raphy of the crude product (dry column, chloroform) gave the expected anthraquinone **25** (20 mg, 29%): mp 185–186 °C (toluene–petroleum ether, bp 90–120 °C) (lit.[21] 185–186 °C;[22] 189–190 °C); UV λ_{max} (chloroform) 280 and 406 nm (log ϵ 3.96 and 3.38); IR ν_{max} (KBr) 1665 (C=O) cm^{-1}; NMR δ (60 MHz, $CDCl_3$) 2.40 (3 H, s, 6-CH_3), 3.90 and 3.95 (3 H and 9 H, 2s, 1,3,4,8-OCH_3), 6.75 (1 H, s, 2-H), 7.00 (1 H, br s, 7-H), and 7.50 (1 H, br s, 5-H); *m/e* 342 (M^+). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.77; H, 5.35. Demethylation of this compound (17 mg) according to Tanaka and Kaneko[21] gave xanthorin (6 mg, 40%), mp 247–248 °C (acetic acid) (lit.[21] 244–246 °C; lit.[22] 253 °C; lit.[23] 250–251 °C) indistinguishable from a sample of the authentic material (mmp, IR spectra, and TLC in four solvent systems).

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Registry No.—**1**, 61539-63-7; **2**, 65120-61-8; **3**, 41501-60-4; **4**, 922-69-0; **5**, 65120-62-9; **6a**, 65120-63-0; **6b**, 65120-64-1; **7**, 13719-93-2; **8**, 65120-65-2; **9**, 65120-66-3; **10**, 65120-67-4; **11**, 65120-68-5; **12**, 57165-99-8; **13**, 65120-69-6; **14**, 65120-70-9; **15**, 32013-63-1; **16**, 65120-71-0; **17**, 65120-72-1; **18**, 65120-73-2; **19**, 65120-74-3; **20**, 32013-66-4; **21**, 1989-44-2; **22**, 52431-64-8; **23**, 52431-72-8; **24**, 37567-67-2; chloroacetyl chloride, 79-04-9; 1,1-dichloroethylene, 75-35-4; methoxyacetyl chloride, 38870-89-2; chlorotrimethylsilane, 75-77-4; xanthorin, 17526-15-7.

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Biosynthesis of α-Naphthocyclinone[1]

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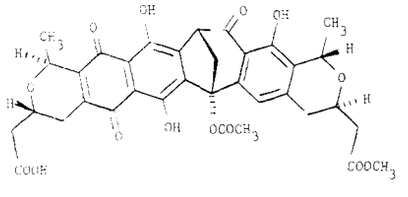
The biosynthesis of α-naphthocyclinone in *Streptomyces arenae* was studied by feeding experiments with sodium [1-^{13}C]- and [2-^{13}C]acetate and diethyl [2-^{13}C]malonate followed by ^{13}C NMR analysis. The compound is derived entirely from acetate/malonate units by the polyketide pathway, and the labeling pattern is consistent with its formation from two benzoisochroman quinone units. Surprisingly, [2-^{13}C]malonate labels both the starter and the chain extension units, but not the acetoxy group. Possible explanations of the latter finding are suggested.

The naphthocyclinones are a series of closely related pigments which were isolated from cultures of *Streptomyces arenae*, strain Tü 495.[2] Some of the compounds, β- and γ-naphthocyclinone (I and II, Scheme I), exhibit antibacterial activity against gram-positive organisms. Their structure elucidation by Zeeck's group showed[2,3] that the naphthocy-

Scheme I. Structures of Naphthocyclinones



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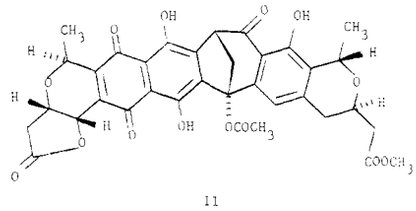
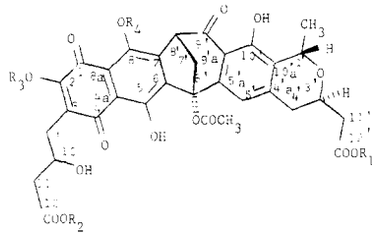


II



III: R_1=CH_3, R_2=R_3=R_4=H, α-naphthocyclinone
IV: R_1=R_2=R_3=R_4=H, α-naphthocyclinone acid
V: R_1=R_2=R_3=CH_3, R_4=H
VI: R_1=R_2=R_3=R_4=CH_3

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Table I. Incorporation of Precursors into α-Naphthocyclinone and α-Naphthocyclinone Acid

Expt no.	Precursor fed	Feeding time after inoculation, h	No. of cultures	Amount fed, mg	Spec. radioactivity of precursor, dpm/mmol	Product isolated (III + IV), mg	Spec. radioactivity of product, dpm/mmol	Incorporation,[a] %	Dilution factor[b]	Relative enrichment per labeled atom[c]
1	Sodium [1-^{14}C]acetate	24	2	50	7.05×10^6	0.1		Not determined		
2	Sodium [1-^{14}C]acetate	32	2	50	7.05×10^6	18.7	1.03×10^6	0.7	6.9	0.9
3	Sodium [1-^{14}C]acetate	40	2	50	7.05×10^6	30.9	1.58×10^6	1.7	4.5	1.4
4	Sodium [1-^{14}C]acetate	48	2	50	7.05×10^6	22.0	2.55×10^6	2.0	2.8	2.3
5	Sodium [1-^{14}C]acetate	48	4	200	2.16×10^7	41.0	8.39×10^6	1.0	2.6	2.4
6	Sodium [1-^{13}C,-1-^{14}C]acetate	48	10	300	1.55×10^7	106	5.94×10^6	1.6	2.6	2.4
7	Sodium [2-^{13}C,-2-^{14}C]acetate	48	10	300	1.51×10^6	98	3.55×10^5	1.0	4.3	1.5
8	Diethyl [2-^{14}C]malonate	48	10	500[d]	1.69×10^7	64.3	4.31×10^5	0.08	39	0.2
9	Diethyl [2-^{14}C]malonate	48	5	250[e]	1.30×10^7	29.6	3.40×10^6	0.5	3.8	1.7
10	Diethyl [2-^{13}C,-2-^{14}C]malonate	48	10	500[e]	2.08×10^7	44.1	3.64×10^6	0.3	5.7	1.2

[a] (Radioactivity in product/radioactivity in precursor) × 100 (%). [b] Specific radioactivity of precursor/specific radioactivity of product. [c] 100/(no. of precursor units in product molecule × dilution factor). [d] Precursor added neat. [e] Precursor fed as solution in Me_2SO.

clinones are related to the general class of isochroman quinone antibiotics, which also includes granaticin,[4,5] frenolicin,[6] kalafungin,[7] actinorhodin,[8] and the nanaomycins[9] and griseusins.[10] The naphthocyclinones are dimers in which one of the naphthoquinone units has been modified to an aryl ketone moiety. Furthermore, in α-naphthocyclinone (III), the major metabolite, the other unit contains only 14 rather than the usual 16 carbon atoms.

In this communication we report some results which establish the biogenetic origin of α-naphthocyclinone.

Results and Discussion

Inspection of the structure of the naphthocyclinones suggests that their biosynthesis might proceed via the polyketide pathway from acetate as the precursor. This hypothesis was examined in a series of feeding experiments with ^{13}C-labeled acetate and malonate followed by determination of the ^{13}C distribution in the resulting α-naphthocyclinone using ^{13}C NMR analysis.

In a series of preliminary experiments, optimum conditions for the ^{13}C experiments were determined. By following the time course of naphthocyclinone formation in *Streptomyces arenase*, strain Tü 495, determined by measuring the absorption at the λ_{max} of α-naphthocyclinone at 488 nm, it was established that the pigment concentration reached a maximum 72 h after inoculation (Figure 1). Chromatography of the pigment mixture showed that α-naphthocyclinone (III) and α-naphthocyclinone acid (IV) were the two most prevalent compounds. Next, a series of experiments with sodium [1-^{14}C]acetate were carried out in which the precursor, in a concentration of 25 mg per flask, was added at different times after inoculation (Table I, expt 1–4). Each set of cultures was harvested 24 h later, and the specific radioactivity and yield of α-naphthocyclinone (III and IV) were determined. It is evident that the best result is obtained when the precursor is added at 48 h. Doubling the amount of precursor per flask (Table I, expt 5) does not give higher isotope enrichment in the product. Based on this exploratory work, feeding at a concentration of 30 mg of sodium acetate per flask at 48 h and harvesting at 72 h were chosen as the set of standard conditions for the ^{13}C feeding experiments.

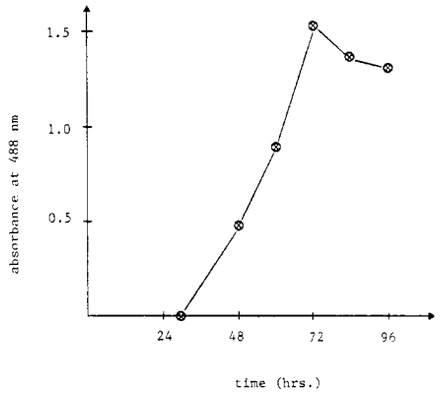


Figure 1. Time course of production of α-naphthocyclinone and α-naphthocyclinone acid.

The results of the experiments with sodium [1-^{13}C,1-^{14}C]- and [2-^{13}C,2-^{14}C]acetate are shown in Table I (expt 6 and 7). It is evident that both C-1 and C-2 of acetate are efficiently incorporated. For the analysis of the ^{13}C distribution of the products from these experiments, α-naphthocyclinone and α-naphthocyclinone acid were combined and the mixture was methylated with diazomethane[3] to give α-naphthocyclinone methyl ester methyl ether (V). This compound was subjected to ^{13}C NMR spectroscopy, and the normalized peak heights of the spectra were compared to those of the natural abundance spectrum. A complete ^{13}C NMR analysis of V and other naphthocyclinones has been carried out in Zeeck's laboratory, and the signal assignments were made available to us.[11] The relative ^{13}C abundance values for the products of experiments 6 and 7 (Table II) show that all carbon atoms of α-naphthocyclinone, with the exception of the *O*-methyl group, originate from acetate and that C-1 and C-2 of acetate label the molecule in the alternating pattern predicted by the polyketide pathway. This same kind of labeling pattern has

Table II. ^{13}C Distribution in the α-Naphthocyclinone Ring System Biosynthesized from [^{13}C]Acetate and [^{13}C]Malonate

Carbon atom no.	α-Naphthocyclinone methyl ester methyl ether (V)			α-Naphthocyclinone methyl ester dimethyl ether (VI)	
	Chemical shift, ppm	^{13}C abundance[a] from: [1-^{13}C]acetate	[2-^{13}C]acetate	Chemical shift, ppm	^{13}C abundance[a] from diethyl [2-^{13}C]malonate
1	183.5	4.0	1.1	178.9	1.3
2	158.9	1.2	2.6	159.9	2.5
3	131.8	4.4	1.2		
4	188.7	1.2	2.8	190.9	2.8
4a	111.9	3.5	1.2		
5	152.3	1.2	2.8	153.0	3.0
6	146.1	4.3	1.2		
7	134.8	1.0	2.6	122.6[b]	2.6
8	154.6	4.3	1.1	151.0	1.2
8a	112.3	1.2	2.8		
9	30.0	1.2	2.6	29.6	3.1
10	66.9	4.2	1.3	66.9	1.1
11	40.9	1.0	3.0	40.8	2.6
12	172.5	4.4	1.2	172.5	1.0
1′	67.9	3.9	1.1	67.9	1.1
3′	63.2	3.5	1.3	63.2	1.2
4′	34.5	1.0	2.6	34.5	2.6
4′a	142.1	4.1	1.2	142.3	1.1
5′	113.1	1.2	3.3	113.0	3.1
5′a	140.2	4.1	1.2	140.6	1.5
6′	84.8	1.1	2.8	84.7	2.9
7′	49.5	4.0	1.1	49.5	1.1
8′	51.0	1.2	2.4	51.9	2.5
9′	198.6	3.7	1.0	199.5	1.2
9′a	107.9	1.0	2.5	108.0	3.0
10′	159.6	4.0	1.1	159.5	1.1
10′a	128.8	1.0	2.3	128.6	3.0
11′	40.6	1.2	2.6	40.5	2.6
12′	171.1	4.2	1.2	171.1	0.9
1′-CH_3	18.6	1.1	2.6	18.6	2.8
Acetyl CO	169.5	4.3	1.0	169.3	1.1
Acetyl CH_3	21.1	1.1	2.3	21.1	1.3
Ester OCH_3	51.6	1.2	1.2	51.6	1.3
2-OCH_3	61.8[c]	1.1	1.1	61.3[c]	1.1
8-OCH_3				62.2	1.1

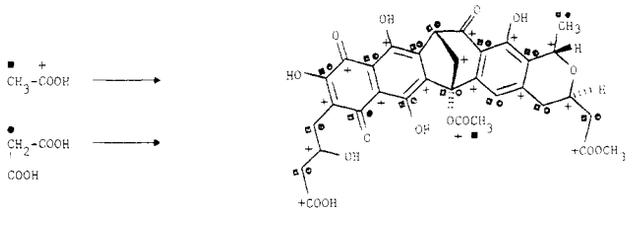
[a] Natural abundance = 1.1; values are subject to an error of approximately ±15%. [b] Tentative assignment. [c] Reference signal used for normalization of peak heights.

been demonstrated for the simpler benzoisochroman quinones, nanaomycin A and B.[12]

Since the "left hand" half of α-naphthocyclinone contains only a 14-carbon skeleton, it could either have arisen from a 16-carbon unit by loss of the two carbon atoms representing the starter unit of the polyketide chain or it could represent a 14-carbon polyketide chain with either C-2 and C-1 or C-4 and C-4a as the starter unit. We attempted to distinguish between these possibilities by feeding [2-^{13}C]malonate, which we expected to label predominantly the chain extension units and not, or only to a lesser extent, the starter units. Since malonic acid or its salt frequently cannot penetrate the cell membrane, the diethyl ester was administered to the culures.[13] The results (Table II) surprisingly indicate that with the exception of the *O*-acetyl group the same carbon atoms were labeled by [2-^{13}C]malonate as by [2-^{13}C]acetate. Derivatization of the sample from this experiment accidentally gave a new compound instead of V, to which structure VI was tentatively assigned, although an alternative structure carrying the extra methyl group at C-5 cannot be excluded. Only partial ^{13}C NMR assignments could be made for this compound, but the signals for the critical carbon atoms 2 and 4 and for the methyl group at C-1′ were assigned unambiguously. As would be predicted if the starter unit from the left hand portion of the molecule is lost, both C-2 and C-4 are enriched. However, any conclusion to that effect is invalidated by the fact that the C-1′ methyl group is enriched to the same extent. Paradoxically, the methyl group of the acetoxy function is not enriched.

The results clearly show that the skeleton of α-naphthocyclinone is built up entirely from acetate and/or malonate units via the polyketide pathway (Scheme II). In agreement with the proposal of Zeeck et al.,[2] we believe that the biosynthesis of α-naphthocyclinone involves the dimerization of two 16-carbon polyketides followed by loss of a 2-carbon unit from one of them. However, experimental verification of this hypothesis is still lacking. At least two reasonable explanations can be offered for the finding that malonate labels both the starter unit and the chain extension units, but not the acetoxy group. Malonyl-CoA may serve both as starter and as chain

Scheme II. Labeling Pattern of α-Naphthocyclinone from [^{13}C]Acetate and [^{13}C]Malonate



extension unit, with the starter unit undergoing decarboxylation at a subsequent stage. Alternatively, assembly of the polyketide chain may occur much earlier in the fermentation period than O-acetylation, and acetyl-CoA and malonyl-CoA may be in rapid equilibrium, resulting in labeling of both pools at the time of polyketide assembly, whereas the added precursor may be largely consumed at the time O-acetylation occurs. Further experimentation will be necessary to clarify these and other details of this biosynthesis.

Experimental Section

General. NMR spectra were measured on a Jeol PFT-100 system interfaced to an EC-100 Fourier transform computer with 20K memory. ^{13}C NMR spectra were generally recorded at a pulse width of 20 μs and a repetition time of 5 s using $CDCl_3$ as solvent. IR spectra were obtained on a Beckman IR 4230 spectrometer and UV spectra on a Perkin-Elmer 124 or a Cary 17 instrument. Mass spectra were measured on a DuPont 21-492 BR mass spectrometer.

Analytical as well as preparative chromatographic separations were carried out on thin-layer plates or columns of oxalic acid treated silica gel prepared as described by Zeeck and Mardin.[3] Radioactivity determinations were made by liquid scintillation counting in a Beckman LS-250 spectrometer using Bray's solution[14] as the scintillation fluid. Counting efficiences were determined with [^{14}C]toluene as an internal standard. ^{14}C-Labeled compounds were purchased from Amersham-Searle and ^{13}C-labeled substrates from Merck Sharp and Dohme.

Culture Conditions. *Streptomyces arenae,* strain Tü 495, was maintained on slants of M2 agar (1% malt extract, Difco, 0.4% dextrose, 0.4% yeast extract, Difco). Production cultures of 100 mL of medium (2% soy flour, 2% mannitol, pH 7.2) in 500-mL baffled Erlenmeyer flasks were inoculated with 2 × 2 cm pieces of mycelium cut from agar slants and were incubated at 27 °C and 350 rpm on a rotary shaker. The time course of the fermentation was determined by inoculating a series of four cultures and removing 2-mL aliquots from each flask at 24, 30, 48, 56, 72, 83, and 95 h after inoculation. These samples were each shaken with 2 mL of ethyl acetate, and the absorbance of the ethyl acetate solution at 488 nm was determined after appropriate dilution. The points shown in Figure 1 are the averages of the four determinations.

Labeled precursors were added as sterile aqueous solutions (except in expts 8–10) at the times and in the amounts indicated in Table I, and the cultures were harvested 24 h later. In all ^{13}C experiments the precursor was mixed with a small amount of ^{14}C-labeled material to allow determination of the overall dilution factor.

Isolation and Purification of Products. At the end of the fermentation period the mycelium was separated from the culture medium by filtration with the aid of Celite. The damp mycelium was extracted with acetone, the extract was concentrated in a vacuum, and the resulting aqueous suspension was combined with the culture filtrate. The mixture was acidified to pH 3 with 1 N HCl and extracted with ethyl acetate. The extract was dried and evaporated to dryness; the residue was taken up in a small volume of ethyl acetate, and the crude pigment mixture was precipitated out with petroleum ether and collected by centrifugation. This material was then chromatographed on a column (1.8 × 24 cm) of 30 g of oxalic acid treated silica gel. Elution with chloroform/ethyl acetate (1:1) gave α-naphthocyclinone and α-naphthocyclinone acid (IV), in addition to traces of other pigments which were not collected. The α-naphthocyclinone and α-naphthocyclinone acid were combined and suspended in chloroform. A solution of diazomethane in ether was added dropwise at −20 °C until a clear red solution was obtained and no more starting material was detectable by TLC analysis (acetone/chloroform, 1:9; oxalic acid treated silica gel). The solvent was evaporated, and the residue was chromatographed on a column (1.8 × 48 cm) of 60 g of oxalic acid treated silica gel. Elution with chloroform containg 2% acetone gave α-naphthocyclinone methyl ester methyl ether (V). In the feeding experiment with diethyl [2-^{13}C]malonate, α-naphthocyclinone acid was formed almost exclusively and the diazomethane methylation was carried out on a solution of this material (44.1 mg) in 30 mL of chloroform/methanol (2:1). Workup as above gave a new derivative (37 mg) which was tentatively identified as α-naphthocyclinone methyl ester dimethyl ether (VI). The material was identical to the product from the methylation of V.

α-Naphthocyclinone Methyl Ester Dimethyl Ether. α-Naphthocyclinone methyl ester methyl ether[3] (213 mg) was dissolved in 50 mL of chloroform/methanol (1:1), and excess diazomethane solution in ether was added at room temperature. The solution was evaporated to dryness, and the residue was chromatographed on 100 g of oxalic acid treated silica gel (chloroform containing 2% acetone) to give 149 mg of product, which was recrystallized from carbon tetrachloride/cyclohexane (2:3), mp 116 °C; IR (KBr) 1738, 1670, 1637 sh, 1623, 1579 cm^{-1}; UV (EtOH) λ_{max} 427 nm (ε 4300), 332 (5900), 247 sh, 228 (44 500); UV (EtOH/NaOH) λ_{max} 555 nm (ε 5400), 378 (12 100), 276 sh, 232 (44 500); UV ($CHCl_3$) λ_{max} 438 nm (ε 3400), 328 (5300), 286 sh, 249 sh, end absorption; ^1H NMR ($CDCl_3$) δ 12.74 (5-OH), 11.98 (10′-OH), 6.97 (5′-H), 5.00 (1′-H), 4.35 (3′-H), ca. 4.2 (10-H), 4.12 (2-OCH_3), 3.88 (8-OCH_3), ca. 3.8 (7′-H_a), 3.73 (12′-OCH_3), 3.69 (12-OCH_3), 2.93 (7′-H_b), 2.4–3.0 (4 CH_2 groups), 2.29 (6′-$OCOCH_3$), 1.52 (1′-CH_3).

Molecular weight for $C_{36}H_{36}O_{15}$: calcd, 708.204; observed (by EI mass spectrometry), 708.219.

Acknowledgment. We are greatly indebted to Dr. Axel Zeeck of the University of Göttingen for providing *Streptomyces arenae* Tü 495, reference samples of the naphthocyclinones, and the ^{13}C NMR assignments for these compounds.

Registry No.—I, 55050-83-4; II, 55095-58-4; III, 54826-93-6; IV, 54367-38-3; V, 54367-41-8; VI, 65015-70-5; sodium acetate, 127-09-3; diethyl malonate, 105-53-3.

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