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MODE OF ANTIBACTERIAL ACTION OF RETROCHALCONES FROM *GLYCYRRHIZA INFLATA*

HIROYUKI HARAGUCHI,* KATSUHIKO TANIMOTO, YUKIYOSHI TAMURA,† KENJI MIZUTANI† and TAKESHI KINOSHITA‡

Faculty of Engineering, Fukuyama University, Gakuen-cho, Fukuyama 729-02, Japan; † Biology and Chemistry Laboratory, Maruzen Pharmaceutical Co., Ltd, Sanada Takanishi-cho, Fukuyama 729-01, Japan; ‡ Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko-machi, Tsukui-gun, Kanagawa 199-01, Japan

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Key Word Index—*Glycyrrhiza inflata*; Leguminosae; retrochalcone; licochalcone; echinatin; antimicrobial activity; respiratory inhibition; electron transport.

Abstract—Licochalcone A–D and echinatin, retrochalcones isolated from the roots of *Glycyrrhiza inflata*, showed antimicrobial activity. Among them, licochalcone A and C had potent activity against some Gram-positive bacteria. These retrochalcones inhibited oxygen consumption in susceptible bacterial cells. The oxidation of NADH in bacterial membrane preparations was also inhibited by them. NADH-cytochrome *c* reductase was inhibited by licochalcones, while cytochrome *c* oxidase was not. NADH-CoQ reductase and NADH-FMN oxidoreductase were not inhibited. The site of respiratory inhibition of licochalcones was thought to be between CoQ and cytochrome *c* in the bacterial respiratory electron transport chain.

INTRODUCTION

Liquorice (root and rhizome of *Glycyrrhiza* spp.) is currently used in the tobacco, confectionery, and pharmaceutical industries. It has been used for centuries as a medicine because of its wide-ranging therapeutic properties including relief of rheumatic and other pain and its healing effect on ulcers [1]. Liquorice is known to contain the sweet principle glycyrrhizin, a biologically active triterpene glycoside. It also contains various flavonoids, isoflavonoids, chalcones and coumarins [1]. In a search for new bioactive components from the roots of *Glycyrrhiza inflata* [2–5], one of the main botanical source of liquorice, two new chalcones, licochalcone C and D, were isolated together with the three known chalcones echinatin, licochalcone A and B [5]. These chalcones are distinguished from ordinary chalcones by the absence of an oxygen-function at the 2-position, and are called retrochalcones [5]. This paper reports on the antimicrobial activity of these retrochalcones and studies on their mechanism of action. The structures of the retrochalcones found in *G. inflata* are listed in Fig. 1.

* Author to whom correspondence should be addressed.

RESULTS

The antimicrobial activity of retrochalcones in *G. inflata* against various bacteria, yeasts and fungi was examined by the broth dilution method. Their minimum inhibitory concentrations (MIC) are listed in Table 1. Licochalcone A–D and echinatin inhibited the growth of Gram-positive bacteria. Licochalcone A and C showed a small effect on fungal growth. Among the retrochalcones from *G. inflata*, licochalcone A showed the most potent antibacterial activity.

The effects of licochalcone A on macromolecule (DNA, RNA, and protein) biosynthesis were examined using radioactive precursors according to the method previously described [6, 7]. Licochalcone A inhibited the incorporation of radioactive thymidine, uracil and leucine into the corresponding macromolecules in *Micrococcus luteus* cells to a similar extent (data not shown). This suggested that licochalcone interfered with energy metabolism in the same way as several respiratory inhibiting antibiotics [8–10], since energy is required for the active uptake of various metabolites and for the biosynthesis of macromolecules.

The effects of retrochalcones from *G. inflata* on respiratory activities in various bacterial cells are

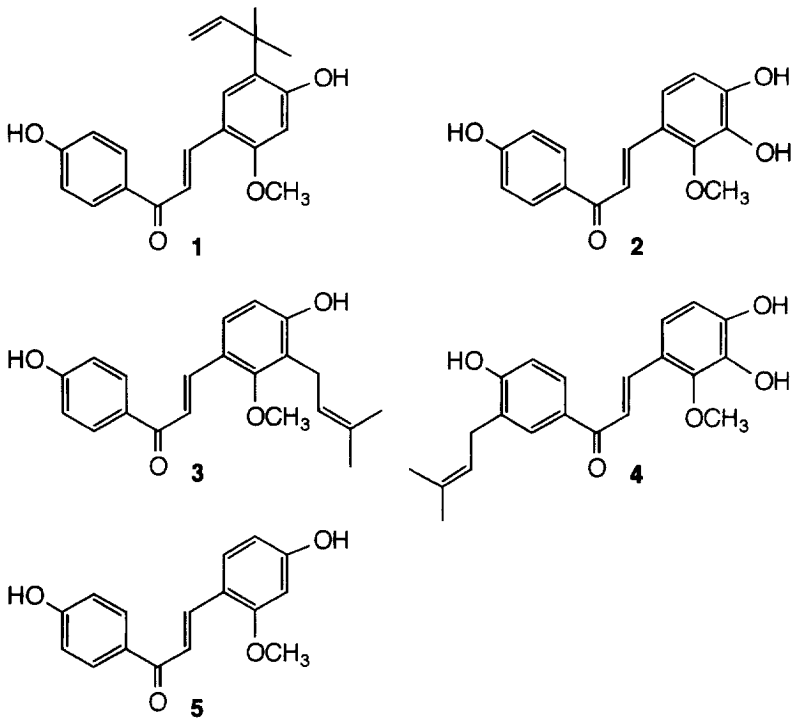

Fig. 1. Structure of retrochalcones isolated from *G. inflata*. 1, licochalcone A; 2, licochalcone B; 3, licochalcone C; 4, licochalcone D and 5, echinatin.

Table 1. Antimicrobial activity of retrochalcones from *G. inflata*

Microorganisms	MIC (μg/ml)				
	1	2	3	4	5
Bacillus subtilis IFO 3060	3.13	100	12.5	50	25
Staphylococcus aureus IFO 3007	1.56	100	6.25	50	25
Micrococcus luteus IFO 3333	1.56	50	6.25	50	25
Escherichia coli HUT 215	>100	>100	>100	>100	>100
Pseudomonus aeruginosa JCM 2776	>100	>100	>100	>100	>100
Saccharomyces cerevisiae IFO 0203	>100	>100	50	>100	>100
Candida albicans TIMM 0134	>100	>100	>100	>100	>100
Mucor pusillus HUT 1185	12.5	>100	100	>100	>100
Asperigillus niger IFO 4343	>100	>100	>100	>100	>100

shown in Table 2. Freshly harvested cells of *M. luteus*, *E. coli* and *S. aureus* have high respiratory rates. When the suspensions were incubated with licochalcone A, oxygen consumption by *M. luteus* and *S. aureus* was strongly inhibited. The respiration of *E. coli* was insensitive to licochalcones. This result is in agreement with the antimicrobial spectrum (Table 1).

The effects of licochalcone A and C, which showed potent antibacterial and respiratory inhibitory activity, on the oxidation of NADH by the respiratory chain of membrane fractions prepared from various bacterial cells were examined. Licochalcone A and C were both effective inhibitors of bacterial NADH oxidase. Their 50% inhibitory concentrations are listed in Table 3. NADH oxidase in the membranes of Gram-negative bacteria, *E. coli* and *P. aeruginosa*, whose cell growth and cellular respiration were unaffected by licochalcones, was also inhibited by them. This suggests these retrochalcones are not able to permeate into Gram-negative bacterial cells.

The inhibition of respiratory electron transport by antimicrobial licochalcones was prior to cytochrome *c*. As shown in Table 4, NADH-cytochrome *c* reductase in the membrane fraction of *M. luteus* was inhibited by licochalcone A and C, which showed potent inhibition on bacterial growth and cellular respiration. On the other hand, cytochrome oxidase, which was measured as ascorbate-TMPD oxidase [11].

Table 2. Effect of retrochalcones from *G. inflata* on respiratory activity of bacterial cells

Microorganism	IC_{50} (μM)*				
	1	2	3	4	5
M. luteus IFO 3333	3.0±0.4	>100	53.3±3.5	>100	>100
Staph. aureus IFO 12732	22.7±1.6	>100	58.6±2.9	74.9±5.2	>100
Staph. aureus 11D 671	23.6±2.1	>100	55.9±4.1	>100	>100
E. coli IFO 3545	>100	>100	>100	>100	>100

* Inhibitory activity was expressed as the mena of 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration-inhibition curves.

Table 3. Effect of retrochalcones from *G. inflata* on NADH oxidase in bacterial membrane

Microorganism	IC_{50} (μM)*	
	1	3
M. luteus	6.2±0.5	3.0±0.2
Staph. aureus	8.0±0.7	23.4±2.0
P. aeruginosa	18.9±1.3	13.9±1.4
E. coli	9.5±0.7	7.4±0.5

* Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration-inhibition curves.

Table 4. Effect of retrochalcones from *G. inflata* on respiratory chain in bacterial membrane

Enzyme activity	IC_{50} (μM)*	
	1	3
NADH oxidase	6.2±0.5	3.0±0.2
NADH-cytochrome *c* reductase	14.8±1.6	29.6±2.7
Ascorbate-TMPD oxidase	>100	>100
NADH-CoQ reductase	>100	>100
NADH-FMN oxidoreductase	>100	>100

* Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration-inhibition curves.

was not affected by these licochalcones. These observations suggested that the site of electron transport inhibition by antimicrobial retrochalcones in *G. inflata* is prior to cytochrome *c*. To clarify the site of inhibition of licochalcones, NADH-CoQ reductase in the membrane fraction was measured. Licochalcone A and C showed no effect on NADH-CoQ reductase in *M. luteus* (Table 4). This result suggested the site of respiratory inhibition of antimicrobial licochalcones is not before coenzyme Q. This was confirmed by the effect on NADH-FMN oxidreductase from *Vibrio harveyi*. Retrochalcones from *G. inflata* had no effect on this enzyme preparation.

DISCUSSION

The respiratory chains of bacterial membranes consist of a primary dehydrogenase, coenzyme Q, cytochrome *b*, *c*, *o*, and *a* [12, 13], and constituents unique to bacteria. Figure 2 shows the respiratory electron transport chains of various bacteria and the sites of action of known inhibitors. Antibacterial retrochalcones from *G. inflata* inhibited NADH-cytochrome *c* reductase, but not cytochrome oxidase. Furthermore, they did not affect NADH-CoQ reductase. These results suggested that the inhibition site of retrochalcone from *G. inflata* would be between CoQ and cytochrome *c* in the bacterial respiratory chain.

In many cases, rotenone, amytal and antimycin, potent inhibitors of mitochondrial respiratory chain in mammalian cells, are not effective in bacterial electron transport system [14, 15]. In our experiments, rotenone and antimycin A at 10 μg/ml had no effect on either cellular respiration or membrane NADH oxidation in *M. luteus* and *P. aeruginosa*. However, retrochalcones from *G. inflata* were effective inhibitors of the bacterial respiratory chain. Among the retrochalcones from *G inflata*, licochalcone A and C showed potent antibacterial activity. These two compounds each have a prenyl chain in ring B, whereas other chalcones do not. Licochalcone A and C were also effective inhibitors of bacterial respiration in whole cells and NADH oxidase in bacterial membranes. However, licochalcone B and D were also effective as inhibitors of membrane NAHD oxidase (data not shown). These findings may indicate that the permeability of the cell to the test compounds is one of the determining factors to their exhibiting antibacterial activity. In this context the presence of a hydrophobic prenyl moiety would be important since it provides enough hydrophobicity for molecules to penetrate into the cell membrane. The finding that licochalcone D is less active than licochalcones A and C, might be due to its reduced hydrophobicity as a consequence of the prenyl group being located in ring A, as a consequence of which the B ring is more hydrophylic than that of licochalcones A and C due to the presence of two hydroxyl groups. The prenyl group is biogenetically derived from mevalonate, and a large number of flavonoids possessing this group

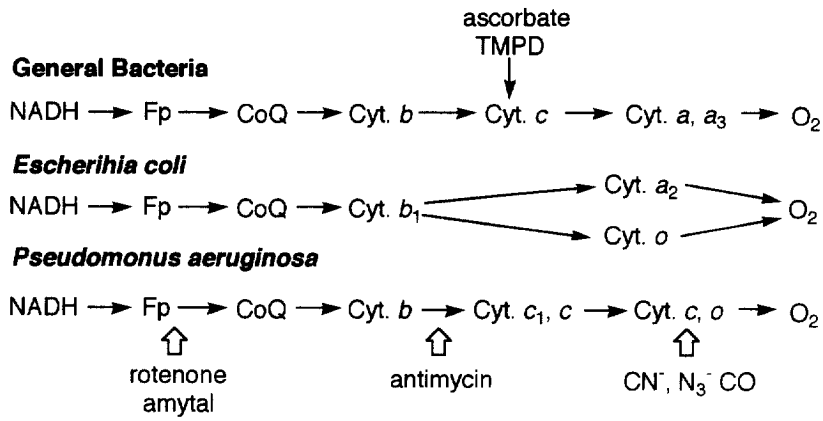

Fig. 2. Respiratory chains of various bacteria and inhibition site of electron transport inhibitors. Fp; flavoprotein (NADH dehydrogenase), CoQ; coenzyme Q, Cyt; cytochrome.

have been reported [16]. Though the prenyl group contributes to the structural diversity of phenolic compounds, it has not so attracted so much attention in terms of its influence on the biological activity of host compounds. Thus the results reported in this paper are of particular significance.

The inhibition of the bacterial respiratory chain may lead to some anxiety that the retrochalcones in *G. inflata* could affect mammalian mitochondria. However, the respiratory chains of bacteria differed from each other and from those of mitochondria [17, 18]. Therefore, some drugs which act as electron transport inhibitors show different effectiveness towards different organisms [19, 20].

EXPERIMENTAL

Chemicals

Licochalcones A–D and echinatin were isolated from the roots of *Glycyrrhiza inflata* [5]. Cytochrome *c*, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and CoQ_0 were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). NADH-FMN oxidoreductase was obtained from Toyobo Co. (Osaka, Japan). Other chemical reagents were of commercial grade.

Assay of antimicrobial activity

The minimum growth inhibitory concentrations (MIC)were measured by the 2-fold serial broth dilution method. Bacteria were cultured in 3% nutrient broth at 37°. Yeasts and filamentous fungi were cultured in 2.5% malt extract at 25°. After 2 days, the growth of bacteria and yeasts was measured spectrometrically (A at 660 nm), and that of filamentous fungi by the naked eye. MIC was defined as the lowest concentration of the test compounds at which growth was below 0.03 A or not visible [21].

Measurement of oxygen uptake

Exponentially growing bacterial cells were harvested and washed with saline by centrifugation. The cells were suspended in 50 mM phosphate buffer (pH 7.0) to give approximately 1 mg dry cells/ml. The test compound dissolved in dimethyl sulphoxide was added to the reaction mixture and the O_2 consumption was measured polarographically at 30° with an OBH 100 oxygen electrode (Otsuka Electronics Co.) [6].

Preparation of bacterial cell membrane

Bacterial cells were harvested by centrifugation and then washed twice with distilled water. The cell paste was suspended in 50 mM Tris–Hcl buffer (pH 7.4) containing 0.5 M sucrose and 20 mM $MgCl_2$, and then disrupted by ultrasound using a Sonifier 450 (Branson) at 10 kc for 2 min at 4°. After centrifugation at $15\,000 \times g$ for 20 min, the supernatant was centrifuged at $105\,000 \times g$ for 90 min. The resultant precipitate was washed by centrifugation at $105\,000 \times g$ for 60 min with 10 mM Tris–Hcl buffer (pH 7.4) containing 0.5 M sucrose and 10 mM $MgCl_2$. The precipitate was resuspended in the same buffer [22].

Assay of enzyme activity

NADH oxidase activity was assayed by measuring the decrease in the absorbancy at 340 nm. The reaction mixture contained 0.1 M Tris–Hcl buffer (pH 7.5), 200 μM NADH and membrane fraction (equivalent to 2 mg protein) [23]. NADH–cytochrome *c* reductase was measured by the increase in the absorbancy at 550 nm resulting from the reduction of cytochrome *c* [24]. The reaction was carried out in the same soln as the assay of NADH oxidase supplemented with 5 mM NaN_3 and 0.7 mg/ml horse heart cytochrome *c*. Ascorbate–TMPD oxidase was assayed by measuring the O_2 consumption polarographically [25]. The reac-

tion mixture contained 0.1 M Tris–Hcl buffer (pH 7.5), 0.1 mM TMPD, 2 mM ascorbate and membrane fraction. NADH–CoQ reductase was assayed by measuring the decrease in the absorbance due to NADH oxidation at 340 nm [26]. The reaction was carried out in the same soln at the assay of NADH oxidase supplemented with 20 mM NaN_3 and 0.1 mM CoQ_0. NADH–FMN oxidoreductase was measured by the decrease in the absorbance at 340 nm. The reaction mixture was composed of 44.2 mM phosphate buffer (pH 7.0), 17 μM FMN, 130 μM NADH and enzyme preparation [27].

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