J. Pesticide Sci. 2, 453–456 (1977)

Notes

Isolation and Identification of Biologically Active Constituents of *Leucothoe catesbaei* A. Gray

Kanzo SAKATA, Makoto HATTORI, Akira SAKURAI and Toyoji HOSOTSUJI

The Institute of Physical and Chemical Research, Wako, Saitama 351, Japan

(Received April 11, 1977)

From extracts of fresh leaves of *Leucothoe catesbaei*, grayanotoxin I (**1**) and III (**2**) and poriolide (**3**) and isoporiolide (**4**) were isolated as insecticidal and antimicrobial constituents, respectively. This is the first report to show the presence of these compounds in the plant.

During the screening search for biologically active substances to insects in fresh drug plants, Murakoshi *et al.* found that extracts of some kinds of the plants showed significant activities on the growth of silkworm larvae.[1] Then, we tested activities of extracts from these plants against agricultural insect pests such as green rice leafhopper (*Nephotettix cincticeps* U.), smaller brown planthopper (*Laodelphax striatellus* F.), rice stem borer (*Chilo suppressalis* W.) and carmine mite (*Tetranychus teralius* L.). As the results, extracts of *Leucothoe catesbaei* A. Gray revealed insecticidal activity against the rice stem borer. Further, the extract showed antifungal activity against *Pyricularia oryzae*.

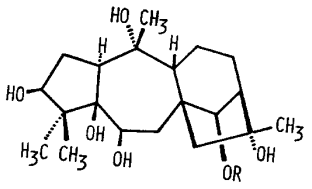
Then, we attempted isolation of active principles from this plant and succeeded in obtaining two insecticidal substances together with antifungal constituents. The insecticides were identified as grayanotoxins I (**1**) and III (**2**), and the fungicides as poriolide (**3**) and isoporiolide (**4**) (Fig. 1). This is the first report on the isolation of **1**–**4** from *L. catesbaei*.

Although many kinds of grayanotoxins[2,3] and their related compounds[2] have been isolated from plants of *Ericaceae* and have been reported or supposed to have insecticidal activity, details on their activities have, to our knowledge, not yet been reported. In this paper we wish to present the details of the isolation and biological activities of the active components.

Leaves of *L. catesbaei* were extracted with methanol and the extracts were separated into ethyl acetate-soluble and *n*–butanol-soluble fractions. During the isolation procedure, behaviours of active principles were monitored by biological activities against *C. suppressalis* and *P. oryzae*.

As the results, grayanotoxins I (**1**) and III (**2**) were isolated as insecticidal constituents. The contents of **1** and **2** in fresh leaves amounted to about 0.036 and 0.005%, respectively. The insecticidal activities against a few kind of agricultural insect pests are shown in Table 1. Both **1** and **2** exhibited considerably potent insecticidal activities against *C. suppressalis* in almost the same degree, but did not show any significant activities against *N. cincticeps*, *L. striatellus* and *T. telarius* even at a dose of 500 ppm.

From the ethyl acetate fraction were isolated two antifungal components, which were identified as poriolide (**3**) and isoproriolide (**4**) by comparison of their spectrometric data with those of authentic samples. Compounds **3** and **4** have already been isolated by Ogiso *et al.*[4] from *Leucothoe keiskei* M. as potent toxic constituents. However, our experiment is the first to reveal the antimicrobial activities of these compounds (Table 2). Their antimicrobial



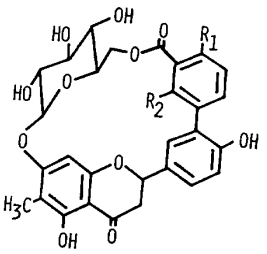


Fig. 1 **1**: R=$COCH_3$ **3**: R_1=OH, R_2=H
2: R=H **4**: R_1=H, R_2=OH

activities seem rather specific: they are active against *Xanthomonus oryzae* and *Piricularia oryzae,* but almost inactive against bacteria and fungi tested here. It is noticeable that **3** and **4** are active against phytopathogenic bacterium and fungus which live upon rice.

The leaves of *L. catesbaei* are known to be seldom attacked by phytopathogenic microorganisms or insect pests. This will be mainly attributed to the fact that the leaves are covered with wax and cuticulated epithelium, but the occurrence of the insecticidal and antimicrobial constituents in considerable amounts might be taken into consideration to some extent for the healthiness of the plant.

EXPERIMENTAL

Carbon–13 NMR spectra were recorded on JEOL–FX 100 FT–NMR spectrometer using pyridine–d_5 as a solvent and TMS as an internal standard.

Table 1 Insecticidal activity of grayanotoxins I (**1**) and III (**2**).

Conc. (ppm)	Mortality (%)[a] of *Chilo suppressalis*	
	1	**2**
1.6	0	0
3.1	17.4	4.3
6.3	39.1	34.8
12.5	60.9	43.5
25	95.6	78.5
50	100	100

Compounds **1** and **2** did not show any significant activities against green rice leafhopper (*Nephotettix cincticeps*), smaller brown planthopper (*Laodelphax striatellus*) and eggs and adults of carmine mite (*Tetranychus telarius*) even at a dose of 500 ppm.

[a] Details of the bioassay method are described in the text. Averages of repeated experiments are presented after correction using the Abbotto's formula.

1. Isolation of Insecticidal Constituents

Fresh leaves (10 kg) of *Leucothoe catesbaei* A. Gray harvested at the Kyoto Herbal Garden of Takeda Chemical Industries, Ltd. in July,

Table 2 Antimicrobial activities of poriolide (**3**) and isoporiolide (**4**)[a]

Test organism	Poriolide				Isoporiolide			
	500[b]	100	50	25	500	100	50	25
Staphylococcus aureus 209P	(23)[c]	—	—	—	(33)	(27)	(21)	(16)
S. aureus 209P resistant strain	—	—	—	—	—	—	—	—
Bacillus subtilis	—	—	—	—	22.0	17.0	±	—
Esherichia coli	(28)	—	—	—	(25)	(17)	—	—
Xanthomonus oryzae	32.5	24.5	21.5	14.0	39.5	30.0	26.5	20.5
X. citri	20.5	—	—	—	21.5	—	—	—
Pseudomonus aerginosa	+	—	—	—	+	—	—	—
Mycobacterium phlei	—	—	—	—	+	—	—	—
Piricularia oryzae	22.0	19.5	15.0	14.0	13.0	—	—	—

Compounds **3** and **4** did not show any significant activities against the following fungi and yeasts even at 500 ppm. *Colletotricum lagenaria, Altanaria mali, Ophiobolus miyabeanus, Botrytis cinerea, Penicilium chrysogenum, Pericularia sasakii, Aspergillus niger, Diaponthe citri, Saccharomyces cerevisiae* and *Candida albicans.*

[a] Activities were determined by a paper-disk method (see the text).

[b] Concentration (ppm) of acetone solution.

[c] Inhibitory zone was obscure.

1976, were soaked in 50 liter of methanol for a month and then in 60 liter of 80% aq. methanol for a week. The combined extracts were concentrated and extracted with ethyl acetate at pH 7. The aq. layer was adjusted to pH 4 and extracted with *n*-butanol. The butanol extracts were concentrated and subjected to eight transfer countercurrent distributoon (CCD) (*n*-butanol–*n*-propanol–water, 2: 1: 4; 1.5 liter of each phase). Fractions No. 5–8* were combined, concentrated and applied to eight-transfer CCD(*n*-butanol–*n*-propanol–0.1 N ammonia, 3: 1: 5; 1 liter of each phase). Fractions No. 5–8 were combined and lyophilized to give 17 g of light brownish powder. A half of the powder was dissolved in a small volume of ethyl acetate–methanol–water (35: 5: 5) and charged onto a silica gel 60 column (500 g, Merck for column chromatography), which was developed with the solvent. Fractions showing insecticidal activity were collected, concentrated and applied onto a Sephadex G–50 column (3.5×95 cm) which had been packed with the lower phase of benzene–ethyl acetate–methanol–water (2: 8: 2: 3) and equilibrated by developping the upper phase. The column was developed with the upper phase. Insecticidal activities were found in two separate fractions. The active substance eluted earlier was crystallized from ethyl acetate to yield 426 mg of colorless crystalls: C–13 NMR δ 19.74 (*q*), 21.20 (*q*), 22.37 (*t*), 23.29 (*q*), 23.88 (*q*), 27.24 (*t*), 28.21 (*q*), 35.72 (*t*), 43.95 (*t*), 50.92 (*s*), 51.22 (*d*), 51.65 (*s*), 54.92 (*d*), 55.50 (*d*), 61.01 (*t*), 73.63 (*d*), 77.82 (*s*), 78.50 (*s*), 82.50 (*d*), 82.65 (*d*), 84.35 (*s*), 170.21 (*s*). Found: C, 63.88; H, 8.72. Calcd. for $C_{22}H_{36}O_7$: C, 64.05; H, 8.80%. The compound was identified as grayanotoxin I by comparison of its PMR and mass spectra with those of the authentic specimen.

Crystallization of another active compound from ethyl acetate gave 90 mg of colorless crystalls: C–13 NMR δ 19.81 (*q*), 22.52 (*s*). 23.28 (*q*), 23.87 (*q*), 27.07 (*t*), 28.30 (*q*), 35.78 (*t*), 44.27 (*t*), 51.63 (*d*), 51.78 (*s*), 52.52 (*s*), 55.16 (*q*), 56.36 (*d*), 60.30 (*t*), 74.18 (*d*), 78.09 (*s*), 79.32 (*d*), 79.73 (*s*), 82.60 (*d*), 84.60 (*s*). Found: C, 64.48; H, 9.22. Calcd. for $C_{20}H_{34}O_6$: C, 64.84; H, 9.25%. The compound was identified as grayanotoxin III.

From the ethyl acetate fraction described above grayanotoxin I was isolated as insecticidal principle. The overall yields of grayanotoxins I and III amounted to 0.036 and 0.005% of the fresh weight, respectively.

2. Isolation of Antimicrobial Constituents

A part (1/10) of the ethyl acetate fraction described above was fractionated by nine-transfer CCD (*n*-hexane–ethyl acetate–methanol–water, 2: 3: 2: 2; upper and lower phases, each 200 ml). Fractions No. 1–5 were combined and evaporated to dryness. The residual material was dissolved in a small volume of methanol and absorbed on 50 g of Silica gel 60. After evaporation of the methanol, the silica gel was suspended in a small volume of chloroform and introduced onto the top of a column of Silica gel 60 (500 g). The column was developed with chloroform–methanol the content of methanol being increased (2, 4, 10 and 25%: 1 liter each).

From the fractions showing activity against *P. oryzae* was obtained 1.7 g of yellow solid. Crystallization from aqueous ethanol gave 0.93 g (*ca.* 0.093% of fresh weight) of fine needles. It was identified as poriolide by comparison of its PMR and mass spectra with those of the authentic sample.

The mother liquor was chromatographed on Florisil (200 g, Wako Pure Chemicals) using acetone as developing solvent to yield 0.59 g (*ca.* 0.059% of fresh weight) of isoporiolide as needles.

3. Assay of Antimicrobial Activity

Each sample was dissolved in acetone and diluted to the definite concentration. Each sample solution was subjected to bioassay by the paper-disk method using a paper-disk (8 mm in diameter and 1.6 mm thick).

4. Assay of Insecticidal Activity

Each sample was dissolved in a small volume of methanol and diluted with water to the definite concentration. Newly hatched larvae (15 each) were placed on rice seedlings (*ca.* 0.5 cm high) in a plastic cup (6 cm in diameter and 3 cm high) and allowed to feed for 24 hrs.

* Fraction No. 1 is the most polar fraction.

The sample solution (2.5 ml) was sprayed on the infested plants in the cup, and then the cup was covered with a cap with a 1.0 cm hole in the center which was plugged with cotton. The larvae were reared at 25°C± 2°C with a photoperiod of 16L/8D. After 12 days, survival larvae were counted.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. T. Takahashi and T. Kamikado of Takeda Chemical Industries, Ltd. for the supply of *L. catesbaei* leaves. They are indebted to Drs. S. Suzuki and M. Uramoto and their coleagues of this Institute for the assay of antimicrobial activity. They are also grateful to Prof. Dr. J. Iwasa of Department of Agricultural Chemistry of Okayama University and Dr. A. Ogiso of Central Research Laboratories of Sankyo Co., Ltd., for the gift of authentic specimens of grayanotoxins I and III and poriolide and isoporiolide, respectively. They wish to thank Dr. S. Tamura for his encouragement throughout this work.

要　　約

アメリカイワナンテン生葉のメタノール抽出物は殺虫および抗菌活性を示す．ニカメイチュウに対する殺虫作用を指標にして，grayanotoxin I, III を単離同定した．抗菌性成分の単離はイモチ菌を用いて行ない，その活性成分は poriolide, isoporiolide と同定した．アメリカイワナンテンよりこれらの物質が単離同定されたのはこれが最初である．本報告は，これらの物質の単離とその生理活性の詳細に関するものである．

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