Biosynthesis of Actinorhodin. Determination of the Point of Dimerization

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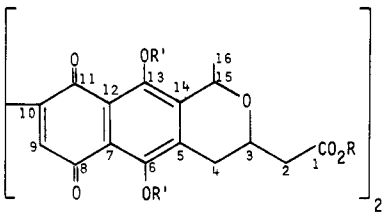
Actinorhodin (**1a**) is a dimeric isochromanequinone antibiotic produced by *Streptomyces coelicolor* A3(2).[1] Its structure has been elucidated[2] but the points of connection of the two identical molecular halves remained undetermined. In this note we present results establishing the biosynthesis of this dimeric antibiotic, from which the point of dimerization may be deduced.

Results and Discussion

After preliminary experiments with sodium [1-^{14}C]-acetate, feeding experiments were conducted with sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]acetates. The results of these studies together with the assignment of the natural abundance proton noise-decoupled ^{13}C NMR spectrum of actinorhodin dimethyl ester tetraacetate (**1c**) are presented in Table I. The use of the derivatized actinorhodin was necessitated by the poor solubility of the metabolite itself in organic solvents. The derivative (**1c**) could be obtained from actinorhodin (**1a**) via the dimethyl ester (**1b**) as has been detailed previously.[3]



1a R = R' = H

1b R = CH_3; R' = H

1c R = CH_3; R' = $COCH_3$

The unequivocal assignment of every signal in the spectrum was made by using the characteristic chemical shifts[4] and multiplicities, analysis of one-bond carbon–carbon couplings of pairs of carbon atoms, and comparison with related compounds, especially granaticin[5] and 5,8-diacetoxynaphthoquinone.[6] The aliphatic carbon signals are in close agreement with their granaticin counterparts, while the aromatic carbon signals do not differ too greatly from their analogues in 5,8-diacetoxynaphthoquinone, with the exception of the signals assigned to C-5, C-9, C-10, and C-14. The assignment of the C-9 signal was apparent from its multiplicity arising from directly bonded C–H coupling, whereas the C-5 and C-10 signals could be assigned by analysis of their carbon–carbon coupling constants in the spectrum of (**1c**) obtained from sodium [1,2-$^{13}C_2$]acetate.

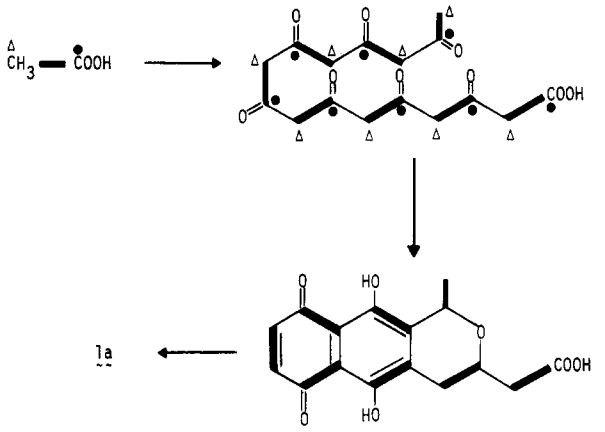
The biosynthesis of the basic carbon skeleton of the isochromanequinones proceeds from acetate units via the polyketide pathway as has been demonstrated for the nanaomycins,[7] α-naphthocyclinone,[8] and granaticin.[9] This was confirmed for actinorhodin by the results shown in Table I, the spectrum of the [1-^{13}C]acetate-derived compound (**1c**) showing eight enhanced carbon signals corresponding to C-1, C-3, C-5, C-7, C-9, C-11, C-13, and C-15. Conversely, [2-^{13}C]acetate increased the intensity of the eight carbon signals corresponding to C-2, C-4, C-6, C-8,

Table I. ^{13}C NMR Spectral Data for Actinorhodin Dimethyl Ester Tetraacetate (1c), Including Relative Enrichment Factors from Sodium [1-^{13}C]- and [2-^{13}C]Acetates

carbon no.	δ[a]	multiplicity[b]	enrichment factors[c] [1-^{13}C]	enrichment factors[c] [2-^{13}C]	$^1J_{C-C}$, Hz
1	170.8	Sm	5.22	1.13	58.4
2	40.4	T	0.99	4.90	58.5
3	63.3	Dt	6.53	1.28	37.9
4	27.5	T	0.89	4.19	37.8
5	141.0	Sm	5.49	1.10	53.7
6	146.7	S	0.99	3.25	53.5
7	124.4	Sd	4.21	0.99	*d*
8	181.5	Sd	0.77	3.40	54.6
9	131.4	D	6.40	1.50	69.2[d]
10	146.7	S	1.06	3.51	69.6
11	180.9	Sd	5.53	2.88	*d*
12	125.0	S	1.31	3.38	*d*
13	144.8	Sd	6.09	1.09	*d*
14	136.5	Sd	0.85	3.56	*d*
15	67.1	D	5.58	1.18	37.1
16	19.0	Qd	1.21	3.91	37.1
ester CH_3	51.8		1.00	1.00	
acetate CO	169.1				
acetate CH_3	20.9				
	20.7				

[a] Chemical shifts are given in parts per million downfield from internal Me_4Si in $CDCl_3$. [b] Multiplicities in the gated decoupled spectrum. Capital letters refer to directly bonded C–H couplings, small letters refer to >1 bond C–H couplings. S = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. [c] The enrichment is relative to the methyl ester signal as 1.0. [d] The long relaxation times of these carbon atoms and the overlap of signals rendered the determination of coupling constants difficult.

Scheme I. Biosynthesis of Actinorhodin, 1a



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C-10, C-12, C-14, and C-16. Incorporation of eight intact acetate units in the polyketide chain was indicated by analysis of the derivative (1c) obtained from feeding experiments with [1,2-$^{13}C_2$]acetate. Although the long relaxation times of some of the carbon atoms and the overlap of certain signals precluded the complete determination of all the carbon–carbon coupling constants, the available data were consonant with a polyketide pathway with the chain extending from C-16 through the ring systems to C-1, in the direction of decreasing numbers of the carbon atoms as shown in the Scheme I.

The only remaining ambiguity in the structural elucidation of actinorhodin, viz., whether the two monomeric units are connected at C-9 or at C-10, could be neatly resolved from the biosynthetic enrichment studies. The only sp^2 carbon atom bearing a directly bonded hydrogen atom arises from C-1 of acetate, thus identifying it as C-9 and placing the point of dimerization at C-10. The same conclusion has been reached by Zeeck and co-workers[10] on purely chemical grounds.

Experimental Section

^{13}C NMR spectra were recorded on Varian FT-80, JEOL PFT-100, and Nicolet NT-360 spectrometers. The spectra were generally recorded at a repetition time of 5 s from a 90° pulse, using $CDCl_3$ as solvent.

Incorporations of Sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-Acetates. Preliminary experiments on cultures of *Streptomyces coelicolor* A3(2) grown in shake cultures (300 rpm) at 28 °C on complete medium[11] showed that actinorhodin production reached a maximum on the tenth day after inoculation.

To each of eight 500-mL Erlenmeyer flasks containing the 4-day-old growth of *Streptomyces coelicolor* on the complete medium (150 mL) was added [1-^{13}C]-, [2-^{13}C]-, or [1,2-$^{13}C_2$]acetate (1 g; 90% enriched) in portions every 24 h from day 4 to day 7. After 10 days the cultures were filtered, and the mycelium was washed with hydrochloric acid (0.1 N, 2 L) and stirred with 2 N hydrochloric acid (100 mL) for 2 h and then with acetone (100 mL) for 30 min. The residue was filtered, dried, and stirred with sodium hydroxide (2 N, 100 mL). After filtration the filtrate was acidified with hydrochloric acid (2 N). The crude actinorhodin obtained on centrifugation was dried in vacuo. A typical yield was 640 mg.

Actinorhodin Dimethyl Ester Tetraacetate (1c). The crude finely powdered actinorhodin obtained above was suspended in methanol–dioxane (1:1 v/v, 200 mL). Dry hydrogen chloride was bubbled through the suspension until saturation, and the resulting mixture refluxed for 3 h. The solvent was removed under reduced pressure and the residue partitioned between chloroform and water. The chloroform layer was dried (Na_2SO_4) and the solvent removed under reduced pressure. The crude actinorhodin dimethyl ester (1b) was dissolved in acetic anhydride–pyridine (1:2 v/v, 15 mL) and left at 20 °C for 24 h. The solution was poured into water (150 mL) and extracted with chloroform (2 × 50 mL). The chloroform layer was dried (Na_2SO_4) and the solvent removed under reduced pressure. Purification by chromatography on silica gel, eluting with chloroform–methanol (95:5 v/v), and crystallization from benzene gave actinorhodin dimethyl ester tetraacetate (1c) (40 mg) as yellow needles, whose identity was confirmed by comparison of its properties with the published data.[3]

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Registry No. 1a, 15428-92-9; 1c, 15429-00-2.

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Synthesis of Calcitroic Acid, a Metabolite of 1α,25-Dihydroxycholecalciferol[1]

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1α,25-Dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) is the most potent known metabolite in the vitamin D series for the regulation of calcium and phosphate homeostasis.[2] Recently it was discovered that rats rapidly metabolize 1,25-$(OH)_2D_3$ to a compound having an acid function on the side chain.[3] This metabolite was isolated as the methyl ester and identified as methyl 1α,3β-hydroxy-24-nor-9,10-secochola-5,7,10(19)-trien-23-oate or calcitroic acid methyl ester.[3] The synthesis of **1a** was of interest to confirm the structure of the biologically generated compound and to provide a route for obtaining a sufficient quantity of the metabolite for examining its biological activity. A convenient synthetic route yielding **1** and a comparison of spectral and chromatographic properties of synthetic and biologically generated **1b** are presented herein.

By use of the general method of Ryer and Gebert,[4] an Arndt–Eistert homologation sequence starting with commercially available **2** provided the side chain desired in the final product. Use of methanol in the silver oxide catalyzed Wolff rearrangement directly yielded the methyl ester, **3**, which was recovered in approximately 60% yield after recrystallizations; **3** was converted to the 5,7-diene, **4a**, by allylic bromination and dehydrobromination.[5,6] Retention of configuration at C-20 is expected[7] and was confirmed by 270-MHz NMR spectra. Mild hydrolysis yielded **4b** which was purified and irradiated to form previtamin **5**. After separation from the other photoisomers by high-pressure liquid chromatography (high-pressure LC), **5** was thermally isomerized to vitamin D ester **6**.

Introduction of the 1α-hydroxy function was achieved by the method fo Paaren et al.[8] Intermediate **6** was converted to the 3,5-cyclovitamin **7** by bicarbonate-buffered methanolysis of the 3-tosylate.[9] Allylic oxidation of **7** with selenium dioxide and *tert*-butyl hydroperoxide in dichloromethane[8] gave the desired 1α-hydroxy derivative. Cycloreversion of the oxidized cyclovitamin with glacial acetic acid gave **1c**, which after high-pressure LC purification, was hydrolyzed to **1b**. This compound was fully characterized and used for comparison with the methylated biological metabolite. Vigorous alkaline hydrolysis yields the natural product, **1a**, for use in biological testing.

Synthetic **1b** demonstrated UV and mass spectra identical with those found for the methyl ester of the isolated metabolite[3] and was found to comigrate with the biological material in analytical high-pressure LC. These findings

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