STUDIES ON LANKACIDIN-GROUP (T-2636) ANTIBIOTICS. IX

PREPARATION OF ^{14}C-LABELED LANKACIDIN C 14-PROPIONATE

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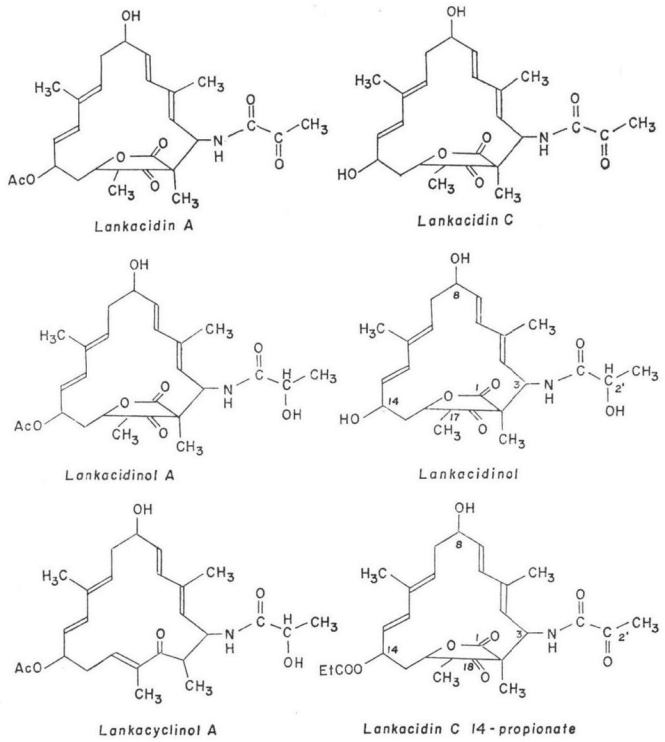
To investigate the metabolic fate of lankacidin C 14-propionate in experimental animals, the ^{14}C-labeled antibiotic was prepared by the fermentation of *Streptomyces rochei* var. *volubilis* in the presence of various ^{14}C-labeled organic carboxylic acids, amino acids and carbohydrates. Significant incorporation (20~40 %) was observed with L-methionine-methyl-^{14}C. Lankacidin C 14-propionate-^{14}C (specific activity 49.6 μCi/mg) was obtained from lankacidin C-^{14}C and ethyl propionate by the action of an acylase of the streptomyces.

The lankacidin-group (T-2636) antibiotics were isolated from the culture filtrate of *S. rochei* var. *volubilis*[1~3] and found to be mainly active against Gram-positive bacteria including a number of resistant strains.[4] Their chemical structures are shown in Chart 1.[5] Lankacidin C 14-propionate obtained by chemical modification of lankacidin C displayed the most desirable biological properties.[6] The labeled antibiotic was used to investigate its metabolic fate in experimental animals[7] and its mode of action in microorganisms.

Chart 1. Chemical structures of lankacidin-group (T-2636) antibiotics.



This paper describes the preparation of lankacidin C-^{14}C by the fermentation of *S. rochei* var. *volubilis* and its conversion[8] to lankacidin C 14-propionate-^{14}C. Some mention is made of possible biosynthetic routes to lankacidin C.

Materials and Methods

Microorganism and culture conditions: *Streptomyces rochei* var. *volubilis* strain No. 19M-8-85 derived from the original strain, an efficient producer of the lankacidin-group antibiotics was cultured in the following medium; 3% glycerine, 2% corn steep liquor, 1% Proflo (Traders Protein Division), 0.5% peptone, 0.5% NaCl, 0.1% $FeSO_4 \cdot 7H_2O$ (pH 7.0). A spore suspension (1.8×10^9 spores/ml) prepared from the slant (BENNET's agar) was stored at 4°C and 1 ml of this suspension was used to inoculate to the culture medium in Erlenmeyer flasks sterilized by autoclaving (120°C, 15 minutes). The fermentation was carried out on a rotary shaker (200 r.p.m.) (New Brunswick Scientific Co. Model G-25) for 66 hours at 28°C using a ^{14}C-CO_2-trapping apparatus. Five μCi of ^{14}C-labeled compound was added to the culture medium at 0, 24 and 48 hours post inoculation, respectively.

^{14}C-Labeled compounds: ^{14}C-Labeled compounds used in this experiment were as follows: glycerine-U-^{14}C (13.3 mCi/mmole), sodium acetate-U-^{14}C (56 mCi/mmole), sodium acetate-2-^{14}C (55.1 mCi/mmole), sodium propionate-1-^{14}C (46 mCi/mmole), sodium propionate-2-^{14}C (12 mCi/mmole), sodium pyruvate-U-^{14}C (31.5 mCi/mmole), L-leucine-U-^{14}C (180 ~ 270 mCi/mmole), glycine-U-^{14}C (108 mCi/mmole) purchased from The Radiochemical Centre Amersham, sodium propionate-3-^{14}C (50 μCi/0.965 mg) purchased from New England Nuclear, and L-methionine-methyl-^{14}C (50.5 mCi/mmole) purchased from Service Molécules Marquées Fabriqué par CEA-France.

Measurement of radioactivity: Radioactivity was measured with a liquid scintillation counter, Aloka model LSC-50 (Nihon Musen Co.). The liquid scintillator[9] consisted of naphthalene (60 g), ethyleneglycol (20 ml), PPO (2,5-diphenyloxazole) (4 g), POPOP 1,4-bis(5-phenyloxazolyl) benzene (0.2 g), and methanol (100 ml) in 1,000 ml of dioxane. The samples were counted in 10 ml of this scintillation liquid. Radioactivity on thin-layer plates was measured by autoradiography using a thin-layer chromatogram scanner, Aloka model TRM-IB.

Isolation and purification: The lankacidin-group antibiotics were isolated according to a procedure described previously.[2]

Thin-layer chromatography (TLC): The culture filtrate was extracted with methylisobutylketone (MIBK) and the extract was concentrated *in vacuo*. The residue was applied to a silica gel plate (20×20 cm, HF_{254}, Merck Co.) and developed with benzene-acetone (1:1) (solvent system 1) or benzene-ethyl acetate (1:1) (solvent system 2). The antibiotics, T-2636 B*, lankacidin C, lankacidinol A, lankacyclinol A, and lankacidinol were separated with the solvent system 1 and lankacidin C 14-propionate and lankacidin C were separated with the solvent system 2. The Rf-values of the various components compared with those of standard samples were detected with UV-light, by spraying with conc. H_2SO_4, or by radioautogram and bioautogram using *Sarcina lutea* PCI 1001 as the test organism (Fig. 1).

Preparation of lankacidin C 14-propionate-^{14}C: To the culture filtrate of *S. rochei* var. *volubilis* was added ethyl propionate (1/2 volume of the filtrate). After stirring at room temperature for 3 hours the reaction mixture was extracted with MIBK and the extract washed with 0.1 N HCl, 2% $NaHCO_3$ and H_2O. A concentrate of this extract was applied to a preparative TLC plate and developed with the solvent system 2. The crude powder obtained by extraction of the appropriate band was crystallized from ether as colorless needles of lankacidin C 14-propionate.

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* T-2636 B[2] is a neutral macrolide similar to lankamycin.[10]

Results

Incorporation of ^{14}C-Labeled Compounds into Lankacidin C

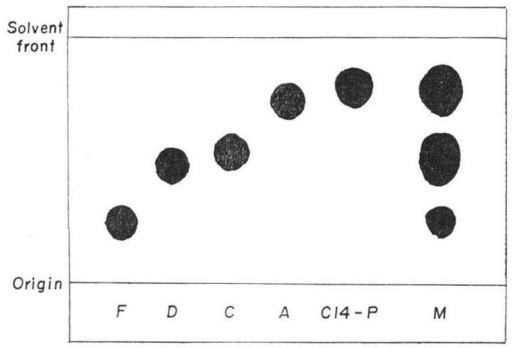
Table 1 shows that L-methionine-methyl-^{14}C was efficiently incorporated into lankacidin C (about 25 %). The other ^{14}C-labeled compounds *i.e.* sodium acetate, sodium propionate, sodium pyruvate, glycine and glycerine were incorporated at much more moderate levels (1.5~6.0 %). L-Leucine-^{14}C was incorporated into the mycelium (approximately 70 %), but not into lankacidin C.

Table 1. Incorporation ratio of various ^{14}C-compounds into lankacidin C.

^{14}C-Compound	Specific activity of ^{14}C-compound (mCi/mmole)	Amount of radio-activity added to culture (μCi)*	Lankacidin C formed				
			Amount of lanka-cidin C (μmole)	Specific activity (μCi/μmole)	Total activity (μCi)	Incorpo-ration ratio (%)**	Molar incorpo-ration ratio (%)***
Glycerine-U-^{14}C	13.3	2	0.93	0.004	0.030	1.51	0.03
Na-Acetate-U-^{14}C	56	5	7.63	0.014	0.108	2.17	0.03
Na-Acetate-2-^{14}C	55.1	5	8.71	0.009	0.075	1.50	0.02
Na-Propionate-1-^{14}C	46	5	4.79	0.047	0.223	4.47	0.10
Na-Propionate-2-^{14}C	12	5	8.71	0.016	0.139	2.78	0.13
Na-Propionate-3-^{14}C	4.8	5	8.50	0.019	0.163	3.26	0.40
Na-Pyruvate-U-^{14}C	31.5	5	5.23	0.002	0.092	1.84	0.01
Glycine-U-^{14}C	108	5	4.58	0.064	0.290	5.80	0.06
L-Leucine-U-^{14}C	180~270	2.5	1.09	0	0	0	0
L-Methionine-methyl-^{14}C	50.5	5	20.70	0.06	1.238	24.76	0.12
		2,500	16.67	33.89	564.923	22.60	67.11
		5,000	43.57	29.15	1,270.0	25.40	57.72

* ^{14}C-Compounds except glycerine-U-^{14}C were added at 48 hours after inoculation (in the case of glycerine-U-^{14}C, immediately after inoculation) and incubated for 66 hours.
** Radioactivity of lankacidin C/Added radioactivity×100.
*** Specific activity of lankacidin C/Specific activity of ^{14}C-compounds×100.

Fig. 1. Bioautogram of lankacidin-group antibiotics by TLC.
Silica gel HF_{254} (Merck), Benzene-acetone (1:1)
Test organism: *Sarcina lutea* PCI 1001
C 14-P: Lankacidin C 14-propionate
A: Lankacidin A
C: Lankacidin C
D: Lankacidinol A
F: Lankacidinol
M: Mixture (the mixture of lankacidin C 14-propionate, lankacidin C, lankacidinol A and lankacidinol)



Effects of Incubation Time and Addition Time on the Incorporation of L-Methionine-methyl-^{14}C into Lankacidin-group Antibiotics

One ml of a sterilized solution of L-methionine-methyl-^{14}C (5 μCi/ml) was added to the culture medium following inoculation and this was then incubated for 90 hours. The labeled lankacidin-group antibiotics in the culture filtrate at 18, 42, 66 and 90 hours were extracted with MIBK and separated by TLC. The radioactivity of each component

Table 2. Effects of incubation time and addition time on the incorporation ratio of L-methionine-methyl-^{14}C* into the four components of the lankacidin-group antibiotics.

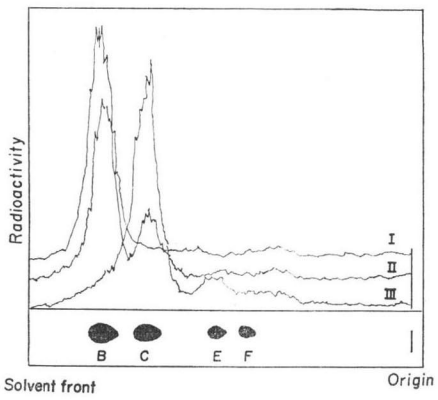
Addition time (hrs)	Incubation time (hrs)	Incorporation ratio (%)				
		Lankacidin group antibiotics (%)	T-2636 B (%)	Lankacidin C (%)	Lankacyclinol A (%)	Lankacidinol (%)
0	18	12.2	54.6	24.3	—	—
	42	33.3	63.4	26.3	—	—
	66	28.2	51.8	18.2	—	—
	90	17.8	77.0	0	—	—
24	66	42.8	50.3	31.5	7.3	4.3
48	66	28.1	9.6	79.1	8.1	—

* Amount of radioactivity added was 5 μCi.

Fig. 2. Radioautograms of ^{14}C-labeled lankacidin-group antibiotics produced by *Streptomyces rochei* var. *volubilis* in various incubation time and addition time of ^{14}C-methionine.

Silica gel HF_{254} (Merck), Benzene-acetone (1:1)
B: T-2636 B, C: Lankacidin C,
E: Lankacyclinol A, F: Lankacidinol

	Addition time (hrs)	Incubation time (hrs)
I	0	90
II	0 or 24	18, 42 or 66
III	48	66



was measured with a TLC-radioautogram scanner.

^{14}C-Labeled methionine was also added at 24 and 48 hours post inoculation and the radioactivity of each component of the lankacidin-group antibiotics measured after a total incubation time of 66 hours. As can be seen from Table 2 and Fig. 2, the radioactivity derived from the added ^{14}C-labeled methionine (0 and 24 hours post inoculation) was observed mainly in T-2636 B (50 %) together with lankacidin C (20~30 %) after 66 hours incubation. On the other hand no radioactive lankacidin C was observed after 90-hour incubation (0 hour post inoculation), whereas the addition of ^{14}C-labeled methionine 48 hours post inoculation led to lankacidin C possessing approximately 80 % of the incorporated activity (66 hours incubation). In conclusion, the optimal conditions for the production of lankacidin C-^{14}C are the addition of labeled methionine 48 hours after inoculation with a total incubation period of 66 hours.

Preparation of Lankacidin C 14-Propionate-^{14}C

Lankacidin C-^{14}C was prepared from L-methionine-methyl-^{14}C (8 mCi added, 50 μCi/ml of medium) under the above-mentioned conditions. Table 3 is a summary of the purification procedures together with a distribution of the radioactivity in each fraction. Most of the radioactivity existed in the filtrate of the culture broth and one half of this was extracted with MIBK. Among the lipophilic components, ^{14}C-labeled carbon was mainly incorporated

Table 3. Distribution of radioactivity in the extraction steps of the lankacidin-group antibiotics from the culture broth.

	Distribution of radioactivity (%)	Distribution of radioactivity in components of lankacidins (%)
Filtrate	84.0	
Methylisobutylketone extract	35.2	100
T-2636 B	3.7	10.5
Lankacidin C	22.7	64.5
Lankacyclinol A or lankacidinol	1.6	4.5
Aqueous layer	40.8	
Washed water	8.0	
Mycelium	14.0	
CO_2	1.6	
Total recovery	99.6	

into lankacidin C. Lankacidin C-^{14}C was converted into lankacidin C 14-propionate-^{14}C with an acylase of the producing organism and ethyl propionate.[8] The specific activity of the lankacidin C 14-propionate-^{14}C (34.3 mg) obtained was 49.6 μCi/mg (yield 25 %) and its purity was 98 % by TLC-radioautogram.

Discussion

Studies on the biosynthesis of methymycin[11] and magnamycin[12] using ^{14}C-labeled compounds, demonstrated that the aglycone moiety of these macrolide antibiotics was derived from acetate and propionate.[13] Preliminary data on the incorporation of ^{14}C-labeled acetate and propionate into lankacidin C, leads us to suspect that they are also incorporated into the basic carbon skeleton of lankacidin C. The increase of incorporation of methionine into lankacidin C at 48 hours post inoculation compared with 0 hour post inoculation suggests that the introduced methyl functions are incorporated into lankacidin C after the formation of its basic skeleton. It appears likely that the two allylic methyl moieties (C-5 and C-11) of lankacidin C are derived from propionate, and that the C-2 or C-17 methyl group is derived from methionine.

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