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Biosynthesis of Monensin

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The biosynthesis of monensin by *Streptomyces cinnamonensis* was studied by using ^{14}C-labeled glucose, acetate, propionate, butyrate, and methionine. The results indicated that the antibiotic is synthesized from five acetate, seven propionate, and one butyrate molecules. The *o*-methyl group of monensin is derived from methionine, whereas the terminal hydroxymethyl group is incorporated from acetate.

Monensin, an antibiotic produced by *Streptomyces cinnamonensis* (ATCC 15413), was first described by investigators from Eli Lilly and Co. (4). Initial fermentation studies were presented by Stark (7). The structure of the major component, factor A (Fig. 1), was determined by X-ray crystallographic analysis of the silver salt by Agtarap et al. (2). In addition to factor A, three additional factors have been recognized (3): in factor B the ethyl group on ring C is replaced by a methyl group, in factor C the methyl at the carboxyl end is replaced by an ethyl group, and in factor D the methyl group on the B ring is replaced by an ethyl group. The structures of factors C and D are tentative proposals. Factors B, C, and D are minor constituents of the fermentation broth. Factor A, hereafter designated monensin, and its sodium salt are only slightly soluble in water but are very soluble in organic solvents.

This study is concerned with the biosynthesis of monensin and the incorporation of labeled intermediates into the antibiotic.

MATERIALS AND METHODS

Organism and cultural conditions. The ingredients of the medium used during these studies with *S. cinnamonensis*, ATCC 15413, were (in mg/ml): glucose (16.7), L-tyrosine (3.3), L-valine (6.6), L-lysine (1.0), $CaCO_3$ (1.0), $FeSO_4 \cdot 7H_2O$ (0.17), K_2HPO_4 (0.17), KCl (0.05), $MgSO_4 \cdot 7H_2O$ (0.67), biotin (0.025), and folic acid (0.025). Cultures were grown at 32 C in shaken (250 rpm) flasks (100 ml of medium per 500 ml wide-mouth flask), or in 1.5-liter glass fermentors. Fermentors were aerated at 0.0015 m^3/s with an agitator speed of 1,750 rpm and were inoculated with 150 ml of a vegetative culture grown in shaken flasks at 32 C for 16 h. This medium consisted of glucose (0.6%), corn starch (2.0%), soybean flour (2.2%), yeast extract (0.12%), and $CaCO_3$ (0.15%). All media were steam-sterilized at 120 C for 30 min.

Sterile radioactive intermediates were added aseptically in 50-μCi quantities 24 h postinoculation, or in five 10-μCi samples at 24-h intervals starting at 24 h postinoculation. The total incubation time was 144 h.

Assays. Monensin in culture broth was assayed as described by Stark et al. (8).

Isolation of monensin. The whole broth was harvested and the pH was adjusted to 9.0 with NaOH, after which it was extracted twice with one-half volume of chloroform. The extracts were combined and washed through a column containing carbon (Pittsburgh 12 by 40 mesh). The column was washed with excess chloroform, and the combined extracts were evaporated. The residue was dissolved in methanol and chilled. Cold deionized water was added until the monensin crystallized. The monensin was collected by filtration, washed with cold water, recrystallized from petroleum ether, and assayed for radioactivity.

Degradation of labeled monensin. The periodate oxidation of monensin is illustrated in Fig. 2. A sample of ^{14}C-labeled monensin (100 mg) was dissolved in *t*-butanol (4 ml) with stirring. To this solution was added 0.2 M aqueous sodium *meta*-periodate solution (2 ml). The final mixture was allowed to stand overnight. The reaction mixture was distilled into a receiver containing a dimedone solution (100 mg of dimedone in 2 ml of 50% ethanol-water). The distillate was allowed to stand a few minutes and then concentrated under reduced pressure to induce crystallization. Crystals of the dimedone derivative of formaldehyde (melting point 189 to 191 C) were collected by filtration and dried. The residue remaining after distillation was diluted with water and extracted three times with ether. The combined ether extracts were washed once with water and once with saturated sodium chloride solution, and dried with anhydrous sodium sulfate. Evaporation of the solvent gave the dehydroxymethyl derivative of monensin (2) as a foam. A typical experiment yielded 69 mg of the latter and 8 mg of the crystalline dimedone derivative.

The chromic acid oxidation (Fig. 3) was carried out as follows. The reaction mixture was prepared by dissolving a sample of ^{14}C-labeled monensin (0.40 to 0.50 g) in glacial acetic acid (15 ml) along with an equal amount of unlabeled monensin (labeled monen-

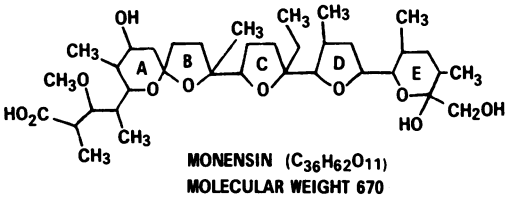

FIG. 1. *Structure of monensin.*

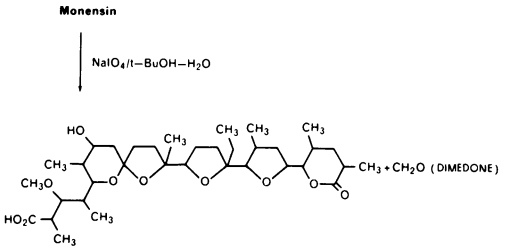

FIG. 2. *Products of periodate oxidation of monensin.*

sin obtained by using propionate-2-^{14}C as precursor was diluted 9:1 for this degradation). A solution of chromium trioxide (1.36 g) in glacial acetic acid (12 ml) and water (3 ml) was added and the mixture was allowed to stand overnight. The reaction mixture was diluted with saturated sodium chloride solution (200 ml) and extracted four times with chloroform. The combined extracts were washed three times with saturated sodium chloride solution and dried with anhydrous sodium sulfate. Evaporation of the solvent gave a bluish gum residue. The latter was treated with ether and filtered to remove a bluish, insoluble solid. The ether filtrate was extracted three times with 50-ml portions of saturated sodium carbonate solution to remove acidic material. The ether layer was dried (Na_2SO_4) and evaporated to give the neutral fraction. The combined sodium carbonate extracts were acidified with concentrated hydrochloric acid and extracted with ether to obtain the acidic fraction. In a typical experiment, there were obtained 270 mg of neutral material and 240 mg of the acidic fraction, both of which contained several components. Before separation by chromatography, the acidic material was treated with excess diazomethane in ether.

The neutral fraction (270 mg) was chromatographed on silica gel (Woelm, activity grade I, 22 g). Elution with ethyl acetate-benzene (1:3) gave 62 mg of the dilactone (1, 2) which was crystallized from hexane (50 mg) (melting point 96 to 97 C). Similarly, the esterified acidic fraction was chromatographed (24 g of silica gel, ethyl acetate-benzene, 1:9) to obtain the ester-lactone (2) (melting point 102 to 104 C [hexane]).

The isolation of the methoxyl methyl group from the ester-lactone was achieved by a standard microanalytical procedure for methoxyl determination (6). This procedure was applied to a sample of the ester-lactone obtained from monensin labeled with L-methionine-$^{14}CH_3$. Methyl iodide formed from the methoxyl groups was trapped as tetramethylammonium iodide. A yield of 3 mg of the crystalline iodide was obtained from 7 mg of the ester-lactone and was submitted for radioactivity assay.

The Kuhn-Roth oxidation of monensin was carried out to cleave the methyl groups from the molecule and the ethyl group of ring C. These were isolated as acetate and propionate, respectively. The general procedure followed for Kuhn-Roth oxidation of ^{14}C-labeled monensin was as follows (5). The antibiotic sample (0.500 g) was refluxed for 15 min in a standard Kuhn-Roth oxidizing medium (chromic acid in sulfuric acid). The volatile acids were steam-distilled and neutralized (phenolphthalein) with 1 N sodium hydroxide. The solution was concentrated under reduced pressure to about 20 ml, and the pH was adjusted to a faint pink color on blue litmus with 0.1 N hydrochloric acid. A solution of *p*-bromophenacyl bromide (360 mg) in ethanol (5 ml) was added, and the mixture was refluxed for 1 h. The mixture was allowed to cool, diluted with H_2O (200 ml), and extracted three times with $CHCl_3$. The combined extracts were washed twice with H_2O and dried (Na_2SO_4). Evaporation of the solvent gave a pale yellow solid which amounted to about 325 mg. Comparison with authentic samples by thin-layer chromatography (silica gel, benzene) showed the presence of *p*-bromophenacyl esters of acetic and propionic acids. The esters were isolated by preparative gas chromatography (Hewlett-Packard 775-Prepmaster instrument, 31.5-cm, W98 silicone gum column at 130 C). The separated esters were recrystallized from ethanol-water. Melting points were in agreement with those of authentic samples.

RESULTS

Before utilization of ^{14}C-labeled intermediates, a study was made of stimulation of monensin synthesis using possible precursors (Table 1). Whereas acetate did not stimulate the amounts of monensin synthesized, propionate and butyrate at levels of 0.1% markedly increased the levels of monensin. Methyl malonic acid stimulated synthesis to a lesser degree.

The results of the incorporation of some ^{14}C-labeled precursors into the monensin molecule are summarized in Table 2. Propionate, in this experiment, showed a high level of incorporation, as did the methyl from [*methyl*-^{14}C]-methionine. Butyrate was also incorporated; however, glucose and acetate were not as well incorporated.

Monensin labeled from various ^{14}C precursors was then subjected to the degradative schemes described under Materials and Methods in an attempt to locate the radioactivity within the molecule. The periodate oxidation of the monensin molecule cleaves the terminal hydroxymethyl group which is isolated as the dimedone derivative of formaldehyde. If this terminal 2-carbon unit is incorporated as acetate, the C-1 would be part of the E ring and the C-2 would be the hydroxymethyl carbon. The data in Table 3 indicate that the most signifi-

TABLE 1. *Stimulation of monensin synthesis in synthetic medium by various precursors*

Addition	Monensin (μg/ml) at 7 days	Increase or decrease (%)
None	630	
0.1% Sodium propionate added at 42 h	1,365	+117
0.1% Sodium propionate added at 0 h	700	+11
0.2% Sodium propionate added at 0 h	265	−60
0.3% Sodium propionate added at 0 h	<50	−100
0.1% Sodium acetate added at 0 h	640	None
0.5% Sodium acetate added at 0 h	<25	−100
0.05% Sodium butyrate added at 0 h	830	+31
0.1% Sodium butyrate added at 0 h	1,165	+85
0.2% Sodium butyrate added at 0 h	<140	−100
0.1% Methyl malonic acid added at 0 h	715	+14
0.5% Methyl malonic acid added at 0 h	115	−82

TABLE 2. *Incorporation of ^{14}C-precursors into monensin*

Precursor	Incorporation (%)	Molar incorporation
Glucose-*U*-^{14}C	0.70	0.025
Sodium acetate-*1*-^{14}C	1.11	0.074
Sodium acetate-*2*-^{14}C	1.86	0.124
Sodium propionate-*2*-^{14}C	19.80	1.320
Sodium butyrate-*2*-^{14}C	3.18	0.189
L-Methionine-*methyl*-^{14}C	6.40	0.280

TABLE 3. *Radioactivity in degradation products of periodate oxidation of monensin*

Precursor	Disintegrations per min per mmol of monensin ($\times 10^{-6}$)	Disintegrations per min per mmol of formaldehyde ($\times 10^{-4}$)	Radioactivity in formaldehyde (%)
Acetate-*1*-^{14}C	0.88	0.30	0.34
Acetate-*2*-^{14}C	2.45	19.80	8.10
Propionate-*2*-^{14}C	3.80	0.63	0.17
Butyrate-*2*-^{14}C	2.15	6.66	3.10
Glucose-*U*-^{14}C	0.89	3.27	3.70
Methionine-$^{14}CH_3$	4.62	0.33	0.07

cant degree of labeling in the formaldehyde arises from acetate-*2*-^{14}C. Both glucose and butyrate carbons are probably incorporated at this point after oxidation to acetate. Propionate and the methyl of methionine do not contribute to the hydroxymethyl group.

The chromic acid oxidation cleaved the monensin molecule into a C_{11} acidic fragment and a C_{23} neutral fragment (Fig. 3). Two carbon atoms of monensin were lost in this reaction, these being the terminal hydroxymethyl carbon and one carbon from ring A. The C_{11} fragment was esterified with diazomethane to give a C_{12} ester. The resulting fragments contained the radioactivity shown in Table 4. These data are consistent with the hypothesis that there are three propionate units in the C_{11} fragment and four propionate units in the C_{23} fragment (3:4 ratio). Consideration of the results of acetate-*1*-^{14}C labeling and butyrate-*2*-^{14}C labeling indicate one acetate in the C_{11} fragment and four acetate units in the larger fragment. This conclusion is made on the postulate that butyrate is going through β-oxidation to acetate before its incorporation into the antibiotic. Due to a loss of sample during preparation, the acetate-*2*-^{14}C results are not available to support this theory. However, if acetate is being incorporated in this manner, very little radioactivity should be observed in the C_{11} fragment when labeled with acetate-*2*-^{14}C.

The almost total incorporation of the radioactivity from [*methyl*-^{14}C]methionine into the C_{11} fragment indicates that the methyl of the methoxy group adjacent to the carboxyl group arises from methionine.

When the methoxyl methyl group from the ester-lactone was isolated as the methyl iodide [trapped as $(CH_3)_4NI$] from monensin labeled with L-methionine-$^{14}CH_3$, about 92% of the radioactivity was found to be associated with the tetramethylammonium iodide. With this precursor, disintegrations per minute per millimole were: monensin, 21.40×10^{-5}; C_{11} frag-

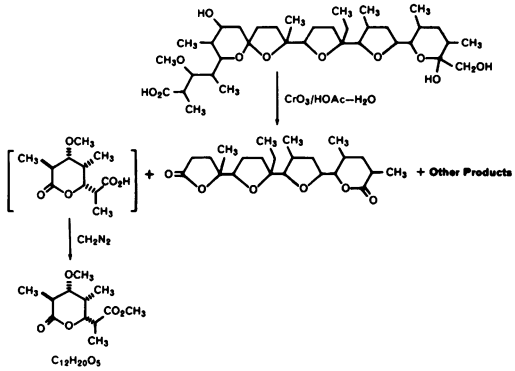

FIG. 3. *Products of chromate oxidation of monensin.*

TABLE 4. *Radioactivity in degradation products of chromate oxidation of monensin*

Precursor	Disintegrations per min per mmol of monensin ($\times 10^{-5}$)	Disintegrations per min per mmol of C_{11} fragment ($\times 10^{-5}$)	Radioactivity in C_{11} (%)	Disintegrations per min per mmol of C_{23} fragment ($\times 10^{-5}$)	Radioactivity in C_{23} (%)
Acetate-*1*-^{14}C	4.40	0.78	18.7	3.68	83.6
Acetate-*2*-^{14}C	12.20	[a]	[a]	8.29	68.0
Propionate-*2*-^{14}C	41.50	18.60	44.8	23.90	57.6
Butyrate-*2*-^{14}C	10.80	1.87	17.3	8.98	83.0
Glucose-*U*-^{14}C	4.46	1.20	26.9	2.48	55.6
Methionine-$^{14}CH_3$	21.40	20.60	96.2	0.85	3.9

[a] Sample lost during preparation.

TABLE 5. *Radioactivity in acetate and propionate after Kuhn-Roth oxidation of monensin labeled with ^{14}C-precursors*

Precursor	Radioactivity[a]		
	Monensin ($\times 10^{-3}$)	Acetate	Propionate
Propionate-*2*-^{14}C	6.80	2.11×10^3	1.01×10^3
Butyrate-*1*-^{14}C	5.70	0.53×10^2	2.55×10^2
Butyrate-*2*-^{14}C	2.87	2.24×10^2	2.45×10^3
Butyrate-*3*-^{14}C	2.11	0.98×10^3	1.79×10^3

[a] Expressed as disintegrations per minute per milligram.

ment, 20.60 $\times$ $10^{\times 5}$; and $(CH_3)_4NI$, 19.01 $\times$ $10^{\times 5}$.

The Kuhn-Roth degradation cleaves the methyl groups and the adjoining carbon from the monensin molecule as acetic acid. The same reaction cleaves the ethyl group on ring C with the adjoining carbon giving propionic acid. Monensin was labeled with propionate-*2*-^{14}C, butyrate-*1*-^{14}C, butyrate-*2*-^{14}C, or butyrate-*3*-^{14}C, and subjected to this degradation. This was done to determine the labeling of the methyl and ethyl groups from ^{14}C-propionate and -butyrate. The results (Table 5) indicate that the ethyl group of ring C arises from butyrate and that the methyl groups are labeled by propionate. When propionate-*2*-^{14}C was used as the precursor, a significant level of the radioactivity was found in the acetate after Kuhn-Roth oxidation, approximately twice that found in propionate. Labeling with *1*-, *2*-, and *3*-^{14}C-butyrate resulted in higher levels of radioactivity in the propionate from the Kuhn-Roth degradation. Butyrate-*1*-^{14}C resulted in the lowest level, and this would be expected since the labeled carbon would not be cleaved from the C ring by the Kuhn-Roth oxidation. Butyrate-*2*-^{14}C would result in labeled propionate after oxidation, and this was observed. The same is true for butyrate-*3*-^{14}C.

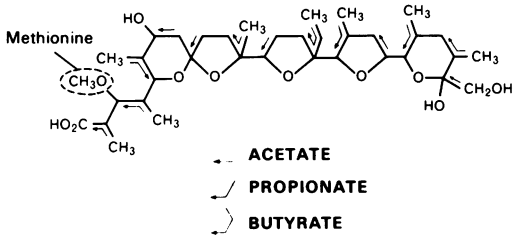

FIG. 4. *Location of acetate, propionate and butyrate within the monensin molecule.*

DISCUSSION

The results of this study are in agreement with the scheme proposed in Fig. 4. The monensin molecule is synthesized from five acetates, seven propionates, and one butyrate. The *o*-methyl group arises from methionine. Monensin methyl groups are derived from the "tails" of propionate, and the ethyl is contributed by the butyrate. We have attempted to label monensin with acetate-*1*-, and -*2*-^{13}C and to localize these carbons within the molecule through ^{13}C-nuclear magnetic resonance spectroscopy. However, the degree of incorporation was not high enough to detect ^{13}C enrichment.

The biosynthesis of monensin is very similar to that of antibiotic X-537A (9, 10). These workers, through similar methods, have shown the incorporation of acetate, propionate, and butyrate into this antibiotic. In addition, they have verified the incorporation of butyrate by using butyrate-*1*-^{13}C (10). The X-537A molecule contains three ethyl groups, all of which arise from butyrate.

LITERATURE CITED

1. Agtarap, A., and J. W. Chamberlin. 1968. Monensin, a new biologically active compound. IV. Chemistry. Antimicrob. Ag. Chemother. 1967, p. 359-362.
2. Agtarap, A., J. W. Chamberlin, M. Pinkerton, and L. K. Steinrauf. 1967. The structure of monensic acid, a new biologically active compound. J. Amer. Chem. Soc. 89:5737-5739.
3. Gorman, M., J. W. Chamberlin, and R. L. Hamill. 1968. Monensin, a new biologically active compound. V.

Compounds related to monensin. Antimicrob. Ag. Chemother. 1967, p. 363-368.
4. Haney, M. E., Jr., and M. M. Hoehn. 1968. Monensin, a new biologically active compound. I. Discovery and isolation. Antimicrob. Ag. Chemother. 1967, p. 349-352.
5. Manwaring, D. G., R. W. Rickards, G. Gaudiano, and V. Nicolella. 1969. The biosynthesis of the macrolide antibiotic lucensomycin. J. Antibiot. **22**:545-550.
6. Roth, H. 1958. Pregl-Roth quantitative organic microanalysis, p. 245. Springer-Verlag, Vienna.
7. Stark, W. M. 1968. Monensin, a new biologically active compound produced by a fermentation process, p. 517-540. *In* D. Perlman (ed.), Fermentation advances. Academic Press Inc., New York.
8. Stark, W. M., N. G. Knox, and J. E. Westhead. 1968. Monensin, a new biologically active compound. II. Fermentation studies. Antimicrob. Ag. Chemother. 1967, p. 353-358.
9. Westley, J. W., R. H. Evans, Jr., D. L. Pruess, and A. Stempel. 1970. Biosynthesis of antibiotic X-537A. J. Chem. Soc. Chem. Commun. p. 1467-1468.
10. Westley, J. W., D. L. Pruess, and R. G. Pitcher. 1972. Incorporation of [1-^{13}C] butyrate into antibiotic X-537A: ^{13}C nuclear magnetic resonance study. J. Chem. Soc. Chem. Commun. p. 161-162.