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New Metabolites of Two Hybrid Strains ME 0004 and 0005 Derived from *Penicillium citreo-viride* B. IFO 6200 and 4692

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6-Hydroxy-8-methoxy-3S,5-dimethylisochroman and 6-hydroxy-8-methoxy-3S,5-dimethyl-3,4-dihydroisocoumarin have been isolated from the mycelium of a hybrid strain ME 0004 derived from *Penicillium citreo-viride* B. IFO 6200 and 4692. The latter has also been produced by the hybrid strain ME 0005 in addition to 11-β-hydroxy-12-oxocurvularin.

In connection with citreoviridin, citreoviranol and related compounds,[1] as reported in the previous paper,[2] more than ten hybrid strains were produced by means of cell fusion technique[3] using two different strains IFO 6200 and 4692, and several curvularin-type metabolites were obtained from the mycelium of the hybrid strain ME 0005. We describe herein isolation and structures of three new metabolites produced by two hybrid strains ME 0004 and 0005, as follows.

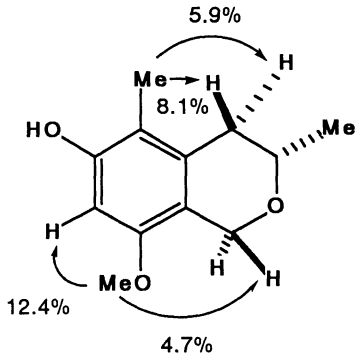
According to essentially the same procedure as previously described,[2] the polished rice (*ca.* 600 g), which was inoculated with a suspension of mycelium of the hybrid strain ME 0004 in a sterilized water, was incubated stationarily at 25 °C for 23 days and extracted with acetone and then EtOAc. The combined extracts were partitioned between EtOAc and water. The EtOAc extract was chromatographed on silica gel using a gradient solvent of MeOH - $CHCl_3$ (1 - 50%). Elution with $CHCl_3$ - MeOH (50 : 1) afforded a brown oil, which was further separated by repeated preparative TLC (Kieselgel PF_{254}) using hexane - EtOAc (1 : 1) and then hexane - EtOAc (2 : 1) to give rise to 6-hydroxy-8-methoxy-3S,5-dimethylisochroman (**1**) and the known conjugated γ-lactone (**2**)[4] in 0.42 and 0.13% yields, respectively.[5] Further elution with $CHCl_3$ - MeOH (100 : 3 - 7) gave a brown oil, which was also separated by repeated preparative TLC (Kieselgel PF_{254}) using hexane - EtOAc - acetone (7 : 2 : 1), EtOAc - acetone (9 : 1), CH_2Cl_2 - acetone (9 : 1) and $CHCl_3$ - MeOH (9 : 1) to afford 6-hydroxy-8-methoxy-3S,5-dimethyl-3,4-dihydroisocoumarin (**3**), in 0.16% yield.[5] The spectral data of the newly isolated compounds are shown below.

1 as a white powder: $C_{12}H_{16}O_3$ [m/z 208.1112 (M^+)]; $[\alpha]_D^{25}$ +102° (c 0.154, EtOH); IR (film) 3330, 1600 and 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 1.37 (3H, d, J = 5.9 Hz), 2.05 (3H, s), 2.42 (1H, dd, J = 10.7, 16.6 Hz), 2.61 (1H, ddd, J = 1.5, 3.4, 16.6 Hz), 3.72 (1H, m), 3.74 (3H, s), 4.57 (1H, dd, J = 1.5, 15.1 Hz), 4.66 (1H, s, OH), 4.88 (1H, d, J = 15.1 Hz) and 6.26 (1H, s).

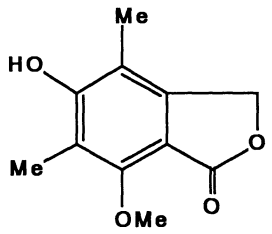
3 as colorless needles: mp 212 - 213 °C; $C_{12}H_{14}O_4$ [m/z 222.0892 (M^+)]; $[\alpha]_D^{25}$ +106° (c 0.118, MeOH); IR (film) 3270, 1685, 1590 and 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 1.49 (3H, d, J = 6.3 Hz), 2.10 (3H, s),

2.66 (1H, dd, J = 11.5, 16.4 Hz), 2.91 (1H, dd, J = 2.7, 16.4 Hz), 3.82 (3H, s), 4.47 (1H, m), 6.43 (1H, s) and 6.48 (1H, s, OH).

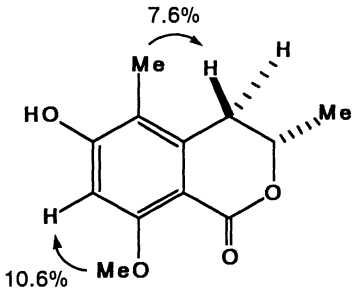
The NMR spectral data of both **1** and **3** are similar to each other, indicating the presence of a pentasubstituted benzene ring (δ 6.26 in **1**; δ 6.43 in **3**) and a partial structure CH_3-CH-CH_2- (δ 1.37, 3.72, 2.42 and 2.61 in **1**; δ 1.49, 4.47, 2.66 and 2.91 in **3**). However, some remarkable differences are observed in the following points: the former has the isolated methylene group (δ 4.57 and 4.88), but instead 3 has the CO group (1685 cm^{-1}). On the basis of NOE experiments, finally, these two new metabolites are regarded as 6-hydroxy-8-methoxy-3S,5-dimethylisochroman and 6-hydroxy-8-methoxy-3S,5-dimethyl-3,4-dihydroisocoumarin, as seen in **1** and **3** respectively, wherein the absolute configuration at C_3-position in **3** was determined by comparing its optical rotation ($[\alpha]_D^{25}$ +106°) with that of the known compound (**4**) ($[\alpha]_D$ -56°).[6] This is one of the rare examples of naturally occurring 3-methyl-3,4-dihydroisocoumarins which have 3S configuration instead of 3R configuration. From a biogenetic point of view, **1** must also have the same absolute configuration as that of **3**. Interestingly, any curvularin-type compound has not been detected in the case of the hybrid strain ME 0004. In contrast, several ones were obtained from the mycelium of the hybrid strain ME 0005, including 12-oxocurvularin (**5**), 11-β-hydroxy-curvularin (**6**) and 11-α-hydroxycurvularin (**7**), which show remarkable activity against sea urchin embryo cells.[2]



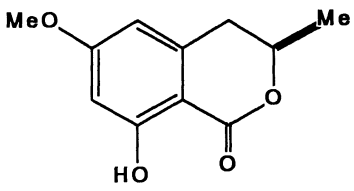
1



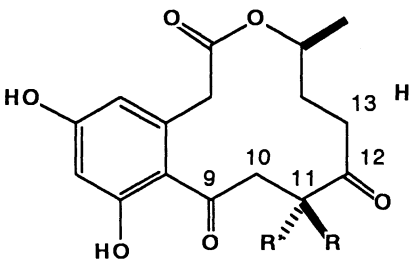
2

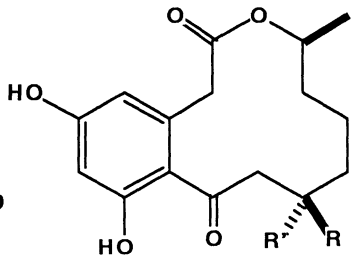


3



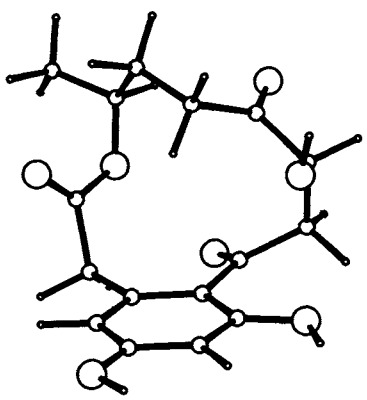
4



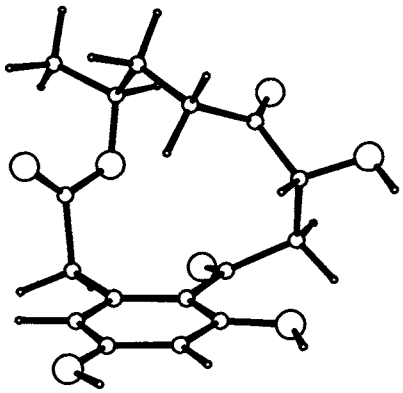


5 R = R' = H
8 R = OH, R' = H
9 R = H, R' = OH

6 R = OH, R' = H
7 R = H, R' = OH



[A] (S. E. 26.3520 kcal/mol)



[B] (S. E. 26.4025 kcal/mol)

Table 1. Comparison of the observed J values with the calculated ones

J (Hz)	C_{10}-H(α) / C_{11}-H	C_{10}-H(β) / C_{11}-H	C_{13}-H(α) / C_{14}-H(α)	C_{13}-H(α) / C_{14}-H(β)	C_{13}-H(β) / C_{14}-H(α)	C_{13}-H(β) / C_{14}-H(β)
8	5.2	4.0	4.6	4.2	12.8	4.4
9	13.0	3.1	5.0	3.8	12.6	4.7
observed	4.4	4.4	6.0	3.3	10.8	3.3

In the case of the hybrid strain (ME 0005), as previously reported,[2] the EtOAc extract was chromatographed on silica gel using a gradient solvent of MeOH - $CHCl_3$ (1 - 50%). The fraction eluted with $CHCl_3$ - MeOH (100 : 3 - 4)[7] was further separated by repeated preparative TLC (Kieselgel PF_{254}) using hexane - EtOAc (3 : 1), hexane - EtOAc (1 : 1) and benzene - acetone (2 : 1) to afford 6-hydroxy-8-methoxy-3S,5-dimethyl-3,4-dihydroisocoumarin (**3**) and 11-β-hydroxy-12-oxocurvularin (**8**) in 0.10 and 0.16% yields[5] respectively. The newly isolated curvularin-type compound as a white powder has the following spectral data: $C_{16}H_{16}O_6$ [m/z 304.0961 (M^+ - H_2O)]; $[\alpha]_D^{24}$ +41° (c 0.09, EtOH); IR (film) 3300, 1712, 1608 and 1530 cm^{-1}; ^1H NMR ($CDCl_3$) δ =1.14 (3H, d, J = 6.2 Hz), 1.48 (1H, m), 2.00 (1H, m), 2.43 (1H, ddd, J = 3.3, 10.8, 20.1 Hz), 2.67 (1H, dddd, J = 1.8, 3.3, 6.0, 20.1 Hz), 2.88 (1H, dd, J = 4.4, 12.5 Hz), 3.19 (1H, dd, J = 4.4, 12.5 Hz), 3.45 (1H, d, J = 17.4 Hz), 4.28 (1H, d, J = 17.4 Hz), 4.70 (1H, m), 4.89 (1H, t, J = 4.4 Hz), 6.32 (1H, d, J = 1.5 Hz) and 6.55 (1H, d, J = 1.5 Hz).

The NMR spectrum of **8** is similar to that of 12-oxocurvularin (**5**)[2] except for the following points: the three NMR signals coupled to one another are observed at δ 2.88, 3.19 and 4.89, indicating the presence of a partial structure -CH_2-CH(OH)- in **8**. In the light of these data together with co-occurrence of the curvularins (**5** - **7**), the stereostructure of the newly isolated compound is represented by **8**, wherein the stereochemistry at C_{11}-position was determined on the basis of the NMR spectral data coupled with molecular mechanics calculations successively using Still's RINGMAKER and BAKMOD programs based on Allinger's MM2 and their MMP2. Fortunately, two possible epimers (**8** and **9**) at C_{11}-position adopt only one stable conformer ([A]

in **8**; [B] in **9**).[8] Thus, the observed J values of the NMR signals were compared with the calculated ones based on [A] and [B], as seen from Table 1,[9] indicating that the secondary OH group at C_{11}-position is in a β-configuration. Of these curvularins, 11-β-hydroxy-12-oxocurvularin (**8**) is the most highly oxygenated one and its physiological properties will be examined in due course.

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References

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2) S. Lai, Y. Shizuri, S. Yamamura, K. Kawai, Y. Terada, and H. Furukawa, Tetrahedron Lett., **30**, 2241 (1989).

3) Each protoplast corresponding to *Penicillium citreo-viride* B. IFO 6200 and 4692 was prepared by enzymatic treatment of these two strains, which were incubated on potato sucrose agar (25 °C, 7 days), using cellulase, chitinase, pectolyase and sulfatase (30 °C, 60 min). And then, these two protoplasts in 0.05 M Ca solution (pH 10.5) were subjected to cell fusion experiments using polyethylene glycol (PEG 6000) as usual and incubated on potato sucrose agar (25 °C, 3 days) to give a number of colonies, from which many new hybrid strains including *Penicillium citreo-viride* ME 0004 and 0005 were obtained. These experiments were reported briefly (H. Furukawa, K. Kawai, M. Niwa, M. Yogo, S. Yamamura, and Y. Shizuri, 109th National Meeting of The Pharmaceutical Society of Japan, Nagoya, April 1988, Abst., No. 4FF 1 - 6.).

4) H. Achenbach, A. Muhlenfeld, and G. U. Brillinger, Liebigs Ann. Chem., **1985**, 1596.

5) Based on the weight of the EtOAc extract.

6) F. Kurosaki and A. Nishi, Phytochem., **22**, 669 (1983); the corresponding methyl ether also shows negative optical rotation ($[\alpha]_D$ -153°).

7) Citreofuran and related dehydrocurvularins have been obtained from the same fraction eluted with $CHCl_3$ - MeOH (100 : 3 - 4).

8) Of the possible conformers of **8**, relative population of **[A]** is more than 98%. In the case of **9**, relative population of **[B]** is also more than 98%.

9) From a biogenetic point of view, two possible 12-oxocurvularins having one OH group at C_{10}-position seem to be ruled out. In fact, the calculated J values are considerably different from the observed ones in the natural compound (**8**), because of some difference in their 12-membered ring conformations depending on location of the OH group at C_{10}- or C_{11}-position.

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