Determination, by NMR Spectroscopy, of the Structure of Ciceritol, a Pseudotrisaccharide Isolated from Lentils

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An unknown pseudotrisaccharide was isolated from *Lens culinaris* seeds. NMR spectroscopy of this substance established unambiguously its structure as α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-*chiro*-inositol. This compound has received the trivial name of ciceritol. It has been previously identified as manninotriose.

INTRODUCTION

α-D-Galactopyranosylcyclitols have been described in soybeans (Schweizer et al., 1978; Schweizer and Horman, 1981). The mass spectral analysis of the products obtained by hydrolysis and acetylation of trideuteromethylated derivatives was used to assign the structure of these compounds, namely 1D-2-*O*-(α-D-galactopyranosyl)-4-*O*-methylinositol, 1D-5-*O*-(α-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol, and 1D-2-*O*-(α-D-galactopyranosyl)-*chiro*-inositol. Since α- but not β-D-galactosidase hydrolyzed these compounds, it was assumed that α-linkages were present. The ^{13}C NMR spectra of these compounds were also assigned by analogy with published models.

More recently, Quemener and Brillouet (1983) described the isolation of a new pseudotrisaccharide from an aqueous ethanolic extract of chickpea by preparative PC. The new compound was named ciceritol (1) and assigned the structure of *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-1D-4-*O*-methyl-*chiro*-inositol, because the mass spectra fragmentation pattern of the product obtained by pertrideuteromethylation of ciceritol and subsequent hydrolysis followed by alditol acetate derivatization was similar to the one described earlier (Schweizer and Horman, 1981) for 1D-2-*O*-(α-D-galactopyranosyl)-4-*O*-methylinositol. Ciceritol was also detected (HPLC) in lentil, white lupin, bean, and soybean (Quemener and Brillouet, 1983). These authors also showed that manninotriose was absent in chickpea and the other legumes under study.

We have recently isolated a pseudotrisaccharide from *Lens culinaris* seeds. Considering that NMR spectroscopy can be successfully used to establish the structure and stereochemistry of oligosaccharides in an unambiguous manner, we decided to apply this methodology to obtain the full structure of this compound.

EXPERIMENTAL PROCEDURES

Apparatus. A Waters Associates liquid chromatograph equipped with a M-510 pump, a Model U6K injector, and a Model R-410 differential refractometer (Waters) was used. The chromatographic system was controlled by a PC Power Mate 1 (NEC) with a database Baseline 810 version 3.10 (Waters). A precolumn (3.2 mm i.d. × 4.0 cm) packed with phenyl-Corasil (Waters) was used. The chromatographic column was a 3.9 mm × 30 cm stainless steel μBondapack carbohydrate column (Waters), and the elution was carried out with 75:25 acetonitrile–Milli-Q water.

TLC Analysis. This was carried out by comparison of standards (glucose, fructose, sucrose, raffinose, stachyose, and verbascose) with lentil extract on silica gel (Merck) plates impregnated with 0.03 M boric acid. The monodimensional chromatography was carried out by eluting with 1-butanol–ethyl acetate–2-propanol–water (35:100:60:30). The sugars were identified by treating the plate with a solution of naphthoresorcinol (80 mg) in absolute ethanol (40 mL) and concentrated sulfuric acid (0.8 mL) and heating them in an oven at 110 °C (detection limit 5 μg).

Extraction of Lentil Seeds. Finely ground *L. culinaris* seeds (cv. Castellana) (200 g) were mixed with ethanol–water (8:2 v/v) (500 mL) for 1.5 h with stirring. This mixture was filtered through a sintered glass funnel, and the solution was evaporated under vacuum.

Chromatographic Separation. The residue obtained above was mixed with sea sand and a mixture of methanol–ammonia (9:1 v/v) until a fluid mass was obtained. This mass was transferred to the top of a glass column (3 cm i.d.) filled with silica gel G60 (100 g), and the column was eluted with the methanol–ammonia mixture. Fractions (40 mL) were collected, and their content was monitored by TLC. Those containing the unknown pseudotrisaccharide and raffinose were pooled together and evaporated under vacuum. The residue was dissolved in water, and the solution was freezed-dried. The unknown compound was finally separated from raffinose by flash chromatography using 40 μm of silica–amino stationary phase (Baker) and eluting with acetonitrile–water (75:25 v/v). The content of these fractions was monitored by TLC and HPLC as indicated above. Fractions containing the unknown compound were pooled together and evaporated under vacuum. Finally the residue (6 mg) was dissolved in D_2O and subjected to NMR spectroscopy using the conditions stated below.

NMR Spectroscopy. NMR spectra were recorded at 30 °C on a Varian Unity 500 spectrometer. Proton chemical shifts were referenced to residual HDO at δ 4.71. Carbon chemical shifts were referenced to external dioxane at δ 67.4.

The double quantum filtered DQF-COSY experiment (Piantini et al., 1982) was performed in the phase-sensitive mode using the method of States et al. (1982). A data matrix of 256 × 2K points was used to digitize a spectral width of 1500 Hz. Sixteen scans were used per increment with a relaxation delay of 2 s. The 90° pulse width was 7.5 μs. Prior to Fourier transformation, zero filling was used in F_1 to expand the data to 1K × 2K.

The triplet quantum filtered TQF-COSY experiment was collected in the phase-sensitive mode (Piantini et al., 1982). A data matrix of 256 × 1K points was used to digitize a spectral width of 1500 Hz. Twenty-four scans were used per increment with a relaxation delay of 2 s. Zero filling was used to expand the data to 1K × 2K.

The clean 2D-TOCSY experiment (Griesinger et al., 1988) was

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carried out in the phase-sensitive mode using MLEV-17 for isotropic mixing (Bax and Davis, 1985). The mixing time was set to 150 ms. A data matrix of 256 × 1K points was used to resolve a spectral width of 1500 Hz. Sixteen scans were used per increment with a relaxation delay of 2 s. The 90° pulse width during the mixing period was 22.5 μs. Squared cosine bell functions were applied in both dimensions, and zero filling was used to expand the dat to 2K × 2K.

The 2D rotating frame NOE (ROESY, CAMELSPIN) experiment was recorded in the phase-sensitive mode (Bothner-By et al., 1984). The spin-lock period consisted of a train of 30° pulses (2.5 μs), separated by delays of 50 μs (Kessler et al., 1987). The total mixing time was set to 300 ms. The radio frequency carrier was set 325 Hz downfield from the most deshielded anomeric proton to minimize spurious Hartmann–Hahn effects. A data matrix of 256 × 2K points was used to resolve a spectral width of 2500 Hz. Sixteen scans were used per increment with a relaxation delay of 2 s. Prior to Fourier transformation, squared sine bell functions shifted by $\pi/3$ were applied in both dimensions and zero filling was used in F_1 to expand the data to 2K × 2K.

The pure absorption one-bond proton–carbon correlation experiment was collected in the ^{1}H detection mode using the HMQC pulse sequence and a reverse probe (Bax and Subramanian, 1986). A data matrix of 256 × 2K points was used to resolve a spectral width of 1500 Hz. Sixteen scans were used per increment with a relaxation delay of 2 s and a delay corresponding to a J value of 145 Hz. A BIRD pulse was used to minimize the proton signals bonded to ^{12}C. ^{13}C decoupling was achieved by the WALTZ scheme. Prior to Fourier transformation, squared cosine bell functions were applied in both dimensions and zero filling was used in F_1 to expand the data to 2K × 2K.

The multiple-bond proton–carbon correlation experiment was carried out in the ^{1}H detection mode using the HMBC pulse sequence and a reverse probe (Bax and Summers, 1986). A data matrix of 256 × 2K points was used to digitize a spectral width of 1500 Hz. Sixty-four scans were used per increment with a relaxation delay of 2 s and a pulse delay of 60 ms. The processing was performed in the absolute mode after zero filling in F_1 to a 2K × 2K data matrix.

RESULTS AND DISCUSSION

The high-resolution ^{1}H NMR spectrum and proton-decoupled ^{13}C NMR spectra of 1 in D_2O solution showed 2 anomeric signals with the same relative intensity, but 15 signals were observed in the ^{13}C NMR spectrum, all of them in the region between δ 60 and 100, with several resonances presenting double or multiple intensity. The peaks at δ 99.1 and 96.1 indicated the presence of 1,2-cis pyranoside type of linkages, in agreement with the values of $J_{1,2}$ measured in the proton spectrum (ca. 4.0 Hz). The resolution of the ^{1}H NMR spectrum at 30 °C was good enough to allow DQF-COSY, TQF-COSY, and TOCSY experiments to be performed in a satisfactory way. Almost all of the resonances in the proton spectrum were assigned by combining these techniques. The proton chemical shifts are listed in Table I. TQF-COSY permitted us to assign H-5 and the methylene protons of the two different hexopyranose moieties. The TOCSY experiment indicated the presence of three different residues, one lacking anomeric signals, suggesting the presence of a cyclitol ring. The two hexopyranose residues were labeled A and B, while the cyclitol ring was named C. The TOCSY subspectra through the anomeric signals of A and B residues showed clear connectivities to H-2, H-3, and H-4 of both pyranoid rings. Coherence transfer from H-4 to H-5 and further was not observed, indicating a rather small value for both $J_{4,5}$ coupling constants. From the highest field signal in the proton spectrum (δ 3.37) it was possible to ascertain the position of the protons belonging to the third spin network C. The large values of both $^3J_{H,H}$ couplings for this signal (ca. 9.5 Hz) indicate a double trans diaxial arrangement with its neighbors.

Table I. ^{1}H NMR Chemical Shifts (δ) and $^3J_{H,H}$ (Hz) Coupling Constants for 1 in D_2O Solution at 30 °C

	residue		
proton	A	B	C
H-1	4.96	5.12	4.20
H-2	3.80	3.85	3.85
H-3	3.88	3.95	3.80
H-4	3.94	4.04	3.37
H-5	3.98	4.39	3.83
H-6a	3.72	3.67	4.03
H-6b	3.72	3.87	
CH_3			3.60
$J_{1,2}$	4.0	4.0	3.5
$J_{2,3}$	>7.0	>7.0	>4.0
$J_{3,4}$	nd[a]	nd	9.5
$J_{4,5}$	<2.0	<2.0	9.5
$J_{5,6a}$	nd	4.5	<5.0
$J_{5,6b}$	nd	8.0	
$J_{6a3,6b}$	nd	−11.0	

[a] nd, not determined.

Table II. ^{13}C NMR Chemical Shifts (δ) for 1 in D_2O Solution at 30 °C

	residue		
carbon	A	B	C
C-1	99.1	96.1	68.2
C-2	69.2	69.2	76.1
C-3	70.2	70.2	71.6
C-4	70.2	70.2	83.7
C-5	71.8	70.1	70.7
C-6	62.1	67.8	71.9
CH_3			60.6

It was possible to identify and to assign the rest of both the proton and carbon resonances by using an HMQC experiment, which maps the connectivities between carbon atoms and their directly bonded protons. The assignment of most of the signals in the ^{13}C NMR spectrum was straightforward, since almost all of the ^{1}H NMR chemical shifts were already known. Carbon chemical shifts are gathered in Table II. Thus, C-6 of residue B appeared at low field (δ 67.8) indicating substitution at that oxygen. An *O*-methyl signal that had been previously guessed from the proton spectra was also detected. The carbon atom coupled to the proton at δ 3.37 was also strongly deshielded, indicating *O*-substitution at that position. The carbon chemical shifts along with the previously discussed proton ones allowed us to conclude that residue A is an unsubstituted α-galactopyranosyl residue, while B is a 6-O-substituted α-galactopyranosyl moiety.

Some information on the glycosyl sequence was obtained from a 2D-CAMELSPIN (ROESY) experiment which shows cross-peaks due to dipolar coupling (distance smaller than ca. 3.5 Å). Thus, the anomeric proton of residue A showed NOE to both H-6's of galactose B, while H-1 of this ring is in close proximity to the protons at δ 4.20 and 3.85 of the cyclitol C. On the other hand, the signal corresponding to the methyl group showed a cross-peak with the proton at δ 3.37 of residue C. Since NOE cross-peaks are dependent on the conformation around the glycosidic bonds, the existence of these cross-peaks does not guarantee the exact position of the linkages.

These positions were unambiguously identified by using an HMBC experiment which shows long-range connectivities between carbon atoms and their coupled protons through two or three bonds. Using this technique, couplings across the glycosidic linkages can be unequivocally detected. Thus, cross-peaks between H-1 of galactose A and C-6 of ring B and between H-1 of ring B and the carbon signal of residue C at δ 76.1 were observed.

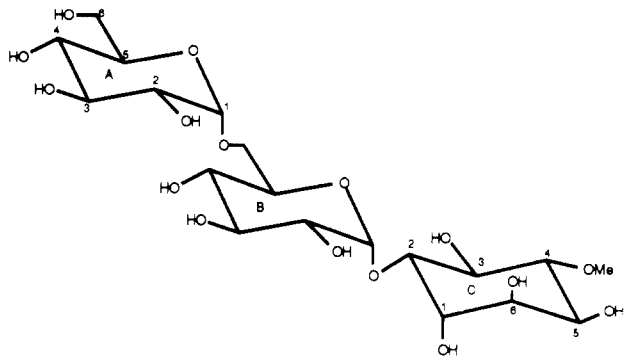

Figure 1. Structure of ciceritol.

Moreover, a correlation between the highest field proton at δ 3.37 and the carbon methyl group was deduced. Several intraresidue connectivities were also detected. Therefore, at this point, it can be concluded that the two galactopyranose rings are connected via a α-(1→6) type of linkage and that galactose B is also α-linked to a cyclitol residue. The arrangement of the groups in this moiety came from the analysis of the data mentioned above along with the values of the vicinal proton coupling constants.

The *O*-methyl group is at an equatorial position which is flanked by two unsubstituted equatorial oxygens (from *J* values, ca. 9.5 Hz, see above). Galactopyranose B is connected to a position which bears a proton (δ 3.85) coupled to the lowest field proton of this ring (δ 4.20). This proton shows two small coupling constants (ca. 3.5 Hz), the second one with a proton at δ 4.03, which is in turn coupled to the proton at δ 3.83 with a $^3J_{H,H} < 5$ Hz. This proton is coupled to the one at the *O*-methyl-substituted carbon. Thus, the circle is completed, and the stereochemistry of the protons starting from the *O*-methyl-substituted carbon is H_{ax} (*O*-methyl)-H_{ax}(OH)-H_{eq}(OH)-H_{eq}(OH)-H_{ax}(*O*-galactose)-H_{ax}(OH). This stereochemistry corresponds to a 2-O-substituted D-pinitol residue. The ^{13}C and ^1H NMR chemical shift values are fairly consistent with those previously reported for the pinitol (1D-4-*O*-methyl-*chiro*-inositol) moiety.

According to these results, the primary structure proposed for the pseudotrisaccharide is α-D-Galp-(1→6)-α-D-Galp-(1→2)-D-*chiro*-pinitol (see Figure 1). The absolute configuration of the cyclitol ring may be guessed from the chemical shift of C-1B. If it is assumed that this chemical shift depends on, among other factors, the stereochemistry around the glycosidic linkage (Baumann et al., 1988), δ C1-B for the α-D-Galp-(1→2)-D-pinitol fragment should be similar to that of an α-D-Galp-(1→3)-α-D-Galp moiety (ca. δ 96), while δ C-1B for a putative α-D-Galp-(1→2)-L-pinitol linkage could be correlated with an α-D-Galp-(1→3)-α-D-Manp block (ca. δ 102) (Baumann et al., 1988). The observed chemical shift for C-1B (δ 96.1) (see Table II) agrees then with the presence of a D-pinitol ring. Additional evidence for this absolute configuration is provided by the NOE cross-peaks for H-1B (see above), which are similar to those for α-D-Galp-(1→3)-α-D-Galp-type linkages (Baumann et al., 1988). Only derivatives of 1-D-*chiro*-inositol have been isolated from legumes.

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