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6-Ethyl-5-hydroxy-2,7-dimethoxy-1,4-naphthoquinone from *Hendersonula toruloidea*: A Biosynthetic Study Using ^{13}C Labels Detected by Nuclear Magnetic Resonance and ^{14}C Tracers[1]

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Production of 6-ethyl-5-hydroxy-2,7-dimethoxy-1,4-naphthoquinone was obtained by growth of *Hendersonula toruloidea* on Czapek–Dox broth supplemented with malt extract. Stationary cultures were grown at 28°C for 21–22 days yielding about 6 mg of metabolite per 700 ml of culture fluid. The best incorporations of isotopic tracers were obtained by addition at the 20th day of growth, followed by harvest 24–48 hr later. With [2-^{14}C]acetate, incorporation values were in the range of 0.1–0.3% with dilution values from 2000 to 5900. With [1-^{14}C]propionate, incorporations were much lower (0.04%) and dilutions much higher (120,000). Activity from [$^{14}CH_3$]methionine was incorporated only into the OCH_3 groups (incorporation values, 0.5–0.7%). Nuclear magnetic resonance studies confirmed that propionate was not a precursor. Using [1,2-^{13}C]acetate, substantial enrichments were obtained at all carbon atoms except those of the OCH_3 groups. The following pairs of carbon atoms were shown to be derived from acetate units: C-1 + 2, C-3 + 4, C-5 + 10, C-6 + 7, C-8 + 9, C-11 + 12. The biosynthetic pathway is clearly that of acetate plus polymalonate. Experiments with [2-$^{13}C^2H_3$]acetate suggested that the "starter" acetate unit was located at positions C-12 + 11.

INTRODUCTION

The biosynthetic routes to naturally occurring naphthoquinones and related compounds are of interest since this group of compounds includes one important primary metabolite (vitamin K) as well as many secondary metabolites. Several interesting biological properties, for example, the use of naphthoquinones in the defensive secretions of certain beetles, are associated with the secondary metabolites. Many of these naphthoquinones are derived by polyketide biosynthetic pathways (*1*). Frequently, the presence of appropriately positioned methyl and carboxyl groups, together with carbonyl and/or OH (or OCH_3) groups, provides a good preliminary indication of a biosynthetic pathway involving acetate and malonate residues. In many cases, the predictions have been confirmed experi-

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mentally. While methyl groups are commonly present in natural naphthoquinones, ethyl substituents are uncommon: of approximately 220 naphthyl aromatics listed in an authoritative compilation (*2*), only 10 contain ethyl groups.

When ethyl substituents are present in naphthoquinones or any other natural product, biosynthetic speculation is on insecure ground. While the ethyl group is often derived from a normal homopolyketide sequence (acetate plus malonate) as, for example, in diplosporin biosynthesis (*3*), propionic acid sometimes functions as a "starter" unit in a heteropolyketide mechanism and contributes an intact ethyl group. This is the case with ϵ-pyrromycinone (*4*), homoorsellinic acid (*5*), 6-ethylsalicylic acid (*6*), homononactinic acid (*7*), 2-ethyl-1,4-benzoquinone (*8*), and aurovertin B (*9*). Ethyl groups can be derived by the addition of a C_1 unit, presumably from *S*-adenosylmethionine, onto a methyl group, as exemplified by barnol (*10*) and aurovertin B (*9*). In the case of the Streptomycete antibiotic, X-537A, ethyl groups are derived from butyrate molecules (*11*). In aplasmomycin biosynthesis, a 17-carbon chain is apparently constructed from one glycerol unit plus seven acetate units, representing yet one further source for a CH_3CH_2 grouping (*12*).

In view of these challenges with respect to the biosynthetic origin of ethyl groups and our continued concern with naphthoquinones, it was of interest to study 6-ethyl-5-hydroxy-2,7-dimethoxy-1,4-naphthoquinone, which is produced by the dematiaceous fungus, *Hendersonula toruloidea*. The isolation and structural characterization of this material were reported briefly by Howe and Moore in 1969 (*13*). The problem was also of interest since not only are ethyl naphthoquinones rare, but in addition they have been found to a greater extent in Echinoderms (*14*) than in microorganisms and plants. Since the *Hendersonula* metabolite has not been given a trivial name, the abbreviation, EHDN, will be used in this paper; the numbering system for identification of individual carbon atoms is shown in Fig. 1.

EXPERIMENTAL

Hendersonula toruloidea Nattrass, IMI 135205, was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. The original culture had been acquired from Imperial Chemical Industries Ltd., Macclesfield, Cheshire, England, in 1968, and was the organism used in the work of Howe and Moore (*13*). The fungus was maintained on agar slants of the following composition: Czapek–Dox broth mixture (Difco), 35 g; yeast extract (Difco), 10 g; Bacto agar (Difco), 20 g; deionized water, 1 liter. The inoculated slants were incubated at 28°C in the dark; maximum growth was obtained after 7 days, and cultures were transferred every 2 months. For most of the biosynthetic experiments, the organism was grown as stationary liquid cultures for 21 days at 28°C; the medium contained, per liter of deionized water, Czapek-Dox broth mixture (Difco), 35 g, and malt extract (Difco), 10 g. For small-scale experiments, 75-ml portions of medium were used in 250-ml wide-mouth Erlenmeyer flasks, and for large-scale

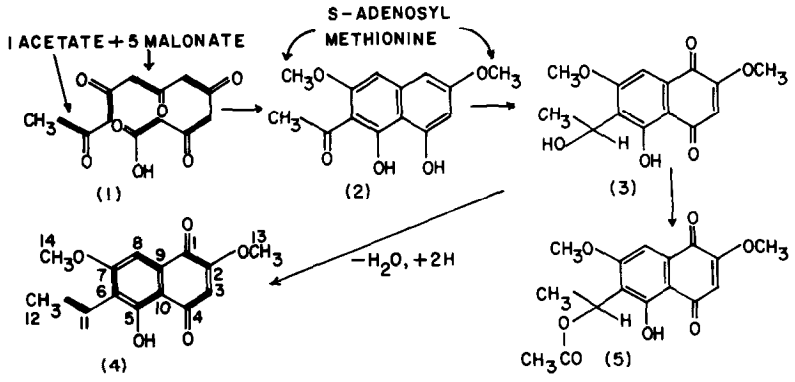

FIG. 1. Postulated biosynthetic pathway for production of EHDN and related compounds. In the initial polyketide (1) and in EHDN (4) the individual acetate units have been emphasized by the use of "thickened" bonds.

work, 700-ml portions in 2.8-liter Fernbach flasks. The contents of the flasks were inoculated with a spore suspension (1 ml); this was prepared by adding 10 ml of sterile deionized water to a tube which contained an agar slant culture of *H. toruloidea* followed by vigorous mixing on a Vortex mixer. After work with the organism had been carried out for about one year, the liquid cultures began to show irregular growth and a low production of the naphthoquinone. It was discovered that this problem could be overcome by supplementing the medium with 1% Casamino acids (Difco). Experiments using this supplemented medium will be indicated in the text.

To isolate EHDN from the 75-ml cultures, the mycelial mat and remaining medium from a single flask were placed in a 1-liter blender (Waring, explosion proof) with 75 ml ethyl acetate acidified to pH 2 by the addition of 1 ml of concentrated HCl. After running the blender at full speed for 1 min, the resulting slurry was placed in a 125-ml centrifuge tube; the blender was rinsed with 25 ml ethyl acetate and this wash was added to the first extract. The tube was centrifuged for 10 min at 5000 rpm using the HL-8 rotor in a Sorvall RC 3 centrifuge at 4°C. The organic layer was carefully removed, and was evaporated to dryness in vacuum. The crude extract was subject to preparative thin-layer chromatography on silica gel GF plates (Fisher Scientific) using benzene as solvent; the dried plate was then run a second time in the same solvent. The yellow band was scraped off the plate, and the pigment was extracted into boiling acetone using 3 × 20-ml portions. Evaporation of the acetone in vacuum yielded the pure EHDN.

Cultures from 2.8-liter Fernbach flasks were treated as follows. The mycelial mats were separated from the medium by filtration through glass wool and were washed with 100 ml of deionized water. The combined filtrate and washings were acidified to pH 2 with concentrated HCl and were continuously ether extracted for 48 hr. Following evaporation of the ether, the residue was added to the mycelial extract, just prior to the column chromatography to be described. The mycelial mats were dried for 24 hr at 130°C, then ground to a fine powder. The powder was subject to Soxhlet extraction using Whatman cellulose extraction thimbles, with

acetone (500 ml) acidified to pH 2 with concentrated HCl (3 ml). After extraction for 24 hr, the acetone was removed under reduced pressure. The resulting black gum was dissolved in a minimum amount of acetone, about 3 ml, and was dripped onto a Soxhlet extraction thimble with continuous blow drying. The thimble was placed in the Soxhlet apparatus and was extracted with chloroform for 4 hr; the extract was then evaporated to dryness in vacuum. This extract, plus that obtained from the medium by ether extraction, was dissolved in the minimum amount of chloroform and the solution was layered onto the top of a column (3.5 × 15 cm) containing 75 g of Unisil silicic acid in chloroform. The column was eluted with chloroform, collecting 125-ml fractions. The yellow band of EHDN usually eluted in fractions 4 to 6; these fractions were combined and taken to dryness in vacuum. The crude product was redissolved in a small amount of benzene:chloroform (4:1, v/v) and rechromatographed in this solvent on a column (2.2 × 22 cm) of Unisil, 200–325 mesh. The yellow band was again collected, a total volume of about 1500 ml of solvent being required. The residue obtained on evaporation (usually 10 mg/flask) was recrystallized from boiling absolute ethanol.

The material obtained by these operations had a mp of 186–187°C corresponding to that reported for 6-ethyl-5-hydroxy-2,7-dimethoxy-1,4-naphthoquinone (*13*). On thin-layer chromatography on silica gel GF, 250 μm, with chloroform:methanol (9:1) as solvent, a single yellow spot, R_f = 0.81, was observed; the spot became colorless on treatment with sodium borohydride and purple on staining with ethanolic $FeCl_3$ and addition of base. Ultraviolet (EtOH) λ_{max} 220, 264, 304, and 425 nm; shoulder, 257 nm; log ϵ 4.36, 4.15, 4.13, and 3.81, respectively; ir (KBr pellet) ν_{max} 1675, 1627, 1595, 1360, 1315, 1255, 1140, and 1120 cm^{-1}; mass spectrum, eight most intense peaks at *m/e* 262 (M^+), 247, 233, 263, 215, 217, 219, and 248 with relative intensities of 100, 38, 19, 16, 13, 8, 6, and 6, respectively. All of these data are consistent with the assigned structure.

For spectrophotometric assays of EHDN, samples were dissolved in 1 ml absolute ethanol; 0.1 ml of the solution was pipetted into a cuvette and 3.0 ml of absolute ethanol was added. The absorbance was determined at 425 nm, and the amount of EHDN was then calculated using the value ϵ = 14.75 mg^{-1} cm^{-1} ml.

[2-^{14}C]Acetic acid, [1-^{14}C]- and [2-^{14}C]propionic acid, as sodium salts, were obtained from Amersham-Searle; [3-^{14}C]propionic acid, sodium salt, and *L*-[^{14}C-CH_3]methionine were obtained from New England Nuclear; [1-^{13}C]propionic acid, sodium salt, 63.2 atom% ^{13}C, [3,3,3,-$^2H_3$]propionic acid, 98 atom% ^{2}H, and [2-$^{13}C_{0;1}$, 2-$^2H_{3;3}$]acetic acid, sodium salt, 90 atom% ^{13}C and 98 atom% ^{2}H (hereafter called sodium [2-$^{13}C^2H_3$]acetate),[2] were obtained from Merck, Sharp and Dohme; and [1-$^{13}C_{0;1;1}$, 2-$^{13}C_{1;0;1}$]acetic acid, 90 atom% ^{13}C (hereafter called [1,2-^{13}C]acetic acid), was obtained from Bio-Rad. The ^{13}C nmr spectrum of the [2-$^{13}C^2H_3$]acetate, obtained with {^{1}H, ^{2}H} decoupling, confirmed that the material was deuterated to greater than 98%.

Incorporation (*I*%) and dilution (*D*) values in experiments with radioactive

[2] The nomenclature used for isotopically modified compounds is that of "Nomenclature of Organic Chemistry," Sect. H, "Isotopically Modified Compounds," 1st ed. Pergamon, New York. *Pure Appl. Chem.* **51**, 353 (1979).

tracers have the meanings in general use (*15*). In determining radioactivity in samples of EHDN, an appropriate quenching factor was used in view of the pronounced yellow color of the solutions.

For the work with stable isotopes, the precursors (75- to 85-mg amounts for acetate, 100 mg for propionate) were added to each of 10 cultures of *H. toruloidea* (700 ml). The additions were made at Day 20 of growth and the contents of the flasks were worked up after a further 24-hr period. In all cases, the Casamino acid-supplemented medium was used, and the yields of pigments (total amounts between 10 and 22 mg) were lower than those obtained in the earlier experiments.

^{13}C and ^{2}H nmr spectra were recorded at 30°C with a Varian XL-100/15 Fourier-transform spectrometer equipped with a Varian 620L computer and Diablo disk accessory. The conditions that varied were sample, solvent, sample tube, spin rate, frequency of observation, ν_0, concentration of relaxation reagent $Cr(acac)_3$, spectral width (SW), acquisition time (AT), flip angle (FA) (90° pulse length 44 μsec for ^{13}C, 55 μsec for ^{2}H), delay (PD) between acquisitions, time constant (TC) for weighting the free induction decay, decoupling field $\gamma H_2/2\pi$, decoupler phase modulation frequencies (ν_M) for broadband irradiation, internal field-frequency lock conditions (to ^{2}H or ^{19}F), and internal reference compound. These variables are listed below for the individual samples.

(1) EHDN, natural abundance (60 mg in 08 ml C^2HCl_3, 12-mm microcell, 10-Hz spin) and [1,2-^{13}C]acetate enriched (21.5 mg in 0.3 ml C^2HCl_3, 5-mm tube, 25-Hz spin), ^{13}C spectrum, ν_0 25.16 MHz, without and later with 10 mg/ml $Cr(acac)_3$, SW 5120 Hz, AT 1.6 sec, FA 40° (16° for one experiment), PD 1.6 sec for high-resolution spectrum with decoupler on during PD only to retain nuclear Overhauser enhancement (nOe), TC −0.8 or −1.6 sec, $\gamma H_2/2\pi$ (^{1}H, 100 MHz) ca. 3800 Hz, ν_M 200 Hz, ^{2}H pulse lock $(CH_3)_4Si$ reference.

(2) EHDN, [2-$^{13}C^{2}H_3$]acetate-enriched, 3 mg in 0.2 ml 4:1 C^1HCl_3:C_6F_6, 5-mm tube, 15 mg/ml $Cr(acac)_3$ added, 25-Hz spin, ^{13}C spectrum, ν_0 25.16 MHz, SW 5120 Hz, AT 1.6 sec, FA 55°, PD 0 or 2.4 sec with decoupler off during PD to suppress nOe, TC −0.8 sec or −1.6 sec, $\gamma H_2/2\pi$ (^{1}H, 100 MHz) ca. 3500 Hz, ν_M 150 Hz; $\gamma H_2/2\pi$ (^{2}H, 15.4 MHz) ca. 310 Hz, ν_M 40 Hz; ^{19}F lock, $(CH_3)_4Si$ reference.

(3) EHDN, [3,3,3-$^{2}H_3$]propionate-enriched, 16 mg in 0.25 ml 4:1 C^1HCl_3:C_6F_6, 5-mm tube, 25-Hz spin, no $Cr(acac)_3$ added, ^{2}H spectrum, ν_0 15.4 MHz, SW 500 Hz, AT 2 sec, FA 90°, PD 0 sec, TC −0.2 or −0.8 sec, $\gamma H_2/2\pi$ (^{1}H, 100 MH_z, where used) ca. 3800 Hz, ν_M 200 MHz, ^{19}F lock, C^1HCl_3 reference.

RESULTS

Howe and Moore obtained approximately 10 mg/liter of the yellow pigment when *H. toruloidea* was grown, with shaking, on a medium containing glucose, ammonium tartrate, KH_2PO_4, $MgSO_4$, yeast extract, and a trace elements mixture (*13*). However, with this medium, we obtained none of the desired pigment; the addition of further amounts of yeast extract and/or corn steep liquor, and use of both shaken and stationary cultures was equally unproductive. After various

trials, it was found that some pigment was produced on unsupplemented Czapek–Dox medium in stationary culture; of various supplements tried (malt extract, yeast extract, corn steep liquor, Marmite) the best results were obtained on addition of 1% malt extract. Production of EHDN began slowly from Day 7 onward and usually reached 0.6 mg per gram mycelium dry weight at the end of the growth period (21 days). From the combined mycelium and medium from one 700-ml culture, the yield of purified pigment was usually about 6 mg. Over the growth period, the pH of the medium slowly declined from an initial value of 6.4 to 5.5; the major change occurred in the first 5 days.

Although there was a sharp increase in naphthoquinone production over the 12- to 16-day period, a preliminary incorporation study with [2-^{14}C]acetate added on each of these days, followed by harvest at Day 21, gave low incorporations with low specific activities and high *D* values. To determine the optimum period for addition of labeled precursors, 10-μCi portions of [2-^{14}C]acetate (as sodium salt, specific activity = 58 mCi/mmol) were added to cultures beginning on the sixth day after inoculation and on each successive day until Day 20. All cultures were harvested on Day 21; the amounts of EHDN were determined by the spectrophotometric assay, and radioactivity was determined by scintillation counting. The highest *I* value (0.11%) was obtained when the tracer was added on Day 20, and this was accompanied by the lowest dilution (*D* = 1990). Prior to this time point, the *I* values had ranged from 0.002 to 0.056%, and the *D* values from 24,700 to 2700. These experiments confirmed that the best incorporation of activity did not coincide with the sharp increase in actual production (12–16 days). Since the relatively short 24-hr exposure had given the best incorporation in this experiment, the effect of addition of the same amount of tracer for the final 1-hr period was examined. The incorporation was substantially lower (*I*% = 0.03) than that obtained in the 24-hr experiment and all further isotopic incorporations were, therefore, carried out over the 20- to 21-day period. As expected, there was some variation from culture to culture; the results of three experiments with [2-^{14}C]acetate (100-μCi portions, specific activity = 57.7 mCi/mmol) added under the standard conditions gave *I* values of 0.11, 0.22, and 0.28% with corresponding *D* values of 1990, 1970, and 5890.

To investigate a possible role for propionate, a similar screening experiment was carried out with 10-μCi additions of [1-^{14}C]propionate (sodium salt, specific activity = 57 mCi/mmol) from Day 6 onward; a slightly longer overall growth period of 23 days was used in this work. As with the acetate experiments, an exposure of 24 hr toward the end of the growth period gave the highest incorporation (*I*% = 0.04); the observed dilution was, however, rather high (*D* = 13,030). At the earlier time points, the *I* value had ranged between 0.001 and 0.019%, and the *D* values between 33,800 and 124,540. When experiments were carried out with all three of the possible labeled propionates (addition at Day 20, harvest at Day 21) the *I* values for the [2-^{14}C]- and [3-^{14}C]propionates were somewhat higher (0.082 and 0.077%, respectively) than for the carboxyl-labeled sample. All of the propionate incorporations were lower than for [2-^{14}C]acetate.

In a further experiment, L-[^{14}C-CH_3]methionine was administered to *H. toruloidea* cultures over the 20- to 21-day period. As shown in Table 1 excellent

TABLE 1

INCORPORATION OF ACTVITY FROM L-[^{14}C-CH$_3$]METHIONINE INTO 6-ETHYL-5-HYDROXY-2,7-DIMETHOXY-1,4-NAPHTHOQUINONE BY *H. toruloidea*

	EHDN				Tetramethylammonium iodide		
Expt No.	Specific activity as isolated (dpm/μmol)	*I* (%)	*D*	Specific activity on dilution (dpm/μmol)[b]	Specific activity as determined (dpm/μmol)	Specific activity, calculated for two methylations (dpm/μmol)	Specific activity calculated for three methylations (dpm/μmol)
1[a]	106220	0.50	1056	4631	2418	2316	1544
2	137653	0.69	816	3916	2117	1958	1305

[a] To each of four 700-ml cultures was added 30 μCi of L-[^{14}C-CH$_3$]methionine.

[b] The samples were diluted with carrier EHDN prior to carrying out the Zeisel degradation.

incorporations were obtained (*I*% from 0.5 to 0.7). To determine whether activity was present in positions other than the two OCH_3 groups, Zeisel degradations were performed; the liberated methyl iodide from the OCH_3 groups was converted to tetramethylammonium iodide for determination of radioactivity (see Table 1). These results indicate that methionine labeled only the OCH_3 groups.

Chemical degradations to locate atoms of the naphthoquinone ring proved difficult on a small scale, and we therefore turned to the use of stable isotopes and nmr studies. Since such experiments require the addition of higher amounts of precursors than do radioactive experiments, and particularly in view of the known antifungal properties of propionate, the tolerance of *H. toruloidea* to both acetate and propionate was first examined. The addition of sodium acetate to 20-day-old cultures resulted in a marked increase of pH by Day 21 as higher amounts were used. In control flasks, the pH remained at 4.7 at both Days 20 and 21, but with the indicated sodium acetate additions (700-ml cultures in Fernbach flasks) the following pH values were observed: 25 mg, pH 4.7; 50 mg, pH 4.8; 100 mg, pH 4.9; 200 mg, pH 5.0. Since there was little or no effect on metabolite production, acetate feeding levels of 60–70 mg per Fernbach flask with 700 ml of culture medium were chosen as appropriate. Sodium propionate was reasonably well tolerated with additions on Day 20 and there was only a 32% decline in metabolite production at a level of 1000 mg per Fernbach flask. When additions of sodium propionate were made prior to inoculation, there was a surprising stimulation of metabolite production, with a three-fold increase noted at the level of 500 mg per Fernbach flask.

In an experiment using [1-^{13}C]propionate as precursor, the nmr spectrum of the isolated EHDN showed no evidence for ^{13}C enrichment at any position. When [3,3,3-$^{2}H_3$]propionate was administered, the only ^{2}H resonances observed corresponded to the methyl of the ethyl side chain and a broad impurity peak. The ^{2}H enrichment at the methyl was estimated to be about 0.4 $\pm$ 0.2% above natural abundance. Apart from a very small enrichment in the methylene of the ethyl

TABLE 2

[1,2-^{13}C]ACETATE INCORPORATION INTO 6-ETHYL-5-HYDROXY-2,7-DIMETHOXY-1,4-NAPHTHOQUINONE BY *H. toruloidea*; nmr DATA[a]

Carbon no.	Chemical shift δ_c[b]	Spin–spin coupling constant, J_{CH}[c] (Hz)	Spin–spin coupling constant, $^1J_{CC}$[d] (Hz)	Enrichment[e] (% above natural abundance)
1	179.45	dd[b] $^3J_{CH}$ 4.5, 7.8	58.25 ± 0.08	0.68
2	160.39	q $^3J_{CH}$ 4.8	58.26 ± 0.08 } 58.26 ± 0.07	0.73 } 0.71
3	109.24	d $^1J_{CH}$ 164.3	57.50 ± 0.08	0.75
4	189.92	d $^2J_{CH}$ 2.5	57.10 ± 0.36 } 57.30 ± 0.32	0.64 } 0.70
5	160.54	t $^3J_{CH}$ 3.8	63.70 ± 0.05	0.65
10	108.89	dd[f]	63.66 ± 0.06 } 63.64 ± 0.09	0.70 } 0.68
6	127.45	bm-q $^3J_{CH}$ 4.7	70.98 ± 0.14	0.68
7	162.31	bm-q $^3J_{CH}$ 4.0	70.88 ± 0.19 } 70.93 ± 0.16	0.64 } 0.66
8	102.80	d $^1J_{CH}$ 164.6	64.38 ± 0.02	0.70
9	129.83	d $^2J_{CH}$ 2.3	64.18 ± 0.06 } 64.30 ± 0.11	0.70 } 0.70
13 }	{ 56.54 }	q $^1J_{CH}$ 146.7		
14 }	{ 56.12 }	q $^1J_{CH}$ 145.1		
11	16.26	tq $^1J_{CH}$ 130.1, $^2J_{CH}$ 4.0	AB 33.5 ± 0.02	0.72 } 0.71
12	12.84	qt $^1J_{CH}$ 127.4, $^2J_{CH}$ 5.3		0.69 }

[a] The ^{13}C assignments were based on identification of the doubly labeled pairs, chemical shifts, and J_{CH} values from the high-resolution (hr) ^{13}C spectrum, as follows. The five resonances at low field (δ_c 189.9 to 160.4) correspond to carbons bonded to oxygen. The hr spectra of the two carbons bearing OCH_3 groups would be expected (*27*) to show quartets ($^3J_{CH}$ = 4 Hz) due to coupling with OCH_3 hydrogens (the resonance of C-2 should be a simple quartet; that of C-7 a triplet of quartets due to additional coupling to the hydrogens on C-11). The multiplicity of the other three low-field resonances should be less: C-1 a doublet of doublets ($^3J_{CH}$ to H-3 and H-8), C-4 a doublet ($^2J_{CH}$ to H-3) and C-5 a triplet ($^3J_{CH}$ to H-11). Thus C-1 (δ_c 179.5), C-2 (δ_c 160.4), C-4 (δ_c 189.9), C-5 (δ_c 160.5), and C-7 (δ_c 162.3) are assignable on the basis of multiplicity in the hr spectrum. The assignments are confirmed by the ^{13}C–^{13}C couplings. Two of the carbons resonating at low field form a ^{13}C–^{13}C-coupled unit, the only possible pair being C-1 and C-2. The ^{13}C–^{1}H coupling locates the resonances of the two-ring carbons bearing hydrogen. One of these (δ_c 102.8) must be C-8 since it is coupled to a carbon (C-9) resonating at δ_c 129.8. This would not be possible for C-3. The other (C-3, δ_c 109.2) is coupled as expected to a carbon resonating at low field (C-4, δ_c 189.9). The carbon identified above as C-5 is coupled to a carbon (δ_c 108.9) with chemical shift appropriate for C-10. The remaining aromatic carbon (δ_c 127.5) forms a ^{13}C–^{13}C pair with C-7 and has a multiplicity in the hr spectrum (a broad multiplet including a quartet; ^3J to CH_3; ^3J to H-8) consistent with its position. The carbons of the ethyl group form an AB-coupled ^{13}C–^{13}C pair and are readily identified from their chemical shifts and multiplicities in the hr spectrum. The two methoxyl carbons were not assigned individually, and were not enriched by [1,2-^{13}C]acetate.

[b] Solvent C^2HCl_3, reference to internal $(CH_3)_4Si$.

[c] Abbreviations are as follows: d, doublet; dd, doublet of doublets; q, quartet; t, triplet; bm-q, broad multiplet including quartet. Error, ca. ± 0.6 Hz.

[d] Averaged from three spectra.

[e] Measured from integrals of spectrum obtained with $Cr(acac)_3$ addition to sample. Enrichment (%) = $1.1 I_s/(I_c - fI_s)$; I_s is satellite intensity, I_c is central peak intensity of resonance for other carbon of a given pair. f = 1/9 is fraction of singly labeled acetate in sample of doubly labeled acetate. The error is ca. ± 0.04%.

[f] Low-intensity precluded measurement of J_{CH}.

group, no other position showed ^2H. No broadening of the resonances was observed when the spectrum was recorded without ^1H decoupling, so there was no evidence for $C^1H^2H_2$ or $C^1H_2{}^2H$ species. The experiment lacked sensitivity due to a poor signal/noise ratio. In any event, the low ^2H or zero ^{13}C incorporations observed in these experiments confirmed the earlier conclusion that propionate does not function significantly as a source of ethyl groups.

To pinpoint the two-carbon units contributed by acetate, proton-decoupled Fourier transform ^{13}C nmr spectra were obtained from a sample of the metabolite produced in the presence of [1,2-^{13}C]acetate. In contrast to the results with propionate, substantial enrichments were observed at all positions except the methoxyl groups (C-13 and C-14) (see Table 2). The overall average enrichment was 0.69 ± 0.03%. Measurements of $^1J_{CC}$ for the resonances of each of the labeled carbons showed which pairs formed doubly labeled units; the magnitudes of $^1J_{CC}$ were sufficiently different (see Table 2) to eliminate any ambiguity in the pairing. The following pairs of carbon atoms are each derived from an acetate unit: C-1 + 2; C-3 + 4; C-5 + 10; C-6 + 7; C-8 + 9; C-11 + 12.

In the experiment just described, no one doubly labeled unit showed a significantly higher enrichment than the others; it was not possible, therefore, to identify which acetate unit functioned as the "starter" for the polyketide chain. To provide information on this point, cultures of *H. toruloidea* were treated with [2-$^{13}C^2H_3$]acetate; the results from this experiment may be summarized as follows. (i) Six positions were enriched with ^{13}C. C-1 (5.6%), C-3 (7.2%), C-6 (6.3%), C-8 (6.6%), C-10 (7.5%), and C-12 (5.4%). The enrichment was uniform within the comparative error of ± 1.5% for any two enriched carbons, averaging 6.4 ± 0.9 SD%. The absolute error of each measurement was ± 3.0% due to the difficulty of measuring the weak natural abundance ^{13}C resonances used as internal standards for determining ^{13}C enrichments. This labeling pattern agrees with the results previously reported with the use of [1,2-^{13}C]acetate. (ii) The resonance for C-12 in the {^1H, ^2H}-decoupled ^{13}C spectrum contained a $^{13}C^1H_3$ component and isotopically chemically shifted peaks for $^{13}C^1H_2{}^2H$, $^{13}C^1H^2H_2$, and $^{13}C^2H_3$ in the ratio of 19 : 28 : 45 : 8% (±4%), after subtracting the natural ^{13}C abundance contribution to the intensity of the $^{13}C^1H_3$ signal. When the ^2H-decoupling field was removed only the $^{13}C^1H_3$ peak was unaffected, the others giving multiplets due to ^{13}C–^2H spin–spin coupling which were undetectable because of a poor signal/noise ratio. The signal assigned to $^{13}C^2H_3$ was only apparent in expanded plots and its relative intensity was estimated by fitting Lorentzians to the $^{13}C^1H_3$, $^{13}C^1H_2{}^2H$, and $^{13}C^1H^2H_2$ signals and subtracting the sum from the experimental spectrum. It was not possible to confirm that this low-intensity peak was due to $^{13}C^2H_3$, and it may in fact be due to an impurity in the EHDN sample (see Discussion). (iii) The C-3 and C-8 resonances in the {^1H, ^2H}-decoupled ^{13}C spectrum did not possess isotopically chemically shifted components due to deuteromethine groups. Since ^2H was associated only with C-12, it appears likely that the "starter" acetate unit is represented by C-12 + C-11.

DISCUSSION

Although it is not clear why the strain of *H. toruloidea* used by Howe and

Moore (*13*) no longer produces EHDN under the culture conditions described by them, we were usually able to obtain a sufficient amount of the metabolite by growth as surface cultures on a simple Czapek–Dox plus malt extract medium. Poor metabolite production encountered in the late stages of this investigation was overcome by supplementing the medium with 1% Casamino acids. While our work was in progress, van Eijk and Roeymans (*16*) reported the isolation of another naphthoquinone, as well as EHDN itself from a clinical isolate of *H. toruloidea* (CBS 145.78 = IMI 198935). This material was identified as 2,7-dimethoxy-5-hydroxy-6-(1-acetoxyethyl)-1,4-naphthoquinone. We have often noted other yellow pigmented materials in crude extracts from our strain, and one of them may well be this new naphthoquinone.

Our results indicate that EHDN is produced by a simple polyketide pathway and suggest that the "starter" acetate unit is represented by C-12 and C-11. These conclusions rest on the following arguments.

(i) It is clear from the experiments with labeled methionine that this precursor is used only for the two OCH_3 groups, and does not contribute carbon to other positions in the molecule. Hence, any mechanism requiring a carbon methylation process is eliminated.

(ii) The incorporation and dilution values observed in tracer experiments with labeled acetates were strongly supportive of a polyketide pathway. The best *I* values obtained with [2-^{14}C]acetate approached 0.3%, a value typical of those observed for polyketide pathways in other fungi. Much lower incorporation values were obtained with propionate suggesting that there is a nonspecific utilization of this material, perhaps by way of acetate. The situation is somewhat complex since activity from [1-^{14}C]propionate has no ready access to acetate; however, the incorporation values with [1-^{14}C]propionate were themselves significantly lower than with the 2- and 3-labeled material. The observation that propionate, added prior to inoculation, stimulated EHDN production approximately threefold also suggests an ability to use propionate in some indirect fashion.

(iii) The conclusions as to the relative roles of acetate and propionate derived from the radioactive experiments were fully supported by the use of precursors labeled with stable isotopes. Experiments with [1-^{13}C]- and [3,3,3-$^{2}H_3$]propionate gave either no enrichment (^{13}C experiment) or at best an incorporation (^{2}H) which was difficult to interpret on account of the signal to noise problem. On the other hand, a comparable experiment with [1,2-^{13}C]acetate gave substantial enrichment at all carbon atoms (12 positions) except those known to be contributed by methionine. Likewise, with [2-$^{13}C^{2}H_3$]acetate, substantial enrichment of six-carbon positions was observed. The use of the doubly labeled acetate sample, and determination of the coupling constants (see Table 2) showed unequivocally which pairs of carbon atoms are derived from acetate; the incorporation of these doubly labeled acetate units and or methionone is diagrammed in Fig. 1.

(iv) Attempts to determine which of the acetate units functions as the "starter" for the polyketide chain encountered some difficulties. When [2-$^{13}C^{2}H_3$]acetate was examined as a precursor of EHDN, the nmr spectra indicated that ^{2}H was associated only with C-12. At first glance, this result would be consistent with the

identification of C-12 + 11 as the "starter" unit. There is ample precedent for the complete or partial loss of ^2H at the "malonate" positions (*17*); in the present case, the absence of ^2H at positions C-3 and C-8 (the only ones in EHDN which still retain hydrogen) identifies them as originating in malonate. To account for the formation of an ethyl group from a polyketide chain, the most reasonable postulate is that an acetyl naphthoquinone is one of the first products of polyketide cyclization and that the following steps would likely occur after cyclization:

$$CH_3CO\text{-} \rightarrow CH_3CHOH\text{-} \rightarrow CH_2{=}CH\text{-} \rightarrow CH_3CH_2\text{-}.$$

The plausibility of these reactions is reinforced by the isolation of the acetate derivative of the CH_3CHOH compound related to EHDN (*16*). The cooccurrence of 2-ethyl and 2-acetyl naphthoquinones (e.g., echinochrome A and spinochrome A) and 6-ethyl and 6-acetyl juglone derivatives has been described in echinoderms (*18*). Similarly, in the plant *Lomandra hastilis,* ethyl and hydroxyethyl compounds are found—lomandrone is 2-ethyl-8-hydroxy-3,6,7-trimethoxy-1,4-naphthoquinone and lomazarin is 2-(α-hydroxyethyl)-5,8 dihydroxy-3,6,7-trimethoxy-1,4-naphthoquinone (*19*). If these reactions occur in EHDN biosynthesis in *H. toruloidea,* the use of [2-$^{13}C^2H_3$]acetate as the labeled precursor should lead to EHDN in which the ethyl groups would contain predominantly $^{13}C^1H^2H_2C^1H_2$ and in which the species $^{13}C^2H_3C^1H_2$ should be absent. As noted under Results, a $^{13}C^2H_3$ component was apparently present to the extent of about 8%; the most prevalent species was, however, the expected $^{13}C^1H^2H_2$ (45%). The presence of species containing less than two atoms of deuterium can result from exchange reactions; in the absence of other information, the presence of the $^{13}C^2H_3$ component is ascribed to some unidentified impurity. Despite these difficulties, it seems reasonable to assign C-12 and C-11 as the "starter" acetate unit and to conclude that C-5 represents the COOH group from the terminus of the hexaketide chain. The overall biosynthetic pathway based on this conclusion is shown in Fig. 1. The condensation of one acetate and five malonate units leads to the initial polyketide (**1**) which undergoes cyclization to the naphthalene derivative (**2**) (or the nonmethylated precursor). Following reduction of the acetyl side chain at position 6, the hydroxyethyl intermediate (**3**) can give rise to EHDN (**4**) or, if it undergoes acetylation, to the derivative (**5**) reported by van Eijk and Roeymans (*16*). Other reaction sequences are, of course, possible.

Apparently the only other study of the biosynthesis of an ethyl naphthoquinone is that on the production of echinochrome A (2-ethyl-3,5,6,7,8-pentahydroxy-1,4-naphthoquinone) by the sea urchin, *Arbacia pustulosa* (*20*). In this work, [2-^{14}C]acetate, [$^{14}CH_3$]methionone, and [3-^{14}C]propionate were all poorly incorporated in feeding experiments lasting 10 days (*I* values respectively 0.0054, 0.0016, and 0.0013—recalculated from original data). On Kuhn–Roth oxidation of the three echinochrome samples, those derived from methionine and propionate gave acetic acid (i.e., from the ethyl group) which was essentially inactive. The Kuhn–Roth acetic acid from the [2-^{14}C]acetate experiment was labeled—paradoxically in both the CH_3 and COOH groups—but contained much less activity than expected for a complete polyacetate origin (7.1% vs expected 16.6% for 2 out of 12 carbons). A similar randomization of activity had occurred in a sample of palmitic

acid from the same organism. Despite these difficulties the authors proposed that the 10 carbons of the naphthalene ring were derived from acetate—and discretely remained silent concerning the origin of the ethyl group (the only relevant statement is "Des pigments naphthoquinoniques sans chaîne latérale pourraient donc être des intermédiaires"). There does not appear to be any way to rationalize these results.

Tentative evidence has been gained that the nonquinonoid ethyl naphthalenones produced by *Aspergillus parvulus* also originate by a polyketide pathway (*21*). In experiments with [2-^{14}C]actetate, *I* values of 1.68 and 1.75% were observed for asparvenone and 6-*O*-methyl asparvenone, respectively. Materials with an α-hydroxyethyl side chain were also present (*22*) and incorporated activity from [2-^{14}C]acetate (*23*).

The general biochemistry of *Hendersonula toruloidea* has received little attention. As found in nature, it is primarily a saprophytic organism, invading old tree bark and injured wood of citrus and other trees. Strains infecting humans have also been reported (*24*). When grown on glucose–peptone medium, the organism produced a surprising number of phenolic compounds in the culture solution (orsellinic acid, 6-methylsalicylic acid, orcinol, 3,5-dihydroxybenzoic acid, 2,3,5-trihydroxytoluene, *m*-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid plus small amounts of other identified and unidentified materials) (*25*). Many of these metabolites are also typical polyketide products. This organism can utilize and transform many phenolic products, and it produces a high yield of a humic acid-type polymer. The organism growing on *Citrus limon* bark produces another phenolic compound, xanthoxylin (*26*).

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