Incorporation of Labeled Precursors into A16886B, a Novel β -Lactam Antibiotic Produced by *Streptomyces clavuligerus*

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The incorporation of C-14 from amino acids into A16886B, 7-(5-amino-5-carboxyvaleramido)-7-methoxy-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid, by *Streptomyces clavuligerus* is reported. As with cephalosporin C biosynthesis by the mold *Cephalosporium acremonium*, labels from cysteine, valine, and α -amino-adipic acid were incorporated. Unlike cephalosporin C, in A16886B label from lysine was incorporated into the α -aminoadipic acid side chain. Label from methionine- ^{14}C -methyl was incorporated into the methoxyl group. The relative percentage of incorporation of ^3H and ^{14}C from doubly labeled cystine suggests the improbability of the C-7 methoxyl group arising from an intermediate containing a double bond between C-6 and C-7.

Since the discovery of the β -lactam antibiotics penicillin and cephalosporin C (1, 9), the chemistry, biological activity, and biosynthesis of these compounds have been investigated extensively.

Attempts to use the eukaryotic *Cephalosporium* and *Penicillium* mold cultures to study β -lactam antibiotic biosynthesis have met with considerable difficulty. However, the available data indicate that these peptide antibiotics are products of secondary metabolism, being synthesized principally from constituents of intracellular pools. To date, studies with isotopically labeled α -aminoadipic acid, cysteine, and valine suggest that these amino acids are incorporated into the antibiotics in a manner consistent with the tripeptide theory of Abraham et al. (2, 5). Strict data concerning the exact mechanism of β -lactam biosynthesis are still lacking.

More recently, Nagarajan et al. (11), Gorman et al. (Belgium Patent 754,693, 1971; Abstr. 11th Intersci. Conf. Antimicrob. Ag. Chemother., p. 7, 1971), and Hamill et al. (Abstr. 11th Intersci. Conf. Antimicrob. Ag. Chemother., p. 7., 1971) have reported the occurrence of three new β -lactam antibiotics from two species of *Streptomyces*. Structure elucidation studies have shown these compounds to be chemically related to cephalosporin C (Fig. 1, reference 8). Using the prokaryotic *Streptomyces* culture, we have conducted preliminary studies on the biosynthesis of A16886B, 7-(5-amino-5-carboxyvaleramido)-7-methoxy-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid.

The following is a report on the incorporation of C-14 label from various amino acids into antibiotic A16886B by *S. clavuligerus*.

MATERIALS AND METHODS

Microorganisms. All cultures were obtained from the Lilly collection of lyophilized microorganisms. The strain of *S. clavuligerus* used throughout this study was Lilly Culture A16886.7, derived from the original culture described by Higgins et al. (10). In microbiological assays for antibiotic activity, Lilly Culture X142, a strain of *Salmonella gallinarum*, was used. A strain of *Pseudomonas solanacearum* (60-RE-3B), Lilly Culture X185, was used to prepare bioautograms.

Media and growth conditions. The media employed throughout this study are listed in Table 1. Incubation of A16886.7 for 7 to 9 days at 26 C on Bennett's modified agar provided a well-sporulated slant culture which was lyophilized. A vegetative inoculum was grown in wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of vegetative medium. Flasks were inoculated from either lyophilized pellets or sporulated slant cultures and were incubated for 48 hr at 30 C on a G-53 New Brunswick shaker, rotating through a 5-cm arc at 250 rev/min. Fermentations were conducted in wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of fermentation medium. Flasks were inoculated with 5% (v/v) of vegetative inoculum and incubated as described above.

Antibiotic assays. Mycelia were removed from the flasks of *S. clavuligerus* by centrifugation. The total antibiotic titer of the resulting broth was determined by the standard disc-plate technique with culture X142 as the assay organism. Antibiotic activity was recorded

TABLE 1. Growth media used for *S. clavuligerus*

Medium component	Concn (g/liter)		
	Bennett's agar ^a	Vegetative ^b	Fermentation ^c
Dextrine.....	10.0	—	—
Yeast extract.....	1.0	—	—
NZ Amine type A.....	2.0	—	3.0
Beef extract.....	1.0	—	—
Glycerol.....	—	10.0	7.5
Sucrose.....	—	20.0	—
Nutrisoy grits.....	—	15.0	—
Amber BYF 300.....	—	5.0	—
Tryptone.....	—	5.0	—
Cornstarch.....	—	—	45.0
Nutrisoy flour.....	—	—	20.0
Nadrisol ^d	—	—	1.0
CoCl ₂	0.01	—	—
K ₂ HPO ₄	—	0.2	—
FeSO ₄ ·7 HOH.....	—	—	0.1
Meer agar.....	20.0	—	—

^a The pH was adjusted to 7.0 prior to autoclaving.

^b The pH was adjusted to 6.5 prior to autoclaving.

^c The pH was unadjusted.

^d Corn distillers dried solubles, National Distillers Products Co.

as units per milliliter of culture broth, 1 unit being equal to 0.33 μ g of purified antibiotic.

Paper chromatography and bioautograms. The two major antibiotic factors in the *S. clavuligerus* fermentation (A and B) were resolved by descending chromatography on Whatman no. 1 paper. The chromatograms were developed in propanol-pyridine-acetic acid-CH₃CN-water (45:30:9:40:36) for 16 hr at 25 C. Bioautograms were prepared by applying developed chromatograms to the surface of agar plates seeded with culture X185.

Radiochemistry. The incorporation of radioactivity into antibiotic A16886B was conducted under standard fermentation conditions. Radioactive compounds were added to 72-hr fermentations to the final concentrations indicated in Table 2. After 24 hr of additional incubation, the fermentations were terminated, the mycelia were removed from the growth medium by filtration, and the labeled A16886B was isolated according to the procedure of Gorman et al. (Abstr. 11th Intersci. Conf. Antimicrob. Ag. Chemother., p. 7, 1971). Paper chromatograms were scanned for radioactive factors with a Vanguard Autoscanner 880. Quantitative counting of radioactive material was conducted with a Packard Tri-Carb liquid scintillation counter.

Acid hydrolysis of labeled A16886B. Samples of ¹⁴C-labeled A16886B antibiotic were dissolved in 6 N HCl (ca. 1 mg/ml), and the solutions were refluxed for 21 hr. The hydrolysates were taken to dryness under vacuum, approximately 1 ml of water was added, and the solutions were again taken to dryness.

The dried samples were dissolved in buffer (2% thiodiglycol, 0.2 N Na⁺, pH 2.2) and analyzed on a Beckman model 120C amino acid analyzer equipped with a BioCal model BC 501 automatic sample injector. Amino acid separation was achieved on a column of Durrum DC-1A resin (0.9 by 52 cm) at 56.5 C, with a 70 ml/hr flow of buffer (0.5% thiodiglycol, 0.2 N Na⁺, pH 3.23). A ninhydrin flow of 35 ml/hr was used when a ninhydrin response curve was desired. When the amino acids were eluted for determination of radioactivity, 1-min fractions (1.1 ml) were collected directly from the column.

Hydroiodic acid cleavage. Samples of ¹⁴C-labeled A16886B were subjected to Zeisel degradation by dissolving 2.14 mg of antibiotic in 2 ml of hydroiodic acid (specific gravity, 1.70), and heating for 30 min. The resulting methyl iodide was swept into an alcoholic solution of 10% trimethylamine. A sample of the trimethylamine solution was analyzed for radioactivity by scintillation counting in diatol.

RESULTS

Prior to the isolation of A16886B, incorporation of label from a variety of amino acids into the antibiotic was determined qualitatively. Paper chromatograms of broths from fermentations conducted in the presence of ¹⁴C-labeled substrates were prepared. These chromatograms were scanned for radioactivity, and were also used to prepared bioautograms. The coincidence of biological activity and radioactivity on chromatograms developed in a variety of solvent systems was accepted as preliminary evidence for incorporation.

Those amino acids which showed some degree of incorporation of label were selected for more quantitative studies in which labeled A16886B was partially purified. Because of the difficulty in obtaining a crystalline preparation of A16886B, purity of the antibiotic was determined by the number of units of biological activity per milligram of sample. All calculations were based on 3,000 units/mg of pure A16886B. These data are presented in Table 2.

That, indeed, the radioactivity in each labeled preparation of A16886B resided only in the antibiotic is based on the following arguments. In none of the extensive number of paper chromatography systems employed could the antibiotic activity be separated from the radioactivity. Also, the paper chromatogram of the *N*-phthaloyl derivative of labeled A16886B gave a peak of radioactivity that coincided with its antibiotic activity.

To examine the involvement of lysine in the synthesis of the α -amino adipic acid side chain of A16886B, labeled lysine and α -amino adipic acid were added to fermentations, and the resulting labeled A16886B was purified. Samples of A16886B labeled with DL-lysine-¹⁴C-1 and DL- α -

TABLE 2. Incorporation of ^{14}C into cephalosporin A16886B by addition of C-14 labeled amino acids to *S. clavuligerus*

Amino acid ^a	Amt (mg)	Radio-activity (μCi)	Radioautogram incorporation ^b	Isolated A16886B		
				Units per mg of A16886B prepn ^c	DPM ^d per mg of A16886B in prepn	Per cent incorporation
DL-Cysteine- ^{14}C -3	0.12	50	yes	918	2.8×10^4	8.8
DL-Cysteine- ^{14}C -1	1.2	50	yes	635	4.2×10^4	10.9
DL-Valine- ^{14}C -1	0.21	50	yes	840	1.9×10^4	6.09
DL- α -Aminoadipic acid- ^{14}C -1	0.65	50	yes	645	4.3×10^4	10.9
L-Methionine- ^{14}C -methyl	0.60	77	yes	1,580	9.2×10^4	23.6
L-Lysine- ^{14}C -1	4.8	77	yes	720	6.5×10^4	12.47
DL-Serine- ^{14}C -3	2.1	102	yes	1,580	1.3×10^4	3.96
DL-Aspartic acid- ^{14}C -4	1.14	100	no			
L-Citrulline- ^{14}C -ureido	0.85	25 ^e	no			
Glycine- ^{14}C -1	0.05	25 ^e	no			
L-Leucine- ^{14}C -1	0.05	5 ^e	no			
L-Methionine- ^{14}C -1	0.32	5 ^e	no			
L-Threonine- ^{14}C -U	4.2	5 ^e	no			

^a Added to 1 liter of culture at 72 hr with further incubation to 96 hr.

^b Broth samples were chromatographed on paper, and the resulting chromatograms were scanned for radioactivity.

^c Based upon a unit assay in which 3,000 units represents 1 mg of A16886B.

^d Disintegrations per minute.

^e Added to 50 ml of culture.

aminoadipic acid- ^{14}C -1 were hydrolyzed with hydrochloric acid, and the resulting hydrolysates were subjected to amino acid analysis. One portion of the hydrolysate was chromatographed, and the resulting ninhydrin-positive products were quantitated and identified by elution times. A second portion of each hydrolysate was chromatographed by the same procedure except without ninhydrin addition, and the column eluate was collected in 1-min fractions. A 0.1-ml sample of each fraction was assayed for radioactivity. The specific activity of the α -aminoadipic acid from each hydrolysate is presented in Table 3.

The incorporation of label into A16886B from L-methionine- ^{14}C -methyl was indeed suggestive that the methoxy function of this antibiotic was derived from methionine. To examine this possibility, L-methionine- ^{14}C -methyl was added to fermentations, and the resulting A16886B (21.4 mg, 9.2×10^4 disintegrations per min per mg) was purified and hydrolyzed with hydroiodic acid. After hydrolysis, 1.66×10^5 disintegrations/min were recovered in the residue and 1.05×10^6 were in the ^{14}C -methyl iodide. The percentage of label recovered as ^{14}C -methyl iodide was 93.6%.

In a double labeling experiment, DL-cystine- ^{14}C -3,3' (0.189 mg, 54.5 μCi) and L-cystine- ^3H -3,3' (0.120 mg, 100 μCi) were added to 1 liter of culture at 72 hr with further incubation to 96 hr. Differential counting of ^{14}C and ^3H , accomplished by use of a double isotope external standard cor-

rected, 4 quadratic fit (DIESC 4QF) computer program, showed that 9.16% of the ^{14}C and 7.14% of the ^3H were incorporated into A16886B.

DISCUSSION

The formal molecular units that comprise cephalosporin antibiotics are well known. Cephalosporin C, A16884, and the A16886 antibiotics,

TABLE 3. α -Aminoadipic acid obtained from hydrolysis of A16886B precured by the addition of DL- α -aminoadipic acid- ^{14}C -1 and DL-lysine- ^{14}C -1 to *S. clavuligerus*

Amino acid	Labeled A16886B hydrolyzed		α -Aminoadipic acid recovered	
	Amt ^a (mg)	DPM ^b per mg	Amt (μmoles /mg of A16886B)	DPM ^b per mg
DL- α -Aminoadipic acid- ^{14}C -1	0.262	4.3×10^4	1.24	1.27×10^5
DL-Lysine- ^{14}C -1	0.264	6.5×10^4	1.49	1.5×10^5

^a Amount of A16886B in the A16886B preparation as determined by the described unit assay.

^b Disintegrations per minute.

are essentially heterocyclic tripeptides composed of modified α -amino adipic acid, cysteine, and valine. The isolation of a noncyclic tripeptide from *Penicillium* and *Cephalosporium* containing these amino acids has led to the tripeptide theory, which implies that the three amino acids from the cellular pool of amino acids combine to initiate penicillin and cephalosporin biosynthesis. Considerable evidence has been found that supports the tripeptide theory. Bauer (6) recently reported the synthesis of the tripeptide by cell-free extracts of *P. chrysogenum*. However, conversion of tripeptide to antibiotic has not been accomplished.

The discovery of two cephalosporin-producing streptomycete cultures offers hope that the biosynthetic difficulties encountered with the β -lactam-producing mold cultures can be overcome.

Preliminary to a detailed investigation of the biosynthesis of the A16884 and A16886 antibiotics by *S. lipmanii* and *S. clavuligerus*, a general comparison of the incorporation of label from labeled amino acids between cephalosporin C and A16886B seemed necessary. Labels from α -amino adipic acid, valine, and cysteine were incorporated into A16886B by *S. clavuligerus* to a significant extent.

The origin of α -amino adipic acid in *C. acremonium* has been well studied. The cycling of acetate through the tricarboxylic acids of the Krebs cycle produces α -keto-glutarate which condenses with acetyl coenzyme A eventually to give α -amino adipic acid. In such a scheme, lysine is derived from α -amino adipic acid. This is in contrast to the bacterial system in which lysine, derived from 2,6-diaminopimelic acid, serves as the immediate precursor of α -amino adipic acid.

The discovery of 2,6-diaminopimelic acid in the cell wall of *S. clavuligerus* (10) prompted an investigation of the origin of the α -amino adipyl side chain of A16886B. The incorporation of label from DL-lysine- ^{14}C -1 into A16886B by *S. clavuligerus* indicates that the origin of α -amino adipic acid in this culture is different from that in *C. acremonium* and is probably related to the bacterial pathway. Degradation of the DL-lysine- ^{14}C -1-labeled A16886B showed that most of the label resided in the side chain.

Arnstein (3) and Arnstein and Crawhall (4) found that tritium from DL-cystine- ^3H -2,2' and DL-cystine- ^3H -3,3' was incorporated, respectively, into the C-5 and C-6 positions (penicillin numbering system) of benzylpenicillin. They therefore discounted the possibility of β -lactam ring formation involving a double bond between C-5 and C-6 of the cysteinyl residue of the antibiotic. Unsaturation between C-5 and C-6 might, however, seem an attractive means for the introduction of the 7-methoxyl of A16886B by *S. clavu-*

ligerus. Preliminary information does not support such a mechanism. Addition of DL-cystine- ^{14}C -3,3' and L-cystine- ^3H -3,3' simultaneously to *S. clavuligerus* resulted in 9% ^{14}C and 7% ^3H incorporation. It seems unlikely, but not impossible, that so much tritium would be retained during double-bond formation and ring closure. The acid degradation of A16886B labeled with cysteine- ^{14}C -1 and cysteine- ^{14}C -3 has been reported (8).

Sulfate and methionine are alternative sulfur sources for cysteine. In fungi, cysteine is used for β -lactam antibiotic production. In *Penicillium*, the sulfate reduction pathway has been shown to play a major role, and stimulation of cephalosporin production in *C. acremonium* by methionine has been attributed to the formation of cysteine by a reverse trans-sulfuration pathway. As indicated in Table 2, label from DL-serine- ^{14}C -3 was incorporated into A16886B. This observation, combined with preliminary findings that label from DL-methionine- ^{35}S was also incorporated into A16886B, indicates that a trans-sulfuration route to cysteine may be operative in *S. clavuligerus*.

S. clavuligerus is unique in its production of a cephalosporin containing a C-7 methoxy group and cephalosporins with C-3-O-carbamoyloxy-methyl. Label from DL-methionine- ^{14}C -methyl was readily incorporated into A16886B. However, the sequence of biosynthetic steps leading to the carbamoyl function are unknown. Label from L-citrulline- ^{14}C -ureido was not detected in A16886B. The possible role of a deacetylcephalosporin C in the synthesis of A16886A by *S. clavuligerus* has been discussed (7).

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