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New anti-trypanosomal active prenylated compounds from African propolis

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ABSTRACT

The secondary metabolite composition of propolis has always been corroborated on the vegetation surrounding the bee hive. The plants chosen by the bees seem to exhibit activity to protect the hive from the surrounding environmental pressures which include protozoal attack. Two new stilbene compounds were isolated from Ghanian propolis which were formed either via geranylation or prenylation. These compounds exhibited moderate activity against *Trypanosoma brucei brucei*. While a new phloroglucinonone analogue was isolated from a Cameroon propolis that was found to possess high potency comparable to that of suramin.

1. Introduction

Propolis is a resinous bee product composed of beeswax and resin collected by honey bees from exudates of various plants within their environment and is then carried to the hive then blended with the beeswax. The substance is utilised to seal and maintain hives and may be generally used as an anti-infective substance (Burdock, 1998 and Bertelli et al., 2012). Propolis has been widely used as folk remedy for a long time with a range of pharmacological activity being reported, including: antifungal, antioxidant, antimicrobial, and anti-inflammatory activity (Abu-Mellal et al., 2012; Tran et al., 2012). Also, propolis has been shown to be active against honey bee parasites (e.g. *Paenibacillus larvae*) (Salatino et al., 2011). While flavonoids and phenolic acids are the major classes of compounds in propolis from temperate regions, the compounds in propolis from tropical regions especially those from the African continent are often less well known (Bertelli et al., 2012). To date, about 300 compounds have been identified in propolis (Salatino et al., 2011; Marcucci, 1995) from different geographical origin. Propolis is composed of 50% resin and balsams, 30% wax and fatty acid, 10% aromatic and essential oil and 5% of pollen and various other substances (Burdock, 1998). However, the secondary metabolite composition of propolis depends on its geographical origin and the vegetation surrounding the bee hive. Although it has never been proven, it would seem likely that the plants chosen by the bees would exhibit activity against the environmental pressures encountered by the bees which include protozoal attack (Schwarz and Evans, 2013). Within this context, our study investigated the effect of African propolis on the parasite *Trypanosoma brucei brucei*, which is the etiologic agent of sleeping sickness or human African trypanosomiasis (HAT), an endemic parasitosis in sub-Saharan Africa infecting 500,000 people who may die if left untreated.

2. Methods and materials

2.1. General procedures

All solvents were from Merck, Darmstadt in HPLC grade. All sample extracts and fractions were dried under N_2. Optical rotation was measured in $CHCl_3$ at 20 °C using a Perkin–Elmer 241 polarimeter with a sodium lamp. The NMR spectroscopic data [1D: ^1H and ^{13}C NMR, DEPT 135; 2D: COSY, heteronuclear single quantum correlation (HSQC), HMBC, NOESY] were recorded with a JEOL-LA400 FT-NMR spectrometer system (^1H NMR at 400 MHz,

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^{13}C NMR at 100 MHz) (JEOL Ltd., UK) with an AS400 magnet using a Pulse Field Gradient "Autotune" 40TH5AT/FG broadband high sensitivity probe using the appropriate deuterated solvents. Chemical shifts are given in ppm, and coupling constants are in Hz. MestReNova 8.1.2 was used to process the NMR spectroscopic data. HRMS (ESI) were measured with an LTQ Exactive from Thermo Scientific, Dreieich, Germany. TLC was performed on silica glass plates (Si60 F254 from Merck) and RP18 silica glass plates (RP18-F254 from Merck). The chromatograms were observed under short and long wavelength UV light at 254 and 366 nm, respectively. The plates were subsequently sprayed with anisaldehyde/H_2SO_4 reagent.

HPLC–MS analysis was carried out using a Dionex UltiMate® 3000 HPLC system (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) A silica type 75.0 × 3.0 mm^2 C18 ACE HPLC column (HiChrom Ltd., UK), particle size of the silica was 5 mm, pore size 100 Å with a pore volume of 1.0 ml/g and a surface area of 300 m^2/g was used. The column was eluted employing a linear gradient from 90% A (0.1% v/v formic acid in water) and 10% B (0.1% v/v formic acid in acetonitrile) to 100% B mobile phases at a flow rate of 0.4 mL/min over 0–35 min then for a further 5-min for isocratic elution, and a return to starting conditions at 40 min for re-equilibration for the last 5 min, using up a total of 45 min for the run. MS identification and analysis were carried out using a ThermoScientific Exactive (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) with a 10 μl injection volume and the mass spectrometer was operated with UV detection at 254 nm using both positive and negative ion modes of detection. Full scan data were collected from 50 to 2000 *m/z* (mass to charge ratio) with a scan time of 0.1 s. Capillary and cone voltages were set at 3.5 kV and 35 V, respectively; desolvation and source temperatures at 300 °C and 120 °C, respectively. The presence of triterpenoids was analysed with Thermo Quest CE instruments, Trace GC 2000 series GCMS. Initial temperature was at 100 °C, initial time of 1.00 min, with 1 ramp at a rate of 20.0 deg/min to a final temperature of 320 °C. EIMS is set at detector voltage of 500.0 V with a source temperature of 200 °C while the GC interface temperature is at 250 °C with an emission current of 150 μV. Full scan acquisition is at 2.5 scans per second where the scan range starts at 65 amu to end at 485 amu. Syringe volume is at 10 μl. The column was a Factory Four™ capillary (VF-1ms 30 m × 0.25 mm, 0.25 μm).

Preliminary fractionation of the crude extract was done through a Büchi flash chromatography subsequently employed for the fractionation process. The flash system consisted of a Büchi Pump Manager C-615 coupled to binary pumps Modules C-601. The extracts were dissolved in suitable solvent (EtOAc and dichloromethane, respectively), and incorporated with diatomaceous earth or kieselguhr (Celite) to serve as sample carrier for dry loading. The homogenous mixture was subsequently dried. The extracts were then subjected to chromatographic separation over a silica gel column (40 × 150 mm, 96 g, Silica VersaPak cartridge, Bellefonte, U.S.A) with linear gradient elution with hexane (A) and EtOAc (B) at a flow rate of 100 ml/min. This was achieved with the elution of 100% A which was held for 5 min followed by100%A to 100%B for the next 40 min and held at 100%B for a further 5 min, altogether a total run of 45 min. The column was finally flushed with 100% EtOAc and 500 ml of 50:50 acetone/MeOH for another 5 min. Fractions were collected at 100 ml volumes. Flash chromatography was carried out on fractions by using the Grace Reveleris® and Reveleris® iES Flash Chromatography System equipped with a dual-UV wavelength at 210 and 280 nm and ELSD (3 mV) detector and fraction collector. The column used was a 24 g Grace Resolv Silica. Semi-preparative HPLC was performed with a SpectraSYSTEM AS3000 equipped with a Type 7010-150 Rheodyne injection valve (100 μl loop), a SpectraSYSTEM P2000 gradient pump and a SpectraSYSTEM UV1000 detector (Thermo Separation Products, Inc.). The column used was an ACE 5 SIL semi-preparative HPLC column 250 mm × 10 mm I.D., 5 μm particle size, (HiChrom Ltd). Samples were injected using a concentration of 50 mg/ml. The detection wavelength was set at 295 nm and 320 nm. Data were acquired and processed using ChromQuest software.

2.2. Materials

Propolis resin samples from Ghana and Cameroon coded S87 and S263, respectively were provided by BeeVital.

2.3. Extraction and isolation

Propolis resins (108 g for S87 and 50 g for S263) were coarsely ground and were extracted three times each for 4 h with 250 mL acetone and methanol with stirring. The total crude extracts were dried under vacuo. The obtained extracts were subjected to liquid–liquid fractionation by partitioning between EtOAc and water. The ethyl acetate was dried to yield a sticky material (28.5 g for S87 and 35 g for S263). Initial fractionation was done on the Büchi Flash Chromatography to provide nineteen fractions from each of the extract. Fractions were tested for inhibitory activity against *T. brucei brucei*.

From S87, polar fractions F9 and F13 were found to be most active. Fraction 9 (330.9 mg) was further purified by semi-preparative HPLC with isocratic elution of ethyl acetate:hexane (10:9) for 60 min at a flow rate of 5 ml/min which yielded compound **1** (4.5 mg; UV_{max} 244,289, and 328; $[\alpha]_D$ +2.7° (*c*. 0.10, $CHCl_3$)) at a retention time of 36.25 min. Fraction 13 (2.7 g) was further purified with the Grace Reveleris® iES Flash Chromatography System equipped with a dual-UV wavelength at 210 and 280 nm and ELSD detector. Fraction 13 was fractionated over Silica with hexane and ethyl acetate as solvents system. The gradient started 10% EtOAc then went to 20% EtOAc after 12 min. EtOAc was increased by 5% for every 12 min till 45% EtOAc then for the next 40 min, EtOAc was increased by 20% for every 10 min till it reaches 100%. This furnished thirteen fractions and the compound (**2**, 55 mg; UV_{max} 231 and 325) of interest was found in fraction 11 with a retention time 59.30 min.

For S263, it is the non-polar fractions 3 (F3, 12.5 g), 4 (F4, 6.7 g), and 6 (F6, 3.3 g) that were found to exhibit highest bioactivity against *T. brucei brucei*. Fraction 3 was purified over a Versa Pak, spherical Silica (23 × 110 mm, 23 g) column employing gradient elution of hexane: EtOAc from 0% to 85% EtOAc in 35 min at a flow rate of 20 ml/min on the Büchi MPLC set-up. The more non-polar fractions yielded ursolic acid (393 mg) and keto-α/β-amyrin (273 mg). Prenylated phloroglucinone, **3** (14 mg) was obtained as colourless oil from fraction 11, the relatively more polar sub-fraction of F3 with Rf of 0.25 in hexane: EtOAc (85:15) on silica plates. [α]D +75.2° (*c*. 0.13, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.40 (t, $^3J_{H28,H27} = {}^3J_{H28,H29}$ = 7.2 Hz, 1H, H-28), 7.28 (t, $^3J_{H27,H26} = {}^3J_{H27,H28} = {}^3J_{H29,H28} = {}^3J_{H29,H30}$ = 8.0 Hz, 2H, H-27 and H-29), 7.16 (d, $^3J_{H26,H27} = {}^3J_{H30,H29}$ = 8.0 Hz, 2H, 26-H and H-30), 5.09 (t, $^3J_{H32,H31A} = {}^3J_{H32,H31B}$ = 6.9 Hz, 1H, 32-H), 4.94 (dd, $^3J_{H3,H2A}$ = 11.7 Hz, $^3J_{H3,H2B}$ = 2.9 Hz, 1H, H-3), 3.52 (dd, $^3J_{H2A,H3}$ = 11.7 Hz, $^2J_{H2A,H2B}$ = 15.0 Hz, 1H, H-2A), 2.78(dd, $^3J_{H7,H8A}$ = 8.2 Hz, $^3J_{H7,H8B}$ = 10.7 Hz, 1H, H-7), 2.59 (d, $^3J_{H31A,H32}$ = 6.5 Hz, 1H, H-31A), 2.57 (dd, $^3J_{H15A,\ H9}$ = 6.6 Hz, $^2J_{H15A,H15B}$ = 16.3 Hz, 1H, H-15A), 2.39 (m, 1H, H-8A), 2.10 (m, 1H, 9-H), 1.88 (m, 1H, H-8B), 1.85 (d, $^2J_{H15A,\ H15B}$ = 16.3 Hz, 1H, H-15B), 1.69 (s, 3H, H-34), 1.68 (s, 3H, 35-H), 1.58 (m, 1H, H -2B), 1.48 (s, 3H, H-22), 1.35 (s, 3H, H-23), 1.29 (s, 3H, H-20), 1.25 (m, 1H, H-31B), 1.20 (s, 3H, H-18), 1.16 (s, 3H, H-19), 1.09 (s, 3H, H-21). ^{13}C NMR (101 MHz, CDCl3) δ = 208.4 (C-12), 205.2 (C-13), 204.4 (C-16), 192.2 (C-24), 135.3 (C-25 and C-33), 132.4 (C-28), 128.9 (C-26 and C-30), 128.2 (C-27), 128.1 (C-29), 118.9 (C-32), 88.6 (C-6),