Biosynthetic Studies on Citreohybridones, Metabolites of a Hybrid Strain KO 0031 Derived from *Penicillium citreo-viride* B. IFO 6200 and 4692

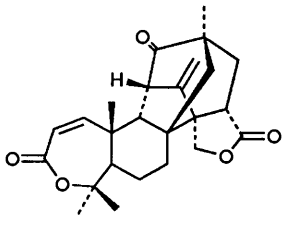
Seiji Kosemura,[a] Hiroshi Miyata,[a] Shosuke Yamamura,*[,a] Kumyul Albone,[b] and Thomas J. Simpson*[,b]

[a] *Department of Chemistry, Faculty of Science and Technology, Keio University, Hiyoshi, Yokohama 223, Japan*

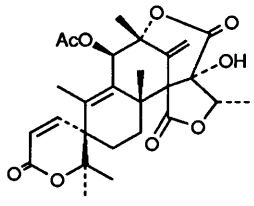
[b] *School of Chemistry, University of Bristol BS8 1TS, UK*

Incorporation of ^{13}C-labelled acetate, formate and ethyl 3,5-dimethylorsellinate into citreohybridones using a hybrid strain KO 0031 derived from *Penicillium citreo-viride* B. IFO 6200 and 4692 has established that their biosynthesis proceeds *via* a mixed polyketide-terpenoid (meroterpenoid) pathway.

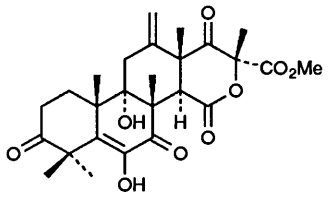
The novel metabolites, anditomin **1**,[1] austin **2**[2] and terretonin **3**,[3] were isolated from *Aspergillus variecolor*, *Aspergillus ustus*, and *Aspergillus terreus* (NRRL 6278) respectively. Based on stable isotope incorporation studies involving labelled acetate and methionine, it has been shown that these metabolites



Anditomin **1**



Austin **2**



Terretonin **3**

originate from a mixed polyketide-terpenoid (meroterpenoid) biosynthetic pathway.[4] The key step in their biosynthesis involves *C*-alkylation of the tetraketide-derived intermediate, 3,5-dimethylorsellinic acid **4**, with farnesyl pyrophosphate, giving **5** as shown in Scheme **1**. This intermediate is then cyclized and undergoes oxidation and other modifications to produce the above metabolites.[4]

Recently, we isolated seven new metabolites named citreohybridones A **8** and B **9**,[5] isocitreohybridones A **10** and B **11**,[6] citreohybriddiones A **12** and B **13**,[6] and citreohybridonol **14**, from the mycelium of the hybrid strain KO 0031 derived from *Penicillium citreo-viride* B. IFO 6200 and 4692. Interestingly, they display antifeedant and insecticidal activities against *Plutella xylostella*. Initial biogenetic analysis suggested that these metabolites could be formed by successive methyl migration and skeletal rearrangement of a sesterterpenoid containing five isoprene units or alternatively from a degraded triterpenoid. However, we now report biosynthetic experiments on citreohybridonol **14**[7] using sodium [1,2-$^{13}C_2$]acetate, sodium [^{13}C]formate, and ethyl [*carboxy*-6-$^{13}C_2$]-3,5-dimethylorsellinate, which indicate that this metabolite also is formed *via* a mixed polyketide-terpenoid (meroterpenoid) biosynthetic pathway (see Scheme 1).

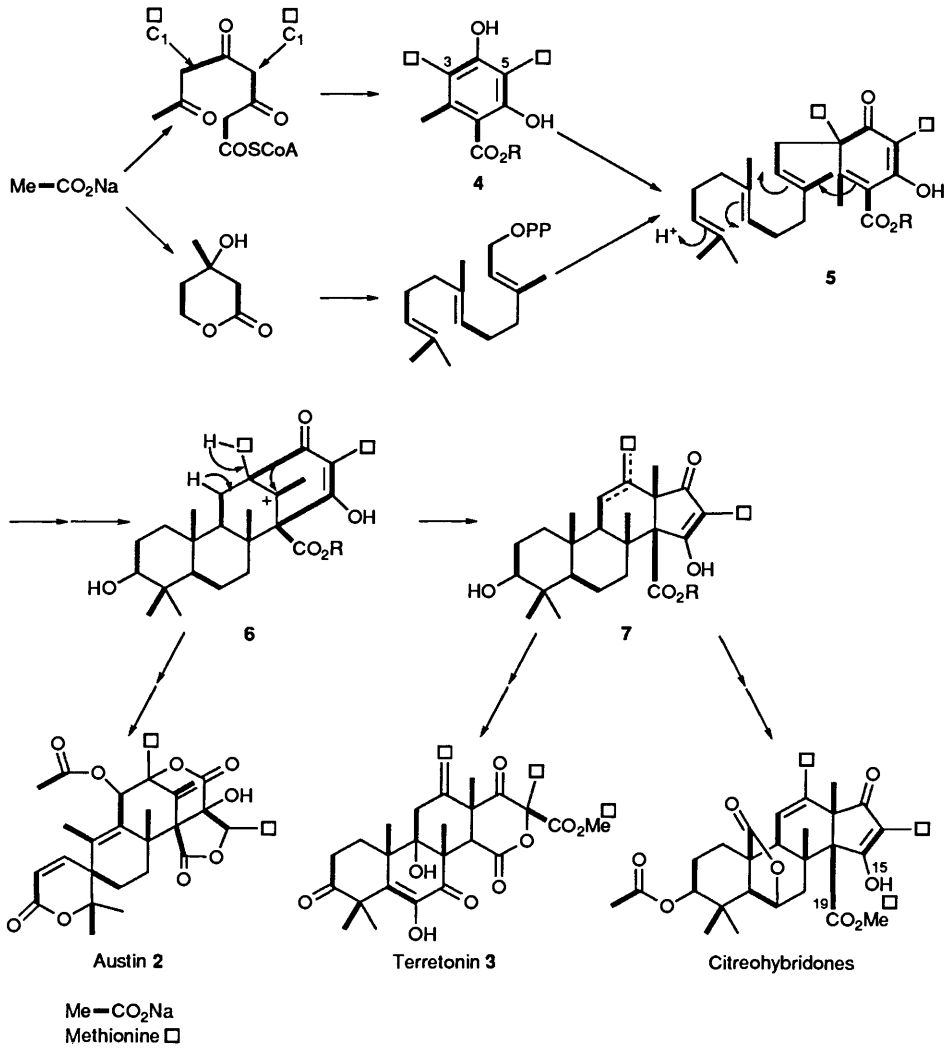
Results and Discussion

Initial biosynthetic experiments on citreohybridones were carried out using sodium [1,2-$^{13}C_2$]acetate and sodium [^{13}C]formate. According to essentially the same procedure described previously,[5-7] the ethyl acetate extract of the culture medium was directly chromatographed on silica gel. Further separation and purification by repeated preparative TLC afforded citreohybridonol **14** (0.92%). Citreohybridonol **14** exists as an equilibrium between two different ring D tautomers in both $CDCl_3$ as well as CD_3OD, which causes difficulties in signal assignments in the ^{13}C NMR spectrum. Therefore, **14** was treated with acetic anhydride–pyridine to afford citreohybridone A **8** and the ^{13}C NMR assignment (Table 1) is based on complete ^1H NMR decoupling experiments coupled with 2D-INADEQUATE experiments on [1,2-$^{13}C_2$]acetate-labelled citreohybridone A **8**. The average level of enrichment was estimated to be 12.7% based on the relative heights of the

Table 1 ^{13}C NMR data[a] for the incorporation of [1,2-$^{13}C_2$]acetate into citreohybridone A **8**

Carbon	δ	J/Hz	Carbon	δ	J/Hz	Carbon	δ	J/Hz
1	20.89		11	123.13	41.5	21	19.23	
2	22.12	36.0[c]	12	134.04		22	22.12	36.0[c]
3	75.78	36.4	13	59.88	37.6	23	178.74	49.0
4	34.52	35.3	14	69.23	57.0	24	26.47	
5	55.35	33.2	15	169.45	82.0	25	22.12	36.0[c]
6	76.71	33.2	16	131.96	82.9	26	170.62	59.5
7	37.70		17	198.96		27	20.89	59.5
8	41.10	36.5	18	9.52		28	52.32	
9	51.64	41.5	19	169.65	57.0	MeCO	164.91	
10	43.70	49.0	20	17.31	36.6	*Me*CO	21.55	

[a] ^{13}C NMR spectra were taken on a JEOL JNM-GX 400 NMR spectrometer. [b] Relative to TMS in $CDCl_3$. [c] Overlapped with other satellites' signals.



Scheme 1

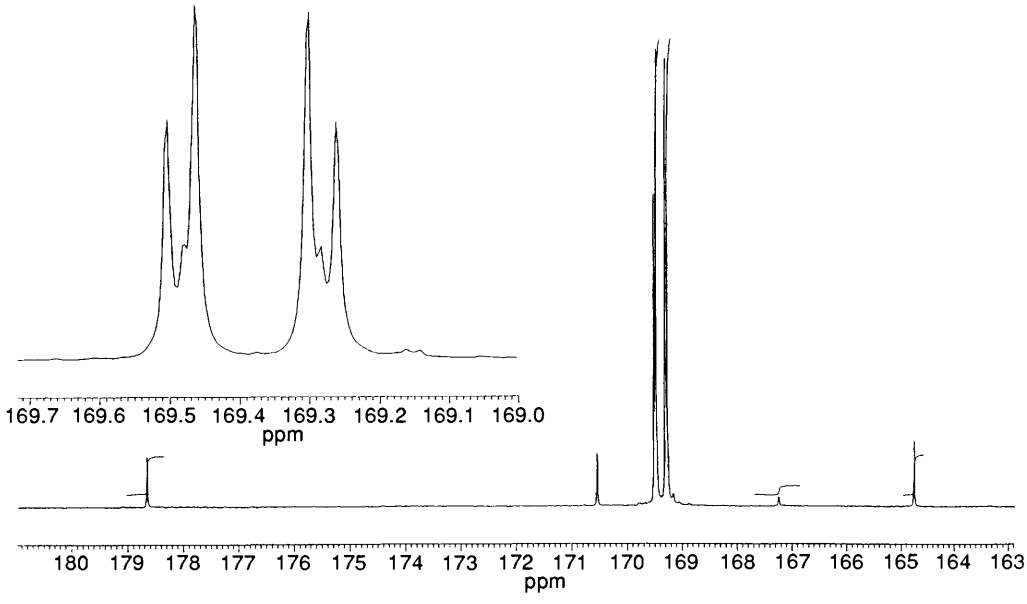
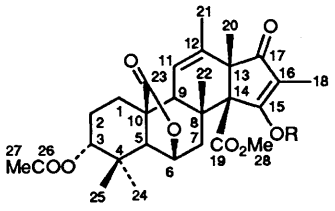


Fig. 1 100.4 MHz ^{13}C NMR spectrum of citreohybridone A labelled from ethyl [*carboxy*-6-$^{13}C_2$]-3,5-dimethylorsellinate

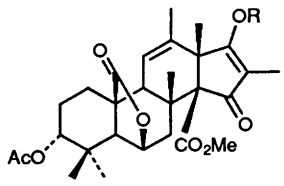
coupling satellites and natural abundance signals.† No significant differences in enrichment levels between the farnesyl and orsellinate derived carbons were observed. The [^{13}C]formate-labelled sample showed signals resulting from three highly enriched carbons (300%) corresponding to C-18 (δ 9.52), C-21 (δ 19.23) and C-28 (δ 52.32). The resulting labelling pattern of citreohybridone A is summarized in Scheme 1, and suggests that this metabolite is formed *via* a mixed polyketide-terpenoid biosynthetic pathway with the same biosynthetic intermediate **7** proposed as a precursor of terretonin **3**. Further evidence for this was provided from incorporation of ^{13}C-labelled ethyl 3,5-dimethylorsellinate[4] into citreohybridonol **14** which was converted into citreohybridone A **8** as above. The [*carboxy*-6-$^{13}C_2$]-3,5-dimethylorsellinate-labelled citreohybridone A **8** showed signals resulting from two highly enriched carbons (1100%) corresponding to C-15 (δ 169.45, $^2J_{CC}$ 3.9 Hz) and C-19 (δ 169.65, $^2J_{CC}$ 3.9 Hz) (see Fig. 1).

The labelling pattern of citreohybridone A **8** is summarized in Scheme 2 and confirms the intact incorporation of 3,5-dimethylorsellinate. The pathway shown in Scheme 2 is consistent with the pathway proposed for the biosynthesis of terretonin **3**.[4f] Ring contraction of the tetracyclic carbocation **6** is common to both pathways with the subsequent alternative proton losses indicated in Scheme 2 producing the exocyclic methylene observed in terretonin or the endocyclic double bond in the citreohybridones. Thus the citreohybridones represent a further extension of the meroterpenoid pathway[8] of which andibenin **15**, a co-metabolite of anditomin **1** in *A. variecolor*, was the first representative. Interestingly, austin **2** has also been isolated from *A. variecolor*. Metabolites related to austin have been isolated from *Penicillium diversum*[4c] and *Emericella dentata*,[9] and two unrelated metabolites which are almost certainly further products of the meroterpenoid pathway, fumigatonin **16** and paraherquonin **17** have been isolated from *Aspergillus fumigatus*[10] and *Penicillium paraherquei*,[11] respectively. The meroterpenoid pathway can now be seen to be relatively widespread in fungi.

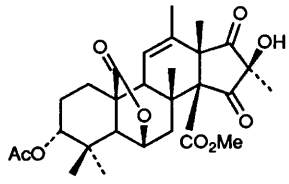
† % Enrichments were calculated by dividing the total intensity of the coupling satellites by the intensity of the non-coupled, natural abundance resonance. No systematic variation of intensities was observed.



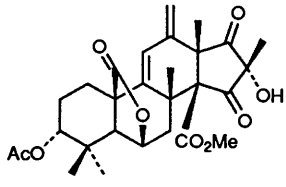
R = Ac; Citreohybridone A **8**
R = Me; Citreohybridone B **9**



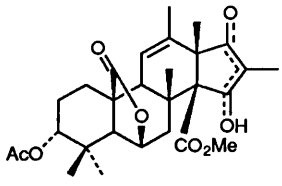
R = Ac; Isocitreohybridone A **10**
R = Me; Isocitreohybridone B **11**



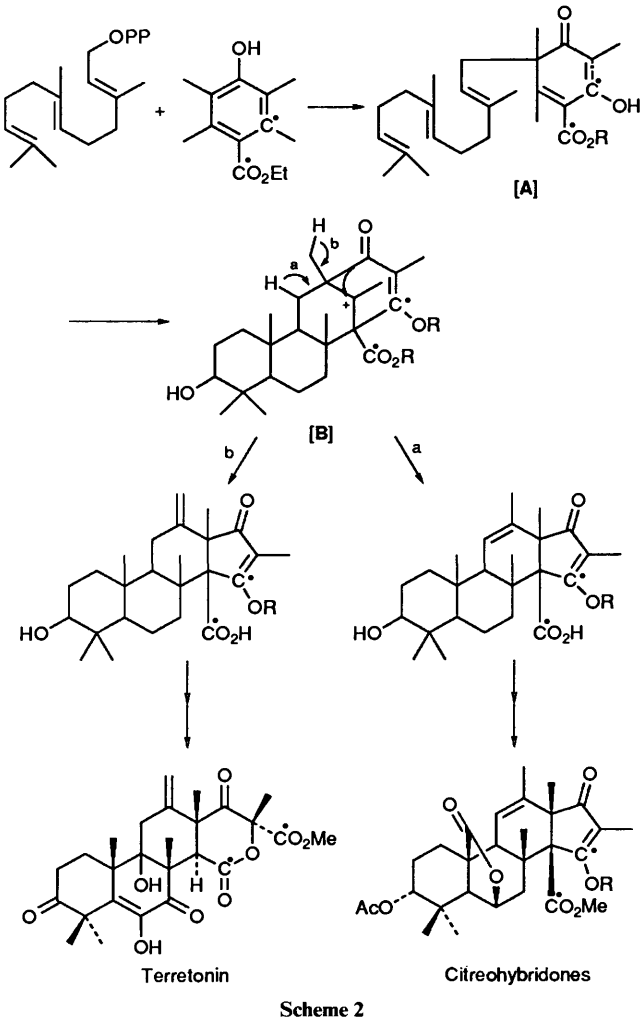
Citreohybriddione A **12**



Citreohybriddione B **13**



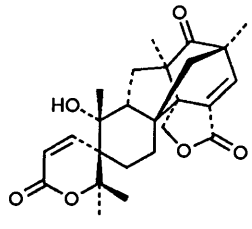
Citreohybridonol **14**



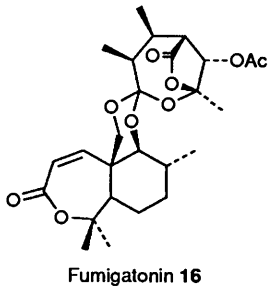
Terretonin

Citreohybridones

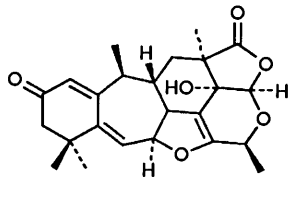
Scheme 2



Andibenin **15**



Fumigatonin **16**



Paraherquoinin **17**

Experimental

Dimethyl [1,3-$^{13}C_2$]malonate was obtained from Amersham International plc UK, ^{13}C-labelled formate and acetate from ISOTEC inc., USA. Melting points were determined on a Mitamura Riken apparatus and uncorrected. Optical rotations were measured with a JASCO DIP-360 polarimeter and are recorded in units of 10^{-1} deg cm^2 g^{-1}. IR spectra were recorded on a JASCO A-202 spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-GX 400 NMR spectrometer in [$^2H_4$]methanol or [$^2H_1$]chloroform; J values are recorded in Hz. Thin layer chromatography was performed using preparative (20 × 20 cm) glass plates coated with a 0.5 mm layer of silica gel (Merck Art. 5744 Kieselgel 60 PF_{254}. UV light of wavelength 254 nm was used to visualize chromatograms.

Incorporation of Sodium [1,2-$^{13}C_2$]*Acetate and Sodium* [^{13}C]*Formate into Citreohybridonol.*—Polished rice (540 g) in deionized water (1.4 dm^3) including sodium [1,2-$^{13}C_2$]acetate (1 g) or sodium [^{13}C]formate (1 g) was cooked using an electric rice cooker (100 °C, 20 min), and then transferred into an Erlenmeyer flask (3 dm^3 × 5). This was sterilised (121 °C, 20 min at 2.1 atm) and then inoculated with a suspension of mycelium of the hybrid strain KO 0031 in sterilised water and incubated at room temperature for 30 days. The culture was extracted with acetone and then ethyl acetate. The combined extracts were partitioned between ethyl acetate and water. The ethyl acetate extract (10.0 g) was directly chromatographed on silica gel (40 g, silica gel 60 KO70, 70–230 mesh, Katayama Chemical). After elution of higher fatty acids and their esters with benzenes, further elution with benzene ethyl acetate (3:1) afforded a pale yellow oil (180 mg), which was further separated by repeated preparative TLC (Kieselgel PF_{254}) using acetone–$CHCl_3$ (1:20 ~ 30), acetone–hexane (1:1.5 ~ 2) and then ethyl acetate–benzene (1:3) to give citreohybridonol **14** as a colourless oil (92.2 mg, 0.92%): α_D^{20} +67.3 (*c* 0.066, $CHCl_3$); (M^+, 500.240; $C_{28}H_{36}O_8$ requires *M*, 500.241); ν_{max}(film)/cm^{-1} 3200, 1770, 1740 and 1620; major tautomer (60%): δ($CDCl_3$) 5.67 (1 H, br s, 11-H), 4.72 (1 H, d, *J* 3.9, 6-Hα), 4.65 (1 H, dd, *J* 2.5, 3-Hβ), 3.67 (3 H, s, 28-H_3), 3.63 (1 H, d, *J* 14.2, 7-Hα), 2.51 (1 H, dd, *J* 14.2, 4.4, 7-Hβ), 2.02 (3 H, s, 3-OAC), 1.87 (3 H, s, 21-H_3), 1.33 (3 H, s), 1.32 (3 H, s), 0.94 (3 H, s) and 0.89 (3 H, s); other signals (δ 2.25–1.25, 9 H) are overlapped one with another; minor tautomer (40%): δ($CDCl_3$) 5.83 (1 H, br s, 11-H), 4.78 (1 H, d, *J* 3.9, 6-Hα), 4.68 (1 H, dd, *J* 2.5, 2.5, 3-Hβ), 3.61 (3 H, s, 28-H_3), 2.93 (1 H, d, *J* 14.2, 7-Hα), 2.75 (1 H, dd, *J* 14.2, 4.4, 7-Hβ), 2.40 (1 H, dd, *J* 2.4, 9-H), 2.07 (3 H, s, 3-OAc), 1.87 (3 H, s), 1.43 (3 H, s), 1.25 (3 H, s), 0.97 (3 H, s) and 0.91 (3 H, s); other signals (δ 2.25–1.25, 8 H) are overlapped one with another.

Incorporation of Ethyl 3,5-*Dimethylorsellinate into Citreohybridonol.*—Ethyl [*carboxy*-6-$^{13}C_2$]-3,5-dimethylorsellinate (96.0 atom % ^{13}C; 90.8 mg) was dissolved in hot distilled water (80 cm^3) containing 'Tween 80' detergent (8 cm^3). This sterilised solution was distributed evenly, by injection through the mycelial mat, into 4-day old stationary cultures of the hybrid strain KO 0031 (360 g of cooked rice in each of four 3-dm^{-3} Erlenmeyer flasks). The cultures were then incubated for a further 31 days at 25 °C.

The acetone extract was concentrated under reduced pressure to an acetone-free aqueous solution (1 dm^3) and then extracted with ethyl acetate (8 dm^3). The combined extracts (dark brown syrup, 4.9 g) were partitioned between ethyl acetate and water. The ethyl acetate extract (4.9 g) was directly chromatographed on silica gel (60 g, silica gel 60 K070, 70–230 mesh Katayama Chemical). After elution of higher fatty acids and their esters with benzene, further elution with benzene–ethyl acetate (3:1) afforded a pale yellow oil (560 mg), which was further separated by repeated preparative TLC (Kieselgel PF_{254}) using acetone–$CHCl_3$ (1:20–30), acetone–hexane (1:1.5–2) and then ethyl acetate–benzene (1:3) to give citreohybridonol in 1.5% yield.

Acetylation of Citreohybridonol **14**.—A solution of compound **14** (74.5 mg) in acetic anhydride (10 cm^3)–pyridine (20 cm^3) was stirred at 0 °C for 16 h after which work-up gave an oil which was subjected to preparative TLC using benzene–ethyl acetate (5:2) to afford citreohybridone A (54%) and isocitreohybridone A (11%), respectively. Citrephybridone A **8** formed colourless prisms, m.p. 261.5–263 °C in a sealed tube (from benzene–hexane); α_D^{19} −25.4 (*c* 1.00, $CHCl_3$) (M^+, 542.249. $C_{30}H_{38}O_9$ requires *M*, 542.251); ν_{max}(film)/cm^{-1} 1775, 1740, 1715, 1660 and 1240; δ_H($CDCl_3$) 0.90 (3 H, s, 25-H_3), 0.93 (3 H, s, 24-H_3), 1.27 (3 H, s, 20-H_3), 1.14 (3 H, s, 22-H_3), 1.42 [1 H, ddd, *J* 13.4, 13.4, 6.2, 1-Hα (ax)], 1.63 (3 H, s, 18-H_3), 1.71 [1 H, s, 5-Hα (ax)], 1.65–1.82 (2 H, m, 2-H_2), 1.75 [3 H, dd, *J* 2.4, 1.5, 21-Hβ (eq)], 2.16 (1 H, m, 1-H), 2.16 (3 H, s, 3-OAc), 2.29 [1 H, d, *J* 14.7, 7-Hα (ax)], 2.31 (3 H, s, 15-OAc), 2.51 [1 H, dq, *J* 2.4, 2.4, 9-Hα (ax)], 2.74 [1 H, dd, *J* 14.7, 4.3, 7-Hβ (eq)], 3.66 (3 H, s, 14-CO_2Me), 4.65 (1 H, dd, *J* 3.4, 1.8, 3-H), 4.71 (1 H, d, *J* 4.3, 6-H) and 5.68 (1 H, s, 11-H); δ_C($CDCl_3$) 9.52 (q, C-18), 17.31 (q, C-20), 19.23 (q, C-21), 20.89 (t and q, C-21 and C-27), 21.44 (q, C-30), 22.12 (t, q and q, C-2, C-22 and C-25), 26.47 (q, C-24), 34.52 (q, C-4), 37.70 (t, C-7), 41.10 (s, C-8), 43.70 (s, C-10), 51.54 (d, C-9), 52.32 (q, C-28), 55.35 (d, C(5), 59.88 (s, C-13), 69.23 (s, C-14), 75.68 (d, C-3), 76.71 (d, C-6), 123.13 (d, C-11), 131.96 (s, C-16), 134.0 (s, C-12), 164.91 (s, C-29), 169.45 (s, C-15), 169.65 (s, C-19), 170.62 (s, C-26), 178.74 (s, C-23) and 198.96 (s, C-17).

Isocitreohybridone A **10** was a colourless oil: $\alpha_D^{19.6}$ +22.6 (*c* 1.0, $CHCl_3$) (M^+, 482.228. $C_{30}H_{38}O_9$–CH_3CO_2H requires 482.230); ν_{max}(film)/cm^{-1} 1770, 1740, 1710, 1670, 1245, 1225, 1175 and 1135; δ(C_6D_6) 0.73 (3 H, s, 25-H_3), 0.76 (3 H, s, 24-H_3), 1.19 (1 H, ddd, *J* 13.4, 13.4, 6.2, 1-Hα), 1.25 (3 H, s, 20-H_3), 1.58 (3 H, s, 17-OAc), 1.62 (3 H, s, 18-H_3), 1.65 (3 H, s, 3-OAc), 1.67 (2 H, m, 2-H_2), 1.69 (3 H, dd, *J* 2.4, 1.6, 21-H_3), 1.80 (3 H, s, 22-H_3), 1.99 (1 H, s, 5-H), 2.26 (1 H, ddd, *J* 14.3, 4.8, 2.0, 1-Hβ), 2.39 (1 H, dq, *J* 2.4, 2.4, 9-H), 2.77 (1 H, dd, *J* 14.3, 4.4, 7-Hβ), 3.25 (3 H, s, 19-OMe), 3.70 (1 H, d, *J* 14.4, 7-Hα), 4.46 (1 H, d, *J* 4.4, 6-H), 4.69 (1 H, dd, *J* 3.3, 1.8, 3-H) and 5.91 (1 H, s, 11-H); δ($CDCl_3$) 7.91 (q), 17.61 (q), 20.67 (q), 20.98 (t), 21.04 (q), 21.13 (q), 22.07 (t), 22.33 (q), 25.23 (q), 26.27 (q), 34.30 (s), 36.51 (t), 43.61 (s), 43.96 (s), 51.54 (d), 51.72 (q), 52.74 (s), 54.49 (d), 72.39 (s), 76.05 (d), 77.67 (d), 124.97 (d), 125.82 (s), 137.00 (s), 164.96 (s), 169.19 (s), 170.17 (s), 174.18 (s), 179.05 (s) and 203.90 (s).

Ethyl[2,7-$^{13}C_2$]-2,4-*Dihydroxy*-3,5,6-*trimethylbenzoate* (3,5-*Dimethylorsellinate*).—The [$^{13}C_2$]-labelled 3,5-dimethylorsellinate was prepared as previously described [12] from diethyl [1,3-$^{13}C_2$]malonate (1.0 g, 6.17 mmol) and 4-methylhex-4-en-3-one (730 mg, 6.52 mmol). The crude brown oily product was purified by flash chromatography eluting with a 3–7% ethyl acetate in light petroleum (b.p. 40–60 °C) to give a pale yellow solid (230 mg, 1.02 mmol, 16.5%); δ_H($CDCl_3$) 1.40 (3 H, t, *J* 7, CH_2CH_3), 2.11 (3 H, s, S-ArCH_3), 2.16 (3 H, s, 5-ArCH_3), 2.42 (3 H, s, 2-ArCH_3), 4.39 [2 H, q (d), *J* 7 ($^2J_{CH}$ 3), CH_2CH_3], 5.30 (1 H, br s, 4-ArOH) and 11.56 (1 H, s (d) $^2J_{CH}$ 4, 2 ArOH]; δ_C($CDCl_3$), 7.9 (2 $ArCH_3$), 11.8 (5 $ArCH_3$), 14.2 (3 $ArCH_3$), 18.7 (CH_2CH_3), 61.2 (CH_2CH_3), 106.1 (C-1), 107.2 (C-2), 115.0 (C-3), 137.0 (C-5), 159.4 (C-4), 159.5 (C-6) and 172.2 ($CO_2C_2H_5$); *m*/*z* 226 (M^+, 30%), 180 (91), 152 (10), 151 (100) and 120 (11)

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