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Separation and NMR structural characterisation of singly branched α-dextrins which differ in the location of the branch point

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Abstract

A series of singly branched α-dextrins with dp ≥ 5 was prepared by treating amylopectin with an α-amylase (Termamyl) and then with β-amylase. The dextrins were separated by gel filtration chromatography and the composition of the fractions was checked by HPAEC. Each fraction gave essentially a single peak by HPAEC but NMR spectroscopy indicated that only the fraction corresponding to dp5 consisted of one compound. The fractions corresponding to dp6, 7 and 8 were found to be mixtures of isomers. Three isomers of dp6 were separated by reversed phase HPLC and were characterised by NMR spectroscopy. The isomers were identified as 6^2-α-maltotriosyl-maltotriose, 6^3-α-maltosyl-maltotetraose and 6^2-α-maltosyl-maltotetraose. ^{1}H and ^{13}C NMR parameters of these compounds and of dp5, 6^2-α-maltosyl-maltotriose, are discussed in relation to the linkage pattern of the different glucose units and compared with similar parameters in the linear maltodextrins.

Keywords: α-Dextrins; structure and NMR; Dextrins, branched; Malto-oligosaccharides; α-Amylase, limit dextrins; Amylopectin, limit dextrins

1. Introduction

The preparation and structural characterisation of α-dextrins is desirable for several reasons. For different α-amylases, the dextrin structures reveal the restrictions imposed on hydrolysis of α-(1→4) linkages by a neighbouring α-(1→6) linkage. The dextrins may also be valuable substrates or inhibitors for the testing of newly discovered or engineered starch degrading enzymes, or provide a starting point for the synthesis of such molecules. Larger, multiply branched dextrins can be a source of information on the distribution of branch points in the original biopolymer, and can potentially be used to compare structures of amylopectins and glycogens from a variety of sources.

Many previous studies have succeeded in identifying the smallest limit dextrins produced from

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Abbreviations: BSA, bacterial saccharifying α-amylase; dp, degree of polymerisation; DQF-COSY, double quantum filtered correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum correlation; HPAEC, high performance anion exchange chromatography; PAD, pulsed amperometric detection; RELAY, relayed correlation spectroscopy; RPC, reversed phase chromatography; t, terminal; TOCSY, total correlation spectroscopy.

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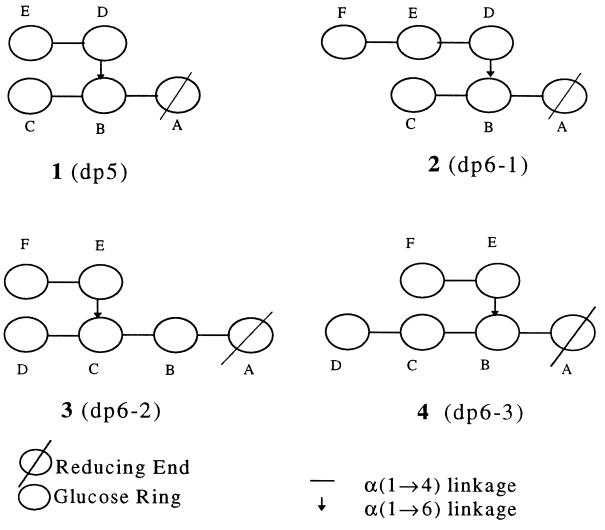
amylopectin by action of individual mammalian, plant, fungal or bacterial α-amylases. One group of α-amylases (which includes those from human saliva [1], porcine pancreas [2], malted barley [3] and *A. oryzae* [2]) has been found to produce 6^3-α-glucosyl-maltotriose as the smallest dextrin; the second group (including the α-amylase from *B. subtilis* [4], and the commercial product, Termamyl, from *B. lichenformis* [5,6]) produces 6^2-α-maltosyl-maltotriose.

Detailed preparative and structural studies of α-dextrins with dp >5 are much less numerous. Umeki and Yamamoto deduced the structures of singly [7,8] and multiply [9,10] branched α-dextrins with dp >5 using an elegant but complex sequence of enzymic degradations (involving pullulanase, β-amylase, isopullulanase, glucoamylase and saccharifying α-amylase from *B. subtilis*). They analysed both the α-dextrins and the degradation products by paper chromatography. A major complicating factor identified by them was the inability of chromatographic methods to separate α-dextrin isomers with the same number of branch points. Abdullah et al. [11] identified a number of singly branched dextrins with dp ≥ 5 produced by action of salivary α-amylase on amylopectin.

In this paper we report the application of modern methods of carbohydrate analysis, principally reversed phase HPLC, high performance anion-exchange chromatography with pulsed amperometric detection (HPAE–PAD) and two-dimensional NMR spectroscopy, to the separation and structure determination of singly branched α-dextrins of dp5 and dp6.



HPAE-PAD chromatography is now a well established method for the analysis of linear malto-oligosaccharides, and is routinely used to determine chain length distributions of oligosaccharides released by enzymic debranching of amylopectins [12] and glycogens [13]. Recent attempts to extend the method to branched malto-oligosaccharides [14] have illustrated the need for unambiguously characterised reference standards for use with HPAE–PAD. In the present investigation we found that HPAE chromatography readily resolved branched and linear dextrins with the same dp, but it failed to separate singly branched isomers of a given dp. NMR spectroscopy indicated that fractions of singly branched dp6, dp7 and dp8 contained mixtures of isomers, but the spectra were too complex for a full interpretation. We have found, however, that reversed phase HPLC [15] may be used for the separation of individual isomers, and that their structures may be established by NMR methods. The procedure is reported here for the case of dp6 together with the determination of the ^{1}H and ^{13}C chemical shifts for each of the isomers by HMQC–TOCSY, HMBC and other 2D NMR experiments.

2. Experimental

Materials.—Amylopectin from corn (A7780) was purchased from Sigma Chemical Co., St. Louis, MO as was the enzyme β-amylase (EC 3.2.1.2. from sweet potato, 21,600 u/mL). The α-amylase Termamyl 120 L (EC 3.2.1.1, from *B. lichenformis*) was kindly given by Novo Nordisk.-Pullulanase (EC 3.2.1.41, from *Klebsiella pneumoniae*, 40 u/mg) was obtained from Megazyme. Saccharifying α-amylase, BSA, (from *B. subtilis*, 20,000 u/g) was a gift of Nagase Biochemicals Ltd (Japan). Enzymes were used without further purification. Enzyme activity units are as defined by the suppliers. All the other chemicals were analytical-grade commercial products.

Gel filtration chromatography.—The Bio-Gel P-2 column (90×5.2 cm) was operated at room temperature using 0.1 M pyridine acetate buffer, pH 3.5, as eluent.

HPAE–PAD chromatography.—The HPAEC was performed on a Dionex chromatograph equipped with a gradient pump system, an eluent degas module, a pulsed amperometric detector (PAD), a Carbopac PA-1 column (250×4 mm) and

a Carbopac PA-1 guard precolumn (25×4 mm). The temperature of the column was controlled at 32 °C using a column block heater and the results were analysed using Gilson 715 HPLC software. The sample injection was 20 μL. Eluent A was 100 mM sodium hydroxide and eluent B 100 mM sodium hydroxide solution containing 600 mM sodium acetate and the flow rate was 1 mL/min. The eluents were degassed with helium gas. The elution programme was as follows: 0–1 min, 92% A, 8% B; linear gradient from 1 to 42 min ending at 60% A, 40% B; return to 92% A, 8% B by 42.1 min; hold until 50 min.

Reversed phase C18 chromatography.—The HPLC system consisted of a Spectra Physics SP8700 gradient pump, rheodyne 7125 injector, Gilson 132 RI detector with a Spherisorb ODS2 column (25 cm×4.6 mm), 5 μm silica C18. The temperature was stabilised at 5 °C using a water jacket. The chromatograms were recorded on a Spectra Physics SP4600 integrator. The sample injection was 200 μL. The solvent used was purified water (Elga Maxima ≥ 18MΩ) at a flow rate of 1 mL/min.

Enzymic preparation of singly-branched glucose oligosaccharides.—The following is a modified version of the procedure given by Motawia et al. [6] for the preparation of 6^2-α-maltosyl-maltotriose (dp5). Amylopectin was treated twice with α-amylase (Termamyl) as described and applied to the Bio-Gel P-2 column (operated at room temperature instead of 60 °C). Peaks corresponding to dp >4 (comparison with linear standards) were collected, combined and then treated with β-amylase as described. The digest was applied to the P-2 column, fractions with mobility corresponding to dp5–8 were collected separately and further purified by applying each one again to the P-2 column. Each fraction was analysed by HPAEC as described for dp5 [6]. Then the dp6 mixture was freeze-dried, dissolved in distilled water (within the concentration range 0.5 to 5 mg/mL) and applied to the reversed phase column. The four peaks were collected separately. The first and fourth peaks provided samples 6-1 and 6-3, respectively, for NMR and enzymic analysis. Sample 6-2 was prepared by combining the second and third peaks after their repeated application to the column had allowed progressive removal of residual first and fourth peaks (see Results). Small portions of the mixture and the purified samples were also borohydride reduced and applied to the column for analysis.

Enzymic analysis using pullulanase and BSA.—The singly-branched oligosaccharides were investigated using the debranching enzyme, pullulanase, and the saccharifying α-amylase, BSA. Samples of dextrins (dp5, dp6 mixture and purified 6-1, 6-2 and 6-3 isomers) were dissolved in water (200 μL) to give a concentration of 1–3 mg/mL. 50 μL acetate buffer, pH 5.6, and 2 μL of pullulanase solution were added to each sample and the solutions were incubated for 24 h at 40 °C. Solutions of the same dextrins (200 μL, concentration 1–3 mg/mL) were treated with BSA. 50 μL acetate buffer, pH 5.4, and 200 μL of BSA solution (concentration 9 mg/mL or 180 u/mL) were added to each sample and the solutions were incubated for 24 h at 40 °C. All the digested samples were filtered through a 0.45 μm filter and diluted to ~0.1 mg/mL for HPAEC.

NMR Spectroscopy.—Spectra were obtained on a JEOL GX400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C or on a Bruker ARX500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts were expressed in ppm from TMS using acetone (2.217 ppm) as internal reference for ^{1}H. The ^{13}C resonances were measured via an external test sample containing D_2O and dioxan (67.4 ppm relative to TMS). A combination of 1D and 2D NMR experiments [16], the latter including DQF–COSY, Double RELAY, TOCSY, C/H shift correlation, HMQC, HMQC–TOCSY and HMBC was needed to characterise each sample. "Inverse" experiments were carried out on the 500 MHz spectrometer, other experiments at 400 MHz. Data processing was performed on a Silicon Graphics workstation using the FELIX 95 software (MSI). Samples (1–4 mg for the single isomers, 10–20 mg for mixtures) were dissolved in 0.6 ml D_2O, placed in 5 mm o.d. NMR tubes, and the temperature was regulated at 27 °C for all experiments.

For the DQF–COSY, Double RELAY and TOCSY experiments, the spectral width was 1250 Hz and matrix size 256(t_1)×2048(t_2). For the C/H correlation experiments, the spectral widths were 5555 Hz (^{13}C) and 1250 Hz (^{1}H) with 4096 points (t_2) and 32 or 64 points (t_1) depending upon the amount of sample and number of scans required at each t_1 value. For the HMQC, HMQC–TOCSY and HMBC experiments the spectral widths were 2264 and 7576 Hz for ^{1}H and

^{13}C, respectively, with typical matrix size 1024(t_2)× 720(t_1). The HMBC experiments were recorded in absolute value mode using the basic 4-pulse sequence with water suppression and a delay of 70 ms between the first two pulses. HMQC and HMQC–TOCSY experiments were carried out in phase sensitive mode with a spin-lock time of 100 ms for HMQC–TOCSY. Appropriate zero filling (in t_1) was employed for all 2D Fourier transformations.

3. Results

Enzymic preparation, chromatography and enzymic analysis of branched dextrins.—Preparation of branched dextrins from amylopectin was based upon the enzymic method of Motawia et al. [6] for preparation of 6^2-α-maltosyl-maltotriose. After enzyme treatment, fractions with dp4–15 from gel filtration chromatography were combined and then treated with β-amylase. This treatment ensured that the products eventually collected would contain no more than two main chain glucose units to the non-reducing side of the (1,4,6)-linked branch point, and not more than three glucose units in the side chain. The digest was applied to a gel filtration column and the fractions of nominal dp5,6,7 and 8 were collected separately.

The fractions were analysed by HPAEC, as shown for dp5 and dp6 in Fig. 1(a), and each was found to give essentially a single peak. Retention times for dp5–8 and for linear maltodextrin standards of comparable dp under the same conditions are given in Table 1. In accordance with other authors [14] it was found that, within the range investigated, the presence of a branch point reduced the retention time such that the peak for a branched dextrin of dp*n* was intermediate between those for linear dextrins of dp($n-1$) and dp*n*. NMR results (see below) confirmed that the oligosaccharides in each fraction had the expected number of glucose units and that dp5 was a single compound. The NMR experiments also showed that the fractions labelled dp6, 7 and 8 were mixtures of isomers. The remainder of this paper deals with characterisation of dp5 and 6. Characterisation of dp7 and 8 will be described elsewhere.

Structural analysis of branched dextrins has conventionally relied upon enzymic hydrolysis to give mixtures of linear and simpler branched dextrins. HPAEC was used here to analyse mixtures of hydrolysis products formed in this way. Response

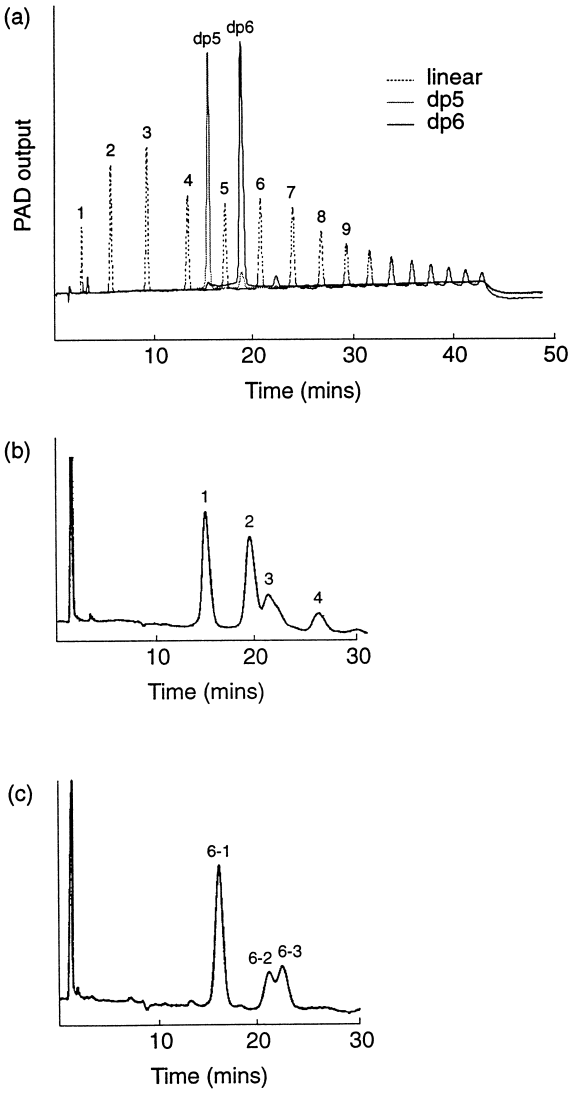


Fig. 1. (a) HPAE–PAD chromatograms of three samples: (- - -) mixture of linear maltodextrin standards, number indicates dp; (····) singly branched dp5 (main peak, minor peak due to singly branched dp6); (——) mixture of singly branched dp6 compounds (major peak, minor peaks due to singly branched dp5 and dp7). (b) Separation of components in the untreated dp6 mixture by RP HPLC (Spherisorb ODS2). (c) Separation of components in same dp6 mixture after borohydride reduction (also Spherisorb ODS2). See text for relationship between peaks in (b) and (c).

factors for HPAEC were calculated using solutions containing known amounts of maltose, maltotriose and maltotetraose. Treatment of dp5 with the debranching enzyme, pullulanase, gave an equimolar mixture of maltose and maltotriose whereas similar treatment of dp6 gave maltose, maltotriose and maltotetraose in a molar ratio of 1.1: 2.7: 1.0. The latter result implies that dp6 contained a mixture of two groups of isomers, the first (56% of the total) with three glucose units in both main and side chains and the second (44%) with four

Table 1
HPAEC–PAD retention times of linear and singly branched dextrins

dp	Linear (min)	Branched (min)
2	5.7	
3	9.3	
4	13.4	10.9 [a]
5	17.1	15.4
6	20.6	18.7
7	23.9	22.2
8	26.8	25.2
9	29.3	

[a] 6^3-glucosyl-maltotriose.

(main chain) and two (side chain) units or vice–versa. The number of possible dp6 isomers is, however, reduced as a result of the β-amylase treatment. A more complicated sequence of enzymic degradations would be required to determine the exact composition of the mixture and the location of the branch point in each of the compounds.

In principle the same information could be obtained by NMR spectroscopy but in practice a full interpretation of the NMR spectra of the dp6 mixture could not be achieved. Reversed phase chromatography of the mixture with water as eluent proved to be an effective method for separation of the isomers on both analytical and preparative scales. The method was previously used by Abe et al. [15] for separation of an isomeric mixture of α-(1→6)-linked dimers of maltotriose (in borohydride-reduced form). Chromatography of the dp6 mixture in its original non-reduced form gave four main peaks [Fig. 1(b)] but only three peaks after borohydride reduction [Fig. 1(c)]. The difference arises because RPC gives separation of α- and β-anomers for the non-reduced form. The three peaks obtained after reduction were labelled 6-1, 6-2 and 6-3. Slightly better resolution could be obtained for both reduced and non-reduced samples by running the column at 5 °C rather than at room temperature.

The composition of the peaks in Fig. 1(b) was then determined and the correspondence with Fig. 1(c) was established. Following collection of fractions 1–4 a small portion of each fraction was reduced. Each fraction was run again (both non-reduced and reduced forms) on the column. After reduction fractions 1 and 4 gave single peaks corresponding to 6-1 and 6-3, respectively. Without reduction (and following anomerisation) fractions 1 and 4 gave two peaks each, with retention times equal to those of peaks 1 and 2 (fraction 1) and peaks 3 and 4 (fraction 4). Fractions 2 and 3 gave more complicated chromatograms which indicated the presence of all three compounds. With repeated passes, after allowing time for anomerisation to occur (4–5 h), it was possible to deplete the amounts of 6-1 and 6-3 via removal of fractions 1 and 4 and eventually to combine fractions 2 and 3 to give purified 6-2. From these results it was concluded that the four peaks in Fig. 1(b) corresponded to: peak 1 (6-1β), peak 2 (6-1α and 6-2β), peak 3 (6-2α and 6-3β), peak 4 (6-3α). It can be seen from Fig. 1(b) that the difference in retention times for the anomeric forms was similar for 6-1 and 6-3 but was much less for 6-2. The structures determined below suggest an explanation: the branch point and the reducing end are adjacent in compounds 6-1 and 6-3 but are separated by another glucose unit in 6-2.

Portions of the isolated fractions were hydrolysed with pullulanase and with BSA and the products identified by HPAEC retention times. Treatment of 6-1 with pullulanase gave only maltotriose whereas 6-2 and 6-3 gave maltose and maltotetraose. BSA is an enzyme which can attack the α-(1→4) linkages, in either the main-chain or the side-chain, that are closest to the branch point on its non-reducing side; to the reducing-end side, however, it does not attack the two α-(1→4) linkages adjacent to the branch point [9]. Under the conditions used 6-1and 6-3 were completely degraded and both gave maltose plus a branched tetraose (retention time intermediate between maltotriose and maltotetraose) as major products. 6-2 was only partly degraded and the major products were glucose and a branched pentaose. These results are consistent with the structures shown below for 6-1, 6-2 and 6-3, provided that the two branched dp4 dextrins from 6-1 and 6-3 are not the same. HPAEC was not able to distinguish them but Umeki and Yamamoto [7] have described a further enzymic analysis of the hydrolysis products which showed that in one case (corresponding to 6-1 in our nomenclature) the maltose was cleaved from the side-chain, whereas in the other case (6-3) it was cleaved from the main-chain, giving two different dp4 products. Rather than pursue this route we have obtained proof of the structures from NMR spectroscopy, as described in the following sections.

^1H NMR spectra.—Individual glucose units are coded **A**, **B**, **C**... starting from the reducing end and continuing first along the main chain and then along the side chain, as shown. It is clear from the

context whether **B**1, **C**2 etc., refers to a hydrogen or carbon atom. Normal type (H-1, C-4 etc.) is used to denote hydrogens or carbons when no specific unit is cited, and H-1(→4), H-1(→6) when it is important to specify whether an anomeric proton is involved in an α-(1→4) or an α-(1→6) linkage.

Figure 2 shows the anomeric region (except for **A**β1 reducing end signals at δ 4.64) of the ^{1}H NMR spectra of dp5 and the three dp6 isomers. The number of glucose units in each molecule was established by integration of the summed **A**α1 and **A**β1 reducing end signals (total 1 unit) relative to the remaining anomeric signals. It is known from previous work [17] that anomeric doublets with chemical shifts between 5.31 and 5.40 ppm are from H-1 protons involved in α-(1→4) glycosidic linkages whilst the doublets at 4.97 ppm are from H-1 protons in α-(1→6) linkages. The integral of the latter doublet in each spectrum showed that there was just one α-(1→6) linkage in each molecule.

Numerous possible structures can be proposed for dextrins of dp5 and dp6 with a single branch point. For example, Bock [5] considered 10 such structures for dp5 and Umeki and Yamamoto [7] considered nine structures of dp6 dextrins susceptible to pullulanase hydrolysis (i.e., with at least two glucose units in the side-chain). In order to determine the structures and, in particular, the location of the branch points by NMR it was necessary first to assign the signals to the correct sugar units, and then to use signal connectivity between units to establish the sequence. Correct assignment of the signals from protons (or carbons) involved in linkages is especially important, since the oligosaccharides contain only glucose and the chemical shift differences are rather small.

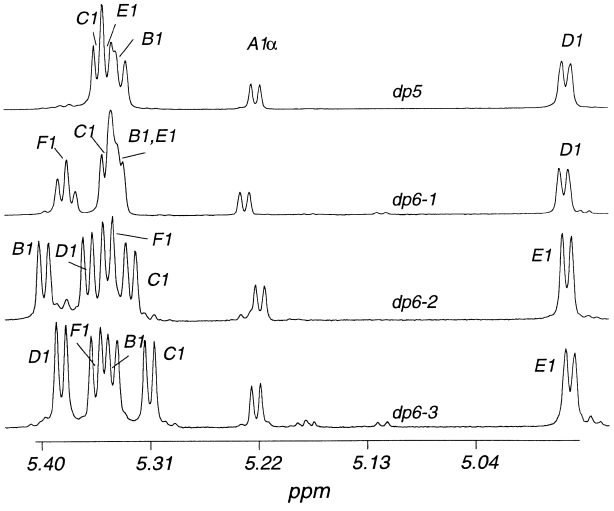


Fig. 2. 400 MHz ^{1}H NMR spectra (anomeric region, except β-reducing end) of dp5 and separated dp6 compounds. See displayed structures for key to spectrum labelling.

The number of different H-1(→4) protons was obvious in dp6-2 and dp6-3, where all four doublets were resolved (Fig. 2), but less so in dp5 and dp6-1. The position was made clearer by examination of the COSY spectra in Fig. 3. These show the region of the H-1/H-2 cross-peaks for the H-1(→4) protons with the **A**α1/**A**α2 cross-peak at the top right of each panel. Among glucose units with an α-(1→4) linkage, four different types of unit can be distinguished in dextrins, each with its characteristic set of ^{1}H and ^{13}C NMR parameters. These are terminal (t), (1,4)-, (1,4,6)-, and (1,6)-linked units. If the full set of ^{1}H and ^{13}C chemical shifts can be obtained for a ring, then the ring may easily be classified as one of the above types. The H-1 chemical shifts of these units may overlap, as in dp6-1, but it was found that the H-2 shifts in all compounds followed a regular pattern, δ(H-2) [t < (1,6) < (1,4) < (1,4,6)], which helped to resolve and identify the signals in 2D spectra. The behaviour is illustrated in Fig. 3 for three of the four linkage patterns [the (1,6)-linked unit was not present in any of the dextrins discussed here, although it is a common feature of other dextrin structures, to be discussed elsewhere].

Further proton assignments were obtained by using standard 2D techniques. The chemical shift of H-3 for all rings was obtainable from the COSY spectrum, but the close similarity of H-4 chemical shifts in the different types of 4-linked unit made a complete assignment of H-4 impossible by this route. Terminal units (δ H-4∼3.40) were, however readily distinguished from 4-linked ones (δ H-4∼3.64). Assignment of H-4, H-5 and H-6 by correlation to H-1 signals in double RELAY and TOCSY experiments was straightforward for those rings with well resolved H-1 resonances, i.e., the two reducing end units and the unit with H-1 at δ 4.97 in an α-(1→6) linkage. For other rings, with closely overlapping H-1 resonances, it was found that proton correlation experiments alone were not enough to provide a complete assignment. It was necessary to consider the ^{13}C spectrum and to use the different methods (via one bond or multiple bond coupling) available for carbon/hydrogen shift correlation.

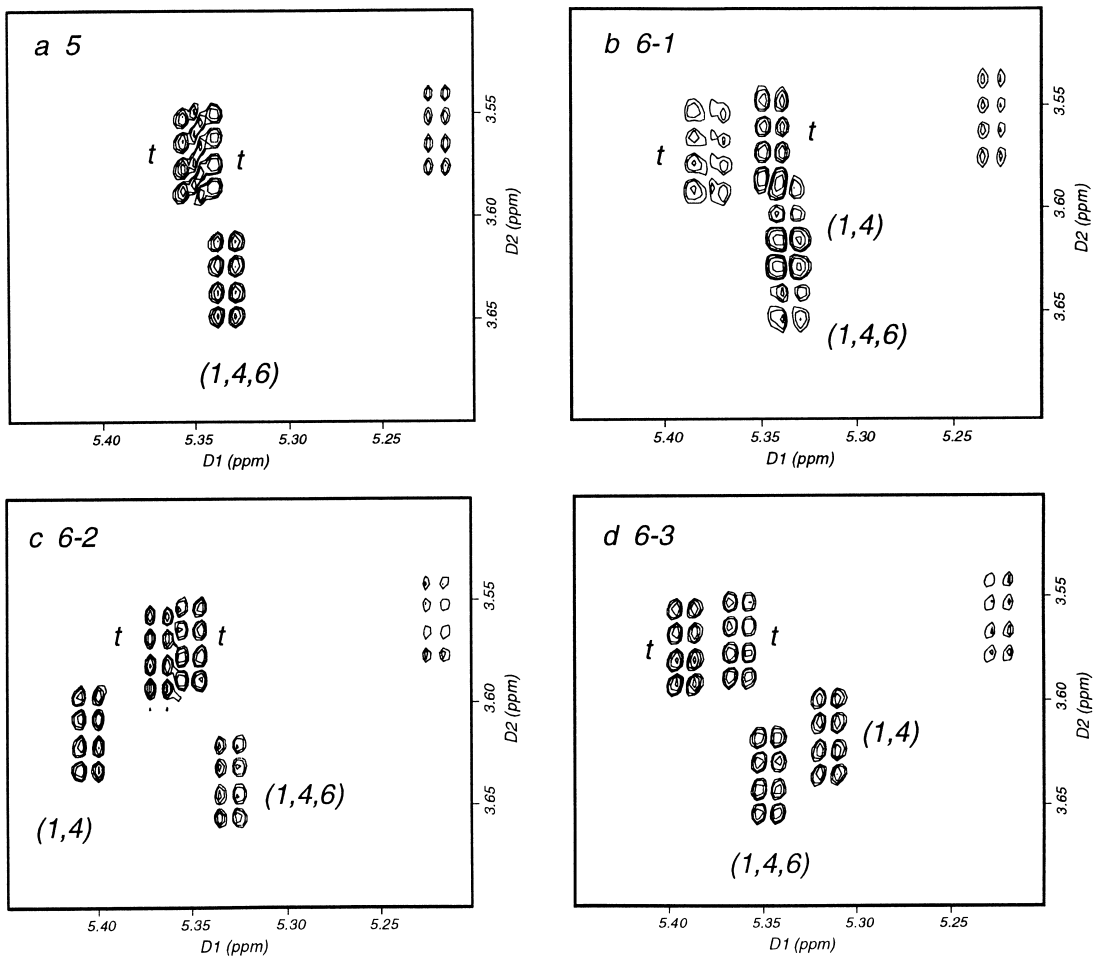


Fig. 3. 400 MHz ^{1}H DQF–COSY spectra of (a) dp5 and (b,c,d) dp6 compounds: H-1/H-2 cross peak region for H-1(→4) protons and the α-reducing end (top right in each panel). H-1 chemical shifts on D1, H-2 chemical shifts on D2 axis. Each cross peak gives a basic 2×4 multiplet pattern although overlaps may modify the appearance. Labelling indicates the origin of each cross peak and shows correlation between H-2 chemical shift and Glc ring type.

^{13}C NMR spectra, HMQC–TOCSY experiments and dp5.—The ^{13}C spectra of the dextrins are shown in Fig. 4. All signals are displayed with the exception of C-1 for the two reducing end units at δ 92.7 (α) and δ 96.6 (β), and C-6 (of rings with CH_2OH groups) at δ∼62. Studies of related disaccharides [18,19] and smaller dextrins [20,21] allowed several distinctive individual signals or signal groups to be recognised. These include C-1(→4) at δ∼100.7; C-1(→6) at δ∼99.4; C-4 of 4-linked rings centred at δ∼78.5; C-4 of terminal groups at δ∼70.1; C-6 of 6-linked rings at δ∼68. Integration of the region at δ∼68 in the ^{13}C spectrum of the original mixture allowed the relative amounts of the three dp6 isomers to be determined: 6-1 (49%), 6-2 (24%), 6-3 (27%).

^{13}C assignments were obtained from ^{13}C detected C/H correlation experiments (when sufficient material was available) or from HMQC and HMQC–TOCSY experiments with ^{1}H detection. The last experiment proved particularly convenient (as long as the H-1 doublets were resolved) since by taking an appropriate cross-section through the 2D spectrum, it was possible to correlate all the ^{13}C resonances of a particular ring with the H-1 signal of the same ring. This was useful, for example, in the case of the C-4 resonances at δ∼78.5. Although these resonances were reasonably well resolved in the ^{13}C spectra (Fig. 4) they were very difficult to assign by C/H or HMQC correlation since all the directly attached H-4 hydrogens had practically the same chemical shift. The problem was avoided by using the longer range correlations (e.g., C-4–H-1) provided by the HMQC–TOCSY experiments. Thus it was possible to assemble a near complete set of assignments for each ring and, using the

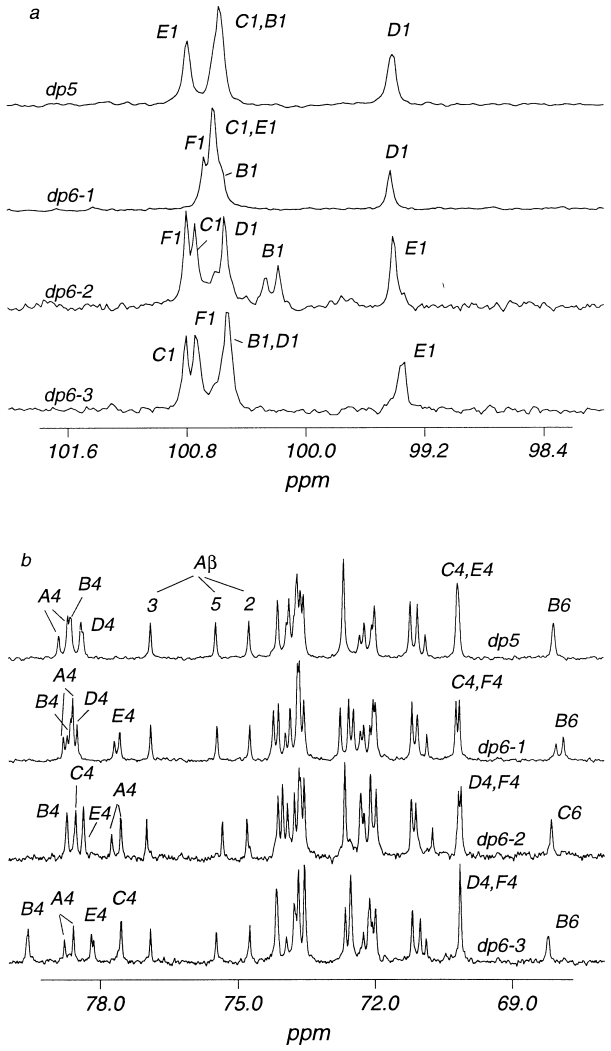


Fig. 4. 100 MHz ^{13}C NMR spectra of dp5 and dp6 compounds: (a) anomeric region, except reducing end C-1; (b) main carbohydrate region, except C-6 (CH_2OH). See displayed structures for key to spectrum labelling.

various characteristic H-1, C-1, C-4 and C-6 chemical shifts mentioned above, to allocate each set to its appropriate ring type: 4-linked reducing end (α and β); (1,4,6→ 4); (1,4→6); (t→4); (1,4→4). It was found that all the compounds possessed a single (1,4,6→4) ring, and furthermore, that none of them had a (4,6)-linked reducing end, (1,6→4) or (t→6) ring. In other words, the side-chain was not linked to either the reducing or non-reducing ends of the main chain and no glucosyl stubs were present. The only structure of dp5 to satisfy these requirements is that of 6^2-α-maltosyl-maltotriose, **1**, which has been characterised previously by enzymic [4] and NMR [5] methods. The NMR parameters of the two (t→4) units were distinguished by an HMBC experiment, described below.

HMBC experiments: sequence determination.—To determine the structures of the dp6 isomers it was necessary to establish the sequence of the glucose units in each isomer. One of the conventional NMR approaches, observation of the NOE between protons across a glycosidic linkage, was not applicable to these compounds. This was because each isomer contained several α-(1→4) linkages and there was close overlap between H-4 chemical shifts in the different 4-linked rings, as mentioned above. However, taking advantage of the greater spread of C-4 shifts, the linkages were determined from long range H/C chemical shift correlations in HMBC experiments, using the coupling ^3J(H-1-C-4) across the glycosidic linkage. Results for all four compounds are shown in Fig. 5. The cross-peaks of interest for sequence determination, correlating H-1 and C-4 chemical shifts in *different* rings, are located in the lower half of each panel and have been labelled. The anomeric region is the same as that displayed in the COSY spectra of Fig. 3. The HMBC spectra may also be compared with the ^1H spectra of Fig. 3 (region centred on δ 5.35) and the ^{13}C spectra of Fig. 4 (region centred on δ 78). The upper half of each panel contains cross-peaks arising from two- and three-bond J(HC) couplings between H-1 and C-3, C-5 and (at a much lower contour level) C-2 of the *same* ring. Thus the upper half provided, in part, the same information as the HMQC-TOCSY experiment and was useful in confirming the assignments.

In **1** the only NMR parameters to distinguish the main chain and side chain terminal residues were the ^1H and ^{13}C shifts of the anomeric signals. The C-4 resonances of the three 4-linked residues in **1** could be assigned by HMQC–TOCSY since the three residues have different linkage patterns. The H-1/C-4 cross-peaks in the HMBC experiment [Fig. 5(a)] then allowed the order of H-1(→4) chemical shifts to be established as α(H-1) [**C**1 > **E**1 > **B**1], where **C** and **E** are main and side chain terminal residues, respectively. To complete the assignment, it was found by C/H shift correlation that the low field ^{13}C anomeric signal was from **E**1. ^1H and ^{13}C chemical shifts for **1** are summarised in Table 2.

Dp6-2 and 6-3.—Dp6-2 and dp6-3 will be discussed before dp6-1 because, for the first two compounds, all four H-1(→4) doublets were well resolved. This simplifies the assignment and structure determination. The H-1/C-4 cross-peaks

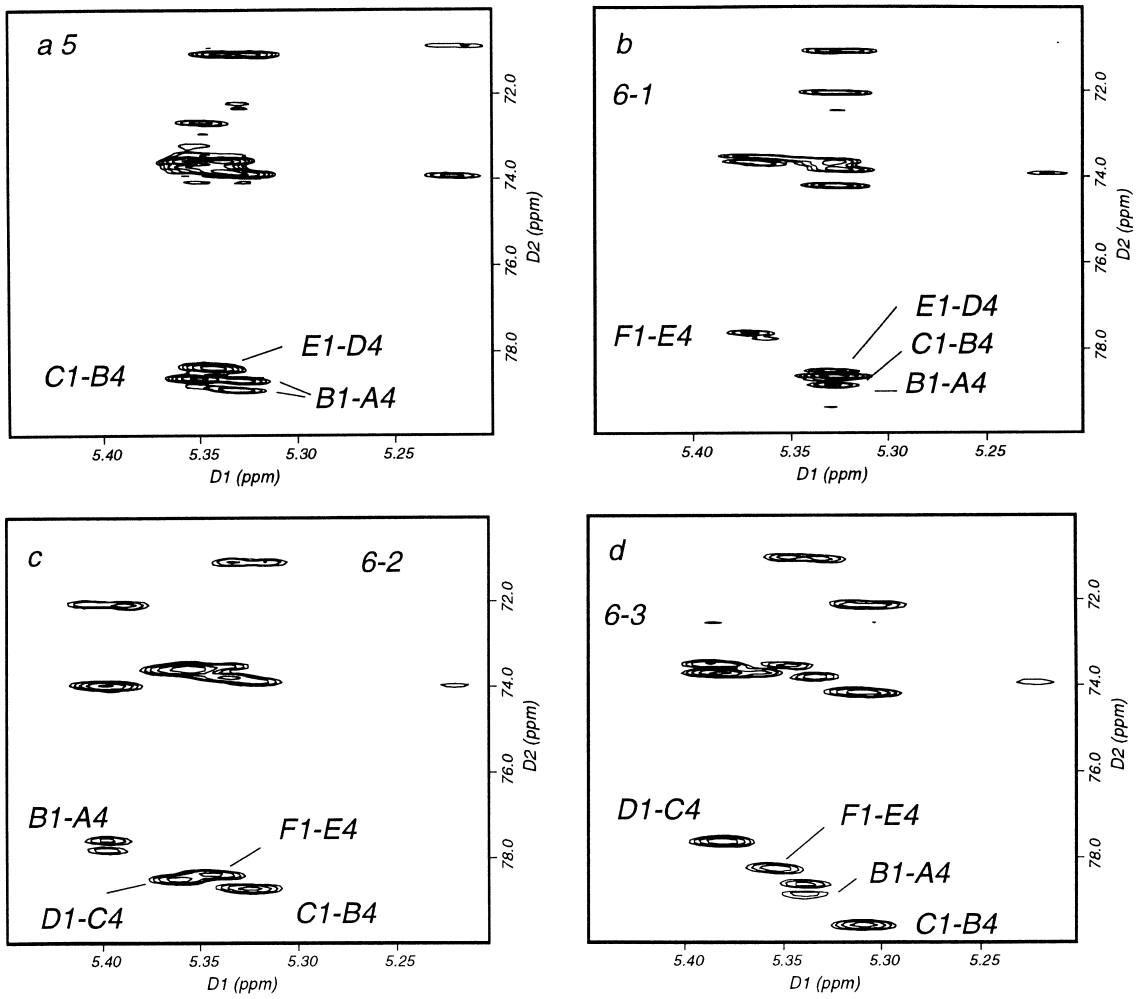


Fig. 5. 500/125 MHz HMBC spectra of (a) dp5 and (b–d) dp6 compounds. H-1(→4) and α-reducing end chemical shifts (D1 axis) and ^{13}C chemical shifts (D2 axis). *Inter*-ring H-1/C-4 cross peaks are labelled (see displayed structures for key to labelling).

Table 2
^{1}H and ^{13}C chemical shifts for dp5 (**1**)

		Chemical shift (δ) [a]					
Residue		1	2	3	4	5	6 [b]
A(α-re)	H	5.22	3.56	3.96	3.59	3.94	3.84/3.88
	C	92.70	72.07	73.94	78.92	70.90	61.62
A(β-re)	H	4.64	3.26	3.76	3.59	3.59	3.80/3.95
	C	96.60	74.76	76.92	78.70	75.49	61.75
B(1,4,6→4)	H	5.33	3.63	3.96	3.65	4.01	3.94/3.86
	C	100.58	72.24/.33	73.88/.77	78.63/.66	71.07	68.10/.12
C(t→4)	H	5.35	3.57	3.69	3.39	3.72	3.75/3.86
	C	100.60	72.68	73.70	70.19	73.63	61.45
D(1,4→6)	H	4.96	3.57	4.00	3.64	3.86	3.81/3.86
	C	99.40/.43	72.01	74.14	78.37/.42	71.23	61.27
E(t→4)	H	5.34	3.57	3.67	3.40	3.72	3.75/3.84
	C	100.81	72.68	73.73	70.19	73.56	61.33

[a] Two ^{13}C chemical shift values are reported (e.g., for **B**2) where anomeric doubling splits the signal.
[b] H-6 chemical shifts reported as H-6(*R*)/H-6(*S*). Identification [22,23] according to qualitative estimate of $J_{5,6}$ values from COSY spectrum.

linking rings **B** and **A** were easily recognised by the appearance of two ^{13}C signals (**A**α4 and **A**β4) at the same ^{1}H chemical shift. In all the compounds, the ^{13}C signal of **A**α4 was ~0.2 ppm to low field of the **A**β4 signal. Identification of this cross-peak [see labelling of Fig. 5(c) and (d)] revealed the ^{1}H chemical shift of **B**1, the anomeric proton of the neighbouring ring to the reducing end. Assignments from the HMQC–TOCSY and other experiments showed that in dp6-2 ring **B** was of the (1,4→4) type whereas in dp6-3 it was the (1,4,6→4) ring, i.e., the branch point was next to the reducing end in dp6-3 (but not in dp6-2). Continuing this procedure, the ^{1}H chemical shift of **C**1 was found from the **C**1/**B**4 cross-peak and ring **C** was identified as (1,4,6→4) in dp6-2 and as (1,4→4) in dp6-3. Then the ^{1}H shift of **D**1 was given by the **D**1/**C**4 cross-peak and, since **D** was a terminal residue (in both compounds) it had to be the non-reducing terminus of the main chain in dp6-2 and 6-3. Again in both compounds, ring **E** was of the (1,4→6) type and was the first unit of the side chain with **F** the terminal side chain residue (**F**1/**E**4 cross peak). Dp6-2 and 6-3 therefore both have four glucose units in the main chain and two in the side chain, but differ in the location of the branch point. Dp6-2 is 6^3-α-maltosyl-maltotetraose, **3**, and dp6-3 is 6^2-α-maltosyl-maltotetraose, **4**. Chemical shifts of the two compounds are given in Tables 3 and 4.

Dp6-1.—Interpretation of the HMBC spectrum of dp6-1 was more complicated because two of the H-1 chemical shifts were identical. The order of the H-1(1→4) chemical shifts [Fig. 3(b)] was δ(H-1) [t > t > (1,4,6) = (1,4)]. In the ^{13}C spectrum an order was established from the HMQC–TOCSY experiment for the C-4 chemical shifts of the 4-linked rings: δ(C-4)[reducing end (α) > (1,4,6→4) ~reducing end (β) > (1,4→6) > (1,4→4)]. Starting from the reducing end ^{13}C signal, **A**α4, and using the above order of ^{13}C chemical shifts we have labelled [Fig. 5(b)] the three tightly clustered H-1/C-4 cross-peaks **B**1/**A**4, **C**1/**B**4, **E**1/**D**4, where **B** was identified with the (1,4,6→4) unit and **C** with the (t→4) unit linked to **B** (a close examination of the HMBC cross-peaks showed that the ^{1}H chemical shift for **C**1/**B**4 was slightly higher than that of the **B**1/**A**4 and **E**1/**D**4 cross-peaks. Therefore, from the order of the ^{1}H shifts given above, **C** was the terminal residue of the main chain). **E** was the (1,4→4) unit linked to the first glucose in the side chain, **D**; and ring **F** was the non-reducing terminus of the side chain linked to **E** (**F**1/**E**4 cross-peak: **E**4 is to high field of the main group of C-4 resonances). Thus dp6-1 is 6^2-α-maltotriosyl-maltotriose, **2**, and its chemical shifts are given in Table 5.

4. Discussion

Chemical shifts and linkage conformation.—It is widely accepted that the chemical shifts of hydrogen and carbon atoms involved in glycosidic

Table 3
^{1}H and ^{13}C chemical shifts for dp6-2 (**3**)

Residue		Chemical shift (δ) [a]					
		1	2	3	4	5	6 [b]
A(α-re)	H	5.22	3.56	3.96	3.65	3.93	n.d.
	C	92.70	72.11	73.92	77.75	70.74	61.30
A(β-re)	H	4.64	3.26	3.76	3.65	3.59	3.76/3.91
	C	96.57	74.81	76.99	77.55	75.33	61.47
B(1,4→4)	H	5.40	3.61	3.95	3.60	3.84	3.76/3.91
	C	100.28/.19	72.32/.24	74.02	78.72	72.10	61.47
C(1,4,6→4)	H	5.33	3.64	3.97	3.63	4.02	3.95/3.86
	C	100.75	72.30	73.92	78.53	71.10	68.14
D(t→4)	H	5.37	3.57	3.68	3.41	3.73	n.d.
	C	100.55	72.65	73.65	70.16	73.65	61.42
E(1,4→6)	H	4.97	3.58	4.01	3.65	3.86	n.d.
	C	99.41	71.97	74.12	78.36	71.21	61.22
F(t→4)	H	5.35	3.57	3.68	3.41	3.73	n.d.
	C	100.81	72.65	73.76	70.10	73.55	61.30

[a] Two ^{13}C chemical shift values are reported (e.g., for **B**2) where anomeric doubling splits the signal.
[b] H-6 chemical shifts reported as H-6(*R*)/H-6(*S*) (see Table 2) where possible.
n.d., not determined.

Table 4
^{1}H and ^{13}C chemical shifts for dp6-3 (**4**)

Residue		Chemical shift (δ) [a]					
		1	2	3	4	5	6 [b]
A(α-re)	H	5.22	3.56	3.96	3.59	3.95	3.85/3.89
	C	92.70	72.06	73.94	78.78	70.89	61.60
A(β-re)	H	4.65	3.27	3.76	3.61	3.63	3.81/3.96
	C	96.57	74.75	76.92	78.59	75.47	61.72
B(1,4,6→4)	H	5.35	3.64	3.97	3.65	4.02	3.94/3.87
	C	100.52	72.26/.15	73.77	79.57	71.01	68.22
C(1,4→4)	H	5.31	3.62	3.94	3.65	3.88	3.80/3.88
	C	100.81	72.53	74.15	77.54	72.12	61.29
D(t→4)	H	5.39	3.58	3.68	3.41	3.72	3.75/3.85
	C	100.52	72.53	73.68	70.13	73.55	61.29
E(1,4→6)	H	4.96	3.58	4.01	3.67	3.86	3.83/3.87
	C	99.34	71.98	74.15	78.19/.15	71.18	61.21
F(t→4)	H	5.36	3.57	3.70	3.41	3.72	3.75/3.85
	C	100.75	72.65	73.65	70.13	73.55	61.39

[a] Two ^{13}C chemical shift values are reported (e.g., for **B**2) where anomeric doubling splits the signal.
[b] H-6 chemical shifts reported as H-6(*R*)/H-6(*S*) (see Table 2).

Table 5
^{1}H and ^{13}C chemical shifts for dp6-1 (**2**)

Residue		Chemical shift (δ) [a]					
		1	2	3	4	5	6 [b]
A(α-re)	H	5.23	3.56	3.96	3.59	3.94	~3.85
	C	92.70	72.11	73.94	78.80	70.87	61.62
A(β-re)	H	4.65	3.27	3.76	3.62	3.59	3.80/3.94
	C	96.59	74.75	76.89	78.59	75.46	61.65
B(1,4,6→4)	H	5.33	3.63	3.96	3.63	4.02	3.95/3.85
	C	100.59	72.33/.24	73.85	78.71/.63	71.07	68.06/67.89
C(t→4)	H	5.34	3.57	3.69	3.39	3.70	3.73/3.86
	C	100.63	72.74	73.70	70.22	73.65	61.46
D(1,4→6)	H	4.97	3.58	4.00	3.64	3.85	~3.84
	C	99.43	71.99	74.11	78.59/.50	71.20	61.24
E(1,4→4)	H	5.33	3.61	3.95	3.63	3.84	3.74/3.84
	C	100.63	72.45	74.21	77.69/.57	72.05	61.31
F(t→4)	H	5.38	3.57	3.68	3.40	3.70	3.74/3.84
	C	100.69	72.56	73.69	70.16	73.55	61.31

[a] Two ^{13}C chemical shift values are reported (e.g., for **B**2) where anomeric doubling splits the signal.
[b] H-6 chemical shifts reported as H-6(*R*)/H-6(*S*) (see Table 2) where possible. Otherwise single (mean) value given.

linkages are influenced by glycosidic torsion angles. For complex carbohydrates, accurate values of the chemical shift are more easily measured than either NOE values or long range coupling constants. However, the theoretical relationship between three-dimensional structure and chemical shifts is not yet well developed. Nevertheless it is worth examining selected chemical shifts in this series of compounds as they may reflect chemical shift trends and conformational influences to be found in larger dextrins, amylopectin and glycogen.

For the α-(1→4) linkage various empirical correlations [24,25] have been proposed between C-1′ and C-4 chemical shifts measured by solid state NMR and the torsion angles available from crystal structures. An explanation [26] has recently been proposed in terms of exo-anomeric and pseudo-anomeric effects on electron densities at the linkage carbons. Ab initio calculations [27] on a model α-(1→4) linkage fragment predicted that the nuclear shielding of C-1′ and C-4 should be a minimum when both torsion angles

ϕ (H-1′–C-1′–O-1′–C-4) and ψ (H-4–C-4–O-1′–C-1′) were zero. Any increase in $|\phi|$ or $|\psi|$ should lead to increased shielding (i.e., to upfield shifts for C-1′ and C-4), a result in accord with the limited experimental evidence that is available [27]. Such conformational effects may explain the chemical shift differences noted below although conformational averaging will make the variations smaller in solution than in the solid state.

^{13}C chemical shifts.—Chemical shifts of the atoms involved in (1→4) linkages will be discussed, focussing on the differences between branched and linear dextrins (maltotriose, maltotetraose, see Table 6). C-1 chemical shifts in the branched dextrins are generally downfield of those in the linear compounds. Figure 4 shows that the most highly shielded (~100.2 ppm) of the C-1(→4) carbons is **B**1 in dp6-2. It has just the same chemical shift as the comparable carbon in the linear dextrins and its signal is split by the neighbouring reducing end. Therefore observation of a (split) signal with this chemical shift is indicative of a (1,4)-linked ring (rather than a branch point) next to the reducing end. For the branched ring the C-1 signal is at lower field (~100.6 ppm) when the ring is adjacent to the reducing end and C-1 is even less shielded (100.75 ppm) when the branch point is further along the main chain as in dp6-2. Linkage to the branch point within the main chain gives rise to a downfield shift of C-1 (e.g. **C1** of dp6-3 compared with maltotetraose). However the C-1 shifts of the main chain termini depend little on whether the terminus is attached to a branch point (dp5, 6-1, 6-2) or not (dp6-3, linear dextrins). C-1 chemical shifts of the side chain termini are downfield of those of the main chain termini with a fairly constant (0.2 ppm) difference. The exception is for dp6-1 in which the difference is smaller. This dextrin has three sugar units in the side chain, and there is evidence (see below) that the shifts of the **F**–**E** glycosidic linkage are affected by an interaction between the side chain and the reducing end.

Table 6
^{1}H (H-1, H-4) and ^{13}C (C-1, C-4) chemical shifts for maltotriose (dp3) and maltotetraose (dp4)

		Chemical shift (δ) [a]			
		dp3		dp4	
Residue		1	4	1	4
A(α-re)	H	5.21	3.61	5.22	3.64
	C	92.71	77.97	92.72	77.86
A(β-re)	H	4.63	3.61	4.64	3.64
	C	96.59	77.75	96.59	77.64
B	H	5.38	3.63	5.39	3.64
	C	100.26/.35	77.55/.60	100.32/.22	77.80/.78
C	H	5.37	3.39	5.38	3.64
	C	100.57	70.15	100.45	77.55
D	H	—	—	5.39	3.41
	C	—	—	100.56	70.15

[a] Two ^{13}C chemical shift values are reported (e.g., for **B**1) where anomeric doubling splits the signal.

Introduction of a branch point in the maltotriose or maltotetraose chain brings about a general downfield shift of the C-4 resonances. In linear maltodextrins the 4-linked units have δ C-4 < 78 ppm but in the branched dextrins, only **E**4 (6-1), **A**4 (6-2) and **C**4 (6-3) have such low values (see Fig. 4). The (1,4,6)-linked ring and two of its neighbours, the preceding main chain residue and the first residue of the side chain, all have δ C-4 > 78 ppm, the largest downfield shift being observed for **B**4 (6-3), i.e., in a (1,4,6)-linked ring not adjacent to the non-reducing end. A further difference between branched and linear dextrins may be seen in the region immediately downfield of the C-5α signal at 70.9 ppm. Branched dextrins have two additional signals at 71.2 and 71.1 ppm. These originate from C-5 of the first side-chain unit and from C-5 of the (1,4,6)-linked residue, respectively. Other glucose rings, including glucosyl stubs, have δ C-5 > 72 ppm so that observation of both signals indicates presence of a branch point and a side chain with at least two Glc units. The signals are observable minor features in the ^{13}C spectra of amylopectins.

^{1}H chemical shifts.—H-1(→4) signals of branched dextrins (Fig. 2) show a general upfield displacement relative to the corresponding signals in linear dextrins, opposite to the trend noted above for C-1(→4) signals. H-1 of the (1,4,6)-linked ring (**B**1 in three compounds, **C**1 in 6-2) shows an upfield shift of ~0.05 ppm compared with the inner glucose rings of maltotriose and maltotetraose and the main chain terminal rings linked to *O*-4 of the branched residue also show a small upfield displacement of δ H-1 relative to the linear equivalents. When the linked ring is not terminal, however, the upfield displacement is greater making **C**1 (6-3) the most shielded of all the H-1(→4) protons. For compounds with two sugar units in the side chain the H-1 shifts of the side chain termini are almost constant and are consistently upfield of the δ H-1 for the main chain termini. For dp6-1, however, δ H-1 of the terminal residue (**F**) is further downfield and, unusually, considering its distance

from the reducing end the **F**1 signal exhibits anomeric doubling. Anomeric doubling is also seen in the ^{13}C spectrum of 6-1 for linkage positions **E**4 and **B**6. This long range influence of the anomeric form on chemical shifts must be related to the branch point location and side chain length in dp6-1 but the explanation is not clear. It could be a conformational effect transmitted through several linkages, or it could indicate the presence of conformers in which the shift is directly affected by a through-space interaction between the reducing end and a side chain unit.

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