Comparison of Larvicidal, Adulticidal and Acaricidal Activity of Two Geometrical Butylidenephthalide Isomers

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Insecticidal and acaricidal activities of two geometrical isomers, (*E*)- and (*Z*)-butylidenephthalide isolated from *Angelica acutiloba*, against larvae and adults of fruit fly (*Drosophila melanogaster*), cat fleas (*Ctenocephalides felis*) and house dust mites (*Dermatophagoides farinae* and *Tyrophagus putrescentiae*) were investigated and compared with that of positive controls. (*E*)- and (*Z*)-Butylidenephthalide exhibited 50% lethal concentration (LC_{50}) values of 2.07 and 0.94 μmol/ml of diet concentration against larvae of *D. melanogaster*, respectively. This indicated that two isomers of butylidenephthalide have geometrical stereoselectivity for larvicidal effect. Even though both (*E*)- and (*Z*)-butylidenephthalide also showed potent adulticidal and acaricidal activity against adults of *D. melanogaster* and two mites, there was no significant difference between two isomers. Insecticidal activity of both (*E*)- and (*Z*)-butylidenephthalide toward adults of *C. felis* was not detected even at the maximum concentration of 200 μg/cm^2.

Key words butylidenephthalide; geometrical isomer; insecticidal activity; acaricidal activity

Butylidenephthalide, the components of *Angelica acutiloba* var. sugiyamae HIKINO (Apiaceae) has the biological activities, such as centrally acting muscle relaxant[1] and antispasmodic effects[2] with characteristic odor. In our previous work,[3] (*Z*)-butylidenephthalide was isolated as the potent insecticidal compound against adults and larvae of *Drosophila melanogaster.* Recently, Ahn *et al.* reported that butylidenephthalide was active constituent of *Cnidium officinale* against *Dermatophagoides farinae* and *Tyrophagus putrescentiae*, and more effective than acaricide, *N*,*N*-diethyl-*m*-toluamide (DEET).[4,5] Therefore, butylidenephthalide is the potential candidate for new natural pesticide. However, above report was unclear on stereochemistry of butylidenephthalide even though both (*E*)- and (*Z*)-butylidenephthalide were found from natural resources, and (*E*)-butylidenephthalide has not been tested against insects and mites.

Geometrical stereoselectivity is often crucial on biological activities. Asahina *et al.* reported that synthetic fluoroquinolone derivatives were tested for antibacterial activity, and (*Z*)-isomers exhibited 2- to 32-fold more potent than corresponding (*E*)-isomers.[6] Against insects, the importance of geometry was discussed that binding affinity of juvenile hormone carrier protein from the hemolymph of *Manduca sexta* was decreased as (*Z*)-double bond are substituted for (*E*)-double bond in synthetic juvenile hormone analogues.[7] Therefore, evaluation of biological activity for geometrical isomers is required.

Biological activity of (*E*)-butylidenephthalide against insects and mites has not been previously reported. In this paper, we investigated biological activities of (*E*)-butylidenephthalide against larvae and adults of *D. melanogaster*, adults of *Ctenocephalides felis* and two house dust mites, *D. farinae* and *T. putrescentiae*, and compared with (*Z*)-butylidenephthalide to investigate geometrical stereoselectivity. Rotenone (positive control for insecticidal activity) and DEET (positive control for acaricidal activity) were also tested.

MATERIALS AND METHODS

GC and GC-MS Analysis GC was performed on a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (FID). The column was a fused silica capillary column (DB-5, 30 m length, 0.25 mm i.d.). Chromatographic conditions were as follows: oven temperature was programmed from 80 to 250 °C at 4 °C/min and held at 250 °C for 15 min; injector and detector temperature were 270 and 280 °C, respectively; carrier gas, helium at a flow rate of 40 cm/s. GC-MS was performed on a Hewlett-Packard 5972A mass selective detector interfaced with a Hewlett-Packard 5890A gas chromatograph fitted with a capillary column (HP-5MS, 30 m length, 0.25 i.d.). Chromatographic conditions were the same for the GC analysis.

Chemicals (*Z*)-Butylidenephthalide was previously isolated from *Angelica acutiloba*.[3] (*E*)-Butylidenephthalide was isolated from chloroform extract of *A. acutiloba* by SiO_2 (200 mesh) column chromatography (hexane : ethyl acetate = 93 : 7), repeatedly. The purity and structure of two isomers

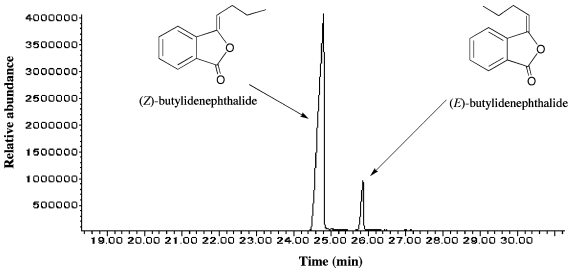

Fig. 1. GC-MS Analysis and the Structure of Two Geometrical Butylidenephthalide Isomers

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were determined by GC-MS and NMR spectroscopic analysis according to Fischer and Gijbels.[8] Whereas total amount of (*E*)-butylidenephthalide isolated from *A. acutiloba* was too small to assess biological activity, we isolated (*E*)-butylidenephthalide from commercially available butylidenephthalide (*E*/*Z* mixture) purchased from Lancaster Synthesis Ltd. (Lancashire, U.K.) using SiO_2 column chromatography. Their identity to the natural compounds was determined compared to the spectral data (NMR, GC, GC-MS) of isolated compounds from *A. acutiloba* and literature data.[8] Both (*E*)- and (*Z*)-butylidenephthalide used for assay was >99% purity determined by GC and GC-MS analysis. Rotenone was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). DEET was purchased from API corporation (Osaka, Japan).

Insects *D. melanogaster* used in insecticidal test originated from The University of Tokyo (Tokyo, Japan). The colony of *D. melanogaster* has been maintained without exposure to any insecticides at 25 °C, relative humidity (RH) >60%, and 12 : 12 light : dark condition. *C. felis* used for insecticidal test were collected at Sakai city (Osaka, Japan), and maintained using previous method[9] at Osaka Seiyaku Co. Ltd. (Osaka, Japan).

Mites *D. farinae* and *T. putrescentiae* used for acaricidal test were obtained from Tokyo Women's Medical University (Tokyo, Japan), and maintained at Osaka Seiyaku Co. Ltd. (Osaka, Japan) using previous method.[10] The colony of two mites has been maintained without exposure to any acaricide and mites at 25 °C and RH>60%.

Bioassay Larvicidal test was carried out according to previous method.[3] Volatility and stability of the two isomers was checked by the medium without insects during the larvicidal test (8 d). After extraction of the medium, almost all the sample was recovered and two isomers were not decomposed. Therefore, larvicidal activity was not affected by the volatility and stability of the two isomers. Adulticidal activity was determined by topical appication on the abdomen of both sexes (males : females=1 : 1) of adults (5—7 d old) *D. melanogaster* according to the previous method.[3] Acaricidal activity was tested according to contact assay using filter paper.[10] The LC_{50} values of the test compounds were determined at several concentrations (50.0, 20.0, 10.0, 5.0, 2.5 $\mu g/cm^2$ for two isomers of butylidenephthalide; 100.0, 50.0, 40.0, 30.0, 20.0, 10.0 $\mu g/cm^2$ for acaricide). Each test compound dissolved in ethanol was applied to filter paper (100 cm^2, Toyo Roshi No. 1). Control was treated with ethanol. After drying the solvent, the filter paper was folded and fixed by a clip. This filter paper was used in acaricidal test. Filter papers applied samples were placed in the plastic container (60 mm diameter×20 mm) equipped with nylon wire (16 mesh) at the bottom of the container, and 25 mites were placed on the filter paper. Then container was covered with a lid (200 mesh wire). Test container was held at 26 °C and RH >75%. Mortality was determined 24 h after treatment using microscope. Mortality was defined as inability to move. All tests were replicated five times, and the LC_{50} values were determined by log-probit analysis. Insecticidal activity against *C. felis* was assessed according to contact assay using filter paper reported previously.[9] The concentrations tested were 200, 100 and 50 $\mu g/cm^2$. However, there was no insecticidal activity both (*E*)- and (*Z*)-butylidenephthalide against adults of *C. felis* even at the maximum concentration of 200 $\mu g/cm^2$.

Table 1. Larvicidal Activity of (*E*)- and (*Z*)-Butylidenephthalide and Rotenone against *D. melanogaster*

Compound	LC_{50} *a)* (μmol/ml of diet)	95% confidence limit	RT *b)*
(*Z*)-Butylidenephthalide	0.94±0.13	0.78—1.20	0.023
(*E*)-Butylidenephthalide	2.07±0.12	1.75—2.44	0.010
Rotenone	0.02±0.01	0.02—0.03	1.000

a) LC_{50} is the lethal concentration for 50% mortality, determined by log-probit analysis. LC_{50} values mean±S.E. of three replicates. *b*) RT means relative toxicity, and calculated by following equation; RT=LC_{50} value of rotenone/LC_{50} value of each test compound.

Table 2. Adulticidal Activity of (*E*)- and (*Z*)-Butylidenephthalide and Rotenone against *D. melanogaster*

Compound	LD_{50} *a)* (μg/adult)	95% confidence limit	RT *b)*
(*Z*)-Butylidenephthalide	0.84±0.10	0.75—0.94	4.38
(*E*)-Butylidenephthalide	0.94±0.23	0.84—1.05	3.91
Rotenone	3.68±0.14	3.33—4.06	1.00

a) LD_{50} is the lethal concentration for 50% mortality, determined by log-probit analysis. LD_{50} values mean±S.E. of three replicates. *b*) RT means relative toxicity, and calculated by following equation; RT=LD_{50} value of rotenone/LD_{50} value of each test compound.

RESULTS AND DISCUSSION

Larvicidal Activity of Test Compounds The larvicidal activity of (*E*)- and (*Z*)-butylidenephthalide and rotenone is given in Table 1. (*E*)-Butylidenephthalide showed larvicidal activity, with an LC_{50} value of 2.07 μmol/ml although (*Z*)-butylidenephthalide exhibited LC_{50} value of 0.94 μmol/ml. This indicated that butylidenephthalide was geometrical stereoselectivity against larvae of *D. melanogaster*. Previously, we investigated structure–activity relationships of the alkylphthalide derivatives on larvicidal activity.[3,11] Then we found that conjugation with the carbonyl group, the numbers and position of the conjugation, and aromaticity in the phthalide appeared to play an important factor in the larvicidal activity. This result also suggested that (*Z*)-conformation of the phthalide was involved for enhanced the larvicidal activity. Nair *et al.* reported that hexane extract from *Delphinium* ×*cultorum* cv. Magic Fountains flowers including both (*E*)- and (*Z*)-butylidenephthalide displayed mosquitocidal activity against larvae of *Aedes aegyptii* but insecticidal principles were not determined because of its volatility.[12] This paper suggested that two isomers of butylidenephthalide might be involved in the mosquitocidal activity.

Adulticidal Activity of Test Compounds The adulticidal activity of (*E*)- and (*Z*)-butylidenephthalide and rotenone is given in Table 2. (*E*)- and (*Z*)-Butylidenephthalide showed larvicidal activity, with 50% lethal dose (LD_{50}) values of 0.94 and 0.84 μg/adult, respectively. This indicated that (*E*)- and (*Z*)-butylidenephthalide showed no specificity for adulticidal activity. Two isomers of butylidenephthalide were more active than rotenone (LD_{50}=3.68 μg/adult). Previously, we investigated structure–activity relationships of the alkylphthalide derivatives on adulticidal activity.[3,11] Then we

Table 3. Acaricidal Activity of (*E*)- and (*Z*)-Butylidenephthalide and DEET against *Dermatophagoides farinae* (Expressed as Mortality Rate)[a)]

Compound	Concentration (μg/cm^2)							LC_{50}[b)] (μg/cm^2)	95% confidence limit	RT[c)]
	50.0	40.0	30.0	20.0	10.0	5.0	2.5			
(*Z*)-Butylidenephthalide	100.0[a)]	NE[d)]	NE	100.0	77.8	32.1	15.6	5.76	5.21—6.35	2.25
(*E*)-Butylidenephthalide	100.0	NE	NE	100.0	94.7	51.6	12.2	4.67	4.27—5.10	2.78
DEET	100.0	100.0	100.0	72.2	33.2	NE	NE	12.98	11.79—14.10	1.00

a) Mortality percent was calculated from following equation {(survival rate of control mites−survival rate of tested mites)/survival rate of control mites}×100. *b*) LC_{50} is the lethal concentration for 50% mortality, determined by log-probit analysis. *c*) RT means relative toxicity, and calculated from following equation; RT=LC_{50} value of DEET/LC_{50} value of each test compound. *d*) NE=not experiment.

Table 4. Acaricidal Activity of (*E*)- and (*Z*)-Butylidenephthalide and DEET against *Tyrophagus putrescentiae* (Expressed as Mortality Rate)[a)]

Compound	Concentration (μg/cm^2)							LC_{50}[b)] (μg/cm^2)	95% confidence limit	RT[c)]
	100.0	50.0	30.0	20.0	10.0	5.0	2.5			
(*Z*)-Butylidenephthalide	NE[d)]	74.1[a)]	NE	56.3	22.8	12.9	7.8	20.37	17.15—24.86	0.80
(*E*)-Butylidenephthalide	NE	57.2	NE	52.9	33.0	NE	12.3	25.56	19.08—37.54	0.64
DEET	100.0	100.0	81.1	NE	20.8	NE	NE	16.28	14.56—18.03	1.00

a) Mortality percent was calculated from following equation {(survival rate of control mites−survival rate of tested mites)/survival rate of control mites}×100. *b*) LC_{50} is the lethal concentration for 50% mortality, determined by log-probit analysis. *c*) RT means relative toxicity, and calculated from following equation; RT=LC_{50} value of DEET/LC_{50} value of each test compound. *d*) NE=not experiment.

found that aromaticity in the phthalide appeared to play an important role in the adulticidal activity. This result suggested that geometrical stereoselectivity of butylidenephthalide was not involved in adulticidal activity.

Acaricidal Activity of Test Compounds The acaricidal activity of (*E*)- and (*Z*)-butylidenephthalide and DEET against two mites, *D. farinae* and *T. putrescentiae* is given in Tables 3 and 4. Against *D. farinae*, (*E*)-butylidenephthalide (LC_{50}=4.67 μg/cm^2) was slightly more active than (*Z*)-butylidenephthalide (LC_{50}=5.76 μg/cm^2). But there was no significant difference between two isomers. Both (*E*)- and (*Z*)-butylidenephthalide was about 2.5-fold more potent acaricidal activity than that of DEET (LC_{50}=12.98 μg/cm^2). (*Z*)-Butylidenephthalide (LC_{50}=20.37 μg/cm^2) was slightly more active than (*E*)-butylidenephthalide (LC_{50}=25.56 μg/cm^2) against *T. putrescentiae*. Both (*E*)- and (*Z*)-butylidenephthalide was less active than DEET (LC_{50}=16.28 μg/cm^2). Ahn *et al.* reported that butylidenephthalide derived from rhizome of *Cnidium officinale* exhibited pronounced acaricidal activity against *D. farinae* and *T. putrescentiae*.[4,5)] However, geometry of butylidenephthalide was not identified so that it has been unknown that wheather (*E*)- or (*Z*)-butylidenephthalide was main principles of the acaricidal activity from *C. officinale*. From this results, acaricidal principles of *C. officinale* were possibly considered to be both (*E*)- and (*Z*)-butylidenephthalide.

Phthalides, natural volatile compounds present in apiaceous plants, have several biological activities against insects and mites. Although some terpenoids and other natural-based products have been investigated for their use as pesticides, phthalide derivatives have not been well-investigated. Our result suggested that commercially available butylidenephthalide was the promised compound as new natural pesticide. In this paper, we studied comparison of larvicidal, adulticidal and acaricidal activity between (*E*)- and (*Z*)-butylidenephthalide. Further studuies for insecticide mode of action of butylidenephthalide are needed.

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