THE SCREENING OF β-LACTAMASE INHIBITORS: INHIBITION BY FATTY ACIDS PRODUCED BY BACTERIA

YOUXIN SONG,* TSUTOMU SAWA, MASAMI TSUCHIYA, YUKIO HORIUCHI, SHINICHI KONDO,** MASA HAMADA and HAMAO UMEZAWA

Institute of Microbial Chemistry
14–23 Kamiosaki 3-Chome, Shinagawa-ku, Tokyo 141, Japan

(Received for publication March 31, 1981)

An active principle inhibiting β-lactamases, which was found in the culture of a bacillus strain was a mixture of known $C_{14}\sim C_{17}$ fatty acids. The mixture was separated into five components by high-performance liquid chromatography. Among these components, 12-methyltetradecanoic acid showed the strongest activity (I_{50}: 20～146 μM). The anteiso fatty acids having the 1-methylpropyl group exhibited an interesting activity inhibiting β-lactamases.

In our screening study for new β-lactamase inhibitors, fatty acids produced by a Gram-positive, endospore-forming bacillus, strain BMG287-AF7 (isolated from a soil sample collected in Nagano Prefecture, Japan) were found to inhibit β-lactamases. These fatty acids were first obtained as a mixture of $C_{14}\sim C_{17}$ fatty acids.[1] From this mixture 12-methyltetradecanoic acid (a-C_{15}),[2] 12-methyltridecanoic acid (i-C_{14}), 14-methylpentadecanoic acid (i-C_{16}), hexadecanoic acid (n-C_{16}) and 14-methylhexadecanoic acid (a-C_{17}) were isolated by high-performance liquid chromatography (HPLC). The main component was a-C_{15}. The structure of each fatty acid in the mixture was determined by comparison of their behaviors in gas chromatography (GC) with authentic samples supplied by Dr. T. KANEDA[1] and their structures were confirmed by mass spectrometry and ^{1}H- and ^{13}C-NMR. In this report, we will report the activity of these fatty acids and their analogs as inhibitors of penicillinases (PCases) and cephalosporinases (CSases).

Experimental

Chemicals and Microorganisms

Potassium benzylpenicillin (PCG), ampicillin (ABPC), carbenicillin (CBPC), and cephaloridine (CER), supplied as standards for assay from National Institute of Health, Japan, were employed as substrates for β-lactamase reactions. Tetradecanoic (n-C_{14}), pentadecanoic (n-C_{15}) and heptadecanoic (n-C_{17}) acids were purchased from Sigma Chemical Co., Ltd.

13-Methyltetradecanoic acid (i-C_{15}) was isolated during a previous search for inhibitors of reverse transcriptase (unpublished). The fatty acid was assayed as an inhibitor of β-lactamases in the study reported here.

Escherichia coli ML2825 producing PCase TEM type, *E. coli* K-12 W3630/Rms212 producing PCase type I, *E. coli* K-12 W3630/Rms213 producing PCase type II, *Pseudomonas aeruginosa* M1/Rms139 producing PCase type IV, *Citrobacter freundii* GN346 producing CSase, *C. freundii* GN7391 producing CSase and *Enterobacter cloacae* GN5797 producing CSase were supplied by Prof. S. MITSUHASHI, Gunma University, Japan, and they were used as β-lactamase producers. *Staphylococcus aureus* FDA209P was used as the assay organism.

* Y. SONG is a visiting researcher (1979～1981) from Institute of Microbiology, Academia Sinica, Beijing, People's Republic of China.

** To whom correspondence should be addressed.

Preparation of β-Lactamases[3]

To 100 ml of heart infusion broth (Difco Laboratories) placed in a 500-ml Sakaguchi flask 10 ml of a 18-hour culture of a β-lactamase-producing strain was added. The culture was then incubated for 5 hours at 37°C on a reciprocal shaker (120 strokes/minute). To induce CSase production, PCG (0.5 mg/ml) was added to the 2-hour culture of *C. freundii* GN346, *C. freundii* GN7391 or *Ent. cloacae* GN5797 and the incubation was continued for 3 hours. Following incubation, bacterial cells were collected by centrifugation, washed twice with saline water and resuspended in 0.1 M phosphate buffer (pH 7.0), at a concentration of 40 to 60 mg (wet cell weight)/ml. The suspension was passed through a French pressure cell (70 kg/cm^2) and then centrifuged at 100,000 *g* for 2 hours. The clear supernatant (β-lactamase solution) was divided into small portions and stored at −80°C. One unit of β-lactamase activity was defined as the amount of enzyme which hydrolyzed one micromole of substrate per minute at 25°C in pH 7.0 buffer solution.

Determination of β-Lactamase-inhibiting Activity by Plate Method

A paper disc (8 mm in diameter), which absorbed 40 μl of sample solution, was placed on a test plate (90 mm in diameter). The nutrient agar medium in the test plate was prepared by mixing 0.5 ml of the β-lactamase solution, 0.5 ml of an aqueous solution of a substrate, 9.0 ml of nutrient agar and 0.1 ml of an overnight culture of *Staph. aureus* FDA209P. To assay for inhibition of PCase activity, PCG (62.7 μg/ml) was used as the substrate and PCase (0.0068 unit/ml) obtained from *E. coli* ML2825 was used as the β-lactamase. CER (104 μg/ml) and CSase (0.01 unit/ml) obtained from *C. freundii* GN346 were used for the assay of CSase-inhibiting activity. After incubation of the test plate at 37°C for 16 hours, the inhibition zone against *Staph. aureus* FDA209P was measured. The activity of an inhibitor was expressed by taking the activity of *a*-C_{15} as the standard (1,000 μg units/mg). In the assay for PCase-inhibiting activity, *a*-C_{15} at 1,000 μg/ml showed about a 17-mm zone of inhibition.

For reference, the antibiotic activity of an inhibitor was determined by using a plate containing 10 ml of nutrient agar and 0.1 ml of a culture of *Staph. aureus* FDA209P.

Determination of β-Lactamase-inhibiting Activity by UV Method

β-Lactamase solution was diluted in 0.05 M phosphate buffer (pH 7.0) until it gave 80% hydrolysis of the substrate in 20 minutes at 25°C. β-Lactamase-inhibiting activity (I_{50}) was determined by the spectrophotometric method at 25°C.[4,5] The decrease in optical density at 240 nm for PCG, ABPC and CBPC, and at 255 nm for CER was recorded. The enzyme was pre-incubated with each inhibitor at 25°C for 15 minutes and thereafter the enzyme reaction was started by the addition of a substrate.

Isolation of Fatty Acids from Cultured Broth of a Bacillus

Strain BMG287-AF7, a Gram-positive, endospore-forming bacillus, was cultured at 28°C on a rotatory shaker (180 rpm) in a 500-ml Erlenmeyer flask containing 110 ml of a medium (2.0% galactose, 2.0% dextrin, 1.0% soy peptone, 0.5% corn steep liquor, 0.2% $(NH_4)_2SO_4$ and 0.2% $CaCO_3$, adjusted to pH 7.4). The 72-hour culture was inoculated to fresh medium in 45 flasks at 2.0% by volume and incubated for 24 hours. The cultured broths were combined and 3.0 liters of the broth were obtained. The activity of 1 ml was 140 μg units. Inhibitors in the combined broth were extracted with a mixture of ethyl acetate (5.0 liters) and 1-butanol (1.5 liters), and the extract was concentrated to dryness (9.4 g). The residue was extracted with acetone (250 ml) and the extract was evaporated to yield a crude powder (6.87 g, 34 μg units/mg). The crude powder was subjected to column chromatography of silica gel (Wakogel C-200, 300 g) developed with a mixture of toluene and ethyl acetate (7: 1 by volume). The evaporation of the active eluate yielded a syrup (1.28 g). Further purification of inhibitors in the syrup (1.0 g) was carried out by preparative TLC using silica gel (Merck No. 5717, 20×20 cm, 9 plates) and a mixture of chloroform and acetone (20: 1 by volume). The area around Rf 0.3 was cut and extracted with a mixture of chloroform and methanol (40: 1 by volume). Concentration of the extract under reduced pressure gave a colorless syrup (460 mg). Inhibitors in this syrup (350 mg) were separated by preparative HPLC (Waters ALC/GPC 244, column; μ-Bondapak C_{18}, 0.95×30.5 cm, solvent; 85% aqueous methanol, flow rate; 2 ml/minute, pressure; 56 kg/cm^2, detection; refractive index). The following five components were thus obtained: component 1; 11 mg, 540 μg

units/mg, 2; 86 mg, 1,000 μg units/mg, 3; 4 mg, <200 μg units/mg, 4; 26 mg, <200 μg units/mg and 5; 9 mg, 260 μg units/mg.

Identification of Fatty Acids by GC

After methylation of each component with diazomethane in a methanol solution, each ester was analyzed by GC (Shimadzu GC-4CM, column; 15% DEGS-Neopak 1A 60~80, 0.3×200 cm, temperature; 165°C, carrier gas; N_2, flow rate; 30 ml/minute, detector; FID).

Results and Discussion

An active principle which inhibits β-lactamases, found in the culture broth of a bacillus strain, has been shown to be a mixture of fatty acids. The mixture of fatty acids was separated into five components by preparative HPLC. These components 1~5 were converted into their methyl esters and the esters of the components 1~5 were identified by GC to be the methyl esters of i-C_{14}, a-C_{15}, i-C_{16}, n-C_{16}, and a-C_{17}, respectively. Properties of these fatty acids are shown in Table 1. Prior to this work, a-C_{15} (sarcinic acid) had been reported as a major acid in lipids of *Sarcina* sp.,[2] *Bacillus subtilis*,[1] *Micrococcus lysodeikticus*[6] and rumen bacteria.[7]

Table 1. Properties of fatty acids obtained from a bacterial culture.

Component	Average retention time of HPLC (min.)	MS (m/z)		Retention time (min.) of GC of methyl ester	Identified with
		M^+	Characteristic fragmentation		
1	15.5	228	43, 185	8.5	i-C_{14}
2	19.3	242	57, 185	12.2	a-C_{15}
3	24.7	256	43, 213	15.7	i-C_{16}
4	26.5	256		18.3	n-C_{16}
5	31.0	270	57, 213	23.0	a-C_{17}

Table 2. I_{50} values (μM) of fatty acids in β-lactamase reactions.

Enzyme	PCase						CSase		
	E. coli ML 2825		*E. coli* K-12 W3630/Rms212		*E. coli* K-12 W3630/Rms213	*P. aeruginosa* M1/Rms139	*C. freundii* GN346	*C. freundii* GN7391	*Ent. cloacae* GN5797
Substrate	PCG	ABPC	PCG	ABPC	ABPC	CBPC	CER	CER	CER
i-C_{14} (Comp. 1)	83	73	107	81	<200	39	128	123	<200
n-C_{14}	<200	<200	<200	<200	<200	<200	<200	<200	<200
a-C_{15} (Comp. 2)	20	25	50	25	146	38	71	116	73
i-C_{15}	101	82	<200	<200	<200	24	<200	<200	151
n-C_{15}	<200	<200	<200	<200	<200	<200	<200	<200	<200
i-C_{16} (Comp. 3)	80	36	<200	<200	200	27	69	100	40
n-C_{16} (Comp. 4)	88	<200	<200	<200	<200	105	<200	78	37
a-C_{17} (Comp. 5)	41	131	<200	<200	<200	41	<200	<200	78
n-C_{17}	136	131	<200	<200	<200	<200	<200	99	<200

As shown in Table 2, this acid (a-C_{15}, component 2) showed the strongest activity in inhibiting β-lactamases, in comparison with i-C_{14} (component 1), n-C_{14}, i-C_{15}, n-C_{15}, i-C_{16} (component 3), n-C_{16} (component 4), a-C_{17} (component 5) and n-C_{17}. Although the activity was weaker, the other fatty acids except n-C_{14} and n-C_{15} exhibited an activity in inhibiting β-lactamases. n-C_{14} and n-C_{15} were inactive. It is interesting that a-C_{15} exhibited a relatively strong activity against PCases produced by *E. coli* ML-2825, *E. coli* K-12 W3630/Rms212 and *P. aeruginosa* M1/Rms139. Moreover, the 1-methylpropyl structure was suggested to be an interesting structure in inhibiting β-lactamases, because a-C_{17} was more active than n-C_{17} and a-C_{15} than i-C_{15} or n-C_{15}.

All fatty acids described above had no activity in inhibiting the growth of *Staph. aureus* FDA209P, when they were tested at concentrations of 4 mg/ml.

Acknowledgement

We wish to express our sincere thanks to Dr. T. KANEDA, Research Council of Alberta,Canada, for providing bacterial fatty acids.

References

1) KANEDA, T.: Biosynthesis of branched chain fatty acids. I. Isolation and identification of fatty acids from *Bacillus subtilis* (ATCC 7059). J. Biol. Chem. 238: 1222~1228, 1963
2) AKASHI, S. & K. SAITO: A branched saturated C_{15} acid (sarcinic acid) from sarcina phospholipids and a similar acid from several microbial lipids. J. Biochem. 47: 222~229, 1960
3) YAGINUMA, S.; M. INOUE & S. MITSUHASHI: Inhibition of cephalosporin β-lactamase by M4854-I and M4854-II. J. Antibiotics 33: 337~341, 1980
4) FU, K. P. & H. C. NEU: Comparative inhibition of β-lactamases by novel β-lactam compounds. Antimicr. Agents Chemoth. 15: 171~176, 1979
5) FU, K. P. & H. C. NEU: Beta-lactamase stability of HR756, a novel cephalosporin, compared to that of cefuroxime and cefoxitin. Antimicr. Agents Chemoth. 14: 322~326, 1978
6) MACFARLENE, M. G.: Composition of lipid from protoplast membranes and whole cells of *Micrococcus lysodeikticus*. Biochem. J. 79: 4P, 1961
7) KEENEY, M.; I. KATZ & M. J. ALLISON: On the probable origin of some milk fat acids in rumen microbial lipids. J. Am. Oil Chem. Soc. 39: 198~201, 1962