Plant Physiol. (1984) 74, 989-992
0032-0889/84/74/0989/04/$01.00/0

Characteristics of Galacturonic Acid Oligomers as Elicitors of Casbene Synthetase Activity in Castor Bean Seedlings[1]

Received for publication November 1, 1983 and in revised form December 27, 1983

DONALD F. JIN AND CHARLES A. WEST*
Department of Chemistry and Biochemistry, University of California-Los Angeles, Los Angeles, California 90024

ABSTRACT

Partial digestion of polygalacturonic acid with polygalacturonase isolated from *Rhizopus stolonifer* produces a mixture of α-1,4-D-galacturonide oligomers which act to elicit casbene synthetase activity in castor bean (*Ricinus communis* L). These oligomers were separated by anion exchange chromatography on DEAE Sephadex A-25 into discrete sizes and their degrees of polymerization were analyzed by fast atom bombardment mass spectrometry. A minimum degree of polymerization of nine units appears to be required for elicitor activity; trideca-α-1,4-D-galacturonide was the most active of the oligomers tested. Methylesterification of the carboxylate groups greatly diminishes the elicitor activity of the oligomers, a finding which suggests a requirement for the polyanionic character of the oligomers for full activity. The fact that a number of other polyanionic polymers tested as casbene synthetase elicitors did not show significant activity indicates that structural features other than the polyanionic character are also necessary for activity.

Castor bean (*Ricinus communis L.*) seedlings respond to challenges from a variety of fungi by producing an antifungal diterpene hydrocarbon, casbene (8). Casbene synthetase activity in castor bean seedlings increases dramaticlly in response to a heat-labile elicitor which was purified to homogeneity from culture filtrates of the fungus, *Rhizopus stolonifer*. The elicitor activity was found to be associated with the enzyme endopolygalacturonase, an endo α-1,4-galacturonide hydrolase (6), and was subsequently shown to be absolutely dependent on the catalytic activity of this enzyme (2). Several lines of evidence support the idea that heat-stable pectic fragments released from isolated castor bean cell walls through the action of the enzyme serve as obligate intermediates in the elicitation process (2).

These results suggested that products released by degradation of the plant cell wall might act as signals for the elicitation of stress metabolites under certain circumstances. A majority of the work on elicitors has focused on molecules of fungal origin as the primary signals in eliciting the plants's response (9). However, recent work in several laboratories has called attention to molecules originating in the plant as elicitors. Hargreaves *et al.* (4, 5) have described the elicitation of phaseollin in hypocotyls of *Phaseolus vulgaris* in response to 'constitutive metabolites' released from damaged bean cells. The chemical nature of these constitutive elicitors has not been elucidated. Hahn *et al.* (3) found that partial acid hydrolysis of cell walls of soybeans and other plants released 'endogenous elicitors' that acted to stimulate the production of phytoalexins in soybean tissue. The major active components of the endogenous elicitors produced from soybean cell walls and citrus pectin have recently been characterized and shown to be oligomers of α-1,4-linked galacturonosyl residues (7).

Mixtures of oligomers produced by the partial digestion of citrus pectin and polygalacturonic acid by *R. stolonifer* endopolygalacturonase proved to be heat-stable elicitors (2). The work described in the present paper characterizes the structural requirements for the elicitor activity of these heat-stable fragments generated by the partial digestion of polygalacturonic acid by this fungal endopolygalacturonase.

MATERIALS AND METHODS

Chemicals. Thin layer silica gel plates were purchased from E. Merck Laboratories. Sephadex molecular sieving gel (G-10) and ion exchange resin (DEAE Sephadex A-25) were from Pharmacia Fine Chemicals. Diazald was from Aldrich, and Polyclar AT from General Aniline and Film Corp. The following materials were purchased from Sigma: Dowex AG50W-X8, 20 to 50 mesh and 200 to 400 mesh, polygalacturonic acid grade III, dextran sulfate, polyvinyl sulfate, chondroitin sulfate, hyaluronic acid, heparin and polyglutamate. *Meta*-hydroxy biphenyl was purchased from Pfaltz and Bauer, Stamford, CT.

Biological Materials. Castor bean seeds (*Ricinus communis L.*) were obtained from plants growing in the botanical gardens at the University of California, Los Angeles. Endopolygalacturonase from *R. stolonifer* culture filtrates was purified as previously described (6).

Elicitor Bioassay. The elicitor bioassay originally described by Lee and West (6) was slightly modified for the work described here. Four hundred castor bean seeds were mechanically freed from their coats and surface-sterilized in 0.01% NaOCl (10 ml commercial bleach in 500 ml of sterile distilled H_2O). The seeds were rinsed 6 times in a total volume of 3.5 L of sterile distilled H_2O, placed in a sterile 19-cm crystallizing dish lined with 3 layers of Whatman No. 1 filter paper, 8 layers of cheesecloth and containing 80 ml of water. The seeds were allowed to germinate for 55 h at 27°C. Ten seedlings with well developed radicles and no signs of necrosis were used for each assay. The radicles were removed and the seedlings were split in half in a plane parallel to the cotyledons. The half-seedlings were placed cut-side down in a 15 × 100 mm Petri plate with 1 layer of cheesecloth and 5 ml of the solution to be tested for elicitor activity, and incubated for 10 to 12 h at 25°C under aseptic conditions. The pooled seedlings from one treatment were weighed, Polyclar AT in the amount of one-third the weight of the seedlings was added along with cold buffer (50 mM Tris·HCl, 50 mM $NaHCO_3$, pH 6.8, containing 10 mM freshly added 2-mercaptoethanol) in the ratio of 2.5 ml buffer per gram of seedling. The suspension was homogenized for 1 min in an ice bath at top speed in a VirTis

[1] Supported by National Science Foundation Grant PMC 79-23142 and United States Department of Agriculture Grant 82 CRCR-1-1090.

"23" homogenizer. The homogenate was filtered through 8 layers of cheesecloth and the filtrate centrifuged at 37,000*g* at 40°C for 15 min. A small portion of the supernatant was carefully removed from the floating lipid layer for the assay of casbene synthetase activity. One hundred μl of the supernatant was mixed with 50 μl of H_2O and 250 μl of buffer (200 mM Tris·HCl, pH 9.0, containing 20 mM $MgCl_2$). The reaction was initiated by the addition of 100 μl of 50 μM [^{3}H]geranylgeranyl pyrophosphate (10 mCi·mmol^{-1}) and allowed to proceed for 30 min at 30°C before it was terminated by addition of ethanol:petroleum ether (1:3, v/v). The radioactivity associated with [^{3}H]casbene was extracted into petroleum ether and the extract was subjected to TLC on $AgNO_3$-impregnated plates developed with benzene:petroleum ether (3:7, v/v). The radioactivity associated with casbene was measured in the 1-cm section at the $AgNO_3$ origin by liquid scintillation spectrometry as described previously.

Preparation of α-1,4-D-Galacturonide Oligomer Samples. Two grams of high mol wt polygalacturonic acid (degree of polymerization = 40 to 50) was dissolved in 400 ml of 10 mM sodium acetate and the pH was adjusted to 4.7. Endopolygalacturonase isolated from *R. stolonifer* (17.6 units) was added to the solution which was then incubated at 30°C for 20 min. The enzyme was heat inactivated by placing the solution into a boiling water bath for 20 min after which the sample pH was readjusted to 7.0. To generate oligomers for the esterification experiment, 5 g of polygalacturonic acid was dissolved in 1 L of buffer and digested with 44 units of polygalacturonase under the same conditions described above. A 660-ml (2.5 × 135 cm) column of DEAE Sephadex A-25 was equilibrated with starting buffer (100 mM KCl, 10 mM imidazole, pH 7.0). The mixture of oligomers generated by digestion was adjusted to a conductivity slightly less than that of the starting buffer by dilution with H_2O and slowly loaded onto the column. After washing the column with 2 L of starting buffer, the oligomers were eluted with an 8-L nonlinear concave salt gradient: 100 mM KCl, 10 mM imidazole (pH 7.0) to 300 mM KCl, 10 mM imidazole (pH 7.0). Thirteen-ml fractions were collected at a flow rate of 20 ml·h^{-1}·cm^{-2}. The uronic acid content of fractions was monitored by the Blumenkrantz and Hansen method with *m*-hydroxybiphenyl (1). Peaks from the anion-exchange column were pooled individually, lyophilized and desalted on a Sephadex G-10 column.

FAB[2]-MS Analysis. A 1-mg sample of each desalted oligomer fraction to be analyzed was passed through a 1-ml column of Dowex AG50W-X8, 200 to 400 mesh, in the H^+ form to convert carboxyl groups to their protonated forms. The samples were analyzed at the NSF Mid-Atlantic MS Facility at Johns Hopkins University. Samples were dissolved in thioglycerol and analyzed by FAB-MS in the positive mode.

Methylation of Oligomers. A mixture of oligomers with degrees of polymerization of 12 to 15 was prepared by partial digestion of 5 g of polygalacturonic acid with *R. stolonifer* endopolygalacturonase, and fractionation of the mixture on a DEAE Sephadex A-25 column as described above. The profile obtained here showed slightly poorer resolution of the peaks than with 2 g of polygalacturonic acid separated under the same conditions (Fig. 1). The fractions corresponding to degrees of polymerization of 12 to 15 units were pooled, lyophilized and desalted on a Sephadex G-10 column. The mixture of oligomers in the void volume fractions was then converted to the protonated form by passage through a 182-ml Dowex AG50W-X8, 20 to 50 mesh column (2.5 × 37 cm) in the H^+ form. The protonated oligomers were lyophilized to dryness. Diazomethane (CH_2N_2) was generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) in an Aldrich diazomethane generating kit as described by Aldrich. Approximately 3 g of CH_2N_2 was generated in a volume of 250 ml in diethyl ether; 550 mg (by weight) of the protonated oligomers was suspended in 250 ml of redistilled methanol in a 500-ml round bottom flask. A very fine suspension resulted. A 75-ml portion of the CH_2N_2/Et_2O solution was added slowly dropwise with stirring; a yellow color persisted after addition of the first 50 ml. The sample was concentrated by rotary evaporation to approximately 20 ml. Redistilled methanol was added back to a total volume of 250 ml and 25 ml of the CH_2N_2/Et_2O was quickly added and allowed to stir for 5 min. Another 25 ml of the CH_2N_2 solution was added and after 10 min a third 25-ml portion was added, following which the solution was allowed to stir for 15 min. The sample was concentrated to dryness by rotary evaporation and redissolved in 50 ml of distilled H_2O. The solution of methyl ester was passed through a DEAE Sephadex A-25 column (75 ml bed volume); partially esterified oligomers were presumably bound to the column while fully esterified oligomers with no net charge passed through. Approximately 43% of the original sample was recovered in the fully esterified fraction. Analysis of this sample by ^{1}H-NMR revealed the presence of a sharp unsplit peak at a chemical shift of 3.77 ppm which is characteristic of a carboxylate methyl ester. This fraction was used as the sample to test the elicitor activity of the methyl ester.

De-esterification of Methyl-Esterified Oligomers. Methyl-esterified oligomers generated in the previous step were de-esterified by mild acid hydrolysis. Six ml of 4 N trifluoroacetic acid was added to 70 mg of esterified oligomers in 6 ml of H_2O. The solution was sealed in a 25-ml screw top test tube with a Teflon cap and incubated at 85°C for 2 h. The solution was evaporated to dryness by rotary evaporation, and the residue was resuspended in methanol and taken to dryness by rotary evoporation 3 times. ^{1}H-NMR analysis of the product showed the absence of the characteristic methyl ester signal that was present in the esterified sample.

NMR Spectrometry of the Oligomers. ^{1}H-NMR was performed on a 200 MHz Bruker WP200 spectrometer. The samples were prepared by lyophilizing them to dryness twice from 99% D_2O and then dissolving them in D_2O. A co-axial capillary tube containing tetramethylsilane in deuterated chloroform was used as the reference.

RESULTS

Generation and Fractionation of α-1,4-D-Galacturonide Oligomers. A series of α-1,4-D-galacturonide oligomers of known sizes were generated in order to characterize the structural requirements for elicitor activity of polygalacturonic acid fragments. High mol wt polygalacturonic acid was partially digested by treatment with *R. stolonifer* endopolygalacturonase and the resulting mixture of oligomers was fractionated by DEAE Sephadex A-25 anion exchange chromatography as described in "Materials and Methods". Figure 1 illustrates the fractionation of the digest into a regular series of distinct but progressively more closely spaced peaks. It was presumed that this represented a series of oligomers differing in size by one galacturonosyl unit with the smallest oligomer emerging first from the column.

To confirm this, peak fractions from selected peak regions were desalted, converted to the protonated form, and analyzed by FAB-MS. Typical FAB-MS analysis of individual peaks generated very clean spectra revealing major peaks corresponding to the parent ion associated with either sodium or ammonium ions and minor peaks corresponding to loss of whole galacturonide residues due to fragmentation. For example, peaks 8 and 10 from the column gave spectra of masses 1621 and 1972 corresponding to molecular ions (M + Na^+) for a nonamer and undecamer of D-galacturonide, respectively. Of the fractions of interest analyzed, only fraction 11 showed spurious ions in addition to the parent ion corresponding to the dodecamer; we

[2] Abbreviation: FAB, fast atom bombardment.

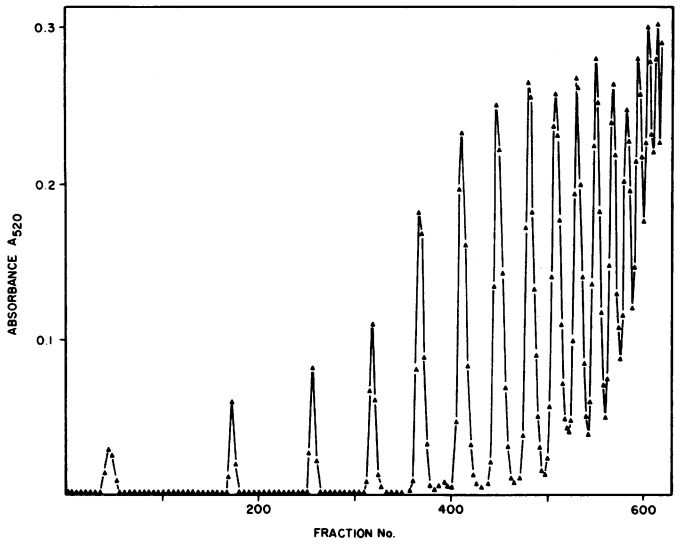

FIG. 1. Anion exchange chromatography of a mixture of oligogalacturonides. Polygalacturonic acid was partially hydrolyzed with endopolygalacturonase and the resulting mixture of oligomers separated on Sephadex DEAE A-25 with a KCl salt gradient as described in "Materials and Methods". The first peak corresponds to a dimer with each successive peak one galacturonosyl unit longer as determined by FAB-MS.

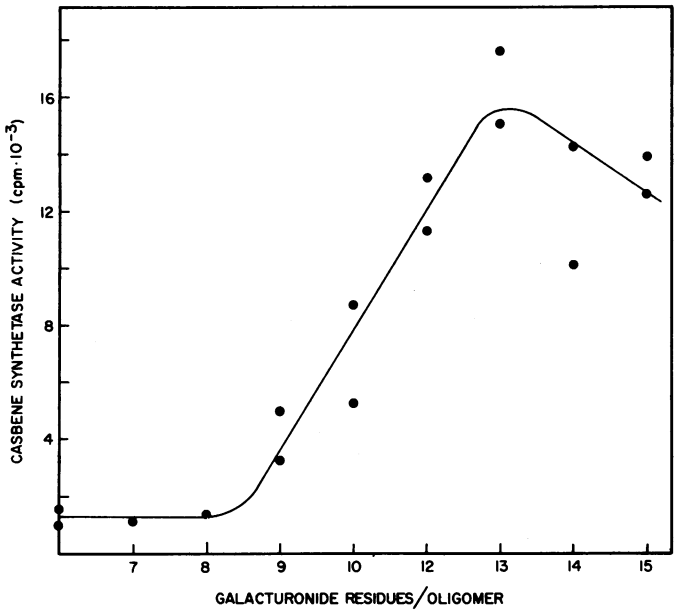

FIG. 2. Casbene synthetase activity as a function of α-1,4-D-galacturonic acid oligomer length. Peaks from the anion exchange chromatography of a mixture of oligomers of galacturonic acid were individually pooled, desalted, and tested in the castor bean bioassay at a concentration of 1 mg·ml^{-1}.

believe these must derive from a contaminant(s) of unknown origin in this fraction. We conclude that the peaks in Figure 1 represent a series of oligomers of α-1,4-D-galacturonide differing in size by one unit beginning with the dimer as the first fraction eluted.

Elicitor Activity of Individual Oligomers. Individual oligomers obtained from the anion exchange column were desalted and assayed for casbene synthetase elicitor activity with the bioassay described in "Materials and Methods". The oligomers were assayed at a concentration of 1 mg uronic acid·ml^{-1} with the results shown in Figure 2. The fractions corresponding to octomers and smaller oligomers showed no significant elicitor activity, while fractions corresponding to nonamers through pentadecamers were active, with the tridecamer eliciting the greatest response. Homogeneous fractions of larger sizes were not obtained for testing. This experiment was performed three times with different preparations of oligomers with very similar results each time. The small decrease in elicitor activity of fractions larger than the tridecamer may be due, at least in part, to a decrease in the number of moles of material tested since the assay samples were of equal weight rather than equimolar.

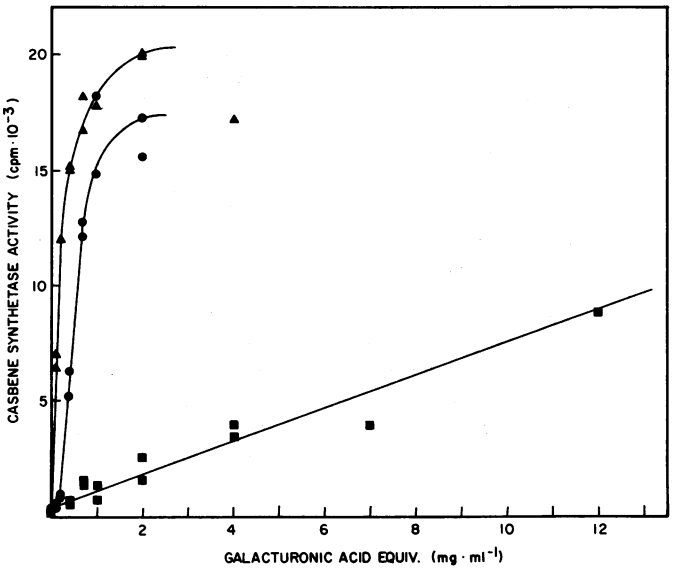

FIG. 3. Effect of esterification on the elicitor activity of oligomers of galacturonic acid. Dose response curves were generated for a mixture of active oligomers of α-1,4-D-galacuronic acid (ED_{50} = 0.6 mg·ml^{-1}), the same mixture of oligomers after methyl esterification of the carboxylate groups (ED_{50} = 13.5 mg·ml^{-1}), and a sample after removal of the ester groups from the derivatized sample by mild acid hydrolysis (ED_{50} = 0.15 mg·ml^{-1}). The samples were prepared by the procedures described in "Materials and Methods".

Table I. *Test of Various Polyanions for Elicitor Activity*

Substance	Concentration	Elicitor Activity
	mg/ml	
Polygalacturonic acid	0.5	+
Chondroitin sulfate	10	–
Hyaluronic acid	1.7	–
Dextran sulfate	10	–
Polyglutamic acid	1.0	–
Heparin	1.0	–
Polyvinyl sulfate	10	–

A dose response curve was performed for the elicitor activity of the fraction containing predominantly the tridecamer (data not shown). The concentration required for 50% of the maximum response (ED_{50}) was 0.1 mg uronic acid·ml^{-1}.

Effect of Methyl Esterification of Carboxylate Groups in the Oligomers on Elicitor Activity. The elicitor activity of a pool of desalted, protonated active oligomers was compared with that of a fully methylated sample of oligomers prepared from the same pool in order to assess changes in elicitor activity due to the conversion of the carboxylate groups to a nonionizable form. As a control, unsubstituted oligomers regenerated from the fully methylated sample were also tested. Dose response curves for these three samples are presented in Figure 3. The starting material gave an ED_{50} of 0.6 mg·ml^{-1}, a value about two times higher than expected based on other experiments with similar

samples. The fully methylated sample, with an estimated ED_{50} of 13.5 mg uronic acid·ml^{-1}, was more than 20 times less effective than the nonderivatized reference sample. The elicitor activity of the demethylated sample (ED_{50} = 0.15 mg uronic acid·ml^{-1}) was fully restored. Although the demethylated sample appeared to be more active than the starting material, it is not clear that this difference is significant. Two other experiments gave results very similar to the one reported here. In one instance, a methylated sample appeared to be nearly as active as a nonderivitized sample. The reason for the discrepancy in that case is not known.

Test of Other Polyanions. A number of different polyanionic polymers were tested in the elicitor bioassay in order to provide some information on the specificity of response (Table I). None of the other polyanions were able to generate a significant response at the concentrations tested. It should be noted that these polymers were tested as obtained without fragmentation or other modification.

DISCUSSION

It was proposed on the basis of earlier observations that pectic fragments from the plant cell wall might serve as obligate intermediates in the elicitation of casbene synthetase activity in response to fungal endopolygalacturonase action on castor bean seedlings (2). Support of this proposal comes from the observations that commercially prepared polygalacturonic acid, which is composed of >98% α-1,4-linked D-galacturonide residues, possessed elicitor activity and that partial digests of this material with endopolygalacturonase generated greatly increased levels of elicitor activity. On the other hand, limit digests of polygalacturonic acid with the enzyme retained little or no elicitor activity. The present work has confirmed and extended those preliminary observations and supports the idea that linear α-1,4-D-galacturonide oligomers of appropriate size are responsible for the elicitor activity seen from the partial digests. The possibility that some other minor component of the polygalacturonic acid sample might be responsible for the elicitor activity has not been rigorously excluded by this work; however, the results obtained make that possibility appear unlikely.

The bioassay of the individual oligomer lengths (Fig. 2) demonstrated that the tridecamer was the most active elicitor of casbene synthetase on a weight basis and that oligomers smaller than the nonamer possessed no significant activity. Oligomers larger than the pentadecamer were not well resolved by the anion exchange chromatographic methods employed and therefore were not tested individually. It appears that oligomers larger than the pentadecamer are active; however, solubility and the ability to penetrate to the appropriate site are likely to become limiting factors. It is not clear why the nonamer is the smallest oligomer possessing activity in this assay system. The nonamer may be the smallest unit that can bind tightly enough to whatever type of receptor is present in the castor bean seedling. The possibility exists, however, that oligomers smaller than nine are degraded to inactive fragments by endogenous enzymes in the assay seedlings too rapidly for their intrinsic activity to be expressed.

Parallel studies by Albersheim and his associates of endogenous elicitors have provided additional support for the role of pectic fragments of the plant cell wall in the elicitation of stress metabolites. Hahn *et al.* (3) described the release of endogenous elicitors of phytoalexins in soybeans by partial acid hydrolysis of plant cell wall fractions and provided initial indications that these elicitors contained galacturonic acid as an essential constituent. More recently, Nothnagel *et al.* (7) purified these elicitors from partial acid hydrolysates of citrus pectin and soybean cell walls and provided convincing evidence that the most active elicitor in the soybean is a dodeca-α-1,4-D-galacturonide. Thus, the most active component detected for the soybean system differs by only a single unit from the most active component found in the present work. However, it should be kept in mind that natural elicitors generated *in vivo* may possess other structural features, such as branching or neutral sugar residues that could alter their size requirements for optimal activity in these assay systems from those determined for the D-galacturonide oligomers.

The polyanionic character of the galacturonide oligomers appears to be important for their activity as elicitors. Modification of the oligomers by methyl-esterification produces a derivative which has less than one-twentieth of the activity of the parent substance in the elicitor bioassay (Fig. 3). The loss of activity was presumably the result of the methyl-esterification rather than some other effect of the methylation conditions on the structure of the oligomer since it was possible to regenerate full activity by de-esterification of the derivative by mild acid hydrolysis. It does not seem likely that the residual activity of the methylated derivative was a consequence of the failure to obtain complete methylation of the oligomer since the sample tested had freely passed through an anion exchange column without being adsorbed. The low residual activity of the derivative might be an intrinsic property of the fully methylated derivative or might be due to partial demethylation of the fully methylated derivative by the seedlings during the bioassay to produce some active substances.

The failure of any of a variety of other polyanionic polymers to give a positive response (Table I) indicates that polyanionic character alone is not a sufficient basis for activity. Other structural features of the galacturonide oligomers must also be important. However, the nature of the other structural features required for activity has not been investigated.

It should be noted that the galacturonide oligomer elicitors produced in this investigation are derived from an acid-modified pectic substance (polygalacturonic acid) and thus may be artificial or unnatural elicitors. The pectic components of plant cell walls are structurally complex substances that contain neutral sugars both interspersed in the polygalacturonide backbone and in side chains. There is also partial methylation of the galacturonosyl carboxylate groups. It is likely that the fragments released from plant cell walls by the action of endopolygalacturonase will be structurally complex and heterogeneous. At present the structural relationship of the oligogalacturonide elicitors released from polygalacturonic acid to the elicitors released from plant cell walls *in situ* is not known. Work on the nature of these elicitors which are released from isolated pectic cell wall material by treatment with endopolygalacturonase is in progress.

Acknowledgments—We wish to thank David Heller at the NSF Mid-Atlantic MS Facility for the FAB-MS analysis of the oligomers and Robert Bruce for the [^{3}H]geranylgeranyl pyrophosphate.

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