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# Biosynthesis of Scytalone[1]

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The biosynthesis of scytalone, a simple derivative of tetralone, has been studied by using [1,2-$^{13}C_2$] and [2-$^{13}$C, 2-$^{2}H_3$]-acetate. Scytalone labelled by [1,2-$^{13}C_2$]-acetate showed an unusual coupling pattern in the $^{13}$C-nuclear magnetic resonance (NMR) spectrum, indicating that all the carbon atoms coupled to both of the adjacent carbons. The results clearly demonstrate that scytalone is biosynthesized *via* a symmetrical aromatic intermediate, 1,3,6,8-tetrahydroxynaphthalene. Incorporation of $^{2}$H into C-4 and -5 from [2-$^{13}$C, 2-$^{2}H_3$]-acetate was demonstrated by $^{1}$H and $^{2}$H decoupled $^{13}$C-NMR. In contrast, $^{2}$H was not observed on the other potential sites of labelling, C-2 and C-7. It has been shown that the use of [2-$^{13}$C, 2-$^{2}H_3$]-acetate is effective in tracing the fate of acetate hydrogen in polyketide biosynthesis.

**Keywords**——biosynthesis; scytalone; polyketide; $^{13}$C, $^{2}$H; [2-$^{13}$C, 2-$^{2}H_3$]-acetate; coupling; isotopic shift; *Phialophora lagerbergii*; fungus

## Introduction

Since $^{13}$C-NMR was first applied to biosynthetic studies of natural products by Tanabe,[2] much new information regarding the formation of carbon skeletons has been obtained.[3] Double labelled acetate, [1,2-$^{13}C_2$]-acetate, was particularly effective in elucidating the mode of skeletal formation of polyketides and terpenoids.[3b,3c,4,5] The labelling pattern of acetate is easily determined by $^{13}$C-$^{13}$C coupling in $^{13}$C-NMR. $^{13}$C-$^{13}$C coupling indicates the presence of $^{13}$C in an adjacent position, that is, coupling in $^{13}$C-NMR spectrum gives information concerning the atom bonded to the $^{13}$C carbon showing coupling. This suggests a further possibility for the utilization of other multiple labelled precusors in biosynthetic studies. Multiple labelled compounds containing $^{13}$C and other nuclei which cause coupling or isotopic shift in $^{13}$C-NMR can be used as precursors. In biosynthetic studies of polyketides, $^{2}$H,[6] $^{17}$O[7] and $^{18}$O[7,8] are the nuclei to be used in combination with $^{13}$C. The fate of acetate hydrogen can be traced when [2-$^{13}$C, 2-$^{2}H_3$]-acetate is used as a precursor, since $^{13}$C bearing $^{2}$H shows isotopic shift and unique $^{2}$H-$^{13}$C coupling.[1,6] This paper mainly deals with the biosynthesis of scytalone (**1**), which is a simple derivative of tetralone produced by imperfect fungi such as *Scytallidium sp.*,[9] *Phialophola lagerbergii*,[10] and *Verticillium dahliae*.[11] Its polyketide nature has been demonstrated by incorporation of [1-$^{13}$C] and [2-$^{13}$C]-acetate into the expected carbons of scytalone (**1**).[10] On the other hand, it was reported that scytalone (**1**) was easily synthesized from 1,3,6,8-tetrahydroxynaphthalene (**2**) by reduction with sodium borohydride.[12] Our


**1** **2**

Fig. 1

initial interest in the biosynthesis of scytalone (**1**) was in the possibility that scytalone (**1**) might be formed from a symmetrical intermediate (**2**) by the reduction of an aromatic ring. We first investigated this hypothesis by using [1,2-$^{13}C_2$]-acetate and then applied [2-$^{13}$C, 2-$^{2}H_3$]-acetate to clarify the details of the mode of polyketide cyclization.

## Results and Discussion

Cultures of *Phialophora lagerbergii* (IMI 96745) supplemented with either [1-$^{13}$C] or [2-$^{13}$C]-acetate in this study produced scytalone showing enhancement in the expected carbon signals. The $^{13}$C-NMR spectrum of scytalone labelled with [1,2-$^{13}C_2$]-acetate, however, showed unusual $^{13}$C-$^{13}$C coupling patterns. Expanded NMR signals revealed that there were four large satellites in all the carbon signals except for C-3 and -5 indicating that all the carbons coupled to both of the adjacent carbons. In the case of C-3 and -5, the two kinds of coupling constant are almost the same, so that the two pairs of satellites were not resolved. A long range coupling between C-2 and -8a ($^{2}J_{C-C}$) and the values of $^{1}J_{C-C}$ coupling of C-6 and -8 made it possible to assign the C-2, -4, -6 and -8 signals which had not been assigned unambiguously.[10] The $^{13}$C-NMR data of scytalone labelled with [1,2-$^{13}C_2$]-acetate are summarized in Table I. The results clearly demonstrate the presence of two different mode of aceta tearrangement in scytalone (**1a** and **1b**) labelled with [1,2-$^{13}C_2$]-acetate. As it appears in Fig. 2, the signal due to C-1 shows four large satellites arising from $^{13}$C-$^{13}$C couplings with both of the adjacent carbons and four small satellite signals arising from further $^{13}$C-$^{13}$C couplings with another adjacent carbons simultaneously labelled by natural abundance $^{13}$C (**1c** and **1d**) or by other [1,2-$^{13}C_2$]-acetate molecules (**1e** and **1f**). The presence of the two different modes of acetate arrangement clearly demonstrates that scytalone is biosynthesized *via* a symmetrical intermediate (**2**), in which the reduction of aromatic ring occurs with an equal probabilitiy in both of the aromatic rings. Scytalone (**1**) lacks a definite starter unit in its structure. If it is biosynthesized from one acetylCoA and four malonyl CoA *via* a $C_{10}$ polyketo-intermediate (**9**), the starter methyl group should be involved in a cyclization reaction to give the symmetrical intermediate (**2**). Another possibility is that the intermediate (**2**) is formed by the cleavage of an acyl group from cyclized $C_{12}$-polyketide (**10**). In fact, a tetralone derivative possessing an ethyl side chain, asparvenone (**11**), was isolated from *Aspergillus parvulus*.[13] An incorporation experiment with [$^{13}$C]-malonate would solve this problem and we tried the incorporation of [$^{14}$C]-malonate into scytalone as a preliminary experiment to check whether malonate could be used as a precursor. The specific incorporation ratios in repeated feeding experiments were 2.0—2.9% and it was evident that [$^{13}$C]-malonate could not be used as a precursor in this

Table I. $^{13}$C Chemical Shifts and $^{13}$C Coupling Constants of Scytalone labelled by [1,2-$^{13}C_2$]-Acetate

| Carbon | Multiplicity in off-resonance spectrum | ppm[a] | $^{1}J_{C-C}$ Hz | $^{2}J_{C-C}$ Hz | $^{1}J_{C-H}$ Hz |
|---|---|---|---|---|---|
| C-1 | s | 202.0 | 40, 55 | — | — |
| C-2 | t | 47.1 | 38, 40 | 9 | 129 |
| C-3 | d | 66.3 | 37 | — | 144 |
| C-4 | t | 38.8 | 38, 40 | — | 129 |
| C-4a | s | 145.7 | 40, 63 | — | — |
| C-5 | d | 108.9 | 63 | — | 161 |
| C-6 | s | 165.4 | 63, 67 | — | — |
| C-7 | d | 101.3 | 67, 70 | — | 160 |
| C-8 | s | 165.9 | 61, 71 | — | — |
| C-8a | s | 111.4 | 54, 59 | 9 | — |

a) Relative to TMS.

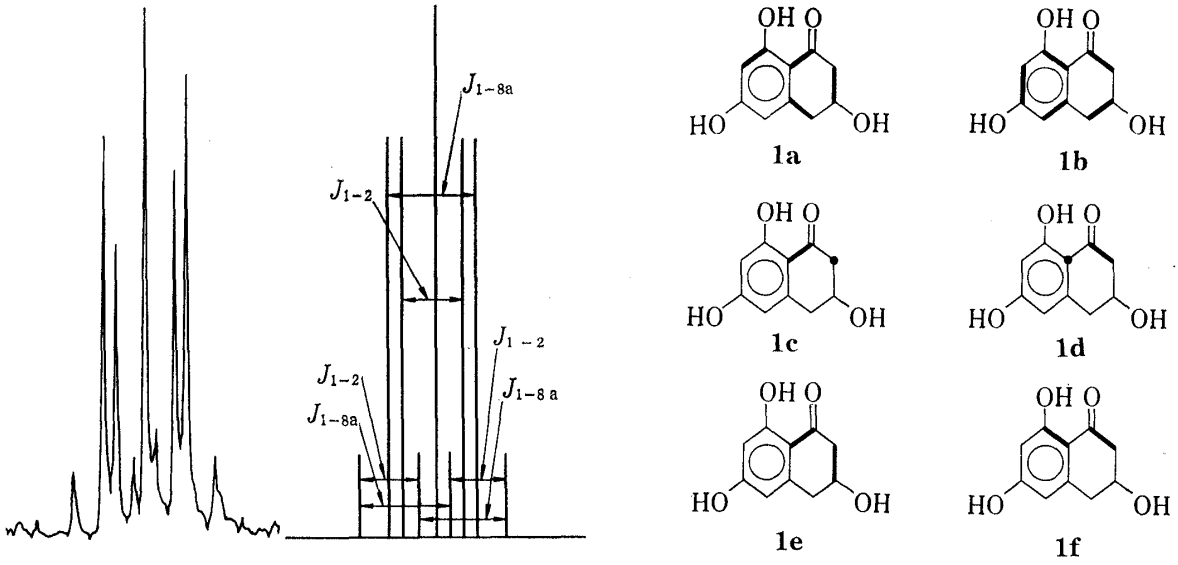


Fig. 2. C-1 Signal of Scytalone labelled with [1,2-$^{13}C_2$]-Acetate and Labelling Patterns

study. As a next approach, we attempted to trace hydrogen incorporation from acetate. The application of $^2$H- and $^3$H-NMR to biosynthetic studies of polyketides has been reported[6] and, for example, the labelling patterns of hydrogen isotopes in penicillic acid[14] and griseofulvin[15] were unambiguously established by using $^2$H- and $^3$H-NMR. We employed [2-$^{13}$C,2-$^2H_3$]-acetate as a tracer to detect the incorporation of acetate hydrogen by using $^{13}$C-NMR instead of $^2$H- or $^3$H-NMR. The incorporation of $^2$H from the multiple labelled acetate can be detected by $^{13}$C-$^2$H coupling or as a decrease of signal intensity. If we assume that

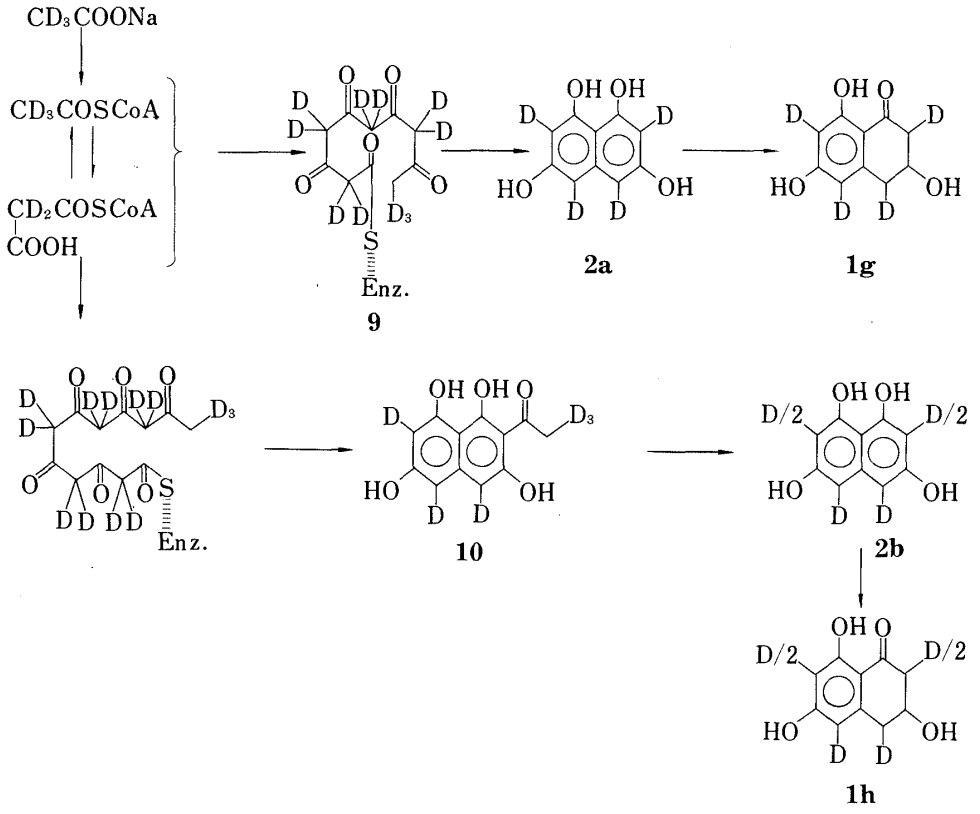


Chart 1

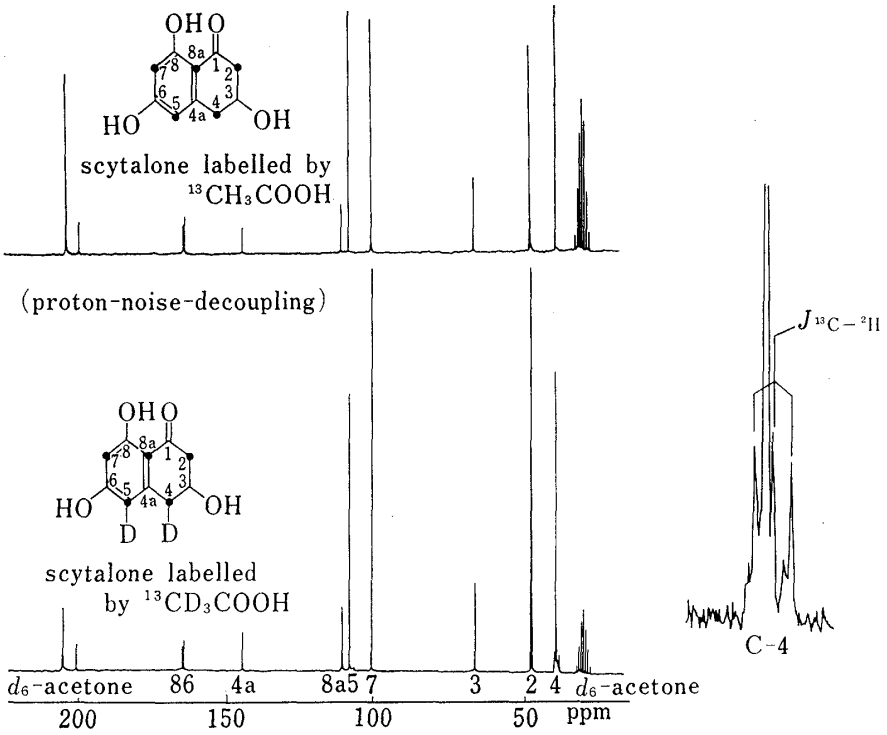


Fig. 3. Proton Noise-decoupled $^{13}C$-NMR Spectra of Scytalone labelled by [2-$^{13}C$] and [2-$^{13}C$, 2-$^{2}H_3$]-Acetate

the deuterium of acetate was retained in scytalone (**1g** and **1h**) as shown in Chart 1, we could show by which pathways scytalone is biosynthesized. The $^{1}H$-decoupled $^{13}C$-NMR spectrum of scytalone labelled by [2-$^{13}C$, 2-$^{2}H_3$]-acetate showed only one $^{13}C$-$^{2}H$ signal arising from C-4 (Fig. 3). The signal was observed as a triplet (1:1:1; $J_{^{13}C-^{2}H}$=20 Hz) centered at 38.5 ppm, 0.3 ppm higher than the corresponding $^{13}C$-$^{1}H$ signal, clearly demonstrating that a part of $^{13}C$ present at C-4 was labelled by one $^{2}H$. As $^{13}C$ bearing $^{2}H$ does not contribute to the enhancement of signal intensity in the corresponding $^{13}C$-$^{2}H$ signal, the presence of $^{2}H$ can be detected as a decrease of signal intensity. Although the intensities of the C-5 signal indicated that $^{13}C$ at C-5 was labelled by $^{2}H$ to a significant extent, no signal with $^{13}C$-$^{2}H$ coupling was observed. C-2 and -7, the other possible labelling sites, did not show any indication of the presence of $^{2}H$. As a $^{13}C$-$^{2}H$ signal might not be observed because of low

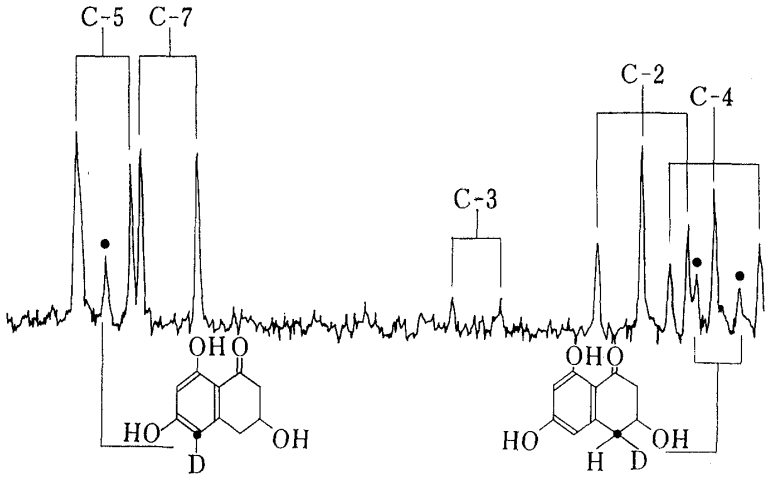


Fig. 4. Deuterium Noise-decoupled $^{13}C$-NMR Spectrum of Scytalone labelled by [2-$^{13}C$,2-$^{2}H_3$]-Acetate

sensitivity, we measured the $^{13}C$-NMR under deuterium noise-decoupled conditions (Fig. 4). In this spectrum, the $^{13}C$-$^{2}H$ signal of C-4 appeared as a doublet ($J_{^{1}H-^{13}C}$=127 Hz) centered at 38.4 ppm among the triplet signals of $^{13}C$-$^{1}H$ and that of C-5 as a singlet at 108.7 ppm between the doublet of $^{13}C$-$^{1}H$, all observed at higher field than the corresponding $^{13}C$-$^{1}H$ signals. No indication of the presence of $^{2}H$ at C-2 and C-7 was obtained in this spectrum. The results so far obtained demonstrate the incorporation of one $^{2}H$ from the multiple labelled acetate into C-4 and C-5. Comparison of signal intensities between the $^{13}C$-NMR spectra of scytalone labelled with [2-$^{13}C$] and [2-$^{13}C$, 2-$^{2}H_3$]-acetate revealed that *ca.* 40% of $^{2}H$ was retained on $^{13}C$ at C-4 and C-5 (Fig. 3). We cannot draw any positive conclusion regarding the cyclization of the polyketo-intermediate from the incorporation study with [2-$^{13}C$, 2-$^{2}H_3$]-acetate. The loss of $^{2}H$ from C-2 and -7 would be accounted for by two different possibilities. One is the involvement of the starter unit in the cyclization, in which $C_{10}$ polyketo-intermediate had lost all the $^{2}H$ except for the starting methyl prior to the cyclization.[5b] The other is the loss of $^{2}H$ at the stage of the symmetrical intermediate (**2a** or **2b**), in which the rate of exchange of $\alpha$ and $\beta$ hydrogen atoms is different. The loss of $^{2}H$ from potential sites of labelling requires further study. Next we tried to isolate cometabolites of scytalone in the hope of obtaining potential intermediates. Ethyl acetate extracts of cultured broth were chromatographed on silica gel. In addition to known metabolites, scytalone (**1**) and flaviolin (**12**), four new metabolites, *cis*-4-hydroxyscytalone (**13**),[11] *cis*-4-hydroxy-6-deoxyscytalone (**14**), 5-hydroxyscytalone (**15**) and *cis*-4-hydroxyscytalone-3,4-acetonide (**16**), were isolated and identified. The last compound seems to be an artifact formed during the isolation, and the other compounds do not appear to be intermediates in the biosynthesis of scytalone (**1**).

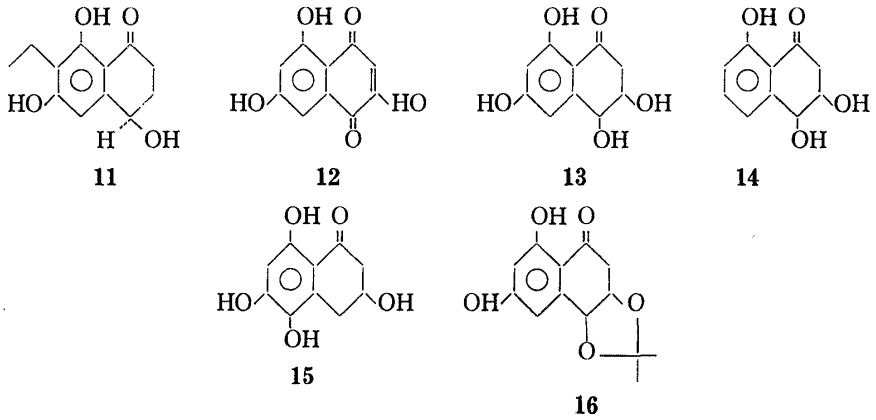


Fig. 5

Since we first used [2-$^{13}C$, 2-$^{2}H_3$]-acetate in polyketide biosynthesis, this method has become one of the standard methods to trace the fate of acetate hydrogen. This method is particularly effective in detecting the number of $^{2}H$ atoms at labelling sites and cannot be replaced by other methods.[6]

## Experimental

Proton noise decoupled $^{13}C$-NMR spectra were measured with a JEOL FX-100 and the deuterium noise decoupled $^{13}C$-NMR spectrum was obtained with a Varian XL-100. [1-$^{13}C$], [2-$^{13}C$] and [1,2-$^{13}C_2$]-acetate were obtained from Merck Sharp and Dohme Canada, Ltd., and [2-$^{13}C$]-malonate from British Oxygen Company, Ltd. [$^{14}C$]-Acetate and malonate were purchased from Radioisotope Association. A strain of *Phialophora lagerbergii* was obtained from CMI.

**Synthesis of [2-$^{13}C$, 2-$^{2}H_3$]-acetate**——[2-$^{13}C$]-Malonate (90.6 atom %) (255 mg) was dissolved in $^{2}H_2O$ (99 atom%) (3 ml) and $^{2}H_2O$ was removed *in vacuo*. This procedure was repeated three times. Deuterated malonate obtained by this exchanging reaction was decarboxylated by heating at 160—180°C for 15 min and distilled under reduced pressure. The distillate was condensed in a trap cooled with dry ice and acetone.

[2-$^{13}$C, $^{2}H_4$]-Acetic acid thus obtained was dissolved in $H_2O$ and neutralized with 2 N NaOH. On removal of $H_2O$ by evaporation, [2-$^{13}$C, 2-$^{2}H_3$]-sodium acetate (195 mg) was obtained in a yield of 95%.

**Incorporation of Labelled Compounds**——Incorporation experiments with [$^{13}$C] and [$^{13}$C, $^{2}$H]-acetate were carried out after the establishment of feeding conditions by repeated experiments with [$^{13}$C]-acetate. *Phialophora lagerbergii* (IMI 96745) maintained on Czapec-Dox slant was inoculated in 500 ml Erlenmeyer fllasks each containing 150 ml of Czapec-Dox medium supplemented with 0.1% yeast extract (DIFCO) and 5% sucrose and grown on a shaker at 200 rpm for 7 days. Five ml aliquots of precultured medium were inoculated in the fresh medium. On the 5th day of production culture [1,2-$^{13}C_2$]-sodium acetate (99.5 mg; 90 atom%) and [1-$^{14}$C]-sodium acetate ($9.66\times10^5$ dpm; $8.16\times10^5$ dpm/mM) dissolved in 5 ml $H_2O$ were added to two flasks. The culture was harvested on the 9th day of production culture and filtered to remove mycelia. The filtrate was adjusted to pH 2 with 2 N HCl and extracted with EtOAc. EtOAc was removed and the residue was chromatographed on silica gel which had been treated with 0.5 N oxalic acid and reactivated. Elution with $CHCl_3$: MeOH (95: 5) gave scytalone (**1**) (140.8 mg; $1.35\times10^5$ dpm/mM). The culture administered [2-$^{13}$C, 2-$^{2}H_3$]-sodium acetate (123.8 mg) and [1-$^{14}$C]-sodium acetate ($2.72\times10^7$ dpm; $1.60\times10^7$ dpm/mM) gave labelled scytalone (**1**) (57.6 mg; $5.35\times10^6$ dpm/mM).

**Isolation of Scytalone and Other Cometabolites**——A large-scale cultivation of *Phialophora lagerbergii* (IMI 96745) was performed in 65 flasks with the same medium as used in the incorporation experiments. After a 7 day preculture, production culture was continued for 11 days. The filtrate of broth (9.75 l) was acidified with 1 N HCl and extracted with EtOAc. The extract was chromatographed on silica gel with $CHCl_3$–MeOH (95: 5—90: 10) to give scytalone (4.43 g) (**1**), flaviolin (312 mg) (**12**), *cis*-4-hydroxyscytalone (100 mg) (**13**), *cis*-4-hydroxy-6-deoxyscytalone (10 mg) (**14**), 5-hydroxyscytalone (10 mg) (**15**) and *cis*-4-hydroxyscytalone-3,4-acetonide (8 mg) (**16**). *cis*-4-Hydroxyscytalone (**13**), mp 168—170°C (benzene–acetone). MS *m/z*: Calcd for $C_{10}H_{10}O_5$: 210.0528. Found: 210.0548. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3540, 1695, 1633, 1600, 1490, 1375. UV $\lambda_{max}^{EtOH}$ nm(log $\varepsilon$): 216 (3.08), 235 (2.60), 285 (3.02), 324 sh (2.60). $^1$H-NMR (acetone-$d_6$) $\delta$: 2.70 (d, $J=5$, C-2 $H_2$), 4.28 (m, C-3 H), 4.76 (d, $J=3$, C-4 H), 6.15 (d, $J=2$, C-7H), 6.58 (d, $J=2$, C-5H), 12.64 (s, C-8 OH). *cis*-4-Hydroxy-6-deoxyscytalone (**14**), mp 83—85°C (benzene–acetone–hexane). MS *m/z*: Calcd for $C_{10}H_{10}O_4$: 194.0579. Found: 194.0582. IR $\nu_{max}^{KBr}$ cm$^{-1}$ 3480, 3020, 1640, 1450, 1310, 1236. UV $\lambda_{max}^{EtOH}$ nm (log $\varepsilon$): 260 (3.62), 355 (2.96). $^1$H-NMR (acetone-$d_6$) $\delta$: 2.92 (m, C-2 $H_2$), 4.38 (m, C-3 H), 4.90 (d, $J=3$, C-4 H), 7.10 (dd, $J=2$, 9, C-5 H), 7.49 (t, $J=9$, C-6 H), 6.80 (dd, $J=2$, 9, C-7 H), 12.40 (s, C-8 OH). 5-Hydroxyscytalone (**15**), mp 164—165°C (dec.) (benzene–acetone). MS *m/z*: Calcd for $C_{10}H_{10}O_5$: 210.0528. Found: 210.0581. IR $\nu_{max}^{KBr}$ cm$^{-1}$: 3260, 3200, 1675, 1663, 1545, 1502, 1463. UV $\lambda_{max}^{EtOH}$ nm (log $\varepsilon$): 243 (3.44), 288 (3.42), 356 (2.76). $^1$H-NMR (acetone-$d_6$) $\delta$: 2.5—3.2 (m, C-2 $H_2$, C-4 $H_2$), 4.28 (m, C-3 H), 6.18 (s, C-7 H), 12.66 (s, C-8 OH). *cis*-4-Hydroxyscytalone-3,4-acetonide (**16**), mp 157—158°C (benzene–hexane). MS *m/z*: Calcd for $C_{13}H_{14}O_5$: 250.0841. Found: 250.0866. IR $\nu_{max}^{EtOH}$ cm$^{-1}$: 3240, 2980, 2910, 1652, 1625, 1602, 1458. UV $\lambda_{max}^{EtOH}$ nm (log $\varepsilon$): 218 (2.83), 235 (2.30), 283 (2.60), 326 sh (2.02). $^1$H-NMR (acetone-$d_6$) $\delta$: 1.16, 1.42 (s.Me$\times$2), 2.96 (m, C-2 $H_2$), 4.64 (dd, $J=4,5$, C-3 H), 5.07 (d, $J=4$, C-4 H), 6.50 (dd, $J=1$, 2, C-5 H), 6.28 (d, $J=2$, C-7 H), 12.62 (s, C-8 OH).

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