STUDIES ON CANDICIDIN BIOGENESIS

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The biosynthesis of candicidin has been studied with various carbon-14 labeled precursors. The aromatic moiety of candicidin is evidently synthesized from glucose via shikimate to *p*-aminobenzoic acid, which is then incorporated into candicidin. The synthesis of candicidin was partially inhibited by the presence of L-phenylalanine, L-tryptophane and L-tyrosine in the growing culture. The implication of this inhibitory effect in relation to *p*-aminobenzoic acid biosynthesis by the organism is briefly discussed.

Candicidin is a heptaene macrolide antifungal antibiotic produced by *Streptomyces griseus* IMRU 3570 originally described by LECHEVALIER *et al*[1]. The chemical structure of this antibiotic has not yet been elucidated. Structural studies[2] of the antibiotic revealed that candicidin contains in addition to the polyene macrolide lactone ring an amino sugar, mycosamine, and an aromatic moiety. The latter was obtained from candicidin by alkaline retro-aldolization and isolated and characterized as *p*-aminoacetophenone[2]. From the biosynthetic point of view, the formation of candicidin is of particular interest because of the complex chemical composition of the antibiotic. The polyene aglycone lactone ring could be visualized as a result of polyketide synthesis biogenetically derived from acetate and probably propionate, as in the case of nystatin[3]. There is evidence to indicate that mycosamine is derived from D-glucose metabolism without changing the carbon skeleton in the sugar[4]. The aromatic moiety of candicidin could be derived biogenetically from the aromatization of the polyketide acetate condensation product which is to become the aglycone lactone ring of candicidin or it might result from the shikimic acid pathway. It was our intention in the work described here to determine the exact pathway of biogenesis of the aromatic moiety of candicidin.

Materials and Methods

Production of candicidin:

The organism *Streptomyces griseus* IMRU 3570 was used. The fermentation medium contained (in grams per liter); Cerelose (technical glucose, Corn Products Co., Argo, Ill.), 55; Soyapeptone (starch-free, DeLamar and Son, Inc., Chicago, Ill.), 25; magnesium carbonate (approx. $4MgCO_3 \cdot Mg(OH)_2 \cdot 4H_2O$, Mallinckrodt Chemical Works, St. Louis, Mo.), 1. The medium was adjusted to pH 7.6 with NaOH before sterilization. Triple baffled Erlenmeyer flasks of 300 ml volume (Bellco Glass, Inc., Vineland, N. J.) containing 50 ml of the medium were employed throughout the entire series of experiments. With butanol extractions the concentrations of candicidin in the culture were determined spec-

trophotometrically at 383 mμ and calculated on the basis of optical density of 1.0=10 μg of candicidin/ml.

Preparation of carbon-14 labeled candicidin:

Various ^{14}C-labeled precursors were used for the study. Shikimic acid-(GL)-^{14}C, sodium acetate-1-^{14}C, sodium acetate-2-^{14}C, methionine-methyl-^{14}C and D-glucose-(UL)-^{14}C were purchased from New England Nuclear, Boston, Mass. *p*-Aminobenzoic acid-(ring, UL)-^{14}C and *p*-aminobenzoic acid-7-^{14}C were obtained from Mallinckrodt/Nuclear, St. Louis, Mo.). All of the radioactive compounds were dissolved in 95% ethanol without adding carrier. Labeled candicidin was prepared by adding to the growing culture the ethanol solutions of ^{14}C-labeled precursors (0.4 ml each time). They were incubated at 34°C in a Gyrotory incubator-shaker (model G-25, New Brunswick Scientific Co., Inc., New Brunswick, N. J.) connected with CO_2 traps for the exhaust gases. The radioactive materials in ethanol were not sterilized before addition. None of these ethanol solutions was found to affect the yield of candicidin.

Extraction and purification of candicidin:

At the end of the fermentation or 5 days after inoculation, the culture broth was acidified to pH 5.0 with 1 N H_2SO_4 and filtered with the aid of 6% (w/v) of Hyflo Super-Cel. The Supercel-mycelium cake was washed twice with distilled water adjusted to pH 5.0 with H_2SO_4, then extracted three times with a mixture of chloroform and 95% ethanol (1 : 1, v/v). To the chloroform-ethanol extract was added an equal volume of acetone with shaking. Candicidin precipitated and was collected by centrifugation. The product was washed once with acetone, then with petroleum ether, and finally twice with diethyl ether.

Further purification of candicidin was done by thin-layer chromatography (TLC) using the following solvent systems:

#1 Chloroform - 95% ethanol - water (50 : 50 : 8, v/v/v)

#2 Chloroform - methanol - water (50 : 50 : 4, v/v/v)

Degradation of candicidin and isolation of *p*-aminoacetophenone:

To about 5 mg of the purified candicidin-^{14}C was added 1 ml of 10% aqueous sodium hydroxide solution. The mixture was heated on a steam bath for 10 minutes. After cooling, the mixture was extracted twice with equal volumes of chloroform. The chloroform was then washed twice with distilled water. The extract was subjected to thin-layer chromatography with fluorescent silica gel plates (Mallinckrodt, Silicar TLC 7 GF) using benzene - acetone (4 : 1, v/v) as the solvent system. Here, *p*-aminoacetophenone, which exhibits an Rf value of 0.49, was detected under UV light and was identified by co-chromatography with an authentic sample and by spectral analysis. For the determination of the specific activity of the degradation product, the compound was eluted from TLC plates with chloroform and its quantity determined spectrophotometrically.

Aromatic amino acids and candicidin biosynthesis:

Two flasks were inoculated simultaneously and grown at 34°C in an incubator-shaker as described earlier. After 40 hours of fermentation, a mixture of L-tyrosine, L-phenylalanine and L-tryptophane was added to one of the flasks so that the final concentration of each of the amino acids added to the flask was 5×10^{-3} M. Sixty minutes later, 5.3 μc of *p*-aminobenzoic acid-(ring UL)-^{14}C (PABA-^{14}C) was added to both flasks, and aliquots of culture broth from the flasks were taken for the analysis of PABA-^{14}C incorporation into candicidin. Radioactivity in candicidin was determined as follows: One ml sample of broth was adjusted to pH 4.5 with 0.1 N H_2SO_4 in the presence of 0.1 g of Hyflo Super-Cel. The resulting mycelial cake after centrifugation was washed twice with distilled water, adjusted to pH 4.0 with H_2SO_4, then extracted with 1 ml of chloroform - 95% ethanol (1 : 1, v/v). An aliquot of the chloroform-ethanol extract was then chromatographed on thin-layer plates and developed in solvent system #1.

A yellow spot on the thin-layer plate corresponding to a major component of

candicidin was scraped from the plate, extracted with solvent system #1, and counted for radioactivity.

Radioactivity measurements:

For the determination of radioactivity in candicidin-^{14}C, the candicidin was eluted from TLC plates with chloroform - 95% ethanol - water (50 : 50 : 8, v/v/v) and counted for activity by employing a liquid scintillation spectrometer (Packard Instrument Co., Inc.). Aquasol (New England Nuclear, Boston, Mass.) was used in the counting solution. Color quenching due to candicidin was corrected by the dilution method. Radioactivity in ^{14}C-labeled *p*-aminoacetophenone was similarly determined.

Results and Discussion

Candicidin Fermentation and Recovery

The time course of candicidin production in shake flasks is shown in Fig. 1. The rate of antibiotic synthesis was proportional to the rate of sugar utilization. The yield of candicidin after 5 days was about 1.5 mg/ml adjusted to initial volume. At least three candicidin components could be separated on TLC plates with solvent system #1. The Rf values at 28°C for these components were: 0.34, 0.74 and 0.95 respectively. The first component of Rf 0.34 was predominant in the whole fresh broth and was isolated by the procedures outlined. The candicidin recovery was about 70% of the original antibiotic titer in broth. The purity of the product was about 70% based on spectral analysis. Further purification of this product was achieved by thin-layer chromatography with solvent systems #1 and #2. The major candicidin component so prepared exhibited an $E^{1\%}_{1cm}$ of about 850 at 383 mμ. These purification procedures were used for the precursor incorporation studies. The purified carbon-14 labeled candicidin appeared to be radioactively pure as was indicated by the radiochromatographic analysis employing the Packard Radiochromatogram Scanner (Model 385). Other minor candicidin components were not used for these incorporation studies.

The Incorporation of Precursors into Candicidin

The effect of time of precursor addition on the efficiency of its incorporation into candicidin was determined by the use of the precursor, sodium acetate-1-^{14}C. Inspection of the kinetics of candicidin formation in shake flasks (Fig. 1) indicates that the onset of antibiotic production was about 10 hours after inoculation. Up to this period, the growth rate of mycelium appeared to be maximum. Thus a higher efficiency of incorporation was expected to occur at around 20 hours of fermentation. At this time, the antibiotic synthesis was at its maximum rate while mycelial growth began to slow down. It was found that there was practically no difference in efficiency of incorporation if the precursor was added anytime between 10 to

Fig. 1. Time course of candicidin fermentation in shake flasks.

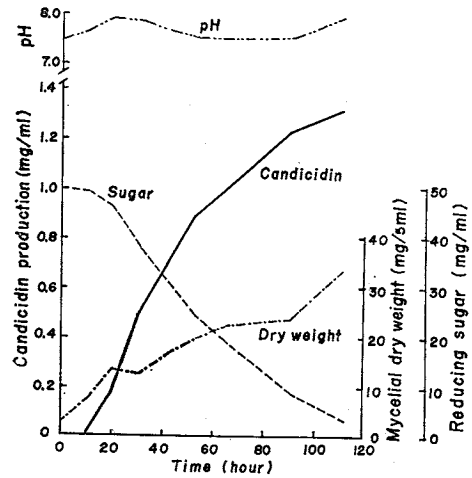


Table 1. Incorporation of various ^{14}C-precursors into candicidin

Precursor*	Amounts of precursor added	Specific activity of candicidin (cpm/μmole)	Efficiency of incorporation (%)	Specific activity of *p*-aminoacetophenone (cpm/μmole)	Percent radioactivity in aromatic moiety
Na-acetate-1-^{14}C	300 μc	1.95×10^5	2.17	1×10^3	0.5
Na-acetate-2-^{14}C	90 μc	9.6×10^4	3.67	3.6×10^3	3.8
Methionine-methyl-^{14}C	104.6 μc	no significant activity	—	—	—
Glucose-(UL)-^{14}C	50 μc	2.72×10^4	2.14	3.66×10^3	13.5
Shikimic acid-(GL)-^{14}C	50 μc	6.6×10^3	1.02	6.66×10^3	101.0
p-Aminobenzoic acid-(ring UL)-^{14}C	98.5 μc	1.06×10^6	33.3	1.04×10^6	98.0
p-Aminobenzoic acid-7-^{14}C	8 μc	7.78×10^4	40.0	7.66×10^4	98.5

* Precursors were added as 95% ethanolic solutions first at the time of inoculation followed by two more additions at 24-hour intervals.

50 hours after inoculation. Cumulative addition of the precursor during this period increased the specific activity of the labeled product but did not improve the efficiency of incorporation. The efficiency of carbon-14 incorporation into candicidin depended on the precursors used (Table 1).

As shown in Table 1, L-methionine-methyl-^{14}C failed to incorporate into candicidin. This indicates that none of the methyl or methylene groups in the candicidin molecule is derived from methionine. It also suggests that the methyl side-chain, if there is any, in candicidin aglycone could be expected to be derived from propionate as in the case of nystatin[9]. The precursor, *p*-aminobenzoic acid (PABA) was more efficiently incorporated than any other precursors tested. More than 30% of the radioactivity added to the flask was found in candicidin-^{14}C. The table also shows the percent of radioactivity recovered from the aromatic moiety of candicidin-^{14}C labeled with different precursors. About 13.5% of the radioactivity in candicidin-^{14}C was found in the aromatic moiety of the molecule when glucose-(UL)-^{14}C was used as the precursor. If the aromatic moiety of the antibiotic were synthesized via the polyketide pathway, it would be expected that about 14%* of the activity would be recovered in the aromatic group when acetate-^{14}C is used as a precursor. Actually, less than 4 percent was recovered from the candicidin-^{14}C produced with either of the two acetate-^{14}C precursors used. The percentage of acetate-^{14}C incorporated into the aromatic moiety of candicidin was lower than that when glucose-(UL)-^{14}C was used as a precursor. The fact that 100% of the radioactivity in candicidin was recovered from *p*-aminoacetophenone when shikimate-^{14}C and PABA-^{14}C were used as precursors clearly indicates that the aromatic moiety in candicidin is biogenetically derived from glucose via the shikimic acid pathway. The precursor, *p*-aminobenzoic acid, appears to be a direct intermediate of the pathway and is used directly as a building block of the aromatic moiety of candicidin. All carbon atoms in the PABA molecule are exclusively incorporated with great efficiency into candicidin as was revealed when PABA-7-^{14}C was employed as a precursor.

* Carbons in sugar moiety, mycosamine, were not included for the calculation, since in the presence of an excess amount of glucose in the culture, it was assumed that the amino sugar could not be synthesized from acetate.

Fig. 2. Inhibition of candicidin production by aromatic amino acids.

Fermentation conditions are described in the text. A mixture of L-tyrosine, L-tryptophane and L-phenylalanine was added to the growing culture at zero, 21 and 43 hours after inoculation to give a final concentration of 5×10^{-3} M in the culture. The antibiotic concentration was assayed spectrophotometrically.

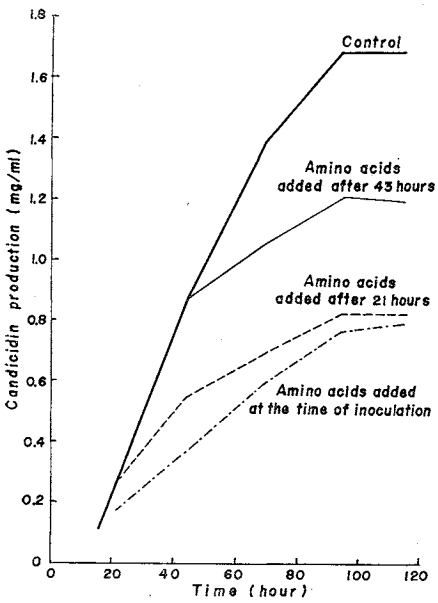
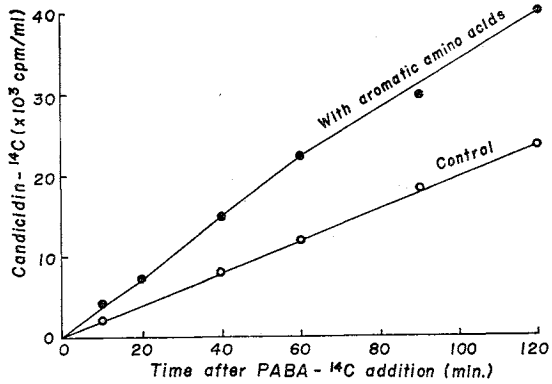


Fig. 3. Time course of *p*-aminobenzoic acid-^{14}C incorporation into candicidin.

Details of the experiment are described in the text. The candicidin concentration at the time of PABA-^{14}C addition (*i.e.*, after 40 hours of fermentation) was about 800 μg/ml. The rate of synthesis during this period was about 20 μg/ml/hour.



Effect of Aromatic Amino Acids on the Synthesis of Candicidin

The synthesis of candicidin was partially inhibited by the presence of L-tryptophane, L-tyrosine and L-phenylalanine in the culture (Fig. 2). In their presence at 5 millimolar concentrations, there was about 50 % inhibition. At this concentration, little effect of these amino acids on the mycelial growth was observed. Thus it appeared that the inhibition was not due to the adverse effect of the amino acids on the primary metabolism of the organism, but was a selective inhibition of a metabolic pathway leading to the synthesis of candicidin. PABA at concentrations higher than 10^{-2} M was also inhibitory to candicidin biosynthesis, although at 10^{-3} M it somewhat stimulated the rate of antibiotic production. The specific inhibition of candicidin biosynthesis by aromatic amino acids was more clearly demonstrated by the kinetic study of PABA-^{14}C incorporation into candicidin. The incorporation took place rapidly without lag in the actively growing culture as shown in Fig. 3.

The rate of PABA-^{14}C incorporation was stimulated by the presence of 5×10^{-3} M aromatic amino acids in the culture. The rate was about 50 % higher initially, then slightly lower after 60 minutes. Furthermore, the specific activity of the labeled candicidin produced was about 35 % higher than that of the control at end of fermentation, although the final yield of antibiotic after 120 hours was reduced about 50 %. The faster rate of PABA-^{14}C incorporation into candicidin indicated that PABA biosynthesis in the cell was retarded by the presence of an excess amount of aromatic amino acids in the culture. Therefore, the exogenous supply of PABA was more efficiently utilized by the cell to make candicidin.

The studies mentioned above indicate that candicidin biosynthesis is apparently affected indirectly by the feed-back control mechanism of aromatic amino acid biosyn-

thesis. This implies that PABA synthesis is subjected to the end product inhibition exerted by the presence of an excess amount of aromatic amino acids in the cell. The results also suggest that PABA is probably synthesized in the *Actinomyces* through the shikimic acid pathway by the enzyme system in common with that used in the formation of the aromatic amino acids without specific isoenzymes of its own[5]. Isolation of enzymes of the shikimic acid pathway in the organism or genetic analysis of the organism should clarify this interesting observation.

The finding that PABA is an intermediary metabolite of candicidin biosynthesis suggests that the system could be used for the study of the mechanism of PABA biosynthesis, the details of which are as yet not fully understood[5]. In producing a large quantity of candicidin, the organism undoubtedly has to have a very active enzyme system for the production of PABA. Advantage is being taken by use of this system to study the formation of PABA from chorismic acid with cell-free systems, and to investigate the control mechanism of PABA metabolism in the organism. These studies should contribute further to our knowledge of the regulation of candicidin biosynthesis.

By taking advantage of the more efficient incorporation of exogenous PABA resulting from the inhibition of synthesis of PABA by added aromatic amino acids, labeled candicidin having very high specific activity has been produced. Further use of the precursor, *p*-aminobenzoic acid-7-^{14}C has resulted in the production of a candicidin labeled specifically in a single carbon atom.

Application of such biosynthetic methodology has enabled the production of candicidin particularly suited for a variety of physical, chemical and biological studies.

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