Inhibitory Effect of *Isodon* Diterpenoids on Growth and Mitochondrial Oxidative Phosphorylation in Lepidopterous Insects

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The diterpenes from the plants of the genus *Isodon* (Labiateae) exhibited a relatively specific growth inhibitory activity against lepidopterous larvae. The α -methylene cyclopentanone moiety (D-ring) was essential for this activity. In addition, these compounds strongly inhibited the oxidative phosphorylation in mitochondria isolated from silkworm midgut.

A number of bitter ent-kaurenoid diterpenes have been isolated from the plants of the genus *Isodon* (Labiateae).^{1,2)} These compounds possess a specific antimicrobial activity against gram positive bacteria, cytotoxic activity against KB tissue culture and inhibitory activity on oxidative phosphorylation in rat liver mitochondria.^{3,4)} These bitter principles contain, as a major structural feature, an α -methylene cyclopentanone moiety (D-ring), which apparently is essential for their biological activity since all their dihydro-derivatives are entirely inactive. However, this moiety has little direct relationship to the bitter taste. We have proposed a qualitative theory to relate the bitter taste of these compounds to their chemical structure.⁵⁾ For a compound in this series to taste bitter, it must contain at least one "bitter unit," where the bitter unit consists of hard acid (HA) and hard base (B) moieties positioned so they can form an intramolecular hydrogen bond. In our recent studies of chemical factors in various plants resistant to the larvae of the African army worm, *Spodoptera exempta*, we have found that the *Isodon* bitter diterpenoids show moderate antifeeding activity in a "choice" test and relatively specific growth inhibitory activity in a "non-choice" test. Their bitter dihydro-derivatives still show antifeeding activity but no growth inhibitory activity.

In the present paper, inhibitory activity of

these diterpenes toward lepidopterous insects was investigated in reference to the growth and mitochondrial oxidative phosphorylation.

MATERIALS AND METHODS

Diterpenoids. The *Isodon* diterpenoids and their dihydro-derivatives used in this study were all analytically pure samples obtained from our previous studies.⁶⁻¹⁰⁾ The selection of these compounds was based mainly upon the availability of the samples as well as their characteristic structural types.

Insects. The larvae of the African army worm, *Spodoptera exempta*, were taken from standard stock cultures, which were reared throughout the year in the International Centre of Insect Physiology and Ecology, Nairobi, Kenya. The monophagous, *S. exempta* is well known as a major graminaceous crop pest in East and South Africa.

The larvae of the silkworm, *Bombyx mori* of the F₁ hybrid between two races, Showa and Kinshu, were supplied by Otsuka Pharmaceutical Co., Ltd.

Feeding and growth tests. Antifeeding and growth inhibitory activities against the third instar larvae of army worm were tested by the leaf disk method with *Zea mays*, a normal food-plant for *S. exempta*.¹¹⁾ Leaf disks, 20 mm in diameter, were punched out with a cork borer. Test disks were immersed for 2 seconds in an acetone solution of the sample. Control disks were immersed only in acetone. The disks were then dried in air. Five test and five control disks were placed in a Petri dish with ten third instar larvae. This constituted a "choice" test, whereas deletion of the control disks constituted a "non-choice" test.

Growth test on the third instar larvae of silkworm was conducted according to the method described by Murakoshi *et al.*¹²⁾

Preparation of mitochondrial suspensions. Midgut tissues were collected from the fourth instar larvae of silkworm and the peritrophic membrane containing the midgut content was removed.¹³⁾ The resultant tissues were washed and homogenized with an ice-cold solution of 150 mM KCl and 1 mM EDTA in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $150\times g$ for 3 min, and the supernatant again centrifuged at $8000\times g$ for 8 min. The sedimented mitochondrial pellet was rinsed once and suspended in the same solution.

Protein content of the mitochondrial suspensions was determined by the biuret reagent after the addition of an equal volume of 5% Triton X-100,¹⁴⁾ bovine serum albumin being used as a standard.

Measurement of mitochondrial oxidative phosphorylation activity. The reaction mixture contained 30 mM K_2HPO_4 , 50 mM Tris-HCl, 15 mM KCl, 5 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA, test compound, 0.8~0.9 mg of mitochondrial protein, 20 mM pyruvate and 10 mM malate in a total volume of 2 ml at pH 7.5.

Oxygen consumption was measured polarographically with a Yanagimoto PO-100A oxygen electrode equipped with a YR-101P chart recorder. Oxidative phosphorylation activity was calculated from the oxygen electrode tracings¹⁵⁾ and expressed as a respiratory control ratio and an ADP/O ratio. The phosphorylation rate was calculated as the ADP/O ratio $\times$ the rate of respiration in State 3.

RESULTS AND DISCUSSION

The antifeeding and growth inhibitory activities of several *Isodon* diterpenes (1~5) as well as their dihydro-derivatives (6~10) have

been tested against the third instar larvae of the African army worm. All compounds which taste bitter to humans^{5,16)} prevented feeding for at least 2 hr at 100 ppm concentration (ca. $2 \mu g/cm^2$ leaf disk) in a "choice" test. However, at this concentration none of these bitter compounds showed antifeeding activity after 24 hr in a "non-choice" test. Isodonal (1) and enemini (2) showed only 50% antifeeding activity even at 1000 ppm concentration (ca. $20 \mu g/cm^2$ leaf disk) after 24 hr. The major portion ($>80\%$) of the treated disk was eaten under conditions of severe starvation. However, even when the larvae were returned to normal diet after eating the treated disk, their growth was strongly inhibited and they eventually died. The effect of these compounds was assessed either by weight loss or by reduced rate of weight gain as compared with controls (Fig. 1), as well as by the number of deaths. Interestingly, the α -methylene cyclopentanone moiety (D-ring) was an essential structural feature for this activity since reduction of the methylene group caused loss of the activity.

Recently Hosozawa *et al.* have proposed the term of "relative antifeedant" to the antifeeding substance which retards feeding over a defined time and, after this defined time, quickly loses the antifeeding activity.¹⁷⁾ It seems therefore that this term may be used to describe the *Isodon* bitter substances. However, it should be noted that antifeedants, as defined, do not directly kill insects.¹⁸⁾ Thus, these diterpene compounds are, in fact, growth

FIG. 1. Effect of Isodonal (1) and Dihydroisodonal (6) on the Growth of *S. eximpta*.

In a "non-choice" test, weight of surviving larvae was measured during the period just before control larvae begin to pupate. Each point is mean with S. D. (bar) of 10 insects. ×—×, control; ○—○, 100 ppm isodonal (1); ●—●, 100ppm dihydroisodonal (6).

inhibitors or biological poisons. These diterpenes appear to act slowly since the larvae consume more than 20 times the lethal dose (oral LD_{100} 1~10 μ g per insect) before becoming unable to feed. Similar toxic effect was also observed on the third instar larvae of the silkworm (oral LD_{100} 0.1~1 μ g per insect).

These compounds did not affect the feeding behavior of many other insects (e.g., the housefly, *Musca domestica* and the antestia bug, *Antestiopsis spp*¹⁹⁾). The growth inhibitory properties seem relatively specific for lepidopterous larvae.

Recently we have reported that these diter-

penoids show an inhibitory effect on some energy-linked functions in rat liver mitochondria and the α -methylene cyclopentanone moiety is also essential for this effect.⁴⁾ Accordingly, it is of interest to examine the effect of these compounds on the mitochondrial functions of lepidopterous larvae to understand the biochemical aspects involved. The silkworm midgut was used as a lepidopterous mitochondrial source because its mitochondria were relatively stable.

As shown in Table I, these diterpenes (1~4), at a concentration of 250 μ M, strongly inhibited the mitochondrial oxidative phosphorylation activity (expressed both as a respiratory control ratio and an ADP/O ratio) and the phosphorylation rate. This may indicate that the growth inhibition by these compounds in lepidopterous insects occurs through the inhibitory action on mitochondria.

These compounds significantly depressed the rate of respiration in State 3 and in 2,4-dinitrophenol uncoupled mitochondria (State 4U), though hardly affected or only a little stimulated that in State 4. Thus, they seem to act neither as an uncoupler (e.g., 2,4-dinitrophenol) nor as a phosphorylation inhibitor (e.g., oligomycin), because the former markedly stimulates the respiratory rate in State 3 and State 4, while the latter inhibits the respiratory rate in State 3 but shows no effect in State 4 and State 4U. These diterpenoids seem to have an action on the respiratory chain enzymes

TABLE I. EFFECT OF DITERPENOIDs ON OXIDATIVE PHOSPHORYLATION ACTIVITY IN MITOCHONDRIA ISOLATED FROM SILKWORM MIDGUT

Compounds ^a	Respiratory rate ^b			Respiratory control ratio	ADP/O	Phosphorylation rate ^d
	State					
	3	4	4U ^c			
Control	85.6	21.3	102.7	4.02	2.21	189
Isodonal (1)	40.9	23.8	55.2	1.72	1.09	45
Enemin (2)	71.6	22.5	91.6	3.18	1.61	115
Nodosin (3)	54.9	25.2	74.6	2.18	1.40	77
Oridonin (4)	65.4	23.6	90.3	2.77	1.61	105

^a The final concentration of each compound was 250 μ M.

^b Expressed as natoms O/mg protein/min.

^c In the presence of 2,4-dinitrophenol at 50 μ M.

^d Expressed as nmoles ATP synthesized/mg protein/min.

similar to that of cyanide, rotenone and antimycin A, although detailed studies on the site of action are needed.

We have previously reported that the α -methylene cyclopentanone moiety of these diterpenoids has a high reactivity toward sulfhydryl groups.^{2,3,20)} Also, recent reports show that sulfhydryl groups in the mitochondrial membrane are largely responsible for energy-linked functions.²¹⁻²³⁾ It seems, therefore, to be of interest to investigate whether these diterpenoids directly affect the function of respiratory chain enzymes in which sulfhydryl groups play an important role. Their antimicrobial and antitumor activities may also have a close relation to inhibition in the respiratory chain, as in the case of insect growth inhibitory activity.

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