Notes

LANOMYCIN AND GLUCOLANOMYCIN, ANTIFUNGAL AGENTS PRODUCED BY *Pycnidiophora dispersa*

III. BIOSYNTHESIS OF LANOMYCIN: ^{13}C NMR ASSIGNMENT AND ORIGIN OF THE CARBON SKELETON

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The antifungal antibiotics lanomycin and glucolanomycin were recently isolated from fermentation broths of *Pycnidiophora dispersa*[1]. The structures of these compounds were reported in a previous paper in this issue[2]. The biosynthesis of the major metabolite (**1**) (Fig. 1), was of interest to better understand which precursors, if added to the broth, might increase the fermentation yield. We were also interested in attempting to alter the structure of the molecule by feeding unnatural precursors and a knowledge of the biosynthesis of the antibiotic was seen as a necessary first step.

The biosynthetic origin of the carbon atoms of lanomycin was unambiguously determined by feeding experiments using ^{14}C and ^{13}C labeled precursors followed by measurement of incorporation, if any, by counting the radioactivity or by ^{13}C NMR analysis of the isolated product. A C-H HETCOR experiment gave the unambiguous assignment of each of the resonances in the ^{13}C NMR spectrum of lanomycin and this information allowed the determination of the enriched carbons from the ^{13}C labeling experiments. These studies show that lanomycin is derived from six acetate units, methionine and glycine as depicted in Fig. 2.

Materials and Methods

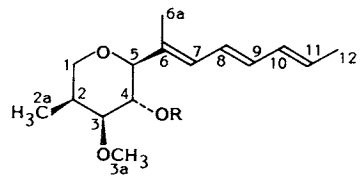
Labeled Compounds

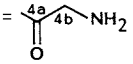
Sodium [*U*-^{14}C]acetate (56 mCi/mmol), L-[*U*-^{14}C]aspartic acid (229 mCi/mmol), [*U*-^{14}C]glycine (113 mCi/mmol), L-[^{3}H-*S*-methyl]methionine (90 Ci/mmol), and L-[*U*-^{14}C]serine (175 mCi/mmol) were purchased from the Amersham Corporation. [*U*-^{14}C]Lactic acid (165.2 mCi/mmol) and sodium [1-^{14}C]propionate (57 mCi/mmol) were obtained from New England Nuclear. [*U*-^{14}C]Glucose (278 mCi/mmol) was obtained from ICN Radiochemicals. Sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate and L-[$^{13}CH_3$-*S*-methyl]methionine were obtained from Cambridge Isotope Laboratories.

Incorporation Studies

The incorporation studies were conducted as follows: A germinator was prepared by loop inoculation of *P. dispersa* into a 250-ml flask containing 50 ml of toasted Nutrisoy flour 1.5%, soluble starch 1.5%, glucose 5.0%, $CoCl_2 \cdot 6H_2O$ 0.0005%, $CaCO_3$ 1.0% (Medium A). This mixture was shaken on a gyratory shaker at 300 rpm and 25°C for three days at which time 5 ml of this cell suspension were transferred to a 500-ml flask containing 100 ml of glucose 2.5%, yeast extract

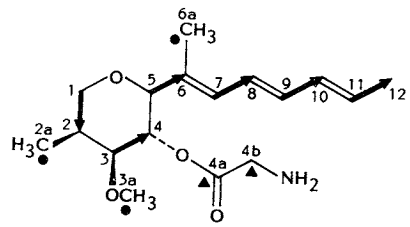
Fig. 1. The structures of lanomycin (**1**) and lanomycinol (**2**).



1 R = 

2 R=H

Fig. 2. The origin of the carbon atoms of lanomycin.



2.0%, NZ amine A 4.0%, K_2HPO_4 0.1%, $Na_2H_2PO_4 \cdot H_2O$ 1.0%, NH_4Cl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.02% (Medium B). Incubation was then continued under the above conditions for an additional two days. The cells from this incubation were harvested by centrifugation (8,500 rpm, 12,220 × *g*) and washed three times with distilled water. Four fermentation flasks were prepared yielding approximately 45 g of wet cells. For the radioisotope studies one μCi of the test compound was added to 5 ml of $NaNO_3$ 0.3%, KH_2PO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001% (Medium C) containing 1.0 g cells and the mixture was incubated as described above for one hour. Two aliquots were removed from each test flask at time zero and counted to determine total radioactivity. After incubation, broth supernatant was recovered by centrifugation, adjusted to pH 5.0 and extracted with ethyl acetate. The lanomycin present in the ethyl acetate concentrate was purified by HPLC using a system consisting of a Hewlett-Packard model 1084B chromatograph and a Whatman 5 micron RAC ODS-3 column. A linear gradient was run starting with a ratio of 55:45, 0.01 M ammonium acetate pH 4.5-acetonitrile to 25:75 at six minutes then a two minute isocratic elution followed by another linear gradient to a final solvent mixture of 15:85 at 9 minutes at a flow rate of 2.0 ml/minute. Eluant absorbance was monitored at 267 nm and under these conditions lanomycin elutes at approximately 9.5 minutes. Fractions containing lanomycin were collected and their radioactivity was determined.

For the ^{13}C labeling experiments the germinator procedures were essentially the same as outlined above. Twenty fermentation flasks were prepared per labeling experiment yielding 200 to 230 g of wet cells. Enriched lanomycin was prepared by combining 10 mg of the labeled precursor with 10 g of cells in a 500-ml flask containing 100 ml of medium C and incubating this mixture as above for 9.5 hours Twenty flasks were prepared per batch and after incubation the broths were recovered by centrifugation, pooled, adjusted to pH 5.0 and extracted with ethyl acetate to concentrate the antibiotic. Final purification of the antibiotic was achieved by column chromatography on silica gel, eluting the column with $CHCl_3$-CH_3OH, 96:4. Fractions shown to contain antibiotic by HPLC were pooled and the solvent evaporated. The ^{13}C incorporation was analyzed by ^{13}C NMR at 67.5 MHz in $CDCl_3$ (each sample was run under identical conditions) and the carbon signal intensities tabulated. A relative response factor was assigned to each carbon signal of the unlabeled molecule using the signal of the glycine methylene carbon (**4b**) as the reference carbon. The ratio of the intensity of each of the other resonances in the spectrum of the unlabeled material to that of the glycine methylene is the response factor given in column 4 of Table 2. The relative response of the carbons of the labeled molecules was calculated by multiplying the intensity of a resonance by its relative response factor (from column 4 of Table 2) and then dividing that number by the intensity of the glycine methylene carbon (**4b**) from that spectrum. In this way if there was no experimental error, each unlabeled carbon in Table 2 would have an intensity of 1.00 and labeled carbons would have an integer value.

Results and Discussion

Table 1 summarizes a group of experiments in which the incorporation of radiolabeled precursors into lanomycin was determined. Only acetate, methionine, serine and glycine labeled the antibiotic to a significant degree. Propionate was not incorporated into the molecule. Glycine predominantly labeled the glycyl carbons of lanomycin as was shown by collecting the lanomycinol (formed by base hydrolysis of glycine radiolabeled lanomycin) as well as the liberated glycine, using HPLC and counting the radioactivity. The radioactivity in the alcohol and in the glycine was 15 and 55% respectively of the total activity in the unhydrolyzed material. It is likely that serine labeled the antibiotic by conversion to glycine and through methylene tetrahydrofolate to methionine which labeled the methyl groups[3,4], however this was not conclusively demonstrated.

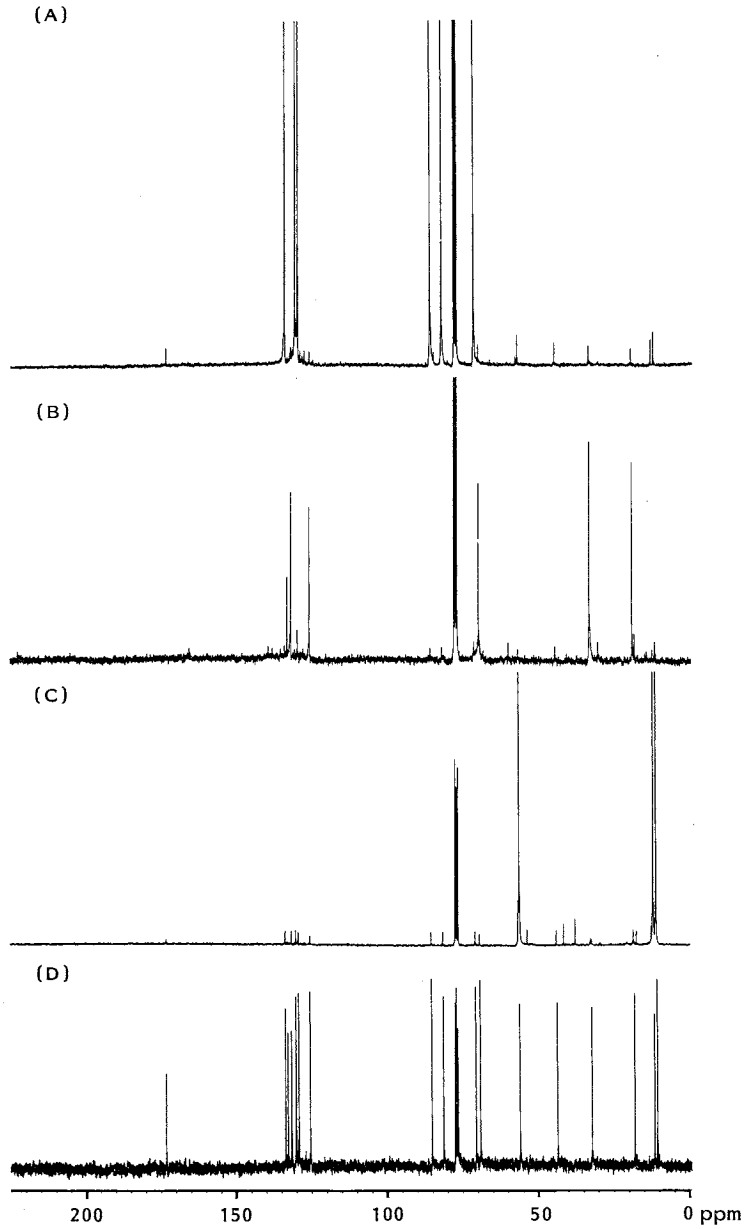
Table 1. Incorporation of radiolabeled precursors into lanomycin.

Isotope-precursor	μ mole in assay	% incorporation
U-^{14}C Acetate	17.2	6.8[a]
C1-^{14}C Propionate	17.5	0.0
U-^{14}C Glycine	22.0	6.05[b]
U-^{14}C Lactic acid	0.61	0.0
U-^{14}C L-Serine	5.7	3.5
U-^{14}C Glucose	3.6	0.0
U-^{14}C L-Aspartic acid	4.4	0.0
^{3}H-*S*-Methyl methionine	0.01	2.0

[a] Average of three experiments.
[b] Average of two experiments.

Fig. 3. The ^{13}C NMR spectra of biosynthetically derived lanomycin; 1-^{13}C acetate, 2-^{13}C acetate, *S*-$^{13}CH_3$ methionine; and the natural abundance spectrum.

(A) 1-^{13}C Acetate, (B) 2-^{13}C acetate, (C) *S*-$^{13}CH_3$ methionine, (D) natural abundance.



Based on the radiolabeling studies, we assumed that acetate, methionine and glycine were the sole direct precursors of lanomycin. We then used these precursors labeled with ^{13}C to study their incorporation by ^{13}C NMR. The results of these biosynthetic studies are given in Table 2 along with the complete ^{13}C and ^{1}H NMR assignments, obtained from the C-H correlation experiment.

These results allowed the assignment of every carbon in the ^{13}C spectrum of **1** using the previously elucidated proton assignments[2]. With the carbon atoms and their chemical shifts assigned as in columns 1 and 2 of Table 2 (numbering of the molecule is for simplicity only and does not correspond to IUPAC standards) the suspected polyketide backbone is immediately apparent.

Table 2. ^{13}C-^{1}H correlation and results of biosynthetic experiments on ^{13}C NMR signal strength.

Carbon No.	Chemical shift		Response factor	1-^{13}C labeled acetate	2-^{13}C labeled acetate	S-$^{13}CH_3$ methionine
	^{13}C (δ)	^{1}H (δ)				
4a	173.18	—	0.62	1.29	ND	0.63
9	133.64	6.20 (m)	0.97	34.73	1.13	1.04
6	132.81	—	0.84	0.43	7.24	ND
10	131.63	6.15 (m)	0.85	0.98	14.4	1.18
11	130.13	5.72 (m)	1.04	34.42	ND	1.03
7	129.24	5.93 (d 10)	1.06	17.97	2.1	0.85
8	125.41	6.25 (m)	1.06	0.59	10.5	0.62
5	85.21	3.56 (d 9)	1.13	18.05	0.78	0.82
3	81.35	3.35 (dd 9, 6)	1.04	34.77	0.92	0.90
1	70.58	3.82 (dd 12, 2), 3.58 (dd 12, 2)	1.09	33.36	1.22	0.88
4	69.14	5.03 (dd=t 9, 9)	1.12	0.83	11.5	0.68
3a	56.07	3.31 (3H, s)	0.99	1.35	0.71	45.21
4b	43.65	3.42 (m), 3.38 (m)	1.00	1.00	1.00	1.00
2	32.25	2.25 (m)	0.97	0.86	16.38	0.48
12	18.05	1.76 (3H, d 6)	1.05	0.67	13.66	1.00
6a	11.46	1.78 (3H, s)	0.94	1.17	0.68	42.57
2a	10.54	1.09 (3H, d 7)	1.13	1.29	1.16	50.28

All spectra were recorded in $CDCl_3$.
ND: Not determined because the resonance had a signal to noise ratio of less than 2.

Starting with carbon 1, the odd numbered carbons are labeled by 1-^{13}C labeled acetate and the even numbered carbons are labeled by 2-^{13}C labeled acetate. The two *C*-methyl groups 2a and 6a as well as the *O*-methyl carbon, 3a were labeled by S-^{13}C-methyl methionine. As expected, no labeling of the glycyl carbons was observed. The ^{13}C NMR spectra of lanomycin derived from feeding the producing organism 1-^{13}C acetate, 2-^{13}C acetate, S-$^{13}CH_3$ methionine and natural abundance media are shown in Fig. 3. These results allow us to postulate the following biosynthetic pathway, with the understanding that not all of the steps occur in the order in which they are given. A hexaketide is formed, followed by methionine mediated alkylation of the appropriate carbons (C-2 and C-6) and methylation of the reduced C-3 oxygen, pyran forming reductive ring closure, oxygenation of C-4, β-keto reduction, enoyl reduction and dehydration of the remaining oxygens, followed by acylation with glycine to complete the biosynthesis.

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