*Monocerin Biosynthesis*

# Synthesis and Incorporation of the First Polyketide Synthase Free Intermediate in Monocerin Biosynthesis**

*Lorraine C. Axford, Thomas J. Simpson,* and Christine L. Willis*

Monocerin (**5**) is a polyketide fungal metabolite that exhibits antifungal, insecticidal, and plant pathogenic properties. It has been isolated from several fungal species, including *Dreschlera monoceras*,[1] *D. ravenelii*,[2] *Exserohilum turcum*,[3] and, along with the related fusarentin ethers, for example, **4**, from *Fusarium larvarum*.[4] Polyketide metabolites are pro-

[*] L. C. Axford, Prof. T. J. Simpson, Prof. C. L. Willis
School of Chemistry
University of Bristol
Cantock's Close, Bristol, BS8 1TS (UK)
Fax: (+44) 117-9298611
E-mail: tom.simpson@bristol.ac.uk

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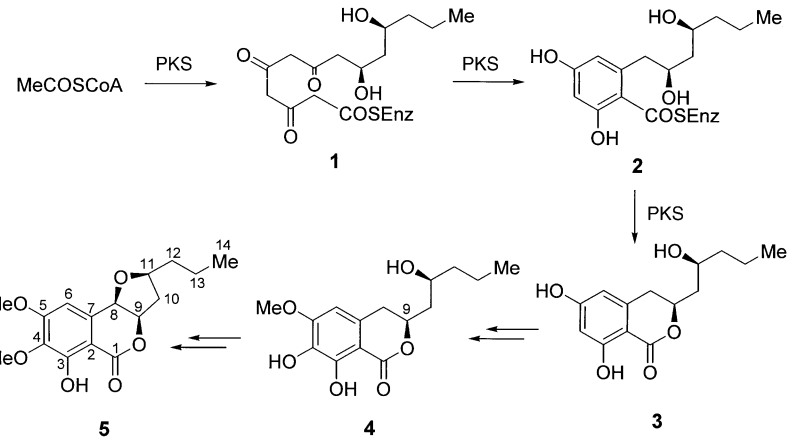
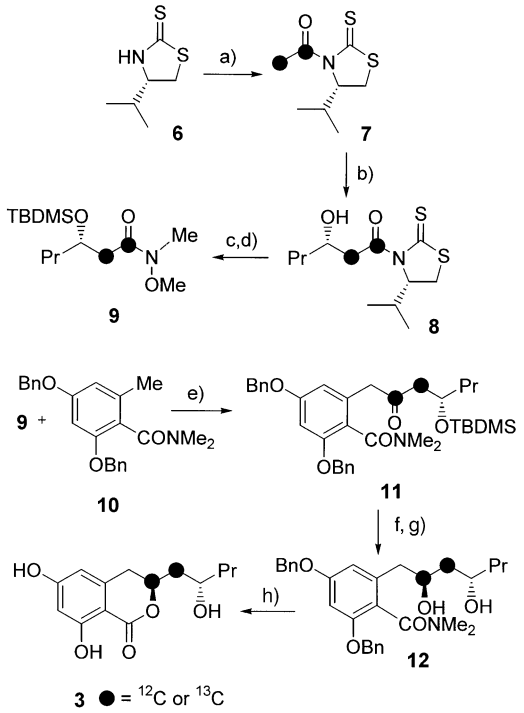
duced via linear, highly functionalized "polyketide" intermediates that are assembled from simple acyl coenzyme A (CoA) precursors by multifunctional enzyme complexes—polyketide synthases (PKSs)—the exact nature of which varies according to the source: bacteria, fungi, or higher plants.[5] In fungi, all PKSs characterized to date belong to the Type I class and consist of a single multidomain protein encoded by a single gene,[6] but the isolation and characterization of fungal PKSs remains a significant challenge. To investigate this problem we have developed a polymerase chain reaction (PCR) based method that uses oligonucleotide primers designed on consensus sequences from the keto-synthase or methyl-transferase domains of known PKS genes.[7] A complication with fungi, however, is that they frequently contain several polyketide pathways and so isolation of the desired PKS gene may still require a reverse genetics approach. Ideally, this would involve an assay based on the product of the PKS of interest, which is not usually the same as the product isolated from fungal fermentations. The isolated metabolite will almost always contain functionality introduced during PKS-catalyzed assembly and cyclization and also structural modifications effected by post-assembly "tailoring" enzymes.[5] As these modifications are not necessarily readily distinguishable, the exact structure of the PKS-derived intermediate is often uncertain.

In the case of monocerin (**5**), stable-isotope labeling studies[2] suggest that the immediate PKS-derived product should be the dihydroisocoumarin **3**. This would be formed as shown in Scheme 1 by cyclization of the linear heptaketide **1** to give the enzyme-bound orsellinate analogue **2**. Lactonization by intramolecular displacement of the thioester linkage to the PKS by the 9-hydroxy group would give **3** as the first enzyme-free intermediate in monocerin biosynthesis. There is much current interest in the engineering and heterologous expression of PKS genes from different sources to permit the rational production of novel structures.[6,8] The monocerin PKS is likely to be of particular interest in this context as it produces an intermediate, **1**, with an initial high level of reductive modification and ending with a more classical poly-β-ketide moiety. Thus, a synthesis of **3** was required for two reasons: first, in isotopically labeled form for incorporation studies to establish its obligate intermediacy on the monocerin biosynthetic pathway, and second as a standard for an assay (for example, HPLC) to facilitate detection of monocerin PKS activity in cell-free extracts of monocerin-producing cultures. Subsequent isolation and purification of the PKS protein will allow partial peptide sequencing and design of specific oligonucleotide probes for isolation of the monocerin PKS gene.

Dihydroisocoumarin **3** was prepared as shown in Scheme 2 by modification of our previously described convergent route[9] with coupling of the Weinreb amide **9** and the benzylic anion derived from benzamide **10** as the key carbon–carbon bond-forming step. To obtain **3** with vicinal $^{13}C$ labels for incorporation studies, sodium [$^{13}C_2$]acetate was converted




***Scheme 1.*** Proposed biosynthesis of monocerin (**5**) via PKS-bound and post-assembly intermediates.




***Scheme 2.*** Synthesis of dihydroisocoumarin **3**. Reagents and conditions: a) $NaN(SiMe_3)_2$, THF, −78 °C; then $MeCO_2COCMe_3$, 5 h, 35%; b) $Sn(OTf)_2$, 1-ethylpiperidine, $CH_2Cl_2$, −40 °C, 4.5 h; then butanal, −78 °C, 20 min, 88%; c) $Et_3N$, MeNHOMe, $CH_2Cl_2$, 25 °C, 72 h, 92%; d) TBDMSOTf, 2,6-lutidine, $CH_2Cl_2$, −25 °C, 1 h; then −25 °C→RT, 12 h, 98%; e) *sec*-BuLi, THF, −72 °C, 15 min; then **9**, −72 °C, 1.25 h, 45%; f) HCl/EtOH (1%), 25 °C, 5 h, 87%; g) AcOH, $Me_4N{\cdot}B(OAc)_3H$, MeCN, 6.25 h, −10 °C, 92%; h) 3 N HCl/dioxane (1:1), reflux, 48 h, 83%. Bn = benzyl, TBDMS = *tert*-butyldimethylsilyl Tf = triflate = trifluoromethanesulfonyl, THF = tetrahydrofuran.

with pivaloyl chloride into the mixed anhydride,[10] which was then used to acetylate the sodium salt of the thiazolidinethione **6**. A stereoselective aldol reaction[11] of the acetylated auxiliary **7** with butanal in the presence of tin triflate and 1-ethylpiperidine gave alcohol **8** as a single diastereoisomer. Displacement of the auxiliary with *N*,*O*-dimethylhydroxylamine and triethylamine[11] followed by TBDMS protection of the secondary alcohol gave the required Weinreb amide **9**.

The benzylic anion, formed by treatment of a rigorously deoxygenated THF solution of benzamide **10**[12] with *sec*-butyllithium under argon, was treated with **9** to give ketone **11** in 45% optimized yield. Deprotection of the silyl ether and stereoselective reduction[13] of the resultant β-hydroxyketone gave the *anti* diol **12** as a single diastereoisomer. Lactone formation and removal of the benzylic ethers were effected in a single step by refluxing in 3 N HCl.

Preliminary fermentation studies showed that monocerin production in *D. ravenelii* commenced after about five days and increased steadily to give a yield of approximately 200 mg $L^{-1}$ after a total of 14 days growth. [9,10-$^{13}C_2$]-Labeled **3** was pulse fed (30 mg in 0.6 mL of dimethyl sulfoxide (DMSO), three aliquots at 2 h intervals) to three flasks of fresh culture after 96 h. After a further 144 h, the cultures were worked up and monocerin (**5**) was isolated by flash column chromatography and further purified by HPLC. Initial analysis of the isolated metabolite by $^1$H NMR spectroscopy showed that exceptionally high-level (60%) incorporation of intact isotopic label from **3** had occurred. The signal assigned to the proton 9-H shows the normal (natural abundance) multiplet at $\delta = 5.06$ ppm flanked by corresponding multiplets due to one-bond $^{13}$C–$^1$H coupling ($^1J = 159$ Hz) from the incorporation of the $^{13}$C label from **3** at C-9. Similar $^1$H–$^{13}$C couplings are seen for the signals due to the diastereotopic methylene protons centered at $\delta = 2.52$ and 2.09 ppm ($^1J = 130$ and 135 Hz, respectively; Figure 1). In addition to the one-bond coupling, the latter signal also shows a clear two-bond $^1$H–$^{13}$C coupling ($^2J = 6$ Hz) to the vicinal $^{13}$C label incorporated at C-9. The high specific incorporation of **3** into monocerin was confirmed in the $^{13}$C NMR spectrum ($\delta_C = 39.1$ and 81.2 ppm, $^1J = 37$ Hz) and the mass spectrum ([$M^+$] 308 (73%), [$M+2$] 310 (100%)).

We have analyzed the fermentation extracts for **3**, but within the limits of sensitivity of HPLC we were not able to demonstrate its presence. Initially this may be surprising, but given the remarkable efficiency with which **3** is incorporated into monocerin, we would not expect it to be present in observable quantities in the cultures. The rate of conversion must be so high that on release from the PKS it is immediately further converted into monocerin, and so the steady-state concentration of **3** will be negligible.

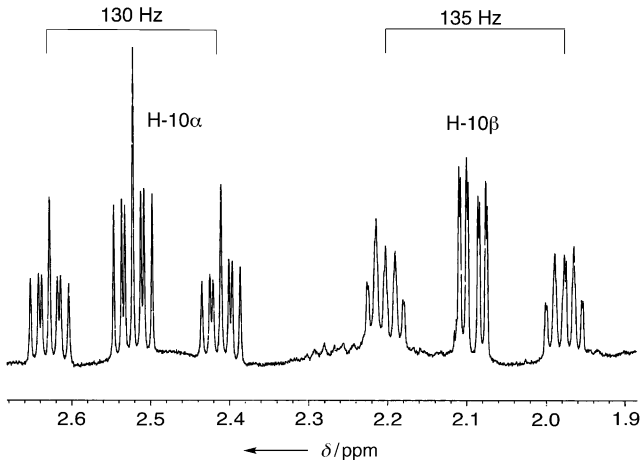



***Figure 1.*** 600 MHz $^1$H NMR spectrum showing the 10-$H_2$ signals of monocerin (**5**) enriched from incorporation of [9,10-$^{13}C_2$]-labeled dihydroisocoumarin **3**.

The results of this feeding study demonstrate that an exceptionally high level of incorporation of dihydroisocoumarin **3** into monocerin occurs, and this provides compelling evidence for the role of **3** as an essential intermediate. The level of incorporation, taken along with the previous stable-isotope labeling experiments[2] is entirely consistent with the proposed role of **3** as the first enzyme-free intermediate on the pathway and, thus, the final product of the PKS-catalyzed part of the pathway. This work has paved the way for the use of **3** in further studies to isolate and sequence the monocerin PKS gene. These are in progress.

## *Experimental Section*

[9,10-$^{13}C_2$]-Labeled **3**: This was prepared according to the route shown in Scheme 2 from sodium [1,2-$^{13}C_2$]acetate (1.50 g, 17.86 mmol) to give dihydroisocoumarin **3** as an oil (169 mg); $[\alpha]_D^{25} = +15.6$ (MeOH, $c = 0.8$); IR (film): $\tilde{\nu}_{max} = 3285, 1629, 1465, 1378, 1255, 1165$ cm$^{-1}$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 10.98$ (s, 1H, 3-OH), 8.35 (br s, 1H, 5-OH), 6.28 (d, $J = 2.2$ Hz, 1H, ArH), 6.14 (br s, 1H, ArH), 4.78 (dm, $J = 140$ Hz, 1H, 9-H), 4.05 (br s, 1H, 11-OH), 4.05 (m, 1H, 11-H), 2.82 (m, 1H, 8-*H*H), 2.71 (dm, $J = 13.9$ Hz, 1H, 8-H*H*), 1.94 (dm, $J = 140$ Hz, 1H, 10-*H*H), 1.69 (dm, $J = 140$ Hz, 1H, 10-H*H*), 1.30–1.55 (m, 4H, 12-$H_2$ and 13-$H_2$), 0.92 (t, $J = 6.8$ Hz, 3H, 14-$H_3$) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$), enriched signals only: $\delta =$ 76.4 (d, $J = 40$ Hz, C-9), 41.8 (d, $J = 40$ Hz, C-10) ppm; MS (CI): *m*/*z* (%): 269 [$M^+ + 1$] (40%), 251 (100), 233 (16), 196 (22), 178 (15), 83 (29); HRMS (CI): calcd for $C_{12}{}^{13}C_2H_{19}O_5$ [$M^+ + 1$]: 269.1300; found: 269.1288.

Isolation of monocerin (**5**): *Dreschlera ravenelii* (CBS 200.29) was stored as a spore suspension in 20% glucose solution at −78 °C. Potato dextrose agar plates were inoculated with spore suspension (200 μL), spread with a glass spreader, and incubated upside down at 28 °C for seven days. A spore suspension prepared from this plate was used to inoculate further potato dextrose agar plates and, after growth, was stored in the dark at 4 °C. *D. ravenelii* was observed as fluffy pale gray mycelia with a dark underside. A spore suspension was made from two agar plates with sterile water (15 mL) and filtered through glass wool. This was used to inoculate liquid culture medium (100 mL) in five Erlenmeyer flasks (500 mL). The liquid medium was made from D-fructose (50.0 g), mycological peptone (2.0 g), sodium nitrate (2.0 g), potassium dihydrogen phosphate (1.0 g), potassium chloride (0.5 g), magnesium sulfate heptahydrate (0.5 g), and iron(II) sulfate heptahydrate (0.01 g) dissolved in MilliQ purified water (1000 mL). The fungus was grown as a static culture at 25 °C for 14 days. The mycelial mat was filtered through Merck filter paper and washed with distilled water. The filtrate was extracted into ethyl acetate (4 × volume), the organic phases were combined and dried over $MgSO_4$, and the solvent was removed in vacuo to yield an orange oil. This was purified by column chromatography on silica, with a gradient eluent of 20→50% EtOAc in petrol, to give monocerin (**5**; 98 mg from 5 Erlenmeyer flasks, corresponding to 196 mg $L^{-1}$) as a yellow oil; $R_f = 0.31$ (silica gel, EtOAc/petroleum ether 40–60 (1:1)); $[\alpha]_D^{21} = +48.2$ (MeOH, $c = 1.1$), literature value[14] $= +53$ (MeOH, $c =$ 0.85); IR (film): $\tilde{\nu}_{max} = 2928, 2856, 2253, 1664, 1375, 1274, 1120, 787, 731$ cm$^{-1}$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 11.29$ (s, 1H, ArOH), 6.60 (s, 1H, 6-H), 5.06 (ddd, $J = 6.2, 3.3, 1.0$ Hz, 1H, 9-H), 4.55 (d, $J =$ 3.3 Hz, 1H, 8-H), 4.12 (m, 1H, 11-H), 3.95 (s, 3H, $OCH_3$), 3.89 (s, 3H, $OCH_3$), 2.60 (ddd, $J = 14.6, 8.6, 6.0$ Hz, 1H, 10-*H*H), 2.16 (ddd, $J =$ 14.6, 6.2, 1.0 Hz, 1H, 10-H*H*), 1.71 (m, 1H, 12-*H*H), 1.57 (m, 1H, 12-H*H*), 1.40 (m, 2H, 13-$H_2$), 0.92 (t, $J = 7.3$ Hz, 3H, 14-$H_3$) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta = 167.8$ (C-1), 158.8 (C-5), 157.5 (C-

3), 137.0 (C-4), 131.2 (C-7), 104.5 (C-6), 101.9 (C-2), 81.3 (C-9), 78.8 (C-11), 74.5 (C-8), 60.7 ($CH_3O$), 56.3 ($CH_3O$), 39.1 (C-10), 38.1 (C-12), 19.2 (C-13), 14.0 (C-14) ppm; MS (EI): *m*/*z* (%): 308 [$M^+$] (100%), 265 (52), 209 (30).

Feeding study: Culture medium (100 mL) in four Erlenmeyer flasks (500 mL) was inoculated with spore suspension (1.2 mL) obtained from one agar plate (with 5 mL of sterile water) and grown as a static culture at 25 °C. After 96 h, the cultures in three flasks were fed with $^{13}$C-labeled compound **3** (30 mg) in DMSO (0.6 mL, divided equally between the three flasks). The feeding was pulsed into three batches, with each culture receiving 67 μL every two hours. After a further 144 h, the mycelia were filtered and extracted into ethyl acetate as usual. The organic extract was purified by column chromatography, with a gradient eluent of 20→50% EtOAc in petrol, to give a mixture of [$^{13}C_2$]-labeled monocerin and **3**. This mixture was then dissolved in ethyl acetate/hexane (ca. 1:1) and purified further by preparative HPLC on a Luna 5μ silica normal-phase column (250 × 21.20 mm) at 254 nm with an ethyl acetate/hexane gradient. [9,10-$^{13}C_2$]-Monocerin was isolated as a pale oil (4 mg); $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 11.28$ (s, 1H, ArOH), 6.59 (s, 1H, 6-H), 5.06 (ddd, $J = 6.1$, 3.4, 1.2 Hz, 0.40H, and ddd, $J = 159$, 5.6, 2.9 Hz, 0.60H, 9-H), 4.55 (m, 1H, 8-H), 4.12 (m, 1H, 11-H), 3.95 (s, 3H, $OCH_3$), 3.90 (s, 3H, $OCH_3$), 2.60 (ddd, $J = 14.7$, 8.7, 6.1 Hz, 0.40H, and dddd, $J = 130$, 14.7, 8.7, 6.1 Hz, 0.60H, 10-H*H*), 2.16 (ddd, $J = 14.7$, 6.6, 1.0 Hz, 0.40H, and dddd, $J = 135$, 14.7, 6.6, 6.0 Hz, 0.60H, 10-*H*H), 1.71 (m, 1H, 12-H*H*), 1.59 (m, 1H, 12-*H*H), 1.38 (m, 2H, 13-$H_2$), 0.92 (t, $J = 7.3$ Hz, 3H, 14-$H_3$) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$), enriched signals only: $\delta = 81.2$ (d, $J = 37$ Hz, C-9), 39.1 (d, $J = 37$ Hz, C-10) ppm; MS (EI): *m*/*z* (%): 310 [*M*+2] (100), 308 [$M^+$] (73), 267 [*M*+2−$C_3H_7$] (62), 265 [$M^+$−$C_3H_7$] (40), 209 (63), 83 (43); HRMS (EI): calcd for $C_{14}{}^{13}C_2H_{20}O_6$ [$M^+$]: 310.1327; found: 310.1328.

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