THE EVERNINOMICINS. BIOSYNTHETIC STUDIES

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The biosynthetic pathways involved in the formation of four everninomicin antibiotics were studied. Carbon-14 labelled substrates added to liquid cultures of *Micromonospora carbonacea* var. *aurantiaca* revealed that acetate, malonate and glucose were good precursors. Degradation of the antibiotics revealed the primary importance of acetate and malonate for the synthesis of the dichloroisoeverninic acid, an aromatic moiety common to all of the four everninomicins studied, thus indicating its relationship to the biosynthesis of orsellinic acid. The incorporation of the methyl group of methionine into the methoxy group of dichloroisoeverninic acid could also be demonstrated. The remainder of the everninomicin molecule is apparently derived principally from glucose.

The formation of the everninomicin antibiotic complex by *Micromonospora carbonacea* and *M. carbonacea* var. *aurantiaca* was first described by WEINSTEIN *et al.*[1] Extractions with water-immiscible solvents followed by chromatographic separation revealed as many as five active components, of which two major components were named everninomicins B and D. Acid hydrolysis of these designated components liberated a fragment, everninocin[2] which a recent study[3] showed to be identical to curacin isolated from curamycin. Alkaline hydrolysis of curacin produced dichloroisoeverninic acid. This paper reports on the incorporation and radioisotope distribution of selected carbon-14 labelled precursors in four antibiotics of the everninomicin complex.

Experimental

Production of everninomicins:

A culture of *Micromonospora carbonacea* var. *aurantiaca* (NRRL-2997)[4] was employed in these studies and maintained on slants of the fermentation medium containing 2% agar. The inoculum was prepared according to the procedure of WAGMAN *et al.*[5] The germination broth consisting of Difco beef extract, 0.3%; Difco tryptone, 0.5%; dextrose, 0.1%; soluble starch, 2.4%; yeast extract, 0.5%; calcium carbonate, 0.1%; and tap water was inoculated from a slant culture and grown for 24 hours at 37°C. A 2.5% inoculum thus prepared was employed for 150 ml fermentation broth in 250 ml Erlenmeyer flasks. The flasks were covered with pads of non-absorbent cotton layered between cheese cloth. These pads were fixed to the flasks by clamps. Previous studies had shown that in 250 ml flasks the production of everninomicin complex per ml broth was identical in 150 ml as in 50 ml of fermentation medium, obviously due to sufficient air supply coming through the cotton pads and gauze.

The fermentation medium employed in these studies consisted of dextrose, 2.0%;

Difco yeast extract, 0.5 %; calcium carbonate, 0.1 %; and tap water. The medium was adjusted to pH 7.2 with 0.5 N sodium hydroxide. The fermentation flasks were incubated at 28°C in a Gyrotory incubator shaker (model G25, New Brunswick Scientific Co., Inc., New Brunswick, N. J.) enabling the control of exhaust gases, particularly carbon dioxide, which was removed by gaseous flow through saturated barium hydroxide solution.

Extraction, purification and separation of everninomicin complex:

In all, 300 ml broth was extracted three times with 300 ml portions of methylene chloride; the combined extracts were dried with anhydrous magnesium sulfate and concentrated under reduced pressure to approximately 30 ml when the crude everninomicin was precipitated by dropwise addition to 300 ml of petroleum ether (b. p. 30~75°C). After several petroleum ether washings the crude precipitate was dried *in vacuo*. For the removal of impurities the crude dry complex mixture was dissolved in the minimum amount of upper phase of the solvent system: petroleum ether – 1-butanol – acetone – water (1 : 1 : 1 : 1, v/v), adjusted to pH 8.0 by the addition of a ammonium hydroxide. The solution was chromatographed on a cellulose column, which was prepared with a slurry of Whatman CF 11 cellulose in the lower phase of the solvent system. The elution of the everninomicin complex with the upper phase solvent was monitored by bioassay on agar plates (Difco Antibiotic Medium) seeded with *Staphylococcus aureus* (ATCC 5638 P).

A final separation of the purified everninomicin complex was achieved by ascending thin-layer chromatography on fluorescent silica gel plates (Mallinckrodt Silicar TLC 7GF) of 40 cm length. The thickness of the layer was 0.5 mm. The solvent system employed was benzene – acetone (3 : 2, v/v). Four major components were extracted from the chromatographic zones with methanol after their detection in ultraviolet light. The four components were designated as E1, E2, E3 and E4, exhibiting in the above chromatographic system Rf values of 0.40, 0.32, 0.25 and 0.18, respectively. Component E1 seems to be identical with everninomicin D. It was the major component of the everninomicin complex.

Degradation of the everninomicins:

A degradation procedure similar to that for curamycin described by GALMARINI and DEULOFEU[6] was carried out. Sufficient unlabelled carrier antibiotic was added to the radioactive separated components to produce a final weight of 6~8 mg of each component. The antibiotic components were dissolved in 0.5 ml methanol to which 8 ml of 0.1 N hydrochloric acid was subsequently added, and the mixture was refluxed for 45 minutes. After cooling four extractions were made with 8 ml portions of methylene chloride. After backwashing once with 4 ml of 0.1 N hydrochloric acid the pooled extracts were dried with anhydrous magnesium sulfate, and the methylenechloride solvent was removed by evaporation *in vacuo*. An aliquot of the residue was subjected to alkaline hydrolysis in 5 ml 0.1 N sodium hydroxide for 12 hours at 37°C. After acidifying with 0.1 N hydrochloric acid to pH 2.0, four extractions were made with 8 ml portions of diethyl ether. The pooled diethyl ether extracts were dried with anhydrous magnesium sulfate, and the solvent was removed by evaporation *in vacuo*. A Zeisel degradation as described by BENTLEY and LAVATE[7] was run with an aliquot of the diethyl ether exract of component E1 after alkaline hydrolysis.

The aqueous solutions remaining after the methylene chloride and diethyl ether extractions were used for the determination of the radioactivity of the residual carbohydrate moieties.

Carbon-14 labelled precursors:

In this study five C-14 labelled precursors were employed. All were obtained from New England Nuclear Corporation. To obtain sterile dilute solutions the content of each ampoule was transferred into a screwcapped test-tube, the final volume adjusted to 10 ml with distilled water and the resultant solution autoclaved for 15 minutes at 121°C.

A comparison of the incorporation at different points of the fermentation was made

employing acetate-2-C-14, diethyl malonate-2-C-14, and D-glucose-uniformly labelled C-14. Two procedures were used for the addition of precursors: in procedure A, 20 μc per flask were added after 36 hours of fermentation; procedure B, 5 μc were added after 48 hours, 10 μc after 72 hours, and 5 μc after 96 hours. The antibiotic mixture was isolated after 114 hours of fermentation. Two precursors, methionine-methyl C-14 and malonic acid-2-C-14 were only used with procedure B.

The radioactivity of intact everninomicin components in methanol, of methylene chloride-extractable matter after acid hydrolysis, of diethyl ether extractable matter after alkaline hydrolysis, and of the residual carbohydrate moieties in neutralized aqueous solutions were measured by liquid scintillation counting. In the measurement of organic solutions the solvent of the sample was removed by evaporation *in vacuo* in the counting vial, whereafter a scintillation solution, containing 3 g PPO and 150 mg dimethyl-POPOP per liter toluene, was added. BRAY's[8] scintillant was used to count 0.5 ml aliquots of the aqueous solutions. Samples were routinely checked for quenching and corrected where necessary. All measurements were performed with a Packard Tricarb Liquid Scintillation Spectrometer (model 3003).

Results

A typical production curve of everninomicin complex in the fermentation broth is illustrated in Fig. 1. Production commences after about 24 hours, rises steeply until 96 hours, and then comes quickly to a halt. It is apparent from the results that the time of the addition of radioactive precursors to the fermentation broth is critical for the level of incorporation of the particular precursor into the antibiotics. The incorporation of acetate into the intact everninomicin is very low, following the single addition at an early point of antibiotic production (procedure A), while the same total amount of precursor added in three portions during later points of the fermentation gives a considerable increase in specific activity (Table 1). Diethyl malonate on the other hand, is better incorporated by the single early addition. Nevertheless, the incorporation of diethyl malonate according to procedure B is greater than that of acetate. The incorporation of glucose is similar in both

Fig. 1

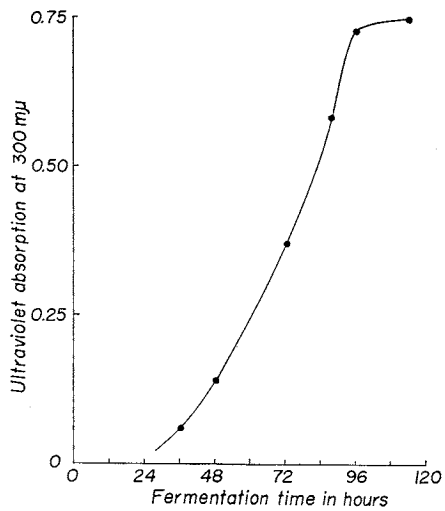


Table 1. Incorporation of radioactivity from C-14 labelled substrates into four everninomicins by *Micromonospora carbonacea* var. *aurantiaca*.

Everninomicins	Radioactive substrates, and specific activities in dpm/mg×10³						
	Procedure A			Procedure B			
	A1*	M2	GUL	A2	M2	GUL	MA2
E1	5.1	47.7	18.7	16.5	21.5	18.6	2.9
E2	4.0	61.9	20.0	27.0	34.5	21.2	3.2
E3	4.6	54.6	17.4	22.8	32.3	21.2	—**
E4	4.8	60.0	20.1	25.5	30.5	17.8	—

The following abbreviations are used for the radioactive substrates:

* A 2, acetate-2-C-14; M 2, diethyl malonate-2-C-14; GUL, D-glucose-uniformly labelled C-14; MA 2, malonic acid-2-C-14.

** not determined.

Table 2. C-14 Distribution in the four everninomicins

Evernino-micin	Substrate	Procedure	Percentages of total radioactivity		
			Aromatic acid ester	Aromatic acid	Carbohydrate fractions
E1	Glucose-UL-C-14	B	26.7	14.5	77.1
E2		A	17.3	8.2	87.6
E2		B	23.5	12.3	78.2
E3		B	24.5	13.7	82.3
E4		B	24.5	14.2	77.7
E1	Diethyl malonate-2-C-14	B	91.6	91.0	7.3
E2		A	91.2	—*	—
E2		B	91.2	90.8	5.1
E3		A	91.2	—	—
E3		B	90.4	88.4	8.6
E4		B	91.2	89.5	7.6
E1	Acetate-2-C-14	B	89.5	86.5	—
E2		B	90.5	86.7	7.4
E3		B	89.7	85.2	10.5
E4		B	91.0	88.0	7.4
E1	Methionine methyl-C-14	B	28.1	18.8**	81.8

* not determined.
** 69.2% of the radioactivity incorporated in the aromatic moiety was found in the methoxy group.

procedures. Some incorporation was also obtained from malonic acid.

Acid and subsequent alkaline hydrolysis of the four everninomicins yielded according to chromatographic studies an identical aromatic moiety, comparable with the degradation product dichloroisoeverninic acid, obtained from a reference sample of everninomicin D. Acetate and diethyl malonate ester were principally incorporated into the dichloroisoeverninic acid moiey (Table 2), whereas relatively poor incorporations were observed in the residual carbohydrate fractions. The methylene chloride extracts of the acid hydrolysates of the four everninomicins contained an aromatic moiety apparently identical by thin-layer chromatographic behavior to everninocin, an aromatic ester originally isolated from everninomicins D and B. The incorporation of radioactivity into this moiety was higher than that in the aromatic dichloroisoeverninic acid itself. The radioactivity from glucose in both procedures A and B is better incorporated into the carbohydrate portion of the everninomicin molecule than into the dichloroisoeverninic acid moiety. Nevertheless, incorporation of glucose into the aromatic acid was more efficient in procedure B than in procedure A.

Methionine was added to the fermentation broth according to procedure B. With methionine as a precursor, most of the incorporation occurred in the carbohydrate portion of the everninomicin molecule. The dichloroisoeverninic acid moiety was isolated from component E1 only. A Zeisel degradation revealed that most of the activity of the aromatic acid was located in the methoxy group.

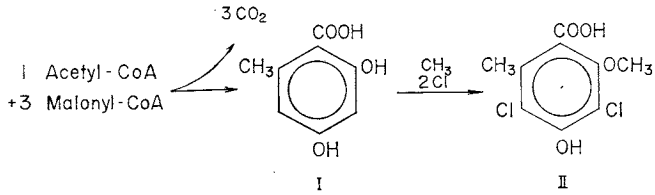
Discussion

Polyacetic aromatic compounds have long been known as secondary metabolites of lichens and molds. Their biosynthetic pathway has been outlined by BIRCH and DONOVAN[9] as a "head to tail" acetate condensation. GATENBECK and MOSBACH[10,11] demonstrated the

same pathway for orsellinic acid. In extending the hypothesis of LYNEN[12] that malonyl-Co-A might be the real condensing agent in the "polyacetic" series, BENTLEY and KEIL[13] and MOSBACH[14] demonstrated that acetate and malonate units in a one to three ratio are required for the synthesis of orsellinic acid.

Our studies on the four everninomicins, produced by an actinomycete, indicate that the aromatic moiety common to these antibiotics, dichloroisoeverninic acid (II), is synthesized via the polyketide pathway as a derivative of orsellinic acid (I).

Fig. 2.



Malonate was incorporated into the antibiotics more effectively than acetate or glucose. It is of interest to note that when acetate was added at an early point during the everninomicin production (procedure A), the incorporation was extremely low. Diethyl malonate, however, was utilized here even more readily than at a later state of the fermentation (procedure B). Probably the "pool" of acetate at that early time is fairly large, thereby diluting considerably the added radioactive acetate. Malonyl-CoA, on the other hand, seems not yet to be formed by the usual CO_2 fixation reaction from acetyl-CoA to the extent it is formed later.

Free malonic acid sometimes cannot enter the cells of microorganisms. With malonic acid as precursor the appearance of activity in the everninomicins indicates that a certain amount of the acid might be used by the organism.

The results of the experiment with methionine methyl-C-14 prove the incorporation of that C_1-unit into the methoxy group of dichloroisoeverninic acid. The high activity in the carbohydrate fraction of E1 could be attributed to the methylation of some of the sugars. A recent description of evernitrose[15], a sugar present in everninomicin D, reports the presence of a methoxy and secondary and tertiary methyl groups in this moiety. Another sugar found in everninomicin D is everninose which contains an O-methyl group[2]. Studies on mycarose, cladinose and desosamine[16] showed the incorporation of the methyl group from methionine into C-methyl, O-methyl and N-methyl groups. Of special interest is observation of CORCORAN[17] that the total activity in cladinose as derived from methyl-labelled methionine was equally distributed between the C-methyl and O-methyl groups both on the C_3 atom.

The carbohydrate fractions of the everninomicins contained the major part of the incorporated glucose. There was also some low activity from diethyl malonate and acetate in these fractions, which may be due to a resynthesis to glucose as proposed by GRISEBACH *et al.*[18] The appearance of glucose in the aromatic acid can be expected as a result of dissimilations by the EMBDEN-MEYERHOF pathway leading to pyruvate and further to acetate.

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