C, 60.50; H, 5.92. Found: C, 60.37; H, 6.03.

**2f**——Colorless needles from MeOH–cyclohexane, mp 231—233 °C. MS *m/z*: 191 ($M^+$), 174, 162, 146, 118, 77. UV $\lambda_{max}^{EtOH}$ nm: 252, 317. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3170—2500, 2232, 1722, 1573. $^1$H-NMR (acetone-$d_6$) $\delta$: 2.52 (3H, s), 4.01 (3H, s), 5.8 (1H, br, exchangeable with $D_2O$), 7.50 (1H, br s), 7.55 (1H, br s). *Anal.* Calcd for $C_{10}H_9NO_3$: C, 62.82; H, 4.75; N, 7.33. Found: C, 62.61; H, 4.65; N, 7.37.

**2g**——Colorless prisms from $C_6H_6$, mp 150—158 °C. MS *m/z*: 208 ($M^+$), 177, 176, 149. UV $\lambda_{max}^{EtOH}$ nm: 239, 290. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 2980—2470, 1729, 1695, 1571. $^1$H-NMR ($CDCl_3$) $\delta$: 2.30 (6H, s), 3.84 (3H, s), 7.58 (2H, s), 8.3 (1H, br, exchangeable with $D_2O$). *Anal.* Calcd for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81. Found: C, 63.30; H, 5.83.

**2h**——Colorless needles from AcOEt–ligroin, mp 125.5—126.5 °C. MS *m/z*: 222 ($M^+$), 193, 177, 176, 149. UV $\lambda_{max}^{EtOH}$ nm: 239, 290. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3000—2480, 1726, 1691, 1571. $^1$H-NMR ($CDCl_3$) $\delta$: 1.37 (3H, t, $J$=7.2 Hz), 2.32 (6H, s), 4.37 (2H, q, $J$=7.2 Hz), 7.67 (2H, br s), 8.3 (1H, br, exchangeable with $D_2O$). *Anal.* Calcd for $C_{12}H_{14}O_4$: C, 64.85; H, 6.35. Found: C, 64.68; H, 6.50.

**2i**——Colorless microcrystals from AcOEt–ligroin, mp 156—158.5 °C. MS *m/z*: 270 ($M^+$), 269, 193, 105, 77. UV $\lambda_{max}^{EtOH}$ nm: 251, 290 (sh). IR $\nu_{max}^{KBr}$ cm$^{-1}$: 2960—2490, 1689, 1673, 1594, 1576, 1481. $^1$H-NMR ($CDCl_3$) $\delta$: 2.16 (3H, s), 3.71 (3H, s), 7.30—7.88 (8H, m, 1H was exchangeable with $D_2O$). *Anal.* Calcd for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22. Found: C, 71.03; H, 5.15.

**2j**——Colorless needles from $C_6H_6$, mp 181—183.5 °C. MS *m/z*: 214, 212 ($M^+$), 199, 197. UV $\lambda_{max}^{EtOH}$ nm: 238, 284 (sh), 292. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3000—2400, 1694, 1558, 624, 565. $^1$H-NMR ($CDCl_3$) $\delta$: 2.29 (3H, s), 2.50 (3H, s), 7.6 (1H, br, exchangeable with $D_2O$), 7.73 (2H, br s). *Anal.* Calcd for $C_{10}H_9ClO_3$: C, 56.49; H, 4.27. Found: C, 56.45; H, 4.14.

**2m**——Colorless solid, high-resolution MS *m/z*: 194.0601 (required for $C_{10}H_{10}O_4$: 194.0578). MS *m/z*: 194 ($M^+$), 163, 162, 149, 135. UV $\lambda_{max}^{EtOH}$ nm: 243, 293. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3000—2500, 1726, 1705. $^1$H-NMR ($CDCl_3$) $\delta$: 2.65 (3H, s), 3.93 (3H, s), 5.7 (1H, br, exchangeable with $D_2O$), 6.6 (1H, br, exchangeable with $D_2O$), 7.96 (3H, br s).

**2n**——Yellowish oil, high-resolution MS *m/z*: 270.0909 (required for $C_{16}H_{14}O_4$: 270.0891). MS *m/z*: 270 ($M^+$), 255. $^1$H-NMR ($CDCl_3$) $\delta$: 2.13 (3H, s), 3.86 (3H, s), 7.26 (5H, br s), 7.53 (1H, br s), 7.63 (1H, br s).

**2o**——Colorless solid, high-resolution MS *m/z*: 245.9737 and 243.9783 ($M^+$) (required for $C_9H_9{}^{81}BrO_3$: 245.9716; $C_9H_9{}^{79}BrO_3$: 243.9735). MS *m/z*: 246, 244 ($M^+$), 231, 229, 227, 201, 199. $^1$H-NMR (acetone-$d_6$) $\delta$: 2.45 (3H, s), 3.96 (3H, s), 7.45 (1H, br s), 7.56 (1H, br s).

**6-Methyl-2-oxo-2*H*-pyran-5-carboxylic Acid**——Colorless needles from MeOH–$C_6H_6$, mp 187—191 °C. MS *m/z*: 154 ($M^+$), 139, 136, 126, 43. UV $\lambda_{max}^{EtOH}$ nm: 244, 297. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3140—2460, 1730, 1704 (sh), 1688, 1674, 1618, 1552. $^1$H-NMR (acetone-$d_6$) $\delta$: 2.59 (3H, s), 5.9 (1H, br, exchangeable with $D_2O$), 6.09 (1H, d, $J$=9.6 Hz), 7.78 (1H, d, $J$=9.6 Hz). *Anal.* Calcd for $C_7H_6O_4$: C, 54.55; H, 3.92. Found: C, 54.47; H, 3.78.

**5-(1-Hydroxyethyl)-4-methoxy-6-phenyl-2-pyrone**——Colorless glass, high-resolution MS *m/z*: 246.0916 (required for $C_{14}H_{14}O_4$: 246.0892). MS *m/z*: 246 ($M^+$), 231, 141, 105, 77. $[\alpha]_D^{18.5}$ +21.8° ($c$=0.40, MeOH). UV $\lambda_{max}^{EtOH}$ nm: 229, 296. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3416, 1701 (br), 1630, 1602, 1552, 1495. $^1$H-NMR ($CDCl_3$) $\delta$: 1.54 (3H, d, $J$=6.8 Hz), 3.88 (3H, s), 4.1 (1H, br, exchangeable with $D_2O$), 4.71 (1H, q, $J$=6.8 Hz), 5.60 (1H, s), 7.35 (5H, s).

**4-Methoxy-6-methyl-3-vinyl-2-pyrone**——MS *m/z*: 166 ($M^+$), 138, 123, 95, 43. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1693, 1641, 1616 (sh), 1552. $^1$H-NMR ($CDCl_3$) $\delta$: 2.25 (3H, s), 3.86 (3H, s), 5.26 (1H, dd, $J$=11.4, 3.4 Hz), 5.96 (1H, s), 6.13 (1H, dd, $J$=17.8, 3.4 Hz), 6.67 (1H, dd, $J$=17.8, 11.4 Hz).

**3-(1-Hydroxyethyl)-4-methoxy-6-methyl-2-pyrone**——Colorless microcrystals from $CCl_4$, mp 144—148 °C. MS *m/z*: 184 ($M^+$), 183, 169, 167, 141, 43. UV $\lambda_{max}^{EtOH}$ nm: 301. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3085—2852, 1696, 1639, 1550. $^1$H-NMR ($CDCl_3$) $\delta$: 1.40 (3H, d, $J$=6.6 Hz), 2.22 (3H, d, $J$=0.8 Hz), 3.79 (3H, s), 4.59 (1H, q, $J$=6.6 Hz), 5.92 (1H, br s).

**5-Acetyl-5,6-dihydro-4-methoxy-2-pyrone**——UV $\lambda_{max}^{EtOH}$ nm: 236. $^1$H-NMR ($CDCl_3$) $\delta$: 2.25 (3H, s), 3.26 (1H, dd, $J$=4.6, 3.8 Hz), 3.77 (3H, s), 4.31 (1H, dd, $J$=12, 4.6 Hz), 4.66 (1H, dd, $J$=12, 3.8 Hz), 5.20 (1H, s).

**Administration of $^{14}$C-Labeled Compounds**——[U-$^{14}$C]Succinic acid (CEA), [U-$^{14}$C]α-ketoglutaric acid (ICN Radiochemicals), [1,5-$^{14}$C]citric acid (ICN Pharmaceuticals) and cyclohexylammonium phosphoenol[1-$^{14}$C]pyruvate (Amersham) were purchased. Each $^{14}$C-labeled compound (*ca.* 50 μCi) diluted with non-labeled compound (1.2 mmol) and **1a** (1.2 mmol) were distributed into five flasks containing 10-d-old washed cells in phosphate buffer solution and incubated at 27 °C for 10 d in a stream of air. The $^{14}CO_2$ evolved during the incubation was passed into a trap containing 10% NaOH. $^{14}$C-Labeled **2a** was extracted from the culture medium and purified with PTLC. The radioactivities of the trapped solution, culture medium and purified **2a** were measured with a liquid scintillation

spectrometer.

**Shaking Culture and Preparation of Pre-incubated Cells**——*M. commelinae* IFO 9570 was cultured under shaking (stroke 10 cm, 150 rpm) at 27 °C for 48 h in 1 l flasks containing 100 ml of a modified Pfeffer medium consisting of corn steep liquor (1.2 g), yeast extract (0.25 g), sucrose (5.0 g), $NH_4NO_3$ (1.0 g), $KH_2PO_4$ (0.5 g), $MgSO_4$ (0.25 g), $FeCl_3$ (0.1 mg) and tap water (100 ml) (the pH was adjusted to *ca.* 7 with 5 N NaOH). In the preparation of pre-incubated cells, a 0.2 M solution of **1a** in dimethylformamide (DMF) was added (final concentration, 2 mM) to the culture at 40 h after inoculation, and incubation was continued for a further 8 h under shaking.

**Experiments with Pre-incubated Cells under Shaking**——Pre-incubated mycelia were collected by filtration, washed with saline, suspended in saline and starved overnight (15 h) at 4 °C with stirring. After filtration, the mycelia (1 g) were suspended in a mixture of 0.1 M potassium phosphate buffer (pH 7.3) (10 ml), saline (10 ml) and **1a** (50 mM solution in DMF, 0.4 ml), and shaken at 30 °C. Every hour, an aliquot of the incubation mixture was withdrawn, filtered with an EKICRODISC 3 (Gelman Sciences Japan, Ltd.) and analyzed by HPLC.

**HPLC Analysis**——A Shimadzu LC-6A instrument equipped with a system controller (SLC-6A), a UV detector (SPD-6A) and a column oven (CTO-6A) was used. Peak areas were calculated by using a Shimadzu C-R3A Chromatopac. Other conditions were as follows; column, Hibar LiChrosorb RP-18 (4 mm i.d. × 250 mm, 5 μm, Merck); mobile phase, solution A [10% $CH_3CN$ in 10 mM phosphate buffer (pH 2.6)] and solution B [40% $CH_3CN$ in 10 mM phosphate buffer (pH 2.6)]; elution, 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); detection wavelength, 250 nm; a.u.f.s., 0.16; flow rate, 1 ml/min; temperature, 50 °C; sample volume, 10 μl; retention times, **1a** 12.7 min and **2a** 21.9 min.

**Administration of Carbon Sources to Pre-incubated Cells**——Pre-incubated mycelia were incubated with **1a** (final concentration, 1 mM) and a carbon source (glucose, sodium pyruvate, sodium citrate, sodium succinate, fumaric acid, malic acid, oxalacetic acid, sodium acetate, phenylpyruvic acid, acrylic acid or malonic acid; final concentration, 10 mM) by the shaking procedure described above. The progress of transformation was examined by HPLC.

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