INCORPORATION OF ^{14}C-LABELED COMPOUNDS INTO SINEFUNGIN (A9145), A NUCLEOSIDE ANTIFUNGAL ANTIBIOTIC

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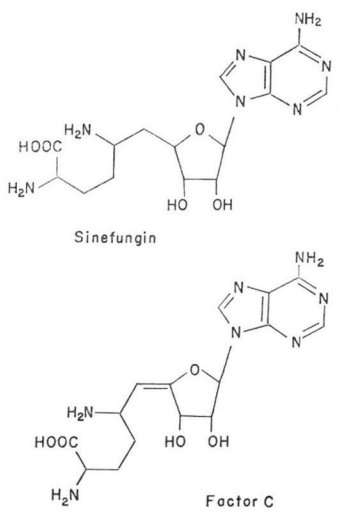
Streptomyces griseolus produces a complex of antifungal nucleoside antibiotics that contain an ornithine residue linked to the ribose moiety of adenosine. ^{14}C-Labeled compounds were added to cultures of *S. griseolus* ($\approx$0.5 μCi/ml culture broth) and the amount of label incorporated into the two major antifungal components (sinefungin and factor C) was measured. Substantial incorporation (16$\sim$52%) was obtained with adenosine [8-^{14}C], ATP [^{14}C(u)], adenine [8-^{14}C], L-ornithine [^{14}C (u)], and DL-citrulline [5-^{14}C]. Glycine, glucose, L-arginine, and acetate were incorporated to the extent of 1.7$\sim$4.7%. Studies were conducted on the fermentation time course and on the time dependence of label incorporation in order to optimize the incorporation of labeled adenine into sinefungin. Adenine [8-^{14}C] incorporation and sinefungin specific activity were highest 48 hours after label addition and both declined during subsequent incubation. As much as 43% of the labeled adenine was incorporated into the antibiotic and sinefungin was produced with a specific activity of 24.8 μCi/mg. The labeling experiments suggest that a preformed adenine derivative (*e.g.*, an adenine nucleotide) and ornithine (or a closely related metabolite) are direct biosynthetic precursors of sinefungin.

Streptomyces griseolus produces a mixture of nucleoside antifungal antibiotics. These compounds are produced as part of a complex of antifungal factors that contain adenosine as the nucleoside component. The two major antibiotics (sinefungin and factor C) contain an ornithine residue linked *via* a carbon-carbon bond to the adenosine moiety of the molecule (Fig. 1).[1] Sinefungin possesses more antifungal activity than factor C and accounts for about 70% of the total antibiotic complex. Sinefungin is as active as amphotericin B in treating experimental *Candida albicans* infections in mice.[2] Factor C is less active against *C. albicans* and displays a much lower LD_{50} in mice.

Fig. 1. Structures of the major antifungal antibiotics produced by *Streptomyces griseolus*.



BOECK *et al.*[3] studied the fermentation conditions for the production of antifungal compounds by *S. griseolus*. The fermentation medium contained soybean grits, cottonseed oil, and low concentrations of $CaCO_3$, Na_2HPO_4, and $CoCl_2 \cdot 6H_2O$.[3] Antibiotic synthesis in this medium was stimulated by the incorporation of one of several amino acids or nucleic acid-related compounds. The most effective additions were tyrosine, ornithine, citrulline, adenine, adenosine, and cytidine.

Our studies were initiated to identify precursors of the antifungal compounds by measuring the incorporation of various ^{14}C-labeled substrates into sinefungin and factor C. A second

objective was to maximize the incorporation of a ^{14}C-labeled precursor into sinefungin in order to produce labeled antibiotic with high specific activity for pharmacological studies.

Materials and Methods

Microorganism:

Streptomyces griseolus NRRL 3739 was obtained from the Lilly Research Laboratories Culture Collection. The culture was preserved by the methods described by BOECK *et al.*[3]

Cultivation:

A standardized inoculum was prepared by growing the microorganism in a medium consisting of: 1% dextrose, 3% dextrin 700, 1% "grit extract," 0.5% edible molasses, and 0.5% Amber BYF 300 in tap H_2O (pH adjusted to 7.2 with 1 N NaOH). Grit extract was prepared by suspending 70 g of Nutrisoy grits in 400 ml deionized H_2O. The suspension was heated at 121°C for 20 minutes and the resulting supernatant was collected by vacuum filtration. The filtrate was lyophilized and 14 g of water-soluble grit extract were obtained.

The medium was dispensed in 200-ml portions into 1-liter Erlenmeyer flasks, stoppered with a cotton plug, and autoclaved at 121°C for 30 minutes. The flasks were inoculated with a mycelia/spore suspension washed from agar slants. After 24 hours of incubation at 30°C on a rotary shaker (250 rpm, 2″ stroke), the contents of the flasks were pooled and centrifuged at 7,000×*g* to recover the biomass. The sedimented cell pellet was washed with tap H_2O and resuspended to 1/3 the original volume with a 20% glycerol in H_2O solution. The latter suspension was transferred in 2-ml portions into sterile 100×13 mm Kimax tubes and stored in the vapor phase of liquid nitrogen.

Fermentations were conducted in cotton-stoppered Erlenmeyer flasks containing the following medium: 4% Proflo Oil, 1.5% nutrisoy grits, 0.00001% $CoCl_2 \cdot 6H_2O$, 0.54% L-tyrosine, 0.2% $CaCO_3$, and 0.02% Na_2HPO_4 in deionized H_2O (pH 7.4). The medium was sterilized at 121°C for 30 minutes and inoculated with liquid nitrogen cell suspensions using 0.1 ml of suspension per 10 ml of medium. The inoculated flasks were incubated on a rotary shaker (250 rpm, 2″ stroke) at 30°C for 7~9 days. Initial labeling studies were performed in 50-ml flasks containing 10 ml of medium. Studies on maximizing adenine incorporation were performed in either 250 ml or 2 liter wide-mouth Erlenmeyer flasks containing 50 ml or 400 ml of medium, respectively. The larger flasks were employed to minimize volume depletion due to sampling during the fermentation.

Antibiotic Assays:

The amounts of sinefungin in the fermentation medium and in column chromatography eluates were determined by turbidimetric assay using *Saccharomyces pastorianus*.[3] The assays were performed on the culture supernatants because <3% of the total activity was associated with the cell biomass.[3] Factor C displayed about 1/100 the activity of sinefungin against *S. pastorianus*. Thus, bioassays on mixtures containing sinefungin and factor C reflect almost exclusively the sinefungin content.

Factor C was assayed by thin-layer scanning densitometry. Samples (4 ml) of fermentation broth were applied to a 2-ml bed volume of Dowex 50W×2 ion-exchange resin in the NH_4^+ cycle (Bio-Rad Laboratories, Richmond, CA). The columns were washed with 8 ml H_2O and the antifungal factors were eluted with 0.5 N NH_4OH. Sinefungin and factor C, which eluted in the same fraction, were concentrated about 4-fold and partially purified by this chromatography. Fractions containing sinefungin and factor C were applied to silica gel 60 F 254 (E. Merck, Darmstadt, Germany) thin-layer plates. The plates were developed with chloroform - methanol - 28% NH_4OH (1 : 3 : 1) to separate the factors. The plates were then scanned by reflectance spectroscopy at 266 nm with a Schoeffel SD-3000-2 Scanning Thin-Layer Densitometer (Schoeffel Instrument Corp., Westwood, NJ). Standard curves were constructed by plotting μg of antibiotic per lane against the peak area recorded by the densitometer. Linear relationships were obtained from 5 μg to 20 μg. Since both antifungal components have essentially identical extinction coefficients ($\varepsilon_{260}^{1cm}=15,100$),[4] similar standard curves were obtained for the two factors. Calculation of the peak area ratio of factor C to sinefungin provided the relative amounts of the two compounds in the fermentation. The ratio of factor C to

sinefungin as measured by thin-layer scanning densitometry was multiplied by the concentration of sinefungin determined by turbidometric biological assay to calculate the absolute concentration of factor C in the fermentation broths and column eluates, *i.e.*, factor C concentration=(sinefungin concentration) ((factor C/sinefungin) ratio). Other factors were also produced in the fermentation but they accounted for less than 10% of the total antibiotic complex and were not considered in the calculations.

DNA Measurement:

The growth rate of the microorganism was determined by measuring changes in the DNA content of the culture. This method was used because the insoluble soybean grits in the medium interferred with measurements of dry cell mass or packed cell volume. DNA was measured by the diphenylamine method of BURTON.[5] The cell pellets from the 4-ml fermentation samples used for the antibiotic assays were washed with tap H_2O and extracted with 8 ml of cold 0.25 N perchloric acid (PCA). The extracts were discarded and the pellets were resuspended in 4 ml of 0.5 N PCA. The suspensions were heated at 90°C for 30 minutes and filtered by gravity through No. 560–15 cm Schleicher and Schuell filter paper to obtain clear filtrates. The cooled filtrates (1 ml) were mixed with 2 ml of diphenylamine reagent[5] and reacted for 16 hours at 30°C to develop a color which was measured at 600 nm on a Beckman DB Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). A standard curve was prepared using calf thymus DNA (Sigma Chemical Co., St. Louis, MO). The curve was linear from 10 μg DNA/ml to 160 μg DNA/ml.

^{14}C-Incorporation Studies:

^{14}C-Labeled compounds were added to the fermentation media 48 hours after inoculation. Immediately after addition and mixing, 10 μl of the culture broth were added to 10 ml of counting fluid and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375 (Packard Instrument Company, Inc., Downers Grove, IL). Five to six days after a labeled compound was added to the culture, the antifungal compounds were recovered from the culture supernatant by the ion-exchange chromatography procedure described previously. The column eluates were assayed for sinefungin content by turbidometric bioassay and the radioactivity was determined by scintillation counting as described above. The column eluates were also subjected to thin-layer chromatography and scanning densitometry to determine factor C concentration. The specific radioactivities of factor C and sinefungin were calculated from the scintillation counts of the column eluates and from the amounts of factor C and sinefungin in the eluate as determined by bioassay and scanning densitometry. Thin-layer chromatograms of the column eluates were scanned for ^{14}C with a Packard Radiochromatogram Scanner Model 7201. These scans established that factor C and sinefungin accounted for at least 90% of the radioactivity in the eluates. The remaining 10% or less of radioactivity detected in the scans was associated with minor factors produced in the fermentation. These minor factors were not considered in the calculations of percent incorporation and specific activity.

Chemicals:

All ^{14}C-labeled compounds were purchased from New England Nuclear (Boston, MA) except for DL-citrulline [5-^{14}C] which was purchased from Research Products International Corp. (Elk Grove Village, IL). Pure standards of factor C and sinefungin were obtained from Dr. R. NAGARAJAN of the Lilly Research Laboratories.

Results

Initial Labeling Studies

^{14}C-Labeled compounds were added to fermentation flasks 48 hours after inoculation. Approximately 5 μCi of radioactivity (based on manufacturer's specifications) were added to 10 ml of fermentation broth. Samples of broth were taken immediately after label addition and radioactivity was determined with a scintillation spectrometer. The results of these analyses are shown in Table 1. Cultures were incubated for 5~6 days in the presence of labeled substrate and then factor C and sine-

Table 1. Incorporation of ^{14}C into sinefungin and factor C by addition of ^{14}C-labeled compounds to *S. griseolus* cultures

^{14}C-Substrate	Initial radioactivity[a] (μCi/ml culture)	Total percent incorporation[b]	Percent incorporation into sinefungin[c]	Sinefungin specific activity[c] (μCi/mg)
1. Adenosine [8-^{14}C]	0.460	52.6	33.1	0.540
2. ATP [^{14}C(u)]	0.500	38.1	28.3	0.797
3. Adenine [8-^{14}C]	0.610	32.0	18.9	0.353
4. L-Ornithine [^{14}C(u)]	0.560	18.6	10.0	0.182
5. DL-Citrulline [5-^{14}C]	0.302	16.8	10.9	0.072
6. L-Glycine [^{14}C(u)]	0.549	4.7	2.7	0.047
7. Glucose [^{14}C(u)]	0.569	3.6	1.9	0.024
8. L-Arginine [^{14}C(u)]	0.563	2.0	1.4	0.030
9. Na acetate [^{14}C(u)]	0.464	1.7	0.97	0.009
10. L-Isoleucine [^{14}C(u)]	0.610	0	0	0
11. L-Citrulline [ureido-^{14}C]	0.580	0	0	0
12. L-Valine [^{14}C(u)]	0.547	0	0	0
13. Choline Cl [1, 2-^{14}C]	0.436	0	0	0
14. L-Glutamic Acid [^{14}C(u)]	0.416	0	0	0
15. L-Tyrosine [^{14}C(u)]	0.458	0	0	0
16. D-Ribose [1-^{14}C]	0.446	0	0	0

[a] Level of radioactivity measured by scintillation counting of broth samples immediately after label addition.
[b] Reflects incorporation into both factor C and sinefungin.
[c] Calculated by procedures described in the Methods section.

fungin were recovered by ion-exchange column chromatography. The column eluates were chromatographed on silica gel thin-layer plates to separate the antibiotics and the plates were scanned for radioactivity. The scans revealed that labeled antibiotic was produced from 9 of the 17 substrates tested. Small amounts of other labeled compounds were detected on some of the plates, but these compounds accounted for less than 10% of the total radioactivity. The 9 labeled substrates also were chromatographed and scanned. These scans demonstrated that the substrates and their impurities did not co-chromatograph with the antibiotic compounds.

Column eluates were assayed by scintillation counting, turbidimetric bioassay, and scanning thin-layer densitometry to determine percent incorporation and specific activity (Table 1). Highest incorporation, 52.6%, was achieved with adenosine [8-^{14}C]. Substantial incorporation was also obtained with ATP [^{14}C(u)], adenine [8-^{14}C], L-ornithine [^{14}C(u)], and DL-citrulline [5-^{14}C]. Correlation of radioactivity and densitometric scans of thin-layer plates indicated that the label was distributed between factor C and sinefungin in proportion to the amounts of each produced in the fermentation, *i.e.*, the specific activities of factor C and sinefungin were about equal.

Adenine Incorporation

The pH, factor content, and DNA content of the culture were measured at 24-hour intervals during the fermentation. A typical time course profile of these parameters is presented in Fig. 2. The same general pattern of changes was observed in other experiments, although 2~3 day differences occurred in the time at which factor C synthesis was initiated. The time course data suggest that

Fig. 2. Time course changes illustrating growth and antibiotic production by *Streptomyces griseolus*.

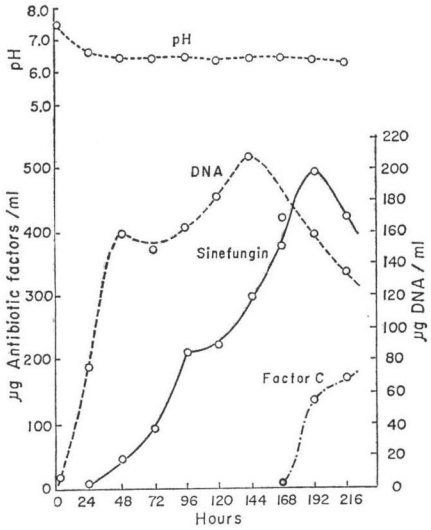
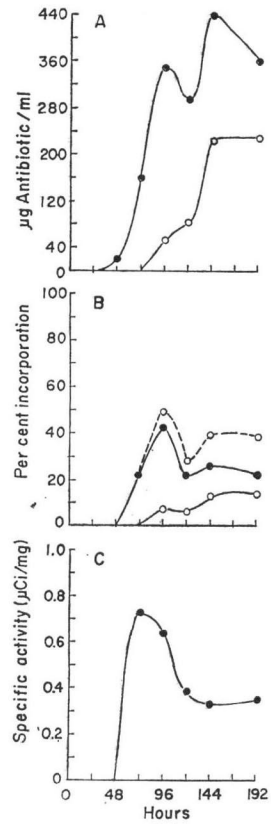


Fig. 3. Adenine-[8-^{14}C] incorporation into sinefungin and factor C as a function of fermentation time.

(A) Concentrations of factor C (○—○) and sinefungin (●—●).

(B) Percent incorporation of labeled adenine into sinefungin and factor C. Symbols: (●—●) sinefungin; (○—○) factor C; (○---○) factor C and sinefungin.

(C) Specific activity of sinefungin.



labeled substrates should be added to the culture about 48 hours after inoculation. Adding substrate at that time should minimize incorporation of the label into cell biomass since the period of rapid growth is over and the population density is within 20% of its maximum value (Fig. 2). The time course data also indicate that labeled sinefungin should be recovered early in the fermentation to limit the amount of label diverted to factor C synthesis.

Labeling studies with adenine [8-^{14}C] were performed to optimize ^{14}C incorporation into sinefungin (Fig. 3). The specific activity of sinefungin and percent incorporation of adenine into sinefungin and/or factor C were measured on fermentation samples taken at 24-hour intervals after adenine [8-^{14}C] addition. Specific activity of sinefungin was near its maximum 24~48 hours after adenine [8-^{14}C] addition (Fig. 3C). The percent incorporation of adenine [8-^{14}C] into sinefungin reached a maximum 48 hours after label addition and then declined (Fig. 3B). Factor C synthesis was initiated early in this fermentation but a relatively small amount of this factor was present when label incorporation into sinefungin was at its maximum (Figs. 3A and 3B).

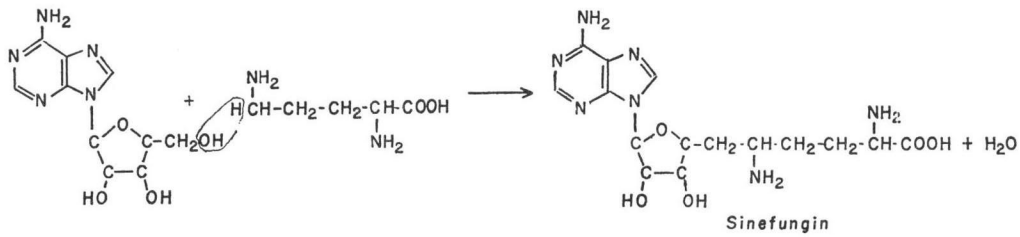
Sinefungin with a high specific activity was prepared by adding 1 mCi (2.56 mg) of adenine [8-^{14}C] to 50 ml of culture 48 hours after inoculation. Factor C and sinefungin were recovered 48 hours after label addition and specific activity and percent incorporation were determined. Approximately 25.1% of the label was incorporated into sinefungin and the specific activity of the antibiotic was 24.88 μCi/mg. Sinefungin was then purified to homogeneity by gradient ion-exchange chromatography and counted in a scintillation counter (R. HAMILL, unpublished data). The specific activity of the purified antibiotic,

24.2 μCi/mg, agreed closely with the activity measured *via* microbiological assays and scanning densitometry.

Discussion

The preceding studies demonstrate the advantages of measuring changes in a fermentation time course as part of a study to maximize the incorporation of a labeled compound into microbial metabolites. The DNA measurements and factor ratio determinations specified the optimum times to add labeled precursor and recover labeled antibiotic. The judicious selection of addition and recovery times resulted in incorporation of as much as 43% of labeled adenine into sinefungin (Fig. 3B). The optimization studies were facilitated by the methodology employed to measure changes in the fermentation time course. Using just 4-ml samples, measurements were made of pH, DNA content, factor C concentration, sinefungin concentration, and specific activities of factor C and sinefungin. Scanning thin-layer densitometry provided a rapid, sensitive method for measuring antibiotic concentration that eliminated the need for tedious isolation and purification procedures. The combination of thin-layer densitometry, biological assays, DNA measurements, and scintillation counting may be applicable (with appropriate modifications) to other multi-factor antibiotic fermentations.

The high percent of label incorporation measured in these studies strongly suggest that a preformed adenine derivative and ornithine (or a closely related metabolite) are close precursors of sinefungin and factor C. The terminal steps in the biosynthesis of sinefungin may have the net effect of condensing adenosine and ornithine as follows:



The mechanism by which the above reaction could occur is not known; and, of course, other reaction sequences cannot be eliminated. The relative amounts of precursor incorporation provide little guidance for identifying the actual reaction substrates because differences in incorporation may be due to differences in the metabolism and rate of uptake of labeled compounds by the microorganism.

Incorporation of adenine into sinefungin reaches a maximum and then declines sharply indicating that sinefungin is not a stable end product of the fermentation. The loss of label from sinefungin was accompanied by an increase in label incorporated into factor C. Thus, the loss of label from sinefungin does not appear to be due to conversion of sinefungin to factor C. This conclusion was also apparent from other fermentation studies (BERRY and ABBOTT, unpublished data) where the sinefungin concentration occasionally fell precipitously without a concommitant increase in factor C concentration. Although factor C may be derived from sinefungin, it appears that sinefungin also is metabolized to other unidentified compounds.

Acknowledgments

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