BIOSYNTHESIS OF BICYCLOMYCIN
II. BIOSYNTHETIC CONDITIONS AND INCORPORATION OF RADIOACTIVE PRECURSORS INTO BICYCLOMYCIN BY WASHED MYCELIUM

MANABU ISEKI, TOSHIO MIYOSHI, TOSHIO KONOMI and HIROSHI IMANAKA

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.,
Osaka, Japan

(Received for publication November 2, 1979)

The biosynthesis of bicyclomycin by *Streptomyces sapporonensis* was studied using suspensions of washed mycelium. Nicotinamide and Fe^{2+} were found to be essential cofactors in the biosynthesis. Production of bicyclomycin was enhanced most effectively in the presence of equal moles of L-leucine and L-isoleucine, which in experiments with radioactively labeled compounds were found to be incorporated into bicyclomycin at equivalent rates. These facts strongly suggest that bicyclomycin biosynthesis involves coupling of equal moles of these two amino acids.

Bicyclomycin has a unique chemical structure[1] with two amide bonds contained in a diketopiperazine ring and four hydroxy groups. This suggests that bicyclomycin is formed by condensation of two amino acids.

The present paper reports the investigation of essential cofactors and precursors for biosynthesis of bicyclomycin using suspensions of washed mycelium.

Materials and Methods

Organism and media

Streptomyces sapporonensis LH9455 strain, previously studied,[2] was used for all experiments. Media for inoculum and vegetative growth contained (per liter) 10 g soluble starch, 10 g nutrient broth (Difco), 5 g yeast extract (Difco), 7.2 g $Na_2HPO_4 \cdot 12H_2O$ and 10.9 g KH_2PO_4. Basal synthetic medium for biosynthesis of bicyclomycin contained 1% soluble starch, 0.1 mM $FeSO_4 \cdot 6H_2O$, 100 μg/ml nicotinamide, 0.72% $Na_2HPO_4 \cdot 12H_2O$ and 1.09% KH_2PO_4.

Preparation of suspensions of washed mycelium

The mycelium of strain LH9455, grown in the inoculum medium in a shake flask for 3 days at 30°C, was inoculated into vegetative growth medium. The vegetative mycelium was harvested at 0~2°C 50 hours after inoculation, washed three times with a basal synthetic medium or 0.1 M phosphate buffer (pH 6.0), and resuspended in the basal medium or 0.1 M phosphate buffer to contain 220 mg/ml dry weight cells.

Antibiotic assay was carried out using *Escherichia coli* ATCC 27166 as previously reported.[2]

Biosynthetic conditions

Various compounds were added to 5 ml of suspension of washed mycelium in a L-tube, and the mixture was incubated with constant shaking for 20~24 hours at 30°C.

Incorporation of labeled precursors into bicyclomycin and purification of labeled bicyclomycin

Labeled precursors were added to the suspensions of washed mycelium in the basal biosynthetic medium and incubated as described above. After incubation, 1.5% (w/v) of activated carbon was added to the culture filtrate to adsorb bicyclomycin. The carbon adsorbate was filtered off and washed

with water. The washed carbon cake was extracted with ethylacetate - methanol (2: 1) by shaking at 30°C. The extracts were concentrated under reduced pressure and dried. Authentic bicyclomycin crystals (100 mg, about 100-times the content of bicyclomycin in the concentrate) were weighed accurately, added to the concentrate and solubilized with an appropriate volume of methanol. The mixture was concentrated again to a small volume. Bicyclomycin was crystallized by adding a small volume of acetone to the second concentrate and storing at 4°C.

Determination of specific activity of labeled bicyclomycin

Five mg of labeled bicyclomycin crystals obtained by the above-mentioned dilution method were weighed accurately and solubilized with 2.5 ml of methanol. The radioactivity of 0.5 ml of the solution was measured in a toluene scintillator by Liquid Scintillation Spectrometer (Packard). Specific activity of labeled bicyclomycin was expressed by the following equation. Specific activity (dpm/μmole)$=(100+a)\cdot b\cdot 0.302/a$. The meaning of the symbols are as follows, a (mg): bicyclomycin content in the purified extract obtained by adsorption onto activated carbon, b (dpm): radioactivity of 1 mg of bicyclomycin crystal isolated by dilution method, 0.302: one μmole of bicyclomycin, 100 (mg): authentic bicyclomycin added to the concentrate.

Autoradiography and autobiography

Active fractions containing bicyclomycin were chromatographed on silica gel thin-layer sheets (Eastman Kodak) with chloroform - methanol (3: 1) or (1: 1), isopropanol - formic acid - water (20: 1: 5), and *n*-butanol - pyridine - water (1: 1: 1) as the developing solvent system and were detected by autobiography on agar plates seeded with *E. coli* ATCC 27166 or by autoradiography on X-film (Sakura film).

Radioisotopes

L-Leucine-^{14}C (U) (298 mCi/mmole) and L-isoleucine-^{14}C (U) (279 mCi/mmole) were purchased from New England Nuclear Corp.

Results

1. Effect of Amino Acids on Bicyclomycin Production of Suspensions of Washed Mycelium in Phosphate Buffer

Eighteen different amino acids were investigated. Each of them was added to suspensions of washed mycelium in phosphate buffer, and the mixture was incubated for 24 hours. L-Leucine, L-isoleucine, L-phenylalanine and L-glutamic acid were found to have a stimulatory effect on bicyclomycin production. However, Casamino acids (Difco) containing all 18 amino acids was more effective than any single amino acid (Table 1).

2. Effect of Carbohydrates on Bicyclomycin Production of Suspensions of Washed Mycelium

Various kinds of carbohydrates were added as a carbon source to the basal medium containing washed mycelium in the presence of Casamino acids as a nitrogen source. The mixture was incubated for 24 hours. Soluble starch, mannose and fructose were most effective of the compounds investigated for bicyclomycin production. In the basal medium without carbohydrate, bicyclomycin production was reduced (Table 2).

3. Effect of Vitamins on Bicyclomycin Production of Suspensions of Washed Mycelium

Nine different vitamins were investigated. Each of them was added to suspensions of washed mycelium in phosphate buffer, and the mixture was incubated for 48 hours. Nicotinamide and nicotinic acid were most effective of the compounds investigated for bicyclomycin production (Table 3).

Table 1. Effect of amino acids on the biosynthesis of bicyclomycin by washed mycelium.

Amino acid (1 mg/ml)	Bicyclomycin (μg/ml)	Amino acid (1 mg/ml)	Bicyclomycin (μg/ml)	Amino acid (1 mg/ml)	Bicyclomycin (μg/ml)
None (control)	±	L-Hydroxyproline	±	L-Phenylalanine	92
L-Alanine	±	L-Serine	±	L-Tryptophan	±
L-Arginine	40	L-Methionine	±	L-Glutamic acid	75
L-Glycine	±	L-Threonine	±	L-Tyrosine	±
L-Histidine	45	L-Valine	±	L-Aspartic acid	±
L-Lysine	±	L-Isoleucine	150	Casamino acids (vitamin free)	215
L-Proline	45	L-Leucine	135		

Each amino acid (1 mg/ml) was added to the suspensions of washed mycelium (220 mg/ml dry weight) in 0.1 M phosphate buffer, and the mixture was incubated for 24 hours.

Table 2. Effect of carbohydrates on bicyclomycin production by washed mycelium.

Carbohydrate (1%)	Bicyclomycin (μg/ml)
None (control)	150
Soluble starch	550
Glycerin	350
Glucose	420
Fructose	580
Lactose	130
Mannose	690
Sucrose	420

Each carbohydrate (1%) was added to the basal biosynthetic medium containing washed mycelium and Casamino acids (1 mg/ml) as a nitrogen source, and the mixture was incubated for 24 hours.

Table 3. Effect of vitamins on bicyclomycin production by washed mycelium.

Vitamin (100 μg/ml)	Bicyclomycin (μg/ml)
None (control)	325
Thiamine	270
Riboflavine	325
Pyridoxal 5-phosphate	380
Biotin	325
Para-aminobenzoic acid	325
Nicotinic acid	725
Nicotinamide	875
Calcium pantothenate	400
Choline chloride	420

Each vitamin (100 μg/ml) was added to the suspensions of washed mycelium in phosphate buffer, and the mixture was incubated for 48 hours.

4. Effect of Mixed Amino Acids on Bicyclomycin Production of Suspensions of Washed Mycelium

As shown in Table 1, bicyclomycin production was high when Casamino acids was used as a nitrogen source. This fact suggested that a combination of two or more amino acids was more effective than any one of the 18 amino acids tested alone. Therefore, effective amino acids such as L-leucine, L-isoleucine and phenylalanine were mixed and bicyclomycin production was examined. The mixture of L-leucine and L-isoleucine, especially in the case of equal moles of each, showed the highest stimulatory effect on bicyclomycin production (Table 4).

5. Effect of Fe^{2+} and Fe^{3+} Ions on Bicyclomycin Biosynthesis

Addition of ferrous sulfate (0.1 mM) stimulated bicyclomycin production (Table 5). To confirm this, the effect of a chelating agent such as α,α'-dipyridyl was examined in biosynthetic medium containing equal moles of L-leucine and L-isoleucine (10 mM each), and nicotinamide. Bicyclomycin production was inhibited almost completely by the addition of α,α'-dipyridyl, but it recovered after further addition of ferrous sulfate (Fe^{2+}) as shown in Table 6. In case of ferric sulfate (Fe^{3+}), the recovery was not remarkable. From these results, it was concluded that Fe^{2+} was an essential factor for bicyclomycin biosynthesis.

Table 4. Effect of mixed amino acids on the biosynthesis of bicyclomycin by washed mycelium.

Amino acid (10 mM each)	Bicyclomycin (μg/ml)
None (control)	120
L-Leucine	315
L-Isoleucine	315
L-Phenylalanine	230
L-Leucine + L-Isoleucine	1,100
L-Leucine + L-Phenylalanine	440
L-Isoleucine + L-Phenylalanine	190
L-Leucine + L-Isoleucine + L-Phenylalanine	570

The effective amino acids (10 mM each) such as L-leucine, L-isoleucine and L-phenylalanine were added in various combinations to the basal biosynthetic medium containing washed mycelium, and the mixture was incubated for 24 hours.

Table 5. Effect of ferrous sulfate on bicyclomycin production by washed mycelium.

$FeSO_4 \cdot 6H_2O$ (mM)	Bicyclomycin (μg/ml)
0	340
0.001	320
0.005	490
0.01	635
0.1	1,225
1.0	730

Ferrous sulfate was added to the suspensions of washed mycelium in phosphate buffer, and the mixture was incubated for 24 hours.

Table 6. Effect of Fe ion on the biosynthesis of bicyclomycin.

Addition, mM	Bicyclomycin (μg/ml)
Fe^{2+}, 0.1	760
Fe^{3+}, 0.1	660
α,α'-Dipyridyl, 0.3	0
α,α'-Dipyridyl, 0.3 + Fe^{2+}, 0.2	500
α,α'-Dipyridyl, 0.3 + Fe^{3+}, 0.2	150

Fe^{2+} (ferrous sulfate) or Fe^{3+} (ferric sulfate) was added to the basal biosynthetic medium containing L-leucine and L-isoleucine (10 mM each) one hour after start of incubation. The mixture was incubated for a further 24 hours.

6. Incorporation of Radioactive L-Leucine and L-Isoleucine into Bicyclomycin

In the presence of equal moles of L-leucine and L-isoleucine, bicyclomycin was biosynthesized most effectively as described above. Thus these two amino acids seemed to be the best precursors for bicyclomycin. To confirm this, the incorporation of radioactive L-leucine and L-isoleucine into bicyclomycin was examined by addition of them to the basal biosynthetic medium containing equal moles (10 mM) of cold L-leucine and L-isoleucine. In the presence of 0.5 μCi/ml or 2.5 μCi/ml of ^{14}C-L-leucine, the radioactivity incorporated into bicyclomycin was 8.85% and 11.15%, respectively, of the total radioactivity supplied to the medium (Table 7). The specific activity of ^{14}C-labeled bicyclomycin produced by addition of 2.5 μCi/ml of ^{14}C-L-leucine was about 5-times higher than that obtained with 0.5 μCi/ml, indicating that incorporation of radioactive label was proportional to the concentration of radioactive precursor in the medium. In the case of ^{14}C-L-isoleucine, the same kind of results were obtained, showing that the incorporation rate of ^{14}C-L-leucine was approximately the same as that of ^{14}C-L-isoleucine. This fact suggested that equal moles of these amino acids were incorporated into bicyclomycin.

The radioactive material isolated from the culture medium by adsorption onto activated carbon was investigated by thin-layer chromatography in four solvent systems. Autoradiography showed that it contained several radioactive components. The most intensive radioactive spot showed antibacterial activity against *E. coli* on bioautography and coincided with the bicyclomycin.

7. Effect of Inhibitors on Bicyclomycin Biosynthesis

Inhibitors of protein synthesis, such as tetracycline, streptomycin and chloramphenicol, suppressed bicyclomycin production in suspensions of washed mycelium almost completely (Table 8). On the

Table 7. Incorporation of radioactive precursors into bicyclomycin.

Precursor supplied				Bicyclomycin synthesized		
Isotope	μCi/ml	L-Leucine μmole	L-Isoleucine μmole	Yield μmole	Incorporation (%)	Specific activity dpm/μmole $\times 10^{-5}$
L-Leucine-^{14}C(U)	0.5	100	100	10.2	8.85	0.95
	2.5	100	100	12.3	11.15	5.09
L-Isoleucine-^{14}C(U)	0.5	100	100	11.4	10.88	1.06
	2.5	100	100	12.5	13.20	5.82
L-Leucine-^{14}C(U)	0.25	50	—	3.2	3.6	0.3
L-Isoleucine-^{14}C(U)	0.25	—	50	4.4	5.8	0.37

Radioactive precursor (0.5 μCi/ml or 2.5 μCi/ml) was added to the basal biosynthetic medium in the presence of L-leucine and L-isoleucine and incubated for 24 hours. Labeled bicyclomycin was crystallized by dilution method as described in Materials and Methods. Incorporation rate (%) was determined as [Specific activity (dpm/μmole) $\times$ Yield of bicyclomycin (μmol)/Total dpm supplied] $\times 100^{-1}$.

other hand, respiratory enzyme inhibitors such as potassium cyanide, sodium azide and sodium arsenite did not reduce, but rather stimulated bicyclomycin biosynthesis.

8. Ability of Aerial Mycelia Negative (am$^-$) Strain to Synthesize Bicyclomycin

The producing strain may degenerate to an aerial mycelia negative (am$^-$), low-producing strain.[2] The ability of the degenerated am$^-$ strain to synthesize bicyclomycin was examined by suspensions of washed mycelium in basal biosynthetic medium containing L-leucine and L-isoleucine. As shown in Table 9, the ability of am$^-$, low-producing strain to synthesize bicyclomycin was only 1/10 to 1/30 of that of the normal strain.

Table 8. Effect of inhibitors on the biosynthesis of bicyclomycin by washed mycelium.

Inhibitor, μg/ml	Inhibition (%)
Tetracycline, 50	79
Streptomycin, 50	86
Chloramphenicol, 50	95
Penicillin G, 50	0
$CuSO_4 \cdot H_2O$, 100	−13
NaN_3, 100	−11
KCN, 100	−10
$NaAsO_2$, 100	−11

Each inhibitor was added to the basal biosynthetic medium in the presence of L-leucine and L-isoleucine and incubated for 24 hours. The rate of inhibition was calculated as percentage of the bicyclomycin control yield.

Table 9. Ability of washed mycelium of the aerial mycelia negative (am$^-$) strain to synthesize bicyclomycin compared with that of normal strain.

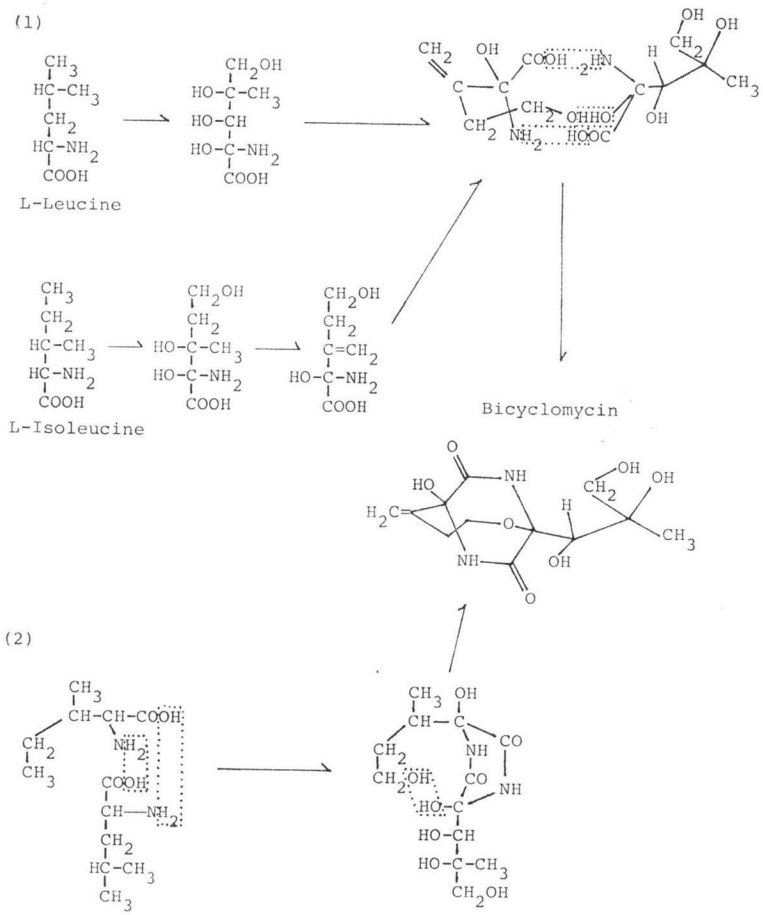
Strain	Bicyclomycin (μg/ml)
Am$^+$ (Normal)	500～700
Am$^-$	25～50

Discussion

The results indicate that *Streptomyces sapporonensis* synthesizes bicyclomycin from one molecule of L-leucine and one molecule of L-isoleucine in the presence of Fe^{2+} ion and nicotinamide as cofactors.

Incorporation of ^{14}C-L-leucine and ^{14}C-L-isoleucine into bicyclomycin molecule support the hypothetical biosynthetic pathways shown in Fig. 1. These pathways consist of hydroxylation or oxygenation of these amino acids and their coupling by peptidation. Oxygenases are known to be widely distributed in nature.[3,4] They require an electron donor such as ascorbate, reduced pyrimidine nucleotide, or glutathione and cofactor such as flavin, haem, non-haem iron or other metals. Ferrous ion

Fig. 1. Hypothetical schematic pathways for biosynthesis of bicyclomycin.



essential for bicyclomycin synthesis may be necessary for hydroxylation of L-leucine and L-isoleucine. Nicotinamide may be required as an electron donor.

Protein synthesis inhibitors, such as chloramphenicol or tetracycline, suppress bicyclomycin synthesis, but involvement of ribosomes in the biosynthesis has not been demonstrated.

Acknowledgement

The authors wish to express their gratitude to Dr. SHIGEATSU KUMADA, Director of Fujisawa Research Laboratories, for his permission to publish their manuscript. They also wish to thank Dr. HATSUO AOKI and colleagues of Research Laboratories for their valuable advice and encouragement.

References

1) KAMIYA, T.; S. MAENO, M. HASHIMOTO & Y. MINE: Bicyclomycin, a new antibiotic. II. Structural elucidation and acyl derivatives. J. Antibiotics 25: 576~581, 1972
2) MIYOSHI, T.; M. ISEKI, T. KONOMI & H. IMANAKA: Biosynthesis of bicyclomycin. I. Appearance of aerial mycelia negative strains (am$^-$). J. Antibiotics 33: 480~487, 1980
3) TAI, H. H. & C. J. SIN: 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione-4,5-deoxygenase from *Nocardia restrictus*. I. Isolation of the enzyme and study of its physical and chemical properties. J. Biol. Chem. 245: 5062~5071, 1970
4) PHO, D. B.; A. OLOMUCKI & N. V. THOAI: L-Arginine oxygenase decarboxylante. IV. Incorporation de ^{18}O dans la γ-guanidinobutyramide. Biochim. Biophys. Acta 118: 311~315, 1966