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Chemical Characterisation of Nigerian Red Propolis and its Biological Activity Against *Trypanosoma brucei.*

Ruwida M.K. Omar,[1] John Igoli,[4] Alexander I. Gray,[1] Godwin Unekwuojo Ebiloma,[2] Carol Clements,[1] James Fearnley,[3] RuAngeli Edrada Ebel,[1] Tong Zhang,[1] Harry P. De Koning[2] and David G. Watson[1*]

1. University of Strathclyde, Strathclyde Institute of Pharmacy and Biomedical Science, 27 Taylor Street, Glasgow, G4 0NR, UK.
2. Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences University of Glasgow, Glasgow G12 8TA, UK
3. BeeVital, Whitby, North Yorkshire, YO22 5JR, UK
4. Phytochemistry Research Group, Department of Chemistry, University of Agriculture Makurdi, Nigeria.

*Author for correspondence:

David Watson,

Strathclyde Institute of Pharmacy and Biomedical Sciences,

161 Cathedral Street,

Glasgow, UK. G4 0RE.

Tel +44-(0)141-548-2651

E mail: d.g.watson@strath.ac.uk

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ABSTRACT:

Introduction - A previous study showed the unique character of Nigerian red propolis from Rivers State, Nigeria (RSN), with regards to chemical composition and activity against *Trypanosoma brucei* in comparison with other African propolis.

Objective - To carry out fractionation and biological testing of Nigerian propolis in order to isolate compounds with anti-trypanosomal activity. To compare the composition of the RSN propolis with the composition of Brazilian red propolis. Methodology - Profiling was carried out using HPLC-UV-ELSD and HPLC-Orbitrap-FTMS on extracts of two samples collected from RSN with data extraction using MZmine software. Isolation was carried out by normal phase and reversed phase MPLC. Elucidation of the compounds with a purity > 95% was performed by 1D/2D NMR HRMS and HRLC-MS^n.

Results - Ten phenolic compounds were isolated or in the case of liquiritigenin partially purified. Data for 9 of these correlated with literature reports of known compounds i.e., one isoflavanone, calycosin (1); two flavanones, liquiritigenen (2) and pinocembrin (5); an isoflavan, vestitol (3); a pterocarpan, medicarpin (4); two prenylflavanones, 8-prenylnaringenin (7) and 6-prenylnaringenin(8); and two geranyl flavonoids, propolin D (9) and macarangin (10). The 10^{th} was elucidated as a previously undescribed dihydrobenzofuran (6). The isolated compounds were tested against *Trypanosoma brucei* and displayed moderate to high activity. Some of the compounds tested had similar activity against wild type *T. brucei* and two strains displaying pentamidine resistance.

Conclusion - Nigerian propolis from RSN has some similarities with Brazilian red propolis. The propolis displayed anti-trypanosomal activity at a potentially useful level.

Keywords: Prenylated flavonoids, benzofuran, pentamidine resistance, Nigerian Red Propolis.

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Introduction

Propolis is produced by honeybees from natural resinous substances collected from plant exudates and different parts of plants, and possesses various biological activities which vary with the phytogeographic characteristics of the collection site and the season of collection (Bankova, 2005). It is used to seal gaps in the hives and to coat the surfaces onto which the honeycomb is laid. Propolis is broadly characterized into two distinct types: temperate zone propolis (poplar type), containing mainly phenolic flavonoid aglycones (flavones and flavanones), phenolic acids and their esters; and tropical propolis, rich in prenylated derivatives of *p*-coumaric acids, diterpenes and lignans, prenylated benzophenones and prenylated flavonoids (Bankova *et al.*, 2002).

The growing use of propolis as a component of pharmaceuticals, cosmetics and food supplements with antimicrobial (Seidel *et al.*, 2008), anti-inflammatory, antiviral, anticarcinogenic and immunomodulatory (Burdock., 1998) activities has increased interest in its composition with the aim of identifying the components responsible for its activity. Propolis has been well-studied worldwide except, according to our own survey of the literature, in Africa. Previously, 22 different samples of propolis collected from nine different sub-Saharan countries were profiled using a combination of high resolution LC-MS, GC-MS and HPLC with evaporative light scattering detection and they were found to present a high diversity in chemical composition but no clear geographic delineation was found for the classification of these samples (Zhang *et al.*, 2014). However, a sample from Rivers State in South Nigeria stood out as being chemically different and demonstrated relatively high activity against *Trypanosoma brucie*. In the current study this propolis type was re-collected from Rivers State Nigeria in order to provide enough material for isolation of individual compounds from the mixture. Its ethanolic extract was chemically compared to the previously collected sample. The samples were also tested against three strains of *T.brucie* including two resistant lines and the

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compositions of the propolis samples were compared against the literature data for Brazilian red propolis.

EXPERIMENTAL

Chemicals and materials

Absolute ethanol, HPLC grade acetonitrile, hexane, methanol and formic acid were obtained from Fisher Scientific (Loughborough, UK). Deuterated chloroform ($CDCl_3$), deuterated dimethyl sulphoxide (DMSO-d6), Silica Davisil® grade 633, pore size 60 A, mesh 200-425 µm and Wilmad nuclear magnetic resonance (NMR) tubes were obtained from Sigma Aldrich (Dorset, UK). AnalaR grade formic acid (98%) was obtained from BDH-Merck (Dorset, UK). HPLC grade water was produced in-house by a Milli Q system (Millipore, UK). An ACE C18 column (3 mm × 150 mm, 3 µm) was purchased from Hichrom (Reading, UK).

Propolis sample collection and preparation

The propolis sample coded RSN was collected in 2003 (exact location of collection unknown) in Rivers State, Nigeria and was supplied by BeeVital (Whitby, UK), while the second, coded BRN, was collected by Dr. John Igoli from Bonny, a riverine town in Rivers State, Nigeria in July 2013. Both samples were reddish and had a very sticky texture. The propolis samples RSN (3.3 g) and BRN (140.0 g) were extracted three times with fresh ethanol (50 mL and 500 mL respectively) by sonication at 40 °C for 3 h each, and the final residue was macerated overnight with ethanol (50 mL and 500 mL respectively). Each of the 4 extracts per sample was filtered through filter paper, combined and the solvent evaporated *in vacuo* to yield the ethanolic extracts of RSN (600 mg, of a red gum) and BRN (15.5 g of a red gum). These ethanolic crude extracts of each sample were prepared at the same time and stored at -20 °C until required.

HPLC-UV-ELSD and HPLC-ESIMS and MS/MS analysis of extracts

An aliquot (2 mg) of crude extract was reconstituted in methanol (1 mL) and injected (10 µL) onto an ACE C18 column (150 x 3 mm, 3 µm particle size) (HiChrom, Reading UK). The mobile phases used

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were 0.1% v/v formic acid in water (A) and 0.1% v/v formic acid in acetonitrile (B) at a flow rate of 0.3 mL/min. The formic acid was omitted from A and B when the evaporative light scattering detector (ELSD) was used in order to reduce the background noise. The gradient elution was programmed as follows: 0–15 min linear gradient from 30% to 50% of B, 15–25 min at 50% of B, 25–40 min linear gradient from 50% to 80% of B, 40–50 min at 80% of B, 50–51 min increasing to 100% of B, 51–59 min at 100% of B with the flow rate increasing to 0.5 mL/min for cleaning the column and 60–70 min back to 30% of B. The HPLC–UV–ELSD analysis was performed using an Agilent 1100 system (Agilent Technologies, Germany) consisting of a quaternary pump, an autosampler, a degasser and a UV detector with two channels (290 and 320 nm), coupled with an evaporative light scattering detector (ELSD) (model: SEDEX75, SEDERE France) set at at 30° C. The HPLC-ESIHRMS analysis was perfomed using an Accela 600 HPLC system combined with an Exactive (Orbitrap) mass spectrometer from Thermo Fisher Scientific (Bremen, Germany). The MS detection range was from *m/z* 100 to 1500 and the scanning was performed under polarity switching mode. The data obtained were split into positive and negative ion files and then processed using MZMine 2.10 (Pluskal *et al.*, 2010). The extracted masses were then searched against the Dictionary of Natural Products (DNP, Buckingham, 1993) electronic database in order to match them against exact masses for known metabolites. In addition Xcalibur 2.2 from Thermo Fisher Scientific was used to check the raw LC–HRMS data manually. Since the Exactive instrument was not capable of isolating individual ions for fragmentation, a HPLC-MS^n data-dependent fragmentation of the purified compounds was accomplished using collision-induced dissociation (CID) at 35 V on an LTQ-Orbitrap Classic mass spectrometer in negative ion mode, since this instrument model does not have positive negative ion switching capability, downstream of a Surveyor HPLC system from Thermo Fisher Scientific (Bremen, Germany). The mass axis of the both MS instruments were externally calibrated according to the manufacturer's instructions just before commencing the experiments.

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Preparative scale chromatography

For reversed phase chromatography, the ethanolic extract of RSN (500 mg) was dissolved in ethyl acetate (5 mL) and was mixed with celite (1 g), dried in a fume cupboard and the sample was packed into an empty "dryloader" cartridge (Alltech, Carnforth, Lancs. UK) allowing it to be transferred onto to a Grace Davison Reveleris® flash chromatography system (Alltech, Carnforth, Lancs, UK) by the mobile phase. The system was equipped with a dual-UV wavelength detector that was set at 290 and 320 nm, an ELSD detector, and an automatic fraction collector which collected peaks according to the threshold set for the ELSD which was set to medium. The gradient elution method used was the same as in the analytical profiling but with a flow rate of 12 mL/min and a C18 (12g) cartridge (Grace Davison, Columbia, USA). Fractions were monitored using HPLC-UV-ELSD to yield compounds **7** (17.1 mg pale yellow powder), **9** (6.6 mg brown powder), **10** (8.3 mg red brown powder) and a mixture of **1** and **2** (10.6 mg, white powder).

For normal phase chromatography, the EEP of RSN (800 mg) was redissolved in ethyl acetate (5 mL) and mixed with celite (1.6 g), blown dry and the sample was packed into an empty "dryloader" cartridge allowing it to be eluted onto the Revelris MPLC system which was fitted with a pre-packed 24 g silica column (Alltech, Carnforth, Lancs.). The detection threshold was set at medium and by using hexane:ethyl acetate ranging from 0-100% over a 57 min linear gradient , 26 fractions of varying volumes were collected according to threshold setting. Fraction GRP11 (188.3 mg) was the largest in weight and was collected at around 60:40 hexane:ethyl acetate. This fraction was rechromatographed using a Reveleris® flash chromatography system fitted with a Grace C18 cartridge (12 g) (Alltech, Carnforth, Lancs.). Compounds were eluted with acetonitrile:water (40:60) at 9mL/min over 30 min followed by linearly increasing acetonitrile to 100% over 30 min. This resulted in the separation of compounds **3** (6.5 mg, white needles) , **6** (5 mg, reddish brown powder) and **8** (4 mg, pale yellow solid).

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The ethanolic extract of sample BRN (7 g) was reconstituted in ethyl acetate (10 mL) mixed with coarse silica (5 g), blown dry and was packed onto a silica gel bed (50 g) in an open glass column (55 x 3cm). Compounds were eluted using 200 mL of mobile phase at each step starting with 100% hexane then stepwise increments of ethyl acetate as follows: 90:10-80:20-60:40-40:60-20:80-100% ethyl acetate and then continuing with increasing amounts of MeOH in presence of ethyl acetate as follows: 90:10-70:30-60:40-50:50-100% MeOH). Fractions (50 mL) were collected and were pooled, based on HPLC-UV-ELSD analysis, to yield 14 fractions. Fraction BRN5 (163 mg), which was eluted with ethyl acetate:hexane (60:40) and fraction BRN9, which was eluted from the open column with 100% ethyl acetate were rechromatographed isocratically with acetonitrile:water (1:1) and acetonitrile:water (3:7) respectively at 12 mL/min over 30 min using the Grace Revelris system fitted with a Grace C18 cartridge (12g). Fraction BRN5 yielded compounds **4** (22.4 mg, colourless needles), **5** (8.6 mg, dark orange powder) and **7** (8 mg, pale yellow solid) and fraction BRN9 yielded compound **1** (17.6 mg, white powder).

Structure elucidation experiments

After testing the purity of the fractions with HPLC-UV-ELSD, the fractions with a purity >90% were characterised by LC-MS^n, and by NMR. The ^1H, ^{13}C and DEPT 135, and 2D ^1H,^1H-COSY, and ^{13}C-^1H HSQC and HMBC NMR spectra were obtained by using a Bruker Avance 600 and JEOL-LA 400 FT-NMR spectrometer system using $CDCl_3$ and d6-DMSO as solvents. MestReNova 8.1.2 was used for processing the NMR spectroscopic data. The melting point of riverinol was measured using a Stuart Scientific melting point apparatus (Bibby,UK).

Anti-trypanosomal testing

Preliminary testing against *T. brucei* in *in vitro* anti-trypanosomal tests was carried out by using an AlamarBlue™ cell proliferation assay according to a modification of the protocol describe by Raz *et al.*, 1997 (Igoli *et al.*, 2011). MIC values for three compounds were determined using this assay as described previously (Igoli *et al.*, 2011). The rest of the compounds were screened using a variant of

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this assay (Rodenko *et al.*, 2007; Wallace *et al.*, 2002). In this screen testing was carried out against a standard drug-sensitive *T. b. brucei* clone and two derived drug resistant lines, in order to assess the potential for cross-resistance with existing drugs. In this case the results were expressed as EC_{50} values based on three replicates at each concentration. The assay is based on viable cells metabolizing the blue non-fluorescent dye resazurin to resorufin, which is pink and fluorescent. The assays were performed using serial dilutions in white opaque plastic 96-well plates (F Cell Star, Greiner Bio-one GmbH, Frickenhausen, Germany), with each compound or mixture doubling diluted over 2 rows of the plate (i.e. 23 doubling dilutions and a no-drug control well), facilitating an optimally-defined EC_{50} value after plotting of the reading to a sigmoid curve with variable slope (GraphPad Prism 5.0). Bloodstream forms of the following clonal strains of *T. b. brucei* were utilized: Lister strain 427 (s427) (De Koning *et al.,* 2000), the standard drug-sensitive control strain; the B48 clone that was derived by *in vitro* adaptation to pentamidine (Bridges *et al.,* 2007); and the aqp2/aqp3 null strain (Baker *et al.,* 2012), from which the gene encoding the High Affinity Pentamidine Transporter (HAPT1) has been deleted. For each strain, the seeding density at the start of the assay was 2×10^4 cells/well, and the cells were exposed for 48 h to the test compounds, at 37 °C/5% CO_2, before the addition of the resazurin dye and a further incubation of 24 h under the same conditions. Fluorescence was determined in a FLUOstar Optima (BMG Labtech) at wavelengths of 544 nm and 620 nm for excitation and emission, respectively.

RESULTS AND DISCUSSION

Structure Elucidation of Compounds Isolated From Nigerian Red Propolis

Nine compounds (Figs. 1 and 2) were isolated from the Nigerian red propolis and their stuctures were determined by NMR. In addition the structure of liquiritigenin (**2**) was determined although it was part of a mixture containing calycosin (**1**) (also isolated in pure form) in approximately equal amounts. The NMR spectra of nine of the compounds were consistent with the literature data for known compounds (full NMR details are given in Supplementary Information S1).

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For the compounds derived from flavanoids the typical fragmentations across the C ring giving the substitutions in rings A and B (Fig. 3) proposed previously were useful for structure elucidation (Hughes *et al.*, 2001). The mass spectrometry and retention time data for the isolated compounds compounds is summarised briefly below.

Calycosin (**1**) Rt 8.0 min. Ratio BRN/RSN 0.976. ^{1}H,^{13}C NMR data in d_6DMSO, were consistent with those previously reported (Du *et al.*, 2006), ESI-MS (negative mode), $[M - H]^-$ *m/z* 283.0614 ($C_{16}H_{11}O_5$, Δ -0.024 ppm). MS^2 *m/z* 268.0378 (100) ($C_{15}H_8O_5$, Δ -0.454 ppm), MS^3(100) *m/z* 240.0430 ($C_{14}H_8O_4$, Δ 0.096 ppm), *m/z* 239.0352(50), *m/z* 224.0482(50), *m/z* 211.0403(60), *m/z* 195.0454(30) none of these fragments were indicative of structure. However, in addition there was a small A ring fragment at *m/z* 135.0090 (4) ($C_7H_3O_3$, Δ 1.5 ppm) indicating one hydroxyl group in ring A.

Liquiritigenin (**2**) Rt 8.0 min. Ratio BRN/RSN 0.807 ^{1}H ,^{13}C NMR data in d_6DMSO were consistent with those previously reported (Ma *et al.*, 2005) ,ESI-MS (negative mode), $[M - H]^-$ *m/z* 255.0664 ($C_{15}H_{11}O_4$, Δ 1.67 ppm); MS^2, *m/z* 237.0559(20) *m/z* 153.0194(30) , *m/z* 135.0087 (100) ($C_7H_3O_3$, Δ -0.424 ppm) *m/z* 119.0502 (20) (C_8H_7O, Δ 0.706 ppm). The ion at *m/z* 135.0087 indicates a single hydroxyl in ring A and there is a corresponding ion at *m/z* 119.0502 which contains the B ring and indicates a single oxygen in the B ring.

Pinocembrin (**3**) Rt 11.5 min. Ratio BRN/RSN 0.724. ^{1}H ,^{13}C NMR data were consistent with those previously reported (Jung *et al.*, 1990), ESI-MS (negative mode), $[M - H]^-$ *m/z* 255.0665 ($C_{15}H_{11}O_4$, Δ 1.24 ppm) MS^2 *m/z* 213.0560(80), *m/z* 211.0767 (30), *m/z* 187.0786 (11), *m/z* 151.0038 (30) ($C_7H_3O_4$, Δ 0.583 ppm), *m/z* 169.0661(10). The fragment ion at *m/z* 151.0038 indicates the presence of two hydroxyl groups in ring A.

Vestitol (4) Rt 13.3 min**.** Ratio BRN/RSN 0.741 ^{1}H ,^{13}C NMR data were consistent with those previously reported (Piccinelli *et al.*, 2005), ESI-MS (negative mode), $[M - H]^-$, *m/z* 271.0977 ($C_{16}H_{15}O_4$, Δ -0.082 ppm). MS^2 *m/z* 109.0295(70) ($C_6H_5O_2$, Δ -0.117) *m/z* 135.0451 (100) ($C_8H_7O_2$, Δ 0.646 ppm) ,

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m/z 147.0452(70) ($C_9H_7O_2$, Δ 1.55 ppm). The fragment at *m/z* 135.0451 is consistent with a fragment containing the B ring while the fragment at *m/z* 109.0295 derives from the A ring.

Medicarpin(**5**) Rt 14.0 min. Ratio BRN/RSN 0.325. ^1H ,^{13}C NMR data were consistent with those previously reported (Piccinelli *et al.*, 2005), ESI-MS (negative mode), $[M - H]^-$ *m/z* 269.0822 ($C_{16}H_{13}O_4$, 1.51 ppm) MS^2 *m/z* 254.0587 (78) ($C_{15}H_{10}O_4$, Δ 0.602 ppm), *m/z* 237.0560 (83) ($C_{15}H_9O_3$, Δ 1.40 ppm) *m/z* 145.0296 (100) ($C_9H_5O_2$, Δ 1.02 ppm).

6-prenylnaringenin(**7**) Rt 23.9 min. Ratio BRN/RSN 0.208. ^1H ,^{13}C NMR data were consistent with those previously reported (Stevens *et al.*, 1997, Nakahara *et al.*, 2003), ESI-MS (negative mode), $[M - H]^-$ *m/z* 339.1236, MS^2 *m/z* 219.0663 ($C_{12}H_{11}O_4$, Δ 0.127 ppm) (100), *m/z* 119.0503 (4) (C_8H_7O, Δ 0.435 ppm), MS^3 *m/z* 175.07669(100) ($C_{11}H_{11}O_2$, Δ 0.897 ppm), *m/z* 151.07656(50) ($C_9H_{11}O_2$, Δ 0.444), *m/z* 133.06593(85) (C_9H_9O, Δ 0.238 ppm). The fragment at *m/z* 219.0663 is consistent with a prenyl group in ring A along with two hydroxyl groups and the small fragment at *m/z* 119.0503 contains the B ring indicating one hydroxyl group in the B ring. A longer retention time in reversed phase mode in comparison with 8-prenylnaringenin is probably due its higher surface area (298.3 $Å^2$ for 8-prenylnaringenin compared to 311.2 $Å^2$ for 6-prenylnaringenin as computed by Chem 3D, CambridgeSoft, Boston, USA) .

8-prenylnaringenin(**8**) Rt 16.1 min. Ratio BRN/RSN 0.086. ^1H ,^{13}C NMR data were consistent with those previously reported (Stevens *et al.*, 1997) , ESI-MS (negative mode), $[M - H]^-$ *m/z* 339.1236 ($C_{20}H_{19}O_5$, Δ 1.39 ppm) , MS^2 *m/z* 219.0663 (100) ($C_{12}H_{11}O_4$, Δ 0.335 ppm) *m/z* 119.0503 (3) (C_8H_7O, Δ 0.351 ppm) , MS^3 *m/z* 175.07669(100) ($C_{11}H_{11}O_2$, Δ1.61 ppm), *m/z* 151.0766 (50) ($C_9H_{11}O_2$, Δ1.17 ppm) *m/z* 133.0659 (90) (C_9H_9O, Δ 1.37 ppm). The fragment at *m/z* 219.0663 is consistent with a prenyl group in ring A along with two hydroxyl groups and the small fragment at *m/z* 119.0503 contains the B ring indicating one hydroxyl group in the B ring.

Propolin D(**9**) Rt 28.6 min. Ratio BRN/RSN 0.293. ^1H ,^{13}C NMR data were consistent with those previously reported (Chen *et al.*, 2004), ESI-MS (negative mode), $[M - H]^-$ *m/z* 423.1819 ($C_{25}H_{27}O_6$, Δ

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0.208 ppm), MS^2 151.0035 (100) ($C_7H_3O_4$, -Δ 0.807 ppm) , *m/z* 405.1701(70), *m/z* 271.1703 (20) ($C_{18}H_{23}O_2$, Δ 0.762 ppm) ,297.1496 (10) ($C_{19}H_{23}O_3$, Δ -1.60 ppm) . The ion at *m/z* 151.0035 indicates two hydroxyl groups in ring A and the ion at *m/z* 271.1703 indicates that the geranyl chain is in the B ring.

Macarangin(**10**) Rt 35.4 min. Ratio BRN/RSN 0.371 ^1H ,^{13}C NMR data were consistent with those previously reported (Hnawia *et al.*, 1990) , ESI-MS (negative mode), $[M - H]^-$ *m/z* 421.1654 ($C_{25}H_{25}O_6$, Δ -1.05 ppm) MS^2 *m/z* 352.09533 (80) ($C_{20}H_{16}O_6$, Δ -1.35 ppm), *m/z* 335.09236 (28) ($C_{20}H_{15}O_5$, Δ 1.18 ppm) *m/z* 309.0404 (100) ($C_{17}H_9O_6$, Δ -1.26 ppm), *m/z* 297.0405 (70) ($C_{16}H_9O_6$, Δ -0.913 ppm). In this case the MS^2 fragments result from losses of portions on the geranyl chain.

Structure elucidation of riverinol (6) Rt 20.9 min.

Of the 9 compounds chromatographically isolated from Nigerian red propolis in this study (Figs. 1 and 2), compound **6** (mp. 137 – 142 °C, did not correspond to previously described compounds. ESI-MS (negative mode), $[M - H]^-$ m/z 285.1134 ($C_{17}H_{17}O_4$, Δ = 0.6 ppm) indicating a molecular formula $C_{17}H_{18}O_4$.

The ^1H and ^{13}C NMR data for **6** (Table 1 and Supplementary file S2) indicated two similar aromatic systems each containing three protons with two protons ortho to each other and one meta-coupled proton. The DEPT 135 spectrum showed 17 carbons; six quaternary aromatic carbons, four of which were attached to oxygens; six aromatic methine carbons; and five aliphatic carbons, two of which corresponded to methoxy groups, two methylene carbons bearing and one methine carbon. A careful analysis of the 1D and 2D NMR spectroscopy data allowed elucidation of **6** as a previously undescribed dihydrobenzofuran herein given the trivial name riverinol. For ease of structure elucidation, an idosyncratic numbering system was used (Fig. 4). In the HMBC spectrum, the most useful carbons for connecting the structure were the 4 and 4' carbons which showed couplings to the aromatic protons within their rings and to the aliphatic protons (i.e., C4 to H6, H8, $H10_d$, $H10_u$ and C-

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4' to $H10_u$, $H10_d$, H3, H2', 3'-OH, H6'). It was possible to assign the position of the hydroxyl group to the 3' position on the basis of a weak four bond coupling of C3' to the C3 proton, this would not be possible for the alternative C1' position for this substituent. Thus the data are consistent with riverinol being 3-(2-hydroxy-4-methoxybenzyl)-6-methoxy-2,3-dihydrobenzofuran (**6**). A literature search revealed a closely related dihydrobenzofuran isolated from *Campylotropis hirtella* (Franch.) Schindl. (Han *et al*., 2008) that presented very similar and supporting NMR data. Further support for the structure was derived from the MS data. MS^2 *m/z* 270.0902 ($C_{16}H_{14}O_4$, Δ 1.56 ppm), *m/z* 255.0667 ($C_{15}H_{11}O_4$, Δ 1.56 ppm), *m/z* 149.0609 (34) ($C_9H_9O_2$, Δ 0.853 ppm), *m/z* 123.0452 (100) ($C_7H_7O_2$, Δ 0.384 ppm) *m/z* 108.0217 (13) ($C_6H_4O_2$, Δ 0.577 ppm). The ion at *m/z* 149.0609 corresponds to cleavage between C10 and C3 with retention of charge on the dihydrobenzofuran portion of the molecule, the ion at *m/z* 123.0452 corresponds to cleavage across the furan ring between the oxygen and C3 and C2 and the aromatic ring and the ion at *m/z* 108.0217 corresponds to loss of methyl from the fragment at *m/z* 123.0452.

HPLC-UV-ELSD and HPLC MS Profiling of RSN and BRN

HPLC-UV-ELSD and HPLC-MS analysis of RSN and BRN showed rich chromatographic responses with the combination of the ELSD and the UV detector (290 and 320 nm), suggesting the absence of ELSD-only responsive compounds such as terpenes and fats. Many compounds were common between the two samples but with different relative abundances which are listed along with the elucidated (above) or partially elucidated structures (discussed below). Like Brazilian red propolis, the Nigerian red propolis contained medicarpin (**5**), liquiritigenin (**2**) and vestitol (**4**) which are probably collected from *Dalbergia ecastophyllum* (Piccinelli *et al*., 2011) The Nigerian sample also contains retusapurpurins (S3 shows the ESI spectra and an extracted ion trace corresponding to two retusapurpurin isomers which are abundant in RSN and BRN) which are responsible for the red colour of the propolis and have only been recorded in *Dalbergia* species. There are a number of *Dalbergia* species found in West Africa (Saha *et al.*, 2013). Thus there is a degree of similarity

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between Nigerian red and Brazilian red propolis based on the compounds found in *Dalbergia* species discussed above. However, there were also compounds, either isolated and fully characterised or putatively identified by mass spectrometry, in the Nigerian samples which have not been reported to be present in Brazilian red propolis and these included: the fully characterised compounds propolin D (**9**), 6- and 8-prenylnarigenin (**7**, **8**) and macarangin (**10**). In addition there were many other abundant compounds in many cases related to the compounds isolated and characterised by NMR. The high resolution mass spectra were generally accurate to within 2 ppm of the proposed formula which means that it is possible to be confident of the elemental composition assigned and indeed the hits in DNP database are all isomers of the proposed compositions. Supplementary file S4 summarises the elemental compositions for the top 200 compounds by mean abundance in the samples. For many of the listed elemental compositions > 100 isomers can be found in the DNP database. The mass spectra of some of the characteristic compounds are discussed in detail below. and are referred to as Unknowns 1-18. Where MS^3 data are reported these are for the base peak in the MS^2 spectrum.

Unknown 1 Rt 9.3 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 331.0822 (composition $C_{17}H_{15}O_7$, Δ -0.139 ppm, 184 matches in DNP). BRN/RSN 1.047. MS^2 *m/z* 316.0589 (100) ($C_{16}H_{12}O_7$, Δ -0.155 ppm), *m/z* 301.0357 (7) ($C_{15}H_9O_7$, Δ 1.11 ppm), *m/z* 151.0039 (1.5) ($C_7H_3O_4$, Δ 1.37 ppm). MS^3 *m/z* 301.0354 (100) ($C_{15}H_7O_6$, Δ -0.180 ppm). Possibly dimethyl quercetin with methylation of the hydroxyl groups in ring B since the small ion at *m/z* 151.0039 indicates a dihydroxylated A ring.

Unknown 2 Rt 10.3 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 285.0765 (composition $C_{16}H_{13}O_5$, Δ 0.537 ppm, 225 matches in DNP). Not in RSN. MS^2 *m/z* 270.0356 (5) ($C_{15}H_{10}O_5$, Δ 0.81 ppm), *m/z* 267.0664 (61) ($C_{16}H_{11}O_4$, Δ 0.37 ppm), *m/z* 257.0819 (100) ($C_{15}H_{13}O_4$, Δ 0.03 ppm). MS^3 *m/z* 242.0583 (100) ($C_{15}H_{10}O_4$, Δ -0.44 ppm), *m/z* 239.0716 (21) ($C_{16}H_{11}O_3$, Δ 1.14 ppm). Consistent with a methylated pterocarpin but fragmentation pattern does not match data reported previously (Piccinelli *et al.* , 2011) for pterocarpins in Brazilian red propolis.

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Unknown 3 Rt 9.9 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 285.0765 (composition $C_{16}H_{13}O_5$, Δ -0.024 ppm, 225 matches in DNP). BRN/RSN (0.623). MS^2 *m/z* 270.0357 (9) ($C_{15}H_{10}O_4$, Δ 1.03 ppm), *m/z* 257.0821 (18) ($C_{15}H_{13}O_4$, Δ 0.964 ppm), *m/z* 241.0871 (100) ($C_{15}H_{13}O_3$, Δ 0.26 ppm), *m/z* 226.0638 (25) ($C_{14}H_{10}O_3$, Δ 1.27 ppm), *m/z* 163.004 (19) ($C_8H_3O_4$, Δ 0.79 ppm), *m/z* 109.0295 (12) ($C_6H_5O_2$, Δ 0.25 ppm). MS^3 *m/z* 226.0637 (100) ($C_{14}H_{10}O_3$, Δ 0.87 ppm). Consistent with a methylated pterocarpin but fragmentation pattern does not match data reported previously (Piccinelli *et al.*, 2011) for pterocarpins in Brazilian red propolis.

Unknown 4 Rt 23.7 min ESI-MS (negative mode), $[M - H]^-$ *m/z* 423.1817 (composition $C_{25}H_{27}O_6$, Δ -2.84 ppm, 137 matches in DNP). Ratio BRN/RSN 0.158. Putative identification geranylated or diprenylated flavanoid. MS^2 *m/z* 287.1285 (100) ($C_{17}H_{19}O_4$, Δ -1.192 ppm). The fragment at *m/z* 287.1285 indicates the presence of a geranyl or two prenyl groups in the A ring; the base peak in the MS^3 spectrum for this ion was at m/z 243.1391 has the formula $C_{16}H_{19}O_2$ (Δ -0.234 ppm) corresponding to a loss of CO_2.

Unknown 5 Rt 32.9 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 423.1817 (Composition $C_{25}H_{27}O_6$, -0.17 ppm 137 matches in DNP). BRN/RSN 0.461. Unknown 5 is an isomer of propolin D but does not give any diagnostic MS^2 fragments since it loses CO to give a base peak at *m/z* 395.1861 ($C_{24}H_{27}O_5$, Δ -0.679 ppm). The MS^3 spectrum of this base peak is more imformative yielding a peak at *m/z* 351.1961 due to loss of CO_2 and and this ion appears to undergo another loss of C7H6 (approximating to benzyl) to give the base peak at *m/z* 261.1493 ($C_{16}H_{21}O_3$, Δ -1.08 ppm). There is also a a fragment at *m/z* 287.1286 ($C_{17}H_{19}O_4$, Δ -1.088 ppm) suggesting that unknown 8 also has a geranyl or two prenyl groups in ring A.

Unknown 6 Rt 29.6 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 407.1868 (composition $C_{25}H_{27}O_5$, Δ -2.5 ppm, 135 matches in DNP). BRN/RSN 0.289. MS^2 *m/z* 287.1282 (100) ($C_{17}H_{19}O_4$, Δ -1.3 ppm). The fragment *m/z* 287.1282 indicates the presence of the geranyl/prenyl groups in the A ring; *m/z*

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119.0502 (0.8) (C_8H_7O, Δ -0.657 ppm) indicates one hydroxyl group in ring B. Putative identification geranylated flavanoid.

Unknown 7 Rt 33.7 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 491.2445 (Composition $C_{30}H_{35}O_6$, Δ -1.83 ppm, 36 matches in DNP). BRN/RSN 0.340. Unknown 7 is related to propolin D but carries an additional prenyl group. The base peak in the MS^2 spectrum of unknown is at *m/z* 219.0663 (100) ($C_{12}H_{11}O_4$, Δ 0.447 ppm) indicating substitution in ring A with two hydroxyl groups and a prenyl group and the additional geranyl group is in ring B as indicated by a fragment at *m/z* 271.1706 (15) ($C_{18}H_{23}O_2$, Δ 0.762 ppm).

Unknown 8 Rt 24.1 min ESI-MS (negative mode), $[M - H]^-$ *m/z* 353.1025 (Composition $C_{20}H_{17}O_6$, Δ -1.562 ppm, 160 matches in DNP). BRN/RSN 0.246. MS^2 *m/z* 335.0926 (68) ($C_{20}H_{15}O_6$, Δ 0.397 ppm), *m/z* 298.0584 (77) ($C_{16}H_{10}O_6$, Δ -0.549 ppm), *m/z* 269.0457 (100) ($C_{15}H_9O_5$, Δ 0.644 ppm). Possibly prenylated flavanoid where loss of C_4H_7- might indicate loss of part of a prenyl chain.

Unknown 9 Rt 12.2 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 267.0666 (Composition $C_{16}H_{11}O_4$, Δ 1.26 ppm, 96 matches in DNP). BRN/RSN 0.893. MS^2 *m/z* 252.0429 (100) ($C_{15}H_8O_4$, Δ 0.21 ppm). MS^3 m/z 223.0403 (100) ($C_{14}H_7O_3$, Δ 0.903 ppm), *m/z* 208.0532 (83) ($C_{14}H_8O_2$ Δ 0.972 ppm) *m/z* 135.009 (2) ($C_7H_3O_3$, Δ 0.761 ppm). Isomer of methylchrysin with single hydroxy in ring A and methoxy in ring B.

Unknown 10 Rt 7.9 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 315.0875 ($C_{17}H_{15}O_6$, Δ 0.294 ppm, 176 matches in DNP). BRN/RSN 1.269. MS^2 *m/z* 297.0766 (100) ($C_{17}H_{13}O_5$, Δ 0.249 ppm), *m/z* 287.0926 (88) ($C_{16}H_{15}O_5$, Δ 0.324 ppm). MS^3 *m/z* 282.0553 (100) ($C_{16}H_{10}O_5$, Δ 0.324 ppm). Possibly dimethyl flavonoid.

Unknown 11 Rt 18.9 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 539.1702 ($C_{32}H_{27}O_8$, Δ -1.81 ppm, 5 matches in DNP) Ratio BRN/RSN 1.22. MS^2 283.0977 (100) ($C_{17}H_{15}O_4$, Δ 0.699 ppm), *m/z* 255.0664 (75) ($C_{15}H_{11}O_4$, Δ 0.501 ppm), *m/z* 240.0431 (32) ($C_{14}H_8O_4$, Δ 1.262 ppm). MS^3 *m/z* 268.0742 (100) ($C_{16}H_{12}O_4$, Δ 0.421 ppm). The molecule fragments into two halves of similar molecular weight

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suggesting an isoflavonoid dimer such related to the daljanelins which are found in Dalbergia species (Saha *et al.*, 2013) .

Unknown 12 Rt 11.4 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 273.0773 ($C_{15}H_{13}O_5$, Δ 1.76 ppm, 217 matches in DNP) Ratio BRN/RSN 1.23. MS^2 *m/z* 163.0400 (100) ($C_9H_7O_3$, Δ -0.475 ppm), *m/z* 109.0294 (56) ($C_6H_5O_2$, Δ -0.94 ppm). The molecule forms two main fragments suggesting a dihydroxybenzene bonded to caffeic acid.

Unknown 13 Rt 10.5 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 283.0632 ($C_{16}H_{11}O_5$, Δ 0.61 ppm, 165 matches in DNP) Ratio BRN/RSN 0.99. MS^2 *m/z* 268.0337 (100) ($C_{15}H_8O_5$, Δ -0.006 ppm). MS^3 *m/z* 224.0480 (100) ($C_{14}H_8O_3$, Δ 0.57 ppm), *m/z* 135.0089 (3) ($C_7H_3O_3$, Δ 1.29 ppm). Isomer of methyl galangin with one hydroxyl group in ring A.

Unknown 14 Rt 49.7 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 601.3528 ($C_{38}H_{49}O_6$, Δ 1.035 ppm, 41 matches in DNP). Ratio BRN/RSN 14.1. Putative identification polyisoprenylated benzophenone isomer of guttiferone A. The fragments in the MS^2 spectra were consistent with those observed previously although shifted by *ca* 2 amu since the previous data were obtained in positive ion mode (Piccinelli *et al*., 2011). MS^2 *m/z* 465.3363 (100) ($C_{31}H_{45}O_3$, Δ -2.04 ppm) which is consistent with loss of the dihydroxybenzoyl group from this molecule. Piccinelli *et al*., 2011, observed this ion in positive ion mode at *m/z* 467 and attributed it to the loss of a geranyl chain which has the same mass as the dihydroxy benzophenone moeity, in the current case it is clear from the accurate mass data that the loss is due to loss of dihydroxy benzophenone as described previously (Yang *et al.*, 2010). An ion at *m/z* 423.3258 ($C_{29}H_{43}O_2$, Δ -2.39 ppm) is consistent with cracking across the ring bearing the dihydroxy benzophenone moeity. An ion at *m/z* 409.1657 (observed by Piccinelli *et al.* 2011, at *m/z* 411 but not interpreted) ($C_{24}H_{25}O_6$, Δ 0.116) would be consistent with a structure such as oblongifolin A where a geranyl group is subsituted onto the bicyclononane ring. These proposed fragmentations are illustrated in figure 5 using oblongifolin A as the example.

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Unknown 15 Rt 50.8 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 601.3544 ($C_{38}H_{49}O_6$, Δ -2.45 ppm, 41 matches in DNP). Not found in RSN. The MS^2 spectrum has the same fragment at *m/z* 465.3362 as Unknown 14 but does not have an ion at *m/z* 409.16. However, a corresponding loss occurs from the ion at *m/z* 465.3362 in the MS^3 spectrum of unknown 15 resulting in an ion at *m/z* 273.1491 (100) ($C_{17}H_{21}O_3$, Δ -1.74 ppm) suggesting that this molecule also has a geranyl tail attached. Loss of the geranyl tail itself from the ion at *m/z* 465.3362 is also observed giving rise an ion at *m/z* 327.1960 (20) ($C_{21}H_{27}O_3$, Δ - 1.8 ppm).

Unknown 16 Rt 52.7 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 669.4147 ($C_{43}H_{57}O_6$, Δ -2.08 ppm 11 matches in DNP). Ratio BRN/RSN 11.1. The MS^2 spectrum gives a base peak at *m/z* 533.3981 ($C_{38}H_{53}O_3$, Δ -1.48 ppm) showing the same loss of dihydroxybenzophenone as was seen for unknowns 11 and 12. The spectrum also has a small ion at m/z 477.2266 ($C_{29}H_{33}O_6$, -3.44 ppm) which indicates the loss of a fragment containing the geranyl tail as was observed for unknowns 10 and 11.

Unknown 17 Rt 38.4 min. *m/z* 515.3158 ESI-MS (negative mode), $[M - H]^-$ ($C_{34}H_{43}O_4$ Δ -1.71 ppm 3 matches in DNP) Ratio BRN/RSN 0.1. Only one of the DNP matches is naturally occuring since two of the matches are polyprenylated benzophenones which were methylated during isolation. Thus the unique match is to schweinfurthin C which is a digeranylated stilbene isolated from *Macaranga schweinfurthii* (Beutler *et al.*, 1998). However, more recently further isomers of this formula, denticulatains A and B, not listed in DNP, have been isolated from *Macaranga denticulata* which are diterpene/stilbenes (Yang *et al.*, 2015). The MS^2 spectrum for unknown 13 gave the following major ions:

m/z 445.2390 (3) ($C_{29}H_{33}O_4$, Δ -2.05 ppm), *m/z* 405.2791 (95) ($C_{28}H_{37}O_2$, Δ -2.4 ppm), *m/z* 379.1915 (4) ($C_{24}H_{27}O_4$, Δ -0.31 ppm), *m/z* 309.1131 (11) ($C_{19}H_{17}O_4$, -0.43 ppm), *m/z* 255.0660 (91) ($C_{15}H_{11}O_4$, -1.4 ppm), *m/z* 253.0504 (66) ($C_{15}H_9O_4$, -0.84 ppm), *m/z* 241.0503 (100) ($C_{14}H_9O_4$, -1.295 ppm). From the fragmentation pattern it seems likely that unknown 13 is one of the denticulatains. The diterpene moeity is attached to one of the rings making it possible to lose an unmodfied dihydroxybenzene ring

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to give the fragment at *m/z* 405.2791. A proposed fragmentation pattern accounting for the major fragments is shown in figure 6 using denticulatain A as an example. Figure 6 also shows the major fragments derived from the *m/z* 241.0503 ion in the MS^3 spectrum. Macarangin (**10**) which was isolated in pure form from the BRN propolis was also isolated from *M.vedeliana* (Hnawia *et al.* 1990) thus it seems likely that the diterpene stilbene was obtained from a *Macaranga* species. There are several minor isomers of unknown 13 present in the extract and one of these has a spectrum which fits more with the digeranylated structure of schweinfurthin C since the ion at m/z 405.2791 is absent from the MS^2 spectrum and ions at *m/z* 377.1754 (100) ($C_{24}H_{25}O_4$, Δ -1.014 ppm) and *m/z* 241.0506 ($C_{14}H_9O_4$, Δ -0.261 ppm) result from successive losses of geranyl chains.

Unknown 18 Rt 34.6 min. ESI-MS (negative mode), $[M - H]^-$ *m/z* 447.2536 ($C_{29}H_{35}O_4$, Δ -1.031 ppm, 9 matches in DNP). BRN/RSN 0.366. MS^2 *m/z* 391.19220 (30) ($C_5H_{27}O_4$, Δ 1.83 ppm), *m/z* 377.1765 (100) ($C_{24}H_{25}O_4$, Δ 1.63 ppm), *m/z* 309.1137 (70) ($C_{19}H_{17}O_4$, Δ 1.44 ppm), *m/z* 241.0511 (18) ($C_{14}H_9O_4$, Δ 2.07 ppm). The compound has fragments in common with unknown 17, suggesting a sesquiterpene stilbene adduct.

As can be seen from table S2 there are many other abundant compounds in the propolis samples which have yet to be characterised.

Anti-trypanosomal activity

The activities of the crude extracts of RSN and BRN and the isolated compounds were tested against *T. brucei*. The crude extracts were more active than the isolated compounds suggesting either that the most active compounds had not been isolated or that mixtures of components together were acting in an additive manner. Preliminary tests were carried out against a wild type strain and MIC values were obtained for: medicarpin (3.12 µg/ml), pinocembrin (12.5 µg/ml) and propolin D (3.12 µg/ml). Testing was carried out for the other isolated compounds against a wild type strain and against two resistant strains (Table 2) of *T.brucei*. The compounds showed strong anti-trypanosomal activity with EC_{50} values against a standardized strain ranging from 4.2 µg/mL for the crude RSN

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fraction to 16.6 µg/mL for riverinol (**6**), confirming the previously observed high activity of the crude extract when compared with other African propolis samples (Zhang *et al*., 2014). Of the purified compounds, 8-prenylnarigenin (**8**) was the most active against the wild type strain at 6.1 ± 0.1 µg/ml but macarangin (**10**) and vestitol (**4**) displayed very similar activities. In order to assess the potential for cross-resistance with current drugs of the diamidine and melaminophenylarsenical classes (Baker *et al*., 2013), the compounds were tested on the multi-drug resistant (MDR) strains B48 (Bridges *et al*., 2007) and aqp2/aqp3 null (Baker *et al.,* 2012) in parallel. Pentamidine, used as a control drug, was 31-fold ($P=5.3 \times 10^{-6}$) and 224-fold (P=0.00028) less active against B48 and aqp2/aqp3 null, respectively. In contrast, none of the fractions or compounds tested displayed a substantially reduced activity towards the MDR strains, with the resistance factors not exceeding 1.5-fold, establishing that the activity of these phytochemicals is not dependent on the same drug transporters that mediate uptake of the currently used drugs (De Koning, 2008).

Source and relative activites of various propolis constituents

It would seem that Nigerian red propolis is very similar to Brazilian red propolis and that bees are collecting from similar plants although the two regions are geographically far apart. Isoflavonoids have a very restricted distribution in the plant kingdom and occur almost exclusively in legumes (Leguminosae family) (Silva *et al.*, 2008), along with isoflavans such as liquiritegenin (**2**) and medicarpin (**5**) which been previously isolated from Cuban and northern Brazilian red propolis, which originates largely from *Dalbergia ecastophyllum* resin (Piccinelli *et al.*, 2005). The Nigerian red propolis, according to LC-MS profiling, also contains polyprenylated benzophenones (unknowns 14-16) that are typically found in Brazilian and Cuban propolis and originate from Clusiaceae species (Yang *et al.*, 2010; Yuliar *et al.*, 2014). However, in Nigeria a likely source would be the African mangosteen (*Garcinia livingstonei*) (Yang *et al.* 2010). A polyprenylated benzophenone has previously been isolated from Cameroonian propolis (Almutairi *et al.*, 2014). Geranylated flavonoids have been isolated before from Japanese propolis collected from Okinawa and also from Pacific propolis

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collected from the Solomon Islands (Kumazawa *et al*., 2007; Raghukumar *et al*., 2008). Macarangin (**10**) has previously been isolated from Kenyan propolis and was reported to originate from Asian *Macaranga denticulata* and New Caledonian *M. vedeliana* (Petrova *et al.*, 2010). *M. barteri* has been reported to occur in mangrove forests in Delta State in Nigeria (Ebigwai and Akomaye, 2014). Prenyl naringenins have not been reported before in propolis and have only been reported to occur in hops (Jung *et al.*, 1990). The strong anti-protozoal activity of propolis appears to be an almost constant feature of the material and strongly suggests that bees collect the material to protect themselves against protozoal attack by chemically treating the surfaces in the hive. The best known protozoal parasite of bees is *Crithidia bombi* which infects bumble bees (Schlüns *et al.*, 2010) and this flagellated kinetoplastid is a quite close relative of the human pathogen *T. brucei*. It has been proposed that *Crithidia mellificae* is responsible for significant winter mortality in Western European beehives (Ravoet *et al.*, 2013). Quite small alterations in structure appear to produce a marked difference in anti-trypanosomal activity with 8-prenyl naringenin (**8**) being more active than its isomer 6-prenyl naringenin (**7**). Propolis can be collected in large quantities and thus has good potential for treating protozoal infections if *in vivo* efficacy can be proved.

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Table 1 NMR data for riverinol. Carbon numbering refers to figure 4.

Carbon	**^{13}C δ** [a] (ppm)	**^{1}H δ** (ppm)	**COSY** (^{1}H – ^{1}H)	**HMBC** (^{13}C -^{1}H)
2	69.77	4.18($H2_d$)[b] , ddd [c], $J^{d}_{2d,2u}$=10.4, $J_{2d,3}$ =3.5, $J_{2d,10u}$ =1.9 3.96 ($H2_u$), t , $J_{2d,2u}$=10.4 $J_{2u,3}$ = 5.2	$H2_u$, H3, $H10_u$ $H2_d$, H3	$H10_d$, $H10_u$
3	31.47	3.35, m	$H10_d$, $H10_u$, $H2_d$, $H2_u$	$H10_d$, $H10_u$
4	114.9	na [e]	na	H6, H8, $H10_d$, $H10_u$
5	155.19	na	na	H6, H9
6	101.52	6.35 (H6) d, $J_{6,8}$ = 2.4	no [g]	H8
7	159.02	na	na	H6, H8, H9, H7-OCH_3
7-OCH_3	55.36	3.70, s	no [f]	H6, H8
8	107.15	6.45 (H8) dd, $J_{8,9}$= 8.2, $J_{8,6}$= 2.4	no[g]	H6
9	128.19	7.01 (H9) d $J_{8,9}$= 8.2	H8, H6	H3
10	30.17	2.95 ($H10_d$)[b] ,dd, $J_{10d,10u}$ =15.45, $J_{10d,3}$ = 10.76	$H10_u$, H3	$H2_u$, $H2_d$, H3
		2.79 ($H10_u$) ddd $J_{10d,10u}$ =15.45, $J_{10u,3}$ = 5.56, $J_{10u,2d}$ =1.9	$H10_d$,H3	
1’	159.3	-	na	H2’, H5’ , 1’-OCH_3

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1'-OCH_3	55.5	3.68	na	H1'
2'	101.8	6.43, (H2') d $J_{2,'6'}$ = 2.7	no[g]	H6'
3'	156.35	OH 9.6s	na	H5', H2 ,H3
4'	120.03	-	na	$H10_u$, $H10_d$, H3, H2', 3'-OH, H6'
5'	130.64	6.99,(H5') d, $J_{5,'6'}$ = 8.5.	H5'H6'	$H10_u$, $H10_d$
6'	104.85	6.37, (H6') dd, $J_{5',6'}$ = 8.5, $J_{2,'6'}$ = 2.7	H5'H6'	H6

[a] all protonated carbon assignments confirmed using a gradient-enhanced ^{13}C – ^{1}H HSQC NMR experiment

[b] d = downfield and u = upfield partner of geminal proton pair

[c] s=singlet, d=doublet, t=triplet, m=multiplet

[d] J: coupling constant (Hz)

[e] not applicable

[f] none observed

[g] Not possible to observe because of overlaid signal for the correlation between 8 and 9.

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Table 2 EC50 values (n=3) values (µg/ml) for some of the compounds isolated from Nigerian red propolis. The strains of *T.brucie* tested were Lister 427 wild-type (standard drug-sensitive control), AQP2-KO (same strain as WT, but with the TbAQP2 gene, coding for the important drug transporter HAPT1, deleted by targeted gene deletion; resistant to pentamidine and melarsoprol), and B48 (adapted from Lister 427WT by *in vitro* exposure to pentamidine; lost both main drug transporters, HAPT1 and TbAT1, and is highly resistant to pentamidine). Averages and SEM are given for n=3. Resistance factor (RF) is the ratio of EC_{50} for resistant strain over WT. Unpaired ttest compared EC_{50} values of each resistant strain versus WT. Units are in µg/ml except for pentamidine, where the EC_{50} value is given in µM. NA = naringenin.

	TbS427WT		**B48**				**aqp2/aqp3 null**			
Fraction No.	**AVG**	**SEM**	**AVG**	**SEM**	**RF**	ttest	**AVG**	**SEM**	**RF**	ttest
RSN	4.2	0.04	3.9	0.16	0.92	0.13	4.4	0.26	1.05	0.46
Riverinol(**6**)	16.6	0.24	4.7	1.83	0.28	<0.001	6.6	1.52	0.40	<0.001
Calycosin/liquiritigenin (**1/2**)	10.0	0.44	14.8	1.61	1.48	0.05	7.4	0.32	0.74	0.01
Vestitol (**4**)	8.3	0.10	10.2	0.66	1.23	0.05	8.1	0.14	0.97	0.18
8-prenyl NA (**8**)	6.1	0.10	7.1	0.18	1.15	0.01	6.5	0.06	1.05	0.05
6-Prenyl NA (**7**)	11.4	0.34	13.7	0.33	1.20	0.01	10.4	0.18	0.91	0.05
Macarangin (**10**)	7.8	0.10	10.2	2.56	1.31	0.40	6.5	0.05	0.83	<0.001
BRN	6.9	0.3	7.2	0.3	1.04	0.58				
Pentamidine	0.0023	0.0002	0.51	0.04	224.78	<0.001	0.071	0.002	31.08	<0.001

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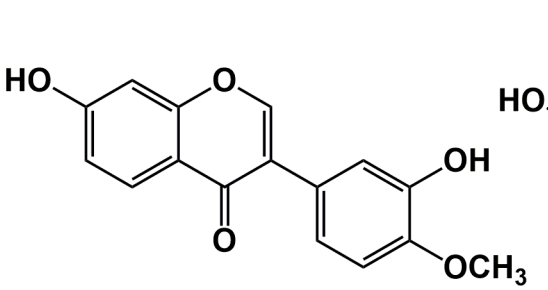
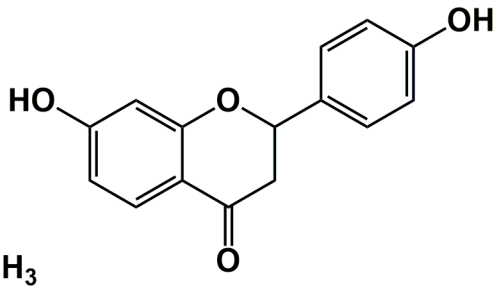
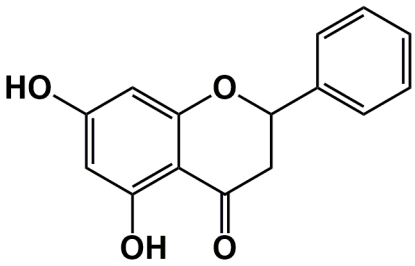
Figure 1 Structures of the flavonoids, isoflavonoids and benzofuran isolated from Nigerian red propolis.



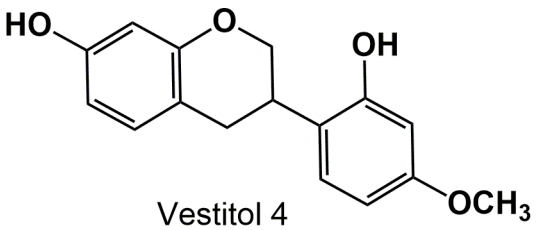
Calycosin 1



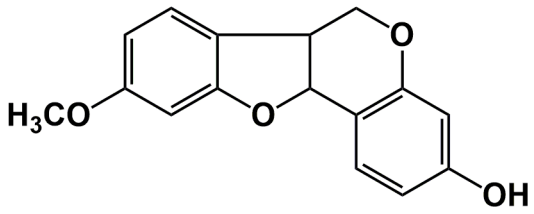
Liquiritigenin 2



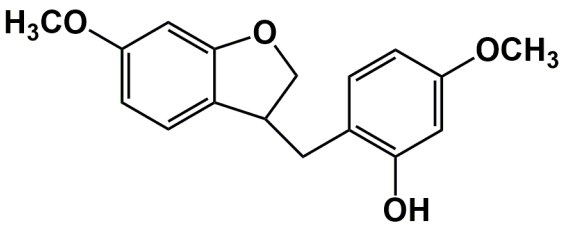
Pinocembrin 3



Vestitol 4



Medicarpin 5



Riverinol 6

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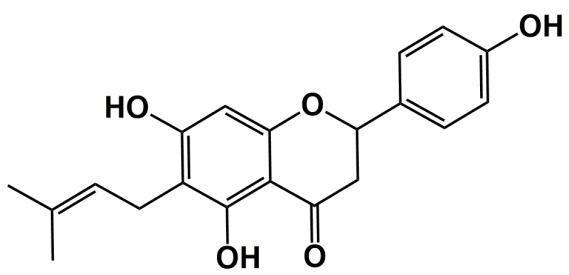
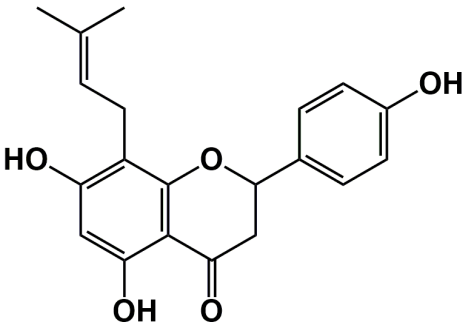
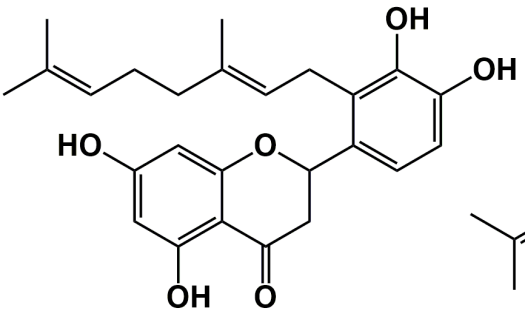
Figure 2 Structures of the prenylated flavonoids isolated from Nigerian red propolis.



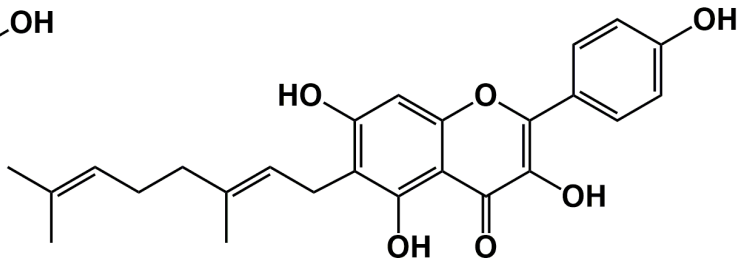
6-prenylnaringenin 7



8-prenylnaringenin 8



Propolin D 9



Macarangin 10

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Figure 3 MS fragmentations across the C ring of a flavanoid.

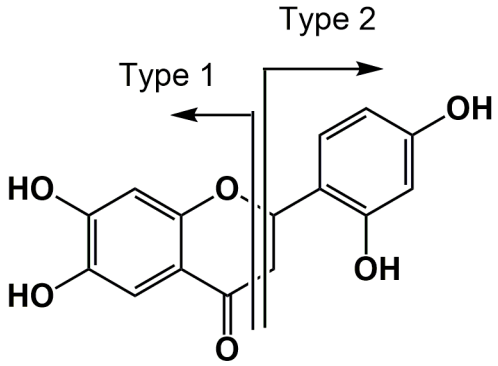

Figure 4 Numbered stucture of riverinol (**6**) for structure elucidation purposes, and a closely related dihydrobenzofuran previously isolated (Han *et al.*, 2008).

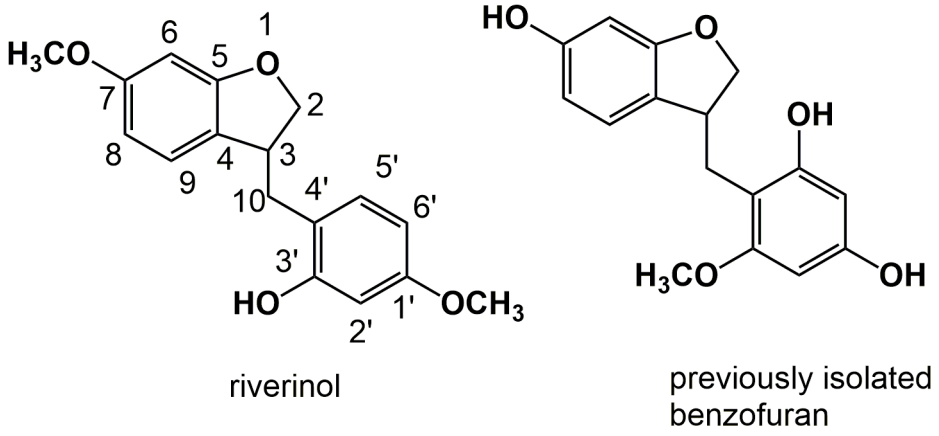

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Figure 5 MS^2 fragmentation of a polyprenylated benzophenone illustrated for oblongifolin A.

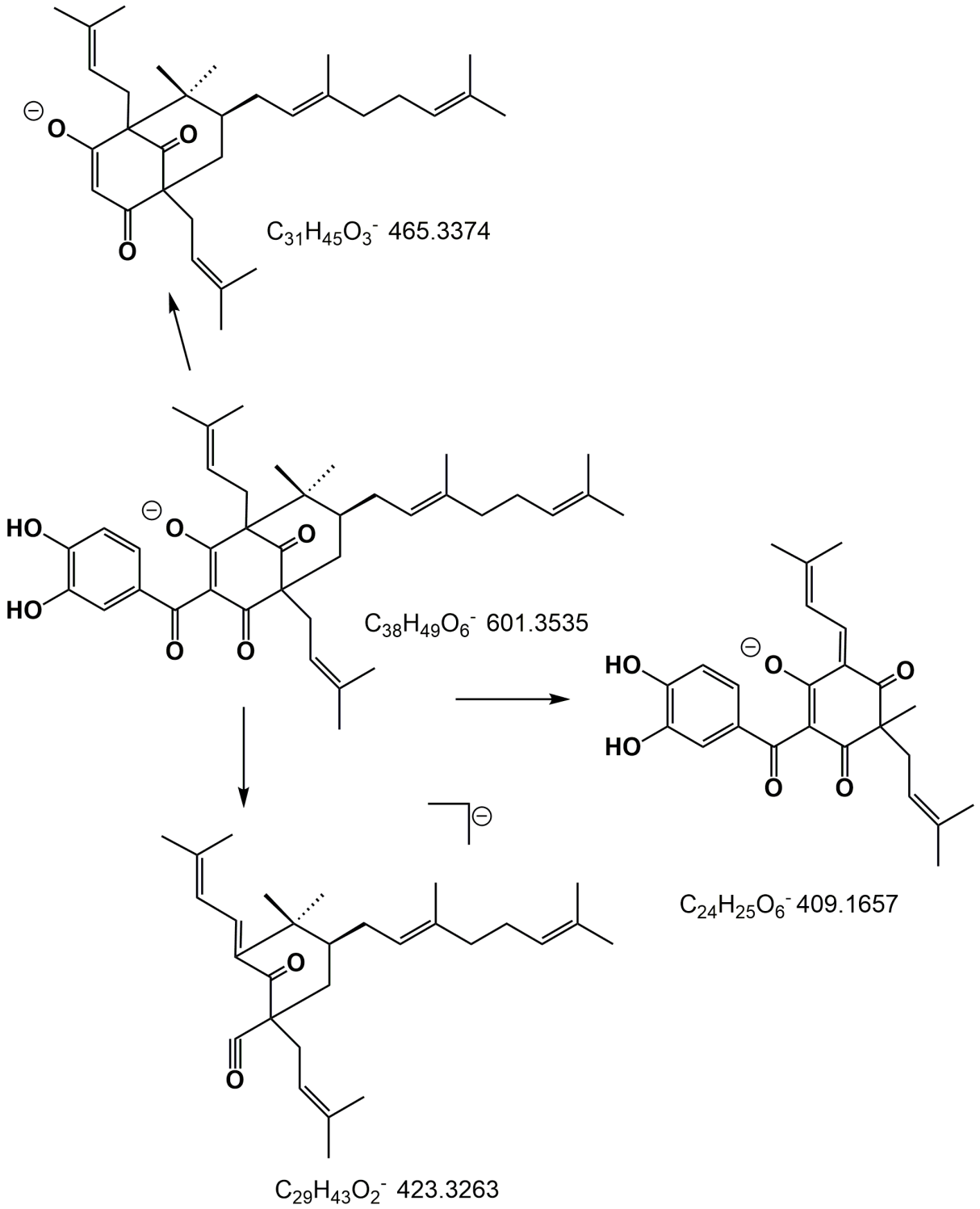

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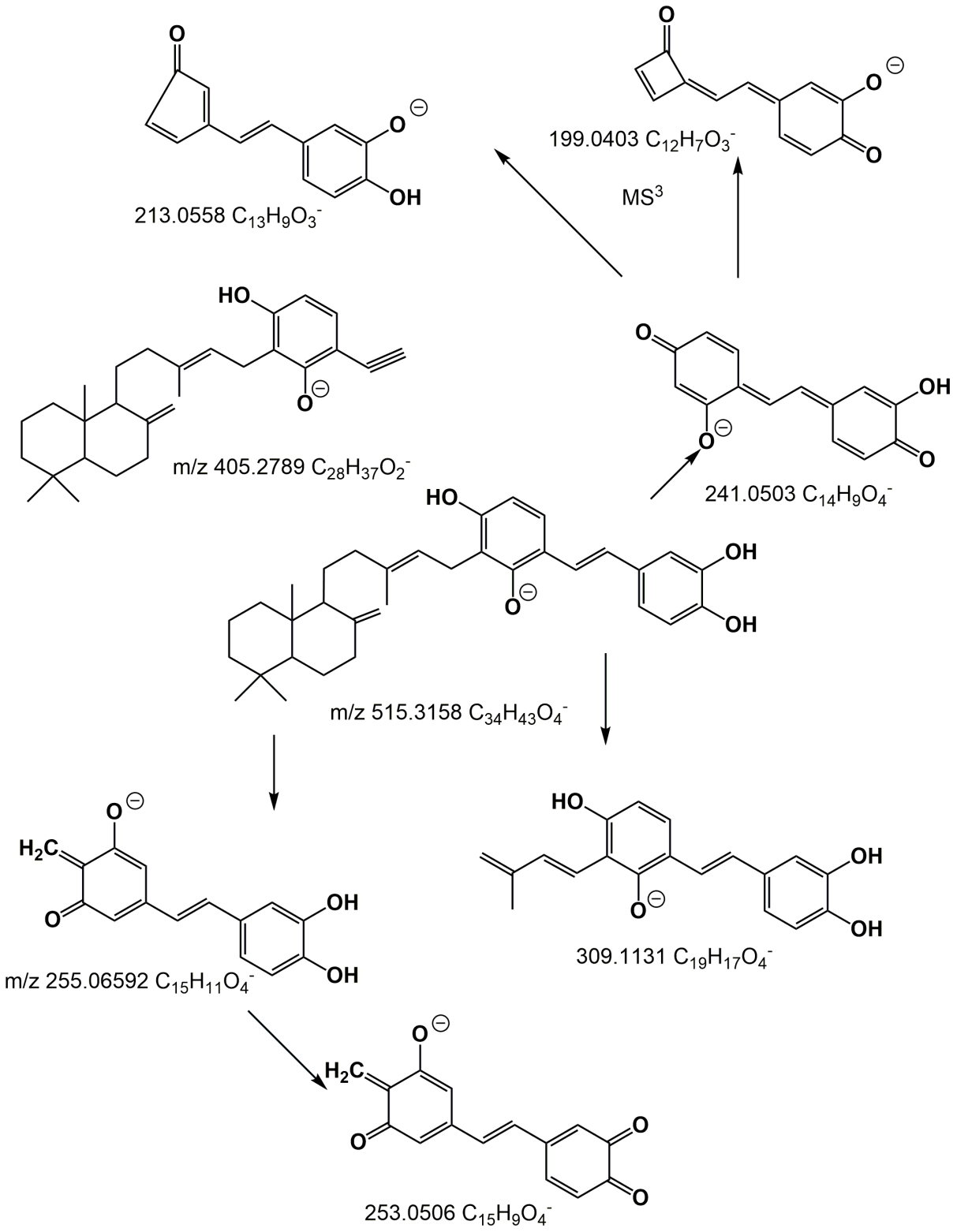
Figure 6 Proposed MS^2 and MS^3 fragmentation of denticulatain A.



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