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ISOLATION AND STRUCTURE DETERMINATION OF TRIGLOCHININ, A NEW CYANOGENIC GLUCOSIDE FROM *TRIGLOCHIN MARITIMUM*

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Abstract—A new cyanogenic glucoside, triglochinin (*O*-[β-D-glucopyranosyl]-1-cyano-1-hydroxy-4,5-dicarboxy-1,3-pentadiene), was isolated from the flowers of *Triglochin maritimum* L. On hydrolysis, the glucoside decomposes (*inter alia*) to a new acid, triglochinic acid (2-butene-1,2,4-tricarboxylic acid). The isolation and structure determination of the glucoside are described.

INTRODUCTION

THE CYANOGENIC properties of sea arrow-grass (*Triglochin maritimum* L.; Juncaginaceae) as well as of marsh arrow-grass (*T. palustre* L.) were originally discovered by Greshoff.[1] Attempts to isolate the cyanogenic compound(s) from *T. maritimum* were unsuccessful, but the presence of linamarin-like glycosides was suggested since small amounts of acetone were detected in distillates of plant material.[1] This idea was rejected by Blanksma[2] who was unable to find any traces of acetone in plant distillates, although the presence of acetaldehyde (and ethyl alcohol) was demonstrated. The detection of oxo compounds in cyanogenic plants is of interest, since most cyanogenic glycosides are known to release sugar(s), HCN and an aldehyde or ketone on enzymic or acid-catalysed hydrolysis.[3-5] In the present case, however, the possibility of acetaldehyde being a cleavage product of a cyanogenic glycoside was considered most improbable, since non-cyanophoric plants were shown to be acetaldehyde-releasing as well.[2]

The chemical nature of the *Triglochin* cyanogen has remained obscure, though some observations have indicated a relatively stable structure for the substance.[6,7] It has also been established, that all aerial parts of the plant are cyanogenic, the largest accumulation of the cyanogen being in flowers and unripe fruits.[2,7]

In the present study, an amorphous glucoside has been isolated in acceptable yield from the flowers of *T. maritimum* L. Although still not obtained in crystalline state, a structure determination has been possible by application of spectroscopic methods along with degradative work. These investigations will be described in terms of the proposed structure of the glucoside (I), for which the trivial name triglochinin is suggested.

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RESULTS

The i.r. spectrum of triglochinin (I) (Fig. 1) shows a broad and intense absorption in the 3600–2500 cm^{-1} region, characteristic of carboxylic acids (the carboxylic νOH absorption is overlapping with νOH for the sugar OH groups). The presence of COOH groups is further indicated by the strong peak centred at 1715 cm^{-1} (νC=O in COOH). The presence of conjugated C=C bonds is revealed by the medium strong band at 1625 cm^{-1}. The absence of CH_3 bands at 1380 cm^{-1} (δ_s CH_3) and 1460 cm^{-1} (δ_{as} CH_3) is of interest, but the presence of active CH_2 is indicated by the broad band at 1410 cm^{-1}. The nitrile $\nu C \equiv N$ band is seen at 2220 cm^{-1}. The intensity of this band is much stronger than normally observed for other cyanogenic glycosides where the $\nu C \equiv N$ band is of very low intensity or scarcely detectable; cf. Ref. 5. This evidence, as well as the low wave number position of the band in the spectrum, strongly favours the presence of an α,β-unsaturated nitrile group.[8] The presence of conjugation in the molecule (I) is evident from broad absorption in the u.v. region with λ_{max} at 275 nm (in H_2O).

The NMR spectrum of the trimethylsilyl derivative of (I) shows two low field doublets centred at δ 5·93 and δ 6·69 due to the vinylic protons H_A and H_B respectively in the aglycone, with coupling constant (J_{AB}) of 12·5 c/s, typical of the system $\rangle C{=}CH{-}CH{=}C\langle$. The methylene protons (H_C) may be assigned to a peak (partially overlapping with glucose protons) at δ 3·63. It would be expected, that the H_B proton could be allylically coupled to the H_C protons, and actually significant broadenings of the respective signals are observed in the spectrum. The anomeric proton of the glucose residue exhibits a multiplet at δ 4·8 and the remaining six sugar protons resonate between δ 4·1 and 2·9.

Triglochinin (I) was shown to be a dibasic acid by alkalimetric titration and esterification using HCl in MeOH gave a di-*O*-Me ester as verified by its NMR spectrum. When treated with β-glucosidase or heated in aqueous solution, (I) was decomposed to glucose, HCN and a non-volatile, tribasic, unsaturated acid (IV), for which the trivial name triglochinic acid is suggested. No evidence for the formation of oxo compounds in these reactions, capable of condensation with 2,4-dinitrophenylhydrazine, could be obtained.

The formation of IV may be explained through the unstable intermediates II and III. Hydrolysis is initiated by cleavage of the glycosidic bond in I yielding glucose and the α,β-unsaturated cyanohydrin (II). On hydrolysis, II releases HCN and gives the α,β-unsaturated oxo compound (III), but this compound, a ketene, will add H_2O immediately yielding the tri-acid (IV). In this respect, I behaves analogously with acacipetalin (*O*-[β-D-glucosyl]-1-cyano-1-hydroxy-2-methyl-1-propene). This glucoside is known to decompose to glucose, HCN and isobutyric acid under similar conditions.[9,10]

Treatment of I with HCl, 3 per cent in MeOH at 40° leads to triglochinin-di-*O*-Me-ester (V), but simultaneously cleavage of the glycosidic bonds in I and V takes place, resulting in formation of HCN, a mixture of α- and β-methylglucopyranosides (VI) and triglochinic acid-tri-*O*-Me-ester (VII).

The mass spectrum of V shows a feeble signal at *m/e* 387 indicative of the molecular ion, but a prominent signal at *m/e* 225 corresponds to the elimination of glucose as $C_6H_{10}O_5$ (162 *m/e*), typical of *O*-glucopyranosides; cf. Ref. 11. Additionally, a peak at *m/e* 198 may be interpreted as a fragment formed by expulsion of HCN (27 *m/e*) from the aglycone fragment $[C_{10}H_{11}O_5N]^+$ (225 *m/e*).

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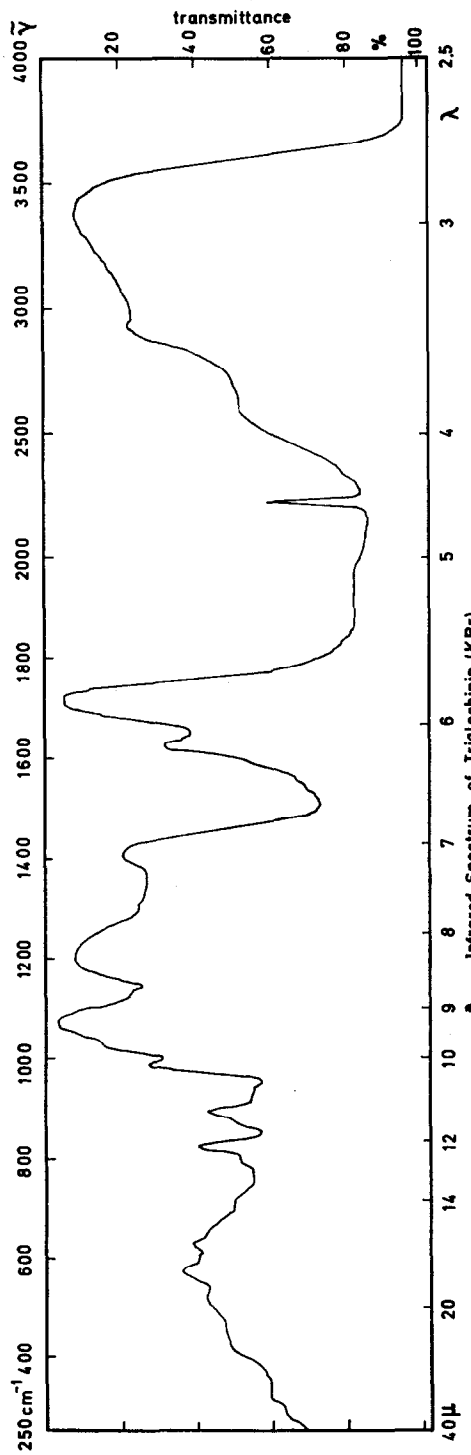

FIG. 1. INFRARED SPECTRUM OF TRIGLOCHININ (KBr).

Ba(OH)2
H+ or β-glucosidase
MeOH
HCl
MeOH
HCl
H2O
H2O
+ NH3
+ Glc
+ HCN
+ HCN
+ H, OMe
(I)
(II)
(III)
(IV)
(V)
(VI)
(VII)
(VIII)

The glucoside is sensitive to basic reagents. Treatment of (I) with excess of $Ba(OH)_2$ results in formation of β-glucosan (1,6-anhydroglucopyranose) (VIII) and NH_3. This feature is not shared by other known cyanogenic glycosides as this reaction normally gives an acidic glycoside due to hydrolysis of the CN group to a COOH group.[3-5]

As evident from the formula (I), four geometrical isomers of triglochinin are theoretically possible. Work on this problem, as well as search for other minor cyanogenic components in the plant, is in progress.

EXPERIMENTAL

Mass spectra were recorded with a MS 9 instrument using direct insertation technique on a ceramic rod; source temp. 200°; 70 eV. NMR spectra were recorded at a fixed frequency of 60 Mc/s. All δ values are relative to TMS (internal). TLC work was done with 8 × 10 cm glass plates; layer thickness *ca.* 0·15 mm. Detection of cyanogenic glycosides on PC followed the method of Butler and Butler.[12] Plant material consisted almost entirely of young flowers and was collected on 21 May 1969 in marshy fields at the peninsula of Enø, Southern Zealand, Denmark. The material was extracted *ca.* 1 hr after collection.

Isolation of Triglochinin (I)

The fresh flowers of *Triglochin maritimum* L. (177·5 g) were thrown into boiling H_2O (1 l.) and the mixture was kept at >85° for 5 min with stirring. The mixture was cooled to *ca.* 2° and homogenized. Solid matter was centrifuged down and extracted with H_2O (3 × 400 ml) by renewed homogenizations and centrifugations. The combined aqueous extracts were filtered through kieselguhr and concentrated *in vacuo* (30°) to a sticky, dark-coloured syrup (15·2 g). PC analysis of this material in a number of solvents indicated the presence of one cyanogenic glycoside only. The syrup was suspended in H_2O (60 ml) and chromatographed on a column of polyamide (BASF) (100 g) in order to remove phenolics. Elution with H_2O and screening of the fractions (10 ml) by TLC [silica gel GF_{254}; solvent, MeCOEt–EtOAc–HCO_2H–H_2O, 5:3:2:1, v/v (solvent A), detection, naphthoresorcinol–H_2SO_4 and heating to 105°, short wave u.v.] showed that I was eluted in the first 350 ml together with large amounts of carbohydrates. The effluent was concentrated to dryness *in vacuo* (30°). The residue (11·5 g) was dissolved in H_2O (50 ml) and placed on a column of a weakly basic anion exchange resin (Amberlite I.R. 45, OH^-) (100 g). The column was eluted with H_2O (775 ml) or until a perfectly neutral and a colourless eluate was obtained. TLC analysis of the eluate showed the presence of carbohydrates, whereas (I) was withheld by the column. The column was now eluted stepwise with 1 M AcOH (340 ml), 2 M AcOH (100 ml), 3 M AcOH (100 ml), 4 M AcOH (100 ml), 5 M AcOH (100 ml) and 5 M HCO_2H (500 ml). The effluent was collected in fractions (10 ml) and monitored by TLC (silica gel GF_{254}; solvent A). Traces of I were eluted with 5 M AcOH but predominantly in 5 M HCO_2H. Concentration of relevant fractions *in vacuo* (25°), at last with frequent addition of EtOH to the concentrate, gave a yellowish, thick syrup (0·64 g). This syrup was dissolved in H_2O (4 ml), mixed with microcrystalline cellulose (Merck) (4 g) and dried *in vacuo* at 25° over $CaCl_2$ for 36 hr. The powder was suspended in EtOAc–AcOH–H_2O, 8:1:1, v/v (solvent B) (3 ml) and transferred to a cellulose column (300 g) packed in solvent B. The column was irrigated with solvent B, that was collected in fractions (25 ml). Analysis of the eluate (TLC; microcrystalline cellulose; solvent B; detection, 2′,7′-dichlorofluorescein, 0·04‰ in the solvent and short wave u.v.,[13] treatment of plates with β-glucosidase followed by aniline hydrogen phthalate and heating) localized I in fractions 51–63. Fractions 55–63 were free of impurities and concentration *in vacuo* yielded a colourless syrup (440 mg). The syrup was concentrated repeatedly *in vacuo* with EtOH:Bz in order to remove water as quantitatively as possible and the syrupy residue was triturated with Et_2O and concentrated to yield a colourless powder which was dried to constant weight *in vacuo* over P_2O_5.

The material thus obtained was a colourless, extremely hygroscopic powder (370 mg or 0·21 % of the fresh plant material) that has not as yet been crystallized satisfactorily from solution. Its homogeneity was verified by PC and TLC in numerous solvents using a variety of detection reagents. Some R_fs follow: PC (Whatman No. 1; descending technique), solvent A, R_f 0·66; EtOAc–Pyr–H_2O, 36:10:11·5, v/v, R_f 0·34. TLC (silica gel GF_{254}), solvent A, $R_{glucose}$ 1·80. TLC (microcrystalline cellulose), solvent B, $R_{malic\ acid}$ 0·46; EtOAc–AcOH–H_2O, 7:1·5:1·5, v/v, $R_{malic\ acid}$ 0·67; EtOAc–AcOH–H_2O, 3:1:1, v/v, $R_{malic\ acid}$ 0·92. The substance gave no reactions with $FeCl_3$ or 2,4-dinitrophenylhydrazine (aqueous solutions). Titration of the material (20·50 mg) with 0·05 N NaOH indicated the presence of two COOH groups. $[\alpha]_D^{16.5}$ (*c*, 0·2 in MeOH) = +5·5°. I.r. spectrum (KBr; Fig. 1), ν_{max}: 3600–2500 (s), 2220 (m), 1715 (s), 1625 (m), 1410 (m), 1200 (s), 1075 (s), 987 (m), 895 (w), 825 (w) and 632 (w) cm^{-1}. U.v. spectrum: Broad absorption, λ_{max} 275 nm (H_2O).

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Hydrolysis of I

(*a*) *Enzymic hydrolysis.* The glucoside (10 mg) and β-glucosidase (Fluka) (2 mg) were dissolved in H_2O (2 ml). The solution was kept in a closed vial at room temp. for 24 hr. The hydrolysate contained HCN (picric acid test,[14] cupric acetate–benzidine test[15] and nitrobenzaldehyde–dinitrobenzene test[16]), glucose (identified by PC; 2 solvents and TLC; 2 solvents) and the presence of a non-volatile acid (triglochinic acid) (IV) was demonstrated by TLC in a number of solvents. The hydrolysate gave no reactions for oxo compounds (2,4-dinitrophenylhydrazine) or volatile acids (distillation of hydrolysate followed by titration of the distillate with 0·01 N NaOH against a blank test).

(*b*) *Acid hydrolysis.* Triglochinin (I) (1·1 mg) was dissolved in H_2O (200 μl) and N_2 was bubbled through the solution that was heated to 100° for 1 hr in a sealed ampoule. Examination of the product gave the same result as described under (*a*).

(*c*) *Hydrolysis in presence of 2,4-dinitrophenylhydrazine.* The glucoside (I) (1·1 mg) was dissolved in 2,4-DNPH reagent (2,4-DNPH, 1‰ in 4 N HCl) (2 ml) and the mixture was heated to 100° for 1 hr. The cooled, turbid solution was extracted with Et_2O (2 × 1 ml) and the Et_2O phase was analysed by TLC (2 solvents). A blank test prepared by treating D-glucose in the same manner served as reference. No differences between the two samples could be observed.

(*d*) *Treatment with $Ba(OH)_2$.* The glucoside (I) (1·1 mg) and $Ba(OH)_2$, 8 H_2O (2·2 mg) were dissolved in H_2O (200 μl) and heated to 50° for 45 min in a sealed ampoule. The cooled solution was treated with an excess of Dowex 50 × 8 (H^+) and the acidic solution obtained was examined by TLC. The presence of a non-reducing, non-u.v.-absorbing carbohydrate (β-glucosan) (VIII) was demonstrated together with undecomposed glucoside (I). The reaction mixture also appeared to contain triglochinic acid (IV) in small amount as well as other unidentified acids. NH_3 was detected in the mixture, prior to treatment with the resin, with red litmus paper and Nessler's reagent. A similar experiment, where the reaction was run at 100° for 30 min, led to the same result, except that no starting material (I) could be detected.

Isolation of Triglochinic Acid (IV)

The glucoside (I) (48·7 mg) was dissolved in H_2O (5 ml), a drop of 4 N AcOH was added and the solution was heated to 100° under N_2 for 1 hr. The pale-yellow hydrolysate was evaporated to dryness *in vacuo* and the residue was mixed with acid-washed silica gel (300 mg). The dry powder was transferred to a column of silica gel (10 g) that was eluted with $CHCl_3$—MeOH—HCO_2H, 96:4:0·5, v/v. Concentration of relevant fractions (1 ml) gave a colourless solid that was crystallized from $CHCl_3$—EtOAc to yield triglochinic acid (IV) (17 mg). M.p.: 160–161° (corr.). Found: C, 44·10; H, 4·33. $C_7H_8O_6$ required: C, 44·69; H, 4·29%. No optical activity. Titration (of 2·296 mg) with 0·02 N NaOH indicated a tribasic acid. I.r. spectrum (KBr), ν_{max}: 3500–2200 (s), 1720 (s), 1650 (s), 1472 (w), 1447 (m), 1423 (m), 1381 (w), 1330 (w), 1270–1200 (s), 1157 (w), 1109 (m), 1046 (w), 1020–950 (m), 875 (w), 829 (w), 720 (m), 670 (w) and 620 (w) cm^{-1}. U.v. spectrum: λ_{max} 209 nm (H_2O). NMR spectrum (12 mg in $(D_3C)_2CO$; 250 μl), δ: 10·38, singlet, 3 H (COOH protons), disappears on addition of D_2O and is replaced by a signal at 4·55 (DOH); 7·21, triplet, 1 H (H_B), $J_{AB} = 7$ c/s; 3·44, singlet, 2 H (H_C); 3·88, doublet, 2 H (H_A), $J_{AB} = 7$ c/s, the low field peak of the doublet overlaps completely with the H_C signal, but the coupling was verified by spin-decoupling experiments.

Treatment of I with HCl–MeOH

The glucoside (I) (95·6 mg) was dissolved in AcOCl–MeOH, 0·5:10, v/v (5 ml) and kept at 40° for 22 hr. The product (in which HCN was easily detected) was evaporated *in vacuo* at 25° over NaOH and the semi-solid residue was dissolved in MeOH (0·5 ml) and mixed with silica gel (0·5 g). The dried powder was placed on a column of silica gel (10% H_2O) (12 g), that was eluted stepwise with EtOAc–MeOH, 95:5, v/v (90 ml); EtOAc–MeOH, 92·5:7·5, v/v (60 ml) and EtOAc–MeOH, 9:1, v/v (100 ml). Analysis of the eluate (TLC) and concentration of relevant fractions (5 ml) resulted in isolation of triglochinic acid-tri-*O*-Me-ester (VII) (32 mg), triglochinin-di-*O*-Me-ester (V) (31 mg) and a fraction of methylglucosides (VI) (27 mg).

Triglochinic acid-tri-O-Me-ester (VII). This compound was purified further by column chromatography (silica gel (10% H_2O), (5 g); solvent: Bz–EtOAc, 8:2, v/v) which gave the ester as colourless, mobile oil (30·5 mg). I.r. spectrum (liq. sandwich), ν_{max}: 3650–3400 (w), 2995 (m), 2945 (m), 2840 (w), 1750–1690 (s), 1650 (m), 1433 (s), 1370 (w), 1320 (sh), 1265 (s), 1165 (s), 1115 (m), 1060 (m), 1010 (m), 955 (w), 887 (w), 830 (w), 770 (m) and 730 (w) cm^{-1}. U.v. spectrum: λ_{max} 217 nm (MeOH). NMR spectrum (25 mg in CCl_4; 300 μl), δ: 7·05, triplet, 1 H (H_B), $J_{AB} = 7·5$ c/s, the signals show fine structure due to allylic coupling to H_C; 3·75, 3·68, 3·64, three singlets, 9 H (O—CH_3 protons); 3·30, singlet, 2 H (H_C), slight coupling to H_B; 3·20, doublet, 2 H (H_A), $J_{AB} = 7·5$ c/s. Mass spectrum: Weak signal at *m/e* 230 (M^+).

Triglochinin-di-O-Me-ester (V). Further purification by column chromatography (silica gel (10% H_2O), (5 g); solvent: EtOAc–MeOH, 9:1, v/v) yielded a colourless syrup (29·6 mg). $[\alpha]_D^{20\cdot1} = +12·7°$ (*c.* 0·6 in MeOH). I.r. spectrum (KBr), ν_{max}: 3600–3100 (s), 3000–2840 (w), 2220 (w), 1720 (s), 1620 (w), 1433 (m),

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[15] F. FEIGL and V. GENTIL, *Microchim. Acta* **1**, 44 (1959).
[16] G. G. GUILBAULT and D. N. CRAMER, *Anal. Chem.* **38**, 834 (1966).

1400 (w), 1200 (s, broad), 1170 (s), 925 (w), 891 (w), 822 (w) and 720 (w) cm^{-1}. U.v. spectrum: broad absorption, λ_{max} 280 nm (MeOH). NMR spectrum (25 mg in $(D_3C)_2CO$; 200 μl), δ: 6·84, doublet, 1 H (H_B), J_{AB} = 12·5 c/s, the signals are slightly broadened due to allylic coupling to H_C; 6·15, doublet, 1 H (H_A), J_{AB} = 12·5 c/s; many bands between 5·2 and 2·9, *ca.* 19 H, two sharp signals at 3·72 and 3·66 (singlets) can be attributed to the O–CH_3 groups in (V), whereas the methylene (H_C) signal is not readily recognizable (probably overlapping with the low field O—CH_3 signal). Mass spectrum: Feeble signal at *m/e* 387 (M^+), cf. Results.

Methylglucoside fraction (VI). The solid residue obtained was investigated by TLC (2 solvents) and shown to contain α- and β-methylglucopyranoside (mostly the former) using authentic reference compounds.

Preparation of VII From IV

Triglochinic acid (IV) (12 mg) was treated with AcOCl–MeOH, 0·5:10, v/v, for 48 hr at 40°. The mixture was concentrated *in vacuo* and the residue was chromatographed (silica gel (10% H_2O), (5 g); solvent: Bz–EtOAc, 9:1, v/v). A colourless oil was obtained (9 mg) and shown to be identical with VII prepared directly from I (TLC, i.r., u.v., NMR).

Identification of β-Glucosan (VIII)

Triglochinin (I) (20 mg) and $Ba(OH)_2$, 8 H_2O (50 mg) were dissolved in H_2O (2 ml). The mixture was heated to 100° for 2 hr in a sealed ampoule and the cooled, light-yellow product was treated with Dowex 50 × 8 (H^+). The resulting acidic solution was evaporated to dryness *in vacuo* and the residue was chromatographed (silica gel (10% H_2O); 5 g). Elution with EtOAc–MeOH, 9:1, v/v, resulted in the isolation of a colourless solid that was crystallized from Bz–MeOH to yield β-glucosan (VIII) as colourless needles (2·3 mg); m.p. 183–184° (m.p.-microscope). Its identity was confirmed by direct comparison with an authentic sample by TLC (4 solvents) and i.r. analysis.

Preparation of the Trimethylsilyl Derivative of I

The method of Mabry *et al.*[17] was used. The glucoside (18 mg) was dissolved in dry pyridine, hexamethyldisilazane (HMDS) (0·5 ml) and trimethylsilylchloride (TMSC) (0·5 ml) were added and the mixture was shaken at room temp. for 1 hr. The solvent and excess reagents were removed *in vacuo* (0·3 mm; 20°) and the residue was extracted with CCl_4 (2 × 2 ml). The CCl_4 extract was filtered, concentrated and the yellowish, solid residue was dissolved in CCl_4 (300 μl). HMDS (5 μl) and TMSC (5 μl) were added and the mixture was analysed immediately by NMR. NMR spectrum, δ: 6·69, doublet, 1 H (H_B), J_{AB} = 12·5 c/s; 5·93, doublet, 1 H (H_A), J_{AB} = 12·5 c/s; 4·8, multiplet, 1 H (glucose H_1); many signals in the 4·1–2·9 region, 8 H (a signal at 3·63, singlet, broadened by coupling to H_B, is due to the H_C protons and the remaining 6 H account for the sugar protons); very strong signals at 0·5–0·0 are due to the trimethylsilyl groups as well as some weak bands (spinning sidebands) in the 1·5–0·5 region.

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