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α-D-Mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-*myo*-inositol, a new and unusual oligosaccharide from cultured rose cells

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Abstract

Cell-suspension cultures of rose (*Rosa* sp.) accumulated approximately micromolar concentrations of a novel, extracellular, non-reducing trisaccharide, characterised here as α-D-mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-*myo*-inositol (MGI). Acid hydrolysis of [glucuronic acid-^{14}C]MGI, isolated after incubation of the cells with D-[6-^{14}C]glucuronic acid, readily yielded [^{14}C]glucuronic acid-inositol, which was relatively resistant to further hydrolysis, as revealed by chromatography and electrophoresis. The complete structure and stereochemistry of MGI was elucidated using one and two dimensional NMR spectroscopic techniques. The structure of MGI suggests a biosynthetic origin from a membrane-bound glycophosphosphingolipid (GPS). We propose that GPSs represent a hitherto unrecognised source of oligosaccharin signalling molecules in plants.

Keywords: *Rosa* sp; Glucuronic acid; Mannose; *myo*-inositol; Oligosaccharide; Oligosaccharin; Trisaccharide; Glycophosphosphingolipid; NMR spectroscopy; 1D TOCSY; DQFCOSY; HMQC; HMBC

1. Introduction

Oligosaccharides that evoke 'hormone-like' responses in plant cells have been termed oligosaccharins (Aldington & Fry, 1993; Darvill et al., 1992). Oligosaccharins can be produced artificially by the partial hydrolysis of various cell wall polysaccharides including pectins (Aldington & Fry, 1994; Darvill et al., 1992), xyloglucans (Warneck, Fulton, Seitz & Fry, 1998; York, Darvill & Albersheim, 1984) and galactoglucomannans (Auxtová et al., 1995). Extraprotoplasmic oligosaccharins may play an important natural role in the regulation of plant metabolism, growth and development (Aldington & Fry, 1993; Darvill et al., 1992): if so, they would be predicted to occur in vivo. Certain extraprotoplasmic oligosaccharins have been detected at biologically relevant concentrations in the spent media of plant cell-suspension cultures, e.g. a xyloglucan-derived oligosaccharin in spinach cultures (McDougall & Fry, 1991), pectic oligosaccharins in some cultures (Tani, Fukui, Shimomura & Tabata, 1992) but not in others (García-Romera & Fry, 1997), xylomannoside oligosaccharins apparently arising by glycoprotein hydrolysis in *Silene* cultures (Priem & Gross, 1992; Priem et al., 1990) and unidentified oligosaccharins in carrot and tobacco cultures (Schröder & Knoop, 1995). More studies are clearly needed of the in vivo production of oligosaccharins to assess their biological role.

We now report the discovery in rose cell cultures of a new oligosaccharide with an unusual structure,

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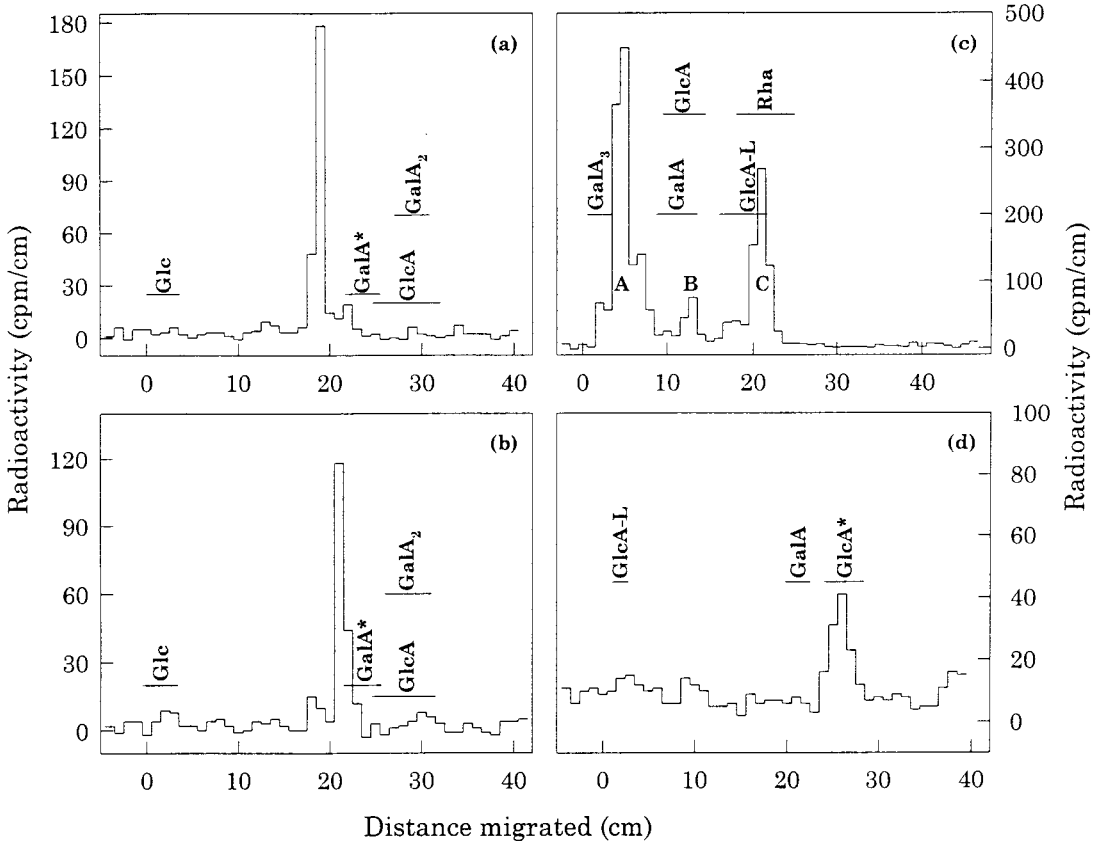

Fig. 1. Paper electrophoresis and paper chromatography of ^{14}C-labelled trisaccharide MGI and its hydrolysis products. (a) Electrophoresis of ~6 Bq of ^{14}C-labelled MGI. GalA was included as an internal marker (*) and stained after scintillation counting; Glc, GlcA and $GalA_2$ were external markers (run alongside the radioactive track). (b) Electrophoresis of ~6 Bq of ^{14}C-labelled MGI after hydrolysis in TFA; markers were as in (a). (c) Chromatography (in BuOH–HOAc–H_2O) of ~70 Bq of ^{14}C-labelled MGI after formolysis/hydrolysis; markers were external. Peaks A–C are discussed in the text. (d) Electrophoresis of peak B from (c). GlcA was an internal marker (*); GalA and glucuronolactone (GlcA-L) were external.

apparently related to a poorly understood group of lipids, the glycophosphosphingolipids (GPSs). Traces of an unidentified glucuronic acid-containing oligosaccharide were shown to be produced by cultured rose cells (García-Romera & Fry, 1997). Recent work (Smith, 1998) has suggested that this oligosaccharide, when added to living plant cells, may evoke biological effects on amino acid metabolism. A completely new naturally occurring oligosaccharin would be of considerable interest. Therefore, the present work was undertaken to elucidate its structure.

2. Results and discussion

2.1. Radiolabelling and chromatography

To facilitate a search for traces of acidic oligosaccharides produced by rose cells, we incubated a suspension-culture in the presence of D-[6-^{14}C]glucuronic acid. This is metabolised to UDP-[^{14}C]glucuronic acid and UDP-[^{14}C]galacturonic acid, the main donors of uronic acid residues; negligible radiolabelling of neutral hexose and pentose residues occurs (Brown & Fry, 1993; García-Romera & Fry, 1997). When rose cells were cultured in the presence of D-[6-^{14}C]glucuronic acid for 7 days, an extracellular radioactive compound, referred to here as MGI, accumulated in the medium to a concentration accounting for 0.048% of the starting ^{14}C. On paper chromatography in two acidic solvents, MGI approximately co-migrated with the α-(1 → 4)-linked trisaccharide of D-galacturonic acid ($GalA_3$), suggesting that it was a trisaccharide. Electrophoresis at pH 3.5 showed that it was anionic but had a relatively low charge:mass ratio (Fig. 1a), indicating the presence of one or two non-anionic residues. Acid hydrolysis efficiently converted ^{14}C-MGI into a radioactive product with a somewhat higher charge:mass ratio (Fig. 1b). This effect is consistent with hydrolysis of the trisaccharide MGI to yield a non-radioactive, neutral monosaccharide and a ^{14}C-disaccharide with one acidic residue, which would be relatively resistant to acid hydrolysis (Redgwell, 1983). Hydrolysis of ^{14}C-MGI under more severe conditions

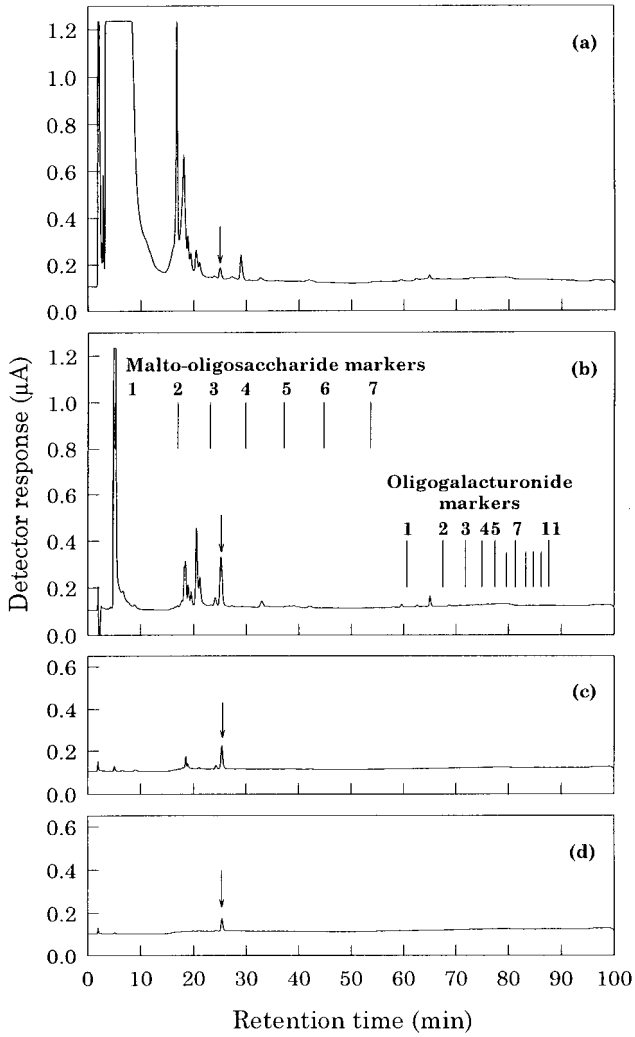

Fig. 2. HPLC of non-radioactive culture filtrate showing purification of MGI. (a) Supernatant after EtOH precipitation, (b) anionic fraction after ion-exchange chromatography, (c) trisaccharide fraction after Bio-Gel P-2 (d) after Sephadex G10. HPLC was performed with programme 2. The arrows indicate the peaks of compound MGI. The vertical lines in (b) indicate the retention times of glucose (1) and malto-oligosaccharides (2–7) and GalA (1) and oligogalacturonides (2–11).

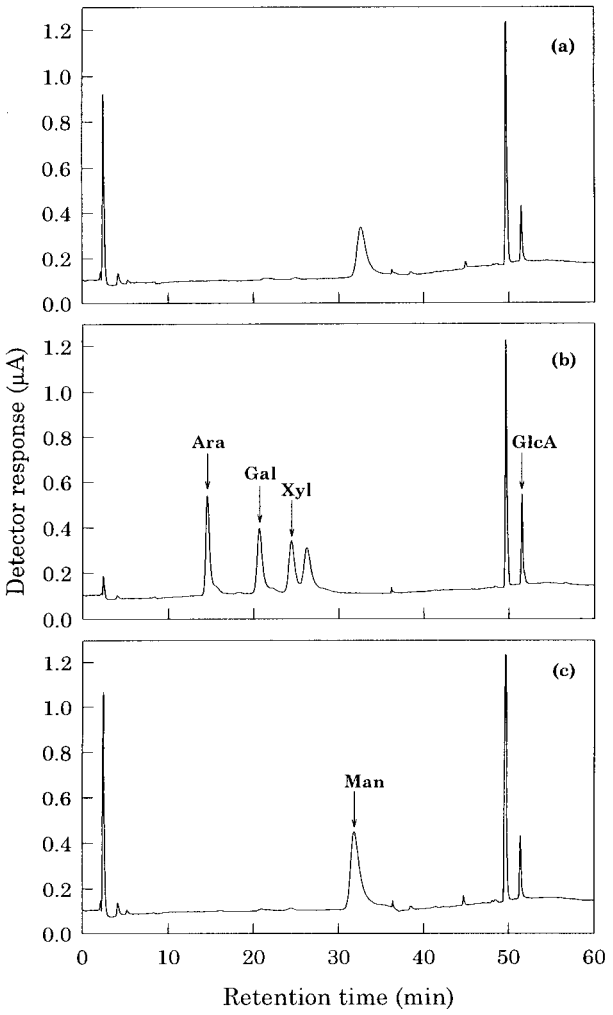

Fig. 3. HPLC of formolysis/hydrolysis products of non-radioactive MGI. (a) Products alone; (b) products with added Ara, Gal, Xyl and GlcA; (c) products with added Man. HPLC was performed with programme 3.

yielded three radioactive products (A–C in Fig. 1c): A was the ^{14}C-disaccharide, B co-electrophoresed with glucuronic acid (Fig. 1d) and C was [^{14}C]glucuronolactone (confirmed by alkaline hydrolysis to a product that co-electrophoresed with glucuronic acid; data not shown). These results indicate that the radioactive residue in MGI was D-glucuronic acid, since there is no pathway by which exogenous D-[^{14}C]glucuronic acid can be converted metabolically to L-[^{14}C]glucuronic acid.

Using ^{14}C-MGI as a marker, we isolated larger amounts of non-radioactive MGI. Anion-exchange and gel-permeation chromatography of the ethanol-soluble fraction from the culture filtrate of 14-day-old rose cells yielded MGI (typically 0.6 mg/l medium) that was judged to be pure by HPLC (Fig. 2). Severe acid hydrolysis of MGI gave four main products (Fig. 3a), which appeared by HPLC to be glucuronic acid (GlcA), an acidic disaccharide, mannose (Man) and a very early-eluting peak, later shown to be *myo*-inositol (Ins). Mannose is assumed to be present as the naturally occurring D-isomer. The identities of GlcA and Man were supported by co-elution with internal markers (Figs. 3b and c). We attempted to reduce MGI with $NaBH_4$ or NaB^3H_4 prior to acid hydrolysis; however, HPLC of the products revealed no difference from the products of non-reduced MGI; specifically, gulonate and mannitol (products expected if GlcA acid or Man, respectively, were the reducing terminus) were absent (Fig. 4). Thus, MGI appeared to be a non-reducing trisaccharide.

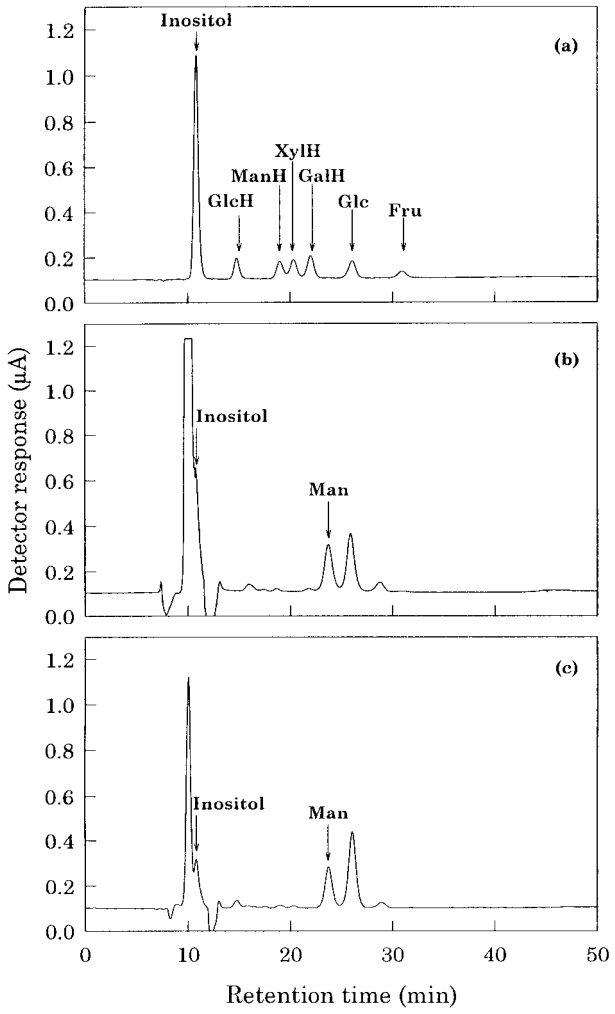

Fig. 4. HPLC of formolysis/hydrolysis products of untreated and $NaBH_4$-treated MGI. (a) Markers alone (GalH = galactitol; GlcH = glucitol; ManH = mannitol; XylH = xylitol); (b) formolysis/hydrolysis products of MGI; (c) formolysis/hydrolysis products of $NaBH_4$-treated MGI. HPLC was performed with programme 1. These samples appeared to be contaminated with Glc.

2.2. Structural elucidation of MGI by NMR spectroscopy

An unambiguous elucidation of the structure of MGI was obtained by NMR spectroscopy using one (1D) and two (2D) dimensional techniques. The standard 1D single-pulse 600-MHz proton spectrum (Fig. 5a) of MGI shows signals from 18 protons (all OH signals having exchanged in the D_2O used as solvent) excluding the residual HOD signal after suppression. To obtain separate proton spectra of individual component monosaccharides and thereby simplify the spectrum, a series of 1D TOCSY spectra was obtained in which the selective pulse was applied at each of the well resolved signals in turn. These spectra, of which those in Fig. 5b–d) are typical, established that only three separate spin systems were present, confirming that MGI was a non-reducing trisaccharide. A 2D TOCSY spectrum (not shown) confirmed, as expected, the presence of only these three spin systems. The individual proton multiplets were sufficiently well resolved in the 1D TOCSY spectra to allow identification of the carbohydrate residues. The individual proton multiplets were also well resolved in a 2D *J* resolved spectrum (not shown).

The 1D TOCSY spectrum (Fig. 5b) of one residue was that of a mannose unit. The unique coupling patterns of resonances H-1, H-2, H-4 and H-5 allowed their assignment directly from the 1D TOCSY spectrum and the remaining resonances were assigned from the 2D DQCOSY spectrum (not shown).

The 1D TOCSY spectrum (Fig. 5c) of a second residue was that of an α-linked GlcA. The unique H-1, H-2 and H-5 resonances of the GlcA residue were assigned directly from the coupling patterns and those of H-3 and H-4 distinguished from the 2D DQFCOSY spectrum. The low (3.3 Hz) value for $^3J_{12}$ showed GlcA (which had been shown from the ^{14}C-incorporation studies to be the D-isomer) to be present in the α-anomeric configuration.

The 1D TOCSY spectrum (Fig. 5d) of the third residue was consistent only with that of a Ins unit and excluded all isomeric inositol configurations. The unique H-2 and H-5 resonances of the Ins residue were assigned directly from the coupling patterns and the remaining connectivity established from the 2D DQFCOSY spectrum. The assignments are presented in Table 1.

These data indicate that MGI is a trisaccharide with Ins in place of a reducing monosaccharide. This is consistent with the borohydride reduction results reported above.

The single-pulse and polarisation-transfer 1D carbon-13 NMR spectra (not shown) are in full agreement with this composition, showing signals attributable to only two anomeric carbons, only one methylene carbon, fourteen methine carbons and a single carbonyl resonance.

Carbon-13 chemical shifts (Table 1) were assigned from the 2D 1-bond proton-carbon heteronuclear multiple quantum correlation (HMQC) spectrum (Fig. 5e). The carbon-13 shifts of the Man residue compare well with those of α-methyl-D-mannoside, indicating that Man was present as the α and not the β anomer. Only C-1 shows a high frequency shift of about 0.5 ppm compared with free mannose suggesting that this is a terminal residue linked via C-1.

The carbon-13 chemical shifts of the GlcA residue compare well with those of the free acid except for C-1 which is shifted by about 5 ppm to high frequency consistent with the presence of a glycosidic link. Apart from that for C-2, the carbon-13 chemical shifts corresponding to the Ins residue lie in the same range (71–

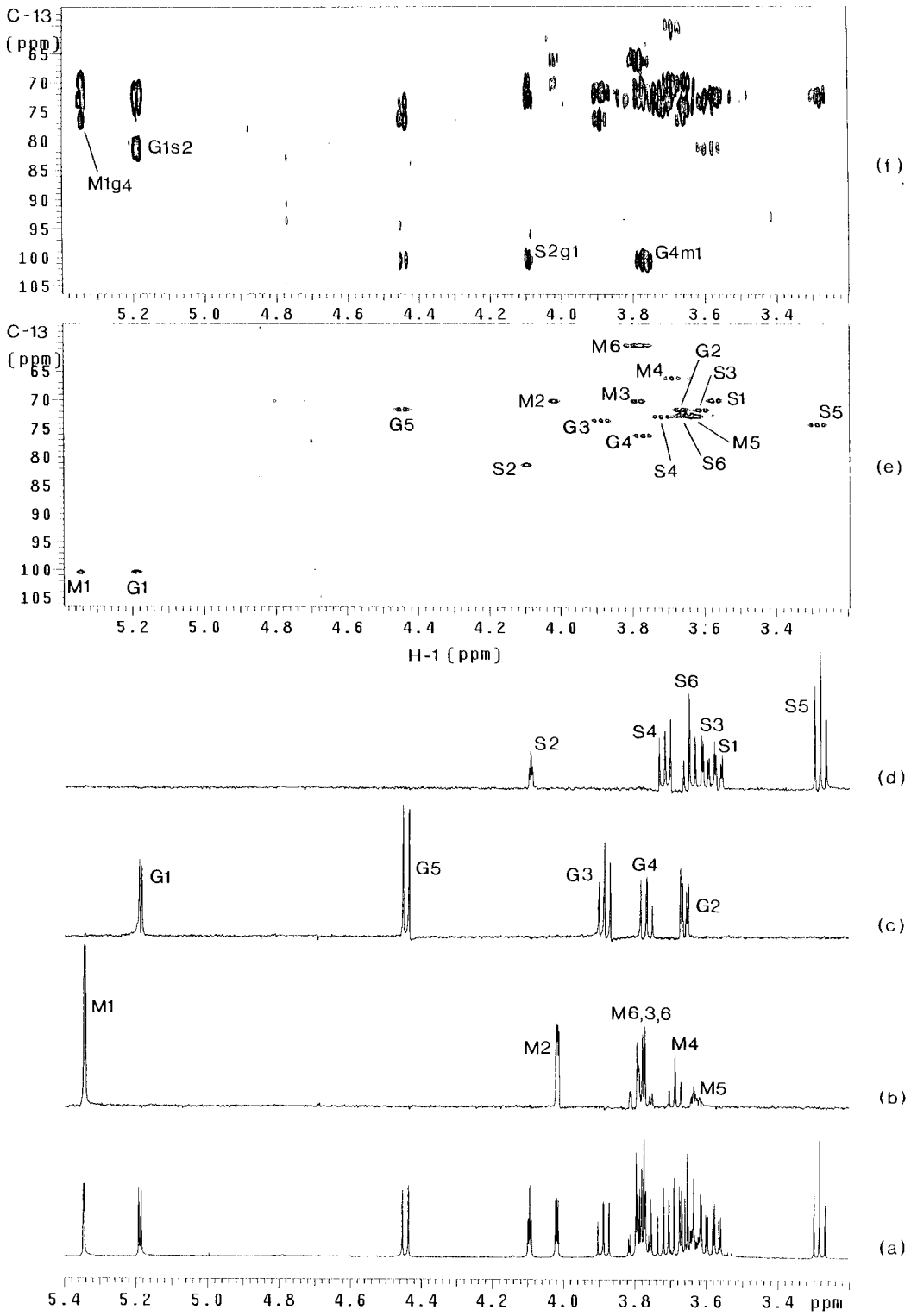

Fig. 5. One and two dimensional NMR spectra of the trisaccharide MGI. (a) Standard 1D single-pulse 600-MHz proton spectrum of MGI; (b)–(d) 1D TOCSY spectra of MGI with selective irradiation applied at M1, G1 and S5, respectively, where Man, GlcA and Ins proton resonances are annotated by M, G and S, respectively, followed by the proton site; (e) 2D 1-bond proton-carbon correlation (HMQC) spectrum with resonances annotated as in (b)– (d); (f) 2D long-range proton-carbon correlation (HMBC) spectrum; resonances annotated e.g. M1g4 where M, G or S denotes the proton co-ordinate and m, g or s denotes the carbon co-ordinate.

Table 1
^{1}H and ^{13}C NMR spectral data of α-D-Man*p*-(1 → 4)-α-D-GlcA*p*-(1 → 2)-Ins (MGI)[a]

Site	δ_C	δ_H	J_{HH} (Hz)	Proton-carbon long-range[b] correlations		Proton NOE enhancements	
				inter-residue	intra-residue	inter-residue	intra-residue
Mannose							
1″	100.4	5.345 (d)	1.7	C-4′	C-2″, C-3″, C-5″	H-4′ (6%)	H-2″ (6%)
2″	70.1	4.019 (dd)	3.4, 1.7		C-3″, C-4″		H-1″ (5%), H-3″ (2%)
3″	70.0	3.782 (dd)	9.8, 3.4		C-1″, C-4″ + obsc.		
4″	66.2	3.689 (t)	2 × 9.8		C-6″		
5″	72.7	3.630 (ddd)	9.8, 4.0, 2.7		obsc.		
6″	60.4	3.765 (dd)	12.4, 4.0		C-4″ + obsc.		
		3.802 (dd)	12.4, 2.7		C-4″ + obsc.		
Glucuronic acid							
1′	100.3	5.188 (d)	3.3	C-2	C-2′, C-3′, C-5′	H-2 (16%)	H-2′ (11%)
2′	71.6	3.664 (dd)	3.3, 9.8		obsc.		
3′	73.5	3.887 (dd)	9.8, 9.1		C-4′		
4′	76.2	3.770 (dd)	10.1, 9.1	C-1″	C-6′ + obsc.		
5′	71.6	4.445 (d)	10.1		C-1′, C-3′, C-4′, C-6′	H-6 (1%)	H-3′ (4%)
6′	174.9	-					
Inositol							
1	70.1	3.570 (dd)	10.0, 2.7		C-2		
2	81.3	4.095 (t)	2 × 2.7	C-1′	C-1, C-3, C-4, C-6	H-1′ (10%)	H-1 (5%), H-3 (5%)
3	71.7	3.607 (dd)	10.0, 2.7		C-2		
4	72.8	3.719 (dd)	10.0, 9.3		obsc.		
5	74.3	3.284 (t)	2 × 9.3		C-4, C-6		
6	72.4	3.652 (dd)	10.0, 9.3		C-5		

[a] dd = doublet of doublets, ddd = doublet of doublets of doublets, obsc = obscured, if present, by other peaks.
[b] From HMBC spectrum.

75 ppm) as those for Ins. Alkylation of one oxygen normally results in a chemical shift increase of 8–10 ppm of the corresponding ring carbon. As the chemical shift of C-2 in the Ins group of MGI is markedly higher than that in free Ins, this suggests that the linkage is at C-2. The H-1/H-3 and H-4/H-6 inositol signal non-equivalence arises from the asymmetry of the group linked through oxygen at C-2.

The Man-(1 → 4)-GlcA link was demonstrated by 3-bond correlations between Man[H-1] and GlcA[C-4] and between Man[C-1] and GlcA[H-4] in the 2D long-range proton-carbon heteronuclear multiple bond correlation (HMBC) spectrum (Fig. 5f). This was supported by nuclear Overhauser enhancement (NOE) (spectra not shown) of GlcA[H-4] on irradiation of Man[H-1]. The absence of enhancement of the Man[H-3] and Man[H-5] resonances in this experiment showed that Man in MGI was present as the α-anomer (under the steady state conditions employed for this experiment, irradiation of Man[H-1] in β-anomers gives significant enhancements of the Man[H-3] and Man[H-5] resonances).

The GlcA-(1 → 2)-Ins link was confirmed by the following data: (a) 3-bond correlations between GlcA[H-1] and Ins[C-2] and between GlcA[C-1] and Ins[H-2] in the 2D long-range proton-carbon HMBC spectrum (Fig. 5e) and (b) A 4-bond correlation in the 2D long-range COSY spectrum (not shown) between GlcA[H-1] and Ins[H-2]. This was further supported by NOE enhancement of GlcA[H-1] on irradiation of Ins[H-2]. All other clearly identifiable long-range proton-carbon correlations and proton NOE enhancements (Table 1) were consistent with the structure for the trisaccharide MGI shown in Fig. 6.

2.3. Electrospray mass spectrometry

An electrospray mass spectrum (not shown) of MGI showed strong peaks at 519.1, 536.1 and 541.2 mass

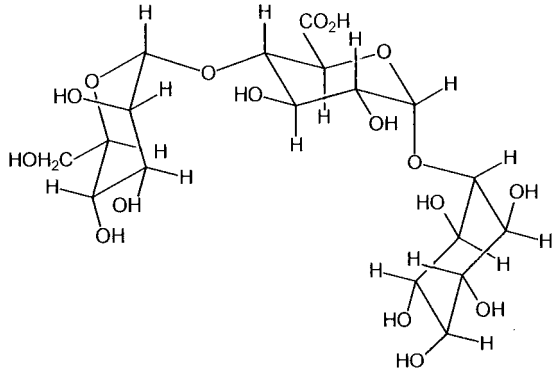

Fig. 6. Structure of the trisaccharide MGI.

units respectively attributed to $(M+H)^+$, $(M+NH_4)^+$ and $(M+Na)^+$ ions corresponding to a molecular formula for MGI of $C_{18}H_{30}O_{17}$ as required by the proposed structure.

2.4. Conclusion

Pulse-labelling experiments with [6-^{14}C]glucuronic acid have suggested (Smith, 1998) that MGI is released into the culture medium relatively slowly from a preformed cellular conjugate. The structural data reported in the present paper lead us to consider the most likely nature of such a conjugate to be a glycolipid rather than a polysaccharide or glycoprotein. The polysaccharides with the closest similarity to MGI are the glucuronomannans, which have a backbone of a repeating disaccharide, [α-D-Man*p*-(1 → 4)-β-D-Glc*p*A-(1 → 2)-]$_n$ (Honda, Inaoka, Takei, Sugimura & Otsuji, 1996; Mori & Kato, 1981; Redgwell, 1983). However, glucuronomannans do not contain inositol or α-glucuronic acid. A more plausible possibility is that MGI arises in vivo by degradation of a glycophosphosphingolipid (GPS). Plants possess a family of GPSs, based on the core structure, oligosaccharide-GlcA-Ins-phosphate-ceramide (Carter, Betts & Strobach, 1964; Carter, Brooks, Gigg, Strobach & Suami, 1964; Carter et al., 1962; Carter, Strobach & Hawthorne, 1969; Hsieh, Lester & Laine, 1981; Laine & Hsieh, 1987). Several GPSs have been characterised; reported structures include

α-D-Man*p*-(1→2)-*myo*-Ins-1-P–ceramide
|
α-D-Glc*p*N-(1→4)-α-D-Glc*p*A-(1→6)

(Carter et al., 1969) and α-D-Glc*p*N-(1 → 4)-α-D-Glc*p*A-(1 → 2)-*myo*-Ins-1-P-ceramide (Hsieh et al., 1981), where Glc*p*N = 2-amino-2-deoxyglucose. No GPS has been reported to contain the exact structure present in MGI, but the similarity is striking, especially the α-anomerism of GlcA and Man and the presence of Ins. The sub-cellular location of GPSs has not been definitively established, but a putative GPS in pea root nodules was immunolocalised to the plasma membrane (Perotto, Donovan, Drøbak & Brewin, 1995). Therefore, we postulate that a GPS, present in the plasma membranes of cultured rose cells, is subject to hydrolysis by a phosphodiesterase to yield the novel molecule with a potential signalling role, MGI.

The idea that a GPS-derived, Ins-containing oligosaccharin serves a signalling role in plants has a precedent in the recent discovery that Ins-phosphoglycans, thought to be released from membrane-bound glycosylphosphatidylinositols, are second messengers that mediate the action of insulin on animal cells (Caro et al., 1997).

3. Experimental

3.1. Materials

$GalA_2$ and $GalA_3$ were isolated from a pectinase digest of commercial homogalacturonan. D-[6-^{14}C]Glucuronic acid (55 Ci/mol) was prepd as described before (Smith, 1998).

3.2. Plant cell-suspension cultures

Cell-suspension cultures of 'Paul's Scarlet' rose (*Rosa* sp.) were maintained in a medium (Fry & Street, 1980), initially pH 6.1, containing inorganic salts, D-glucose (20 g/l), 2,4-dichlorophenoxyacetic acid (1 mg/l), kinetin (0.5 mg/l) and $Na_2EDTA \times 2H_2O$ (7.4 mg/l). No Ins was added. The cells were sub-cultured fortnightly by 4-fold dilution. All manipulations were carried out aseptically.

3.3. Isolation of ^{14}C-labelled MGI from rose culture filtrate

D-[6-^{14}C]GlcA (150 kBq) was filter-sterilised and added to a 1-week-old rose culture (50 ml), which was further incubated under standard growth conditions for 1 week. The culture was then filtered through 53-μm nylon gauze and the filtrate was dried on to three sheets of Whatman 3MM paper and developed in BuOH–HOAc–H_2O (12:3:5). This resolved unused [^{14}C]GlcA from ^{14}C-labelled MGI.

3.4. Purification of non-radioactive MGI from rose culture filtrate

Non-radioactive MGI was isolated from 800 ml of filtrate from 2-week-old cell cultures of rose. EtOH (1 volume) was added to the culture filtrate and incubated overnight at 4°C. Material that remained soluble was concd by rotary evaporation and chromatographed on Dowex-1. The anionic fraction was re-dried and chromatographed on Bio-Gel P-2 (MGI eluted at $k_{av} \approx 0.8$) followed by Sephadex G10 (MGI eluted at $k_{av} \approx 0.25$). At each step, portions were analysed by HPLC (programme 2).

3.5. Reduction and hydrolysis of MGI

For reduction, purified MGI (0.5 mg) was incubated in 0.5 M $NaBH_4$ in 1 M NH_4OH (0.5 ml) at 20°C for 4 h. The reaction was stopped by addition of excess of HOAc and the product was freed of cations on a 1.5-ml column of Dowex-50 (H^+ form). H_3BO_3 was removed by drying 5 times from MeOH–HOAc (9:1, v/v). Hydrolysis was conducted in 2 M TFA at 120°C for 1 h. Formolysis/hydrolysis was conducted in 75%

HCOOH at 100°C for 5 h, after which TFA was added to a final concentration of 2 M and the sample heated at 120°C for a further 1 h.

3.6. Techniques for fractionation of carbohydrates

Descending paper chromatography was conducted on Whatman 3MM paper for 16 h in BuOH–HOAc–H_2O (12:3:5 by volume) or EtOAc–HOAc–H_2O (1:1:1 by volume). High-voltage electrophoresis was performed on Whatman No. 1 paper in HOAc–pyridine–H_2O (10:1:189 by volume, pH 3.5) at 3 kV for 90 min with white spirit as coolant (20–30°C). Samples thought to contain lactones were incubated in 50 mM NaOH at 20°C for 10 min and neutralised with HOAc immediately before electrophoresis. Reducing sugars and oligosaccharides were stained on paper with aniline hydrogen-phthalate (Fry, 1988; Partridge, 1949). Gel-permeation chromatography was on a 270-ml (bed volume) column of Bio-Gel P-2 or on a 150-ml column of Sephadex G10, eluted with HOAc–pyridine–H_2O (1:1:25 by volume, pH $\approx$ 4.7). The columns were calibrated using Blue Dextran ($k_{av}=0$) and $CoCl_2$ ($k_{av}=1$). Anion-exchange chromatography was conducted on Dowex-1 (OAc^- form; 100 ml bed volume) pre-equilibrated with 10 mM pyridinium acetate; neutral material was eluted with 10 mM pyridinium acetate (1.5 bed volumes), then anions were eluted with 1 M pyridinium acetate. Eluted sugars were assayed with phenol–H_2SO_4 (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

Alditols were analysed by HPLC on a CarboPac MA1 column; oligosaccharides and monosaccharides were analysed on a CarboPac PA1 column (Dionex UK). Sample (20 μl) was injected and eluted according to one of the following eluent programmes. Sugars and alditols were detected by a pulsed amperometric detector fitted with a gold electrode. *Programme 1* (on MA1): isocratic 520 mM NaOH at 0.4 mL/min. *Programme 2* (on PA1): 10 min isocratic 20 mM NaOH/10 mM NaOAc, 10 min isocratic 50 mM NaOAc/100 mM NaOH, a 30-min gradient to 100 mM NaOAc/100 mM NaOH, 5 min isocratic 100 mM NaOAc/100 mM NaOH, a 25-min gradient to 500 mM NaOAc/500 mM NaOH and 15 min isocratic 500 mM NaOAc/500 mM NaOH; flow rate 1 ml/min (reduced to 0.8 mL/min for 500 mM NaOAc/500 mM NaOH); post-column base (500 mM NaOH) was added at 0.5 ml/min. *Programme 3* (on PA1): 30 min isocratic H_2O, a 20-min gradient to 800 mM NaOH and 5 min isocratic 800 mM NaOH; flow rate 0.8 ml/min; post-column addition of base as above. *Programme 4* (on PA1): 5 min 10 mM NaOH, 25 min H_2O, a 40-min gradient to 800 mM NaOH and 5-min isocratic 800 mM NaOH; flow rate 1 ml/min; post-column addition of base as above.

3.7. Assay of radioactivity

Radioactive compounds on chromatography paper were assayed by scintillation counting after the addition of ~2 ml of 'OptiScint HiSafe' scintillant (Wallac Chemicals). If the sample was needed for further analysis after scintillation counting, the scintillant was washed off with toluene and the paper allowed to dry; the sugar was then eluted with H_2O. Radioactive sugars in aqueous solution were assayed by scintillation counting after addition of 10 volumes of 'OptiPhase HiSafe' (Wallac Chemicals).

3.8. Mass spectrometry

A positive ion electrospray mass spectrum was obtained on an aqueous solution containing 1 μg per 10 μl using a Micromass Platform II instrument (cone voltage, 20 V). Source and drying gas temperatures were adjusted for optimal diffusion. Data were acquired on a Mass Lynx Windows NT PC Data system.

3.9. NMR spectroscopy

The NMR spectra were acquired at 25°C on ^2H_2O solutions using a Varian INOVA 600 MHz spectrometer operating at 599.9 MHz for protons and 150.9 MHz for ^{13}C nuclei. Sodium trimethylsilyltetradeuteriopropionate (TSP) was used as an internal standard ($\delta_H Me_3Si=0$; $\delta_C Me_3Si=0$).

Proton NOE spectra were obtained with an acquisition time of 2s. Selective irradiation applied on one line of each well resolved multiplet during a 7s preacquisition delay was followed by a non-selective 90° pulse. Blocks of 16 transients were accumulated for each irradiation site and off resonance sites to give a total of 400 transients per site. Application of 0.5 Hz line-broadening to the resulting FIDs was followed by zero filling from 9984 to 128K data points and Fourier transformation. Subtraction of a control spectrum from the on-resonance irradiated spectra gave the enhancements quoted.

In all the following experiments data were processed with optimised shifted sine bell squared functions before transformation unless otherwise stated. In 1D experiments the residual water signal (4.77δ) was removed by digital filtration (ssfilter = 10 Hz) after processing.

1D TOCSY (Kessler, Anders, Glumecker & Steuernagel, 1989) proton spectra were obtained using the sequence: D1–180_{sel}°– 90°– D2–90°– D3–90°– AT with D1 = 2 s (presaturation of residual HOD signal), 180_{sel}° = 100 ms (= 40 Hz), D2 = 0.4 s (pulsed spin lock (Bax & Davis, 1985)), D3 = 0.01 s (z-filter) AT = 2.5 s (acquisition time). Other parameters were SW = 7000

Hz; 32 K data points. A shaped (IBURP) (Geen & Freeman, 1991) selective spin inversion pulse was applied on- and off-resonance on alternate scans and the FID's alternatively added and subtracted to give a 'difference' FID which gave spectra showing only TOCSY responses.

The 2D TOCSY phase sensitive proton spectra were obtained using the sequence (Bax & Davis, 1985): D1–90°–t_1–D2–AT with D1 = 1.5 s (presaturation) D2 = 0.08 s (spin lock) and AT = 0.41 s. The experiment was preceded by 128 dummy scans to establish thermal equilibrium. An 8-step phase cycle (hypercomplex acquisition) was used. Other parameters were SW = 2500 Hz; 2K data points; 256 increments. F_1-data were zero-filled to 1K before transformation.

The 2D DQFCOSY phase sensitive proton spectrum was obtained using the sequence (Pianti, Sorenson & Ernst, 1982): D1–90°– t_1–90°– 90°– AT with D1 = 1.5 s and AT = 0.41 s. An 8-step phase cycle (hypercomplex acquisition) was used. Other parameters were SW = 2500 Hz; 2K data points; 256 increments each with 16 transients per FID were used. F_1-data were zero-filled to 2K before transformation resulting in digital resolution of 3 Hz/pt.

The long range 2D COSY proton spectrum (absolute value) showing 4-bond proton couplings was obtained using the sequence: D1–90°– t_1–D2–90°–D2–AT with D1 = 1.5 s, D2 = 0.1 s and AT = 0.82 s. An 8-step phase cycle was used. Other parameters were SW = 2500 Hz; 4K data points; 512 increments each with 16 transients per FID were used. Data were processed using non-shifted sine-bell squared functions in both dimensions. F_1-data were zero-filled to 2K before transformation.

The 2D *J*-resolved proton spectrum (absolute value) was obtained using the sequence: D1–90°– $t_1/2$–180°–$t_1/2$–AT with D1 = 3 s, D2 = 0.1 s, AT = 1.64 s. A 4-step phase cycle was used. Other parameters were SW = 2500 Hz; 8K data points; 128 increments each with 4 transients per FID were used. Data were processed using non-shifted sine-bell squared functions in both dimensions. F_1-data were zero-filled to 2K before transformation.

The 2D proton detected one-bond ^{1}H-^{13}C correlation (HMQC) spectra were obtained using the sequence (Summers, Marzilli & Bax, 1986): D1–90°(^{1}H)–D2–180°(^{1}H); 180° (^{13}C)–D2–90°(^{1}H)–D3–90°(^{1}H)–D2–90°(^{13}C)–$t_1/2$–180°(^{1}H)–$t_1/2$–90°(^{13}C)–D2–AT. The delays used were D1 = 1.5 s (presaturation), D2 = 3.7 ms ($1/2^1J_{CH}$) and D3 = 0.65 s (to minimise signals from protons bonded to ^{12}C nuclei). The experiment was preceded by 256 dummy scans to establish thermal equilibrium. A 16-step phase cycle (hypercomplex acquisition) was used with ^{13}C broad band decoupling during acquisition of the proton signals. Other parameters were SW(^{1}H) = 2500 Hz; 2K data points; 256 increments; SW(^{13}C) = 25000 Hz, AT = 0.41 s. The F_1 data were zero filled from 256W to 1K before transformation.

The 2D proton detected long-range ^{1}H–^{13}C correlation (HMBC) spectrum (absolute value) was obtained using the sequence (Summers et al., 1986): D1–90°(^{1}H)–D2–90°(^{13}C)–D3–90°(^{13}C)–$t_1/2$–180°(^{1}H)–$t_1/2$–90°(^{13}C)–AT. The delays used were D1 = 1.4 s, D2 = 3.7 ms ($1/2^1J_{CH}$) and D3 = 55 ms (optimised for signals from protons with couplings to carbon of ca. 9 Hz). A 16-step phase cycle was used with no ^{13}C decoupling during acquisition of the proton signals. 128 Increments with 256 scans per FID were obtained. Other parameters were as for the HMQC experiment. The HMBC data were processed using non-shifted sine-bell squared functions in both dimensions. The F_1 data for both the HMQC and HMBC were zero-filled to 1K before transformation.

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