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NEW BIOREGULATORS OF GIBBERELLIN BIOSYNTHESIS IN *GIBBERELLA FUJIKUROI*

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Abstract—A number of new inhibitors of gibberellin (GA) biosynthesis in *Gibberella fujikuroi* are reported, including secondary, tertiary and quaternary amines. Octyltrimethylammonium iodide and 3-chloropropyltrimethylammonium iodide were equally as effective as 2-chloroethyltrimethylammonium chloride (CCC). At least two of the other inhibitors reported, diethyloctylamine hydrochloride and octyltriethylammonium iodide, showed a different pattern of accumulation of GAs and kaurenoic acids than did CCC.

INTRODUCTION

Knowledge of the regulation of gibberellin (GA) biosynthesis remains fragmentary due to the complex nature of the GA pathway and the low endogenous levels of gibberellins in plants. *Gibberella fujikuroi* has been used as a means of studying aspects of GA biosynthesis without the many difficulties that complicate studies in plants [1–3]. We have studied the effects of a wide variety of compounds on the GA pathway using a mycelial resuspension system that allows rapid qualitative and quantitative evaluation of bioregulators. In addition, sufficient quantities of GAs and GA precursors accumulate to permit more detailed analyses of the GA pathway.

Our studies demonstrate the regulation of gibberellin biosynthesis by a number of new inhibitors with secondary, tertiary, and quaternary amine functional groups. Detailed analyses by GC–MS identified different patterns of accumulation of GAs and kaurenoic acids. Two compounds were found that promote substantial accumulation of a trihydroxykauranoic acid.

RESULTS AND DISCUSSION

Since the plant growth retardant CCC (2-chloroethyltrimethylammonium chloride) (**1**) had previously been shown to be an effective inhibitor of the GA pathway in plant cell-free systems and in *G. fujikuroi* [4], it was used as a reference inhibitor in this study. The effects of **1** over a wide concentration range in our mycelial resuspension system are shown in Table 1. Inhibition increased with concentration and was half-maximal at 10^{-6} M for combined GA_1, GA_3, GA_4 and GA_7, while the weight gain of the mycelium was unaffected. Ninnemann *et al.* [5] reported half-maximal inhibition at 6×10^{-7} M in a *G. fujikuroi* growth system. GA_{4+7} production was inhibited to a greater extent than GA_{1+3} above 10^{-7} M **1**.

Another widely used plant growth retardant, AMO 1618 (2′-isopropyl-4′-(trimethylammonium chloride)-5′-methyl-phenyl-piperidine-1-carboxylate) (**2**) was much less effective than **1** as an inhibitor of GA biosynthesis (Table 2). Compound **2** inhibited GA synthesis 31 and 10% at 10^{-3} and 10^{-4} M, respectively. Although some studies [6] showed that **2** was as effective as **1** in the

Table 1. Inhibition of GA biosynthesis in *Gibberella fujikuroi* by CCC

Treatment, CCC concentration (M)	GA_{1+3} (mg/g dry wt)	GA_{4+7} (mg/g dry wt)	Total GA (mg/g dry wt)	% Inhibition
0	1.44	1.04	2.48	0
10^{-3}	0.32	0.08	0.40	84
10^{-4}	0.38	0.16	0.54	78
10^{-5}	0.56	0.24	0.80	68
10^{-6}	1.00	0.33	1.33	46
10^{-7}	1.04	0.89	1.93	22
10^{-8}	1.11	0.93	2.04	18
10^{-9}	1.22	0.96	2.18	12

Cultures were resuspended in nitrogen-free ICI-media containing indicated levels of 2-chloroethyltrimethylammonium chloride (CCC, **1**) and incubated on a shaker for 48 hr at 22°. Quantification of the acidic EtOAc-soluble metabolites from culture filtrates was by TLC.

Table 2. Comparative inhibition of GA biosynthesis in *G. fujikuroi* by bioregulators

Concentration (M)	Bioregulator	% Inhibition
10^{-3}	2′-isopropyl-4′-(trimethylammonium chloride)-5′-methyl-	31
10^{-4}	phenyl-piperidine-1-carboxylate (AMO 1618) (**2**)	10
10^{-4}	2-chloroethyltriethylammonium iodide (**3**)	46
10^{-4}	3-chloropropyltrimethylammonium iodide (**4**)	75
10^{-4}	2-(4-chlorophenylthio)-triethylamine-HCl (**5**)	58
10^{-4}	2-(4-ethylphenoxy)-triethylamine-HCl (**6**)	18
10^{-4}	2-(3,4-dimethylphenoxy)-triethylamine-HCl (**7**)	23
10^{-4}	2-(3,4-dichlorophenoxy)-triethylamine-HCl (**8**)	33
10^{-4}	4-(2-diethylaminoethoxy)-benzophenone-HCl (**9**)	20
10^{-4}	2-(diethylamino)-propiophenone-HCl (**10**)	41
10^{-4}	4-bromobenzylfurfurylamine-HCl (**11**)	43
10^{-3}	benzylfurfurylamine-HCl (**12**)	39
10^{-4}	benzylfurfurylamine-HCl (**12**)	11
10^{-4}	*N*-methylphenethylamine-HCl (**13**)	24
10^{-4}	benzyldecyldimethylammonium iodide (**14**)	40
10^{-4}	benzyldecylmethylamine-HCl (**15**)	2
10^{-4}	octyltrimethylammonium iodide (**16**)	79
10^{-4}	dimethyloctylamine-HCl (**17**)	18
10^{-3}	diethyloctylamine-HCl (**18**)	69
10^{-4}	diethyloctylamine-HCl (**18**)	39
10^{-5}	diethyloctylamine-HCl (**18**)	24
10^{-6}	diethyloctylamine-HCl (**18**)	9
10^{-4}	octyltriethylammonium iodide (**19**)	29
10^{-4}	maleic hydrazide (**20**)	6
10^{-4}	alar (**21**)	0
10^{-4}	kinetin (**22**)	9
10^{-3}	kinetin (**22**)	6
10^{-4}	benzyladenine (**23**)	13
10^{-3}	benzyladenine (**23**)	7
10^{-4}	abscisic acid (**24**)	8

Cultures were resuspended in nitrogen-free ICI-media containing bioregulator compounds and incubated on a shaker for 48 hr at 22°. Gibberellins GA_{1+3} and GA_{4+7} were quantified by TLC and their combined value was used to determine the per cent inhibition relative to the untreated control cultures.

fungus, other studies in plants and in *G. fujikuroi* showed differences in effects [1].

We tested two new CCC analogs, 2-chloroethyltriethylammonium iodide (**3**) and 3-chloropropyltrimethylammonium iodide (**4**). At 10^{-4} M they inhibited GA production by 46 and 75%, respectively. The 3-chloropropyl group was as effective as the 2-chloroethyl group of **1** but substitution of triethyl for trimethyl reduced the effect by one half.

CPTA [2-(4-chlorophenylthio)triethylamine)-HCl] (**5**) was reported to be an effective inhibitor of activity B of kaurene synthetase (conversion of copalyl pyrophosphate to *ent*-kaurene) in a cell-free extract of *Marah macrocarpus* [7]. We found that **5** at 10^{-4} M inhibited GA production 58% in the *G. fujikuroi* resuspension culture. Three oxy-analogs of **5**, 2-(4-ethylphenoxy)-triethylamine-HCl (**6**), 2-(3,4-dimethylphenoxy)-triethylamine-HCl (**7**) and 2-(3,4-dichlorophenoxy)-triethylamine-HCl (**8**) at 10^{-4} M inhibited by 18, 23 and 33%, respectively. Two similar compounds, 4-(2-diethylaminoethoxy)-benzophenone-HCl (**9**) and 2-(diethylamino)-propiophenone-HCl (**10**) were also effective. Unlike the inhibitors reported by earlier workers, these new inhibitors are all tertiary amines with various substituents attached to the diethylaminoethyl function.

A few secondary amines also were found to be inhibitors. The most effective of these was 4-bromobenzylfurfurylamine-HCl (**11**). At 10^{-4} M it inhibited GA production by 43%. A similar compound without the bromo substituent (**12**) had no activity at 10^{-4} M but was

active at 10^{-3} M. *N*-Methylphenethylamine-HCl (**13**) showed weak inhibitory activity.

In general, the *N*-methyl-substituted quarternary amines were more effective inhibitors than their *N*-methyl tertiary analogs. Two examples of this phenomenon are benzyldecyldimethylammonium iodide (**14**) (40% inhibition) vs benzyldecylmethylamine-HCl (**15**) (no effect), and octyltrimethylammonium iodide (**16**) that was as effective as **1** at 10^{-4} M (79% inhibition) vs dimethyloctylamine-HCl (**17**) (18% inhibition).

Comparison of quaternary and tertiary amines (Table 2) with either methyl or ethyl substitution demonstrated that *N*-ethyl substituents are more effective in the tertiary series (**18** vs **17**) whereas *N*-methyl substituents are more effective in the quaternary series (**16** vs **19**). Thus, diethyloctylamine-HCl (**18**) was an effective inhibitor of GA biosynthesis with 69% inhibition at 10^{-3} M and 39% at 10^{-4} M.

We tested a number of plant growth regulators which have been sometimes described [8, 9] as 'anti-gibberellins' or as inhibitors of GA biosynthesis. Maleic hydrazide (1,2-dihydro-3,6-pyridazine-dione) (**20**), Alar (succinic acid-2,2-dimethyl-hydrazide) (**21**), kinetin (6-furfuryl-aminopurine) (**23**), and abscisic acid (3-methyl-5-(1′-hydroxy-4′-oxo-2′,6′,6′-trimethyl-2′-cyclohexen-1′-yl)*cis*, *trans*-2,4-pentadienoic acid) (**24**) did not significantly inhibit GA synthesis in the fungus. The results suggest that the reported growth regulatory effects of these compounds are not due to direct inhibition of GA biosynthesis.

To expand information about the effects of different classes of amine inhibitors, GC–MS identification of metabolites was undertaken. A study of various octylamine inhibitors revealed different patterns of accumulation and inhibition. Table 3 shows the relative peak areas of all major components of the acid fraction from the reconstructed total ion current (TIC) chromatogram. Gibberellins GA_3, GA_4, GA_7, GA_{12}, GA_{13}, and GA_{14} were identified by GC–MS comparison with published mass spectra [10], as were *ent*-6α,7α-dihydroxykaurenoic acid [11] and the diacid (*ent*-7-oxo-6,7-*seco*kaur-16-en-6,19-dioic acid [12], which were reported to accumulate in *G. fujikuroi* cultures. Another kaurenoic acid derivative whose mass spectrum is identical to that of *ent*-6α,7α,17-trihydroxykauranoic acid [13] was found in the control and several of the treated cultures. The 6,7,17-triol has not previously been reported in *G. fujikuroi*.

Two methyl-substituted quaternary ammonium compounds, CCC (**1**) and octyltrimethylammonium iodide (**16**), have essentially identical inhibition patterns for the kaurenoic acids and the GAs (Table 3). All GAs were substantially inhibited, with GA_3 and the diacid showing the least inhibition.

The point of action in the gibberellin biosynthetic pathway of the plant growth retardants CCC and AMO 1618 has been widely studied. A number of investigators [6, 14–16] demonstrated that CCC or AMO 1618 inhibit a 'pre'-kaurene step with no evidence of 'post'-kaurene inhibition or of multiple sites of action in the pathway. AMO 1618 and a number of other plant growth retardants and biosynthetic effectors are inhibitors of kaurene synthetase [17]. Most of the latter compounds have not been tested for other possible sites of action in the pathway. Several quaternary ammonium iodide inhibitors were recently reported to act on kaurene synthetase and to have no inhibitory activity when kaurene or later intermediates were fed to inhibited cultures [18, 19].

A few recent reports indicate inhibition of the first few oxidation steps after *ent*-kaurene. Two alkylimidazoles were found that inhibit the 19-oxidation of *ent*-kaurene

Table 3. GA pattern from GC–MS analysis of bioregulator-treated culutures of *Gibberella fujikuroi*

Metabolite	Metabolites* accumulated in control (peak area)	Metabolites accumulated (% of control) † Treatment 1	16	17	18	19
GA_{12}	54	0	0	59	0	2
6,7-Diol‡	63	0	0	44	13	8
GA_{14}	56	0	0	69	11	7
6,7,17-Triol‡	6	0	17	50	516	250
GA_{13}	99	16	24	100	37	61
GA_4	90	4	11	46	50	34
Diacid‡	91	23	50	106	66	79
GA_7	74	5	9	82	32	80
GA_3	190	30	31	34	46	78

* Area of peaks from MassMax reconstructed total-ion-current chromatograms. Area units uncorrected for response of individual metabolites. Using GA_4 as a reference, 30 area units represent *ca* 1 mg in original sample. Limit of detection for this study was 0.02 mg.

† Compounds added to resuspension medium at 10^{-4} M. Cultures incubated for 48 hr, then filtered, and filtrate extracted to isolate the EtOAc-soluble acids.

‡ 6,7-Diol: *ent*-6α,7α-dihydroxykaurenoic acid, 6,7,17-triol: *ent*-6α,7α,17-trihydroxykauranoic acid, diacid: *ent*-7-oxo-6,7-*seco*kaur-16-en-6,19-dioic acid.

1: CCC, **16**: octyltrimethylammonium iodide, **17**: dimethyloctylamine-HCl, **18**: diethyloctylamine-HCl, **19**: octyltriethylammonium iodide.

and *ent*-kaurenol, thus blocking the conversion of *ent*-kaurene to *ent*-kaurenal, but show no inhibition of the conversion of *ent*-kaurenoic acid to GA_3 [20]. The plant growth retardant ancymidol, a substituted pyrimidine, was reported [21] to block the oxidation of *ent*-kaurenol and *ent*-kaurenal but not *ent*-kaurenoic acid in microsomal preparations of *Marha macrocarpus* seeds. However, it had no effect on these reactions in *G. fujikuroi*.

The ethyl-substituted compounds diethyloctylamine-HCl (**18**) and octyltriethylammonium iodide (**19**) have different patterns of inhibition from those mentioned above. While showing a general inhibition of GA accumulation, **18** and **19** caused a 5 and 2.5 times accumulation of the 6,7,17-triol, respectively (Table 3). None of the other octylamine inhibitors caused an increased accumulation of the 6,7,17-triol. Our GC–MS evidence implies an effect in a 'post'-kaurene step of the pathway after *ent*-kaurenoic acid. The fungus is known to convert *ent*-7α-hydroxykaurenoic acid both to GA_{12} aldehyde (which leads to the main GA pathway) and to *ent*-6α,7α-dihydroxykaurenoic acid [3]. The 6,7,17-triol, in addition to the 6,7-diol, has been reported to accumulate in the *Cucurbita* cell-free system when fed *ent*-7α-hydroxykaurenoic acid [22]. This alternate branch in the pathway is not known to continue on to the gibberellins in either the fungus or cell-free plant systems. This new pattern of accumulation of metabolites of the GA pathway in *G. fugikuroi* warrants further study to assess its implications.

Several compounds shown here to inhibit GA biosynthesis have reported effects on other terpenoid constituents in *Citrus*. In the tertiary amine series, all of the *N,N*-diethylamine-type lycopene inducers [23–26], chlorophyll inhibitors [27], and limonoid inhibitors [28] tested proved to be inhibitors of GA biosynthesis. Based on reported effects of exogenous GA_3 and changes observed in endogenous GA-like substances in *Citrus*, the GAs appear to exert general regulatory control over growth-associated chlorophyll and limonoid biosynthesis and maturation-associated carotenoid biosynthesis [29]. Consequently, the concomitant inhibition of GA biosynthesis by these bioregulators could enhance their ability to induce carotenoid and inhibit chlorophyll and limonoid accumulation.

Knowledge of structure–activity relationships of the bioregulators and their differential effects on the GAs and kaurenoic acids in *G. fujikuroi* should help in the selection of GA bioregulators for studies in higher plants. There is considerable agricultural potential for regulators of anabolic and catabolic pathways [24, 30–32]. This is especially true for bioregulators of the phytohormones since they offer unique possibilities for manipulating crop performance [29, 33].

EXPERIMENTAL

Culture conditions. Strain ATCC 12616 of *Gibberella fujikuroi* maintained on potato–dextrose–agar slants was subcultured in the synthetic medium ICI 20%-N [34] in shake flasks. After 2 weeks at 22°, 2 ml portions were transferred to 300 ml ICI 20%-N and incubated 6 days., Cultures were harvested during the period of rapid, linear gibberellin production (3 g dry wt mycelium, 2 mg total GA/g dry wt mycelium). The mycelium from 12 flasks was collected by filtration, washed 2 × with sterile H_2O divided into 12 equal aliquots, and resuspended in nitrogen-free medium (ICI 0%-N) with and without bioregulator for 48 hr at 22°. Bioregulators were added in aq. soln sterilized by filtration. The final vol. of resuspension medium was equal to that of the original growth cultures. All treatments were done in duplicate. Studies determined that the rate of GA production was linear throughout the 48 hr resuspension period. Controls and 10^{-3} M CCC treatments were included in each experiment to monitor consistency of response to inhibitor and to give a direct comparison between experiments. Reproducibility of response to 10^{-3} M CCC was ±4% (s.d.). The addition of a nitrogen source (such as NH_4NO_3 or diethylaminoethanol) to the N-free resuspension medium was tested for its effect on GA production. Levels of 10^{-4}–10^{-3} M of the N-containing compounds caused *ca* 10% reduction in GA accumulation during the 2-day resuspension period. Bioregulators showing 10% or less inhibition were therefore considered to be ineffective.

Source of bioregulators. Compounds **1**, **2**, **10** and **20**–**24** were used as obtained from commercial sources except **10** was recrystallized from 95% EtOH/Me_2CO. Compounds **5**–**9**, and **18** were synthesized as previously described [25].

Compound **3** was made by treating an Et_2O soln of 2-diethylaminoethyl chloride with a 3-fold excess of EtI and allowing the mixture to stand 13 days. The ppt. was recrystallized from EtOAc. Compound **4** was synthesized in the same way as **3** using 3-dimethylaminopropyl chloride and MeI. The reaction was completed overnight.

Compounds **11** and **12** were synthesized as for 4-bromodibenzylamine [26] using 4-bromobenzylbromide and benzylbromide, respectively, and an excess of furfurylamine. Instead of recrystallizing compounds from H_2O, they were resuspended in Me_2CO, filtered and the ppt. recrystallized from EtOH [35].

Compound **15** was synthesized by reacting decylbromide with a 4-fold excess of benzylmethylamine. The excess benzylmethylamine was removed by distillation at atmos. pres. The work-up was as previously described [25] except that during extraction with 10% HCl, three phases form. The middle phase is the desired material. After drying, **15** was recrystallized twice from EtOAc.

Compound **14** was synthesized by treating neutralized **15** in Et_2O with a 3-fold excess of MeI.

Compound **17** was synthesized from octylamine by the method of ref. [36] and worked up as for **18** after the excess reagent was removed with a rotary evaporator. **18** was isolated as the free amine. The test soln was made by adding an equimolar amount of HCl to the test aliquot and taking up the ppt. in H_2O.

Compound **16** was synthesized from the free amine of **17** and MeI in the same way as **3** and **4**. It was recrystallized twice from 10% $CHCl_3$ in EtOAc.

Compound **19** was synthesized in the same way as **3** and **4** using the free amine of **18** and EtI. It was recrystallized from 1% $CHCl_3$ in EtOAc.

The compounds synthesized for these tests were found to be chromatographically pure by TLC and HPLC. Analytical Si gel HL TLC plates were developed in MeOH–NH_3 (197:3) or $CHCl_3$–*iso*-PrOH–HCO_2H (5:4:1), and were visualized with Dragendorff or iodoplatinate spray reagents. HPLC employed a Whatman Magnum 9 ODS-2 reverse phase C_{18} column with a solvent of 45% MeOH in aq. 0.75 N H_3PO_4 adjusted to pH 3 with triethylamine. A refractive index detector was used in addition to a UV detector at 206 nm.

Extraction of metabolites. The cultures were filtered, the mycelium washed with H_2O (2 × 50 ml), and filtrate and washings combined. The filtrate was adjusted to pH 2 with M HCl and extracted with EtOAc (4 × 100 ml). The EtOAc layer

was extracted with 0.5 M $NaHCO_3$ (4 × 100 ml). The $NaHCO_3$ layer was adjusted to pH 2 with 6 M HCl and extracted with EtOAc (4 × 100 ml) to give the acid fraction. The mycelium was freeze-dried to constant weight. Results of inhibitor studies are included for only those tests that resulted in no significant change in mycelium dry wt or morphology.

TLC conditions. Analytical Si gel G plates were developed in di-isopropyl ether–HOAc (19:1). GAs were visualized as fluorescent spots under UV light with a spray of (a) EtOH–H_2SO_4 (95:5), then heated 10 min at 120°, or (b) H_2O–H_2SO_4 (30:70), viewed before and after heating as in (a) [37]. Minimum GA level detectable on TLC was 0.1 μg. A difference of 0.1 μg could readily be detected in the range 0.5–1.5 μg per spot allowing quantification of extracts to ±5%.

Quantification of gibberellins. TLC spot diameter and intensity were visually estimated by comparison with gibberellin standards GA_{1+3} and GA_{4+7} which were spotted next to each extract at 0.1 μg intervals in the range 0.5–1.5 μg. Triplicate aliquots of each sample were quantified and the values of duplicate samples averaged. GA content was expressed as mg GA/g dry wt mycelium. For the extraction and quantification procedures, recovery was 96% and the s.d. was 4%. The TLC values and identifications were later verified by GLC and GC–MS.

GLC conditions. Silanized glass columns (183 × 0.2 cm) were packed with 2% QF-1 or 2% SE-33. He flow was 30 ml/min, isothermal at 180° for 2 min, then temp. programmed to 230° at 2°/min, with injection temp. 250°, dual FID detector at 275°. Me derivatives were prepared in MeOH with CH_2N_2 in Et_2O; MeTMSi derivatives were prepared in sealed vials with DMCS–TMCS–C_5H_5N (2:1:10).

GC–MS conditions. Columns as above were programmed 100° for 2 min, then 4°/min to 230°. Mass spectra were obtained at 70 eV on VG Micromass 7070F with glass jet separator at 200° and VG data system and software for total ion current (TIC) and mass spectral acquisition and reprocessing. Reconstructed TIC chromatogram using VG MassMax option to eliminate overlapping peaks was used for quantitative estimation of GAs. Area units in Table 3 are uncorrected for response of individual GAs and represent comparative amounts relative to the control.

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