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Application of ^{13}C-NMR to the Biosynthetic Investigations. II.[1] Biosynthesis of Aureothin and Related Nitro-containing Metabolites of *Streptomyces luteoreticuli*[2]

Mikio Yamazaki, Yukio Maebayashi,[3a] Humio Katoh, Jun-ichi Ohishi, and Yasumasa Koyama[3b]

Research Institute for Chemobiodynamics, Chiba University[3a] *and Faculty of Pharmaceutical Sciences, Chiba University*[3b]

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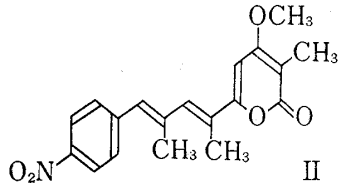
The participation of acetate and propionate to the biosynthesis of aureothin, one of the metabolites of *Streptomyces thioluteus* and *S. luteoreticuli* containing an aryl-nitro group has been proved by the use of ^{13}C tracer technique and ^{13}C-NMR spectrometry.

Aureothin (I), luteoreticulin (II), luteothin (III) isolated from *Streptomyces thioluteus*[4a] and *S. luteoreticuli*[4b] and neoaureothin (IV) from *Streptoverticillium orinoci*[5] are naturally occurring nitro-containing compounds having a particular type of structure, *p*-nitro-aryl-$(C{=}C)_n$-pyrone. Natural products containing an aryl nitro group have not been found so



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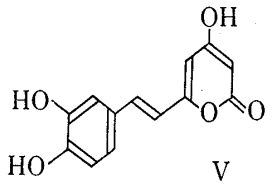

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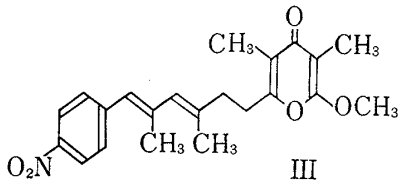

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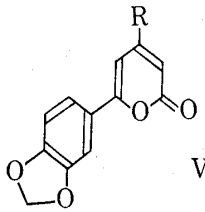

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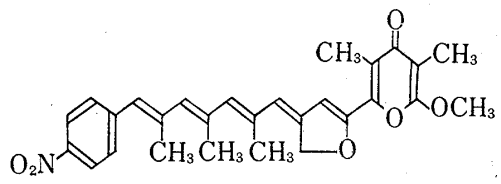

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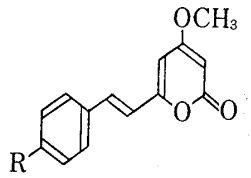


VII

1) Preceding paper in series: Y. Maebayashi, K. Miyaki, and M. Yamazaki, *Chem. Pharm. Bull.* (Tokyo), **20**, 2172 (1972).
2) Presented in part at the 90th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, 1970.
3) Location: *a*) *Izumicho-3-chome, Narashinoshi, Chiba*; *b*) *Yayoicho, Chiba-shi, Chiba.*
4) *a*) K. Maeda, *J. Antibiotics*, **A6**, 137 (1953); F. Washizu, H. Umezawa, and N. Sugiyama, *ibid.*, **A7**, 60 (1954); *b*) Y. Koyama, Y. Fukakusa, N. Kyomura, S. Yamagishi, and T. Arai, *Tetrahedron Letters*, **1969**, 355; S. Yamagishi, Y. Koyama, Y. Fukakusa, N. Kyomura, J. Ohishi, N. Hamamichi, and T. Arai, *Yakugaku Zasshi*, **91**, 351 (1971).
5) G. Cassinelli, A. Grein, P. Orezzi, P. Pennella, and A. Sanfilippo, *Archiv Mikrobiol.*, **55**, 358 (1967); C. Cardani, D. Ghiringhelli, A. Selva, F. Areamone, B. Camerino, and G. Cassinelli, *La Chimica L'Industria*, **52**, 793 (1970).

many but a few from some Actinomycetes and certain plants.[6] Hispidin (V)[7] from *Polyporus hispidus* (Basidiomycetes), paracotoin (VI, R=H)[8] and 4-methoxyparacotoin (VI, R=OCH_3)[8] or kawain,[8] 5,6-dehydrokawain (VII, R=H)[8] and yangonin (VII, R=OCH_3)[8] from higher plants are known examples of such a natural product containing an aryl-$(C=C)_n$-pyrone system.

On the source of the nitro group of this particular type of compound, it was reported that an addition of *p*-aminobenzoic acid to the culture of *Streptomyces thioluteus* increased the production of aureothin, and the ability to oxidize the amino to nitro group of this microorganism was simultaneously recognized.[9] Recently, authors[10] and Cardillo, *et al*[11] have demonstrated some biosynthetic origins of the individual part of aureothin in their preliminary report.

It is described in the present paper that a direct participation of one mole of acetate and four moles of propionate to the biosynthesis of aureothin passing through "Route B" as shown in Fig. 1 has been proved by using the ^{13}C-NMR spectrometry.

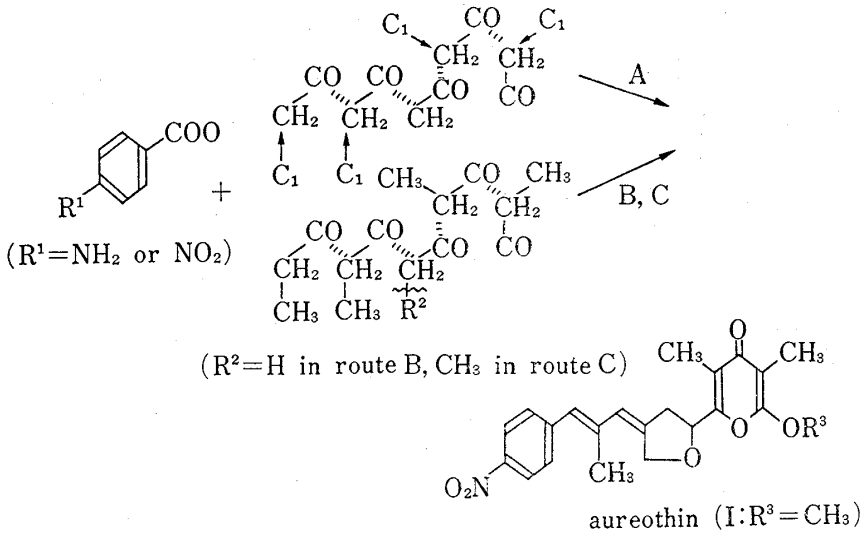


Fig. 1. Possible Routes for the Aureothin Biosynthesis

If the biosynthesis undergoes through "Route A," the participation of C_1-unit to make "extra methyls" would be an important process in the pathway. However, these methyls should be derived from the methyl of propionate but not by the incorporation of C_1-unit when the biosynthesis progressed through "Route B" or "C." The participation of propionate as well as of acetate is known to be involved in the biosynthesis of antibiotic macrolides although the sequence of those acids is not a definitive owing to the kind of microorganisms and metabolites produced by them.

Experimental

Microorganism and Cultivation——*Streptomyces luteoreticuli* Katoh et Arai (Strain KS 2—74) which was isolated from a soil sample collected in Beppu (Kyushu) was used in this experiment through the kindness of Prof. T. Arai of this Institute. Two ml of 48 hr preincubated inoculum was added to each 500 ml Sakaguchi's flask containing 100 ml of glucose-bouillon medium (glucose 10 g, polypeptone 10 g, bouillon 5 g,

6) M. Pailer, *Fortschr. Chem. Org. Naturstoffe*, **18**, 66 (1960); M. Pailer, P. Bergthaller, and G. Schaden, *Monatsh.*, **96**, 863 (1965).
7) R.L. Edwards, *J. Chem. Soc.*, **1961**, 4995 (5003).
8) P.J. van der Jagt, *Tetrahedron*, **27**, 1049 (1971).
9) S. Kawai, K. Kobayashi, T. Oshima, and F. Egami, *Arch Biochem. Biophys.*, **112**, 537 (1965).
10) M. Yamazaki, F. Katoh, J. Ohishi, and Y. Koyama, *Tetrahedron Letters*, **1972**, 2701.
11) R. Cardillo, C. Fuganti, D. Ghiringhelli, D. Giangrasso, and P. Grasselli, *Tetrahedron Letters*, **1972**, 4875.

NaCl 3 g in 1000 ml of water), and incubated for 4—5 days at 28° on a reciprocating shaker at 100—120 cycle/min. The culture broth was filtered and the mycelial cake was extracted with acetone repeatedly. Aureothin and luteoreticulin were isolated from the acetone extract according to the ordinary method as previously reported.[4b]

Feeding of L-Methionine-S-$^{14}CH_3$——L-Methionine-S-$^{14}CH_3$ (0.1 mCi, 1.28×10^{10} dpm/mmole) was added to 600 ml of the medium at the beginning of the incubation. Radioactive aureothin (10.8 mg, 1.77×10^{8} dpm/mmole) was isolated from the $CHCl_3$ soluble part of acetone extract of mycelia through Al_2O_3 column. Added 210.7 mg of non-radioactive aureothin and recrystallized from EtOH to give pure compound, mp 156—157° (206 mg). Specific Activity: 6.31×10^{6} dpm/mmole.

Demethylation of Aureothin——The radioactive aureothin (101.1 mg, 6.31×10^{6} dpm/mmole) obtained above was suspended in 30 ml of EtOH containing 5 ml of conc. HCl. Refluxed for 15 min. The reaction mixture was extracted with ether after cool and the solvent was evaporated. The residual solid was recrystallized from EtOH to give pure desmethylaureothin, mp 195.5—196° (89 mg).[12] Specific Activity: 0.0015×10^{6} dpm/mmole.

Feeding of Sodium Acetate-1-^{14}C——Na acetate-1-^{14}C (100 μCi) was added to 200 ml of the medium at the beginning of the incubation. Aureothin and luteoreticulin were isolated from the acetone extract of mycelia by the preparative thin-layer chromatography. The amount of aureothin and luteoreticulin was determined by measurement of their optical density at λ_{max} 345 and 268 nm in MeOH solution respectively.

Feeding of Sodium Propionate-2-^{14}C——Na propionate-2-^{14}C(50 μCi) was added to 600 ml of the medium at the beginning of the incubation. Crude luteoreticulin (8.5 mg, 4.8×10^{7} dpm/mmole) was obtained from the acetone extract according to the similar method as previously reported.[4b] Diluted with carrier luteoreticulin (34.2 mg) and recrystallized from MeOH to give pure compound, mp 184.5—185° (35 mg). Specific Activity: 4.2×10^{6} dpm/mmole.

Kuhn-Roth Oxidation of Luteoreticulin——Luteoreticulin (8.23 mg, 4.2×10^{6} dpm/mmole) obtained above was oxidized by CrO_3–H_2SO_4 and the reaction mixture was distilled with water. The distillate was titrated with 1/50 N NaOH and then evaporated to dryness. Na acetate obtained was dissolved in water and 1 ml of the solution was mixed with 2 ml of Biosolve No. 3 (Beckman Co.) and 12 ml of toluene scintillator (0.5% 2,5-diphenyloxazol) to measure the radioactivity.

Measurement of Radioactivity——Liquid Scintillation counter, Beckman Model LS-150 was employed.

Feeding of ^{13}C-Labeled Compounds——Na propionate-3-^{13}C (500 mg, 64% enriched, Sharp & Dohm of Canada) was added to 1000 ml of the medium at the beginning of the incubation. Aureothin (12.3 mg) was isolated by the similar method as reported.[4b] Na acetate-2-^{13}C (250 mg, 86.9% enriched, British Oxygen Co., London) was added to the medium and aureothin (43 mg) was obtained.[4b]

Measurement of ^{13}C-NMR Spectra——NMR spectrometer, JNM-PFT-100 (25.15 MHz) with equipped JEC-6 Computer (Japan Electron Optics Laboratory Co. Tokyo) was used for the measurement of ^{13}C-NMR spectra. The single and split signals of each carbon resonance were observed by using proton noise decoupling and off-resonance decoupling technique.

Aureothin (natural abundance): 400 mg/1.0 ml $CDCl_3$. F=6250 Hz. tw=11 μsec. Rep=4 sec×200. Ref=$CDCl_3$, 30 db(4—10). Temp=room temp.

Aureothin (^{13}C-enriched by feeding Na propionate-3-^{13}C): 12.3 mg/0.5 ml $CDCl_3$. F=6250 Hz. tw=11 μsec. Rep=4 sec×2500. Ref=$CDCl_3$. Lock=$CDCl_3$, 30 db(5—10). Temp=room temp.

Aureothin ^{13}C-enriched by feeding Na acetate-2-^{13}C): 42 mg/0.4 ml $CDCl_3$. F=6250 Hz. tw=9 μsec. Rep=2 sec×4000. Ref=$CDCl_3$. Lock=$CDCl_3$. Temp=room temp.

Measurement of Off-Resonance Decoupling Spectrum of Aureothin (natural abundance): 400 mg/1 ml $CDCl_3$. F=6250 Hz. tw=11 μsec. Rep=4 sec×500. Ref=$CDCl_3$. Lock=$CDCl_3$, 20 db(5—10). Temp.=room temp.

Results and Discussion

The result shown in Chart 1, indicated that the methyl from methionine was incorporated solely into the methoxy group but not into "extra methyls" of aureothin. Consequently, it would not seem appropriate that biosynthesis of aureothin undergoes through "Route A."

Acetate-1-^{14}C and propionate-2-^{14}C were well incorporated into aureothin and luteoreticulin in a feeding experiment as summarized in Table I. Acetic acid prepared by Kuhn-Roth oxidation of the radioactive luteoreticulin (4.19×10^{6} dpm/mmole), which was obtained by feeding of propionate-^{14}C, was radioactive (8.3×10^{5} dpm/mmole). The result suggested that propionate was definitely responsible for the formation of luteoreticulin. However, the detailed localization of radioactivity in the molecule was entirely obscure at the time.

12) Y. Hirata, H. Nakata, K. Yamada, K. Okuhara, and T. Naito, *Tetrahedron*, **14**, 252 (1961).

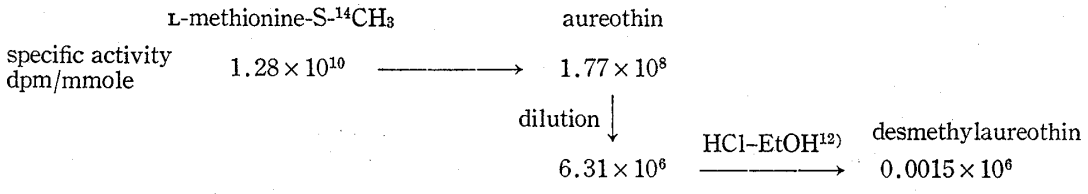


Chart 1. Incorporation of L-Methionine-S-$^{14}CH_3$ into the Methyl Group of Aureothin

TABLE I. Incorporation of Acetate-1-^{14}C and Propionate-2-^{14}C into Aureothin and Luteoreticulin

Metabolite	Precursor	
	Acetate-1-^{14}C (0.1 mCi) specific activity	Propionate-2-^{14}C (0.05 mCi) dpm/mmole
Aureothin	2.4×10^{6}	—
Luteoreticulin	1.08×10^{7}	4.19×10^{6}

TABLE II. The Chemical Shift of ^{13}C-NMR of Aureothin

Peak No.	Peak height	Chemical shift ppm from TMS	Pattern of split[a]	Assignment
1	118	4.90	quartet	CH_3–
2	118	7.47	quartet	CH_3–
3	150	16.02	quartet	CH_3–
4	149	36.95	triplet	–CH_2–
5	136	54.55	quartet	CH_3O–
6	146	69.59	triplet	–CH_2O–
7	170	72.93	doublet	–CH<
8	94	99.93	singlet	–C<
9	92	120.62	singlet	–C<
10	356	124.20	doublet	HC<×2
11	170	126.81	doublet	HC<
12	172	129.11	doublet	HC<
13	365	130.57	doublet	HC<×2
14	147	139.97	singlet	–C–<
15	172	141.97	singlet	–C<
16	138	145.67	singlet	–C<
17	90	147.25	singlet	–C<$_{NO_2}$
18	98	156.47	singlet	–C<
19	74	163.82	singlet	–C<
20	59	183.56	singlet	–C=O

total: 22 carbon resonances
a) off-resonance decoupling

To confirm more conclusive evidence concerning with the participation of acetate and propionate to the aureothin biosynthesis, ^{13}C-labeled acetate and propionate were fed to the Streptomyces and obtained aureothin was subjected to the ^{13}C-NMR spectrometry. The pulse NMR and Fourier transformation by the use of high resolution NMR spectrometer equipped with a programmable radio frequency pulse unit.

In Fig. 2, the ^{13}C-NMR spectra of aureothin; enriched by feeding acetate-2-^{13}C (a), propionate-3-^{13}C (b) to the medium, and natural abundance (c), are shown. The spectra were recorded by the aid of a proton noise decoupling to observe all signals as sharp singlets. In Fig. 2-c, 22 signals of all carbon resonances of aureothin were observed, in which two tall singlets at 124.20 and 130.57 ppm revealed each two carbon resonances. While, only one in Fig. 2-a and four strongly enhanced signals in Fig. 2-b appeared.

Off-resonance decoupling was usefully employed for assignment of these carbon shifts together with comparison of the chemical shifts. As shown in Table II, the presence of three methyls, one methoxy, two methylenes and one methine was apparently recognized by observing the pattern of split which was obtained by off-resonance decoupling. A signal at 72.93 ppm in Fig. 2-a could easily be assigned to the methine (C-6) adjacent to the oxygen atom. Three signals at 4.90, 7.47 and 16.02 ppm of the four appeared in Fig. 2-b were similarly assigned to the methyl and another signal at 69.59 ppm was methylene (C-13) adjacent to the oxygen. Two signals appeared at 124.20 and 130.57 ppm individually revealed two equivalent carbons as mentioned above and were assigned to the ortho and meta carbons in the nitrobenzene ring. It is known that the carbon bonded to the substituent absorbs over widest range but the *meta* carbons are the least affected by substitution. The *ortho* and *para* carbons exhibit appreciable shift for the assortment of substituents. For nitrobenzene, δ ppm from trimethyl silane (TMS): 148.3, 123.4, 129.5 and 134.7 have been reported as the chemical shift of the carbons of C-1, *ortho*, *meta* and *para* of the aromatic ring, respectively.[13] A singlet appeared at 147.25 ppm could be assigned accordingly to be the carbon bearing the nitro group and a singlet at 139.97 ppm could be of the *para* position. A singlet appeared at the lowest field, at 183.56 ppm was assigned to be the carbonyl and the second and third lowest singlets at 156.47 and 163.82 ppm were assigned to be the β-carbon of α,β-unsaturated carbonyl system in the pyrone ring because of the presence of strong polarization of the π-electron toward the carbonyl. In the ^{13}C-NMR spectrum of 2-methyl-6-methoxy-4-pyrone, the chemical shifts of C-2 and 6 of the pyrone ring were observed at 160.623 and 167.236 ppm

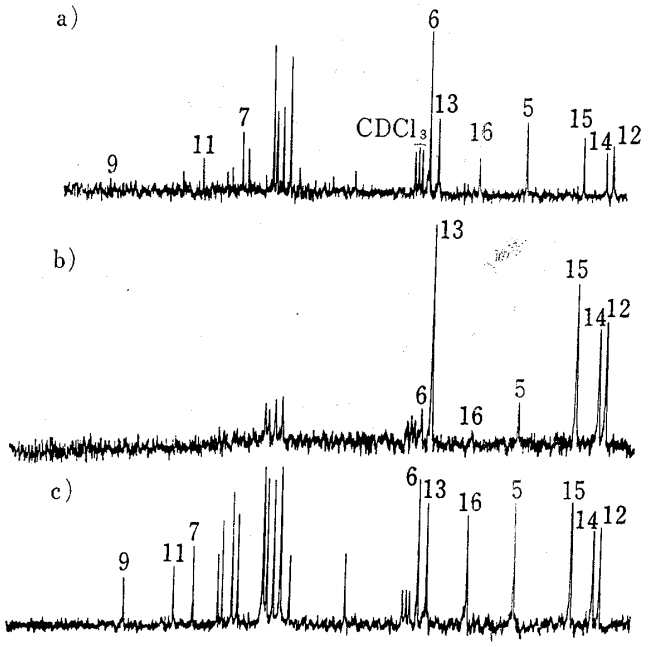
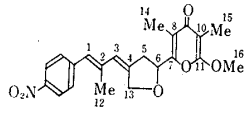


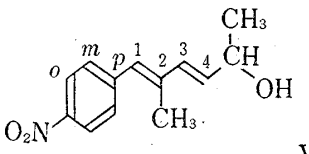
Fig. 2. ^{13}C-NMR Spectra of Aureothin



a) enriched by feeding acetate-2-^{13}C to the culture medium
b) enriched by feeding propionate-3-^{13}C to the culture medium
c) natural abundance

13) J.B. Stothers, "Carbon-13 NMR Spectroscopy," Academic Press, N.Y., London, 1972, p. 197.

and those of C-3 and 5 were at 88.061 and 111.358 ppm while the carbonyl carbon (C-4) appeared at 180.281. The spectrum of a model compound (VIII) showed the chemical shifts of the olefinic carbons (C-1—4) which were observed in comparatively lower range of the magnetic field from 128.959 to 145.40. The signals appeared at 99.93 and 120.62 ppm in Fig. 2-c could accordingly be assigned to the carbons at C-8 and 10 positions in the pyrone ring of aureothin. The remaining signals appeared at 126.81 and 129.11 ppm (doublet by off-resonance decoupling) and 141.92 and 145.92 ppm would be those of four olefinic carbons in the connecting chain, C-1, 3 and C-2 and 4.



VIII

Conclusively, the biosynthesis of aureothin through "Route B" as shown in Fig. 1 has been demostrated from the evidences obtained above by observing the incorporation of ^{13}C to C-6 from acetate-2-^{13}C and to "extra methyls" from propionate-3-^{13}C by the use of the ^{13}C-NMR spectrometry.

Acknowledgement The authors are indebted to Prof. T. Arai of this Institute for providing a strain of *Streptomyces luteoreticuli*. They appreciate the help received from Japan Electron Optics Laboratory Co. Ltd., Tokyo with the ^{13}C-NMR measurement. The research was supported in part by a grant from the Ministry of Education of Japan.