Biosynthetic Origin of the Carbon Skeleton of Simaomicin α,† a Hexacyclic Xanthone Antibiotic‡

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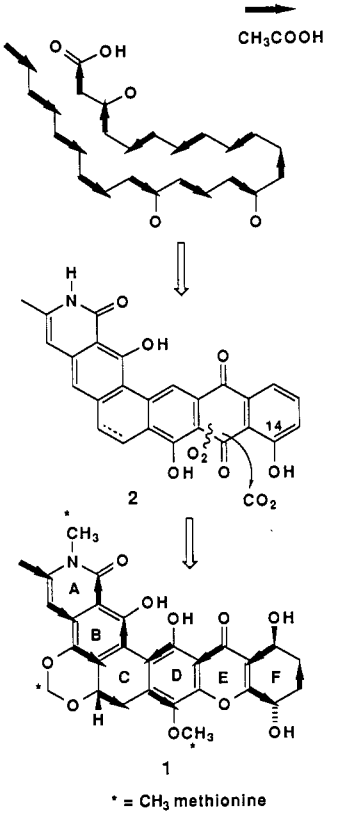
The biosynthetic origin of the hexacyclic skeleton of simaomicin α (1) was determined by incorporation of ^{13}C-labeled precursors. The origin of this ring system was of interest, since it could not be derived from the simple folding of a single polyketide chain. Simaomicin α is the first member of a small family of polycyclic xanthone-type antibiotics whose biosynthesis has been studied. Definitive assignments of the ^{13}C NMR signals for 1 were determined by LR HETCOSY experiments. By feeding ^{13}C-labeled acetates to cultures of *Actinomadura madurae* ssp. *simaoensis*, it was determined that all of the ring carbons were derived from acetate. A biosynthetic scheme is proposed wherein the hexacyclic skeleton is derived from a single polyketide chain via a carbocyclic intermediate (2). It is postulated that the pyrone ring is formed by oxidative cleavage of the E ring of 2, followed by decarboxylation and recyclization through a newly added oxygen atom.

Simaomicin α[1] (1) is a recently described hexacyclic xanthone antibiotic produced by *Actinomadura madurae* ssp. *simaoensis*. As an antibiotic, simaomicin is primarily active against Gram-positive bacteria, giving minimum inhibitory concentration (MIC) values of less than or equal to 0.06 μg/mL for most of these organisms.[2] It is, however, the antiparasitic activity of the compound versus protozoans of the genus *Eimeria*, which has generated the greatest interest. Simaomicin α is the most potent naturally occurring anticoccidial agent ever reported for the treatment of *E. tenella* infections in chickens.[3] Simaomicin α is structurally related to a small group of antibiotics including lysolipin,[4] albofungin,[5] cervinomycin,[6] actinoplanone,[7] and LL-E19085α.[8] Representative structures in this family are presented in Figure 1. The biosynthesis of simaomicin α is of interest since the carbon skeleton cannot be derived from the simple folding of a single polyketide chain. The xanthone portion of the structure is either formed by condensation of two independently formed units or by oxidative cleavage of a carbocyclic intermediate derived from a single polyketide chain, loss of a carbon, presumably as CO_2, and reclosure of the ring with a newly added oxygen atom, as outlined in Scheme I.

In this paper are presented the detailed assignments for the ^{13}C NMR signals of simaomicin α and evidence for the single-chain biosynthetic route for construction of its carbon skeleton. This is the first biosynthetic investigation reported for this family of antibiotics.

Scheme I. Biosynthetic Origin of the Carbon Skeleton of Simaomicin α



Experimental Section

Incorporation Experiments. Inoculum for the fermentation of *A. madurae* ssp. *simaoensis* was built up in two stages from frozen vegetative material in a seed medium consisting of: (g/liter) glucose (10), dextrin (20), NZ Amine A (5), yeast extract (5), and $CaCO_3$ (1) for 3 days at 28 °C. The second stage seed culture (100 mL total) was used to inoculate 2 L of fermentation medium (100 mL in each of 20 500-mL flasks). The fermentation medium consisted of (g/liter): sucrose (30), soy flour (15), corn steep liquor (5), and $CaCO_3$ (1). At 48 and 72 h following inoculation, the labeled compounds were added in equal portions. Enriched acetates were included at a final concentration of 1.0 g/L; labeled methionine was added to a final concentration of 0.5 g/L. These cultures were harvested after 7 days of cultivation at 32 °C, when the titer of antibiotic had reached an average of 29 μg/mL.

† Previously designated as LL-D42067α (see ref 2).

‡ This paper is dedicated to Professor Kenneth L. Rinehart, Jr., on the occasion of his 60th birthday.

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(2) Goodman, J. J.; Labeda, D. P.; Torrey, M. J.; Maiese, W. M.; Korshalla, J. D.; Testa, R. T. Presented at the 26th Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, LA, 28 Sept–1 Oct, 1986; Abstract No. 221.

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(4) Dobler, M.; Keller-Schierlein, W. *Helv. Chim. Acta* **1977**, *60*, 178–185.

(5) Gurevich, A. I.; Karapetyan, M. G.; Kolosov, M. N.; Omelchenko, V. N.; Onoprienko, V. V.; Petrenko, G. I.; Popravko, S. A. *Tetrahedron Lett.* **1972**, 1751–1754.

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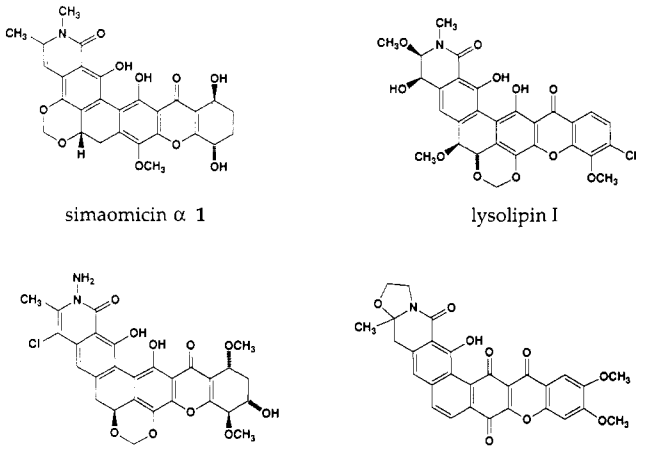

Figure 1. Polycyclic xanthone antibiotics.

Isolation Procedure. The pooled (ca. 1.8 L) fermentation mash was acidified to pH 3.0 with 6 N HCl. Celite 545 (100 g) was added, and the suspension was filtered. The resultant mycelial cake and filtrate were processed separately. The cake was extracted with two 1.0-L portions of acetone. The acetone extract was concentrated to an aqueous residue, and the product was extracted into ethyl acetate. The filtrate was extracted with an equal volume of ethyl acetate. The combined ethyl acetate extract was washed with 5% $NaHCO_3$, 0.1 N HCl, and water prior to concentration under reduced pressure to an oily residue. The bulk of the residue was dissolved in the THF/MeOH (9/1), 2.5 mL, for chromatography on a C_8 reversed-phase HPLC column. The column was 2.5 × 25 cm packed with 10 μm C_8-bonded silica. The mobile phase consisted of 35% acetonitrile/65% 0.1 M NH_4OAc at pH 4.5. The flow rate was maintained at 10 mL/min, and the column effluent was monitored by UV absorbance at 254 and 405 nm. Fractions were combined on the basis of analytical HPLC and neutralized with $NaHCO_3$. The purified product was recovered from the combined fractions by extraction into methylene chloride. Typically, the isolated yield of antibiotic from 2 L of culture was 20 mg.

NMR Experiments. The LR HETCOSY experiments were performed on a Bruker AM400 spectrometer, according to published procedures.[9,10] Initial spectral acquisition parameters were as follows: 18519-Hz sweep width in the F_2 dimension; 128 spectra (104 scans each) were accumulated with a 3.0-s relaxation delay and 0.165-ms increments across the interval 3.70–24.82 ms, with 2.26 Hz/pt resolution in the F_2 dimension and 11.8 Hz/pt in the F_1 dimension. A second experiment with greater resolution in the F_1 dimension revealed crosspeaks for δ 2.45/125.8 and δ 3.62/141.4. In this case, 0.345-ms increments were used across the interval 3.70–47.86 ms, yielding a 2.35 Hz/pt resolution in F_2 and 5.7 Hz/pt resolution in F_1. The ^{13}C NMR measurements used to determine enrichment levels were carried out on a Nicolet NP-300 WB at 75.5 MHz. Intensities were normalized to that of the signal at 61.6 ppm assigned to the OCH_3.

Results and Discussion

In order to use ^{13}C NMR to follow precursor incorporation, the ^{13}C NMR spectrum of 1 had to be unequivocally assigned. Due to the large number of quaternary carbons present in 1, these assignments were not obvious, and two dimensional NMR experiments had to be employed to resolve the ambiguities. Data from the LR HETCOSY spectrum of 1 in DMSO-d_6 allowed clear assignments of all but two of the quaternary carbon resonances. A diagramatic representation of these interactions is presented in Figure 2. Due to slow exchange in this solvent, three crosspeaks were observed for each hydrogen-bonded phenol resonance. Since there were also crosspeaks between δ 109.2 and 6.70 (H-3), and between δ 113.7 and 2.58 (H- 8), these carbon-13 resonances could be assigned to C-25 and C-22, respectively; thus these six (C-20 to C-25) quarternary carbon resonances were assigned.

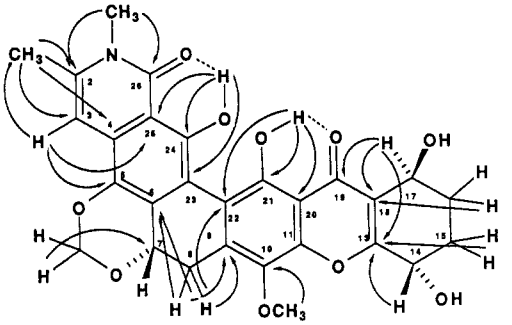

Figure 2. Correlations from LR HETCOSY experiments.

The H-17 resonance at δ 4.80 showed crosspeaks to δ 182.3 (C-19), 119.0, and 165.2; the last resonance also showed a crosspeak to δ 4.55 (H-14). C-13 (δ 165.2) and C-18 (δ 119.0) could therefore be assigned from the chemical shifts and the crosspeaks, and the correct ^1H NMR assignment for H/C-15 and H/C-16 then followed from the observed $^3J_{CH}$ to either C-13 or C-18. C-26 (δ 165.5), C-2 (δ 141.4), and C-10 (δ 136.1) were assigned from crosspeaks with the relevant methyl resonances.

H-3 (δ 6.70) also showed crosspeaks with the *C*-methyl resonance and with δ 135.0, allowing assignment of the latter to C-5. While assignment of C-7 and C-17 from the HETCOR spectrum was ambiguous due to the closeness of the proton chemical shifts, the crosspeak between the protons of the methylenedioxy group (δ 5.55) and δ 71.7, clarified the correct matches.

Four carbons, C-4, -6, -9, and -11, remained to be assigned. Of these four, one (δ 125.8) exhibited a crosspeak with the *C*-methyl, two exhibited crosspeaks with both H-8 resonances, and there were no crosspeaks to the fourth (δ 147.9). Therefore, δ 125.8 has been assigned to C-4, δ 147.9 has been assigned to C-11,[11] and the last two go with either C-9 or C-6. This ambiguity in assignments was removed as a result of the acetate incorporation experiments described below.

Cultures of *A. madurae* ssp. *simaoensis* were supplemented with [1-^{13}C], [2-^{13}C], and [1,2-$^{13}C_2$]acetate as well as $^{13}CH_3$-labeled methionine to determine the origin of the carbon skeleton of simaomicin α. The results of these feeding experiments (Table I) show that all of the ring carbons and the *C*-methyl group are derived from acetate. The extent of incorporation of the labeled acetates was quite variable from experiment to experiment. Thus, the enrichment factors obtained with [2-^{13}C]acetate were quite high (7.2–15.9), whereas those calculated in the [1-^{13}C]-acetate experiment were somewhat lower (1.8–4.5). From the enrichment factors, it is clear that carbons 2a-CH_3, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25 were labeled by the methyl carbon of acetate. The pattern obtained with carboxy-labeled acetate is also clear, even though the en-

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(9) Bleich, H.; Gould, S.; Pitner, P.; Wilde, J. *J. Magn. Reson.* **1984**, *56*, 515.
(10) Sato, Y.; Geckle, M.; Gould, S. J. *Tetrahedron Lett.* **1985**, *26*, 4019.
(11) This is in good agreement with the assignment of the corresponding carbon in the ^{13}C NMR spectra of the actinoplanones (ref 7).

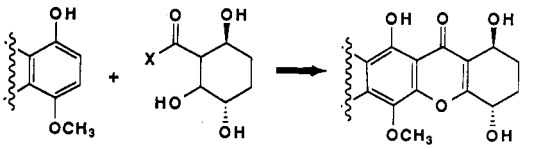
Table I. Acetate Incorporation Data for 1

	δ[a]	[1-^{13}C]Ac EF[b]	[2-^{13}C]Ac EF	[1,2-$^{13}C_2$]Ac J (Hz)
2	141.4	2.2	1.2	48.2
2a-CH_3	20.4	1.1	15.2	48.1
3	100.0	1.0	13.8	54.5
4	125.8	2.4	1.0	54.6
5	135.0	0.9	7.7	69.2
6	126.7	2.8	1.4	70.1
7	71.7	0.9	13.4	38.9
8	29.1	3.6	2.2	38.5
9	135.3	1.0	9.6	AB
10	136.1	4.5	1.8	AB
11	147.9	0.9	7.2	c
13	165.2	0.9	13.4	52.2
14	63.3	3.2	1.8	51.9
15	25.8	1.2	14.7	AB
16	25.4	3.8	2.0	AB
17	58.5	1.2	14.4	48.0
18	119.0	1.8	1.2	48.3
19	182.3	0.5	9.5	54.5
20	109.7	1.8	1.2	55.3
21	152.5	1.2	15.9	72.9
22	113.7	3.8	1.5	72.8
23	111.0	1.0	10.1	75.2
24	151.2	4.5	1.6	75.2
25	109.2	1.3	11.0	68.6
26	165.5	2.6	1.0	66.8
NCH_3	30.4	1.2	1.6	c
OCH_3	61.6			c
OCH_2O	90.4	1.1	1.5	c

[a] In DMSO-d_6, ppm downfield from TMS. [b] Enrichment factor (EF) is expressed as the ratio of normalized signals between labeled and natural abundance spectra. [c] Only a single unsplit resonance was observed.

richment in the experiment was not as great. Thus, carbons 2, 4, 6, 8, 10, 14, 16, 18, 20, 22, 24, and 26 show enhanced signals indicating labeling by the carboxy carbon of acetate. The coupling constants obtained in the doubly labeled acetate experiment (Table I) confirm the labeling pattern of the individual carbons and show which pairs of carbons represent acetate units as assembled in the original polyketide. The coupling observed between the C-5 signal and the resonance at δ 126.7 secured the assignment of the latter to C-6. Similarly, C-10 is coupled to the signal at δ 135.3, which must represent C-9. The remaining carbons, NCH_3, OCH_3, and OCH_2O were all derived from the *S*-methyl of methionine, as extensive incorporation of the $^{13}CH_3$-labeled material was observed.[12] The acetate labeling pattern found for simaomicin α is shown in Scheme I. The intact acetate units, determined by pairing the coupling constants listed in Table I, are represented by bold arrows. Only one carbon, C-11, appears uncoupled as a result of being severed from its corresponding carboxy carbon. This pattern is consistent with oxidative cleavage of a carbocyclic precursor derived from a single polyketide chain, as shown in Scheme I. The hypothetical quinone precursor **2** would be derived from the condensation of 13 acetate units in the normal head to tail fashion, followed by a series of ring-forming condensations, dehydrations, oxidations, and amination. In this proposed intermediate, the oxygen atoms shown are the ones presumed to be derived from acetate, except for the quinone oxygen on C-19. The latter oxygen may or may not be present prior to the formation of the pyrone, but must be introduced subsequent to the elaboration of the polyketide chain. The degree of unsaturation of **2** is shown to be either fully aromatic or nearly so, with the possibility that the 7,8 bond is saturated, as it is in the final product. The pyrone is postulated to be formed by oxidative fission of the quinone ring, loss of C-12 as CO_2, and reclosure of the ring with a newly added oxygen. Ring closure occurs only at C-13 since there is no randomization of the labeling pattern in the F ring.[13]

Scheme II. Two-Chain Route for Pyrone Formation



The possibility that two polyketide chains were condensed to form the E ring, although not absolutely ruled out, is not supported by these data. Mechanistically, the most attractive route for such a condensation would involve the condensation of a separately derived seven-carbon fragment corresponding to C-13 to C-19 onto a tetracyclic precursor, as shown in Scheme II. The operation of this route is precluded by the discovery that C-19 and -20 constitute an acetate unit.

In conclusion, all the ring carbons of simaomicin α were shown to be derived from acetate. Evidence was presented supporting the biosynthetic sequence outlined in Scheme I, wherein the molecule is assembled via a single polyketide chain to give the postulated intermediate **2**. The pyrone ring appears to be formed by oxidative cleavage of the E ring, followed by decarboxylation and reclosure with a newly added oxygen atom.

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(12) Enrichment factors were not calculated for these signals as their enrichment was obvious, having intensities at least 40× those of the remaining signals.

(13) Randomization of the labeling pattern would suggest the intermediacy of a compound with a symmetrically substituted terminal ring, as was observed in the biosynthesis of ravenelin: Birch, A. J.; Baldas, J.; Hlubucek, J. R.; Simpson, T. J.; Westerman, P. W. *J. Chem. Soc., Perkin Trans. 1* **1976**, 898–904.