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The carbohydrate moieties of some flabelliferins (steroidal saponins) of palmyrah fruit pulp

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Abstract

The trifluoro acetic acid hydrolysed flabelliferins were silylated and analyzed by GC/EI/MS. ^{1}H and ^{13}C-NMR. Methylation studies showed that the carbohydrate moiety of F_D (M.W. 722) contained a rhamnosyl residue linked α - 1.4 to a glucosyl residue. which is attached to β-sitosterol. by a β anomeric linkage. Study of chemical shifts and coupling constants showed that glucose (glc) had a β anomeric configuration. F_B was a steroidal triglycoside (M.W. 868) with a rhamnosyl terminus. The carbohydrate moiety of the saponin showed the presence of a branched structure with two rhamnosyl residues linked α-1.2 and α-1,4 to a glucosyl residue, which is attached to the aglycone by a β glucoside linkage.

Flabelliferin F_F was shown to be a monoglucoside with a β linked glucosyl residue. A new triglycoside F_N (MW 884) was shown to have a carbohydrate moiety of two glucosyl and one rhamnosyl residues with a glucosyl terminus, the second glucosyl residue was attached to β-sitosterol. Another flabelliferin (F_E). which was isolated for the First time, was a diglucoside of MW. 738.

KFY WORDS: Palmyrah fruit pulp, Flabelliferins (F_B, F_D, F_F. F_E). Carbohydrate moiety.

1. Introduction

Palmyrah is a palm commonly found in the arid zones of South Asia and South-East Asia. There are a plethora of steroidal saponins named flabelliferins in palmyrah fruit pulp (PFP). These flabelliferins are of importance due to bitterness, which detracts from use as a food and as well as for their bioactivity.

Steroidal saponins from palmyrah were first reported in 1986 (1). These were reported as a monoglucoside and a monorhamrioside said to be of the aglycone spirost-5-en-3-βol (1). A tetraglucoside (F-I) of MW 1062 and the bitter principle of

PFP, a tetraglycoside (F-II) were isolated and assumed to have the same aglycone (2). The isolation was based on dry cellulose chromatography and crystallization. A mixture of flabelliferins was separated, based on flash chromatography. This mixture contained in addition to F-II, other flabelliferins namely. F_B (a triglycoside) F_C (a triglycoside) and F_D (a diglycoside) (3).

F-II inhibited the Na^+/K^+ ATP^{ase} of ghost red blood cells (4) and thereby was probably responsible for the reduced weight gain observed with Institute of Cancer Research mice (5). Debittering of F-II by the enzyme complex naringinase has been reported (2). F_B was found to be the most foam stabilizing hemolytic and anti-yeast agent (a strain of *Saccharomyces cervisiae* SII F-3) (6). F_B also showed anti-bacterial properties against *Staphylococcus epidermidis. Staphylococcus aureus, Escherichia coli. Pseudomonas aeruginosa, Proteus rettigeri* and *Acinetobacter calcoaceticus* (6). F_B also slowed down the rate of alcoholic fermentation (6). F_C and F_D were reported to be inactive flabellilerins (6). Partial structures of some of these flabelliferins have been reported previously (2.3). Glucose and rhamnose were the only sugars present in the carbohydrate moiety of flabelliferins (2). The aglycone was unambiguously worked out as β - sitosterol (7). F_B and F_C which have the same molecular weight of 868, are triglycosides having a carbohydrate moiety consisting of two rhamnosyl and one glucosyl residues both with rhamnosyl termini (3). F-II was found to be a tetraglycoside of MW 1030 with a rhumnosyl terminus having two glucosyl and two rhamnosyl residues in its carbohydrate moiety (2). F_D was separated and found to be a diglycoside of MW 722 having glucose and a rhamonose in its carbohydrate moiety (3).

The objective of this study was to elucidate the structure of the carbohydrate moiety of some flabelliferins of palmyrah by chemical and spectroscopic studies.

2. Materials and methods

The pulp of ripened fresh palmyrah fruits was collected from Ampara, Anamaduwa, Polonnaruwa and Mannar, in Sri Lanka. Palmyrah fruit pulp was extracted with water (1:1) manually (3).

Isolation of flabelliterins from crude flabelliferins

Isolation of flabelliferins was carried out by using Medium Pressure Liquid Chromatography (MPLC) (8). The impure flabelliferins obtained by using medium pressure liquid chromatography (MPLC) (8) was further separated and purified by MPLC (8).

The purification step was carried out to isolate F_B from the UV-active impurity, which was overlapping with the other flabelliferins on TLC even after separation with the MPLC. Solvent gradient from toluene to methanol with dilutions (0%. 0.78%. 3.125%. 6.25%. 12.5%. 25%. 50% and 100%) were used in the separation and 9 ml fractions were collected, the separation was monitored using TLC (2). The UV active compound was found to elute before the flabelliferin at a higher toluene ratio and pure flabelliferin B that did not show the UV active compound was obtained.

Sequencing of sugars by methylation studies.

Sequencing was carried out by methylation analysis using Hakomori method (9). which included methylation, conversion to aldetol acetates and subjecting to GC/MS analysis.

Structural elucidation of the Flabelliferins

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded with JEOL 500 MHz spectrometer using standard pulse sequences. Spectra were recorded (deuterated methanol solutions) at 25°C and at 45°C. Chemical shifts were reported in parts per million (ppm), using sodium 3-trimethylsilylpropanoate-d_4 (δ H 0.00) and acetone (δ C 31.00) as internal references. Proton carbon correlated spectra (HMQC/HSQC) were obtained with decoupling (10).

Electron Spray Ionization - Mass Spectrometry (ESI/MS)

ES1-MS (11.12) was performed using electron spray ionization with an LCQ ion trap mass spectrometer (Thermo Quest. San Jose. CA). Samples were dissolved in methanol/water (50:50. v/v) and introduced to the electron spray at a flow rate of 10 µl/min. Nitrogen was used as a sheath gas.

Gas Chromatograph/Electron Impact-Mass spectrometry (GC/EI/MS)

This was carried out on a Hewlett Packard 2890 gas chromatograph connected to a Nermag R 10-10H quadrupole mass spectrometer with an electron impact ion source. Interface; 280gr, ion source; 280, electron volt bombardment; 70 scan range; 70-7000 m/z.

Gas Chromatography (GC)

Gas chromatography analysis of methylated sugars was carried out on Hewlett Packard 5890 series II plus gas chromatograph. Conditions were as follows. HP 3365 Chem-Station software control, carrier gas. He; Column (carbowax) was 30 m long, 0.25 mm id and 0.25 µm film thickness.

3. Results and Discussion

Separation and purification of flabellifcrins

The separation of PFP (200 g) by methanol extraction, petroleum ether cleaning, acetone extraction and dry cellulose chromatography resulted in crude flabelliferins (88.8 mg) (2). The MPLC (8) resulted in isolation of pure flabelliferins. F_B(300 mg). F_D (150 mg).F_F (50 mg). F_E (23 mg) and F_N(40 mg). The three flabelliferin. F_E, F_F and F_N were new compounds, which have not been isolated before.

Structure of F_D

ES1/ MS data indicated a molecular mass of 722 by $[2MNa^+]^+$ ion at m /z 1467 in the mass spectrum (4) and by the $[MNa^+)$ ion at m/z 745. This is confirmed that the molecular mass obtained previously (5) and shows the presence of the β-sitosterol. GC/E1/MS results of these two are as follows:

1. 1.5-di-O-acetyl-6-deoxy-2.3.4-tri-O-methylhexitol (rha)
   Retention time (min): 16.25
   m/z 59, 72, 87, 89, 101, 115, 131, 175

2. 1,4,5-tri-O-acetyl2.3.6-tri-O- methylhexitol (glc)
   Retention time (min): 24.38
   m/z 58, 59, 71,75,87, 99, 101, 113, 233

Sugar linkages-by methylation analysis

The linkages, by which the sugars are connected, were determined by methylation analysis followed by acetylation formation of 1,5-di-O-acetyl-6-deoxy-2,3,4-tri-O-methylhexitol (rha) (11), and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylhexitiol (glc). It indicated that the rhamnose is 1- 4 linked to glucose.

NMR studies

its ^{1}H-NMR gave a double doublet at δ 4.23 ppm. which corresponded, to H of C-3 of the rhamnosyl residues. A multiplet was given at δ 3.66 ppm. which corresponded to 3H of C-6 of the rhamnosyl residues, which is a characteristic feature of methyl group of rhamnose (12.13). For F_D, 2 anomeric proton signals appeared at δ 4.39 ppm (*J=7.79 Hz*) and at δ 4.85 ppm (J=1.83 Hz) respectively indicating the presence of an α-and a β-pyranoside. Results showed that the rha had a α configuration and the glc having the β configuration.

The HSQC spectrum showed very clearly the rhamnose and the glucose peak (11). The above spectral evidence indicates that the carbohydrate moiety of F_D is β-sitosterol-β-glc-α-1,4 rha.

Structure of F_B

Methylation studies

The linkages by which the sugars are connected, were determined by methylation followed by acetylation (9), which yielded l,5-di-O-acetyl-6-deoxy-2.3.4-iri-O-methylhexitol and l,2,4,5-tetra-O-acetyl-3,6-di-O-methylhexitol (glc). Glc of the sugars indicated that the ratio of glc to rha was 1: 2 and the rha was linked 1.2 and 1,4 to glc.

GC/EI/MS results were as follows:

1. 1.5-di-O-acetyl-6-deoxy-2. 3. 4-tri-O-methylhexitol (rha)
   Retention time (min): 16.25
   m/z: 59.72.89.101.115,117.131.161.175
2. 1.2.4.5- tetra-O-acetyl-3. 6-di-O-methylhexitol (glc)
   Retention time (min.): 27.13
   m/z 71.87.99. 113. 129. 173. 189.

Gas chromatographic and mass spectral data of the methylated F_B, by Hakomori method (9) showed l,5-di-O-acetyl-6-deoxy-2. 3, 4-tri-O-methylhexitol and 1.2,5-tetra-O-acetyl-3,6-di-O-methylhexitol to be in the ratio of 2:1. Showing that the glucosyl residue was linked in the 2 and 4 positions by two rhamnosyl residues.

NMR data

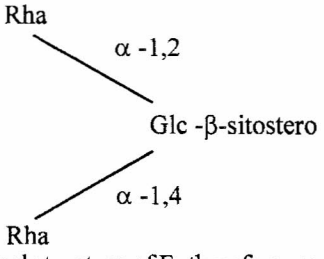
For F_B, 3 anomeric proton signals appeared at δ 4.50 ppm (J = 7.79 Hz), 4.85 ppm (J= 1.83 Hz) and at δ 5.22 ppm (J = 1.83 Hz) respectively indicating two a - and one β links.

Two signals by ^{13}C-NMR of F_B at δ 101.32 ppm and δ 102.01 ppm corresponded to the anomeric carbon of the two rha and the third signal at δ 99.42 ppm corresponded to anomeric carbon of glc. The C-2, C-3. C-4. C-5 and C-6 of β - glc and C-2, C-3, C-4 and C-5 of α-rha were seen in the region of δ 61.01-76.00 ppm. The C-6 for the two rha's gave carbon chemical shifts at δ 16.88 ppm and δ 16.98 ppm. NMR studies showed that the two rha's have an α configuration and the glc has a β configuration.

The ^{1}H-NMR gave a double doublet at δ 4.23 ppm. Which corresponded. to H of C-3 of the two rhamnoses. A multiplet was shown at δ 3.66 ppm. which corresponded to 3H of C-6 of the two rhamnoses. hence rhamnoses moiety is present in F_B

The HMQC spectrum showed very clearly the glc peak. (δ 4.50, 99.42). and the two rha peaks (δ 4. 85, 102. 01:δ 5.22,101.38) ppm (1 1).

The structure of the carbohydrate moiety of F_B was therefore two rha linked α 1,2 and α 1.4 to glc which is β and linked to the aglycone.



The unequivocal structure of F_B therefore, could be a prelude to the synthesis of the compound for clinical trials planned for use of the compound as a topical ointment.

Structure of F_F

Mcthylation studies

The products obtained for the flabellliferin. F_F showed a peak on gas chromatographic which corresponded to 1.5-di-O-acetyl-2. 3. 4. 6-tetra-O-methylhexitol (glc). GC/EI/MS results are as follows;

1.5-di-O-acetyl-2, 3,4,6-tetra-O-methvlhexitol (rha).
Retention lime (min): 24.38
m/z: 59, 71, 87,101, 129, 145, 189, 205

NMR data

^{1}H NMR of F_F showed a signal, which corresponded, to a glc at δ 4.44 ppm (J = 7.79 Hz.) confirming β configuration of glc.

^{13}C-NMRof F_F showed signals corresponding to. C-l (101.8), C-2 (73.76), C-3 (76.72), C-4 (70.31), C-5 (76.54), C-6 (61.38) of glc.

The HSQC spectrum showed glucose peak as the only sugar peak (11). F_F was therefore a β- monogiucoside.

Partial structures of F_N and F_E

ESI/MS of F_N showed a molecular ion peak of m/z 884, which indicated the presence of two glucosyl and one rhamnosyl residues in its carbohydrate moiety. MS-MS on m/z 884. resulted in a molecular ion peak of m/z 722 and m/z 576 confirming the presence of a glc terminus linked to a rhamnosyl residue and the linkage of the second glc to β - sitosterol. Hence F_N is a triglycoside containing 2 glc and 1 rha molecules. F_N is not commonly found in palmyrah fruit pulp. Its structure could not be elucidated due to inadequacy of sample.

E1S/MS of F_F- indicated that its' MW=738 hence F_F contains 2 glc.

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