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Biosynthesis of the Benz[*a*]anthraquinone Antibiotic PD 116198

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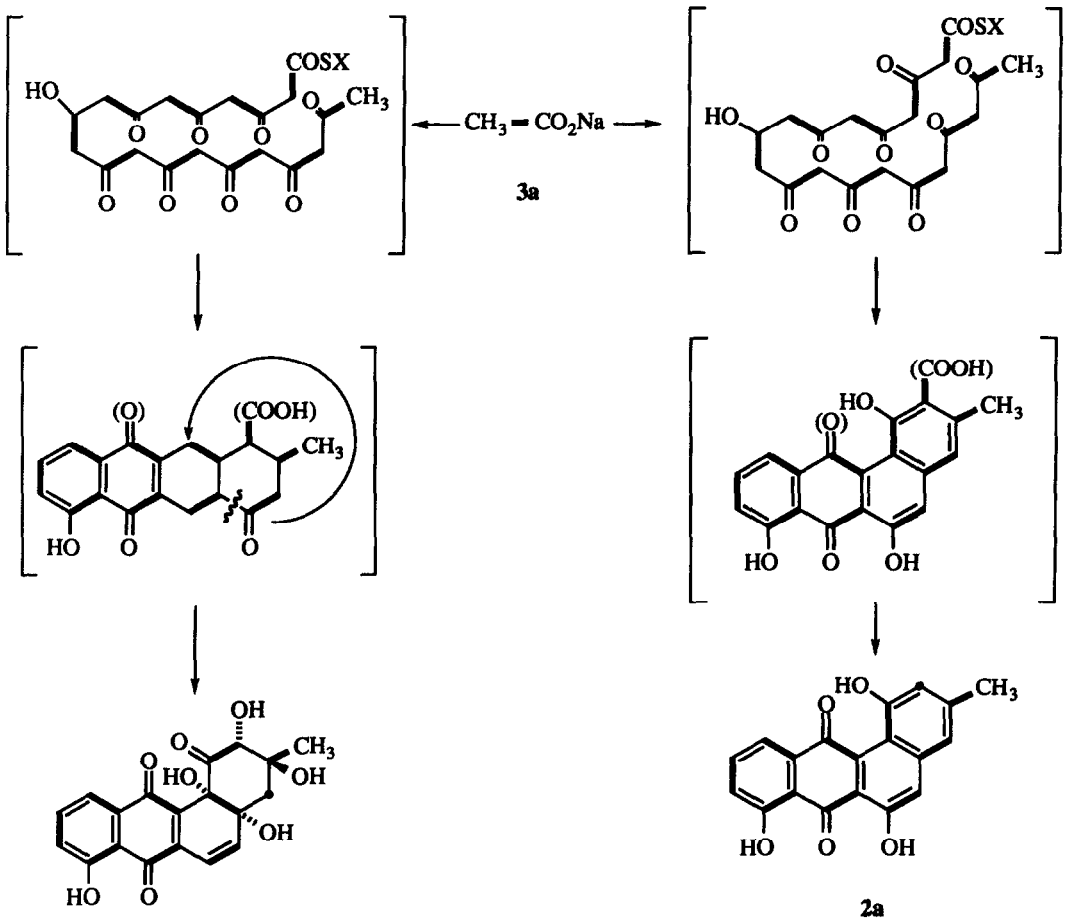
Abstract: The labeling pattern obtained from incorporation of a mixture of sodium [1-^{13}C]- and [2-^{13}C]acetates has confirmed the irregular derivation of the benz[*a*]anthraquinone skeleton of the angucycline antibiotic PD 116198. Subsequent incorporations of sodium [1-^{13}C, $^{18}O_2$]-, and [1-^{13}C, 2-$^{2}H_3$]acetates and of $^{18}O_2$ have revealed the origins of the hydrogen and oxygen atoms of the antibiotic. The possibility of a "two-chain" biosynthesis was tested by feeding ^{2}H-labeled orsellinates; however, no incorporation was detected. PD 116198 seems most plausibly derived by rearrangement of an initially-formed linear tetracyclic intermediate.

PD 116198, **1**, is a benz[*a*]anthraquinone antibiotic produced by *Streptomyces phaeochromogenes* WP 3688.[1] Its relative stereochemistry was recently established from difference NOE and 2D NOESY studies.[2] Based on this data and reported optical rotations, sakyomicin B[3] is the enantiomer of **1**, and yoronomycin[4] is a diastereomer.

During the past decade the known naturally occurring benz[*a*]anthraquinones ("angucyclines"[5]) have become a major class of polyketide metabolites.[5] The biosynthesis of five benz[*a*]anthraquinones have been reported.[6,7,8,9] In each of these cases, a straightforward polyketide origin via derivation from a decaketide intermediate that apparently folds into the angular conformation were consistent with the labeling data (e.g. as shown for dehydrorabelomycin, **2a**, in Scheme 1). However, in a sixth study,[10] an unusual labeling pattern was obtained for **1a** (Scheme 1) derived from sodium [1,2-$^{13}C_2$]acetate, **3a**. We now present results from additional feeding experiments that provide a more detailed understanding of the biosynthesis of **1**.

RESULTS AND DISCUSSION

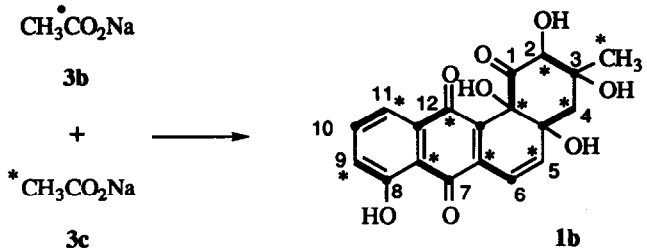
The labeling in **1a** had revealed the connectivities of carbon pairs that were derived from individual intact acetate precursor units. To confirm this unusual result, a mixture of large quantities of sodium [1-^{13}C]- and [2-^{13}C]acetates, **3b** and **3c**, respectively, was fed to *S. phaeochromogenes* WP 3688. If a sufficiently high enrichment were obtained, statistically significant levels could be expected for **1** derived from incorporation of two labeled acetates at adjacent positions within the same molecule. In this case, ^{13}C NMR analysis would reveal spin coupling between carbons from adjacent acetate precursor units.[11] Indeed, a low but measureable level of interunit ^{13}C-^{13}C couplings was observed in the ^{13}C NMR spectrum of the derived **1b**. A 2D INADEQUATE spectrum confirmed the correct pairing for each of the sixteen carbon atoms exhibiting spin coupling: C-1 and C-12b, C-2 and C-3, C-4 and C-4a, C-6 and C-6a, C-7 and C-7a, C-8 and C-9, C-10 and C-11, C-11a and C-12 (Table 1). Fortunately, these included all the D-ring carbons – where the unusual pattern had been observed in **1a** – as well as most of the carbons in the "regular" portion of the structure. These results



Scheme 1

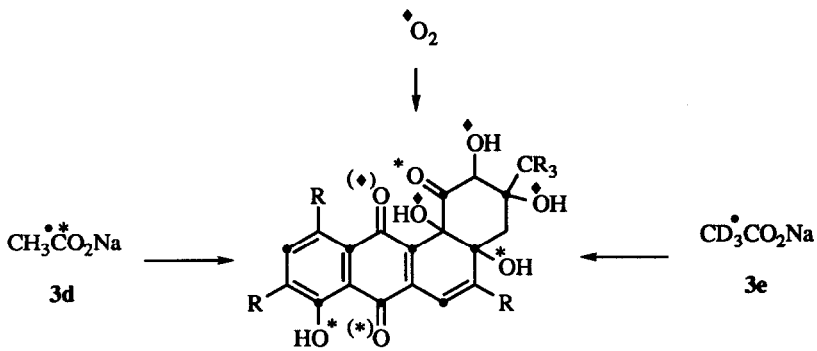
are consistent with the previous findings, and confirm that **1** could not be derived in the manner of "regular" benz[*a*]anthraquinones such as **2**.

The origins of the oxygen atoms of **1** were next addressed by feeding sodium [1-^{13}C, $^{18}O_2$]acetate, **3d**, and with a fermentation in the presence of $^{18}O_2$. The former experiment yielded **1c** (Scheme 2) in which upfield ^{18}O-induced shifts of ^{13}C resonances[12,13] were observed for C-1, C-4a and C-8, while the latter experiment yielded **1d** (Scheme 2), in which isotope induced shifts were observed for C-2, C-3 and C-12b (Table 1). While it is reasonable to have expected **3d** to have labeled the carbonyl at C-7, as well, and to have expected that $^{18}O_2$ should also have labeled the carbonyl at C-12, it is most likely that these labels were lost due to exchange with water during the period of the experiment.[14] The retention of oxygen at C-4a from acetate and



the derivation of the oxygen at C-12b from O_2 are consistent with recent findings in the biosynthesis of the urdamycins.[15] However, the derivation of the C-3 oxygen from molecular oxygen is not, indicating some flexibility in the types and patterns of late-stage modifications possible in this class of natural products.

The fates of the acetate hydrogens were established by feeding sodium [1-^{13}C, 2-^{2}H$_3$]acetate, **3e**. In this case, the ^{13}C NMR spectrum of the derived **1e** (Scheme 2) showed β-isotope shifts[16] at C-3, C-6, C-10 and C-11a (Table 1), which indicated that the hydrogens attached to C-5, C-9, C-11 and C-13 had been retained from acetate.



1c, R = D, • = ^{13}C, * = ^{16}O, ♦ = ^{16}O
1d, R = H, • = ^{12}C, * = ^{16}O, ♦ = ^{18}O
1e, R = H, • = ^{13}C, * = ^{18}O, ♦ = ^{16}O

Scheme 2

Schemes 3 and 4 show two alternative pathways that could lead to 1. The first is an expansion of our previous proposal. This retains the derivation from a single precursor chain in which initial cyclization yields a linear tetracyclic intermediate of the anthracycline class that undergoes subsequent cleavage and recyclization two carbons distant. A biological Baeyer-Villiger Reaction[17] could effect the necessary ring cleavage. The acetate labeling pattern observed for chromomycin A3[18] is fully consistent with an analogous D-ring cleavage of a tetracenomycin intermediate, in this case without a subsequent closure at another site. An alternative derivation from the coupling of products from two precursor chains was also considered. Although most polyketides that

Table I. ^{13}C NMR Spectrum of PD 116198 and Incorporation of Labeled Precursors

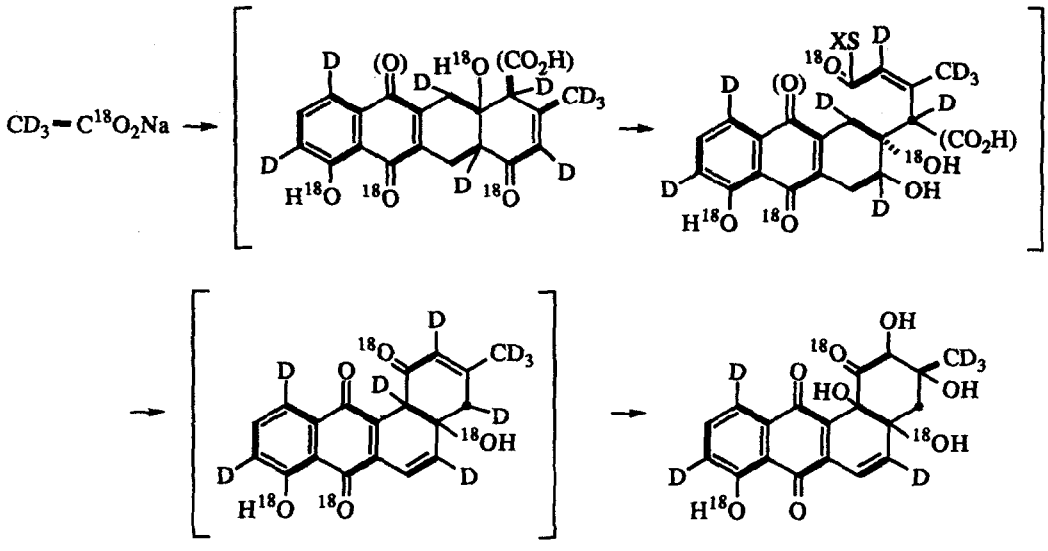
			Precursor[a]			
Carbon	Chemical[b] Shift (δ)	J_{CC} (Hz)	3b + 3c Coupled Pair	3d Δδ	$^{18}O_2$ Δδ	3e Δδ
1	206.3	54.9	a	+0.05		
2	82.8	43.8	b		+0.02	
3	76.1	43.8	b		+0.03	+0.05, 0.09
4	44.0	36.8	c			
4a	76.9	36.8	c	+0.02		
5	147.3					
6	117.1	32.7	d			+0.14
6a	138.5	32.7	d			
7	187.0	31.7	e			
7a	115.9	31.7	e			
8	116.9	44.9	f	+0.01		
9	124.8	44.9	f			
10	137.1	50.1	g			+0.11
11	119.3	50.1	g			
11a	133.0	47.0	h			+0.07
12	183.4	47.0	h			
12a	139.1					
12b	77.5	54.9	a		+0.03	
13	22.4					

[a] Sites of enrichment indicated by J_{CC} coupling constants or by isotope-induced shifts of δ.

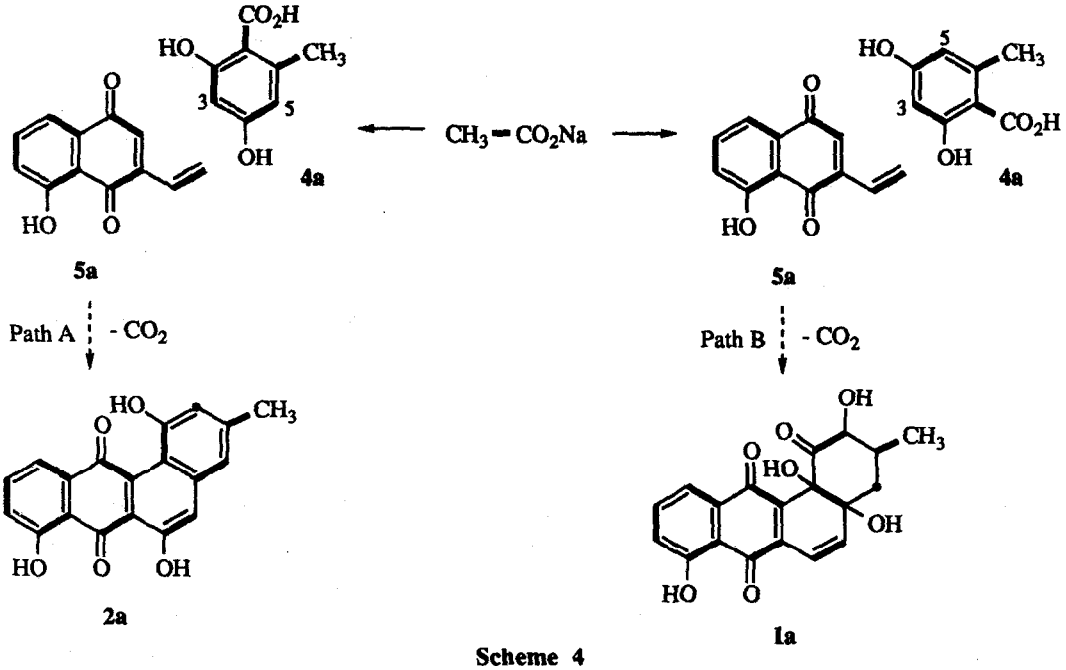
[b] Dioxane-dg, 75 MHz; spectral width 18518 Hz; 128 K data points; 3.53 sec aquisition time; 1 Hz line broadening; 15,000 – 20,000 scans.

had been anticipated to be derived from more than one precursor chain have been subsequently shown to be derived from a single chain, in at least one case[19] the experimental evidence clearly supports a two-chain biosynthesis. As shown in Scheme 4, we recognized that orsellinic acid, **4**, and naphthoquinone **5**, each derived from acetate, could condense in two different ways, and lead to the labeling pattern observed for **2** (Path A) or to the labeling pattern observed for **1** (Path B).

To test Scheme 4, we first synthesized [3,5-$^2H_2$]orsellinic acid, **4a**, and [*methyl*-$^2H_3$]orsellinic acid, **4b**,[20,21] the latter from [3,4,4,4-$^2H_4$]crotonic acid prepared from [$^2H_4$]acetaldehyde.[22] As shown in the Scheme, the deuterium label at H-3 would be lost in each case, but that at H-5 would be retained in **2** and possibly in **1**, while the deuterium-labeled methyl would be retained in both **1** and **2**. **4a** and **4b** were each fed

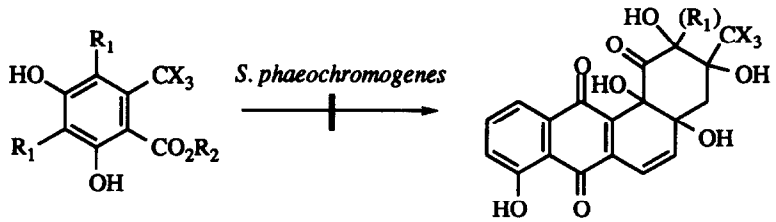


Scheme 3



Scheme 4

to *S. phaeochromogenes*; however, no deuterium enrichment was observed in the ^2H NMR spectrum of either sample of **1**. Polyketide starter and extender units have been identified by successful uptake and *in vivo* utilization of labeled diethylmalonate,[23,24] presumably by either hydrolysis followed by thioesterification with coenzyme A, or direct thioesterification. Similarly, labeled ethyl 2-deoxyorsellinate[25] and ethyl 3,5-dimethylorsellinate[26] have been successfully utilized to identify advanced intermediates in polyketide pathways. We therefore prepared and fed ethyl [3,5-$^2H_2$]orsellinate, **6a**, but again failed to observe any enrichment. However, after the latter two fermentations, neither [methyl-$^2H_3$]orsellinic acid nor [3,5-$^2H_2$]orsellinic acid were detected by HPLC, and only 50% of the ethyl [3,5-$^2H_2$]orsellinate that had been fed was still detected. It would appear that permeability of the cells was not an obstacle.



4a, $R_1 = D, R_2 = X = H$
4b, $R_1 = R_2 = H, X = D$
6a, $R_1 = D, R_2 = Et, X = H$

1

The origins of the carbons, hydrogens and oxygens of **1** have been established by *in vivo* feeding experiments. A novel two-chain biogenesis was recognized which could explain both the irregular acetate labeling patterns for the carbon skeleton of **1**, as well as the regular labeling pattern that has been observed for five other benz[*a*]anthraquinones. Tests of this hypothesis with respect to the formation of **1** were uniformly negative. Although negative data should be viewed cautiously in the absence of positive data consistent with an alternative pathway, it seems more plausible that **1** is, indeed, formed by rearrangement of a linear anthracycline-like intermediate. In an attempt to find intermediates in the pathway to **1**, other metabolites of *S. phaeochromogenes*, grown under normal conditions as well as in the presence of cytochrome P450 hydroxylase inhibitors, were examined. While a number of novel structures were found,[2] no linear decaketides have so far been detected. Work on this problem is continuing.

EXPERIMENTAL SECTION

General Procedures. Sodium [2-^{14}C]acetate was purchased from ICN pharmaceuticals, and sodium [1-^{13}C,$^{18}O_2$]-, [1-^{13}C,$^2H_3$]-, [1-^{13}C]-, and [2-^{13}C]acetates, as well as $^{18}O_2$ gas from Cambridge Isotope Laboratories. Radioactivity was measured in a Beckman LS 7800 liquid scintillation counter, and counting efficiencies were determined automatically using the Beckman DPM program and sealed [^{14}C]-quenched Beckman standards. HPLC analyses for orsellinic acid and ethyl orsellinate in fermentation broths were carried out on a Waters NovaPak C_{18} column using an acetonitrile-water gradient system and UV detection at 220 nm.

Standard Fermentation and Isolation Procedures. The fermentation, extraction and isolation procedures reported previously[10] have been modified. *S. phaeochromogenes* WP 3688 was grown on agar slants composed of cornstarch 1.0%, NZ-Amine type A 0.2%, Difco beef extract 0.1%, yeast extract 0.1%, $CoCl_2$ 0.002%, agar 2.%, pH 7.0. A seed broth (glucose 1.0%, soybean 0.5%, glycerol 0.5%, NaCl 0.3%, $CaCO_3$ 0.3%, pH 7.0; 70 ml in a 250 mL Erlenmeyer flask) was inoculated with a loopful of mycelium from an agar slant and incubatied at 28 °C/250 rpm. After 48 h, the seed culture was used to inoculate production broths of the same composition (150 mL in 1L Erlenmeyer flasks; 1% inoculum; six flasks). Production cultures were incubated at 28 °C/250 rpm, and harvested after 96 h fermentation. These were acidified with 1 N HCl to pH 4.0, and the mycelium removed by filtration. After extraction of the aqueous layer with EtOAc (2 x 0.5 L), the combined organics were dried and concentrated *in vacuo* to give crude **1**. The residue was applied to a column of flash grade SiO_2 (2 x 25 cm, 2% MeOH in $CHCl_3$), and eluted with 2% MeOH in $CHCl_3$ to remove non-polar oils and minor components. Further elution with 4% MeOH in $CHCl_3$ yielded **1** (50 mg) which was recrystallized from acetone to give a sample of pure **1** (15 mg). For feeding experiments with isotopically labeled materials, fermentations were conducted as above and precursors were added as aqueous solutions sterilized by filtration through a filter (Sterile Acrodisc, 0.2 μm, Gelman Sciences).

Incorporation of Sodium [1-^{13}C]- and [2-^{13}C]Acetates. Sodium [1-^{13}C]acetate, **3b**, (99% ^{13}C, 500 mg), [2-^{13}C]acetate (99% ^{13}C, 500 mg), **3c**, and [2-^{14}C]acetate, **3f**, (12 μCi) in water (6 mL) were fed in three portions to production cultures 20, 30, and 40 h after inoculation. Work-up gave **1b** (16 mg, 0.16% incorporation of **3f**).

Incorporation of Sodium [1-^{13}C, 2-^{2}H$_3$]Acetate. Sodium [1-^{13}C, 2-^{2}H$_3$]acetate, **3e**, (99% ^{13}C, 98% ^{2}H, 480 mg) and **3f** (12 μCi) were fed in three portions to production cultures using the same protocol. Work-up gave **1c** (17 mg, 0.29% incorporation of **3f** and 2.5% average ^{13}C enrichment from **3e** for each acetate unit).

Incorporation of Sodium [1-^{13}C, 1-^{18}O$_2$]Acetate. Sodium [1-^{13}C, 1-^{18}O$_2$]acetate, **3d**, (99% ^{13}C, 95% ^{18}O, 480 mg) and **3f** (12 μCi) were fed in three portions to production cultures using the same protocol. Work-up gave **1e** (15 mg, 0.21% incorporation of **3f** and 2.2% average ^{13}C enrichment from **3d**.

Incubation of S. phaeochromogoenes WP 3688 in the Presence of $^{18}O_2$. A 2% inoculum was added to production medium (1 L) and antifoam (1 mL, No. A 5633, Sigma) in the Vederas[27] sterile fermentor apparatus. Fermentation was started with $^{16}O_2$, and after 35 h 1.56 L of $^{16}O_2$ had been consumed. The buret was then charged with 50% $^{18}O_2$, and was replenished as needed during the following 18 h (1.75 L was used). $^{16}O_2$ was now used for the remaining 19 h, and 0.85 L was consumed. Work-up gave 13.7 mg of **1d**.

Syntheses

Ethyl 6-Methylhydroresorcylate:[20,21] Ethyl acetoacetate (4.2 g, 32 mmol) and ethyl crotonate (3.4 g, 29 mmol) were added dropwise to sodium (690 mg, 30 mmol) in abs ethanol (100 mL), and the resulting mixture

was heated at reflux for 2 h. This was then cooled, diluted with H_2O (100 mL), acidified to pH 4 with 2 N H_2SO_4, and extracted with EtOAc (2 x 200 mL). The organic extracts were washed with H_2O and sat brine, and dried and concentrated to give crude product. This was recrystallized from acetone-hexane to yield 3.23 g (56%): ^1H NMR ($CDCl_3$) δ 1.00 (3 H, d, J = 6.8 Hz), 1.20 (3 H, t, J = 7.7 Hz), 2.15 (2 H, AB of ABX), 2.50 (1 H, m), 3.00 (1 H, d, J = 10.5 Hz), 4.20 (2 H, q, J = 7.7 Hz), 5.40 (1 H, s), 9.50 (1 H, brs, exch. D_2O).

Ethyl 3-Bromoorsellinate: Bromine (6.0 g, 39 mmol) in HOAc (10 mL) was added dropwise at RT over 40 min to a mixture of ethyl 6-methylhydroresorcylate (2.5 g, 12 mmol) in HOAc (10 mL). The mixture was stirred for an additional 1 h and quenched with ice, yielding a precipitate that was collected by filtration. Recrystallization from acetone gave 2.0 (72%) of the bromoorsellinate: ^1H NMR ($CDCl_3$) δ 1.40 (3 H, t, J = 7.7 Hz), 2.65 (3 H, s), 4.45 (2 H, q, J = 7.7 Hz), 6.45 (1 H, bs),12.25 (1 H, s, exch. D_2O); ^{13}C NMR ($CDCl_3$) δ 15.1, 24.0, 63.2, 97.0, 106.4, 110.1, 142.0, 156.0, 161.3, 171.4.

3-Bromo[5-^2H]orsellinic Acid: Ethyl 3-bromoorsellinate (347 mg, 0.92 mmol) in conc D_2SO_4 (1 mL) was stirred overnight at RT, quenched with ice, and extracted with EtOAc. The organic extracts were washed with H_2O and sat $NaHCO_3$. The combined aqueous extracts were adjusted to pH 4 with 3 N HCl and extracted with EtOAc, and the organic extracts washed with H_2O, dried and concentrated. Recrystallization from acetone-hexane gave 173 mg (76%) of the acid: ^1H NMR (acetone-d_6) δ 2.60 (3 H, s).

Orsellinic Acid, 4:[21] A mixture of 3-bromoorsellinic acid (300 mg, 6 mmol), 5% Pd-C (300 mg) and Et_3N (1 mL) in MeOH (1 mL) was stirred overnight under an H_2 atmosphere. The catalyst was removed by filtration and washed with MeOH, and the combined organics concentrated to dryness. The residue was dissolved in EtOAc (60 mL) and H_2O (30 mL), the phases separated, and the aqueous phase acidified to pH 3 with 2 N HCl. This was extracted with EtOAc, and the extracts dried and concentrated. The residue was taken up in $CHCl_3$ and chromatographed on silica gel (1.5 x 15 cm) eluted successively with 2% MeOH and 10% MeOH in $CHCl_3$ to yield **4** (110 mg, 87%): mp 175 °C dec; ^1H NMR (acetone-d_6) δ 2.50 (3 H, s), 6.22 (1 H, d, J = 2.6 Hz), 6.27 (1 H, d, J = 2.5 Hz).

[3,5-$^2H_2$]Orsellinic Acid, 4a: A mixture of 3-bromo[5-^2H]orsellinic acid (300 mg, 6 mmol), 5% Pd-C (300 mg) and Et_3N (1 mL) in MeOD (1 mL) was stirred overnight under a D_2 atmosphere. Work-up as above gave **4a** (104 mg, 82%): ^1H NMR (acetone-d_6) δ 2.50 (3 H, s), 6.22 (0.28 H, s), 6.28 (0.28 H, s).

Ethyl Orsellinate, 6:[20] A mixture of ethyl 3-bromoorsellinate (500 mg, 1.45 mmol), 5% Pd-C (500 mg) and Et_3N (500 mg) in MeOH (4 mL) was stirred overnight under an H_2 atmosphere. The catalyst was removed and washed with MeOH, and the combined organics concentraed to dryness. The residue was taken up in EtOAc, washed with H_2O, dried and concentrated, and the residue recrystallized from acetone-hexane to give **6** (255 mg, 90%): mp 128-131 °C, ^1H NMR ($CDCl_3$) δ 1.40 (3 H, t, J = 9.2 Hz), 2.50 (3 H, s), 4.40 (1 H, q, J

= 9.3 Hz), 6.25 (1 H, d, J = 3.0 Hz), 6.20 (1 H, d, J = 3.0 Hz); ^{13}C NMR (acetone-d_6) δ 14.39, 24.40, 61.80, 101.55, 105.30, 112.23, 144.39, 163.08, 166.36, 172.48.

Ethyl [3,5-$^2H_2$]Orsellinate, 6a: A mixture of **6** (200 mg, 0.58 mmol) and DCl (1 mL) in acetone (1 mL) was stirred at RT overnight, and the quenched with ice. This was extracted with EtOAc, and the extracts dried and concentrated to give **6a** (185 mg, 92%): ^1H NMR ($CDCl_3$) δ 1.40 (3 H, t, J = 8.9 Hz), 2.50 (3 H, s), 4.40 (1 H, q, J = 9.2 Hz), 6.25 (0.02 H, s), 6.20 (0.02 H, s); ^{13}C NMR (acetone-d_6) δ 14.40, 24.33, 24.40, 61.80, 101.55, 105.26, 105.28, 112.22, 144.26, 144.36, 163.00, 163.08, 166.31, 166.36, 172.48.

[3,4,4,4-$^2H_4$]Crotonic Acid: [$^2H_{12}$]Paraldehyde (5 g), xylene (1 mL) and conc H_2SO_4 (1 drop) were warmed together at 60 °C, and [$^2H_4$]acetaldehyde (4 g) collected in a flask cooled in an ice bath.[22] This was added dropwise to a mixture of malonic acid (9.5 g, 92 mmol), pyridine (10.7 g) and piperidine (5 drops) at 0 °C. The mixture was stirred at 0 °C for 24 h, then at 90 °C for 4 h, cooled to 0 °C, treated with conc H_2SO_4 (1 mL), and maintained in the cold for an additional 4 h. The mixture was extracted with EtOAc, and the extracts dried and concentrated to give the crotonic acid (4.17 g, 47%): ^1H NMR ($CDCl_3$) δ 1.8 (0.8 H), 5.8 (1 H, s), 7.1 (0.03 H), 11.9 (1 H, brs, exch. D_2O).

Ethyl [3,4,4,4-$^2H_4$]Crotonate: [3,4,4,4-$^2H_4$]Crotonic acid (4.17 g, 39 mmol) and conc H_2SO_4 (0.3 mL) in EtOH (50 mL) was heated at reflux overnight. The excess EtOH was removed and the residue extracted into EtOAc. This was dried and concentrated, and the residue distilled to give the ethyl crotonate (2.99 g, 56%): ^1H NMR ($CDCl_3$) δ 1.2 (3 H, t, J = 8.1 Hz), 1.8 (0.8 H), 4.1 (2 H, q, J = 8.0 Hz), 5.8 (1 H, s).

[methyl-$^2H_3$]Orsellinic Acid, 4b: This was prepared from the deuteriated crotonic acid as described above, ultimately yielding 120 mg of **4b**: ^1H NMR (acetone-d_6) δ 2.50 (0.8 H, s), 6.20 (1 H, d, J = 2.5 Hz), 6.27 (1 H, d, J = 2.5 Hz); ^2H NMR (acetone) δ 2.50; ^{13}C NMR (acetone-d_6) δ 23.9 (m).

Feedings with [3,5-$^2H_2$]Orsellinic acid, 4a, [methyl-$^2H_3$]Orsellinic, 4b, and Ethyl [3,5-$^2H_2$]Orsellinate, 6a: [*methyl*-$^2H_3$]Orsellinic acid (125 mg) was dissolved in acetone (1 mL). To the acetone solution were added Tween 80 (1.6 mL) and sterile water (24 mL). The mixture was fed in three portions as described above, and work-up gave **1** (15 mg), which showed no deuterium enrichment in the ^2H NMR spectrum. [3,5-$^2H_2$]Orsellinic acid (200 mg) and ethyl [3,5-$^2H_2$]orsellinate (100 mg) were each fed in the same manner; again, no deuterium enrichment was observed.

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