ACETYL CoA CARBOXYLASE INHIBITORS FROM THE FUNGUS *GONGRONELLA BUTRELI*

AKIRA ENDO*, HIROSHI TAKESHIMA and KENJI KUWABARA

Department of Agricultural and Biological Chemistry, Tokyo Noko University, 3-5-8 Saiwaicho, Fuchu-Chi, Tokyo 183, Japan

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Four active metabolites that specifically inhibit acetyl CoA carboxylase, the late-limiting enzyme in fatty acid biosynthesis, have been isolated from a strain of *Gongronella butleri* mating type (+). Their chemical structures were determined to be octyl pentanedioic acid, 9-decenyl-1-pentenedioic acid, decanyl-1-pentenedioic acid, decanyl-2-pentenedioic acid, respectively. These compounds inhibited rat liver acetyl CoA carboxylase 50% at a concentration of 30~55 μg/ml.

Acetyl CoA carboxylase (EC 6.4.1.2), the rate-limiting enzyme for fatty acid biosynthesis, is a biotinyl enzyme that catalyzes the formation of malonyl CoA. Its activity is regulated by interconversion of polymerized/depolymerized forms[1,2] and phosphorylated/dephosphorylated forms[3,4] as well as by changes in enzyme quantity[5]. Long-chain acyl CoA thioesters are known to inhibit acetyl CoA carboxylase by tight-binding mechanism[6,7]. Kynurenate and xanthurenate are dead-end inhibitors[8]. Hypolipidemic agents such as 2-methyl-2-(*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxyl)-propionate (TPIA) and 2-(*p*-chlorophenyl)-2-methyl propionate (CPIB) also inhibit polymerization of the enzyme, thereby reducing its activity[9]. None of these compounds, however, are effective in animals.

During the course of search for compounds of microbial origin that inhibit acetyl CoA carboxylase, four active compounds were isolated from cultured broth of *Gongronella butleri* mating type (+) M3180. In this communication, we report the fermentative production, isolation and chemical structure of these metabolites as well as their inhibitory effects on acetyl CoA carboxylase.

Materials and Methods

Materials

$NaH^{14}CO_3$ was obtained from New England Nuclear. Other chemicals were highest grade of commercial products. Microbial strains used in searching for productivity of an inhibitor of acetyl CoA carboxylase were isolated from soil samples collected in central part of Japan.

Assay for Acetyl CoA Carboxylase

Partial purification of acetyl CoA carboxylase and assay for its activity followed the methods of TANABE *et al.*[10]. Ten Wister rats (about 250 g) were fasted for 48 hours and subsequently refed a fat-free and high-glucose diet for 48 hours. The animals were killed by decapitation, and the livers (120 g) were removed. Livers were cut into small pieces and homogenized in two volumes of 0.25 M sucrose under cooling. The homogenate was centrifuged at 13,000×*g* for 45 minutes and the supernatant was collected through cheesecloth. This was further centrifuged at 100,000×*g* for 1 hour, and the supernatant was collected. The crude extract was brought to 30% saturation by adding ammonium sulfate; the pH was maintained at 7.3~7.4 by addition of KOH. After further stirring for 30 minutes, the resulting precipitate was collected by centrifugation at 16,000×*g* for 20 minutes and dissolved in

10 mM phosphate buffer, pH 7.5 (100 ml). This fraction was stirred into 60 ml of a calcium phosphate gel suspension (13.4 mg/ml). After 20 minutes, the gel was collected by centrifugation at $1,000 \times g$ for 5 minutes and washed three times, each time with 42 ml of 33 mM phosphate buffer, pH 7.5. The enzyme was eluted twice, with 42 ml of 0.2 M phosphate buffer, pH 7.5. The enzyme in the eluates was precipitated by 30% saturation with ammonium sulfate and the precipitate was collected by centrifugation. The resulting precipitate was dissolved in 0.1 M phosphate buffer, pH 7.5 containing 0.25 M sucrose and dialyzed against the same buffer. The solution (5 ml; 0.22 unit/mg protein) was kept at −80°C until use. All phosphate buffer described above contained 5 mM 2-mercaptoethanol and 1 mM EDTA.

Enzyme assay followed the $^{14}CO_2$-fixation method[10]. The enzyme was first preincubated at 37°C for 30 minutes in a mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM potassium citrate, 10 mM $MgCl_2$, 3.75 mM glutathione and 0.75 mg of bovine serum albumin per milliliter. The reaction was initiated by adding 40 μl of the preincubated mixture (containing 0.2 mU enzyme) to 760 μl assay mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM potassium citrate, 10 mM $MgCl_2$, 3.75 mM glutathione, 0.75 mg of bovin serum albumin per milliliter, 3.75 mM ATP, 0.125 mM acetyl CoA and 12.5 mM $KH^{14}CO_3$ (0.25 Ci/mol). After incubation at 37°C for 10 minutes, the reaction was terminated with 0.2 ml of 5 M HCl. The mixture was allowed to stand in a vacuum desiccator for 30 minutes and 0.5 ml aliquot was taken to dryness in a counting vial in a vacuum desiccator. After addition of 0.5 ml of distilled water and 5 ml of the scintillator (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-[2-(4-metyl-5-phenyloxazolyl)]benzene in 1 liter of toluene plus 0.5 liter of Triton X-100), the radioactivity was determined. Test samples were added to the mixture at the preincubation.

Results

Screening and Identification of Producing Microbial Strain

Among approximately 1,000 microbial strains tested for productivity of a metabolite that inhibits acetyl CoA carboxylase, one fungal strain was found to produce active metabolites. This fungal strain (M3180), which had been isolated from a soil sample collected in Tanashi-City, Tokyo, was identified as *Gongronella butleri* mating type (+) from its characteristics[11]. This microbial strain was able to form zygospore with *G. butleri* mating type (−) IFO 8081.

Fermentation and Isolation of Substance A

For the production of substance A, *G. butleri* M3180 was grown aerobically at 25°C for 5 days in a medium consisting of 3.5% glucose, 1.0% starch, 2.0% soy bean meal, 0.5% meat extract, 0.5% peptone, 0.2% NaCl, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% CB442 (Nihon Yushi Co.) (pH 5.8). Under these growth conditions, substances B, C and D were not detectably produced (see below). The cultured filtrate (10 liters) was adjusted to pH 3 with HCl and then extracted with ethyl acetate. The extract was dried over Na_2SO_4, concentrated to 100 ml under vacuum and then treated with an equal volume of 5% $NaHCO_3$. The aqueous layer was pooled, adjusted to pH 3 and reextracted with ethyl acetate. The pooled solvent layer was concentrated, and the resulting dried oil was applied onto a silica gel column (Wako gel C-200, 250 g) in C_6H_6 - CH_2Cl_2 (1: 1). The column was developed with C_6H_6 - CH_2Cl_2 (1: 1, 10 liters) and then CH_2Cl_2 - ethyl acetate (8: 2, 10 liters). The active fractions were collected and concentrated to dryness. The dried residue was applied onto a silica gel column (Wako gel C-200, 55 g), and the column was developed with C_6H_6 (2.2 liters), C_6H_6 - CH_2Cl_2 (1: 1, 2.2 liters), and then CH_2Cl_2 (2.2 liters). Under these conditions, greater part of the inhibitory activity was contained in the CH_2Cl_2 fractions. These fractions were submitted to a preparative HPLC (Jasco, silica ODS 10×250 mm) using a solvent of acetonitrile - 0.1% phosphoric acid

(45: 55), yielding 450 mg of substance A.

Fermentation and Isolation of Substances B, C and D

G. butleri M3180 was grown aerobically at 25°C for 4 days in a medium consisting of glycerol, 7.0%, soy bean meal 3.0%, peptone 0.8%, $NaNO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1% and CB442 0.01%. The cultured filtrate (1.7 liters) was adjusted to pH 3 with HCl and extracted with ethyl acetate. After dryness on Na_2SO_4 the extract was concentrated to 100 ml and then treated with 5% $NaHCO_3$. The aqueous layer was pooled, acidified and reextracted with ethyl acetate. The solvent layer was dried, concentrated to dryness and then submitted to silica gel chromatography (Wako gel C-200, 60 g). The column was developed with C_6H_6 (1.8 liters), C_6H_6 - CH_2Cl_2 (1: 1, 1.8 liters) and then CH_2Cl_2 (1.8 liters). The active CH_2Cl_2 fractions were collected and concentrated to dryness, giving 500 mg

Fig. 1. HPLC of acetyl CoA carboxylase inhibitors produced by *Gongronella butleri* M3180. Experimental conditions are described in the text.

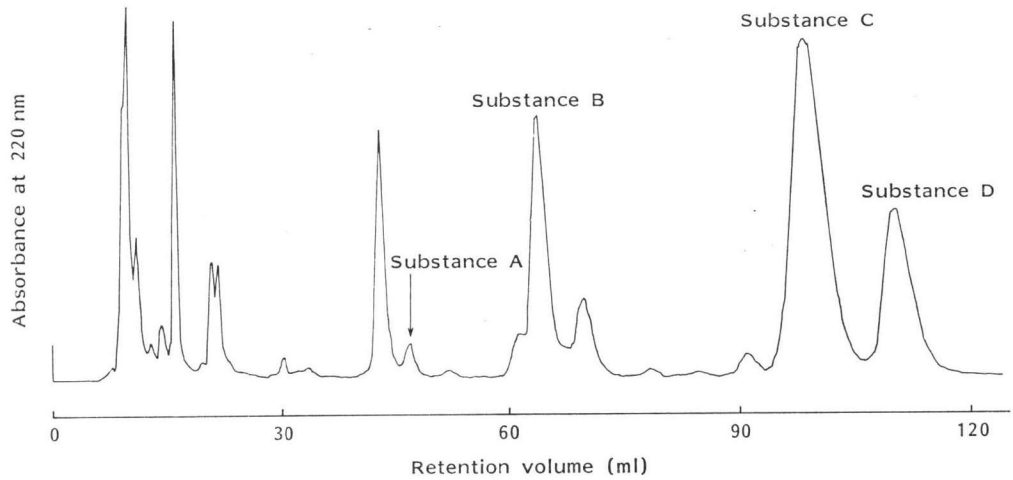


Table 1. ^{13}C NMR of acetyl CoA carboxylase inhibitors.

Substance A δ (ppm)	Substance B δ (ppm)	Substance C δ (ppm)	Substance D δ (ppm)
182.2 (s)	176.4 (s)	176.5 (s)	178.1 (s)
179.6 (s)	172.6 (s)	172.7 (s)	170.9 (s)
44.7 (d)	139.2 (d)	135.3 (s)	147.4 (d)
32.0 (t)	135.1 (s)	134.6 (d)	123.0 (d)
31.9 (t) 2C*	134.7 (d)	33.9 (t)	48.4 (d)
29.5 (—)	114.1 (t)	31.9 (t)	31.9 (t)
29.4 (—)	33.8 (t) 2C	29.6 (—)	31.8 (t)
29.2 (—)	29.5 (—)	29.4 (—)	29.6 (—) 2C
27.2 (t)	29.4 (—) 2C	29.3 (—) 3C	29.3 (—) 2C
26.6 (t)	29.1 (—)	28.9 (t)	29.2 (—)
22.7 (t)	28.9 (—) 2C	27.0 (t)	27.0 (t)
14.1 (q)	26.8 (t)	22.7 (t)	22.7 (t)
		14.1 (q)	14.1 (q)

These spectra were measured with $CDCl_3$ as a solvent, using TMS as internal standard in 50 MHz Jeol Ltd. FX-200 system.

Multiplicities; s, singlet; d, doublet; t, triplet; q, quartet; —, not distinguishable.

* Carbon numbers determined in gated non-NOE decoupling.

of dried residue. This residue was submitted to a preparative HPLC (silica ODS, 10×250 mm) using a solvent system of acetonitrile - 0.1% phosphoric acid (45: 55). Under these conditions, four active peaks (substances A, B, C and D) were observed (Fig. 1), each of which was collected, giving substance B (15 mg), substance C (55 mg) and substance D (30 mg).

Structure of Substance A

The FD-MS spectrum and elemental analysis for substance A established $C_{13}H_{24}O_4$ (molecular weight 244) as the molecular formula. The EI-MS analysis gave a base peak at *m/z* 114 (M−130) indicated $[HOOCCH_2CH_2CO]^+$ and a relevant peak at *m/z* 208 (M−$2H_2O$). The IR spectrum showed a carboxylic acid dimer function at 1710 cm^{-1}. The UV spectrum gave the only end absorption. Methyl ester of this compound, synthesized by diazomethane treatment, gave a molecular ion M^+ 272. These data indicated that substance A is a dicarboxylic acid. The ^{13}C NMR spectrum showed the presence of one methyl carbon, nine methylene carbons, one methine carbon and two carboxylic carbons (Table 1). From these findings it was concluded that substance A is to be octyl pentanedioic acid (Fig. 2). This conclusion was also supported by the data of ^1H NMR spectral analysis (Fig. 3). Further, the structure of substance A was confirmed by direct comparison with synthesized authentic sample of octyl pentanedioic acid[12].

Structure of Substance B

Substance B and its methyl ester gave molecular weights of 268 ($C_{15}H_{24}O_4$) and 296 ($C_{17}H_{28}O_4$), respectively. The IR spectrum showed carboxylic acid at 1732 cm^{-1}, α,β-unsaturated carboxylic acid at 1682 cm^{-1} and vinyl group at 995 and 910 cm^{-1}. The UV spectrum gave a λ_{max} at 216 nm (ε 10,700), indicating the presence of α,β-

Fig. 2. The structure of acetyl CoA carboxylase inhibitors produced by *G. butleri* M3180.

Substance A: $CH_3(CH_2)_7CH(COOH)CH_2CH_2COOH$
Octyl pentanedioic acid

Substance B: $CH_2=CH(CH_2)_5C(COOH)=CHCH_2COOH$
9-Decenyl-1-pentenedioic acid

Substance C: $CH_3(CH_2)_9C(COOH)=CHCH_2COOH$
Decanyl-1-pentenedioic acid

Substance D: $CH_3(CH_2)_9CH(COOH)CH=CHCOOH$
Decanyl-2-pentenedioic acid

Fig. 3. ^1H NMR of acetyl CoA carboxylase inhibitors produced by *G. butleri* M3180.

A, Substance A; B, substance B; C, substance C; D, substance D.

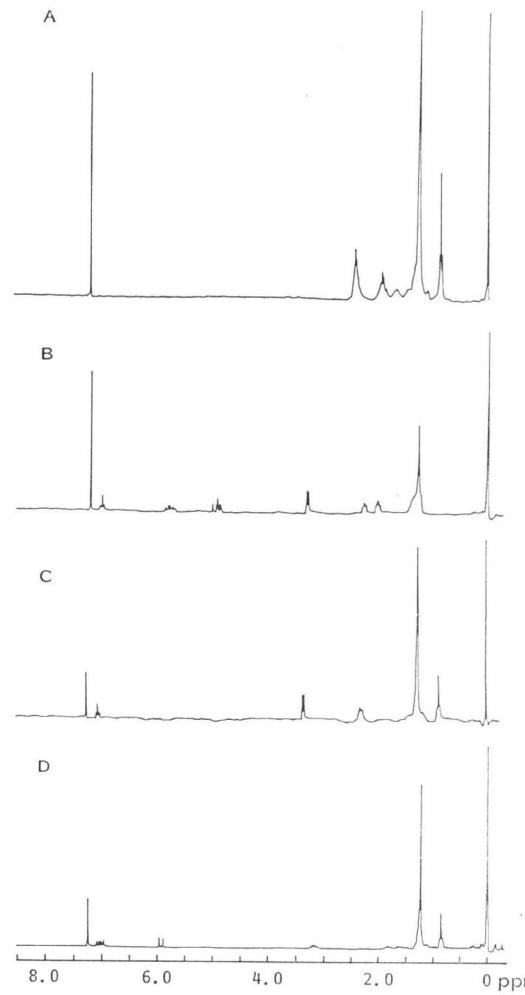
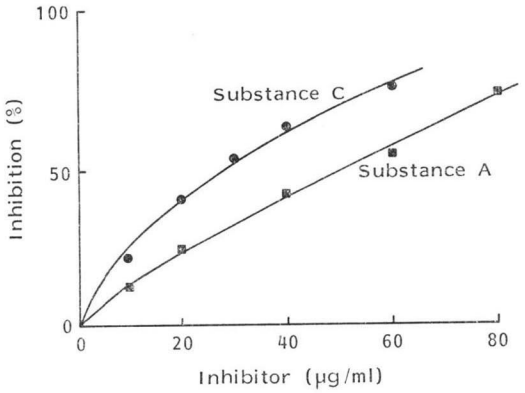


Fig. 4. Inhibition of rat liver acetyl CoA carboxylase by substances A and C.

Assay conditions are described in Materials and Methods, and the indicated concentrations of inhibitors are at preincubation.



unsaturated carboxylic acid. In ^{1}H NMR (Fig. 3), two terminal methylene protons were present at 4.8~5.1 ppm and one proton of α,β-conjugated carboxylic acid at 7.05 ppm. Considered together with the ^{13}C NMR spectrum data (Table 1), substance B was identified to be 9-decenyl-1-pentenedioic acid (Fig. 2).

Structure of Substance C

Molecular formula of substance C and its methyl ester were $C_{15}H_{26}O_4$ (M^+ 270) and $C_{17}H_{30}O_4$ (M^+ 298), respectively. The IR spectrum showed carboxylic acid at 1700 cm^{-1} and α,β-unsaturated carboxylic acid at 1660 cm^{-1}. The UV spectrum gave a λ_{max} at 215 nm (ε 9,000). The ^{1}H NMR spectrum (Fig. 3) and ^{13}C NMR spectrum indicated that substance C is a terminal methylene saturated derivative of substance B, decanyl-1-pentenedioic acid (Fig. 2).

Structure of Substance D

Substance D was converted spontaneouly to substance C on storage in $CHCl_3$, CH_3CN and CH_3OH solution (data not shown). The UV spectrum showed a λ_{max} at 207 nm (ε 9,700), indicating that location of the carbon double bond of substance D was displaced during its conversion to substance C. Two of the three methine carbons present in substance D were converted to one methylene and one quaternary carbon in substance C (Table 1 and Fig. 3). These data suggested the structure of substance D to be decanyl-2-pentenedioic acid (Fig. 2).

Inhibition of Acetyl CoA Carboxylase

The relationship between concentration and inhibition of rat liver acetyl CoA carboxylase by substances A and C are given in Fig. 4. The concentrations required for 50% inhibition were 55 μg/ml for substance A, 40 μg/ml for substance B, 30 μg/ml for substances C and D, respectively.

Discussion

Four active compounds that inhibit acetyl CoA carboxylase have been isolated from cultured broth of *G. butleri* mating type (+) M3180. These compounds are structurally related to each other having a structure of pentanedioic acid or pentenedioic acid with a fatty long chain. All of these compounds are new natural products, although substance A was first synthesized by other group[12].

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