

Glycosylated stigmasterol from the rind of *Napoleonaea imperialis*

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Abstract. *Napoleonaea imperialis* rinds were separated from the seeds and extracted via maceration using methanol. The crude methanol extract was partitioned with *n*-hexane and dichloromethane to yield *n*-hexane fraction (11.01 g), dichloromethane fraction (35.16 g) and methanol fraction (101.75 g). Dichloromethane fraction was chromatographed and purified to give a compound whose structure was elucidated using one-dimension and two-dimension nuclear magnetic resonance (NMR) experiments. The isolated compound was characterized as glycosylated stigmasterol.

Keywords: *Napoleonaea imperialis*; rind; chromatography; saponins; glycosylated stigmasterol.

1. Introduction

Natural products have been the source of most active ingredients of medicine, innovative therapeutic agents for infectious diseases (both bacterial and fungal), cancer, lipid disorders and immunomodulation, and provide starting point for new synthetic compounds with diverse structures and multiple stereocenters [1-3]. The oldest phytochemical-based drugs are analgesics which contain salicin [4], opium with a potent narcotic component (morphine) and taxol a chemotherapeutic drug isolated from *Papaver somniferum* and *Taxus brevifolia* respectively [4-6]. *Napoleonaea imperialis* P. Beauv. (Family Lecythidaceae), commonly called Napoleon's Hat is known as "ukpodu" or "ukpakonrisa" in southern Nigeria and predominantly found in bush fallows and secondary bushes [7]. The bark and fruit rind are used to treat respiratory tract infections while the twigs are used as traditional chew stick for oral hygiene [8, 9]. A molluscicidal triterpenoid saponin (Napoleonaside A) and a novel bioactive triterpenoid saponin (Napoleonaside B) have been isolated from the seed of *N. imperialis* [10, 11]. Two new oleanane-type glycosides, Napoleonaside G and Napoleonaside R, and other compounds of pharmaceutical interest have also been found in the rind extract of *N. imperialis*; while neral and geranial are major compounds reported to be present in the essential oil of *N. imperialis* rind and are responsible for the characteristic aroma of the oil [12, 13]. This paper reports the isolation and structural elucidation of glycosylated stigmasterol from the extract of *Napoleonaea imperialis* rind.

2. Experimental

2.1. Materials

Chemicals used for this work include deuterated dimethylsulphoxide (DMSO-*d*₆), acetic anhydride and sulphuric acid (all products of Sigma-Aldrich). All solvents used were redistilled. Analytical thin layer chromatography (TLC) was conducted on silica gel (Merck F₂₅₃) precoated aluminum plates. Vacuum liquid

chromatography and column chromatography were carried out using silica gel 70-230 and 60-200 mesh ASTM (60H Merck) respectively.

2.2. TLC spots detection

One dimension TLC was done by spotting the isolate on a 5x5 cm TLC plate and developed. Detection of spots on developed TLC plate was done under UV light at wavelength of 254 nm and further detection was done using spray reagent (10 % sulphuric acid in methanol) followed by heating at 105 °C for 2-5 minutes.

2.3. Extraction and partitioning

Fresh fruits of *N. imperialis* were harvested from Owerri, Imo state, Nigeria. The rinds were separated from their seeds, chopped, air-dried, and pulverized at room temperature (25 °C), and subsequently weighed. Methanol (1.4 L) was used to macerate the pulverized rind (617.2 g) for 48 hours. The resulting extract was concentrated to dryness using a rotary evaporator (Labrota 4002) at 40 °C to afford a brown sticky crude extract (148.12 g). The crude extract was dissolved in 200 ml methanol and subsequently partitioned successively between 400 ml *n*-hexane and 400 ml dichloromethane in a separatory funnel. Each of the partitioned fraction was concentrated to yield *n*-hexane fraction (11.01 g), dichloromethane fraction (35.16 g) and methanol fraction (101.75 g) which were preserved for further analysis.

2.4. Chromatography

Vacuum liquid chromatography method was used for this separation [14]. Briefly, 32.97 g of dichloromethane fraction and 20 g of silica gel were properly mixed to obtain a light brown powder. The mixture was poured into a sintered glass funnel (9.8 cm x 10.4 cm) that was already packed with 130 g of silica gel (4.5 cm) and was evenly distributed to a height of 6 cm. Gradient elution using different binary solvent systems of *n*-hexane (*n*-Hex), ethyl acetate (EtOAc) and methanol (MeOH) were carried out and fractions of 200 ml each collected. A total of 25 fractions were obtained and combined based on TLC analysis to give 13 major fractions (F₁ -

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F₁₃). The fractions were subsequently dried at 25 °C, weighed and stored in glass vials.

2.5. Purification

F₉ (light brown powder) which eluted with EtOAc-MeOH (9.5:0.5) mobile phase from VLC weighed 1.9 g. Column chromatography was employed in the purification of F₉. F₉ was chromatographed over 60 g of silica gel packed in a glass column (91 cm x 2.5 cm) and eluted with EtOAc - *n*-Hex (6:4), EtOAc - *n*-Hex (8:2), 100% EtOAc and EtOAc - MeOH (9.5:0.5). A total of 75 fractions of 20 ml each were collected. Fractions 11-38 (which eluted with EtOAc - *n*-Hex, 8:2) were combined to give 500 mg of a white amorphous compound which precipitated out from the solution. TLC analysis using 100% EtOAc as mobile phase, showed that the compound was not visible under the UV light but gave a purple spot with R_f value 0.4 after the developed TLC plate was treated with spray reagent and heated.

2.6. Confirmatory test

Freshly prepared Liebermann-Burchard reagent (1 ml) was slowly added to 5 mg of the isolated compound dissolved in 1 ml dichloromethane [14]. A green colouration was observed.

2.7. NMR spectroscopy

All NMR data were acquired at 25 °C on a Bruker 600 MHz spectrometer (¹H, 600.06 MHz) equipped with a 5 mm cryoprobe. The sample (~ 6 mg) was solubilized in 550 μL DMSO-*d*₆ solution after briefly heating at 45 °C and mixing. The solution was then transferred to a 5 mm NMR tube. ¹H {¹³C} NMR experiment was acquired with spectral width of 9090.9 Hz, 32768 data points, 8 scans and 1.5 s relaxation delay. ¹³C NMR experiments were acquired with spectral width of 35714.3 Hz, 32768 data points, 3840 scans and 1.5 s relaxation delay. 2D NMR data were acquired with ¹H spectral widths of 4424.8 Hz (COSY, TOCSY and NOESY), ¹H/¹³C spectral widths of 4424.8 Hz / 22123.9 Hz (HSQC and HSQC-TOCSY) and ¹H/¹³C spectral widths of 4424.8 Hz / 22123.9 Hz (HMBC). COSY data was acquired with 2 scans and 1536 * 768 (t1 * t2) points, TOCSY data was acquired with 8 scans and 1024 * 384 (t1 * t2) points, NOESY data was acquired with 20 scans and 1280 * 512 (t1 * t2) points, HSQC data was acquired with 8 scans and 1024 * 768 (t1 * t2) points, HSQC-TOCSY data was acquired with 16 scans and 871 * 768 (t1 * t2) points and HMBC data was acquired with 44 scans and 1536 * 256 (t1 * t2) points. Chemical shifts were referenced to the residual DMSO signal at 2.50 ppm (¹H) and 39.5 ppm (¹³C). All spectra were processed and analyzed with MestreNova (version 14.0.1-23559).

3. Results and discussion

The green colour observed when fresh Liebermann-Burchard reagent was added to the compound indicated the presence of a steroidal skeleton [15-17] and the purple spot observed after treatment with the spray

reagent and heating also confirmed the presence of steroidal sapogenin [18]. The isolate's invisibility under the UV light is a strong indication that there is absence of conjugated double bonds in its structure. Structure of the isolate (Figure 1) was determined using ¹H NMR and ¹³C NMR experiments (Figures 2 and 3) aided by connectivities observed in HSQC, HMBC, COSY, HSQC-TOCSY and NOESY experiments (Table 1).

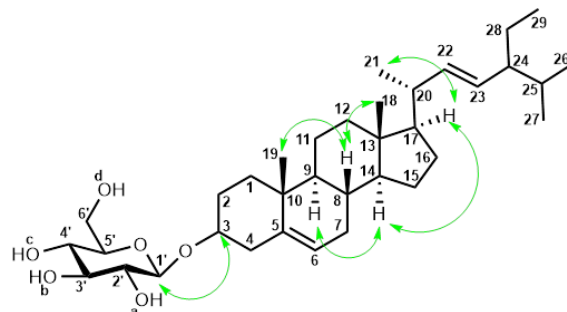


Figure 1. Structure and numbering of glycosylated stigmasterol used in this report. The sugar moiety was identified as β-glucose from TOCSY spin-lock pattern and ¹H/¹³C NMR chemical shifts. NOESY correlations that establish stereochemistry is indicated with green arrows. The stereochemistry at C-3 and C-24 could not be established with the current dataset.

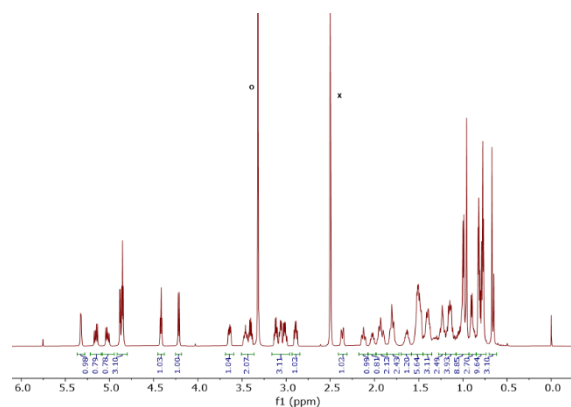


Figure 2. ¹H NMR spectrum of glycosylated stigmasterol acquired in DMSO-*d*₆. Signal from solvent moisture (water) is marked with 'o' while DMSO residual solvent signal is marked with 'x'.

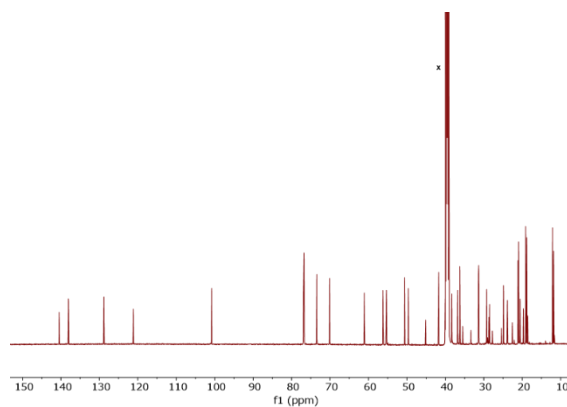


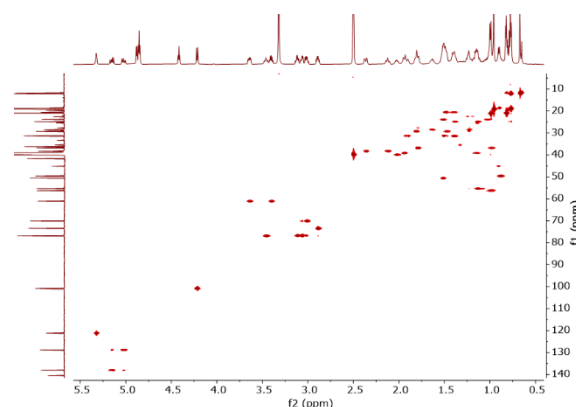
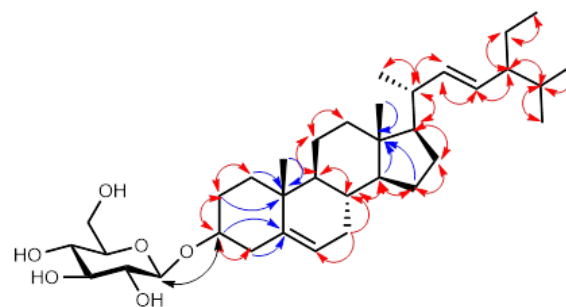
Figure 3. ¹³C NMR spectrum of glycosylated stigmasterol acquired in DMSO-*d*₆. Signal from DMSO is marked with 'x'. Minor peaks are impurity signals of a glycosylated molecule not assigned.

Table 1. ^{13}C and ^1H chemical shift data of glycosylated stigmasterol in $\text{DMSO-}d_6$

Aglycone			Sugar moiety		
Position	$\delta^{13}\text{C}$	$\delta^1\text{H}$	Position	$\delta^{13}\text{C}$	$\delta^1\text{H}$
1	36.8	1.79 (1H, m), 0.99 (1H, m)	1'	101.8	4.22 (1H, d, 7.8 Hz)
2	29.3	1.81 (1H, m), 1.48(1H, m)	2'	73.5	2.89 (1H, ddd, 8.9 Hz, 7.8 Hz, 4.8 Hz)
3	76.9	3.46 (1H, tt, 11.3 Hz, 4.2 Hz)	3'	76.8	3.12 (1H, td, 8.8 Hz, 4.8 Hz)
4	38.3	2.36 (1H, m), 2.12 (1H, m)	4'	70.1	3.01 (1H, td, 9.1 Hz, 5.1 Hz)
5	140.5	-	5'	76.8	3.06 (1H, ddd, 9.9 Hz, 5.9 Hz, 2.1 Hz)
6	121.2	5.32 (1H, m)	6'	61.1	3.64 (1H, td, 11.7 Hz, 5.9 Hz), 3.40 (1H, ddd, 11.5 Hz, 5.7 Hz, 2.0 Hz)
7	31.4	1.92 (1H, s), 1.49 (1H, m)	^a O		4.86 (1H, d, 5.2 Hz)
8	31.3	1.51 (1H, m)	^b O		4.88 (1H, d, 4.8 Hz)
9	49.6	0.89 (1H, m)	^c O		4.85 (1H, d, 5.4 Hz)
10	36.2	-	^d O		4.41 (1H, t, 5.8 Hz)
11	20.6	1.49 (1H, m), 1.40 (1H, m)			
12	39.1	1.94 (1H, m) 1.15 (1H, m)			
13	41.7	-			
14	56.3	0.99 (1H, m)			
15	23.9	1.52 (1H, m), 1.02 (1H, m)			
16	28.5	1.64 (1H, m), 1.23 (1H, m)			
17	55.3	1.13 (1H, m)			
18	11.9	0.67 (3H, s)			
19	19.1	0.96 (3H, s)			
20	39.9	2.02 (1H, m)			
21	21.1	0.99 (3H, d, 6.6 Hz))			
22	138.0	5.15 (1H, dd, 15.2 Hz, 8.7 Hz)			
23	128.8	5.02 (1H, dd, 15.2 Hz, 8.7 Hz)			
24	50.6	1.52 (1H, m)			
25	31.4	1.39 (1H, m)			
26	18.9	0.77 (3H, d*)			
27	20.9	0.82 (3H, d*)			
28	24.9	1.39 (1H, m), 1.13 (1H, m)			
29	12.1	0.77 (3H, m*)			

* Overlapping signals

Thirty-five carbons were identified from ^{13}C NMR and HSQC spectra (Table 1); these include one glucose anomeric carbon signal at $\delta 101.8$ (C-1') and five ring residue signals, $\delta 73.5$ (C-2'), $\delta 76.8$ (C-3'), $\delta 70.1$ (C-4'), $\delta 76.8$ (C-5'), $\delta 61.1$ (C-6') belonging to the sugar moiety. The glucose residue was identified from the corresponding anomeric proton TOCSY spinlock pattern while the glycosyl linkage configuration was determined as beta (β) based on the ^1H chemical shift at 4.22 ppm (Figure 1). ^1H NMR and ^{13}C NMR assignments, aided by heteronuclear single quantum coherence (HSQC) spectrum (Figure 4) and verified with other two dimension (2D) spectra (Figures 5 and 6), show that the isolate (Figure 1) has one unsaturated and two saturated quaternary carbons, $\delta 140.5$ (C-5), $\delta 36.2$ (C-10) and $\delta 41.7$ (C-13); six primary carbons (methyl groups), $\delta 11.9$ (C-18), $\delta 19.1$ (C-19), $\delta 21.1$ (C-21), $\delta 18.9$ (C-26), $\delta 20.9$ (C-27) and $\delta 0.77$ (C-29); ten secondary carbons (methylenes), $\delta 36.8$ (C-1), $\delta 29.3$ (C-2), $\delta 76.9$ (C-3), $\delta 38.3$ (C-4), $\delta 31.4$ (C-7), $\delta 20.6$ (C-11), $\delta 39.1$ (C-12), $\delta 23.9$ (C-15), $\delta 28.5$ (C-16) and $\delta 24.9$ (C-28); and ten tertiary carbons, $\delta 121.2$ (C-6), $\delta 31.3$ (C-8), $\delta 49.6$ (C-9), $\delta 56.3$ (C-14), $\delta 55.3$ (C-17), $\delta 39.9$ (C-20), $\delta 138.0$ (C-22), $\delta 128.8$ (C-23), $\delta 50.6$ (C-24) and $\delta 31.4$ (C-25) assigned to the aglycone. Protons at $\delta 4.86$ (H-^aO), $\delta 4.88$ (H-^bO), $\delta 4.85$ (H-^cO), $\delta 4.41$ (H-^dO) that are not attached to carbons in the HSQC spectrum were assigned as the sugar hydroxy protons.

**Figure 4.** HSQC NMR spectrum (non-edited version) of glycosylated stigmasterol acquired in $\text{DMSO-}d_6$.**Figure 5.** Glycosylated stigmasterol showing COSY and HSQC-TOCSY connectivity with red arrows. Key HMBC connectivity to quaternary carbons are shown with blue arrows.

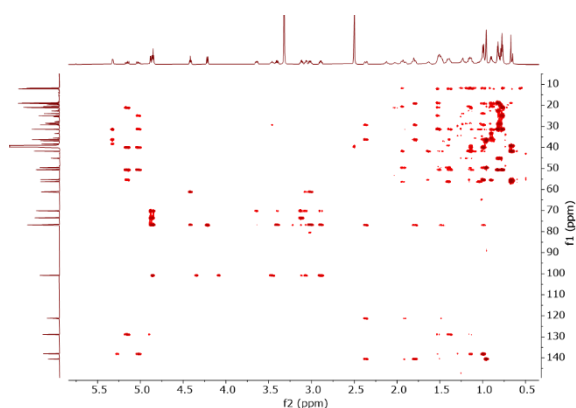


Figure 6. HMBC NMR spectrum of glycosylated stigmasterol acquired in DMSO- d_6 .

Cross peaks from NOESY (Figure 1) show that the anomeric proton $\delta 4.22$ (H-1') correlated with $\delta 3.46$ (H-3) of the aglycone; proton at $\delta 4.86$ (H-^aO) correlated with $\delta 4.22$ (H-1), $\delta 3.06$ (H-5') and $\delta 4.41$ (H-^dO); $\delta 4.88$ (H-^bO) correlated with $\delta 4.22$ (H-1'') and $\delta 3.12$ (H-3') while $\delta 4.85$ (H-^cO) correlated with $\delta 3.01$ (H-4'), $\delta 4.41$ (^dO) and $\delta 2.89$ (H-2''); $\delta 4.41$ (H-^dO) correlated with $\delta 4.86$ (H-^aO), $\delta 4.85$ (H-^cO), $\delta 3.64$ (H-6') and $\delta 3.06$ (H-5'). HSQC-TOCSY (Figure 5) shows that carbon $\delta 101.8$ (C-1') directly correlated with $\delta 4.22$ (H-1') of the sugar moiety is correlated with $\delta 3.46$ (H-3) which is also directly correlated with $\delta 76.9$ (C-3) of the aglycone. Protons at $\delta 4.86$ (H-^aO), $\delta 4.88$ (H-^bO), $\delta 4.85$ (H-^cO), $\delta 4.41$ (H-^dO) showed no correlation in HSQC-TOCSY.

Two olefinic carbon signals at $\delta 140.5$ and $\delta 121.2$ were assigned to C-5 and C-6, while another pair of olefinic carbons $\delta 138.0$ and $\delta 128.8$ were designated C-22 and C-23, respectively, based on connectivity from neighbouring signals in the 2D NMR data. C-6, C-22 and C-23 are trisubstituted olefinic linkages. These peaks are reported as the striking characteristics of stigmasterol [17, 19-22]. Connectivities from long range HMBC (Figures 5 and 6) show that the olefinic carbons $\delta 140.5$ (C-5) and $\delta 121.2$ (C-6) are correlated with $\delta 2.36$ (H-4) and $\delta 1.49$ (H-7) respectively, while the second pair of olefinic carbons $\delta 138.0$ (C-22) and $\delta 128.8$ (C-23) are correlated with $\delta 2.02$ (H-20) and $\delta 1.52$ (H-24). The large coupling of 15.2 Hz between H_{22} and H_{23} (Table 1) is indicative of protons in anti-parallel orientation as is the case for the E-isomer of the double bond shown (Figures 1 and 5). HMBC also shows correlation between $\delta 101.8$ (C-1') and $\delta 3.46$ (H-3) which confirmed that the sugar moiety is attached to C-3 position of the aglycone. COSY (Figure 5) shows that the isopropyl group, $\delta 31.4$ (C-25), $\delta 18.9$ (C-26) and $\delta 20.9$ (C-27) correlated with $\delta 1.52$ (H-24) and $\delta 1.13$ (H-28). Correlations observed in the 2D experiments were similar to those reported for stigmasterol isolated from *Heliotropium indicum* Linn [23]. From 2D experiments, all proton signals belonging to the sugar moiety were assigned and the position of attachment to C-3 of the aglycone was also confirmed. The sugar moiety carbons were similar to that of the steroidal saponin isolated from *Dioscorea panthaica* [24]. All range correlations observed were consistent with the structure in Figure 1. The isolate was confirmed as glycosylated stigmasterol (Figure 1) after comparison with literature [19, 20, 25].

A review on the pharmacological studies of stigmasterols shows their anti-osteoarthritic, anti-hypercholesterolemic, cytotoxicity, anti-tumor, antimutagenic, antioxidant, hypoglycemic, antifungal activities, and analgesic potential [26-30]. Steroidal saponins are generally reported to have anthelmintic, immunomodulatory, anti-inflammatory, anti-melanogenic and anti-thrombotic activities [31-35].

4. Conclusions

Napoleonaea imperialis rind is a rich source of glycosylated stigmasterol, a pharmacologically useful compound. Isolation and characterization of glycosylated stigmasterol were successfully achieved using various chromatographic methods and NMR experiments.

Acknowledgement

The authors are grateful to the research group of Dr. Parastoo Azadi of the Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia, U.S.A. for work on the NMR characterization (supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences, under Award # DE-SC0015662) and the Tertiary Education Trust Fund (TETFund), Nigeria for funding this research.

Conflict of interest

Authors declare no conflict of interest.

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Received: 11.12.2021

Received in revised form: 06.02.2022

Accepted: 11.02.2022