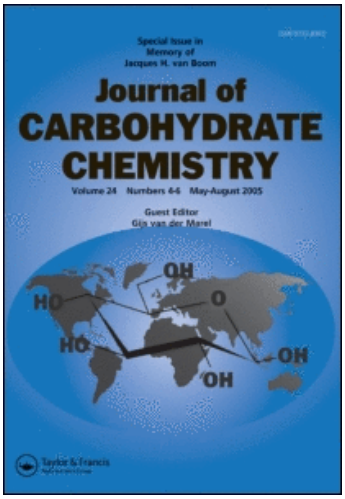

Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

Structural Elucidation of an Elicitor-Active Oligosaccharide, LN-3, Prepared from Algal Laminaran

Akio Kobayashi[a]; Akihiro Tai[a]; Kazuyoshi Kawazu[a]
[a] Department of Agricultural Science, Okayama University, Okayama, Japan

To cite this Article Kobayashi, Akio , Tai, Akihiro and Kawazu, Kazuyoshi(1995) 'Structural Elucidation of an Elicitor-Active Oligosaccharide, LN-3, Prepared from Algal Laminaran', Journal of Carbohydrate Chemistry, 14: 6, 819 — 832
To link to this Article: DOI: 10.1080/07328309508005378
URL: http://dx.doi.org/10.1080/07328309508005378

J. CARBOHYDRATE CHEMISTRY, 14(6), 819-832 (1995)

STRUCTURAL ELUCIDATION OF AN ELICITOR-ACTIVE OLIGOSACCHARIDE, LN-3, PREPARED FROM ALGAL LAMINARAN

Akio Kobayashi,* Akihiro Tai, and Kazuyoshi Kawazu

Department of Agricultural Science, Okayama University,
Okayama 700, Japan

Received October 21, 1994 - Final Form February 17, 1995

ABSTRACT

The primary structure of an elicitor-active oligosaccharide, LN-3, prepared from partially hydrolyzed algal laminaran was determined by means of the analyses of glycosyl-linkage, fragments by acetolysis, and glycosyl-sequence. The elicitor-active oligosaccharide, LN-3, is a pyridylaminated hepta-β-D-glucoside which was shown to have the following linear structure: β-D-Glc*p*(1→6)-β-D-Glc*p*(1→3)-β-D-Glc*p*(1→6)-β-D-Glc*p*(1→3)-β-D-Glc*p*(1→6)-β-D-Glc*p*(1→3)-Glc-PA.

INTRODUCTION

Poly- and oligosaccharides from cell walls of fungi and plants including β-glucans, chitin, chitosan, and pectin, induce various defense responses in plants.[1-6] Such molecules that stimulate any plant defense mechanism are commonly called "elicitors".[7] A hepta-β-D-glucoside from the mycelial walls of a soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* (Pmg) is the only elicitor whose structure is fully understood.[8,9] Recently, the activity as elicitors of a family of chemically synthesized oligo-β-glucosides was examined in soybean cotyledon assay, and the structural elements for elicitor activity of the oligoglucosides were determined.[10] Furthermore, the plant receptor that was presumably responsible for the specific recognition of Pmg elicitor was also found in soybean root.[11]

In most elicitor studies, crude elicitor fractions have been used, because of the limited supply of authentic pure samples. However, for the study of specificity of elicitors on the plant species, the purity of elicitors must be primarily argued.

We have developed a convenient procedure to obtain pure biotic elicitors from naturally occurring polysaccharides.[12] Natural polysaccharides were examined for their activity as elicitors for flavonoid accumulation in alfalfa cotyledons. Laminaran from *Eisenia bicyclis*, which had a prominent elicitor-activity, was hydrolyzed with β-(1→3)-glucanase and the resulting hydrolysate was subjected to chromatography on charcoal and gel filtration columns. Introduction of the pyridylamino (PA) group into the elicitor-active oligosaccharides was attempted in order to facilitate the isolation. These oligosaccharides were separated on HPLC. Three PA-oligosaccharides (LN-1, 2, and 3) were collected each in a pure form and subjected to the alfalfa cotyledon assay. LN-3 showed the highest activity (the minimum effective concentration, 650 nM). ^{1}H NMR and LC-MS analyses suggested that LN-3 was a PA-hepta-β-glucoside.[12]

In this report, we demonstrate the structural elucidation of the elicitor-active PA-oligosaccharide, LN-3.

RESULTS AND DISCUSSION

Glycosyl-linkage analysis. The elicitor-active oligosaccharide, LN-3, prepared from brown-algal laminaran by the action of β-(1→3)-glucanase, followed by pyridylamination, was a pyridylaminated (PA) hepta-β-glucoside.[12] The Pmg elicitor was a branched oligosaccharide.[8,9] However, a preliminary structural elucidation study suggested that LN-3 could have a linear chain. This prompted us to elucidate its structure.

First, glycosyl-linkage analysis was carried out. The per-*O*-methylated LN-3 was hydrolyzed with TFA, reduced with sodium borohydride, and acetylated. The resulting partially *O*-methylated alditol acetates were subjected to GLC-MS.

On the basis of the GLC-MS data, the peaks at *Rt* 11.1, 12.7 and 13.2 were identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol derived from the nonreducing terminal, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol from 3-linked glucosyl residues, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol from 6-linked glucosyl residues,

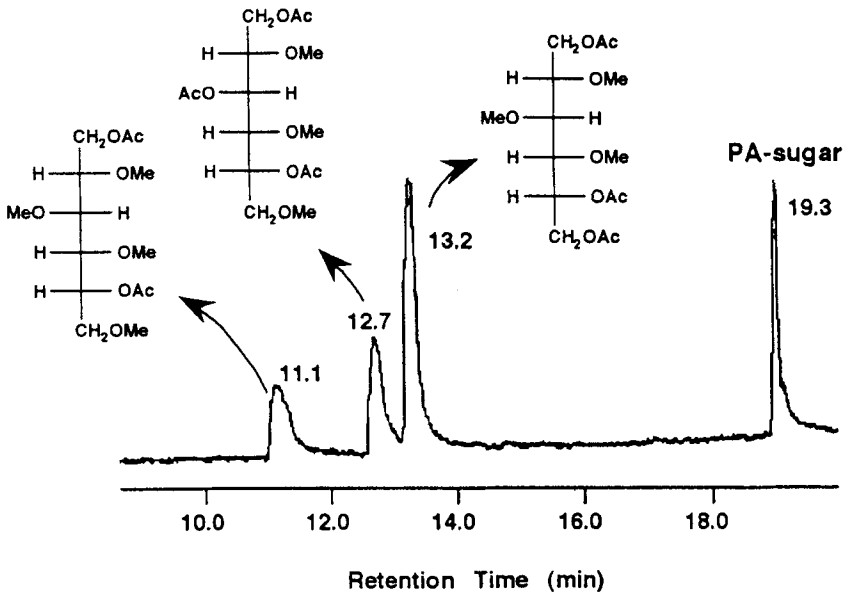

Figure 1. A total-ion chromatographic trace of partially *O*-methylated alditol acetates from per-*O*-methylated LN-3. On the basis of EI-MS data, the peaks at *Rt* 11.1, 12.7 and 13.2 were identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, respectively.

respectively (peak ratio; 1:2:3, Figure 1). The peak ratios show that LN-3 is a linear hepta-β-D-glucoside with two (1→3)- and three (1→6)-linkages, in addition to glucosyl-linkage at the reducing end. The peak at *Rt* 19.3 affords a prominent fragment (*m/z* 121), which is assignable to a daughter ion from a PA-sugar derivative.

Analysis of fragments by acetolysis. On acid hydrolysis, (1→6)-glycosidic bonds are relatively stable, whereas acetolysis can preferentially cleave (1→6)-bonds.[13,14]

A systematic analysis of the structure of LN-3 by acetolysis is summarized in Figure 2. LN-3 was first acetylated and then acetolyzed. The reaction mixture was divided into two groups (experiments A and B).

In procedure A, the sample was *N*,*O*-deacetylated with hydrazine. Only PA-linked fragments from the reducing end were detected by fluorescence spectrophotometry.

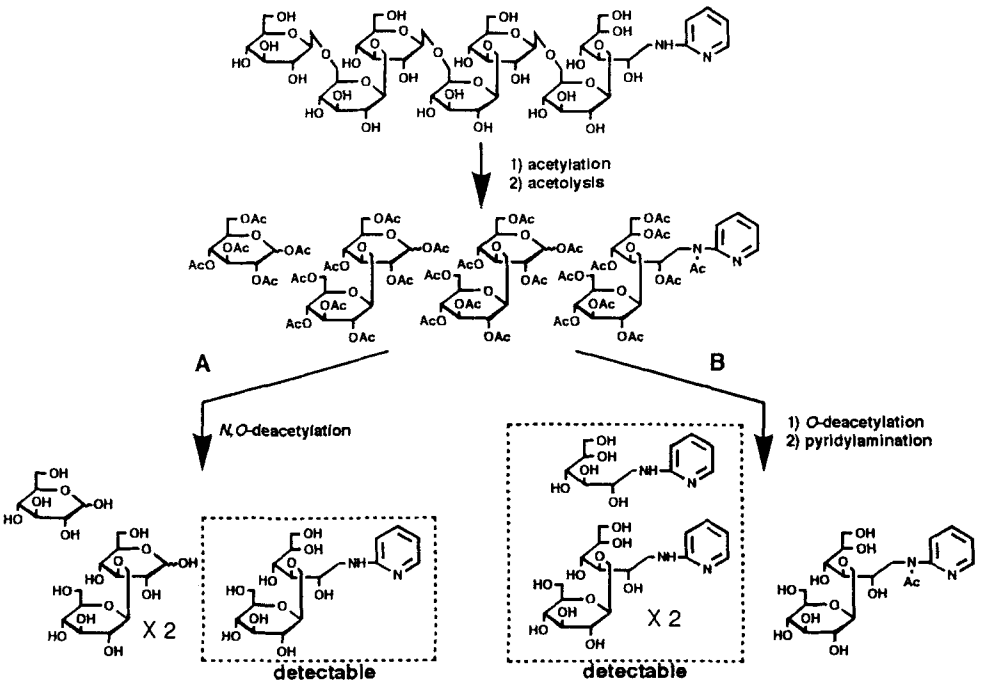

Figure 2. The analysis of the structure of LN-3 by acetolysis.

These PA-fragments obtained by acetolysis were identical to those of the authentic PA-Glc and PA-laminaribiose (Figure 3-A). The ratio of PA-Glc to PA-laminaribiose increased with the time of acetolysis (data not shown), indicating that PA-laminaribiose was further degraded to give PA-Glc. Therefore, it is suggested that the reducing end of LN-3 must be a PA-laminaribiose unit.

In procedure B, complete *O*-deacetylation was achieved by sodium methoxide, but the *N*-acetyl group was retained. The reaction mixture was pyridylaminated. The *N*-acetyl-PA-derivatives have no fluorescence. The PA-derivatives from the nonreducing end are readily detected by fluorescence spectrophotometry (Figure 2). By HPLC analysis, only two fragments, PA-Glc and PA-laminaribiose were detected (Figure 3-B). This analysis as well as the glycosyl-linkage analysis described above suggests that LN-3 possesses a glucose and two laminaribiose units besides a PA-laminaribiose unit at the reducing end.

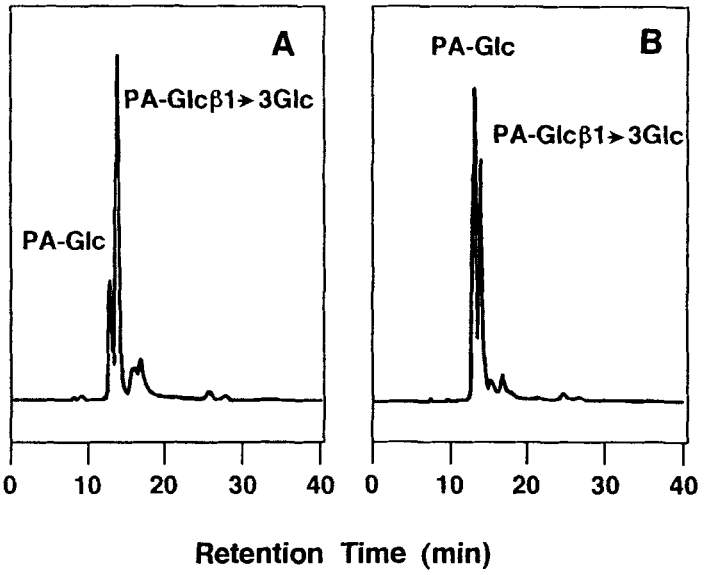

Figure 3. The reversed-phase HPLC elution profiles of A: *N,O*-deacetylated and B: *O*-deacetylated and pyridylaminated acetolysis product mixtures of peracetylated LN-3.

Glycosyl-sequence analysis. The glycosyl sequence of the per-*O*-alkylated oligosaccharide-alditols has been analyzed by GLC-MS.[15-19] Per-*O*-methylated LN-3 was partially hydrolyzed to give several products, which were reduced and then ethylated. The resulting per-*O*-alkylated oligosaccharide-alditols were subjected to GLC-MS. The total ion chromatogram showed five prominent peaks (Figure 4). Selected-ion monitoring at *m/z* 121, 219, and 233 indicated the presence of a characteristic fragment of PA-sugar at *Rt* 7.6 and per-*O*-alkylated oligosaccharide-alditols at *Rt* 9.3, 10.1, and 10.5.

In the EI mass spectrum of a PA-per-*O*-alkylated alditol at *Rt* 7.6, the molecular ion peak at *m/z* 356 was observed and assigned to fragment [a] (Figure 5). The cleavages between C-1 and C-2, and between C-3 and C-4 of the PA-per-*O*-alkylated alditol, reported by Hase *et al.*[20], were clearly detected in EI mass spectra.

A peak, fragment [b] at *Rt* 9.3 shows characteristic ions at *m/z* 88, 101, 201, 233, 263, 323, 377, and 409 (Figure 6). The fragment ion at *m/z* 233 agrees with an A_1 ion and indicates that the nonreducing glycosyl group bears an *O*-ethyl group. A_1 ions from partially methylated, partially ethylated oligosaccharide-alditols can lose methanol or

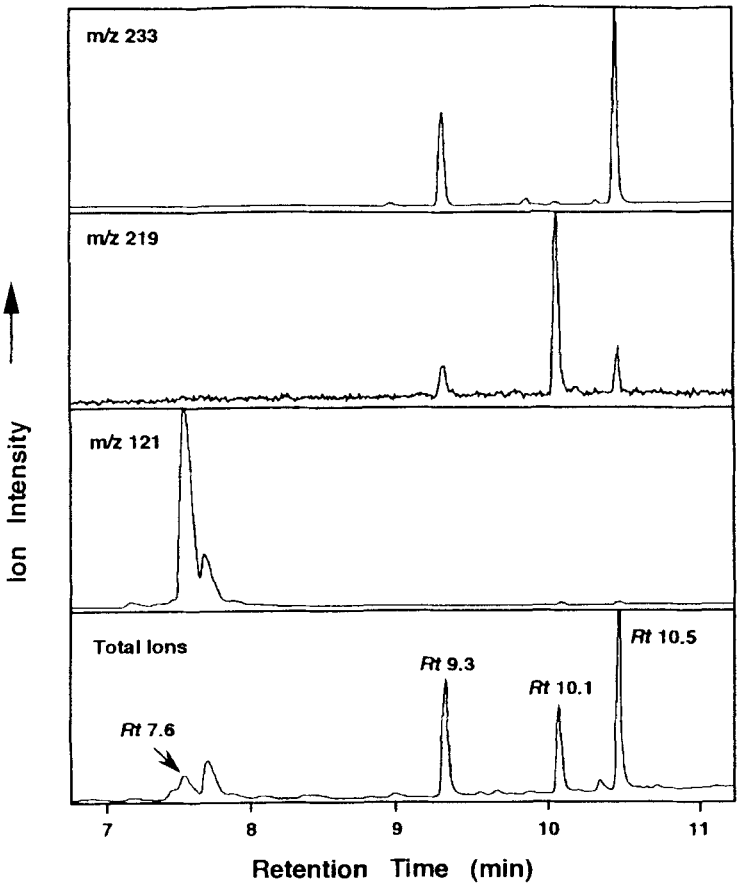

Figure 4. Total-ion and selected-ion chromatographic traces of per-*O*-alkylated oligosaccharide-alditols from reduced and ethylated hydrolysis products of permethylated LN-3.

ethanol to yield A_2 ions. The occurrence of A_2 ion at *m/z* 201 and H_1 ion at *m/z* 88 indicates that an ethyl group is substituted at O-6 of the glycosyl residue and not at O-3. The fact that the per-*O*-alkylated alditol residue bears two *O*-ethyl groups is verified by the occurrence of the J_2 ion at *m/z* 263. The ion at *m/z* 409 is yielded by cleaving the C-C bonds of the alditol portion between C-2 and C-3, and C-4 and C-5. There are two ways to give the ion peak at *m/z* 409. However, the evidence obtained by glycosyl-linkage analysis aforementioned excludes the possibility of the presence of a (1→4)-linked residue. Thus the per-*O*-alkylated disaccharide-alditol is (1→3)-linked.

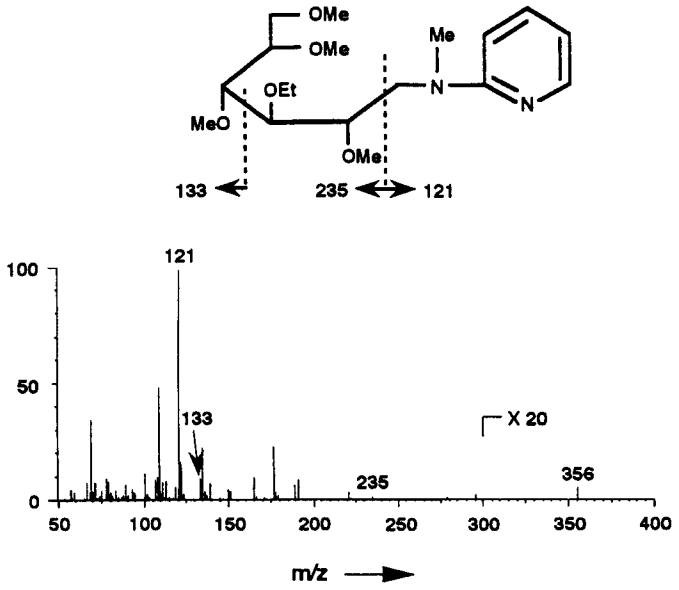

Figure 5. EI mass spectrum of a PA-per-*O*-alkylated alditol, fragment [a] at *Rt* 7.6.

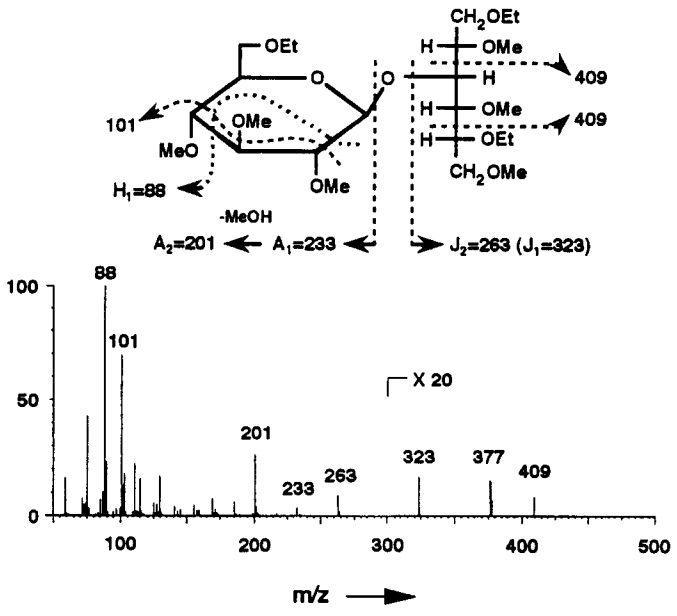

Figure 6. EI mass spectrum of a per-*O*-alkylated disaccharide-alditol, fragment [b] at *Rt* 9.3.

A peak, fragment [c] at *Rt* 10.1 shows characteristic ions at *m/z* 88, 101, 159, 187, 191, 219, 249, 263, 323 and 351 (Figure 7). The fragment ion at *m/z* 219 agrees with an A_1 ion, and corresponds to the nonreducing glycosyl terminal of LN-3 due to the absence of ethyl groups in this fragment. The A_2 ion at *m/z* 187 and the H_1 ion at *m/z* 88 are yielded as shown in Figure 7. The fragment ion at *m/z* 263 is assigned to a J_2 fragment. Ions at *m/z* 191, 249, and 351 are rationally assignable to the fragments from the alditol chain. These ions establish that the per-*O*-alkylated disaccharide-alditol is (1→6)-linked.

A peak, fragment [d] at *Rt* 10.5 shows characteristic ions at *m/z* 101, 102, 159, 187, 191, 201, 233, 263, 337 and 351 (Figure 8). The fragment ion at *m/z* 233 agrees with an A_1 ion, and indicates that the nonreducing glycosyl group bears an *O*-ethyl group. The formation of the predominant A_2 ion at *m/z* 187 from the A_1 ion at *m/z* 233 indicates that O-3 of the glycosyl residue is substituted with an ethyl group. This is further supported by the presence of the abundant H_1 ion at *m/z* 102. The fragment ion at *m/z* 263 is assigned to a J_2 fragment. The presence of an ethyl group on O-3 of the nonreducing glycosyl group is also confirmed by the mass of the J_1 ion[19] at *m/z* 337. Ions at *m/z* 191, 263 and 365 are also rationally assignable to the fragments from the alditol chain. These ions establish that the per-*O*-alkylated disaccharide-alditol is (1→6)-linked.

The structure of LN-3. The elicitor-active oligosaccharide, LN-3 was a PA-hepta-β-D-glucoside.[12] Based on the analyses of glycosyl-linkage and fragmentation by acetolysis, LN-3 is a linear pyridylaminated hepta-β-glucoside which consists of a PA-laminaribiose unit at the reducing end, and a glucose and two laminaribiose units with three β-(1→6)-linkages.

In the glycosyl-sequence analysis, the fragment [c] clearly shows that the glycosyl-linkage at the nonreducing end is 6-linked. This allows us to depict the structure of LN-3 (Figure 9). The fragments, [a], [b] and [d] further support this structure.

LN-3 is the first elicitor prepared from a neutral polysaccharide, which occurs widely in nature. Introduction of the pyridylamino group into the elicitor-active oligosaccharides not only sustained the elicitor activity but enhanced it.[12] The minimum sugar unit required for the exertion of elicitor activity is being examined in our laboratory.

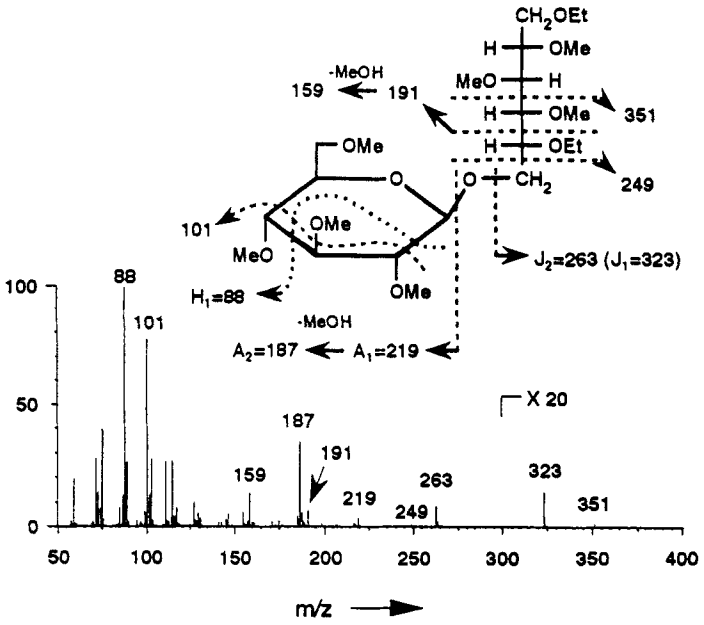

Figure 7. EI mass spectrum of a per-*O*-alkylated disaccharide-alditol, fragment [c] at *Rt* 10.1.

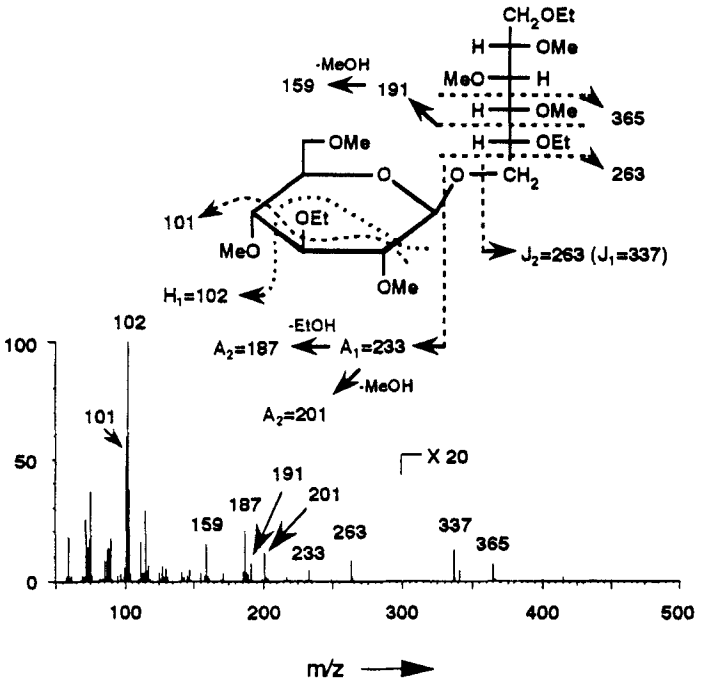

Figure 8. EI mass spectrum of a per-*O*-alkylated disaccharide-alditol, fragment [d] at *Rt* 10.5.

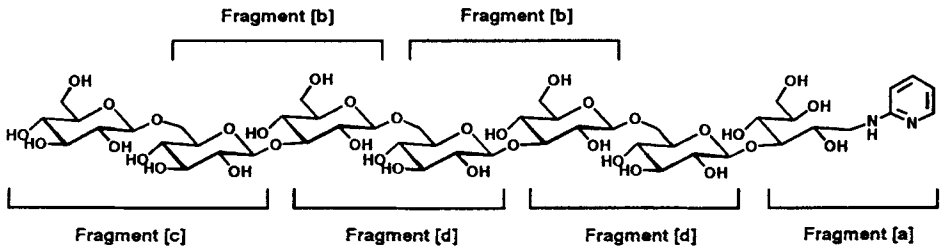

Figure 9. A primary structure of an elicitor-active oligosaccharide, LN-3.

EXPERIMENTAL

Preparation of elicitor-active oligosaccharide, LN-3 from algal laminaran. Laminaran from *Eisenia bicyclis* (Tokyo Kasei Kogyo Co., Tokyo) was commercially available. Laminaran was hydrolyzed with β-(1→3)-glucanase. With the guidance of elicitor activity in alfalfa cotyledons, the hydrolysate was fractionated by means of charcoal and gel-filtration column chromatography. The pyridylamino group was introduced to the elicitor-active oligosaccharides in order to facilitate isolation. An elicitor-active oligosaccharide, LN-3 was purified by ODS-HPLC as described.[12]

Methylation of elicitor-active oligosaccharide, LN-3. Per-*O*-methylation was accomplished by a modified Hakomori procedure.[21] LN-3 (3.0 mg) was lyophilized overnight *in vacuo* at -58 ℃. The LN-3 was dissolved in dry dimethyl sulfoxide (345 μL), and 3.6 M potassium dimethylsulfinyl anion (55 μL) was slowly added. The mixture was stirred for 2 h at room temperature. Methyl iodide (12.3 μL; equimolar to the potassium dimethylsulfinyl anion) was added at ice-bath temperature, and then the solution was stirred for 1 h at room temperature. The addition of potassium dimethylsulfinyl anion and methyl iodide was repeated twice in the same way, except that the last addition of methyl iodide (100 μL). The sample was stirred overnight. The reaction mixture was diluted with water (2.0 mL), and extracted twice with chloroform (2.0 mL). The extracts were combined, washed six times with water (2.0 mL), and concentrated to dryness under a stream of nitrogen. The per-*O*-methylated LN-3 (1.9 mg) was purified by preparative TLC (Kieselgel 60 F_{254}; MERCK; 7:2:1 v/v benzene : acetone : methanol).

Glycosyl-linkage analysis. The per-*O*-methylated LN-3 (100 μg) was hydrolyzed with 2 M TFA (75 μL) for 1 h at 120 °C. The mixture was dried under a stream of nitrogen. Methanol was added and the solution was concentrated to dryness. This procedure was repeated five times. The resulting partially *O*-methylated monosaccharide derivatives were reduced for 3 h at room temperature with a 50-μL aliquot of an ethanol solution of sodium borohydride (10 μg of $NaBH_4$/μL) containing 1 M ammonia. Acetic acid and methanol were added, and the solution was concentrated to dryness. The residue was acetylated with acetic anhydride and pyridine for 12 h at room temperature. The partially *O*-methylated alditol acetates were dissolved in acetone, and injected into the GLC-MS. A JEOL Automass 20 system equipped with a Hewlett-Packard model 5890 gas chromatograph was used for GLC-MS analysis. A DB-1 (J and W Scientific) capillary column (30 m x 0.25 mm i.d.; 0.4 μm) was used for the analysis. The temperature program consisted of holding for 3 min at the injection temperature of 150 °C and then raising the temperature at 6 °C/min to 240 °C. The gas-chromatographic effluent was ionized by electron impact at 70 eV.

Analysis of fragments by acetolysis. A procedure analogous to that reported[22] was employed for the fragment analysis. LN-3 (25 μg) was first acetylated with pyridine and acetic anhydride (40 μL each) for 15 min at 100 °C. Toluene was added to the reaction mixture and then the solution was concentrated to dryness. The acetylated LN-3 was treated with 40 μL of a mixture of acetic anhydride-acetic acid-sulfuric acid (10:10:1; v/v) for 10 h at 37 °C. Then, 8 μL of pyridine was added. The dried residue was dissolved in 800 μL of water, saturated with sodium hydrogen carbonate. The solution was extracted with 1 mL of chloroform (x 3). The extracts were combined and dried over sodium sulfate. The sample was divided into two groups (experiments A and B).

Procedure A was employed to verify the fragments from the reducing end. An aliquot of the sample was placed in a glass tube (ϕ 10 x 100 mm) and 250 μL of anhydrous hydrazine was added. The tube was sealed under vacuum, and heated at 100 °C for 22 h. After *N*,*O*-deacetylation, hydrazine was removed *in vacuo*. The residue was dissolved in 500 μL of $NaHCO_3$-saturated water and the pH was adjusted to 3 by adding a Dowex 50W-X8 (100-200 mesh; The Dow Chemical Co.). The resin was transferred to a minicolumn and then washed with ten bed volumes of water. The sugar fragments

were eluted with six bed volumes of 1.4 M aqueous ammonia, and the eluate was concentrated to dryness. The residue was dissolved in 100 μL of water for HPLC analysis.

Procedure B was used to verify the fragments from the nonreducing end. An aliquot of the sample was placed in a 0.3-mL Mini-Vial (GL Sciences Co., Tokyo). The sample was dissolved in 50 μL dry methanol, and 50 μL of 0.2% sodium methoxide in methanol was added. The mixture was allowed to stand for 1 h at room temperature. Acetic acid (1 μL) was then added and the solution was concentrated to dryness. The residue was pyridylaminated.[23,24] The reaction mixture was chromatographed on a Dowex 50W-X8 column as described above. The eluate was further purified on a TSKgel TOYOPEARL HW-40S (TOSOH Co., Tokyo) column (ϕ 1.0 x 30 cm). The flow rate was 0.4 mL/min and 0.1 M ammonium hydrogen carbonate was used as eluent. The pyridylamino derivatives were obtained by HPLC peak collection. The eluate was concentrated *in vacuo*, and then dissolved in 100 μL of water.

The sample (5 μL each) was subjected to HPLC analysis and identified by co-chromatography with authentic PA-glucose and PA-laminaribiose, prepared from D-glucose and laminaribiose (Seikagakukogyo Co., Tokyo). HPLC conditions were as follows: HITACHI, L-6200 Intelligent Pump; L-1000 Fluorescence Spectrophotometer (excitation: 320 nm, emission: 400 nm); L-5020 Column Oven (at 30 °C); D-2500 Chromato-Integrate; Inertsil ODS (ϕ 4.6 x 250 mm, 5μm; GL Sciences Co., Tokyo); flow rate, 0.5 mL/min; eluent, 1% acetic acid in water (v/v).

Glycosyl-sequence analysis. The per-*O*-methylated LN-3 (600 μg) was partially hydrolyzed with 90% formic acid (200 μL) for 1 h at 80 °C. The formic acid was evaporated under a stream of nitrogen. The sample was then reduced with sodium borohydride. The resulting partially *O*-methylated oligoglucosyl-alditols were desalted on a Sep-Pak C_{18} cartridge (MILLIPORE). The cartridge was washed with 10 mL of water and eluted with 5 mL of 70% methanol in water. The dried sample was dissolved in dry dimethyl sulfoxide (345 μL), and 3.6 M potassium dimethylsulfinyl anion (55 μL) was slowly added. The mixture was stirred for 2 h at room temperature. Ethyl iodide (100 μL) was added, and the solution was stirred for 4 h at room temperature. The mixture was extracted with chloroform as described in the methylation procedure. The solution was concentrated and subjected to preparative TLC (Kieselgel 60 F_{254}; MERCK; 7:2:1

v/v benzene : acetone : methanol). The per-*O*-alkylated oligosaccharide-alditol fraction was dissolved in acetone. The GLC-MS analysis was performed under the previous condition except for the column temperature. The temperature was programmed to remain at 160 ℃ for 2 min, and then rise from 160 to 220 ℃ at 30 ℃/min, and finally from 220 to 300 ℃ at 8 ℃/min.

ACKNOWLEDGEMENT

This work is partly supported by KIBUN Science and Technology Foundation and also by a Grant-in-Aid to A. K. for Scientific Research (No. 04252104) from the Ministry of Education, Science and Culture of Japan. The authors thank Dr. H. Kanzaki and Mr. S. Kajiyama for their valuable help and advice.

REFERENCES

1. N. T. Keen, M. Yoshikawa and M. C. Wang, *Plant Physiol.*, **71**, 466 (1983).
2. C. A. Ryan, *Biochemistry*, **27**, 8879 (1988).
3. J. E. Parker, K. Hahlbrock and D. Scheel, *Planta*, **176**, 75 (1988)
4. A. J. MacDougall, N. M. Rigby, P. W. Needs and R. R. Selvendran, *Planta*, **188**, 566 (1992)
5. A. Kobayashi, K. Akiyama and K. Kawazu, *Z. Naturforsch.*, **49c**, 302 (1994).
6. K. Akiyama, K. Kawazu and A. Kobayashi, *Z. Naturforsch.*, **49c**, 811 (1994).
7. R. A. Dixon, *Biol. Rev.*, **61**, 239 (1986).
8. J. K. Sharp, B. Valent and P. Albersheim, *J. Biol. Chem.*, **259**, 11312 (1984).
9. J. K. Sharp, M. McNeil and P. Albersheim, *J. Biol. Chem.*, **259**, 11321 (1984).
10. J.-J. Cheong, W. Birberg, P. Fügedi, Å. Pilotti, P. J. Garegg, N. Hong, T. Ogawa and M. G. Hahn, *The Plant Cell*, **3**, 127 (1991).
11. J.-J. Cheong and M. G. Hahn, *The Plant Cell*, **3**, 137 (1991).
12. A. Kobayashi, A. Tai, H. Kanzaki and K. Kawazu, *Z. Naturforsch.*, **48c**, 575 (1993).
13. L. Rosenfeld and C. E. Ballou, *Carbohydr. Res.*, **32**, 287 (1974).
14. K. Matsuda, H. Watanabe, K. Fujimoto and K. Aso, *Nature (London)*, **191**, 278 (1961).
15. V. Kováčik, Š. Bauer, J. Rosík and P. Kováč, *Carbohydr. Res.*, **8**, 282 (1968).
16. J. Kärkkäinen, *Carbohydr. Res.*, **14**, 27 (1970).
17. B. S. Valent, A. G. Darvill, M. McNeil, B. K. Robertsen and P. Albersheim, *Carbohydr. Res.*, **79**, 165 (1980).
18. M. McNeil, A. G. Darvill, P. Åman, L.-E. Franzén and P. Albersheim, *Methods Enzymol.*, **83**, 3 (1981).
19. J. K. Sharp and P. Albersheim, *Carbohydr. Res.*, **128**, 193 (1984).
20. S. Hase, T. Ikenaka and Y. Matsushima, *Biochem. Biophys. Res. Commun.*, **85**, 257 (1978).

21. L. R. Phillips and B. A. Fraser, *Carbohydr. Res.*, **90**, 149 (1981).
22. S. Hase in *Toushitsu I, Tou-tanpakushitsu (I)*, 1st Edn., Tokyokagakudojin, Tokyo, 1990, p 274.
23. S. Hase, T. Ikenaka and Y. Matsushima, *J. Biochem.*, **90**, 407 (1981).
24. J. Suzuki, A. Kondo, I. Kato, S. Hase and T. Ikenaka, *Agric. Biol. Chem.*, **55**, 283 (1991).