BIOGENESIS OF CHROMOMYCIN A_3 BY *STREPTOMYCES GRISEUS*

ANTONIO MONTANARI and JOHN P. N. ROSAZZA*

The Biocatalysis Research Group, and the Division of Medicinal and Natural Products Chemistry,
College of Pharmacy, University of Iowa,
Iowa City, Iowa 52242, U.S.A.

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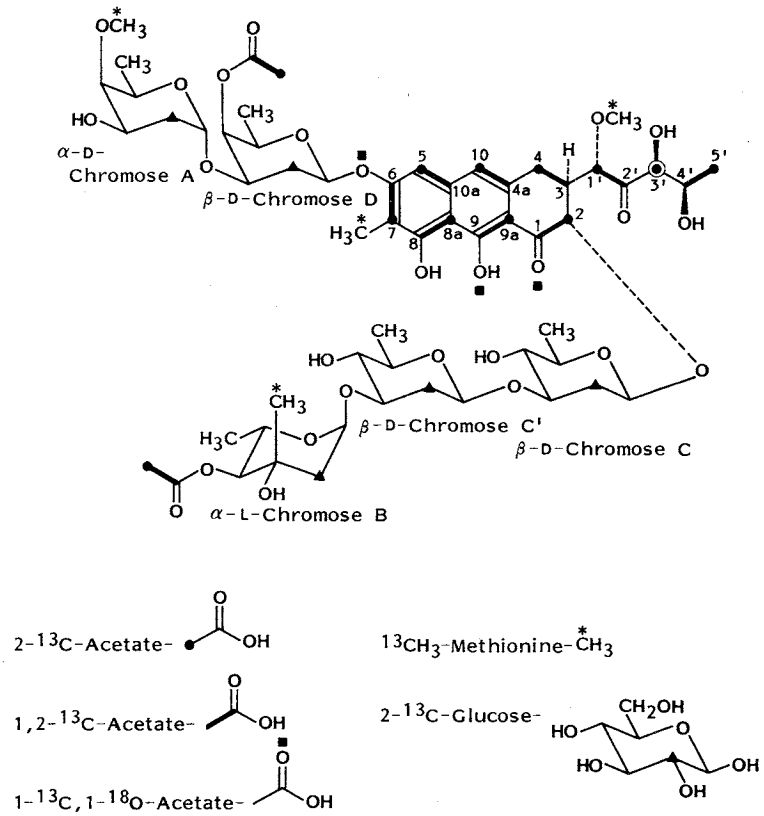
The biosynthesis of chromomycin A_3 was investigated using ^{13}C-labeled acetates, methionine and glucose, and ^{13}C,^{18}O-labeled acetate. ^{13}C NMR spectral analysis demonstrated that: Aglycone assembly occurs by combining at least two polyketide chains; three of nine oxygen atoms of the aglycone originate from acetate precursor oxygen atoms; carbon methylations on the aromatic ring at C-7, on the chromose B sugar, and two *O*-methylations appear to be carried out by *S*-adenosyl-methionine requiring methyl transferases; and glucose is the precursor for all of the sugars.

Chromomycin A_3 (Fig. 1) is an antitumor active[1] member of the aureolic acid family of antibiotics produced by strains of *Streptomyces griseus*[2~4]. Chromomycinone is the highly functionalized aglycone which is substituted at positions-2 and 6 with tri- and disaccharide moieties comprised of 2,6-dideoxy sugars. Early biogenesis studies using ^{14}C-labeled precursors identified methionine, acetate and malonate

Fig. 1. Labeling patterns of chromomycin A_3 observed with stable isotope precursors.



as major precursors for chromomycin A_3[5,6]. However, the positional locations of labeled precursors within the aglycone or sugar portions of the antibiotics were not ascertained. We described the first incorporations of singly and doubly-labeled ^{13}C-acetic acids into chromomycin A_3 by *S. griseus* to clearly demonstrate that the aglycone was a polyketide[4]. We suggested that the assembly of the aglycone involves the incorporation of one long octa- or nona-ketide, and a second shorter chain precursor. The present manuscript describes labeling patterns of chromomycin A_3 enriched by [2-^{13}C]-, [1,2-$^{13}C_2$]-, and [1-^{13}C, 1,1-$^{18}O_2$]acetates, [$^{13}CH_3$]methionine, and [2-^{13}C]glucose, the incorporations of which permit a definition of the pathways by which carbon is involved in the assembly of chromomycin A_3.

Materials and Methods

Microorganism

Streptomyces griseus ATCC 13273 was used to produce chromomycin A_3[4].

General

^{13}C NMR spectra were obtained at a field strength of 90.56 MHz using a Bruker WM-360 FTQ NMR spectrometer equipped with an Aspect 2000 data system and pulse programmer. A Bruker 5-mm carbon/proton probe was employed for all spectra obtained in this work, and 5 mm tubes were used with the samples dissolved in 350 μl of $CDCl_3$.

Chromatography

TLC was carried out using 8% w/w KH_2PO_4 impregnated, 0.25 mm thick silica gel GF_{254} (Merck) plates which were developed in benzene - acetone - formic acid (50 : 50 : 1). Chromomycin A_3 gave an Rf of 0.22 in this system. Reversed phase TLC was performed using 0.25 and 1.0 mm (preparative) ODS, C-18-silica gel GF_{254} plates (Merck) developed in acetonitrile - water - formic acid (70 : 30 : 1) to give Rf 0.50 for chromomycin A_3. Developed chromatograms were visualized by fluorescence quenching under 254 nm UV light, and by spraying developed plates with PAULY's reagent to detect phenolic compounds. PAULY's reagent consisted of three separate solutions: $NaNO_2$ 0.5%, sulfanilic acid 0.5% in HCl 2% and NaOH 5% in 50% ethanol. Equal volumes of sodium nitrite and sulfanilic acid solutions were mixed immediately prior to use and sprayed onto plates, followed by NaOH and warming with a heat gun to give burnt-orange colors to phenolic compounds.

Column chromatography was carried out using silica gel (Baker 3404, 40 ~ 140 mesh) containing 8% w/w KH_2PO_4. Slurries of silica gel and potassium phosphate were dried on a steam bath, and were then oven activated at 120°C for 30 ~ 60 minutes. Columns were slurry packed in benzene, and eluted with a mixture of benzene - acetone - formic acid (50 : 50 : 1), while fractions were collected using an ISCO 1850 or a Fractomette 200 fraction collector.

Reversed phase column flash-chromatography was carried out on 140 g of Baker 40 mesh C-18, ODS (cat. No. 7025-00) in a 3 × 40 cm Baker flash column. The column was pressurized to 0.28 kg/cm^2, and eluted with acetonitrile - water - formic acid (50 : 50 : 1), at a flow rate of 4.5 ml/minute while 9 ml fractions were collected.

HPLC was performed using a Waters M6000-A pump, a U6K Universal Injector, and an ISCO V^4 variable wavelength UV detector recorder set at 280 nm. Samples were resolved on an Alltech, Econosil ODS column (10 μM, 0.46 × 25 cm, i.d.) preceded by a 0.46 × 1 cm guard column of the same stationary phase, using acetonitrile - water - formic acid (55 : 45 : 1) at a flow rate of 1 ~ 2 ml/minute where chromomycin A_3 possessed a retention volume of 4.8 ml. A semipreparative Whatman Partisil M9 10/50 ODS column (1 × 50 cm), preceded by a 0.46 × 5 cm guard column, was used for larger-scale separations and purifications of antibiotic samples. Semipreparative columns were eluted with the same solvent used in analytical-scale separations at a flow rate of 5 ml/minute at 70.3 kg/cm^2.

Fermentations

Fermentations were carried out in a medium consisting of: Glucose 2.5% (Stage I), 5% (Stage II),

soybean meal 1.5%, NaCl 0.3%, $CaCO_3$ 0.3% (w/v), held in 1-liter stainless steel-capped DeLong culture flasks each holding 200 ml of medium. Media were sterilized by autoclaving at 121°C, 0.7 kg/cm^2 pressure for 20 minutes. Stage I cultures were initiated by using the suspended contents of one *S. griseus* slant per flask, followed by incubation on NBS gyrotory shakers (Model G-25, New Brunswick Scientific, Co.) operating at 250 rpm at 27°C for 72 hours. The 72 hours Stage I culture was used as inoculum (10%) for Stage II cultures which were incubated as before. Amberlite XAD-2 polystyrene-divinylbenzene, crosslinked resin, 10 g (Sigma, Cat No. 21, 648-8) was added to each 2-day-old Stage II culture to act as a solid-state extraction system, and to aid in the recovery of antibiotics[7]. After receiving labeled precursors, incubations were continued for 5 days before being harvested.

Additions of ^{13}C-Labeled Precursors to *S. griseus* Cultures

Purities of ^{13}C-precursors were confirmed by NMR spectral analysis. Labeled compounds were routinely added to 48-hour old Stage II cultures when HPLC analysis indicated that chromomycin A_3 production had just started, and when residual medium glucose was 0.1%.

Volumes of Stage II cultures, amounts of labeled precursor added, and the amount of chromomycin A_3 produced were as follows: 2 g of 99 atom% sodium [2-^{13}C]acetate in 2 liters of culture yielded 25 mg of pure chromomycin A_3; 1 g of 99 atom%-^{13}C, 96 atom%-^{18}O sodium [1-^{13}C, $^{18}O_2$] acetate in 1 liter of culture yielded 29 mg of chromomycin A_3; 2 g of 99 atom% [$^{13}CH_3$] methionine in 2 liters of culture yielded 38 mg of chromomycin A_3; and 500 mg of 99 atom% [2-^{13}C]glucose added to 1 liter of culture yielded 38 mg of chromomycin A_3.

Isolation and Purification of ^{13}C-Labeled Chromomycin A_3

Stage II cultures were frozen and lyophilized, and the resulting dry powders were extracted with chloroform (4 × 500 ml) to prepare crude antibiotic extracts. Extracts obtained from fermentations containing sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate, and [$^{13}CH_3$]methionine were purified over phosphate impregnated silica gel columns. Extracts from labeled glucose and doubly labeled [^{13}C,^{18}O]acetate were purified by reversed phase flash column chromatography. TLC was used to identify column fractions containing chromomycin A_3. These were combined, extracted with chloroform and concentrated to dryness.

Results

Chromomycin A_3 isolated in our laboratories[4] was completely characterized and used to verify all ^{13}C and ^{1}H NMR[8,9], mass spectral and UV/visible properties of the antibiotic. Precursors were all added at 48 hours to Stage II cultures when antibiotic production was typically measured at a level of 4 mg/liter, and when residual medium glucose was measured to be 0.1%. The optimum time for harvesting incubations of *S. griseus* was 5 days after labeled substrates were added. The presence of the Amberlite resin aided in increasing yields of chromomycin A_3, and in facilitating antibiotic extractions for chromatographic purifications. ^{13}C NMR signal intensities of labeled-chromomycin A_3, were compared to those of the natural abundance spectrum of unlabeled chromomycin A_3. Relative peak abundances were obtained by comparing the ratios of peak intensities of all carbons to those for position-1[10]. The results of ^{13}C NMR spectral measurements of labeled chromomycin A_3 samples are presented in Table 1, and in Fig. 1.

[2-^{13}C]Acetate was incorporated into aglycone carbons at positions 2, 9a, 8a, 7, 5, 10, 4, 1′, 3′, and 5′, as well as the two sugar acetate moieties. Relative enrichments into the two aglycone carbons at positions 5′ and 7, and the two acetate ester methyl group carbons ranged between 2.1 ~ 2.4, while relative enrichments into the remaining carbons were lower, ranging between 1.3 and 1.9.

Incorporation levels obtained with [1,2-$^{13}C_2$] acetate were similar to those obtained with singly labeled acetate. The ^{13}C NMR spectrum of chromomycin A_3 obtained with doubly labeled acetate revealed 22

Table 1. ^{13}C NMR analysis of chromomycin A_3 enriched with stable isotope precursors.

Carbon	Acetate Fed					[$^{13}CH_3$]-Methionine relative enrichments	[2-^{13}C]-Glucose relative enrichments
	1,2-^{13}C		2-^{13}C Relative enrichments	1-^{13}C,^{18}O			
	ppm	J_{cc} (Hz)		Shift (Hz-ppm)	Relative enrichments		
Aglycone							
1	202.1	43.62	—[a]	3.0~0.03	0.5	[a]	[a]
2	75.9	43.20	1.3	—		—	—
3	43.8	32.26	—	—		—	—
4	27.0	35.19	1.4	—		—	—
4a	134.6	65.74	—	—		—	—
10	117.1	66.01	1.9	—		—	—
5	100.8	58.37	1.4	—		—	—
10a	138.4	58.79	—	—		—	—
6	159.6	68.74	—	1.8~0.02	0.5	—	—
7	111.6	69.29	2.4	—		—	—
8	165.3	64.93	—	—		—	—
8a	108.0	63.68	1.9	—		—	—
9	156.1	63.16	—	1.8~0.02	1.0	—	—
9a	108.0	63.68	1.9	—		—	—
1′	82.0	42.64	1.8	—		—	—
2′	211.2	42.98	—	—		—	—
3′	78.4	NC	1.5	—		—	—
4′	67.9	38.07	—	—		—	—
5′	20.5	38.40	2.4	—		—	—
7-CH_3	8.2		—	—		13	—
1′-OCH_3	59.7		—	—		19	—
D-Chromose A							
2	33.5		—	—	—	—	1.7
4-O-CH_3	62.3		—	—	—	27.5	—
L-Chromose B							
2	44.0		—	—		—	4.0
3-CH_3	23.0		—	—		11.5	—
4-*O*-Acetate							
	20.9	59.69				—	—
	171.4	59.97	2.2	3.3~0.04	0.4	—	—
D-Chromose C							
2	37.5		—	—		—	2.0
D-Chromose C′							
2	37.1		—	—		—	1.6
D-Chromose D							
2	33.0		—	—		—	1.7
4-*O*-Acetate							
	170.9	60.43	—	—		—	—
	20.8	59.6	2.1	—		—	—

[a] Relative enrichments were normalized to peak intensities for the C-1 signal of the aglycone[10].
NC: Not coupled.

spin coupled signals, as well as an enriched, but not coupled signal for C-3′. Labeling patterns obtained using [1-^{13}C,$^{18}O_2$]acetate were evidenced by a splitting of signals for carbons at positions 1, 6, 9, and the chromose B 4-*O*-acetate carbonyl carbon atom[10,11]. Each of the sugar 2-carbon atoms were enriched with [2-^{13}C]glucose, and the four methyl groups clearly labeled by [$^{13}CH_3$]methionine were 1′-O-CH_3 (59.1 ppm, relative enrichment 19), 7-CH_3 (13), chromose A 4-O-CH_3 (27.5), and the chromose B C-CH_3 (11.5).

Discussion

Fermentation conditions for the chromomycin A_3-producing organism, *S. griseus* ATCC 13273 were optimized to yield approximately 30 mg/liter of chromomycin A_3. In our experience, 25 mg samples of pure chromomycin A_3 gave excellent ^{13}C NMR spectral data, usually during spectral acquisition times of $8 \sim 12$ hours. Methods were developed for the rapid extraction and purification of the antibiotic. Amberlite XAD-2 resin (50 g/liter) was added to cultures to serve as a hydrophobic extractant of chromomycin A_3, and resulted in a doubling of antibiotic titers vs. control incubations without resin. While purification of chromomycin A_3 over phosphate impregnated silica gel was vastly better than chromatographic purification over ordinary silica gel, flash-column chromatographic purification of chromomycin A_3 from crude extracts was the best method for obtaining pure samples of chromomycin A_3 for ^{13}C NMR analysis.

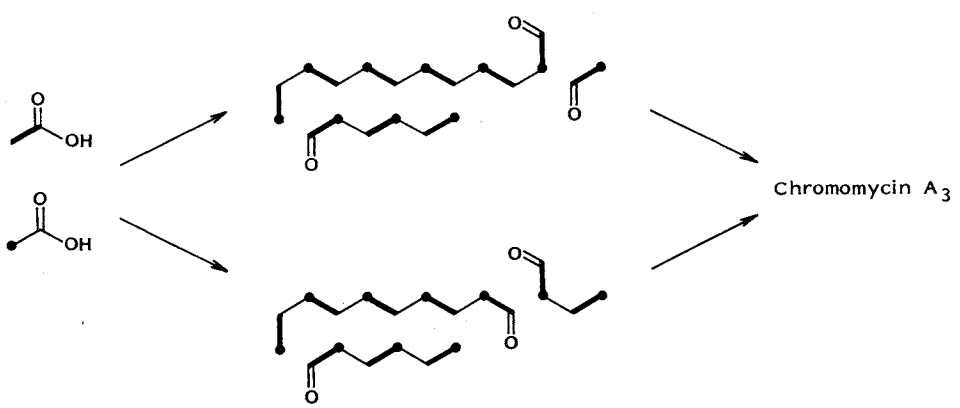
HPLC permitted the ready analysis of cultures to determine that antibiotic biosynthesis commenced 48 hours after Stage II culture inoculation. Labeled precursors were all added in a single dose at the time. This time also coincided with a fermentation point at which glucose titers were less than 0.1%, thus reducing the potential for dilution of ^{13}C-labeled-precursors in metabolic pools.

Chromomycin A_3 derived from singly labeled acetate displayed twelve enhanced signals in the NMR spectrum of the aglycone-portion of chromomycin A_3 indicating an even distribution of label, and a polyketide origin for the aglycone. The relatively high incorporations of [2-^{13}C]acetate into positions 7 and 5′ of the aglycone could imply the involvement of separate polyketide chains with starter-methyl groups at these positions[12~15]. Analysis of carbon-carbon coupling constants indicated that there were eleven coupled doublets arising from doubly labeled acetate incorporated intact. The signal for C-3′ exists as an enhanced singlet, which must arise from double-labeled acetate, split during aglycone assembly. Because of this unusual finding, the doubly labeled acetate incorporation experiment was repeated with the same result.

It is difficult to define the precise means by which the aglycone is assembled from the data obtained in this work. Our incorporation results with single- and double-labeled acetate are similar to those reported for the biogenesis of several other antibiotics[12~15]. The positional labeling of carbons from both precursors could support the assembly of aglycone from either two- or three polyketide chains. The higher incorporations of singly-labeled acetate into carbons 5′ and 7 implies that these could be derived from acetate-starter units. The presence of an enhanced but unsplit signal at C-3′ complicates this picture, and suggests the possible linkage of three different acetate-malonate-derived chains. Incorporating this finding, a two-chain aglycone assembly process could involve either octa- or nona-ketide precursors which link *via* Claisen condensations with mono- or di-ketide units respectively. Alternatively, a three-chain process can be envisioned starting with acetate-chains beginning at carbons 2, 7 and 5′ as illustrated in Fig. 2.

Studies with [1-^{13}C,$^{18}O_2$]acetate were conducted in order to obtain evidence for the possible incorporation of acetate-oxygen atoms into chromomycin A_3. Evidence for the incorporation of ^{18}O can be obtained by measuring subtle differences in ^{13}C-signals attached to normal vs. labeled oxygen[11,16]. Based on the polyketide origin of the aglycone suggested in Figs. 1 and 2, oxygen could derive from acetate

Fig. 2. Possible polyketide pathways for the assembly of chromomycin A_3 aglycone by *Streptomyces griseus*.



at positions 1, 6, 8, 9, 2′ and 4′ in the aglycone, and in the acetate esters attached to the sugars. Three of the oxygen atoms at positions 2, 1′ and 3′ most likely do not arise from acetate oxygen atoms. These could derive from molecular oxygen, *via* separate hydroxylation reactions catalyzed by enzyme systems known to occur in this organism[17]. The ^{13}C-^{18}O-coupled NMR signals for carbons 1, 6 and 9 in the aglycone, and the L-chromose B acetate moiety were observed as doublets with observed shifts ranging from 1.8~4.0 Hz, clearly demonstrating the origin of these oxygen atoms directly from acetate. Signals for carbons at positions 8, 2′, 4′ and the chromose D 4-*O*-acetyl functional groups were broadened, but not actually split in two, suggesting that they too could arise from their respective acetate precursors. Repeated spectral acquisitons, and resolution experiments failed to resolve these broadened peaks, rendering this conclusion speculative.

Three different types of methylation reactions occur during the synthesis of chromomycin A_3. These include etherification of two secondary alcohol functional groups at positions-1′ and -4B of the A chromose sugar; aromatic carbon-methylation at position-7 of the aglycone; and carbon-methylation at position-3 of the B chromose sugar. Each of these carbons derives from methionine as evidenced by the observed high relative enrichments which ranged from 11.5~27.5. The incorporation of methyl groups from methionine implies the probable involvement of three different methyl transferase enzyme systems in *S. griseus*. It is interesting that several demethyl-chromomycins and olivomycins have been discovered[18], and that the potential preparation of demethyl-chromomycins using methylation-inhibitors could afford numerous new aureolic acid analogs.

[2-^{13}C]Glucose was incorporated into each of the sugars attached to the aglycone. The incorporation of glucose into similar 2,6-deoxy-sugars such as mycarose in tylosine has been documented[19,20], and aspects of the mechanism for the formation of 2-deoxy-sugars in other antibiotics have been addressed[12,21]. Our experiment provides the first direct evidence that glucose is incorporated into all of the sugars of chromomycin A_3.

Our results permit a definition of the pathways by which carbon—and some oxygen atoms are biosynthetically incorporated in the assembly of chromomycin A_3.

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