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Characterisation of a sugar fraction from *Sarcocephalus latifolius* stem bark extract

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Abstract

The stem bark extract of the medicinal plant *Sarcocephalus latifolius* was analysed for its qualitative and quantitative carbohydrate content. Preparative high-performance liquid chromatography (HPLC) of the benzoylated sugar fraction, led to the isolation of D-fructose derivatives 1,2,3,4,6-penta-*O*-benzoyl-α-D-fructofuranose, 1,2,3,4,6-penta-*O*-benzoyl-β-D-fructofuranose, 1,2,3,4,5-penta-*O*-benzoyl-β-D-fructopyranose, and 1,3,4,5-tetra-*O*-benzoyl-β-D-fructopyranose, in addition to α- and β-D-pyranose forms of glucose, xylose, and arabinose perbenzoates, glycerol and D-erythriol perbenzoates, and an inseparable mixture of methyl 1,3,4,6-tetra-*O*-benzoyl-α,β-D-fructofuranoside, whose occurrence has never been previously reported from a natural source. The corresponding structures and conformations were fully characterised by extensive one and two-dimensional nuclear magnetic resonance (NMR) experiments. Quantification of the natural free sugars in the extract was achieved by HPLC using refractive-index detection. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Sarcocephalus latifolius*; Carbohydrate analysis; High-performance liquid chromatography

1. Introduction

In earlier publications we reported the evaluation of antibiotic, antitumor, and antileishmanial activity of ethanol extracts of the Rubiaceae *Sarcocephalus latifolius* (Smith) Bruce (*Nauclea latifolia* Sm.) (Abreu et al., 1999), and the isolation and structure determination of several indole alkaloids from its stem bark extract (Abreu & Pereira, 1998). In the coarse of the fractionation procedure, TLC analysis also indicated the presence of sugar constituents, which prompted us to investigate the carbohydrate composition of the extract.

Due to traditionally lengthy and low resolution preparative chromatography of free carbohydrate mixtures, we have opted by previous derivatisation of the sugar fraction with benzoyl chloride, followed by high pressure liquid chromatography (HPLC) isolation of the corresponding benzoylated constituents, monitored by diode-array detection. This method provides a faster alternative for separation of sugar mixtures, with better resolution when compared to the refractive-index detection, which also precludes the use of gradient elution (Daniel, De Feudis, Lott, & McCluer, 1981; Oshima & Kumanotani, 1983). Structural characterisation of the isolated compounds was achieved by extensive nuclear magnetic resonance (NMR) experiments (^{1}H, ^{13}C, DEPT, COSY, HMQC, HMBC, NOESY).

2. Experimental

2.1. General

Mps were determined on a Reichert microscope. Optical rotations were determined on a Perkin–Elmer 241-MC polarimeter, and FTIR spectra were measured on a Perkin–Elmer 157G infrared spectrometer. ^{1}H (400 MHz) and ^{13}C (100.61 MHz) one and two-dimensional NMR spectra were recorded on a Bruker ARX-400 spectrometer. Preparative HPLC of benzoylated carbohydrates was carried out on a D-7000 Merck instrument equipped with a DAD detector L7450A, in a range 200–450 nm, using Lichrospher Si60 and Lichrospher 100 RP18 columns (250×8 mm^2, 10 μm), Rheodyne injector 7725I, loop of 200 μl, flow rate 6.0 ml/min. Analytical HPLC of free carbohydrates was performed in the same instrument equipped with a refractive-index detector L-7490, using a LiChrosorb NH_2 column (250×4 mm^2, 5 μm), Rheodyne injector 7725I, loop of 20 μl, flow rate 1.0 ml/min, elution with MeCN/H_2O (80:20). Gas–liquid chromatography of alditol acetates was performed on a DB-225 column

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(20×0.104 mm^2, 0.1 μm; J&W) fitted to a Carlo Erba GC 6000 Vega Series 2 chromatograph equipped with a flame-ionisation detector and a split–splitless injection-system used in the split mode. Hydrogen was used as carrier gas at a flow rate of 80 kpa. Oven temperature: 200°C raised at 5°C/min to 220°C, where it was kept for 20 min. The injection port and detector were heated to 250 and 300°C, respectively. Normal and reversed-phase column chromatography were conducted on Si gel of 70–230 mesh and LiChroprep RP-18 of 40–63 μm, respectively. Merck Si gel and HPTLC-NH_2 plates 0.25-mm thick were used for TLC. Commercial carbohydrates were obtained from Aldrich Co.

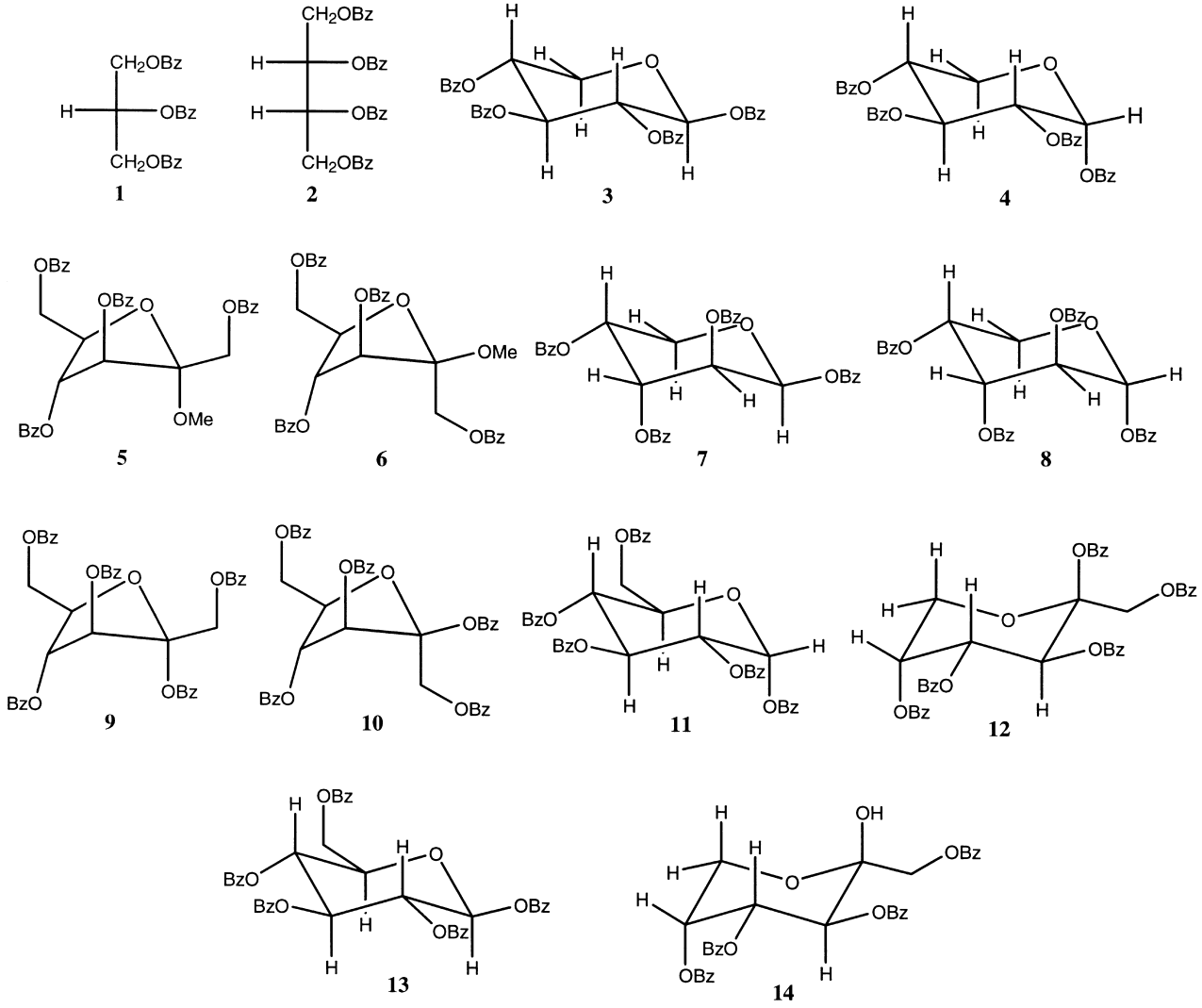
2.2. Plant material

S. latifolius was collected in January 1994 at Contuboel, Guinea-Bissau, and identified at the Herbarium of Botany Centre (LISC), where a voucher specimen is deposited.

2.3. Extraction and isolation

Stem bark of *S. latifolius* (680 g) was previously extracted (Soxhlet) with EtOH, yielding 110 g of brown residue. The extract was fractionated on a celite column using a gradient elution of hexane/$CHCl_3$ (100:0 to 50:50), and the corresponding fractions were monitored for their sugar composition in Si gel and HPTLC-NH_2 plates, using α-naphthol as spray reagent. The sugar fractions were assembled and evaporated, and eluted on a RP-18 column with H_2O, MeOH and $CHCl_3$, successively. The aqueous fraction, whose TLC and NMR analysis revealed a sugar composition, was evaporated to dryness, yielding 3.7 g of a carbohydrate mixture. A portion of this sugar fraction (2 g) was dissolved in pyridine (19 ml) and derivatised with benzoyl chloride (28 ml) at 0°C according to a described procedure (Daniel et al., 1981). The resulting benzoylated mixture (6 g) was partially submitted to normal-phase HPLC, with repeated injections of 250 mg, and using a gradient elution of Hexane/AcOEt (90:10 to 10:90). Eleven fractions (F1 to F11) were collected at each HPLC run, from which compounds **1**–**14** were isolated.

1,2,3-Tri-*O*-benzoyl-glycerol (**1**) — Compound **1**, mp 70–71°C, was isolated from F1. ^{1}H-NMR spectrum



($CDCl_3$) is in accordance with published data (D'Accorso & Thiel, 1986); ^{13}C-NMR ($CDCl_3$): δ 62.9 (C-1, C-3), 69.7 (C-2), 128.4–133.2 (aromatic carbons), 165.6 (CO), 166.0 (CO).

1,2,3,4-Tetra-*O*-benzoyl-D-erythritol (**2**) — Compound **2**, mp 188–189°C, was isolated from F2. ^{1}H-NMR spectrum ($CDCl_3$) is in accordance with published data (D'Accorso & Thiel, 1986); ^{13}C-NMR ($CDCl_3$): δ 62.7 (C-1, C-4), 70.1 (C-2, C-3), 128.4–133.4 (aromatic carbons), 165.3 (*C*O), 166.0 (*C*O).

1,2,3,4-Tetra-*O*-benzoyl-β-D-xylopyranose (**3**) — Compounds **3**–**6** eluted together on the normal-phase HPLC column (F3). Further elution of F3 on a RP-18 column, with MeOH/H_2O (80:20 to 20:80) afforded a fraction (F3a) containing a mixture of **3** and **4**, and a fraction (F3b) containing an inseparable mixture of **5** and **6**. Separation of **3** and **4** was achieved by successive elutions on preparative Si gel TLC, with hexane/AcOEt (90:10). For **3**: mp 175–177°C; $[\alpha]_D$ − 22.7° (*c* 0.8, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): δ 4.01 (dd, 1H, $J_{5,5'}$ 12.5 Hz, $J_{5,4}$ 7.3 Hz, H-5), 4.56 (dd, 1H, $J_{5',5}$ 12.5 Hz, $J_{5',4}$ 3.8 Hz, H-5′), 5.39 (ddd, 1H, $J_{4,5}$ 7.3 Hz, $J_{4,5'}$ 3.8 Hz, $J_{4,3}$ 5.9 Hz, H-4), 5.62 (t, 1H, $J_{2,3}$ 5.4 Hz, $J_{2,1}$ 4.4 Hz, H-2), 5.82 (t, 1H, $J_{3,4}$ 5.9 Hz, $J_{3,2}$ 5.4 Hz, H-3), 6.36 (d, 1H, $J_{1,2}$ 4.4 Hz, H-1), 7.32–8.04 (aromatic protons). ^{13}C-NMR ($CDCl_3$): δ 61.8 (C-5), 68.1 (C-4), 68.5 (C-2), 69.1 (C-3), 92.1 (C-1), 128.4–133.7 (aromatic carbons), 164.5 (*C*O), 165.1 (*C*O), 165.7 (*C*O).

1,2,3,4-Tetra-*O*-benzoyl-α-D-xylopyranose (**4**) — mp 118–120°C; $[\alpha]_D$ + 138.5° (*c* 0.8, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): δ 4.07 (t, 1H, $J_{5',5}$ 11.2 Hz, $J_{5,4}$ 10.0 Hz, H-5), 4.30 (dd, 1H, $J_{5',5}$ 11.2 Hz, $J_{5',4}$ 5.8 Hz, H-5′), 5.54 (ddd, 1H, $J_{4,5}$ 10.0 Hz, $J_{4,5'}$ 5.8 Hz, $J_{4,3}$ 9.8 Hz, H-4), 5.63 (dd, 1H, $J_{2,3}$ 10.1 Hz, $J_{2,1}$ 3.5 Hz, H-2), 6.27 (t, 1H, $J_{3,4}$ 9.8 Hz, $J_{3,2}$ 10.1 Hz, H-3), 6.76 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1), 7.29–8.16 (aromatic protons). ^{13}C-NMR ($CDCl_3$): δ 61.2 (C-5), 69.5 (C-4), 69.9 (C-3), 70.3 (C-2), 90.3 (C-1), 128.4–133.8 (aromatic carbons), 164.6 (*C*O), 165.4 (*C*O), 165.5 (*C*O), 165.9 (*C*O).

Methyl 1,3,4,6-tetra-*O*-benzoyl-α,β-D-fructofuranoside (**5:6**) — oil; $[\alpha]_D$ − 17.2° (*c* 0.4, $CHCl_3$). ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) of **5** (α form) and **6** (β form) are in accordance with published data (Bouali et al., 1992).

1,2,3,4-Tetra-*O*-benzoyl-β-D-arabinopyranose (**7**) — Compounds **7** and **8** eluted together on the normal-phase HPLC column (F4). Further separation was achieved by successive elutions on preparative Si gel TLC, with Hexane/AcOEt (90:10). For **7**: mp 163–165°C; $[\alpha]_D$ + 293.8° (*c* 0.6, $CHCl_3$). ^{1}H-NMR spectrum (acetone-d_6) is in accordance with published data (Durette & Horton, 1971). ^{13}C-NMR ($CDCl_3$): δ 63.8 (C-5), 69.0 (C-2, C-3), 70.6 (C-4), 91.8 (C-1), 129.3–134.8 (aromatic carbons), 166.1 (*C*O), 166.3 (*C*O), 165.4 (*C*O), 165.9 (*C*O).

1,2,3,4-Tetra-*O*-benzoyl-α-D-arabinopyranose (**8**) — mp 158–160°C; $[\alpha]_D$ − 32.4° (*c* 0.5, $CHCl_3$). ^{1}H-NMR spectrum (acetone-d_6) is in accordance with published data (Durette & Horton, 1971). ^{13}C-NMR ($CDCl_3$): δ 64.0 (C-5), 68.7 (C-2), 67.6 (C-3), 68.3 (C-4), 93.4 (C-1), 129.6–130.9 (aromatic carbons), 164.4 (*C*O), 164.5 (*C*O), 165.0 (*C*O).

1,2,3,4,6-Penta-*O*-benzoyl-α-D-fructofuranose (**9**) — Compound **9**, mp 125–127°C, $[\alpha]_D$ + 8.1° (*c* 0.3, $CHCl_3$), was isolated from F5. ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) are in accordance with published data (Bouali et al., 1992).

1,2,3,4,6-Penta-*O*-benzoyl-β-D-fructofuranose (**10**) — Compound **10**, mp 64–66°C, $[\alpha]_D$ − 27.7° (*c* 1.0, $CHCl_3$), was isolated from F6, and further purified on normal-phase HPLC using Hexane/Dioxane (80:20) as eluent. ^{1}H-NMR (DMSO): δ 4.61 (dd, 1H, $J_{6,6'}$ 12.0 Hz, $J_{6,5}$ 5.1 Hz, H-6), 4.70 (dd, 1H, $J_{6',6}$ 12.0 Hz, $J_{6',5}$ 3.1 Hz, H-6′), 4.91 (dd, 1H, $J_{5,6}$ 5.1 Hz, $J_{5,6'}$ 3.1 Hz, H-5), 5.02 (d, 1H, $J_{1,1'}$ 11.5 Hz, H-1), 5.10 (d, 1H, $J_{1',1}$ 11.5 Hz, H-1′), 6.19 (t, 1H, $J_{4,5}$ 6.9 Hz, $J_{4,3}$ 6.9 Hz, H-4), 6.40 (d, 1H, $J_{3,4}$ 6.9 Hz, H-3), 7.21–8.00 (aromatic protons). ^{13}C-NMR (DMSO): δ 67.4 (C-6), 68.8 (C-1), 79.6 (C-4), 81.5 (C-3), 82.6 (C-5), 108.6 (C-2), 128.6–137.9 (aromatic carbons), 167.5 (*C*O), 168.4 (*C*O), 168.7 (*C*O), 169.1 (*C*O), 169.3 (*C*O).

1,2,3,4,6-Penta-*O*-benzoyl-α-D-glucopyranose (**11**) — Compound **11**, mp 164–165°C, $[\alpha]_D$ + 125.4° (*c* 0.3, $CHCl_3$), was isolated from F7, and further purified on normal-phase HPLC using hexane/dioxane (80:20) as eluent. ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) are in accordance with published data (D'Accorso & Thiel, 1986).

1,2,3,4,5-Penta-*O*-benzoyl-β-D-fructopyranose (**12**) — Compound **12**, mp 154–156°C, $[\alpha]_D$ − 198.7° (*c* 0.2, $CHCl_3$), was isolated from F8, and further purified on normal-phase HPLC using Hexane/Dioxane (80:20) as eluent. ^{1}H-NMR spectrum ($CDCl_3$) is in accordance with published data (Lichtenthaler, Klotz, & Flath, 1995). ^{13}C-NMR ($CDCl_3$): δ 63.3 (C-1), 63.7 (C-6), 68.0 (C-3), 69.3 (C-5), 69.4 (C-4), 103.2 (C-2), 125.5–134.0 (aromatic carbons), 163.5 (*C*O), 165.2 (*C*O), 165.3 (*C*O), 165.6 (*C*O), 165.9 (*C*O).

1,2,3,4,5-Penta-*O*-benzoyl-β-D-glucopyranose (**13**) — Compound **13**, mp 138–141°C, $[\alpha]_D$ + 11.1° (*c* 0.4, $CHCl_3$), was isolated from F9. ^{1}H- and ^{13}C-NMR spectra are in accordance with published data (D'Accorso & Thiel, 1986).

1,3,4,5-Tetra-*O*-benzoyl-β-D-fructopyranose (**14**) — Compound **14**, mp 175–177°C, $[\alpha]_D$ − 165.0° (*c* 0.3, $CHCl_3$), was isolated from F11. ^{1}H-NMR and ^{13}C-NMR spectra ($CDCl_3$) are in accordance with published data (Lichtenthaler et al., 1995).

3. Results and discussion

Chromatographic fractionation of the ethanolic stem bark extract of *S. latifolius* afforded an aqueous fraction, whose ^{1}H- and ^{13}C-NMR spectra exhibited a sugar profile (Agrawal, 1992). Preliminary GC analysis of this fraction after derivatisation of the free sugars to alditol acetates (Blakeney, Harris, Henry, & Stone, 1983), indicated

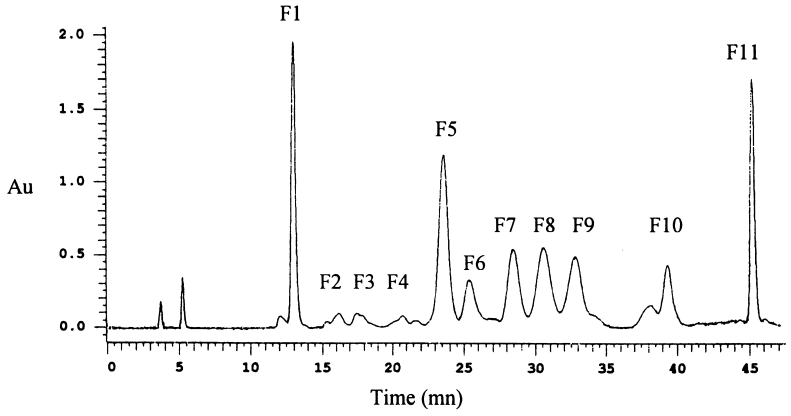


Fig. 1. HPLC fractionation of the benzoylated sugar fraction from the stem bark extract of *S. latifolius*, on a Lichrospher Si60 column (250 × 8 mm^2, 10 μm), flow rate 6.0 ml/min, gradient elution of Hexane/AcOEt (90:10 to 10:90).

D-manitol and D-glucitol as the main constituents, with peak areas of 42 and 39%, respectively. Nevertheless, this result was not conclusive in regarding the identity of the natural constituents, once D-manitol could result from D-fructose and/or D-manose, and D-glucitol could result from D-fructose and/or D-glucose, or D-gulose.

Normal-phase HPLC of the benzoylated mixture afforded eleven fractions (Fig. 1). The first and second fractions (F1 and F2) yielded 1,2,3-tri-*O*-benzoyl-glycerol (**1**) and 1,2,3,4-tetra-*O*-benzoyl-D-erythritol (**2**), respectively, whose NMR data were identical to those of authentic samples. The third fraction (F3) contained a mixture of at least, three compounds, as indicated by the anomeric carbon resonances at δ 90.3, 92.1 and 104.0 ppm. Successive reversed-phase HPLC and preparative TLC of this fraction, allowed the isolation of **3** and **4**, and an inseparable mixture of **5** and **6**. ^{1}H- and ^{13}C-NMR spectra of the fourth fraction (F4) indicated a two component mixture, with anomeric protons at δ 6.61 (d, *J* 3.4 Hz) and 6.86 (s) ppm, linked to carbons at δ 92.1 and 91.2 ppm, respectively, as proved by the HMQC spectrum. Further multiple elutions of F4 on preparative Si gel TLC, yielded **7** and **8**. Fractions F5, F9 and F11 yielded pure **9**, **13** and **14**, respectively, whereas F6–F8 afforded **10**–**12**.

^{1}H- and ^{13}C-NMR spectra of compounds **3**, **4**, **7** and **8** displayed six protons, and five carbon resonances characteristic of peracylated aldopentopyranoses (Bock & Pederson, 1983; Durette & Horton, 1971). Analysis of COSY, DEPT, HMQC and HMBC spectra, as well as proton coupling constant values, led to the identification of 1,2,3,4-Tetra-*O*-benzoyl-β-D-xylopyranose (**3**), 1,2,3,4-Tetra-*O*-benzoyl-α-D-xylopyranose (**4**), 1,2,3,4-Tetra-*O*-benzoyl-β-D-arabinopyranose (**7**), and 1,2,3,4-Tetra-*O*-benzoyl-α-D-arabinopyranose (**8**), confirmed by comparison with authentic samples. Examination of NOESY spectra was also elucidative in regarding the conformation of the benzoates. For compound **3**, H-1 shows intense cross peaks with H-2, H-3 and H-5ax, resulting from two interconverting $^4C_1$ and $^1C_4$ forms (Brakta et al., 1993; Rao, Qasba, Balaji, & Chandrasekaran, 1998). This observation is in accordance with the results of conformational studies on D-aldopentapyranose tetrabenzoates, which indicated an identical proportion of the two chair forms for perbenzoylated β-D-xylose at room temperature (Durette & Horton, 1971). The larger proportion (>98%) of the $^4C_1$ conformation for the α-form of D-xylose perbenzoate (**4**) is illustrated by the intense NOE interaction (Fig. 2) between the two pairs of *cis* axial protons H-2/H-4 and H-3/H-5, and a weak cross peak

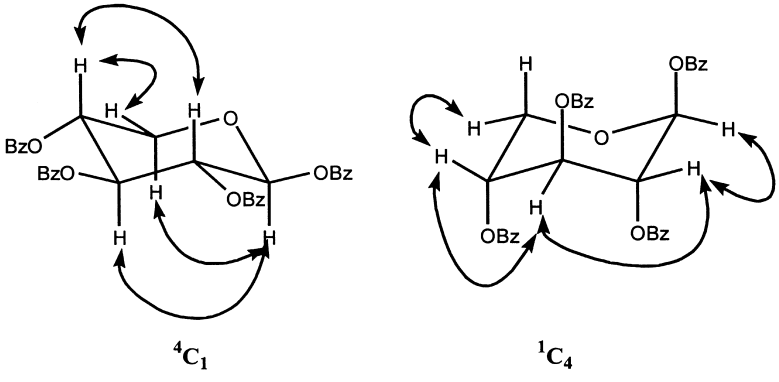


Fig. 2. Observed NOE interactions in chair β-forms of D-xylose.

between the equatorial protons H-2 and H-3 in the $^1C_4$ conformation. Similar observations are extensive to the NOESY spectra of **7** (95% $^1C_4$) and **8** (70% $^1C_4$).

^{13}C-NMR and DEPT spectra of compounds **5**, **6**, **9**, **10**, **12** and **14**, displayed two CH_2, three CH and one tetrasubstituted carbon linked to oxygen, whereas the corresponding ^{1}H-NMR spectra lack the anomeric proton, which suggested cyclic forms of ketoses (Bock & Pederson, 1983). Compounds **5** and **6** had in addition a methoxyl group (δ_H 3.46 ppm) placed at the tetrasubstituted carbon, as indicated by the HMBC spectrum. Full assignement for all carbon and protons followed from the observed HMQC correlations and analysis of proton–proton couplings in the COSY spectra. The IR spectrum of **14** showed the presence of a hydroxyl group (3415 cm^{-1}), which was indicative of an incomplete benzoylation. Comparison of complete NMR data with those of literature (Bouali et al., 1992; Kraska & Lichtel, 1983; Lichtenthaler et al., 1995), confirmed these six compounds as fructofuranosides and fructopyranosides, thus suggesting that various products were obtained from the benzoylation of D-fructose. It is well known that the distribution of acylated products of D-fructose is strongly dependent on experimental conditions (Bouali et al., 1992; Kraska & Lichtel, 1983; Lichtenthaler et al., 1995), and in the case of benzoylation, up to seven products have been reported (Lichtenthaler et al., 1995): the tetrabenzoate β-D-fructopyranoside, two tetrabenzoate D-fructofuranosides (α- and β-forms), two pentabenzoate D-fructopyranosides (α- and β-forms), the pentabenzoate α-D-fructofuranoside, and one acyclic pentabenzoate. The NMR and physical data (mp, $[\alpha]_D$) of compounds **9**, **12** and **14** corresponds to those reported for 1,2,3,4,6-penta-*O*-benzoyl-α-D-fructofuranose (Bouali et al., 1992; Kraska & Lichtel, 1983; Lichtenthaler et al., 1995), 1,2,3,4,5-penta-*O*-benzoyl-β-D-fructopyranose (Kraska & Lichtel, 1983; Lichtenthaler et al., 1995) and 1,3,4,5-tetra-*O*-benzoyl-β-D-fructopyranose (Kraska & Lichtel, 1983; Lichtenthaler et al., 1995), respectively. First order analysis of the ^{1}H-NMR spectra of **10** in DMSO suggested that this compound was the β-form of **9**, which was confirmed by the observed NOESY cross-peak between one methylene proton (δ 5.02) at C-1 and H-3. This interaction is absent in the NOESY spectrum of **9**. To our knowledge, no spectroscopic data has been previously reported in literature for 1,2,3,4,6-Penta-*O*-benzoyl-β-D-fructofuranose. The identity of compounds **9**, **10**, **12** and **14**, was confirmed by comparison with benzoylated products of a commercial sample of D-fructose, formed under the same reaction conditions. HPLC of the reaction mixture displayed a peak corresponding to fraction F10 (Fig. 1), whose NMR spectrum indicated decomposition products of D-fructose.

The NMR data of the mixture **5:6** corresponds to those reported for methyl 1,3,4,6-tetra-*O*-benzoyl-α,β-D-fructofuranoside, which was synthesised from 1,3,4,6-tetra-*O*-benzoyl-D-fructose (Bouali et al., 1992). Nevertheless, its occurrence has never been previously reported from a natural source.

Spectral data of **11** and **13** were in accordance with those reported for the α and β anomers of 1,2,3,4,6-Penta-*O*-benzoyl-D-glucopyranose (D'Accorso, Thiel, & Schüller, 1983), which was confirmed by comparison with authentic samples.

The content of D-fructose (73.4%), D-glucose (16.4%), D-arabinose (0.3%), D-xylose (0.2%), D-erythritol (0.3%), glycerol (9.1%), and methyl α,β-D-fructofuranoside (0.3%), in the natural extract, was deduced from the peak areas of the HPLC chromatogram on amino-bonded silica column, using refractive-index detection. The identity of methyl α,β-D-fructofuranoside was confirmed by co-injection with deacylated **5:6** (Itoh, Takamura, Watanabe, Araki, & Ishido, 1986).

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