SYNTHESIS OF CARBON-13 AND CARBON-14 LABELED PALDIMYCIN TRI-SODIUM SALT

Richard S. P. Hsi, Dennis F. Witz, Jeronimo Visser,
Wayne T. Stolle, and Cathy L. Ditto
Research Laboratories of The Upjohn Company,
Kalamazoo, Michigan 49001, U.S.A.

SUMMARY

Carbon-14 labeled paldimycin trisodium salt was prepared by addition of N-acetyl-L-cysteine to [^{14}C]paulomycin, the radioactive antibiotic produced by fermentation of *Streptomyces paulus* in the presence of L-methionine labeled with carbon-14 in the S-methyl group Carbon-13 nuclear magnetic resonance (NMR) spectra of paulomycin produced when the fermentation was carried out in the presence of L-[S-methyl-^{13}C]methionine showed that isotope incorporation had occurred specifically at the methoxy group of ring C, i.e., the 2-deoxy sugar portion of paulomycin. With sustained slow feed of labeled precursors during the optimum antibiotic production period, carbon-14 isotope yields of up to 17.5% with specific activity of up to 11.4 μCi per milligram of paulomycin, and carbon-13 isotope yields of up to 24% with 17-fold isotope enrichment over natural abundance, were achieved.

Key Words: Biosynthesis, paldimycin trisodium salt, paulomycin, carbon-13, carbon-14, fermentation, L-methionine, N-acetyl-L-cysteine, slow feed, NMR, HPLC, isotope incorporation, isotope enrichment, isolation

INTRODUCTION

Paldimycin, a medium-spectrum antibiotic, is the bis-N-acetyl-L-cysteine adduct of paulomycin (*1*), the fermentation product of *Streptomyces paulus*. As part of a program to develop the trisodium salt of paldimycin (*2*) for therapeutic use, the synthesis of radioisotope labeled *2* was undertaken to facilitate conducting drug disposition studies in test animals and human subjects with this agent. Because of the labile nature of the adduct, we ruled out at the outset the option of locating the label(s) in the N-acetyl-L-cysteine moieties of paldimycin, and chose

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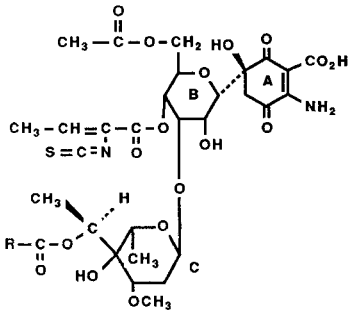
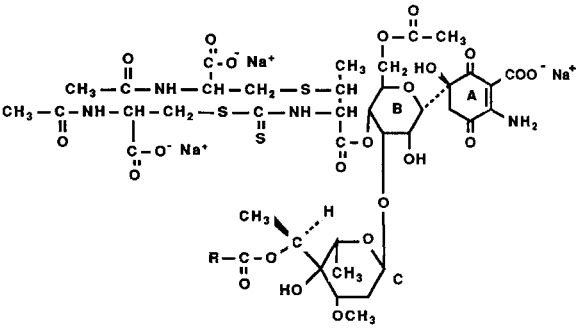
to label paulomycin through biosynthesis and to subsequently convert the latter to paldimycin labeled in the paulomycin moiety.



1 Paulomycin



2 Paldimycin Tri - Sodium Salt

a) R = $CH_3-CH_2-CH(CH_3)-$ Paulomycin A

b) $CH_3-CH(CH_3)-CH_2-$ Paulomycin A_2

c) $CH_3-CH(CH_3)-$ Paulomycin B

d) $CH_3-CH_2-CH_2-$ Paulomycin B_2

e) CH_3-CH_2- Paulomycin C

f) CH_3- Paulomycin D

a) R = $CH_3-CH_2-CH(CH_3)-$ Paldimycin A Tri - Na Salt

b) R = $CH_3-CH(CH_3)-$ Paldimycin B Tri - Na Salt

The term paulomycin (1-4) refers to a mixture of antibiotics produced by *S. paulus*, consisting mainly of paulomycins A (*1a*) and B (*1c*), with A being the more abundant congener of the two. The mixture also contains small amounts of other congeners which include paulomycin A_2 (*1b*), C (*1d*), and D (*1e*). These materials differ from each other only in the carboxylic acid moiety attached to the ring C portion of the molecule. Since the conjugate addition of N-acetyl-L-cysteine to the carbon-carbon double bond of paulomycin creates two new chiral centers in paldimycin, the bis-adduct *2* is a multi-component mixture consisting chiefly of two groups of diastereoisomers, paldimycin A (*2a*), the more abundant group arising from paulomycin A, and paldimycin B (*2b*) arising from paulomycin B.

DISCUSSION AND RESULTS

A) Background

A series of biosynthesis studies has shown that the relative abundance of the

various antibiotics in the paulomycin mixture can be altered by the addition of selected percursors to the fermentation medium. Thus, in a chemically defined medium, use of DL-isoleucine, DL-2-methylbutyric acid (5), L-methionine, L-threonine, and 2-ketobutyric acid (6) increased production of paulomycin A relative to paulomycin B. On the other hand, addition of DL-valine and isobutyric acid (5) favored production of paulomycin B over paulomycin A. L-Methionine, when present in concentrations of 200-1000 mg/l in the medium, also led to elevated yields of paulomycin C (7) in addition to enhancing production of paulomycin A. These results show that the various precursors, when fed in relatively high concentrations in defined fermentation medium, are incorporated into the ring C portion of the paulomycin molecule as the carboxylic ester functions which determine the identity of the congener being produced. Isotopes contained in such precursors would be incorporated into paulomycin. However, the ester functions may be subject to hydrolysis, and thus leave the bulk of the molecule unlabeled and untraceable in a drug disposition study. Also the added precursors at higher concentrations usually altered the ratios of the congeners in paulomycin, thus rendering the labeled material unrepresentative of the substance being developed for therapeutic use.

In one preliminary study in which radioactive precursors were added at trace levels (50 mg/l) to chemically defined medium (7), L-[1-^{14}C]methionine gave no radioactive antibiotics, but L-[S-methyl-^{14}C]methionine did produce radioactive and bioactive materials, although the identities of the antibiotics and the extent of incorporation were unknown since the products were not isolated. These results, coupled with those of some earlier studies showing that larger amounts of added L-methionine enhanced production of paulomycin A and C (6, 7), suggest that L-methionine is capable of at least two separate routes of incorporation into paulomycin. The carbon skeleton of L-methionine can enter the molecule as the carboxylic ester function, while the S-methyl group is incorporated elsewhere in the molecule, and substantially more efficiently than the carbon skeleton. In another experiment, L-[S-methyl-^{13}C]methionine was added to fermentation of *S. paulus* in complex medium. Carbon-13 nuclear magnetic resonance (NMR) spectra of the

paulomycin produced showed that there was an approximately two-fold enrichment of carbon-13 at the O-methyl group of the ring C moiety in both paulomycins A and B, and no detectible enrichment anywhere else in the molecule. This led us to attempt developing the use of L-[S-methyl-^{14}C]methionine as a practical approach to producing [^{14}C]paulomycin. The questions which needed to be addressed included 1) whether the isotope incorporation, when fermentation is carried out in complex medium, could be brought to an efficient enough level to produce labeled paulomycin of adequate specific activity in sufficient quantities, 2) whether the congener distribution in the labeled antibiotic could be made representative of material intended for clinical use, and 3) whether the congeners would be of substantially uniform specific activity and label specificity, particularly with respect to the two major components, paulomycins A and B.

B) Fermentation and Isolation

Because of the low antibiotic titer achievable with chemically defined fermentation media, we elected to use whole beer employed to produce the antibiotics on large scales. The ingredients for the media are shown in Table 1. Two methods of isolation of paulomycin have been developed in conjunction with large scale fermentations, one utilizing polyaklylene glycol (PAG) to extract the antibiotics (as they are formed during fermentation), and the other employing XAD-16 resin beads to absorb the antibiotics. Both of these additives enhance paulomycin production although the mechanism is unknown. The use of PAG was favored in large scale fermentations primarily because of its lower cost and greater ease of handling. However, the plant scale technology for extracting it from the fermentation emulsion could not be successfully adapted to the laboratory scale. The separation of PAG from the rest of the beer proved tedious and hazardous because it required prolonged centrifugation. We therefore turned our attention to XAD-16 resin beads, which proved much more practical and safe for laboratory use, especially in conjunction with radioactive methionine.

Initially, our fermentations were carried out in 500 ml Erlenmeyer flasks containing 60 ml of beer each (in a New Brunswick Psycrotherm incubator-shaker),

since we had carried out radioactive fermentation in this manner previously when we prepared [3H]cyclohexmide (8) and other labeled antibiotics by biosynthesis. In each instance the flasks were connected in series with rubber tubings to form a "closed" system, whereby the air supply and radioactive effluent could be carefully controlled and directed. However, for practical purposes, each run was limited to no more than six flasks in order to ensure uniform quality of the air supplied to all flasks. In the case of paulomycin, it soon became apparent that the antibiotic titer

Table 1. Ingredients for Fermentation Media

A. Primary Seed Medium 273-P-5

Ingredient	Amount
Yeastolac	20 g/l
Dextrose	20 g/l
Beet Molasses	10 g/l

pH adjusted to 6.8 with NH_4OH, then add

$CaCO_3$	2 g/l
Polyalkylene Glycol (PAG)	3 dr/c/s flask

B. Secondary Seed Medium 273-S-5

Ingredient	Amount
Cottonseed Flour	20 g/l
Cornsteep Liquor	10 g/l
Cornstarch	20 g/l
Beet Molasses	10 g/l

pH adjusted to 6.0 with NH_4OH, then add

$CaCO_3$	2 g/l
PAG	1 ml/l

C. Fermentation Medium for *S. paulus,* 273-F-9, modified

Ingredient	Amount
Cornstarch	50 g/l
Dextrin	50 g/l
Beet Molasses	10 g/l
Cottonseed Flour	13 g/l*
Cornsteep Liquor	15 g/l*
Ammonium Sulfate (Tech.)	5 g/l

pH adjusted to 7.2 with NH_4OH, then added

$CaCO_3$	8 g/l
PAG	0.5 ml/l

* Some fermentations were carried out in medium containing cottonseed flour, 11 g/l, and cornsteep liquor, 20 g/l.

achievable was too low ($\leqq$ 800 µg/ml) to make the shake flask method practical. We therefore attempted using a Multigen F-2000 fermentor (New Brunswick Scientific Co.), which can readily accommodate 1.2 l of beer per run. However, this apparatus with its magnetic stirrer is best suited for homogeneous fermentation media. With a whole beer containing solids (particularly the resin), such as the one used for paulomycin production, the reliability of the stirrer became at best uncertain over the three- to four- day fermentation period, and the air sparger invariably became clogged. The original sparger was therefore sealed with Teflon tape and a new air supply tube was inserted through the rubber stopper at the top of the tank (see Figures 1 and 2). An additional impeller was placed near the top of the stirring shaft to serve as a foam breaker. The top and bottom plastic bearings guiding the stirrer shaft, as well as the Teflon washer under the magnet, needed to be carefully fitted and changed after each run to minimize stirrer failures. With

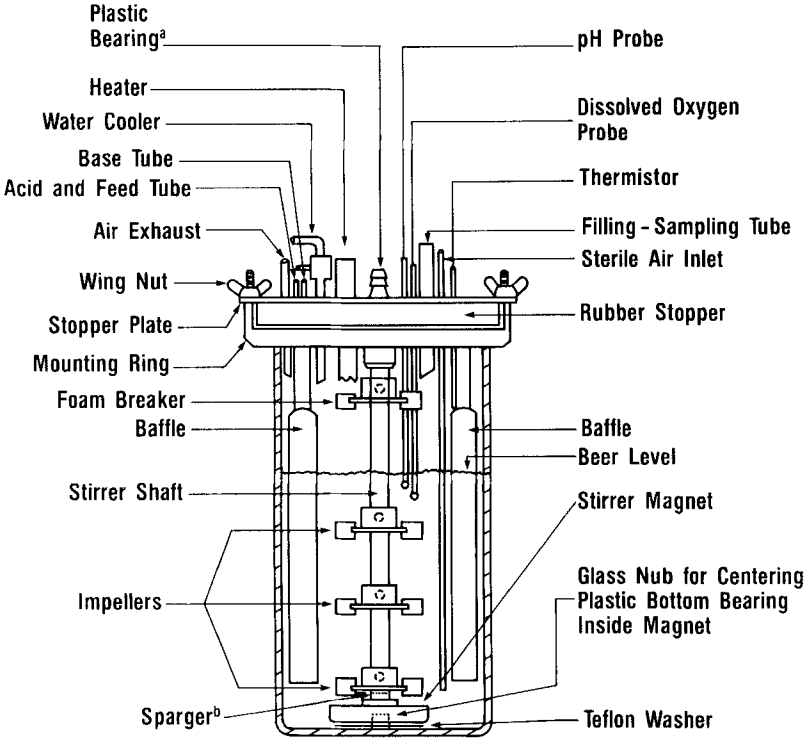
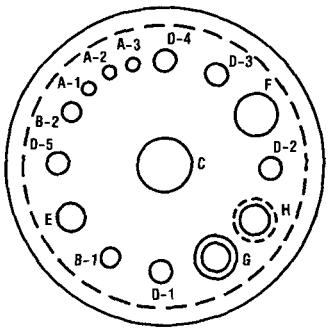


Figure 1. Multigen 2-Liter Vessel Modified for Fermentation with Whole Beer.

[a]Originally the top bearing also served as air inlet.
[b]Sparger holes were sealed with Teflon tape to prevent backing up of beer into shaft interior and fouling the top bearing.



STOPPER PENETRATIONS

LTR.	Hole Diameter	For Insertion of
A-3	11/64	Air Inlet
B-1	15/64	*Thermistor Well*
B-2	15/64	Thermometer Well
C	23/32	Stirrer Shaft Bearing
D-1	23/64	Plug
D-2	23/64	Baffle
D-3	23/64	Air Exhaust
D-4	23/64	Heater Well
D-5	23/64	Baffle
E	7/16	Filling and Sampling Tube
F	9/16	pH Probe
G	27/64 × 19/64	Water Cooler
H	23/64 × 9/16	DO Probe
A-1	11/64	Acid and Feed Tube
A-2	11/64	Base Tube

Figure 2. Stopper Penetration Arrangement for Multigen 2-Liter Vessel for Fermentation with Whole Beer.

these modifications and precautions, the apparatus, though far from being totally reliable, did provide successful fermentations with some regularity.

In the interest of consistency in terms of both the antibiotic titer and product mix, standard production methods were followed as much as possible. Thus the seed growing was carried out in 250-l tanks and the first two days of fermentation proceeded in 250-l or 5000-l tanks before 1.2 liter portions of the beer was chilled in an ice bath for transportation to the radiosynthesis laboratories and transfer into the Multigen vessel, where the remainder of the fermentation was carried out with feeding of L-[S-methyl-^{14}C]methionine. The beer was then screened under vacuum through a 100 mesh stainless steel sieve to collect the resin beads from which the antibiotics were recovered and purified.

C) Fermentations with Labeled L-Methionine.

To ascertain whether paulomycin produced in whole beer with added L-methionine would resemble the normal product with respect to the components,

we analyzed by means of HPLC a sample of paulomycin produced with fermentation medium containing added methionine at 100 mg per liter. This concentration was chosen for anticipated fermentations with L-[S-methyl-^{14}C]methionine in consideration of antibiotic titer as well as precursor cost and availability. We found that the added precursor at 100 mg/l did not change the titer or the main features of the product contents, i.e., paulomycins A and B were the predominant components with A being the more abundant one. We next used L-[S-methyl-^{13}C]methionine to determine the specificity of the label position in paulomycin, and to explore the protocol for the addition of presursor, which would optimize isotope incorporation. The HPLC chromatogram (Figure 3) and carbon-13 NMR spectrum (Figure 5) of a standard unlabeled sample of paulomycin were compared with those of a sample obtained from a fermentation in the presence of carbon-13 labeled L-methionine (Figure 4 for HPLC chromatogram, Figure 6 for ^{13}C-NMR spectrum). Assignment of carbon-13 chemical shifts of the carbon atoms of paulomycin had been previously accomplished and reported (3,4). It is apparent from Figure 6 that ^{13}C enrichment had occurred specifically at only the methoxy carbons at 57.30 ppm associated with ring C portion of both paulomycins A and B.

To determine the extent of ^{13}C enrichment, the signals attributed to the carbons (2''') alpha to the carboxylic carbons attached to ring C, 42.17 ppm for paulomycin A and 34.85 ppm for paulomycin B, were used as internal standards. The relative isotope enrichment Ep at the methoxy carbon in [^{13}C]paulomycin was calculated according to the following formula:

$$Ep = \frac{\dfrac{H_{mL}}{H_{AL} + H_{BL}}}{\dfrac{HmS}{H_{AS} + H_{BS}}}$$

Ep = ^{13}C enrichment in labeled paulomycin relative to standard paulomycin

H_{mL} = peak height of methoxy methyl carbon of labeled paulomycin

H_{AL} = peak height of $C_{2'''}$ of labeled paulomycin A

H_{BL} = peak height of $C_{2'''}$ of labeled paulomycin B

H_{mS} = peak height of methoxy methyl carbon of standard paulomycin

H_{AS} = peak height of $C_{2'''}$ of standard paulomycin A

H_{BS} = peak height of $C_{2'''}$ of standard paulomycin B

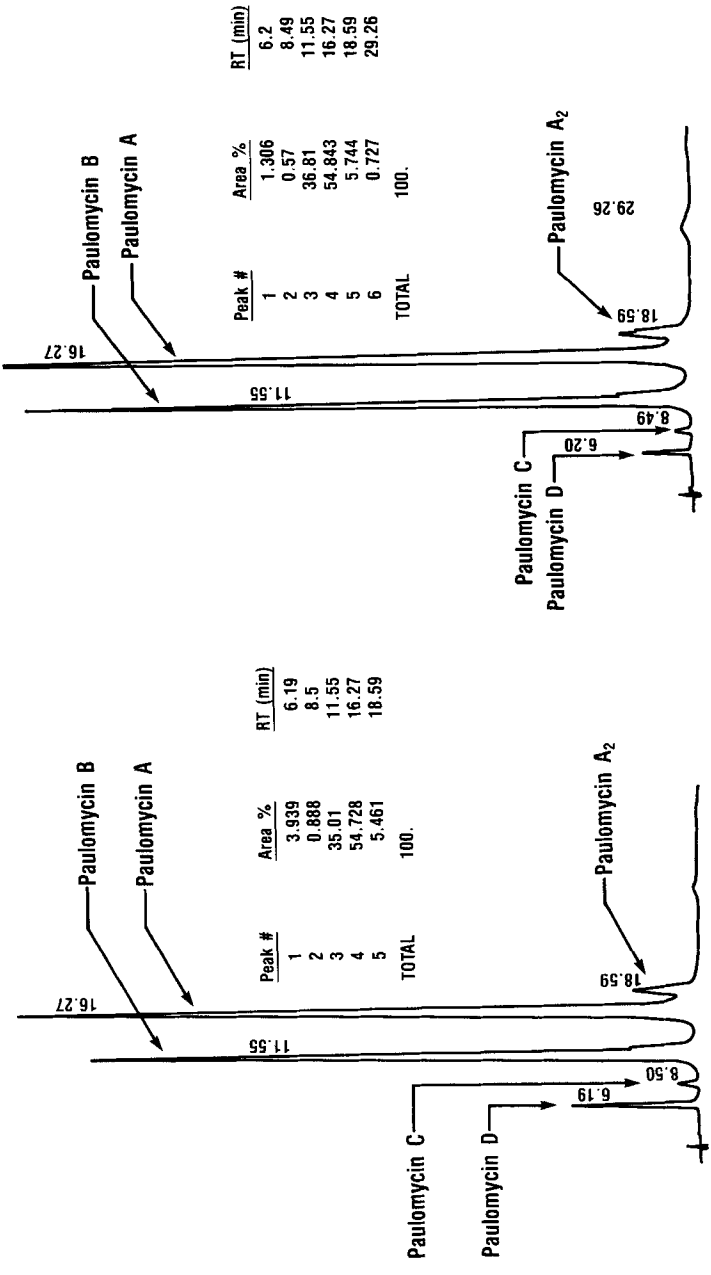


Figure 3. HPLC Chromatogram of a Standard Sample of Paulomycin.

Figure 4. HPLC Chromatogram of a Sample of [^{13}C]Paulomycin.

The isotope yield was calculated according to the following formula:

$$\%\text{Isotope Yield} = \frac{\frac{Wp \cdot Ep \cdot N}{Fp}}{\frac{W_M \cdot A_M}{F_M}} \times 100 \text{ where}$$

W_p = weight of isolated paulomycin

F_p = formula weight of paulomycin, 780

N = natural abundance of ^{13}C (1.1%)

W_M = weight of labeled L-methionine used

A_M = ^{13}C abundance in labeled L-methionine, and

F_M = formula weight of labeled L-methionine, 151

When L-[S-methyl-^{13}C]methionine was introduced in a single addition at the start of fermentation, the paulomycin produced showed an approximately two-fold ^{13}C enrichment at the methoxy methyl group. When the labeled methionine was added in different increments at various intervals over different segments of the presumed peak production period, substantial improvements in the isotope incorporation efficiency were achieved. The results are summarized in Table 2. Addition of the labeled precursor in three increments over 24 hours raised the enrichment to 6.4 fold over unlabeled paulomycin. Further dividing the precursor into six increments over a 48-hour period not only increased the enrichment to 11.9-fold, but also improved the isotope yield from 5.6% to 22.2%. Apparently the addition in smaller increments over a longer period of time reduced consumption of the precursor *via* alternate non-productive pathways. Finally, employment of pumps to deliver the precursor in tiny increments steadily during the peak production period raised the enrichment to 17-19 fold and isotope yields of up to 24%.

Interestingly, the ^{13}C-NMR spectra can also be used to estimate the relative amounts of paulomycins A and B in the sample. Thus, the peak heights corresponding to the C2''' carbons of paulomycins A and B in Figure 4 indicate the sample contains 55.8% A and 44.2% B. The HPLC chromatogram (Figure 3) of the same sample shows 53.2% A* and 45.5% B, in reasonable agreement with the NMR-

*Includes both paulomycin A and paulomycin A_2.

Table 2.

Effects of L-Methionine Feeding Protocol on ^{13}C Enrichment and Isotope Yield in Paulomycin From Representative Fermentations With L-[-S-Methyl-^{13}C]methionione.

Addition Schedule		^{13}C Enrichment Over Natural Abundance	% Isotope Yield
Frequency	Start-End (hrs)		
1	0-0	2	-
3*	66-80	6.4	5.6
6*	66-104	11.9	22.2
SIF**	65-130	16.5	15.5‡
SIF**	76-125	19.5	23.1
SIF**	71-121	17.1	24.3

* Increments at 8-hour intervals.
** Continuous small increment feeding.
‡ Difficulties with stirrer led to low titer and isotope yield.

based estimation. The information gained through the carbon-13 experiments was applied to fermentations with L-[S-methyl-^{14}C]methionine. Typically, an aqueous sterile solution of 35-50 mCi of the radioactive precursor of 55 mCi/mmol was introduced dropwise at regular intervals into the medium over a 30-hour period beginning at the start of the fourth day of fermentation. Fermentation was terminated four to six hours after completion of precursor addition, even though antibiotic production would be still in progress, because further paulomycin synthesis would only serve to isotopically dilute the labeled product already present, and not increase the isotope yield. Sample of [^{14}C]paulomycin thus obtained were all found, as determined by HPLC, to be composed of predominantly [^{14}C]paulomycin A and [^{14}C]paulomycin B, with A being more abundant, and [^{14}C]paulomycin A_2, C and D as minor components. The ratio of the components in a typical sample of [^{14}C]paulomycin as determined by UV absorbance (Figure 7) was in good agreement with that quantified by radioactivity (Figure 8). Thus the paulomycin produced with the Multigen fermentor closely resembled normal

Figure 5. Carbon-13 NMR Spectrum of a Standard Sample of Paulomycin in Acetone-d_6.

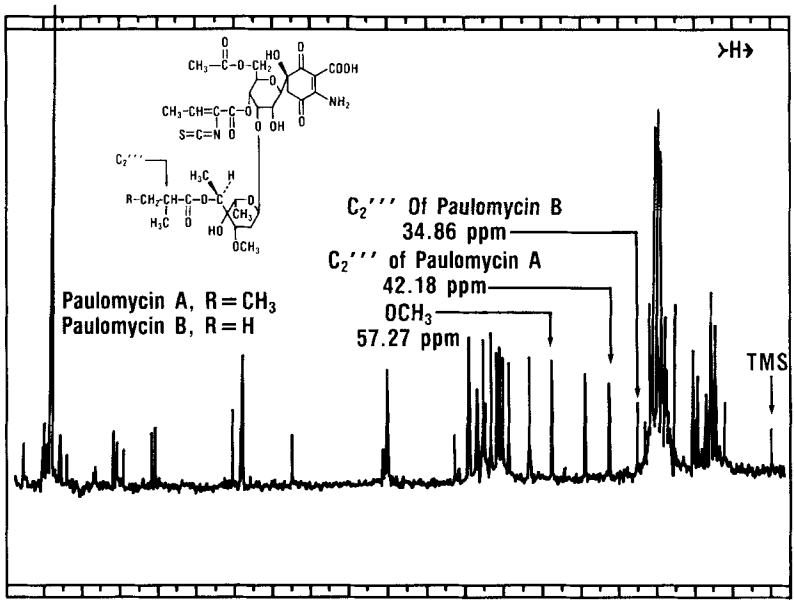
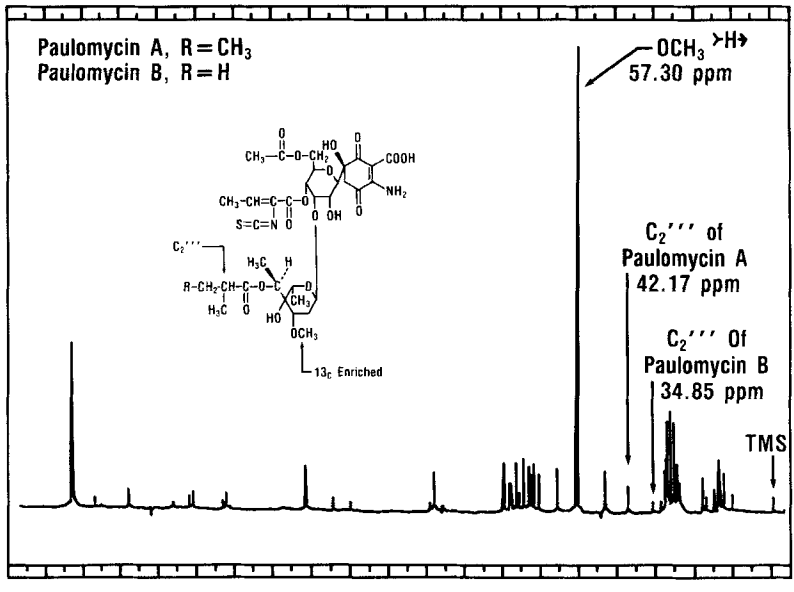
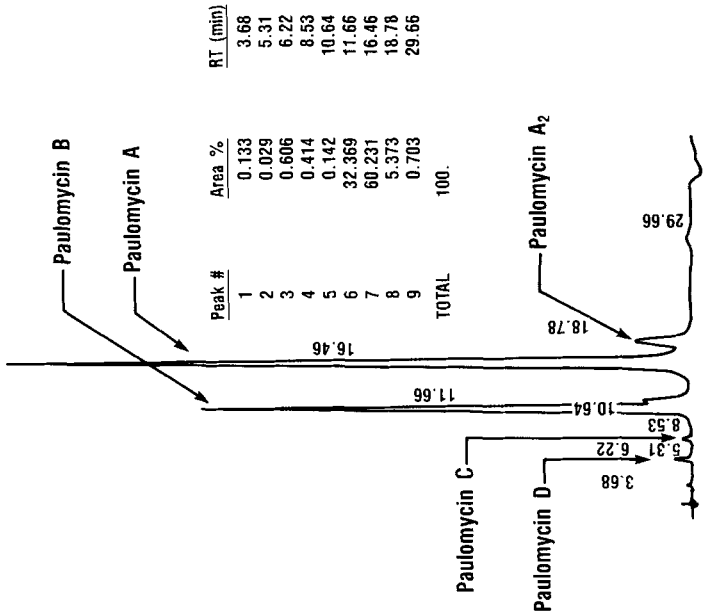


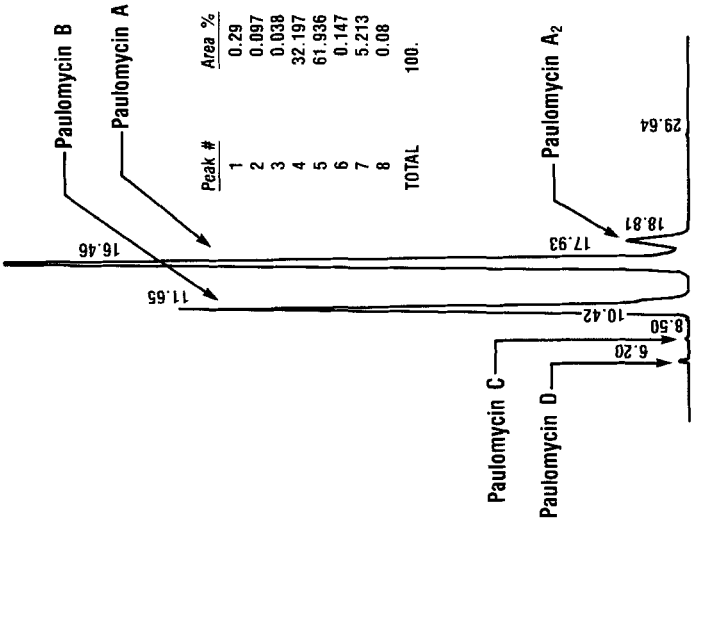
Figure 6. Carbon-13 NMR Spectrum of a Sample of [^{13}C]Paulomycin in Acetone-d_6, with Carbon-13 Enrichment at Methoxy Carbon Atom.





Peak #	Area %	RT (min)
1	0.133	3.68
2	0.029	5.31
3	0.606	6.22
4	0.414	8.53
5	0.142	10.64
6	32.369	11.66
7	60.231	16.46
8	5.373	18.78
9	0.703	29.66
TOTAL	100.	

Figure 7. HPLC Chromatogram of a Sample of [^{14}C]Paulomycin with Detection by UV Absorption.



Peak #	Area %	RT (min)
1	0.29	6.2
2	0.097	8.5
3	0.038	10.42
4	32.197	11.65
5	61.936	16.46
6	0.147	17.93
7	5.213	18.81
8	0.08	29.64
TOTAL	100.	

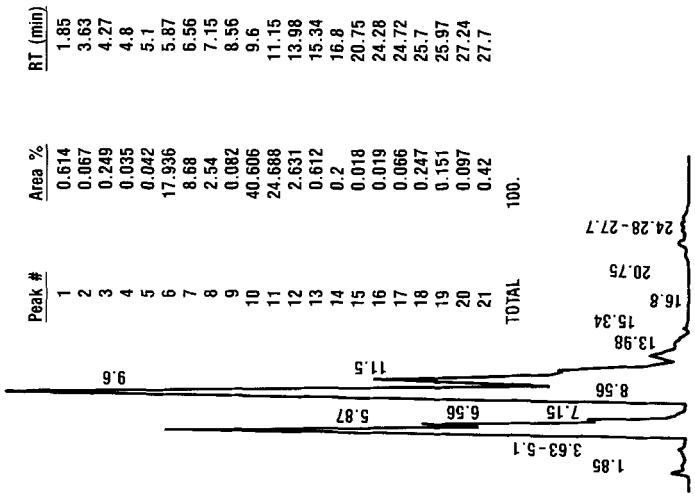
Figure 8. HPLC Chromatogram of a Sample of [^{14}C]Paulomycin with Detection by Radioactivity.

production samples of paulomycin in terms of congener contents, and the components were of largely uniform specific activity. From a typical fermentation, [^{14}C]paulomycin of 9.4 μCi/mg could be obtained in 17.5% radiochemical yield based on 40 mCi of L-[S-methyl-^{14}C]methionine of 55 mCi/mmol used in the radiobiosynthesis.

The conversion to [^{14}C]paldimycin was carried out by treating [^{14}C]paulomycin with a large (20-fold) excess of N-acetyl-L-cysteine at pH 8.7. The resulting bis-adduct was titrated with dilute sodium hydroxide solution to pH 8.3 and the trisodium salt was isolated as an amorphous white solid material. From [^{14}C]paulomycin of 7.8 μCi/mg, [^{14}C]paldimycin of 5.0 μCi/mg was obtained in 83% yield. As in the case with [^{14}C]paulomycin, uniformity of the specific activity of the components in [^{14}C]paldimycin was demonstrated with the good agreement between HPLC analytical results based on UV absorbance (Figure 9) and radioactivity (Figure 10). Radioactive paulomycin and paldimycin are stored in the solid state at -170^{0}C.

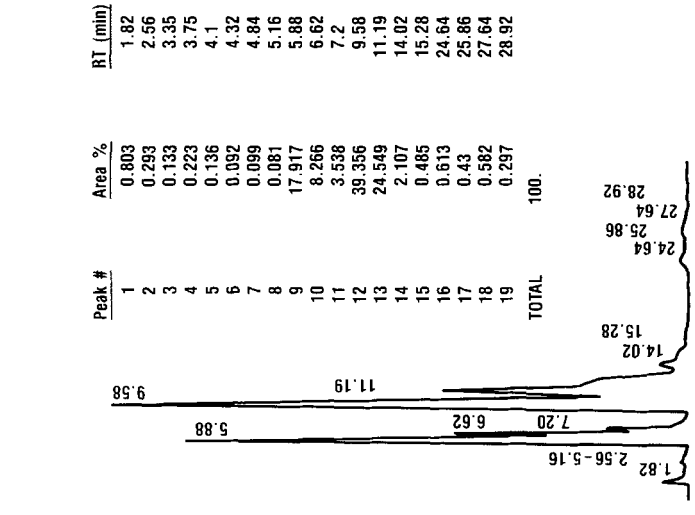
EXPERIMENTAL SECTION

Fermentation of *S. paulus* was carried out in a New Brunswick Multigen F-2000 fermentor modified as described in the Discussion and Results section. Radioactivity determinations were carried out with an LKB Instruments Model 1217 Rackbeta liquid scintillation spectrometer, using the external standard method of counting with Diotol (Burdick and Jackson Laboratories, Muskegon, MI) as the scintillation cocktail. Carbon-13 NMR spectrometer, with solutions of 100 mg of paulomycin in 1 ml of acetone-d_6. HPLC analyses were carried out with a Spectra Physics Model 8700 solvent delivery system with a Supelcosil LC-18 5μ analytical column of 4.6 mm ID x 25 cm. For paulomycin, the mobile phase, delivered at 2 ml/min, was 375:475:90:60 v/v methanol:water:acetonitrile:tetrahydrofuran, adjusted to pH 3.6 with phosphoric acid. For paldimycin the mobile phase was delivered at 1.5 ml/min and consisted of 80:20 v/v pH 6.8 phosphate buffer;acetonitrile. The effluent was monitored for UV activity at 254 nm and for radioactivity with a Berthold Model LB-503 HPLC radioactivity detector with Flo-Scint II (Radiomatric Instruments and



Peak #	Area %	RT (min)
1	0.614	1.85
2	0.067	3.63
3	0.249	4.27
4	0.035	4.8
5	0.042	5.1
6	17.936	5.87
7	8.68	6.56
8	2.54	7.15
9	0.082	8.56
10	40.606	9.6
11	24.688	11.15
12	2.631	13.98
13	0.612	15.34
14	0.2	16.8
15	0.018	20.75
16	0.019	24.28
17	0.066	24.72
18	0.247	25.7
19	0.151	25.97
20	0.097	27.24
21	0.42	27.7
TOTAL	100.	

Figure 10. HPLC Chromatogram of [^{14}C]Paldimycin with Detection by Radioactivity.



Peak #	Area %	RT (min)
1	0.803	1.82
2	0.293	2.56
3	0.133	3.35
4	0.223	3.75
5	0.136	4.1
6	0.092	4.32
7	0.099	4.84
8	0.081	5.16
9	17.917	5.88
10	8.266	6.62
11	3.538	7.2
12	39.356	9.58
13	24.549	11.19
14	2.107	14.02
15	0.485	15.28
16	0.613	24.64
17	0.43	25.86
18	0.582	27.64
19	0.297	28.92
TOTAL	100.	

Figure 9. HPLC Chromatogram of [^{14}C]Paldimycin with Detection by UV Absorption.

Chemical Co., Tampa, FL) as the scintillation cocktail. Quantification of the chromatograms was done with Spectra Physics Model 4270 computing integrators.

Fermentation of *S. paulus*

Liquid nitrogen frozen vegetative culture of *S. paulus*, strain LAP-51-19-2, was used to inoculate (0.5 ml/flask) primary seed medium 273-P-5 (Table 1) in a 1000-ml Erlenmeyer flask containing 300 ml of medium. The flasks were incubated on a rotary shaker (250 rpm) at 28°C for 48 hours. Secondary seed was grown in a 250-l tank containing 250 liters of medium 273-S-5 (Table 1). The 250-l tank was inoculated with 300 ml of 48-hour primary seed. The mixture was incubated at 28°C for 24 hours with agitation and aeration.

The fermentation was started in either 250- or 5000-l fermentors containing 250 or 5000 liters of modified medium 273-F-9 (Table 1), respectively. The fermentors were inoculated with the 24-hour secondary seed. The 250-l fermentations were run with aeration and agitation at 240 rpm for 0-24 hours, then 280 rpm until harvest. The 5000-l fermentations were agitated with aeration at 166 rpm and 26°C. At 42-44 hours, about 1.5 liter of fermentation beer was transferred to a sterile 2000-ml Erylenmyer flask which was chilled on ice during transport to the radiosynthesis facility. The cooled beer (1.2 liter) was transferred aseptically with the aid of a sterile funnel to the Multigen fermentor jar. The jar and accessories had been previously sterilized for 30 minutes at 16 psig in a pressure cooker in the radiosynthesis laboratory. Fermentations in the Multigen fermentor were carried out at 26°C with aeration and agitation at 760-800 rpm. Ucon LB-625 (PAG) was added in small increments (0.1-0.2 ml) when needed to control foaming during the fermentation.

The pH of the fermentation mixture was maintained at 7.4 with H_2SO_4 from 0-3 days and at 7.0 with NaOH from 0-2 days. Once the medium was transferred to the Multigen, a lower pH limit of 6.6 was maintained with NaOH. Dilute solutions were used for pH control in the Multigen fermentor, e.g., 1N H_2SO_4 and 3 N NaOH.

At approximately 48 and 72 hours, 20 ml (166.7 ml per liter of beer) of a sterile slurry of XAD-16 resin (Rohm and Haas) was added to the fermentation with the aid

of a sterile funnel. The resin was sterilized in a total volume of 30-50 ml of distilled water by autoclaving at 121°C for 30 minutes. Prior to use the resin was conditioned by placing it in a column and washing upflow with water for 15-30 minutes. Then five volumes each of acetone, methanol, and methanol:water (1:1), in that order, were passed downflow through the resin. Finally, the resin was washed downflow with 20 volumes of water. The conditioned resin was suspended in distilled water and decanted several times to remove fine particles.

The precursor, L-methionine, was dissolved in 100 ml of distilled water and was sterilized by filtration. The solution was transferred to a sterile 250-ml Erlenmyer flask with an outlet at the bottom of the flask, which was connected via sterile rubber tubing to the acid addition port on the fermentor jar. The solution was fed to the fermentation medium in small increments with the tubing pump used for acid pH control. The methionine addition was started at 72-75 hours and the solution was fed at a rate of 2.5-3 ml/hour for a total of 30-40 hours. The fermentation was generally harvested at five days (114-116 hours).

Isolation and Purification of [^{14}C]Paulomycin

The beer was screened under vacuum through a two-inch 100-mesh stainless steel sieve to collect the XAD-16 resin beads. After thorough washing and rinsing with 1.5 l of water in small portions, the damp resin was placed in a 60-ml filter funnel with fritted glass disk (10-15 μm), and eluted over a one-hour period with 300 ml of ethyl acetate, in 25-30 ml portions, which had been saturated with water. The eluates were removed from the resin by suction, combined, the layers separated, and the organic phase was dried over anhydrous sodium sulfate. The solution was concentrated at reduced pressure at 30°C and the red-brown partially solid residue was chromatographed on a column of 60 g of silica gel packed in and eluted with 2% methanol in methylene chloride. After 250 ml, the eluent was changed to 2.5% methanol in methylene chloride and the eluate was collected in 10 ml fractions at 2.5 ml per minute for 70 fractions. Fractions were analyzed by TLC* and those which contained paulomycin (fractions 6-70) were pooled and

*TLC analyses were done on Analtech glass plates coated with 250 μm layer of silica gel. Plates were developed with 95:5:0:1 v/v methylene chloride:methanol:acetic acid, and developed zones were visualized under UV (254 μm) light.

concentrated. The resulting crude solids were dissolved in boiling methylene chloride (300 mg of solids per ml of solvent). Slow addition with stirring of an equal volume of 1:1 methylene chloride:hexane at room temperature caused crystallization to begin. With continued efficient stirring, another volume of hexane was added to the mixture, so that the final mixture was a 1.1 mixture of methylene chloride and hexane. After thorough mixing, the mixture was filtered and the white crystals were washed with 1:1, then 1:2 mixtures of methylene chloride:hexane, and finally hexane, and dried. From a typical 1.2 l fermentation fed with 40 mCi of L-[S-methyl-^{14}C]methionine (55 mCi/mmol) in 100 ml of sterile water, 745 mg of [^{14}C]paulomycin of 9.4 μCi/mg was obtained in 17.5% radiochemical yield. HPLC analyses of a sample of such material with UV and radioactivity detection, are shown in Figures 7 and 8 , respectively.

[^{14}C]Paldimycin Trisodium Salt

To a stirred slurry of 2.73 g of N-acetyl-L-cysteine (16.7 mmol) in 17 ml of 0.1 M phosphate buffer** (pH 7.85) was added 17.5 ml of 1 N KOH in small portions until the pH of the mixture was 8.71. To the resulting pale straw colored clear solution was added 642 mg of [^{14}C]paulomycin (0.823 mmol, sp. act. 7.8 μCi/mg). The mixture was stirred at room temperature for 1.5 hour, and filtered through a cake of Celite to remove traces of flocculent materials. The filter cake was washed with 5 ml of water. The combined filtrate and washing were adjusted to pH 2.9 with 2N HCl (7.2 ml needed), and extracted with 3 x 40 ml of ethyl acetate. The combined extracts were washed with water, followed by brine and dried over anhydrous sodium sulfate. The dried solution was concentrated at 25 torr and 25^{0}C. The glassy residue was dissolved in 2 ml of acetone and 20 ml of ethyl ether was added in portions with sustained stirring. The precipitates were filtered, washed with ether, and dried under vacuum. There was obtained 803 mg of white solids, sp. act. 5.45 μCi/mg, 87% yield. This material was found to be free of paulomycin and the monoadduct by TLC analysis* and was used in the next step without further purification.

**The phosphate buffer was prepared by adjusting the pH of a mixture of 3.44 ml of H_3PO_4 and 500 ml of H_20 to 7.85 with 4N KOH.

*TLC analysis was carried out on silica gel coated plates developed with 20:30:5 v/v chloroform:ethanol:water. Developed zones were detected under UV (254 nm) light.

A solution of 798 mg of the above described bis-adduct in 46 ml of 2:1 v/v t-butanol:water was adjusted to pH 8.34 with the dropwise addition of 4.1 ml of 0.5 N NaOH. The solution was frozen and lyophilized. The fluffy white residue was triturated with 25 ml of acetone and the mixture was filtered. The collected solids were washed with acetone, followed by ethyl ether, and dried under vacuum to give 839 mg of [^{14}C]paldimycin tri-sodium salt, sp., act. 4.97 μCi/mg. HPLC chromatograms of this material are shown in Figures 9 (UV) and 10 (radioactivity).

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