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Large-Scale Preparation of the Phytoalexin Elicitor Glucohexatose and Its Application as a Green Pesticide

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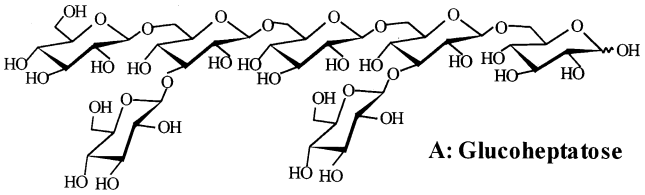
Large-scale preparation of the phytoalexin elicitor was achieved through a highly regio- and stereoselective synthesis using 2,3,4,6-tetra-*O*-benzoyl-D-glucopyranosyl trichloroacetimidate (**1**), 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**2**), and 6-*O*-acetyl-2,3,4-tri-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate (**3**) as the synthons. Coupling of **1** with **2** gave the 1→3-linked disaccharide; subsequent selective removal of 5,6-*O*-isopropylidene to give **5** followed by selective 6-*O*-glycosylation with **1** afforded the trisaccharide **6**. Hydrolysis to remove the 1,2-*O*-isopropylidene was accompanied by ring expansion, giving 3,6-branched pyranosyl trisaccharide. Acetylation, selective 1-*O*-deacetylation, and activation with trichloroacetonitrile gave the trisaccharide donor **7**. The trisaccharide acceptor **9** was prepared from condensation of the disaccharide **5** with **3** and subsequent 6-*O*-deacetylation. Coupling of the trisaccharide donor **7** with the trisaccharide acceptor **9** and subsequent deprotection afforded the glucohexatose elicitor. The cost of the produced glucohexatose should be low enough to allow its applications in agriculture as a green pesticide. At a concentration of 5−10 mg/L, the resultant elicitor was used to treat growing orange trees and harvested oranges, giving very encouraging results, comparable with those obtained using commercial pesticides at a concentration of 1400 mg/L (Topsin-M) for growing trees and 900 mg/L (Tecto) for harvested oranges, respectively. Treatment of tomato leaves against *Botrytis cinerea* with the synthetic elicitor at a concentration of 10 mg/L gave 82% inhibition, comparable with the inhibition of 84% by Wanmeiling at a concentration of 1000 mg/L. Treatment of tea leaves also showed promising results.

KEYWORDS: Phytoalexin elicitor; glucohexatose; green pesticide

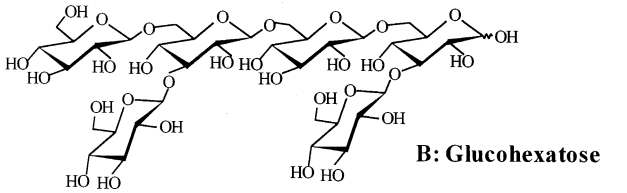
INTRODUCTION

Over the past decades, a variety of methods have been applied to protect growing crops from attack by different pathogens. Generally, various synthetic and naturally derived fungicides, bactericides, and antiviral agents have been used to treat crops, and these agents protect the crops from infection by pathogens without deleteriously affecting the growth and ultimate harvesting of the crops. While many such compositions are effective, there has nonetheless been a growing concern from consumers recently regarding the potential harmful side effects of chemical antipathogenic agents. This, in turn, has led people to pay more attention to products of natural origin, which are far less likely to cause adverse side effects.

It is known that partial acid hydrolysis of mycelial walls of the fungus *Phytophthora megasperma* f. sp. *Glycinea* gives a mixture of oligosaccharides that are capable of stimulating the formation of phytoalexins in soybeans (*1*). The most active heptasaccharide (**A**) is effective in very low doses, approximately 0.1 pmol per cotyledon (*2*). Biological assays of several oligosaccharides revealed that D-glucohexatose (**B**) is the minimum structural element required for high elicitor activity (*3*). It is to be noted that, although much of this work was done with soybean cotyledons, it was established that the glucan elicitor also elicited the synthesis of different phytoalexins in a wide range of other plant species (*4*).



A: Glucoheptatose



B: Glucohexatose

While the use of pathogenic agents on growing crops to elicit natural defenses is theoretically interesting, it has nonetheless

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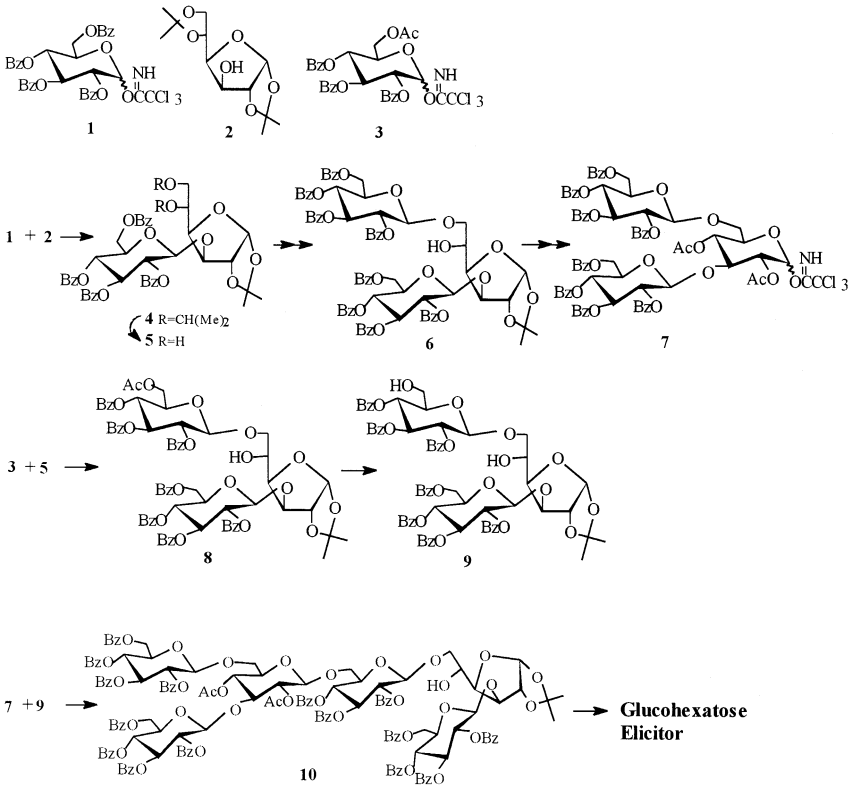
Scheme 1



not yet been a practical reality. There is concern that, by the time the cell material of a growing crop produces the needed antipathogenic agent, the growth of the pathogen has often advanced to the point at which such antipathogenic agents are of little effect. Another obstacle for the practical use of the elicitor is that the successful use of elicitors on plants in an attempt to "trick" a plant into producing antipathogenic agents has been limited to wounded plants (*5*).

We have been engaged in research and development of new, nontoxic, and nonpollutant pesticides for years. In view of its high efficiency and nontoxicity, we selected glucohexatose as a good preventive pesticide against harmful fungi. Provided that regular treatments of "healthy plants" are performed, this should allow the plants to produce enough phytoalexins to inhibit growth and infection of the harmful pathogens. Its relatively low molecular weight also suggested that glucohexatose should have a good ability to penetrate into plants, and therefore wounding of plants before treatment with the elicitor could be unnecessary. Thus, the glucohexatose elicitor could be applied directly to plants without the use of auxiliary agents such as penetration agent, surfactant, and buffer. Simple spraying of a water solution of glucohexatose elicitor onto plants would represent the best approach. A major challenge for practical use of the elicitor is to develop a simple, low-cost method for the large-scale preparation of glucohexatose. This article describes this preparation and the practical application of the elicitor in orange growth and storage.

MATERIALS AND METHODS

Glucohexatose Preparation. Condensation of benzoylated glucosyl trichloroacetimidate **1** with 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose **2** afforded the disaccharide **4**; selective removal of the 5,6-*O*-isopropylidene group to give **5** and selective 6-*O*-glycosylation of **5** with **1** gave the trisaccharide **6** (Scheme 1). Removal of the 1,2-*O*-isopropylidene group of **6** was accompanied by ring expansion. Subesequent acetylation, selective 1-*O*-deacetylation, and trichloroacetimidation gave the trisaccharide donor **7**. The trisaccharide acceptor **9** was obtained by condensation of **5** with 6-*O*-acetyl-2,3,4-tri-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate (**3**) and then selective 6-*O*-deacetylation. It is noted that compound **3** was prepared from benzoylation of 1,6-anhydro-*β*-D-glucopyranose (levoglucosan), an inexpensive material obtained from pyrolysis of cellulose (*6*), followed by acetolysis, 1-*O*-deacetylation, and trichloroacetimidation. Condensation of the trisaccharide donor **7** with the trisaccharide acceptor **9** followed by deprotection gave the target glucohexatose.

Preparation and Characterization of Important Intermediates. *Compound 6*. To a stirred solution of 2,3,4,6-tetra-*O*-benzoyl-*β*-D-glucopyranosyl-(1→3)-1,2-*O*-isopropylidene-α-D-glucofuranose (**5**, 80 g, 0.1 mol) and 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate (**1**, 80 g, 0.108 mol) in dichloromethane (250 mL) was added trimethylsilyl trifluoromethanesulfonate (TMSOTf, 200 μL) at room temperature. After 3 h, triethylamine was added to the solution to quench the reaction. The solution was concentrated, and the residue was subjected to column chromatography with 2:1 petroleum ether−ethyl acetate as the eluent to give the trisaccharide **6** (114.2 g, 83%): $[\alpha]_D$ + 15.3° (*c* 1.0, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 8.06−7.28 (m, 40 H), 5.88 (t, 1 H), 5.87 (t, 1 H), 5.69 (t, 1 H), 5.64 (t, 1 H), 5.53 (dd, 1 H), 5.43 (dd, 1 H), 5.41 (d, 1 H), 4.96 (d, 1 H), 4.93 (d, 1 H), 4.68 (dd, 1 H), 4.48 (dd, 1 H), 4.67 (dd, 1 H), 4.35 (dd, 1 H), 4.34−3.65 (m, 8 H), 1.26, 1.03 (2 s, 6 H).

Compound 7. Compound **6** (50 g, 0.036 mol) was added to 80% aqueous acetic acid solution (500 mL), and the mixture was heated under reflux for 5 h. The mixture was concentrated, and the residue was acetylated with acetic anhydride (250 mL) in pyridine (280 mL) overnight. The resultant trisaccharide was dissolved in 3:1 THF−CH_3-OH (500 mL) containing ammonia (1.2 mol), and the solution was stirred at room temperature for 2 h. The solution was concentrated, and the residue was dissolved in dichloromethane (200 mL). To the solution were added K_2CO_3 (20 g) and CCl_3CN (10 mL), and the mixture was stirred at room temperature overnight. The mixture was filtered, the filtrate and washings were concentrated, and the residue

was subjected to column chromatography, giving the trisaccharide donor **7** as a solid (40.0 g, 71%): $[\alpha]_D$ + 23.3° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (s, 1 H, CN*H*CCl_3), 8.07−7.19 (m, 40 H, 8 Ph*H*), 6.19 (d, 1 H,), 5.91 (t, 1 H,), 5.85 (t, 1 H), 5.62 (t, 1 H), 5.61 (t, 1 H), 5.46 (dd, 1 H), 5.42 (dd, 1 H), 4.97 (d, 1 H), 4.96 (d, 1 H), 4.85 (t, 1 H), 4.67−4.59 (m, 3 H), 4.50−4.37 (m, 2 H), 4.19−4.02 (m, 4 H), 3.91 (dd, 1 H), 3.69 (dd, 1 H), 1.94, 1.78 (2 s, 6 H).

*Compound **9***. Using the same procedure as described for the preparation of **6** from **1** and **5**, the trisaccharide **8** (105.1 g, 80%) was prepared from **3** (73.2 g, 0.108 mol) and **5** (80 g, 0.1 mol). Compound **8** (100 g, 0.076 mol) was dissolved in 250 mL of CH_3OH, 8 mL of acetyl chloride was added, and the reaction was carried out at room temperature for 8 h. After neutralization and concentration, the residue was subjected to column chromatography, giving compound **9** as a solid (87.1 g, 90%): $[\alpha]_D$ + 12.6° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05−7.26 (m, 35 H), 5.91 (t, 1 H), 5.90 (t, 1 H), 5.73 (t, 1 H), 5.56 (t, 1 H), 5.54−5.42 (m, 3 H), 4.99 (d, 1 H), 4.95 (d, 1 H), 4.75−3.77 (m, 12 H), 1.33, 1.05 (2 s, 6 H).

*Compound **10***. A solid hexasaccharide **10** (77.0 g, 90%) was prepared from **7** (50 g, 0.032 mol) and **9** (40.7 g, 0.032 mol) under the same conditions as described for the preparation of **6** from **1** and **5**. For **10**: $[\alpha]_D$ + 6.6° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04−7.18 (m, 75 H), 6.13, 5.88, 5.83, 5.74 (4 t, 4 H), 5.69, 5.65, 5.62, 5.57 (4 t, 4 H), 5.50, 5.48, 5.44, 5.34 (4 dd, 4 H), 5.45 (d, 1 H), 5.07, 4.94, 4.83, 4.80 (4 d, 4 H), 1.95, 1.87 (2 s, 6 H), 1.33, 1.08 (2 s, 6 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.4, 168.2 (2 CH_3*C*O), 166.1−164.7 (15 Ph*C*O), 105.0, 101.5, 101.1, 101.0, 100.9, 100.2 (C-1), 82.9, 82.5 (C-3), 20.85, 20.51 (2 *C*H_3CO), 14.2 (C(*C*H_3)$_2$).

*The Glucohexatose Elicitor (**B**)*. Compound **10** (50 g, 0.0187 mol) was dissolved in 80% acetic acid solution (250 mL), and the mixture was heated under reflux for 6 h. Concentration of the mixture followed by deacylation in ammonia-saturated methanol at room temperature for 24 h gave the target glucohexatose elicitor (**B**) as a powder (17.6 g, 95%): $[\alpha]_D$ −15.1° (*c* 0.2, MeOH); ^{13}C NMR (200 MHz, D_2O) δ 102.6−102.3 (6 C-1), 84.0 (C-3), 69.2 (C-6), 60.4 (C-6); ESMS *m*/*z* 989.5 $[M - 1]^+$; FW = 990.86.

Antifungi Test for Orange Growth and Storage. The test was conducted in an orange garden located in the suburb of Chongqing City of southwest China, where the terrain was smooth, soil was lightly sandy, and there was good sunshine. The tested trees were 16 years old, and there were 66 trees in a 660 m^2 area, with a distance of 4 m between two trees and 2.5 m between two tree lines. The growing posture of the trees was typical. Before the test was started, orange trees were treated once only with mitecide in May. At the time the test began, there was no disease on any of the fruit. Spraying was done using a back-carried MATABI-16 sprayer. The working pressure of the sprayer was 3 Pa, the diameter of the spray nozzle was 1 mm, the rate of solution spraying was 0.5 L/min, and the sprayed fog drops had a diameter of 80−100 μm. The spraying amount was 2 kg of solution per tree, and 1980 kg per hectare. There were four sprayings: on Oct 17, Oct 22, Oct 27, and Nov 2. There were five treatment groups: 5, 3, and 1 mg/L of the glucohexatose, 1400 mg/L of Topsin-M (thiophanate-methyl), and blank water. There were four small districts per treatment, and each district had two trees randomly arranged and isolated by a protection tree. Since no infection had occurred at the time when the test began, the study on infection base was not needed. On Nov 30, 2000, just before the harvesting, a classified investigation of the fruits was carried out. Sampling was conducted on the east, west, north, south, and center portion of each tree, respectively. Four oranges were investigated in each portion, and there were 40 oranges for investigation in each small district. The disease index and prevention effect were calculated on the basis of the investigation using the DMRT method for determination of markedness. There were nine classes: class 0, no disease spot; class 1, one or two disease spots per orange; class 3, three or four disease spots per orange; class 5, five or six disease spots per orange; class 7, seven or eight disease spots per orange, and the spots were partially coupled, occupying one-fifth of the area of the orange surface; class 9, more than nine disease spots per orange, and the spots were coupled, occupying more than one-fourth of the area of the orange surface. The pesticide efficiency was calculated as follows:

$$\text{disease index} = \frac{\Sigma(\text{no. of infected oranges} \times \text{corresponding class order})}{\text{total no. of investigated oranges} \times 9} \times 100$$

$$\text{prevention efficiency} = \frac{\text{disease index in control area} - \text{disease index in testing area}}{\text{disease index in control area}} \times 100$$

A test of the use of the synthetic elicitor as an orange (*Glorioius orange*) antidecay agent was carried out in a storehouse with natural ventilation. In the storehouse there were four layers of iron racks with wooden fruit boxes on each rack. Fifteen days before the test, the storehouse and the boxes were sterlized. The oranges, of uniform size and without mechanical damage, had not been treated with any bactericides before the testing. On Dec 1, the freshly harvested oranges were treated with the synthetic elicitor at concentrations of 1, 5, and 10 mg/L, with Tecto suspension (Monsanto) at a concentration of 173 mg/L, and with blank water. There were four runs for each treatment, and 100 oranges were used for each run. The tested oranges in each

Table 1. Prevention of *Colletotrichum glocosporioides* Penz. in Growing Trees[a]

		disease index after treatment					
		small district					
treatment	concn (mg/L)	1	2	3	4	av	PE (%)
SE	5	3.06	2.78	3.33	2.78	2.99	75.53
SE	3	3.61	3.33	3.06	3.33	3.33	72.75
SE	1	4.72	4.17	4.17	3.89	4.24	65.30
MET	1400	3.06	2.50	2.22	2.50	2.57	78.97
control		13.06	11.67	12.22	11.94	12.22	

[a] SE, synthetic elicitor; MET, Topsin-M (obtained from 70% Topsin by 500 times dilution with water); control, blank water; PE, prevention efficiency.

Table 2. Prevention of Diseases in Oranges during Storage[a]

		treatment				
		SE (1 mg/L)	SE (5 mg/L)	SE (10 mg/L)	Tecto (900 mg/L)	control
applied amount of effective composition (g/ton orange)		0.19	0.96	1.92	173.1	
after 30 days	decay (%)	1.75	1.5	1.25	0.75	3.23
	PE (%)	46.15	53.85	61.53	76.92	
after 60 days	decay (%)	5.75	4.5	2.25	1.75	9.75
	PE (%)	41.03	53.85	76.92	82.05	
after 90 days	decay (%)	13.5	11.75	6.5	3.75	25
	PE (%)	46.02	53.08	74.07	85.03	

[a] SE, synthetic elicitor; Tecto, obtained from 45% Tecto by 500 times dilution with water; control, blank water; PE, prevention efficiency.

Table 3. Prevention Efficiency of Synthetic Elicitor for *Botrytis cinrea* on Tomato Leaves[a]

	treatment					
	SE (0.5 mg/L)	SE (1 mg/L)	SE (5 mg/L)	SE (10 mg/L)	Wanm.	control
concn of effective composition (mg/L)	0.5	1	5	10	500	0
disease index	30.1	26.6	21.6	15.0	16.7	98.3
PE (%)	69.38	72.94	78.03	84.74	83.01	

[a] SE, synthetic elicitor; Wanm., Wanmeiling (obtained from 50% Wanmeiling by 1000 times dilution with water); control, blank water; PE, prevention efficiency.

Table 4. Effect of Synthetic Elicitor on Feeding Amount and Weight of *Ectropic obliqua* Prout

treatment[a]	feed amount[b] (mg/single)	feed decrement (%)	property of difference	weight[b] of the larvae (mg/single)	weight change (±%)	property of difference	weight[b] of the pupae (mg/single)	weight change (±%)	property of difference
SE, 0.1 mg/kg	76.79	1.27	Aa	96.37	8.07	Aa	86.52	7.01	Aa
SE, 1 mg/kg	65.26	16.10	Ab	81.33	–8.79	Ab	73.72	–8.82	BCc
SE, 10 mg/kg	71.85	7.60	Aab	83.98	–5.82	Aab	74.17	–8.26	BCc
Bt, 500 times dilution	75.09	3.46	Aa	56.21	–36.92	Bc	70.44	–12.9	Cc
blank	77.78		Aa	89.17		Aab	80.85		Abb

[a] Test area for each treatment was 32.3 m^2. Spraying used back-carried sprayer at an amount of 750 kg/hectare. Starting from Oct 16, fresh tea leaves were taken for feeding *Ectropic obliqua* Prout. The leaves were replaced every other day with newly collected ones. Each treatment is divided into five groups, and 15 larvae of two instar for each group. [b] Average value for five groups.

run were dipped in a specified solution for 1 min and then taken out to dry under natural air flow and put into a wooden fruit box. The boxes were randomly arranged for different treatments when put onto the iron rack in the storehouse at room temperature. One week later, the oranges were packed in small plastic bags. During the test, the average temperature was 9.8 °C; the highest temperature was 16.2 °C, and the lowest temperature was 5.6 °C. The average relative humidity was 83.8%. Infections by *Pencillium italicum* Wehmer, *Pencillium digitatum* Sacc., *Colletotrichum glocosporioides* Penz., *Phoopsis citri* Fawc., *Phytophthora parasitica* Dastur., and *Oospora citri aurautii* Sacc. were detected. The first detection was conducted 30 days after treatment, and the second and the third, 60 days and 90 days after treatment, respectively. No changes in the appearance and taste of oranges were observed after testing. Calculations were done according to the following equations:

$$\text{decay (\%)} = \frac{\text{no. of decayed oranges}}{\text{no. of totally treated oranges}} \times 10$$

$$\text{prevention efficiency} = \frac{\text{decay in control} - \text{decay in treated}}{\text{decay in control}} \times 10$$

The synthetic elicitor was also tested for prevention of *Botrytis cinerea* on tomato leaves. The tested aqueous solutions were 0.5, 1, 5, and 10 mg/L solutions of the synthetic elicitor, 500 mg/L Wanmeiling, and blank water. Tomato leaves of similar size were taken from the plant body, sprayed with the test solution, and then dried under natural air flow (for about 1 day), inoculated with *B. cinerea*, and incubated at room temperature with a certain moisture and alternative light and dark. Three days after the inoculation, detection was conducted by determination of disease spot diameter and number of infected leaves, and calculations were done according to the following equations:

$$\text{disease index} = \frac{\Sigma(\text{no. of infected leaves} \times \text{corresponding class order})}{\text{total no. of tested leaves} \times \text{the highest class number}} \times 100$$

$$\text{prevention efficiency} = \frac{\text{disease index in control} - \text{disease index in treated}}{\text{disease index in control}} \times 100$$

RESULTS AND DISCUSSION

Large-Scale Production of the Glucohexatose Elicitor Is Possible. The de novo chemical synthesis of the oligosaccharide elicitor (*7*−*9*) is a rapidly developing field; however, this mainly serves the investigation of structure−activity relationships and has not been of practical use. We have reported a highly regio- and stereoselective method to prepare the glucohexatose elicitor through ortho ester formation−rearrangement (*10*, *11*). Substantial improvements have been made to this method to develop a new and efficient way to prepare the elicitor on a large scale (see Scheme 1). This new route contains several multistep one-pot reactions. In terms of simplicity and efficiency, the above-described synthesis can be applied as a practical production method (*12*) at very low cost. Hundreds of grams of glucohexatose have be prepared within a 2-month period in our laboratory, and the construction of a model factory to produce 200 kg/year is under consideration.

The Synthetic Elicitor Has a High Efficiency of Prevention of Fungi Invention. *Colletotrichum glocosporioides* Penz. is one of the major diseases in oranges, occurring usually near harvest time. **Table 1** shows that the synthetic glucohexatose at a concentration of 5 mg/L gave inhibition of *Colletotrichum glocosporioides* Penz. similar to that of Topsin-M at a concentration of 1400 mg/L. Even at concentrations as low as 1 mg/L, the synthetic elicitor was still effective for inhibition of *Colletotrichum glocosporioides* Penz., indicating the high potency of the synthetic elicitor as a pesticide.

Table 2 shows that at a concentration of 10 mg/L (corresponding to 1.92 g/ton of oranges), the synthetic elicitor was an effective antidecay agent. Better results may be possible at higher concentrations.

Table 3 shows that the synthetic elicitor was very effective for prevention of *B. cinerea* on tomato. At a concentration of 10 mg/L it gave 82% inhibition, comparable with the inhibition of 84% by Wanmeiling at a concentration of 1000 mg/L. Large area tests for the prevention of disease in cucumber, eggplant, and tomato are in progress.

Table 4 shows the test of the synthetic glucohexatose against *Ectropis obliqua* Prout in tea leaves using *Bacillus thuringiensis* (Bt) and blank water as comparisons. Before feeding of *E. obliqua* Prout, the tea trees were sprayed on Oct 9 and Oct 16 with 0.1, 1, and 10 mg/L solutions of the synthetic elicitor, 500 times diluted Bt, and blank water, respectively. It was found that at a concentration of 1 mg/L, the synthetic elicitor showed some feed-antagonizing effect for *E. obliqua* Prout (feed decrement 16.1%). However, its influence on body weight of *E. obliqua* Prout was worse than Bt's effect (−8.8 vs −36.9), indicating that the synthetic elicitor is not a good prevention pesticide for *E. obliqua* Prout.

Conclusion. Large-scale production of the glucohexatose elicitor at low cost is possible. The synthetic elicitor can be applied as a green pesticide to protect growing oranges and tomatoes from attack by harmful fungi and can be used as an antidecay agent to keep oranges fresh.

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