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Effects of the new plant growth retardants of quaternary ammonium iodides on gibberellin biosynthesis in *Gibberella fujikuroi*

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The effects of twelve quaternary ammonium iodides, synthesized as plant growth retardants, on the biosynthesis of gibberellins in the culture of *Gibberella fujikuroi* were investigated. Of the two compounds with the strongest growth-retarding activity, *N,N,N*-trimethyl-1-methyl-(3′,3′,5′-trimethylcyclohexyl)-2-propenyl ammonium iodide (**1**) was found to inhibit the biosynthesis, while *N,N,N*-trimethyl-1-methyl-(3′,3′,5′,5′-tetramethylcyclohexyl)-2-propenyl ammonium iodide (**2**) was not. The results on examination of the twelve analogues indicate that their plant growth-retarding activity is not related to the inhibition of gibberellin biosynthesis.

Key words: Gibberellin biosynthesis — *Gibberella fujikuroi* — Growth retardant — Quaternary ammonium iodide.

We have previously reported the syntheses of new quaternary ammonium iodides as plant growth retardants (*1*). The synthesized compounds, **1**–**12** shown in Fig. 1, are structurally related to the growth retardant, **13**, obtained by Haruta et al. (*3*) and showed potent growth retarding activities in rice and cucumber seedlings.

The known synthetic plant growth retardants such as AMO-1618 and CCC have been found to inhibit GA biosynthesis in the culture of *Gibberella fujikuorui* and in the cell-free preparations of certain plants (*5*). Thus, the retardants are considered to suppress plant growth by inhibiting the endogeneous GA biosynthesis (*5*). Recently, Hedden et al. reported that the growth retardant, **13**, also inhibits the GA biosynthesis in *G. fujikuroi* by blocking the pathway between mevalonate and *ent*-kaur-16-ene (*4*).

We investigated the effects of our synthetic compounds **1**–**12** on GA biosynthesis in the culture of *G. fujikuroi* by analyzing the GA production. In this paper, we describe the results and the relationship between their plant growth-retarding and GA biosynthesis-inhibiting activities.

Materials and methods

Culture conditions

G. fujikuroi (NRRL strain) was grown in a 200-ml conical flask containing 40 ml

Abbreviations: AMO-1618, 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl-piperidine-1′-carboxylate; CCC, (2-chloroethyl)trimethylammonium chloride; GA, gibberellins; GA_1, gibberellin A_1; GA_3, gibberellin A_3; Me TMS-, methyl ester trimethylsilyl-.

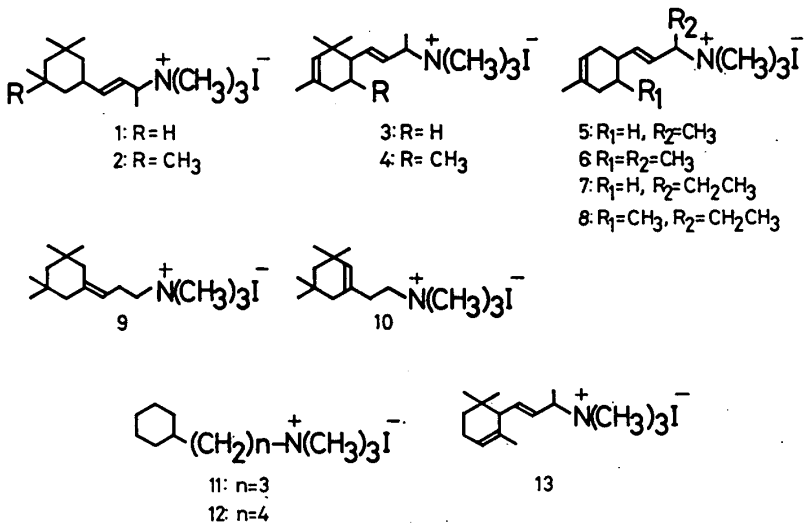

Fig. 1. *Structures of quaternary ammonium iodides.*

of ICI 40%-N medium (*2*) at 27°C under reciprocal shaking (**1st culture**). After 3 days, a 0.8-ml portion of the culture was transferred to a 200-ml conical flask containing 40 ml of the same medium and cultivated for 3 days under the same conditions as above (**2nd culture**). The mycelia were collected by filtration, washed three times with 25 ml each of ICI 0%-N medium (*2*) then cut into four pieces. Each piece of mycelium was resuspended in 10 ml of ICI 0%-N medium and cultivated in a test tube (2.4×20 cm) at 28°C for 2 days under shaking (**3rd culture**). Each ammonium iodide was dissolved in 70% ethanol in definite concentrations, and 100 μl of the solution was added to the culture medium of the 2nd and 3rd cultures or the 2nd culture only prior to inoculation. To the reference culture, 100 μl of 70% ethanol was added. Every experiment was run with two independent cultures and each result represents the average value.

Extraction of acidic metabolites

Two cultures of the 3rd culture under the same conditions were combined and filtered. The mycelia were dried at 100°C for 3 hr and weighed to find the effects on the growth. The culture filtrate was combined with 25 ml of saturated $NaHCO_3$ solution and washed with ethyl acetate (2×30 ml). The aqueous layer was acidified to pH 2.5 with dil. HCl and extracted with ethyl acetate (3×30 ml). The organic extract was dried over anhydrous Na_2SO_4 and evaporated. The residue was dissolved in 2 ml of methanol then the GA content was determined.

Quantitative analyses of GA production

a) Gas chromatography. A portion of each acidic extract (100 μl of the above methanol solution) was converted to methyl esters with diazomethane in ethyl ether solution. After evaporation of the solvent, the residue was dried completely then treated with 10 μl of *N,O*-bis-trimethylsilyl acetamide and 5 μl of trimethylsilyl-chloride in 10 μl of dry pyridine for 5 min giving methyl ester trimethylsilyl (Me TMS) derivatives. The reaction mixture was directly subjected to gas chromatographic analysis on a silanized glass column (200×0.4 cm) packed with 2% OV-1 on

Chromosorb WAW (80–100 mesh) at 230°C with N_2 gas at a flow rate of 75 ml/min using a Shimadzu GC-4BPF gas chromatograph equipped with a hydrogen flame ionizing detector. Under these conditions, the extract of the reference culture gave an easily detectable peak of Me TMS-GA_1 or -GA_3 with a retention time of around 4 min. The quantity of GA was estimated by calculation from the area of the peak using a calibration curve obtained from a 1 : 1 mixture of authentic Me TMS-GA_1 and -GA_3.

b) Bioassay. The amount of biologically active GA in the acidic extracts was determined by bioassay using dwarf rice seedlings (*Oryza sativa* L. cv Tan-ginbozu) according to Murakami's microdrop method (*6*). Each methanol solution of the acidic extract was diluted (1/10–1/200) with acetone-water (1 : 1), and 1 μl of the solution was applied to each seedling. After 3 days of growth under fluorescent light at 30°C, the length of second leaf sheath was measured. The amount of biologically active GA was estimated as the GA_3 equivalent.

Results

In a preliminary experiment, the effect of the known growth retardant CCC on the culture of *G. fujikuroi* was examined. As shown in Table 1, when it was added only to the medium of the 3rd culture, the amounts of GA detected were 3–11% of those of the reference culture. Complete inhibition of GA biosynthesis was observed when the fungus was pre-treated with CCC in the 2nd culture. These facts indicate that the mycelia grown sufficiently without the inhibitor retain their ability of GA biosynthesis in the presence of CCC. On the other hand, the fungus treated with the inhibitor in the 2nd culture did not produce GA in the 3rd culture even without CCC addition.

In each experiment with the quaternary ammonium iodides of **1–13**, the compound was added to the medium of the 2nd culture and the 3rd cultures were carried out with or without an addition of the same concentration. The results are shown in Tables 2 and 3. In every culture, the pH value ranging between 3.4–3.9 was not influenced by the addition of the compound. The weight of the dried mycelia grown in the 3rd culture was almost the same as that of the reference culture, indicating that all the tested compounds did not affect growth. The amounts of GA in the acidic extract of each culture, estimated by gas chromatography were similar to

Table 1 *Effect of CCC on GA biosynthesis in* G. fujikuroi

Concentration of CCC (ppm)		Amount of GA[a] in 3rd culture (% of control)
2nd culture	3rd culture	
0	100	3– 5
0	10	10–11
100	100	<1
100	10	<1
100	0	<1

[a] Estimated by gas chromatography.

Table 2 *GA Production in the culture of* G. fujikuroi *with addition of quaternary ammonium iodides*

Compound	Concentration (ppm)		Dry weight of mycelia[a] (mg)	GA amount in 3rd culture (% of control)	
	in 2nd culture	in 3rd culture		By GC[b]	By bioassay[c]
1	100	100	205	5	7
	10	10	198	11	9
2	100	100	192	89	61
	10	10	172	83	88
3	100	100	185	4	1
	10	10	172	12	6
4	100	100	188	39	27
	10	10	173	79	47
5	100	100	—[d]	6	5
	10	10	213	18	38
6	100	100	—	5	2
	10	10	217	19	13
7	100	100	197	41	24
	10	10	169	102	128
8	100	100	195	78	43
	10	10	196	107	63
9	100	100	192	7	10
	10	10	196	10	18
10	100	100	175	9	12
	10	10	186	62	70
11	100	100	187	0	0
	10	10	187	0	2
12	100	100	178	0	1
	10	10	205	0	2
13	100	100	198	5	5
	10	10	191	11	16

[a] Weight per 10 ml of 3rd culture. In the reference culture, 182 ± 15.5 mg.
[b] Estimated by gas chromatography. In the reference culture as a control, 731 ± 70.6 μg/10 ml culture.
[c] Estimated by bioassay. In the reference culture as a control, 928 ± 236.5 μg/10 ml culture.
[d] Not measured.

those obtained by bioassay. This showed that the fungus produced mainly GA_1 or GA_3 as biologically active GA in every case.

With addition of compound **1**, **3**, **5**, **6** or **9** to each medium of the 2nd and 3rd culture (Table 2), GA production decreased to 4–7% of the reference. This indicates that these compounds strongly inhibit GA biosynthesis. Their activities were comparable to that of **13** which showed the same result as that reported by Hedden et al. (*4*). Compounds **11** and **12** composed of simple cyclohexane derivatives, completely inhibited the biosynthesis even at a concentration of 10 ppm.

On the other hand, compounds **4**, **7** and **10** only weakly inhibited the biosynthesis; at the concentration of 10 ppm, GA was produced in almost the same range as the reference. Compounds **2** and **8** showed little effect on the biosynthesis even at the concentration of 100 ppm.

When the 3rd culture was carried out without the addition of the test compound (Table 3), the mycelia produced almost the same amount of GA as the reference

Table 3 *GA production in the culture of* G. fujikuroi *pre-treated with quaternary ammonium iodides*

Compound	Concentration (ppm)		Dry weight of mycelia[a] (mg)	GA amount in 3rd culture (% of control)	
	in 2nd culture	in 3rd culture		By GC[b]	By bioassay[c]
1	100	0	174	83	115
	10	0	185	88	106
2	100	0	184	91	75
	10	0	191	91	82
3	100	0	183	96	62
	10	0	195	96	111
4	100	0	180	105	78
	10	0	182	111	206
5	100	0	194	87	71
	10	0	161	94	84
6	100	0	177	87	133
	10	0	—[d]	96	100
7	100	0	165	114	80
	10	0	177	108	87
8	100	0	198	101	100
	10	0	198	101	84
9	100	0	183	86	70
	10	0	180	91	121
10	100	0	178	65	87
	10	0	192	96	91
11	100	0	194	6	11
	10	0	179	41	56
12	100	0	187	35	70
	10	0	174	90	111
13	100	0	204	87	81
	10	0	194	88	61

See Table 2 footnotes.

except in the cases of **11** and **12**. This observation suggests that the inhibitory effects of **1**, **3**, **5**, **6**, **9** and **13** are different from that of CCC shown in Table 1. On the other hand, GA production of the mycelia once treated with **11** or **12** decreased similarly to the case of CCC.

Discussion

The growth retardants of quaternary ammonium iodides were found unexpectedly to behave differently in their effects on GA biosynthesis in the culture of *G. fujikuroi*. The synthesized analogues were classified into two types: one with a strong activity for inhibiting the biosynthesis and the other with little activity. No structure-activity relationships could be deduced between these types. Moreover, compounds **1**, **3**, **5**, **6**, **9** and **13** inhibited GA biosynthesis in a different manner from that of **11**, **12** and CCC, since mycelia pre-treated with the former recovered their ability of GA biosynthesis (Table 3).

As reported previously (*1*), every compound described here suppressed the growth of rice seedlings to 50% of the control at the concentration of 60 ppm, and no differences could be observed between their activities. On the other hand, each

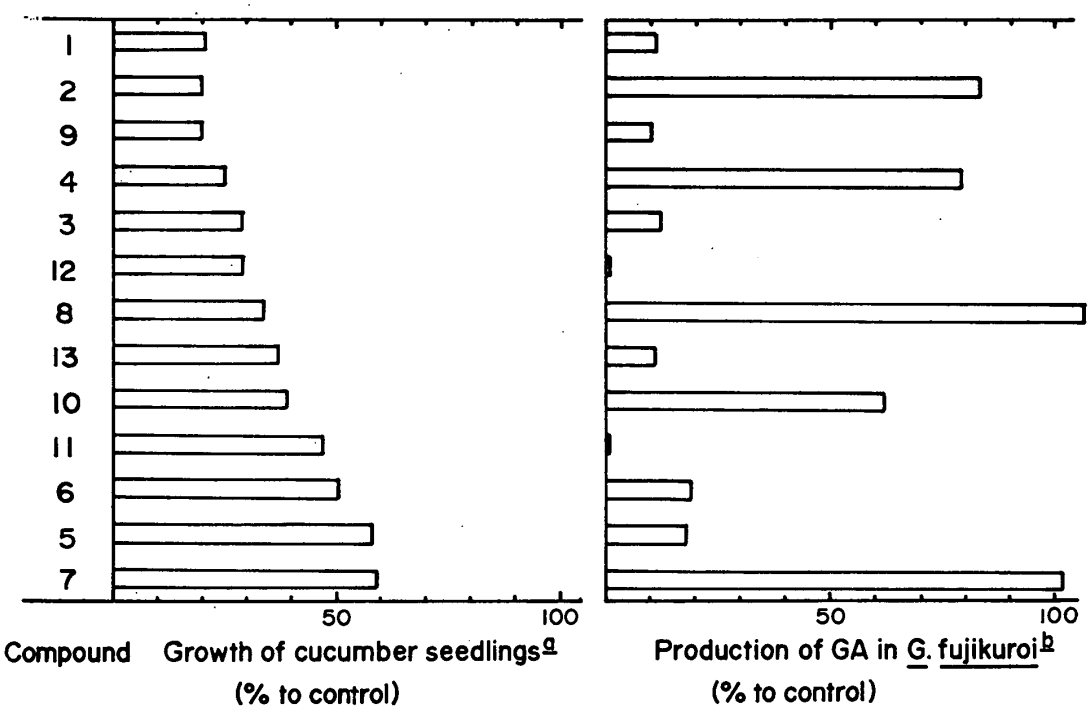

Fig. 2. *Comparison of plant growth-retarding and GA biosynthesis-inhibiting activities of quaternary ammonium iodides.* a) Hypocotyl length of cucumber seedlings grown with 60 ppm of each compound. The bioassay method was described previously (*1*). b) Amount of GA in cultures containing 10 ppm of the test compound. Measurements were made by gas chromatography.

compound showed different effectiveness on the growth of cucumber seedlings. Fig. 2 compares the growth-retarding activity of the compounds on cucumber seedlings and their effects on GA biosynthesis in *G. fujikuroi*. No evident correlations were observed between these two biological activities. A remarkable distinction was found in case of compounds **1** and **2**, both of which showed the strongest growth-retarding activity among the iodides. Compound **1** inhibited GA biosynthesis strongly, while **2**, with an additional methyl on the cyclohexane ring to **1**, did not. Furthermore, compounds **11** and **12**, which completely inhibited GA biosynthesis in a manner similar to that of CCC, showed a relatively weak growth-retarding activity.

These studies revealed that these potent growth retardants having closely related structures do not always inhibit GA biosynthesis in *G. fujikuroi*. Reid and Crozier reported that CCC did not inhibit GA biosynthesis in intact pea seedlings, rather increasing the GA levels in a small dose, and showed that the growth inhibition of pea seedlings induced by CCC was not related to the content of endogeneous GA (*7*). They concluded that the mode of CCC action could not merely be explained as the inhibition of GA biosynthesis in higher plants.

Our results presented here also suggest that the suppression of plant growth by the analogues of quaternary ammonium iodides did not solely depend on the inhibition of GA biosynthesis. These results should be useful for studying the mode of action to examine the effects of these analogues on GA biosynthesis in intact plants.

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