Pergamon

Tetrahedron, Vol. 53, No. 13, pp. 4561-4568, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00226-3

The Biosynthesis of Murayaquinone, A Rearranged Polyketide

Steven J. Gould,* Chris R. Melville, and Jiong Chen

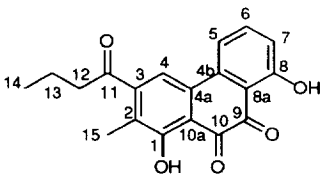
Department of Chemistry

Oregon State University

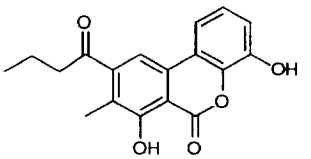
Corvallis, Oregon 97331-4003

Abstract: Incorporations of sodium [1,2-$^{13}C_2$]acetate and sodium [1-^{13}C,$^{18}O_2$]acetate confirmed that murayaquinone, produced by *Streptomyces murayamaensis*, is a polyketide. However, the labeling patterns revealed that a simple folding of the decaketide intermediate does not lead directly to the final phenanthrene skeleton. Instead a different phenanthrene is apparently formed that undergoes an oxidative cleavage, isomerization, and re-closure to a new phenanthrene skeleton. © 1997 Elsevier Science Ltd.

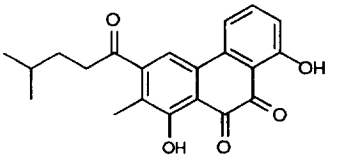
Murayaquinone, **1**, has been isolated from *Streptomyces murayamaensis*[1] and from two unidentified *Streptomyces* species.[2-4] It has been shown to have antibacterial activity against *Mycoplasma* and *Trepanonema* species, to have low oral toxicity in mice, and was recently found to inhibit hepatitis C virus proteinase.[2-4] We have also reported the isolation of an angular lactone, **2**, from *Streptomyces murayamaensis* mutant MC3,[5] (description of mutant will be reported elsewhere) a murayaquinone over-producer. It is presumably derived by oxidative cleavage of **1** or a closely related metabolite. While many phenanthrenes of diterpene origin are known,[6] and at least one phenanthrene is derived from phenylalanine,[7] the oxygenation pattern of **1** and the lack of typical terpenoid side-chains suggested a polyketide biogenesis. Four other phenanthraquinones of similar structure have also been characterized.[8-12] Birch proposed a polyketide biogenesis for one of these.[13,14] Subsequently, sodium [1-^{14}C]acetate, sodium [1-^{14}C]isobutyrate, and [U-^{14}C]valine were incorporated into piloquinone (**3**),[15] which contains a branched side-chain attached to C-3. Degradations indicated an acetate-derived polyketide biogenesis with a valine-derived isobutyryl starter unit. However, the folding pattern for the acyclic intermediate could not be determined from these experiments. We now report on the biosynthesis of **1** and, while we confirmed that **1** is a polyketide metabolite, we also found that a remarkable rearrangement is involved in its formation.



1



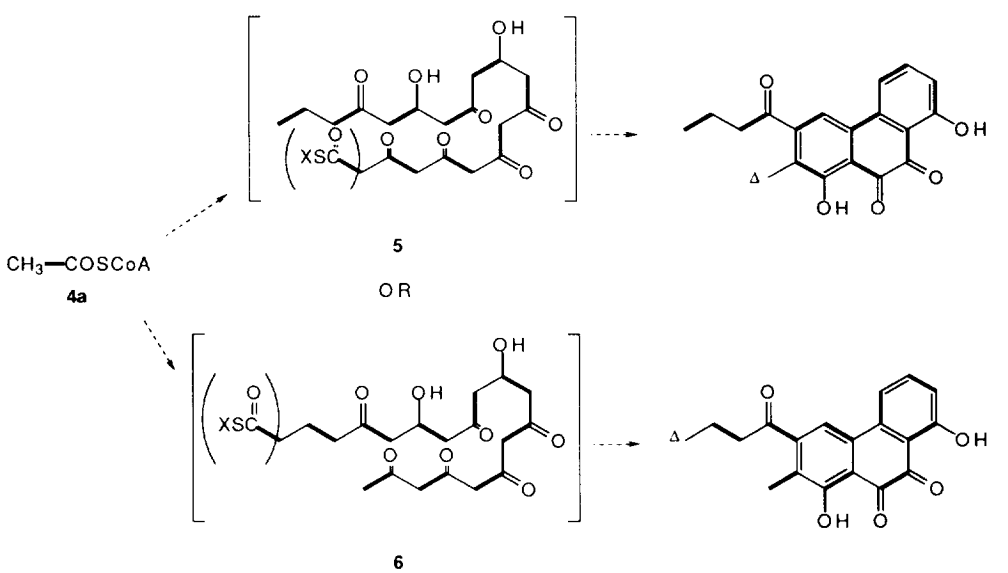
2



3

RESULTS AND DISCUSSION

Murayaquinone contains nineteen carbons, and its structure suggested a polyketide origin derived from of acetyl coenzyme A, **4**. Either of two decaketide intermediates appeared likely, in one case – **5** – a decarboxylation would occur at some point to generate the methyl side-chain, while in the other case – **6** – decarboxylation would generate the butyryl side-chain, as shown in Scheme 1. In each scenario, based on current understanding of aromatic polyketide biosynthesis,[16] a different set of carbonyl reductions would be expected, as shown. Scheme 1 shows how these alternative pathways should be distinguishable if **1** were derived from [1,2-$^{13}C_2$]acetyl coenzyme A, **4a**, with "Δ" indicating the site of decarboxylation.

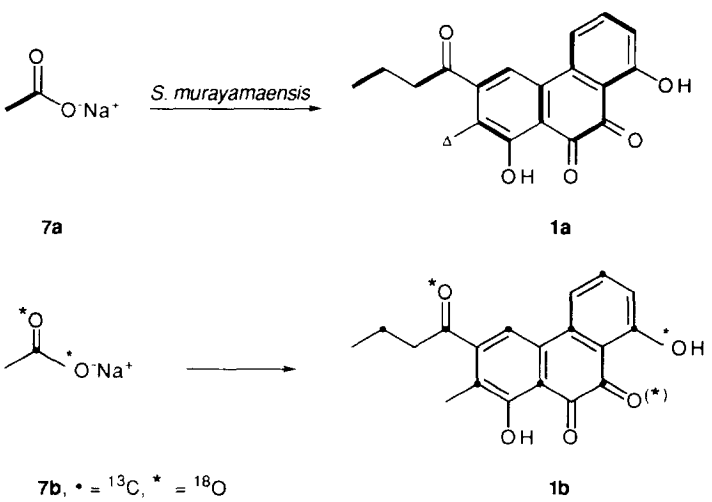


Scheme 1

Streptomyces murayamaensis mutant MC3, which overproduces **1**, was chosen for initial feeding experiments. Test fermentations were done in a variety of growth media with periodic additions of sodium [1-^{14}C]acetate. Highest production and radioactivity incorporation were found using a glycerol-ammonium

sulfate medium,[17] with acetate addition in five doses between 12 and 24 hours after inoculation of the broth. Due to the abundance of murayaquinone produced, the previous isolation protocol[1] could be simplified.

Sodium [1,2-$^{13}C_2$]acetate, **7a**, was fed to mutants MC3 and MC4 using this protocol, and work-up yielded labeled murayaquinone **1a** in each case with very similar levels of enrichment. ^{13}C NMR analysis revealed that except for the C-15 methyl group each carbon showed a substantial level of coupling to one adjacent carbon (Table 1). The methyl resonance appeared as an intense singlet with minor satellites due to a low level of inter-unit coupling. Thus, this is the carbon that had lost its acetate partner during the biosynthesis of **1**. The coupling partners, and therefore the locations of the precursor acetate units, were revealed from a 2D INADEQUATE experiment. Surprisingly, the coupled resonances revealed a highly irregular labeling pattern for the aromatic rings, inconsistent with any straightforward folding of a single decaketide intermediate (cf. Scheme 1). Particularly noteworthy was the origin of the two quinone carbons from the same acetate precursor and the origin of C-4a and C-10a from another. The resonances for the quinone carbons have nearly identical chemical shifts, and a large signal-to-noise ratio was necessary to observe the small outer lines of the second order doublets. The 2D INADEQUATE spectrum revealed the correlation between these two resonances. The fact that murayaquinone is entirely of polyketide origin is in contrast with piloquinone, which has a valine-derived isobutyryl starter unit.



In order to establish the orientations of each of the precursor acetates, as well as to identify which oxygens had been retained from acetate, sodium [1-^{13}C,$^{18}O_2$]acetate, **7b**, was fed next and yielded **1b**. An unusually high average enrichment of 17.4% per site was obtained, which obscured the ^{18}O isotope-shifted resonances. Dilution of a portion of the sample with **1** allowed the resonances to be resolved, and the ^{13}C NMR spectrum now revealed ^{18}O enrichments at C-11 and C-8. Presumably, acetate oxygen had initially been retained at the C-9 quinone carbonyl, as well, but had suffered subsequent exchange with the medium during fermentation or work-up.[18,19]

Table 1. ^{13}CNMR Spectrum of Murayaquinone and Incorporation of Labeled Precursors

			precursor [a]		
	chemical		**7a**	**7b**	
carbon	shift[b](δ)	*J*cc(Hz)	coupled pair	*J*cc(Hz)	Δδ
1	165.0	64.9	a		
2	126.2	64.9	a		
3	150.0	56.8	b		
4	113.1	56.8	b		
4a	132.8	57.0	c		
4b	135.0	62.2	d		
5	115.9	61.8	d		
6	139.4	57.8	e		
7	119.6	57.8	e		
8	166.1	61.8	f		1.01
8a	115.3	61.3	f		
9	182.2	50.0	g	11.4	
10	182.6	50.0	g		
10a	114.6	56.3	c	11.5	
11	205.4	39.8	h		0.06
12	44.9	40.4	h		
13	17.3	34.5	i		
14	13.8	34.5	i		
15	12.0				

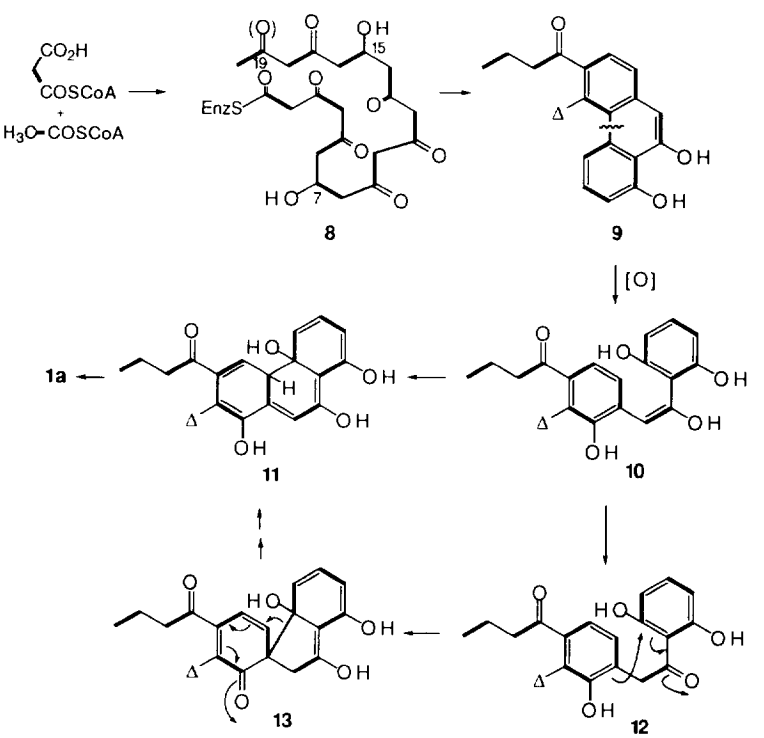
[a]Sites of enrichment indicated by *J*cc coupling constants or by isotope-induced shifts of δ (in ppm). *b* in $CDCl_3$; 75 MHz.

An unexpected and fortuitous result of the very high level of ^{13}C incorporation in **1b** was an inter-unit coupling (J = 11.5 Hz) between C-10a (δ 114.6) and C-9 (δ 182.2) in the ^{13}C NMR spectrum of the undiluted sample: each of the central lines of these resonances was straddled by small doublets. A 2D INADEQUATE experiment showed that these two resonances were coupled to each other. Thus, the resonances for the two quinone carbons could now be individually assigned. More importantly, the magnitude of the coupling is consistent with a two-bond coupling.[20] Therefore, all precursor acetates are linked in a head-to-tail fashion.

The presence of a fully saturated carbon at C-13 suggested the possiblity of a butyryl starter unit for the initial polyketide intermediate. Short-chain fatty acids have been shown to be starters for a number of other polyketides.[21,22] Although attempts to incorporate such moieties have usually resulted in *in vivo* degradation to acetyl CoA prior to incorporation, success has been facilitated by the use of *N*-acetylcysteamine (NAC) thioesters[23,24] that presumably mimic the putative pantothenic acid arm in a polyketide synthase complex, and frequently by co-addition of inhibitors of *beta*-oxidation.[25] Deuterium-labeled butyric acid was prepared by reaction of butyryl chloride with anhydrous trifluoroacetic acid-*d*, which yielded an 83% per position exchange of the *alpha*-hydrogens. Feeding samples of this material alone or in the presence of the *beta*-oxidation inhibitor 4-thiostearic acid[25] failed to label the murayaquinone. The deuteriated butyric acid was then converted to its NAC thioester via a mixed anhydride.[21] However, two separated feedings of this material in the presence of the inhibitor also failed to label murayaquinone.

Although the butyrate feedings were inconclusive, the acetate incorporations reveal an unexpected pathway for murayaquinone biosynthesis. As shown in Scheme 2 acetylCoA and nine equivalents of malonylCoA are condensed to afford a regular decaketide intermediate, **8**, with reductions at C-7, C-15, and C-19. The latter site is completely reduced at some point. A normal cyclization and decarboxylation would yield a phenanthrene, **9**, with a regular head-to-tail organization of precursor acetates. Oxidative fission of the central ring (C-1 and C-4b, murayaquinone numbering) and rotation around the bridging two-carbon unit (**10**, shown in the enol form of the ketone) would then orient the remaining benzene rings for cyclization between C-4a and C-4b (murayaquinone numbering). An electrocyclic closure would afford **11** requiring only dehydration followed by oxidation to yield **1** with the correct labeling. Alternatively, conjugate addition to the keto-form **12** would afford a spiro-intermediate **13** that could ring expand to the same phenanthrene derivative **11**. An analogous rearrangement has been proposed in the biosynthesis of the spirocyclic fredericamycin A.[26]

We have previously reported the biosynthesis of the benz[*a*]anthraquinone PD 116198,[27] which apparently proceeds via ring-cleavage and rearrangement of a linear tetracyclic intermediate to its final angular skeleton. It is likely that other polycyclic aromatic polyketides will prove to be derived from cryptic rearrangements, as well.



Scheme 2

EXPERIMENTAL

General.

NMR spectra were obtained on Bruker AM 400 and AC 300 spectrometers. Samples were analyzed on a Waters 600E HPLC with a Waters NovaPak C_{18} radial compression column (0.8 x 10 cm, 5 μm) eluting at 1.50 mL/min with a linear gradient from 5 to 95% acetonitrile in H_2O (each containing 0.1% HOAc) over 20 min using a Waters 990+ photo diode array detector with 2 nm resolution over the range 200-650 nm. Sodium [1,2-$^{13}C_2$]acetate and [1-^{13}C,$^{18}O_2$]acetate were obtained from Cambridge Isotope Laboratories, Inc. Compounds were dissolved in H_2O and introduced in equal aliquots through a 2 μm syringe filter.

Production and Isolation of ***1****.*

A. From *S. murayamaensis* mutant MC3. A Kinako soybean-glucose seed culture[17] (50 mL in a 250 mL foam-stoppered Erlenmeyer flask) was inoculated from agar plates with actively growing mycelium, and incubated on a rotary shaker at 27 °C, 280 rpm for 48 h. Production media (glycerol-ammonium sulfate[17] 400 mL in each 2 L foam-stoppered flask) were inoculated 5% v/v with seed culture. At harvest (36 hr after inoculation), a sample (10.0 mL) was mixed with an equal volume of EtOAc, acidified to pH 3 with 1 M HCl, and sonicated. After filtration through celite, the organic soluble portion was concentrated to dryness *in vacuo*, redissolved in CH_2Cl_2:MeOH (10:1, 1.00 mL) and analyzed by HPLC; quantitation was based on a standard curve using authentic **1**.

The remainder of the culture was similarly treated through sonication and filtration, and the aqueous fraction was re-extracted twice with EtOAc. The combined organic fractions were dried and concentrated to dryness, and the residue treated with $CHCl_3$. The $CHCl_3$-soluble material was chromatographed on flash silica gel (1.5 x 20 cm) prepared and eluted with MeOH:EtOAc:CH_2Cl_2 = 1:2:97. Fractions containing **1** were combined and concentrated, and **1** was recrystallized from $CHCl_3$-pentane.

B. From *S. murayamaensis* mutant MC4. An aliquot (5% v/v) of Kinako soybean-glucose seed culture was used to inoculate oatmeal-trace metals production media.[17] These cultures were harvested 120 h after inoculation and worked up in the same manner.

Incorporation of sodium [1,2-$^{13}C_2$]acetate into ***1****.*

A. Using mutant MC3. Sodium [1,2-$^{13}C_2$]acetate (248.6 mg), sodium [1-^{14}C]acetate (2.00 μCi), and unlabeled sodium acetate (251.1 mg) in H_2O (10.0 mL) were added to a 400 mL production culture in 5 portions at 12, 15, 18, 21, and 24 h after inoculation. After harvest and work-up, 108 mg of organic extract was obtained, which yielded 57.3 mg after chromatography, and 31.9 mg of **1a** after recrystallization. HPLC analysis of the crude extract indicated 33.4 mg of **1a** had been produced. ^{13}C NMR (100.6 MHz, $CDCl_3$) data given in Table 1, average enrichment of 2.0% per site. A 2D INADEQUATE spectrum was obtained using the Bruker INAD2D.AUR program .

B. Using mutant MC4. Sodium [1,2-$^{13}C_2$]acetate (300 mg) and unlabeled sodium acetate (300 mg) in H_2O (12.0 mL) were added in equal portions to 3 x 400 mL production cultures at 21, 33, 45, and 57 h after

inoculation. Workup after 120 h gave 4 mg of pure **1a** and 25 mg of pure kinamycin D. The sample of **1a** was analyzed as described above.

Incorporation of Sodium [1-^{13}C, $^{18}O_2$]acetate into 1.

Using mutant MC3, sodium [1-^{13}C, $^{18}O_2$]acetate (750 mg) and sodium [1-^{14}C]acetate in H_2O (10.0 mL) were added to two 400 mL production cultures in 5 portions at 14, 17, 20, 23, 27 h after inoculation. The organic extract (214 mg) was chromatographed to yield 71.2 mg which afforded 42.9 mg of pure **1b**. HPLC analysis indicated that 45.5 mg of **1b** had been produced. ^{13}C NMR analysis indicated an average enrichment of 17.4% per labeled position and revealed coupling (J = 11.5 Hz) between δ182.2 (C-9) and δ114.6 (C-10a).

ACKNOWLEDGMENTS

This research was supported by U.S. Public Health Service Grant GM 31715 to S. J. G. The N. L. Tartar Charitable Trust to Oregon State University provided partial support to C. R. M. Dr. Martha Cone is thanked for preparing and characterizing the mutant organism used in this work. Mr. Rodger Kohnert is thanked for assistance with the 2D INADEQUATE spectra. The Bruker AM 400 NMR spectrometer was purchased in part through grants from the National Science Foundation (CHE-8216190) and from the M. J. Murdock Charitable Trust, and the Bruker AC 300 NMR spectrometer was purchased in part through grants from the Public Health Service Division of Research Resources (RR04039-01) and the National Science Foundation (CHE-8712343) to Oregon State University.

REFERENCES

1. Sato, Y.; Kohnert, R.; Gould, S. J. *Tetrahedron Lett.* **1986**, *27*, 143-146.
2. Takagi, M.; Yamazaki, H.; Okada, T.; Takeda, U.; Sasaki, T.; Sezaki, M.; Miyaji, S.; Kojima, M. *Japan Patent JP* 62 30735 *9 Feb 1987* **1987**; *Chem. Abstr.* **1987**, 107:76091d.
3. Takagi, M.; Shimizu, T.; Okada, T.; Takeda, U.; Sasaki, T.; Miyado, S.; Shomura, T.; Sezaki, M.; Kojima, M. *Meiji Seika Kenkyu Nenpo* **1985**, 32-37; *Chem. Abstr.* **1985**, 106:152652j
4. Chu, M.; Mierzwa, R.; Truumees, I.; King, A.; Patel, M.; Berrie, R.; Hart, A.; Butkiewicz, N.; DasMahapatra, B.; Chan, T.-M.; Puar, M. S. *Tetrahedron Lett.* **1996**, *37*, 7229-7232.
5. Melville, C. R.; Gould, S. J. *J. Nat. Prod.* **1994**, *57*, 597-601.
6. Herbert, R. B. *The Biosynthesis of Secondary Metabolites*; Chapman & Hall: London, 1989.
7. Fritzmeier, K.-H.; Kindl, H. *Z. Naturforsch* **1984**, *39c*, 217-221.
8. Polonsky, J.; Johnson, B. C.; Cohen, P.; Lederer, E. *Bull. Soc. Chim. Fr.* **1963**, 1909-1917
9. Lounasmaa, M.; Zylber, J. *Bull. Soc. Chim. Fr.* **1969**, 3100-3103
10. Krone, B.; Hinrichs, A.; Zeeck, A. *J. Antibiot.* **1981**, *34*, 1538-1543
11. Thomson, R. H. in *Naturally Occurring Quinones*, Butterworths Sci. Publ.: London, 1957; pp. 257.
12. Thomson, R. H. in *Comparative Biochemistry*; Academic Press: New York, 1962; Vol. II, pp. 631.
13. Birch, A. J., *Chem. Soc. Symposia*, 1958; 16.

14. Birch, A. J., *Proc. Chem. Soc.*, 1962; 3-13.
15. Zylber, J.; Zissmann, E.; Polonsky, J.; Lederer, E.; Merrien, M. A. *Eur. J. Biochem.* **1969**, *10*, 278-283.
16. Fu, H.; Hopwood, D. A.; Khosla, C. *Chem. & Biol.* **1994**, *1*, 205-210.
17. Cone, M. C.; Seaton, P. J.; Halley, K. A.; Gould, S. J. *J. Antibiot.* **1989**, *42*, 179-188.
18. Erickson, W. R.; Gould, S. J. *J. Am. Chem. Soc.* **1985**, *107*, 5831-5832.
19. Gould, S. J.; Cheng, X.-C.; Melville, C. R. *J. Am. Chem. Soc.* **1994**, *116*, 1800-1804.
20. Simpson, T. J.; Holker, J. S. E. *Phytochemistry* **1977**, *16*, 229-233.
21. Brobst, S. W.; Townsend, C. A. *Can. J. Chem.* **1994**, *72*, 200-207.
22. Harrison, P. H.; Noguchi, H.; Vederas, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 3833-3834.
23. Cane, D. E.; Yang, C.-C. *J. Am. Chem. Soc.* **1987**, *109*, 1255-1257.
24. Cane, D. E.; Ott, W. R. *J. Am. Chem. Soc.* **1988**, *110*, 4840-4841.
25. Li, Z.; Martin, F. M.; Vederas, J. C. *J. Am. Chem. Soc.* **1992**, *114*, 1531-1533.
26. Thomas, R. *Folia Microbiol.* **1995**, *40*, 4-16.
27. Gould, S. J.; Cheng, X.-C. *Tetrahedron* **1993**, *49*, 11135-11144.

(*Received in USA* 6 *January* 1997; *revised* 25 *February* 1997; *accepted* 26 *February* 1997)