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Synthesis and characterization of tyramine-derivatized (1 → 4)-linked α-D-oligogalacturonides

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Abstract

The reducing end C-1 of (1 → 4)-linked α-D-oligogalacturonides (oligogalacturonides), with degrees of polymerization (dp) 3 and 13, was coupled to tyramine via reductive amination in the presence of sodium cyanoborohydride. These derivatives were purified in milligram quantities and structurally characterized. Tyramination of trigalacturonic acid proceeded to completion. The yield of apparently homogeneous tyraminated trigalacturonic acid after desalting was 35%. Derivatization of tridecagalacturonide with tyramine was incomplete. The tyraminated tridecagalacturonide was purified to apparent homogeneity using semipreparative high-performance anion-exchange chromatography (HPAEC) with a yield of 30%. The structures of the derivatized oligogalacturonides were established by ^{1}H NMR spectroscopy and electrospray mass spectrometry. © 1996 Elsevier Science Ltd.

Keywords: Oligogalacturonides; Derivatization; Tyramine; Structural characterization

1. Introduction

Several oligosaccharides derived from plants, fungi, and bacteria have been shown to elicit complex processes in plants, including alteration of morphogenesis and initiation

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of plant defense responses. Some progress is being made towards understanding how plants recognize and respond to these biologically active oligosaccharides (oligosaccharins). Putative receptors for three classes of oligosaccharins have been identified in plant membrane fractions using homogeneous, biologically active, oligosaccharin derivatives labelled to high specific radioactivity (100–1000 Ci/mmol) [1–4]. A binding site specific for the elicitor-active (1 → 3)-(1 → 6)-linked hepta-β-glucoside from *Phytophthora sojae* cell walls has been identified in soybean membrane fractions using ^{125}I-tyramine [1] and ^{125}I-aminophenylethylamine [2] derivatives; *N*-acetylchitooligosaccharide-specific binding sites have been identified in tomato [3] and rice [4] using *tert*-butoxycarbonyl-L-[^{35}S]methionine and ^{125}I-tyramine derivatives, respectively; and binding sites that specifically recognize yeast-derived *N*-linked oligosaccharides have been found in tomato using a *tert*-butoxycarbonyl-L-[^{35}S]methionine-derivatized glycopeptide [5]. These binding sites are present in low abundance and have high affinity for the biologically active oligosaccharins but greatly reduced affinity for their biologically inactive structural analogues [1–4]. These characteristics are consistent with those expected for oligosaccharin receptors.

Binding activity at the soybean cell surface has also been reported for oligogalacturonides. Binding and uptake of ^{125}I-tyramine hydrazone and fluorescein thiosemicarbazone derivatives of a dodecagalacturonide fraction by soybean cells have been investigated [6,7]. The homogeneity and stability of the oligogalacturonide derivatives were not characterized; therefore, the affinity and specificity of the binding activity could not be determined in this study.

We describe the synthesis, purification, and structural characterization of tyramine-derivatized tri- and tridecagalacturonic acid. The tridecagalacturonide elicits a number of responses in plants including synthesis of antimicrobial compounds in numerous plants, alteration of morphogenesis in tobacco explants, and rapid ion flux accompanied by membrane depolarization in tobacco suspension-cultured cells. Trigalacturonic acid does not elicit these responses [8–10]. The apparently homogeneous tyraminated oligogalacturonides described here may be useful in the identification of putative receptors of biologically active oligogalacturonides and in the study of the metabolism of oligogalacturonides in plant cells.

2. Experimental

Polygalacturonic acid (Na^+ salt) and trigalacturonic acid (Na^+ salt) were purchased from Sigma (St. Louis, MO). SpectraPor-7 (2000 MW cutoff) tubing was purchased from Fisher Scientific. All buffers were prepared using ultra-pure water (Continental Ultrafiltration Water System, San Antonio, TX). All other chemicals were obtained from Sigma unless otherwise stated.

Preparation of a tridecagalacturonide fraction.—Oligogalacturonides generated by partial endopolygalacturonase digestion of polygalacturonic acid were size-selectively precipitated in 11% EtOH and further purified by Q-Sepharose anion-exchange chromatography [11]. A fraction containing > 80% tridecagalacturonide was collected and is referred to here as the tridecagalacturonide fraction.

Tyramination of oligogalacturonides.—A fresh reagent solution was required for the tyramination reactions. The reagent solution for derivatization of trigalacturonic acid was made by dissolving 137 mg of tyramine (free base) and 35 mg of sodium cyanoborohydride in 0.4 mL HOAc (10% v/v) in MeOH at 100 °C [12]. H_2O was used in place of MeOH in the reagent solution for derivatization of the tridecagalacturonide fraction. The reagent solution (0.35 mL) was added to 10 mg of the oligogalacturonide in H_2O (0.1 mL) and allowed to react for 1 h at 80 °C in a glass tube fitted with a Teflon-lined cap. The reaction was then cooled to room temperature and glacial acetic acid was added dropwise until the evolution of H_2 gas ceased, ensuring that any remaining sodium cyanoborohydride had been destroyed. This solution was evaporated to dryness under a stream of air at 25 °C, and the resulting residue was dissolved in H_2O (0.5 mL).

The trigalacturonic acid derivatization mixture was desalted using a Sephadex G-25 column (1 × 50 cm) by eluting with H_2O at 0.4 mL/min. Fractions (1.2 mL) were collected and assayed for tyramine by A_{274} and for uronic acid colorimetrically [13]. The fractions containing tyraminated trigalacturonic acid were pooled, concentrated (to 1 mL) by rotary evaporation under reduced pressure, and stored at −20 °C.

The tridecagalacturonide derivatization mixture was desalted by adding 10 vol of absolute MeOH (at −20 °C for 1 h) to precipitate both the derivatized and underivatized oligogalacturonides. The precipitate was collected by centrifugation at 2000 *g* for 20 min at −20 °C. The supernatant, which contained unreacted tyramine and salts, was discarded. The resulting pellet was resuspended in absolute MeOH and collected by centrifugation four successive times to remove any remaining borate and unreacted tyramine. The final pellet was dried under a stream of air and the residue dissolved in H_2O (0.5 mL). The uronic acid content was determined colorimetrically [13], and the solution was stored at −20 °C.

Analytical HPAEC-PAD / UV of the tyraminated oligogalacturonides.—The tyraminated oligogalacturonides were analyzed by HPAEC using a CarboPac PA-1 column with a metal-free BioLC interfaced to an AutoIon series 400 data station (Dionex, Sunnyvale, CA). Tyraminated trigalacturonic acid (20 μg in 0.1 mL H_2O) was eluted at 1 mL/min over 30 min with a linear gradient of 200–500 mM NaOAc, pH 8. The tyraminated tridecagalacturonide (0.1 mg in 0.1 mL H_2O) was eluted at 1 mL/min over 40 min with a linear gradient of 400–800 mM NaOAc, pH 8. The eluting compounds were detected with sequential pulsed amperometric detection (PAD) and UV absorbance. The electrochemical detector, which was equipped with a gold working electrode (Dionex), was operated in the pulsed amperometric mode ($E1$ 150, $E2$ 700, $E3$ −300 mV; $T1$ 480, $T2$ 120, $T3$ 360 ms) at 3 μA sensitivity. To facilitate the pulsed amperometric detection and to minimize baseline drift, NaOH (400 mM) was added post-column at a flow rate of 0.5 mL/min using a pressurized reagent delivery system (Dionex). The A_{274} was monitored with a spectral array detector (Dionex).

Semipreparative HPAEC purification of the tyraminated tridecagalacturonide.—The tyraminated tridecagalacturonide was purified from the desalted tridecagalacturonide derivatization mixture by HPAEC on a semipreparative Carbopac PA-1 column (9 × 250 mm; Dionex). The tridecagalacturonide derivatization mixture (3 mg in 0.5 mL H_2O) was resolved with a linear gradient of 660–760 mM KOAc, pH 8, at a flow rate

of 5 mL/min over 20 min. The UV-absorbing (274 nm) compounds were collected individually, dialyzed at 4 °C against several changes of H_2O, concentrated to 1 mL by rotary evaporation under reduced pressure, and stored at −80 °C.

Electrospray mass spectrometry.—Electrospray mass spectrometry was performed with an API III Biomolecular Mass Analyzer (PE-Sciex, Thornhill, Canada) interfaced to a Macintosh IIfx data station. The mass spectrometer was operated in the positive-ion mode with an ion spray at 5 kV and an orifice potential of 35 V. Solutions of the purified tyraminated oligogalacturonides (1 μg/mL) in 1:3 acetonitrile–H_2O containing 0.1% (v/v) HOAc and 2 mM ammonium formate were introduced into the electrospray source at 2 μL/min using a Harvard 22 syringe infusion pump. The mass range was scanned for 200–2000 amu. At least ten scans were averaged.

^1H NMR spectroscopy.—The purified tyraminated oligogalacturonides were exchanged three times with D_2O (Aldrich 99.96%). ^1H NMR spectroscopy was performed at 25 °C with a Bruker AMX 600-MHz spectrometer. In addition to a one-dimensional (1D) proton spectrum of the purified tyraminated tridecagalacturonide, a 2D TOCSY [14,15] dataset was acquired. The TOCSY experiment contained a DIPSI mixing sequence [16] and was collected in the TPPI phase-sensitive mode [17]. The dataset consisted of 256 free induction decays (FIDs) of 2048 complex data points with 16 scans per FID. The spectral width was set to 6024 Hz and the carrier placed at the residual HDO peak, which was irradiated by a low-power decoupler pulse. Chemical shifts are reported in ppm relative to 3-(trimethylsilyl)-1-propanesulfonic acid (DSS), using the HDO signal as an internal reference of δ 4.789.

3. Results and discussion

Tyramination of oligogalacturonides.—Commercially available, reportedly homogeneous trigalacturonic acid (10 mg) and a Q-Sepharose-purified tridecagalacturonide fraction (10 mg) were separately derivatized with tyramine by reductive amination in the presence of sodium cyanoborohydride (Fig. 1). Trigalacturonic acid and the tridecagalacturonide fraction were derivatized in the same manner, except that MeOH was used as the solvent for the former and H_2O for the latter, since tridecagalacturonide is insoluble in MeOH. The reaction was allowed to proceed for 1 h, after which time any remaining sodium cyanoborohydride was destroyed by addition of glacial acetic acid. The acidified solution was dried under a stream of air, and the residue was dissolved in H_2O.

The trigalacturonic acid derivatization mixture was desalted by Sephadex G-25 gel-permeation chromatography. Fractions were collected and assayed for tyramine and uronic acid. Unreacted tyramine and tyraminated trigalacturonic acid were fully resolved (data not shown). The fractions containing tyraminated trigalacturonic acid were pooled and concentrated to 1 mL. The derivatized reducing end galacturonic acid residue is not detectable by colorimetric analysis of uronic acids (data not shown). The galactosyluronic acid content was corrected accordingly. The yield of tyraminated trigalacturonic acid, based on galactosyluronic acid residues, averaged 35% of the starting material. Much of the loss was probably due to the affinity of Sephadex for aromatic compounds

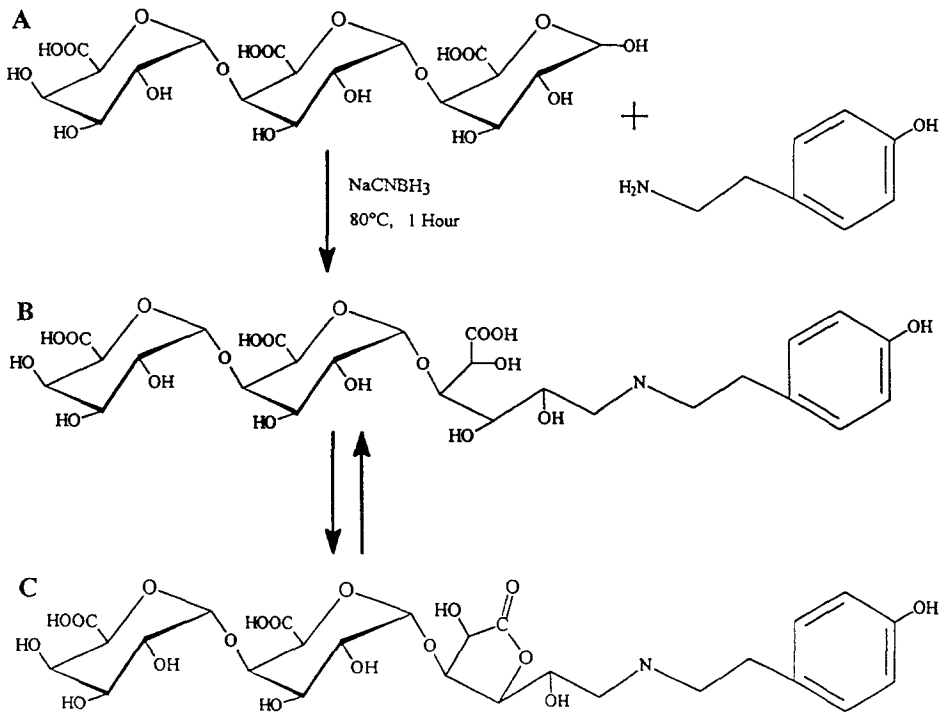

Fig. 1. Tyramination of trigalacturonic acid. L-Galactonic acid is formed when tyramine is reacted with trigalacturonic acid (**A**) and the adduct is reduced with sodium cyanoborohydride. The free-acid (**B**) and lactonized (**C**) forms of L-galactonic acid are in equilibrium.

[18]. The tyraminated trigalacturonic acid was shown to be homogeneous by analytical HPAEC-PAD/UV (see below).

The tridecagalacturonide derivatization mixture was desalted by adding 10 vol of absolute MeOH, which caused the precipitation of both the derivatized and underivatized oligogalacturonides. The supernatant, which contained unreacted tyramine and salt, was discarded. The precipitate was resuspended in absolute MeOH and collected by centrifugation four successive times in order to remove any remaining unreacted tyramine, which is only slightly soluble in MeOH. The pellet of the final centrifugation step was dried, dissolved in H_2O, and the uronic acid content determined colorimetrically [13]. The yield at this step, based on galactosyluronic acid residues, averaged 75% of the starting material.

The desalted tridecagalacturonide derivatization mixture was analyzed by HPAEC-PAD/UV (Fig. 2). Tyramine-derivatized oligogalacturonides can be detected by both PAD and UV absorbance (274 nm), while underivatized oligogalacturonides can only be detected by PAD. One major and three minor tyraminated oligogalacturonides were observed (Fig. 2B and 2C, **1–4**). Small amounts of underivatized dodecagalacturonide and tridecagalacturonide were also detected (compare to Fig. 2A), indicating that tyramine derivatization of the tridecagalacturonide did not go to completion under the

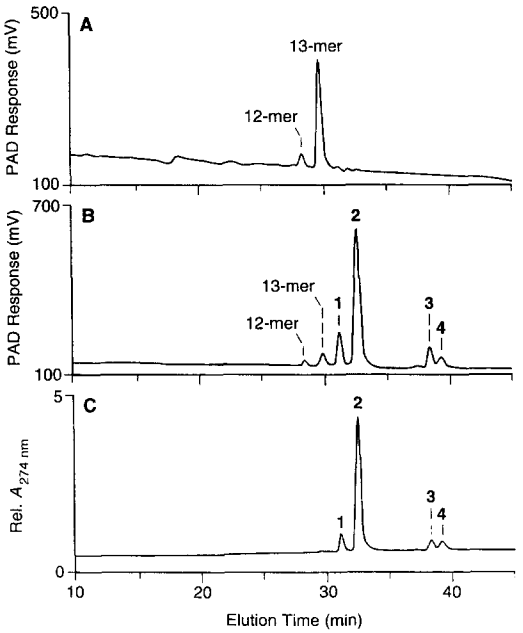

Fig. 2. Analytical HPAE chromatograms of the underivatized tridecagalacturonide fraction (A) and the tridecagalacturonide derivatization mixture (B and C) resolved on a CarboPac PA-1 column with a linear gradient of NaOAc at pH 8 and monitored by PAD (A and B) and UV absorbance (C). The tridecagalacturonide derivatization mixture contains four tyraminated oligogalacturonides (**1–4**), as well as underivatized dodecagalacturonide (12-mer) and tridecagalacturonide (13-mer). The tyraminated oligogalacturonides were purified by semipreparative HPAEC and structurally characterized.

reaction conditions used. Since four tyraminated oligogalacturonides were detected, these compounds (**1–4**) were purified by semipreparative HPAEC and characterized in order to determine their identities. The yield of purified **2**, based on galactosyluronic acid content, was typically 30% of the tridecagalacturonide fraction starting material.

Characterization of the tyraminated oligogalacturonides.—Tyraminated trigalacturonic acid, purified by G-25 gel-permeation chromatography, was analyzed by HPAEC-PAD/UV. Two tyraminated trigalacturonic acid components were detected (Fig. 3C, **5** and **6**), and no underivatized trigalacturonic acid was observed, indicating that the derivatization reaction had proceeded to completion (compare to Fig. 3A). Acidification of the tyraminated trigalacturonic acid (1% HOAc), prior to HPAEC analysis, increased the relative amount of **5** compared to **6** (data not shown). However, upon alkalinization of the tyraminated trigalacturonic acid (10 mM NaOH), only **6** was observed (Fig. 3B). This result, as well as the results of electrospray mass spectrometry (see below), demonstrates that **6** is tyraminated trigalacturonic acid and that **5** is tyraminated trigalacturonic acid in which the L-galactonic acid formed during the derivatization reaction is in the lactone form (Fig. 1). Lactones of aldonic acids, the product of the reduction of the C-1 of uronic acids, form under acidic conditions and hydrolyze under alkaline conditions [19].

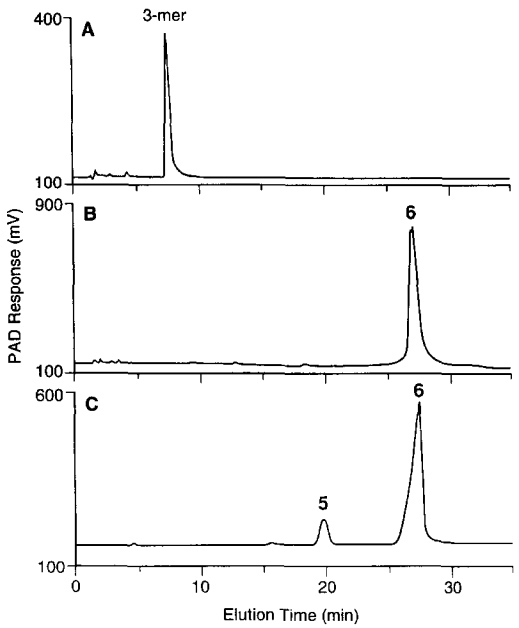

Fig. 3. Analytical HPAE chromatograms of underivatized trigalacturonic acid (A) and tyraminated trigalacturonic acid (B and C) resolved on a CarboPac PA-1 column with a linear gradient of NaOAc, at pH 8, and monitored by PAD. Tyraminated trigalacturonic acid was alkalinized (B) or acidified (C) before being subjected to HPAEC with sequential PAD and UV absorbance detection. The chromatograms generated by UV absorbance detection (not shown) demonstrate the same compounds as observed by PAD. The lactone (**5**) and free-acid (**6**) forms of tyraminated trigalacturonic acid are both present in the acidified sample. The lactone is hydrolyzed by alkaline conditions.

Purified **2** of the tridecagalacturonide derivatization mixture was analyzed by HPAEC-PAD/UV. Alkalinization of **2** (10 mM NaOH), prior to HPAEC analysis, resulted in a single peak as detected by PAD and UV absorbance (Fig. 4), providing evidence that **2** is homogeneous. Acidification of **2** generated a second peak corresponding to **4** (data not shown). This analysis, as well as electrospray mass spectrometry (see below) of purified **2** and **4**, demonstrated that **2** is tyraminated tridecagalacturonide and **4** is tyraminated tridecagalacturonide in which the L-galactonic acid formed during the derivatization reaction is in the lactone form. HPAEC-PAD/UV analysis of purified **1** (data not shown) and electrospray mass spectrometry of purified **1** and **3** (see below) demonstrated that **1** is tyraminated dodecagalacturonide and **3** is its lactone.

The positive-ion electrospray mass spectrum of tyraminated trigalacturonic acid (Fig. 5A) gave major ions at m/z 668, 690, 706, 712, and 728, corresponding to the $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, $[M + 2Na - H]^+$, and $[M + Na + K - H]^+$ ions, respectively, of tyraminated trigalacturonic acid (Fig. 1). Ions were also detected at m/z 650 and 672, corresponding to the $[M + H]^+$ and $[M + Na]^+$ ions of the lactone of tyraminated trigalacturonic acid.

The positive-ion electrospray mass spectrum of purified **2** (Fig. 5B) gave ions at m/z 1215, 1223.5, 1234, and 1251, corresponding to $([M + 2H]^{2+})/2$, $([M + H +$

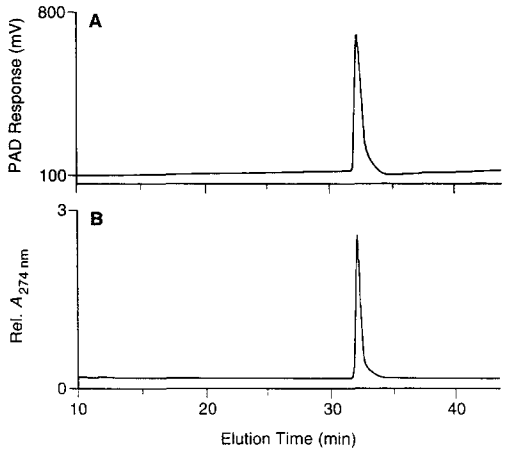

Fig. 4. Analytical HPAE chromatograms of purified tyraminated tridecagalacturonide monitored by PAD (A) and A_{274} (B).

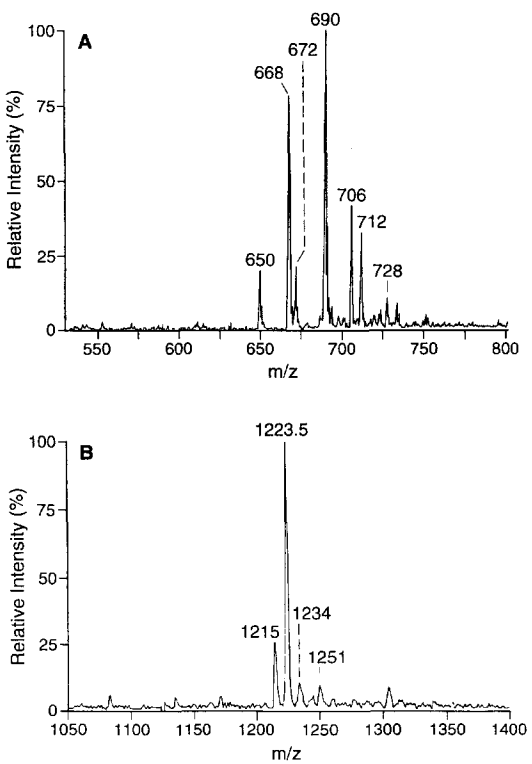

Fig. 5. Electrospray mass spectra of tyraminated trigalacturonic acid (A) and tyraminated tridecagalacturonide (B). Solutions (1 μg/mL) of the tyraminated oligogalacturonides, in 3:1 H_2O–acetonitrile containing 0.1% HOAc and 2 mM ammonium formate, were introduced into the electrospray source with a syringe infusion pump. The signals are identified in the Results section.

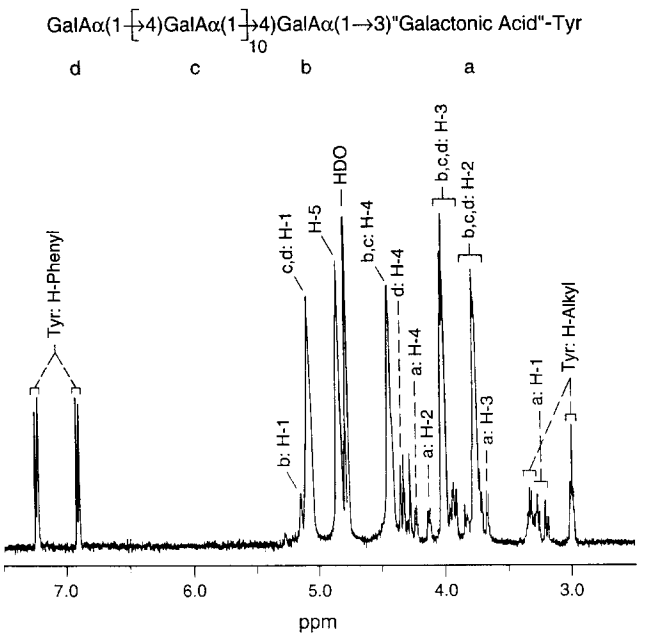

Fig. 6. ^{1}H NMR spectrum of the HPAEC-purified tyraminated tridecagalacturonide.

$NH_4]^{2+})/2$, $([M + H + K]^{2+})/2$, and $([M + 2NH_4 + K - H]^{2+})/2$, respectively, of tyraminated tridecagalacturonide. The positive-ion electrospray mass spectrum of **4** (data not shown) gave the same ions observed for **2** and additional ions corresponding to the lactone of tyraminated tridecagalacturonide. This indicates that **4** is in equilibrium between lactone and free-acid forms. The positive-ion electrospray mass spectrum of **1** (data not shown) gave major ions corresponding to Na^+, NH_4^+, and K^+ adducts of tyraminated dodecagalacturonide. The spectrum for **3** (data not shown) gave identical ions as **1** and additional ions corresponding to the lactone of tyraminated dodecagalacturonide.

The ^{1}H NMR spectra of tyraminated trigalacturonic acid and purified **2** are consistent with oligogalacturonides of dp 3 and 13, respectively, each derivatized with a single tyramine at the C-1 of the reducing end galacturonic acid residue. The 1D ^{1}H NMR spectrum of **2** (Fig. 6, Table 1) shows five large signals corresponding to the protons of the repeating internal galactosyluronic acid residues (Fig. 6, c) [20]. In addition Fig. 6 contains smaller signals generated by the galacturonic acid residues at the nonreducing end (d), the residue proximal to the reducing end (b), the L-galactonic acid residue at the former reducing end (a), and the tyramine group. The latter resonances integrate to one tyramine group per 12 galactosyluronic acid residues, as would be expected for the tridecagalacturonide derivatized with a single tyramine. The signals corresponding to the internal and non-reducing end galactosyluronic acid residues (c and d) can be assigned from the 1D spectrum based on published 1D ^{1}H NMR spectra of oligogalacturonides [16]. The additional signals (a and b) were assigned by a 2D TOCSY experiment. In

Table 1
Assignments of the signals in the ^{1}H NMR spectrum of the purified tyraminated tridecagalacturonide

Residue	Chemical shift				
	H-1	H-2	H-3	H-4	H-5
a [a]	3.19/3.25 [b]	4.13	3.66	4.23	4.84
b	5.15	3.84	3.94	4.43	4.84
c	5.08	3.75	4.01	4.43	4.84
d	5.05	3.71	3.92	4.28	4.84
	H-alkyl	H-phenyl			
Tyr	2.99/3.22	6.90/7.23			

[a] The letters refer to the residues as labelled in Fig. 6.
[b] The protons of the L-galactonic acid residue (a) are numbered according to the D-galacturonic acid residue from which it was derived. Reductive amination leads to two protons on the C-1 of the former reducing end residue.

particular, Fig. 6 contains the expected signals at δ 3.196 and 3.263 corresponding to the methylene protons at C-6 of the L-galactonic acid residue (a) linked to tyramine. The other signals from the L-galactonic acid residue have chemical shifts different from the galactosyluronic acid residues and do not show the coupling patterns expected for a pyranose ring.

The identification and isolation of oligosaccharide-specific binding proteins requires homogeneous biologically active derivatives with high specific radioactivity [1–4]. We have produced homogeneous oligogalacturonide derivatives containing tyramine at the C-1 of the former reducing end galacturonic acid residue. Tyraminated oligogalacturonides can be labelled to a high specific radioactivity with ^{125}I and used to detect low-abundance binding sites [1,3]. The L-galactonic acid formed during the derivatization reaction is in equilibrium between the lactone and free-acid forms. The lactone form of tyraminated oligogalacturonides could potentially interfere with the use of these compounds for identifying binding sites, as the lactone may react with amines or alcohols leading to non-specific covalent binding of tyraminated oligogalacturonides. This possibility is unlikely, however, as we have shown that the lactone form of the tyraminated oligogalacturonides does not react with high concentrations of free tyramine.

We are characterizing the biological activity of purified tyraminated oligogalacturonides to determine their usefulness as reagents for identifying putative receptors for oligogalacturonides. Eventually this line of research should lead to elucidation of the signal transduction pathways elicited by the oligogalacturonides.

Acknowledgements

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