

Characterisation of triterpenes and new phenolic lipids in Cameroonian propolis

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Abstract

Chemical investigation of a sample of propolis originating from North-Western Cameroon led to the isolation of thirteen alk(en)ylphenols (**1–13**) (inseparable mixture) along with α -amyrin (**14**), β -amyrin (**15**), lupeol (**16**), cycloartenol (**17**), mangiferonic acid (**18**), ambonic acid (**19**), mangiferolic acid (**20**), ambolic acid (**21**), isomangiferolic acid (**22**) and nine alk(en)ylresorcinols (**23–31**) (inseparable mixture). All compounds were identified following analysis of their spectroscopic data and comparison with previously published reports. Compounds (**8**), (**12**), (**13**) and (**30**) are new natural products. GC–MS analysis carried out on the alk(en)ylphenol and alk(en)ylresorcinol mixtures (dimethyl disulfide trimethylsilyl derivatives) revealed the presence of saturated and mono-unsaturated compounds with side chain lengths ranging from C11 to C19 and C15 to C19, respectively. The position of the double bond in mono-unsaturated derivatives was established from the characteristic fragments resulting from the cleavage of the bond between the two methylthio-substituted carbons. The most abundant compound in each mixture was 3-(12'-heptadecenyl)-phenol (**10**) and 5-(12'-Z-heptadecenyl)-resorcinol (**29**). This study is the first to report the presence of triterpenes (except for lupeol) and phenolic lipids, including eighteen compounds previously unreported in bee glue, in an African sample.

Keywords: Cameroonian propolis; Triterpenes; Alk(en)ylphenols; Alk(en)ylresorcinols; Phenolic lipids

1 Introduction

Propolis is a natural substance produced by bees upon collection of resins and exudates from plants. Bees use it as antiseptic glue to seal gaps between honeycombs, embalm dead intruders and generally preserve the hive from external contamination (Bankova, 2005a). Propolis has a long history of use in folk medicine and is a popular remedy currently employed to treat a variety of ailments (Castaldo and Capasso, 2002). Numerous scientific studies have been published on the biological properties of propolis and its constituents, including anti-inflammatory (Borrelli et al., 2002) anti-oxidant (Silva et al., 2011), hepatoprotective (Banskota et al., 2001), immunostimulant (Fischer et al., 2007), antitumour (Sawicka et al., 2012), neuroprotective (Nakajima et al., 2009) and antimicrobial activity (Seidel et al., 2008).

The chemical composition of propolis is complex. Different propolis types have been characterised based on the nature of the plant-derived substances present and the geographical origin of collection. Typically, propolis is broadly characterised into (i) samples from temperate regions mainly originating from poplar tree exudates and rich in phenolics such as flavonoids, aromatic acids and esters (Bankova et al., 2002) and (ii) samples from tropical areas, devoid or containing traces of poplar constituents but rich in other substances including prenylated derivatives of *p*-coumaric acids, diterpenes and lignans (Marcucci and Bankova, 1999), prenylated benzophenones (Cuesta Rubio et al., 2002) and prenylated flavonoids (Raghukumar et al., 2010).

There are numerous reports in the literature on the isolation and structural elucidation of phytochemicals from propolis collected in Europe (Hegazi et al., 2000), South America (Trusheva et al., 2004), Asia and the Pacific region (Chen et al., 2004). Much less is known, however, about the exact chemical constituents of African propolis. In Cameroon, propolis is an important traditional medicine which has antibacterial and antiradical activity (Njintang Yanou et al., 2012; Mbawala et al., 2009, 2010; Talla et al., 2013). We carried out a phytochemical analysis of a propolis sample collected in North-Western Cameroon and report herein the characterisation of nine triterpenes, thirteen alk(en)ylphenols and nine alk(en)ylresorcinols.

2 Results and discussion

The sample of propolis was extracted with 70% ethanol and the extract obtained was partitioned with *n*-hexane, ethyl acetate and *n*-butanol, respectively. Fractionation of the *n*-hexane and ethyl acetate extracts using repeated chromatographic procedures led to the isolation of a mixture of thirteen alk(en)ylphenols (1–13), including the three new structures (8), (12) and (13), along with α -amyrin (14) (Hernández Vázquez et al., 2012; Basyuni et al., 2006), β -amyrin (15) (Basyuni et al., 2006; Mahato and Kundu, 1994), lupeol (16) (Basyuni et al., 2006; Thanakijcharoenpath and Theanphong, 2007), cycloartenol (17) (Kamisako et al., 1987; Zhu et al., 2012), mangiferonic acid (18) (Escobedo-Martinez et al., 2012), ambonic acid (19) (Da Silva et al., 2005), mangiferolic acid (20) (Escobedo-Martinez et al., 2012), ambolic acid (21) (Escobedo-Martinez et al., 2012), and isomangiferolic acid (22) (Escobedo-Martinez et al., 2012). Fractionation of the *n*-butanol extract afforded a mixture of nine alk(en)ylresorcinols (23–31), including the new structure (30) (Figs. 1 and 2). All known compounds were identified following analysis of their spectroscopic data and comparison with previously published reports.

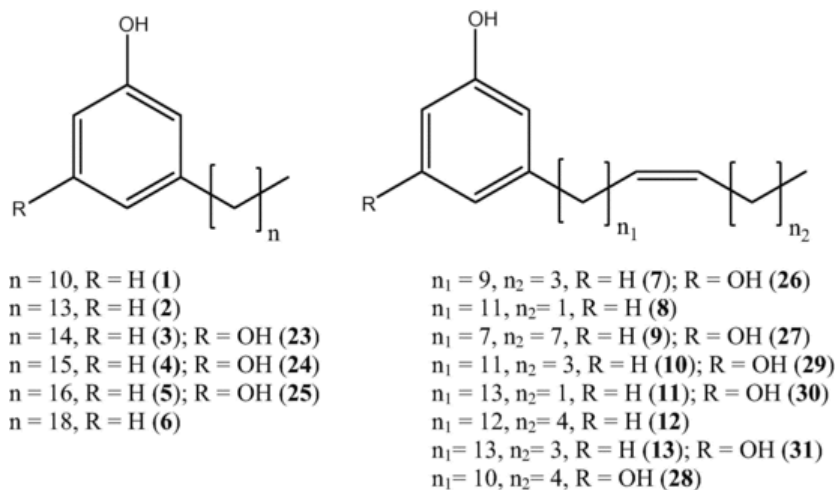
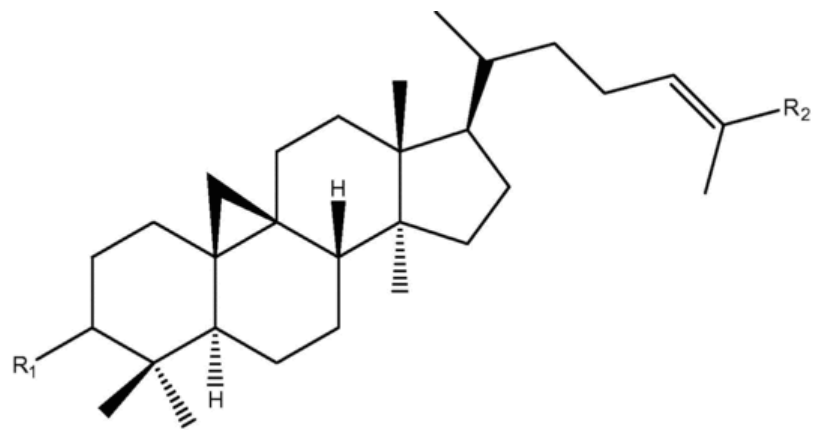


Fig. 1 Structures of alk(en)ylphenols and alk(en)ylresorcinols in Cameroonian propolis.



$R_1 = \beta\text{-OH}$, $R_2 = \text{CH}_3$

$R_1 = =\text{O}$, $R_2 = \text{COOH}$

$R_1 = \beta\text{-OH}$, $R_2 = \text{COOH}$

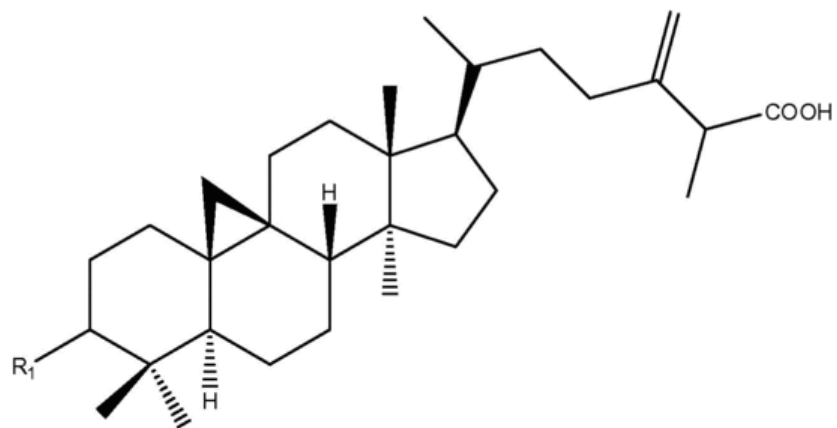
$R_1 = \alpha\text{-OH}$, $R_2 = \text{COOH}$

Cycloartenol (**17**)

Mangiferonic acid (**18**)

Mangiferolic acid (**20**)

Isomangiferolic acid (**22**)



$R_1 = =\text{O}$, Ambonic acid (**19**)

$R_1 = \beta\text{-OH}$, Ambolic acid (**21**)

Fig. 2 Structures of cycloartane triterpenes in Cameroonian propolis.

Compounds (1–13) were obtained as a yellow-coloured oil (R_f 0.49 in hexane–EtOAc, 8:2). The ^1H NMR revealed a 1,3-disubstituted aromatic system with four protons at δ 7.13 (t), 6.74 (d), 6.64 (s) and 6.63 (d); as well as signals typical for a long aliphatic chain including olefinic (δ 5.33, m), benzylic (δ 2.54, t) and allylic (δ 2.04, m) protons. A sharp singlet at δ 4.57, attributable to a phenolic OH group, was also observed. Both ^1H and ^{13}C NMR data showed good correlation with

literature reports on 3-alk(en)ylphenols (Saitta et al., 2009; Silva et al., 2008; Franke et al., 2001). In order to determine the composition of the mixture, the compounds were derivatised to their corresponding dimethyl disulfide-trimethylsilyl derivatives and the mixture was separated by GC–MS. The GC-trace (Fig. 3) allowed the unambiguous identification of a total of 13 different compounds with side chains ranging from C11 to C19 and including six (saturated) alkylphenols and seven alkenylphenols showing mono-unsaturation (Table 1). The EI mass spectrum of all compounds showed predominant fragment ions at m/z 179 (benzylic cleavage) and/or 180 (benzylic cleavage with transfer of one proton) (Franke et al., 2001). The position of the double bond in mono-unsaturated dimethyl disulfide derivatives was established from the characteristic fragments resulting from the cleavage of the bond between the two methylthio-substituted carbons (Christie, 1997). Key fragments observed for mono-unsaturated derivatives are reported in Table 2. The fragmentation of compounds (7), (9) and (10) was in agreement with the literature (Saitta et al., 2009). Key fragments for the dimethyl disulfide trimethyl silyl derivative of (11) are reported here for the first time. The mass spectrum of (11) showed a molecular ion $[M]^+$ at m/z 496 and two fragments, resulting from α -homolytic cleavage directed by the sulphur atom, at m/z 89 (base peak) and 407, corresponding to the structures $[\text{CH}_3\text{-CH}_2\text{-CHSCH}_3]^+$ and $[(\text{CH}_3)_3\text{SiO-C}_6\text{H}_4\text{-(CH}_2)_{13}\text{-CHSCH}_3]^+$, respectively. A fragment at m/z 359 was attributed to neutral loss of a CH_3SH unit (m/z 48) from the fragment ion at m/z 407. The mass spectrum of compound (8) showed a molecular ion $[M]^+$ at m/z 468 and two diagnostic fragments at m/z 89 (base peak) and 379, corresponding to the structures $[\text{CH}_3\text{-CH}_2\text{-CHSCH}_3]^+$ and $[(\text{CH}_3)_3\text{SiO-C}_6\text{H}_4\text{-(CH}_2)_{11}\text{-CHSCH}_3]^+$, respectively. A fragment at m/z 331 was attributed to neutral loss of a CH_3SH unit from the fragment at m/z 379 (Fig. 4A). The mass spectrum of compound (12) showed a molecular ion $[M]^+$ at m/z 524 and two key fragments at m/z 131 and 393, corresponding to the structures $[\text{CH}_3\text{-(CH}_2)_4\text{-CHSCH}_3]^+$ and $[(\text{CH}_3)_3\text{SiO-C}_6\text{H}_4\text{-(CH}_2)_{12}\text{-CHSCH}_3]^+$, respectively (Fig. 4B). The mass spectrum of compound (13) also showed a molecular ion $[M]^+$ at m/z 524 but with two typical fragments at m/z 117 and 407, corresponding to the structures $[\text{CH}_3\text{-(CH}_2)_3\text{-CHSCH}_3]^+$ and $[(\text{CH}_3)_3\text{SiO-C}_6\text{H}_4\text{-(CH}_2)_{13}\text{-CHSCH}_3]^+$, respectively. A fragment at m/z 359 was attributed to a neutral loss of a CH_3SH unit from the fragment at m/z 407 (Fig. 4C). The configuration of the double bond was established as Z based on the ^{13}C NMR resonances of allylic methylenes (δ_c 627.2 and 26.9) in agreement with the literature (Ali Al-Mekhlafi et al., 2012). Analysis of the percentage composition revealed that the majority of the mixture was comprised of mono-unsaturated alkenylphenols (ca. 76%) and of compounds with an odd number of carbons in their side chains (99.6%). 3-(12'Z-Heptadecenyl)-phenol (10) was identified as the most abundant compound (ca. 55% of the mixture) (Table 1).

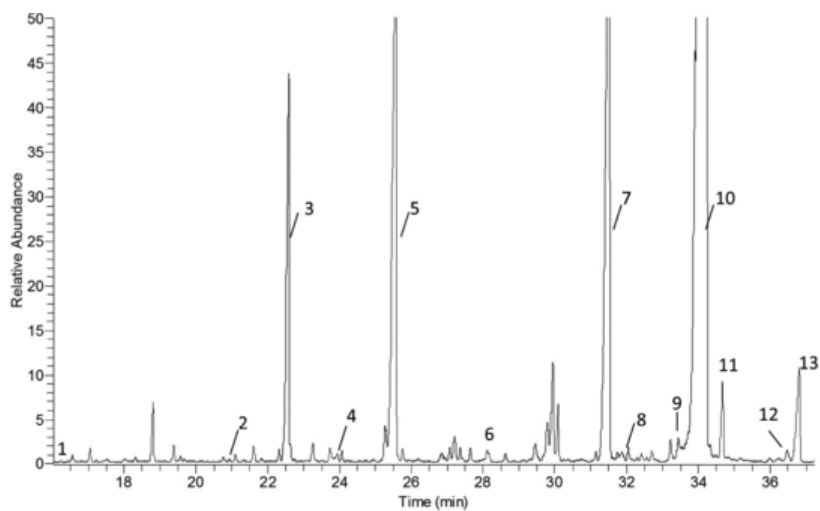


Fig. 3 GC–MS trace of alk(en)ylphenols (dimethyl disulfide-trimethylsilyl derivatives) in Cameroonian propolis. The identity of peaks is reported in Table 1.

Table 1 Percentage composition of identified alk(en)ylphenols (1–13) in Cameroonian propolis.^a

Compound name	Range (%)	Mean ± SD (%)
3-Undecyl phenol (1)	0.07–0.09	0.09 ± 0.01
3-Tetradecylphenol (2)	0.07–0.10	0.09 ± 0.01
3-Pentadecylphenol (3)	7.47–8.72	8.06 ± 0.63
3-Hexadecylphenol (4)	0.16–0.19	0.17 ± 0.02

3-Heptadecylphenol (5)	14.34–15.87	15.16 ± 0.77
3-Nonadecylphenol (6)	0.26–0.32	0.28 ± 0.04
3-(10′Z-Pentadecenyl)-phenol (7)	14.79–17.0	16.16 ± 1.20
3-(12′Z-Pentadecenyl)-phenol (8)	0.28–0.35	0.31 ± 0.03
3-(8′Z-Heptadecenyl)-phenol (9)	0.63–0.80	0.74 ± 0.10
3-(12′Z-Heptadecenyl)-phenol (10)	54.48–55.58	54.98 ± 0.56
3-(14′Z-Heptadecenyl)-phenol (11)	1.10–1.36	1.25 ± 0.13
3-(13′Z-Nonadecenyl)-phenol (12)	0.35–0.39	0.37 ± 0.02
3-(14′Z-Nonadecenyl)-phenol (13)	1.99–2.35	2.17 ± 0.18

^a Determined on the basis of peak areas obtained by GC–MS (three replicates).

Table 2 Key fragments observed on the mass spectra of mono-unsaturated alkenylphenols (**7–13**) (dimethyldisulfide-trimethylsilyl derivatives).

Compound	RT (min)	[M] ⁺ , <i>m/z</i> (%)	Key fragment ions, <i>m/z</i> (%)
(7)	31.51	468 (2)	351 (30), 303 (6), 179 (70), 117 (85)
(8)	32.07	468 (2)	379 (6), 331 (5), 180 (55), 179 (50), 89 (100)
(9)	33.43	496 (3)	323 (24), 275 (14), 179 (60), 173 (60)
(10)	34.20	496 (3)	379 (35), 331 (20), 179 (85), 117 (100)
(11)	34.67	496 (2)	407 (9), 359 (9), 179 (44), 89 (100)
(12)	36.47	524 (4)	393 (26), 179 (32), 131 (50)
(13)	36.80	524 (4)	407 (20), 359 (10), 179 (50), 117 (95)

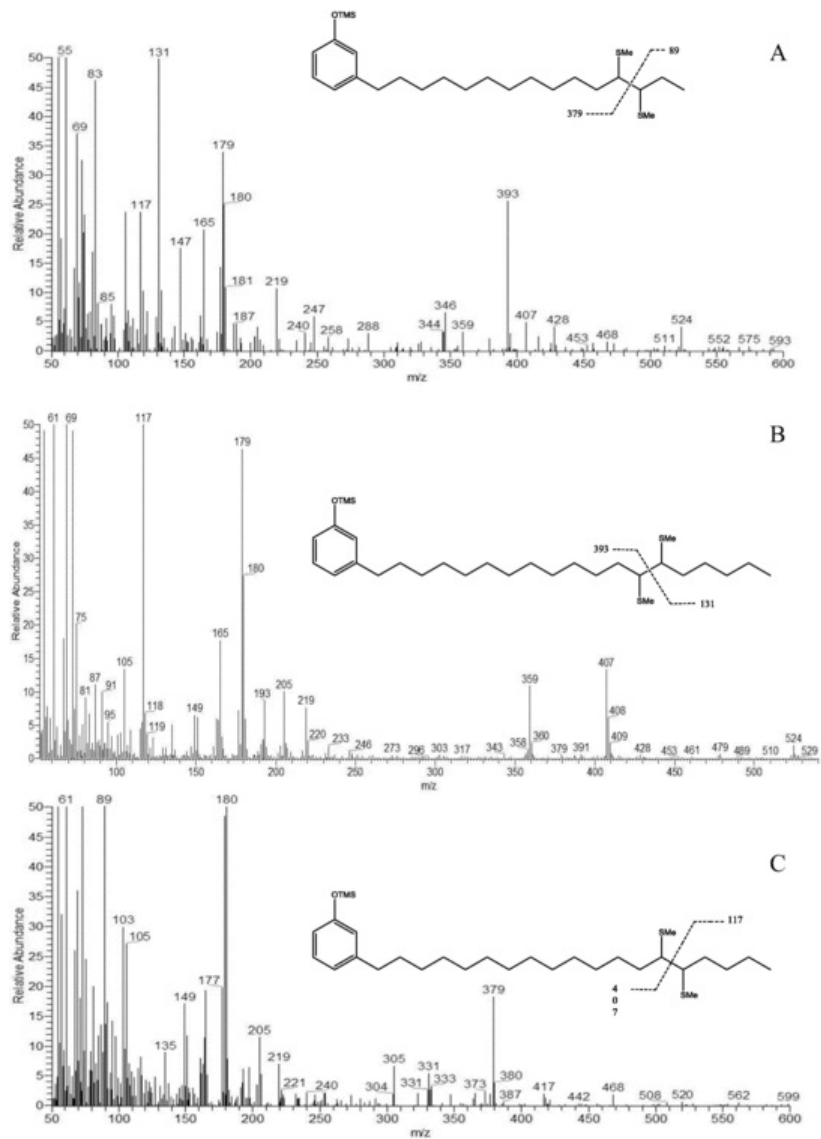


Fig. 4 EI mass spectra including key fragmentation of (A) compound (8); (B) compound (12); (C) compound (13) (dimethyl disulfide-trimethylsilyl derivatives).

Compounds (23–31) were obtained as a yellow-coloured oil (R_f 0.57 in CH_2Cl_2). The ^1H and ^{13}C NMR spectra revealed the presence of a resorcinol moiety with two protons at δ_{H} 6.24 (d, $J = 1.8$ Hz, H4/6) and one at δ_{H} 6.17 (t, $J = 1.8$ Hz, H2) and four carbon signals at δ_{C} 100.1 (C-2), 108.0 (C-4/6), 146.1 (C-5), 156.6 (C-1/3); as well as signals typical for a long aliphatic chain including olefinic (65.34, m), benzylic (62.46, t), and allylic (62.01, m) protons. Both ^1H and ^{13}C NMR data showed good correlation with literature reports on 5-alk(en)ylresorcinols (Silva et al., 2008; Knödler et al., 2008). The configuration of the double bond was established as Z based on the ^{13}C NMR resonances of allylic methylenes (δ_{C} 627.2 and 26.9) in agreement with the literature (Ali Al-Mekhlafi et al., 2012). In order to determine the composition of the mixture, the compounds were derivatised to their corresponding dimethyl disulfide-trimethylsilyl derivatives and the mixture was separated by GC–MS (Fig. 5). Diagnostic key fragments originating from the cleavage of the bond between the two methylthio-substituted carbons in mono-unsaturated derivatives indicated the presence of six mono-unsaturated

alkenylresorcinols (Table 3). The molecular ions observed at m/z 602 (**26**), m/z 630 (**27–30**) and m/z 658 (**31**) were found to correspond to the dimethyl disulfide-trimethylsilyl derivatives of C15:1 (m/z 556), C17:1 (m/z 584), and C19:1 (m/z 612), respectively each showing an additional substituent accounting for +46 amu. This shift, which was also observed in all diagnostic fragments containing the resorcinol moiety, indicated additional substitution on the aromatic ring (Knödler et al., 2008). In order to establish unambiguously what was the nature of this change on the aromatic ring, we prepared some dimethyl disulfide derivatives of commercial orcinol ($C_7H_8O_2$, $[M]^+$, $m/z = 124$) and resorcinol ($C_6H_6O_2$, $[M]^+$, $m/z = 110$).

GC–MS analysis of the products obtained confirmed the presence of $[M+46]^+$ molecular ions at m/z 170 and m/z 156, as well as $[M+92]^+$ ions at m/z 216 and m/z 202 for orcinol and resorcinol, respectively. Further 1H NMR analysis was carried out on the dimethyl disulfide derivative of pure orcinol since the latter, like 5-alk(en)ylresorcinols, is a 5-methyl substituted resorcinol. The spectrum revealed the presence of non-equivalent *meta*-coupled methines at 6.31 (d, 2.5 Hz) and 6.34 (d, 2.5 Hz), a methine singlet at 6.55, sharp phenolic singlets at 7.15 and 7.35, and methyl singlets at 2.77, 2.45, 2.16 and 2.14. Further analysis of the ^{13}C NMR and HMBC spectrum established the presence of two distinct derivatives of orcinol, which were identified as 6-methylmercapto-orcinol (**32**) and 4,6-dimethylmercapto-orcinol (**33**) (Fig. 6, Table 4). Phenols are known to react with alkyl disulfides (under reflux, stirring and in the presence of a strong acid catalyst) to form alkylmercaptophenols. This occurs *via* the formation of a highly-reactive sulfenium ion intermediate which attacks the aromatic ring by electrophilic reaction (Farah and Gilbert, 1963). The presence of two *meta*-coupled phenolic hydroxyls in resorcinols means that the *ortho* and *para* positions relative to each hydroxyl are strongly activated and highly reactive towards electrophilic attack, allowing for reactions to take place under much milder conditions than for phenols and leading to the formation of polysubstituted products (Durairaj, 2005). This explained the addition of one (two) thiomethylated substituent(s) on the aromatic ring as seen in the GC–MS with a +46 (+92) amu shift. Peaks in the GC–MS spectrum observed at m/z 510, 524 and 538 were subsequently identified as C15:0 (**23**), C16:0 (**24**) and C17:0 (**25**) methylmercaptoresorcinol derivatives.

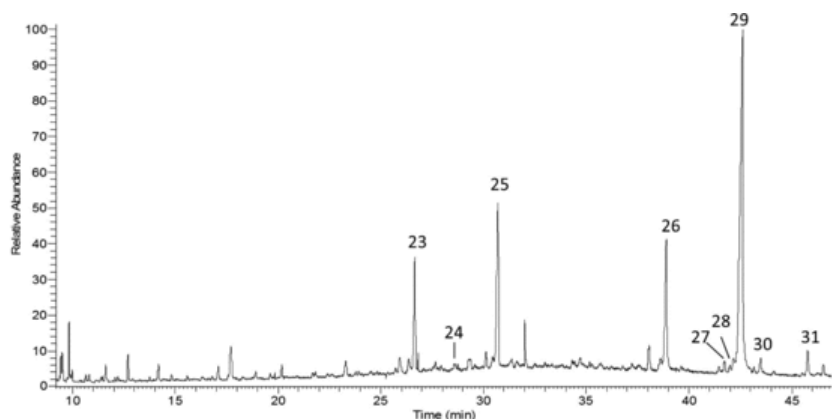


Fig. 5 GC–MS trace of alk(en)ylresorcinols (dimethyl disulfide-trimethylsilyl derivatives) in Cameroonian propolis. The identity of peaks is reported in Table 3.

Table 3 Key fragments observed on the mass spectra of mono-unsaturated alkenylresorcinols (**26–31**) (dimethyl disulfide-trimethylsilyl derivatives).

Compound	RT (min)	$[M+46]^+$, m/z (%)	Key fragment ions, m/z (%)
(26)	38.89	602 (100)	485 (60), 268 (35), 117 (35)
(27)	41.72	630 (85)	457 (70), 267 (35), 173 (95)
(28)	42.16	630 (70)	499 (45), 268 (55), 131 (55)
(29)	42.51	630 (87)	513 (100), 267 (55), 117 (85)
(30)	43.49	630 (90)	541 (55), 268 (55), 89 (90)
(31)	45.78	658 (90)	541 (85), 267 (82), 117 (75)

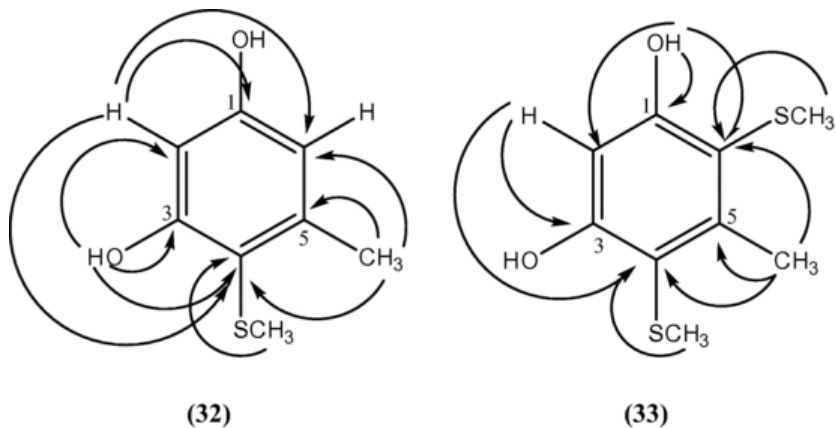


Fig. 6 Important HMBC correlations (H \rightarrow C) for compounds (32) and (33).

Table 4 NMR data for orcinol and compounds (32) and (33) in CDCl₃.^a

Position	Orcinol	(32)		(33)	
	¹ H	¹ H	¹³ C	¹ H	¹³ C
1	—	—	158.2	—	159.0
2	6.16 s	6.34 d (2.5)	99.2	6.55 s	98.4
3	—	—	157.3	—	159.0
4	6.23 s	6.31 d (2.5)	109.5	—	112.3
5	—	—	144.6	—	148.4
6	6.23 s	—	111.7	—	112.5
Me	2.24 s	2.45 s	20.8	2.77 s	19.4
SMe	—	2.15 s	18.7	2.16 s	18.7
1/3-OH	—	7.15 s	—	7.35 s	—

^a ¹H (400 MHz) and ¹³C (100 MHz); assignments were established by DEPT 135 and HMBC data.

Among the nine alk(en)ylresorcinols identified, compound (30) is a new natural product. Its mass spectrum showed a molecular ion [M+46]⁺ at *m/z* 630 and two diagnostic fragments at *m/z* 89 (base peak) and 541, corresponding to the structures [CH₃-CH₂-CHSCH₃]⁺ and [[(CH₃)₃SiO]₂-C₆H₄-(CH₂)₁₃-CHSCH₃+46]⁺, respectively (Fig. 7). Analysis of the percentage composition revealed that the majority of the mixture was comprised of mono-unsaturated alkenylresorcinols (77.8%) and of compounds with an odd number of carbons in their side chains (99.3%). 5-(12'-Z-Heptadecenyl)-resorcinol (29) was identified as the most abundant compound (ca. 58% of the mixture) (Table 5).

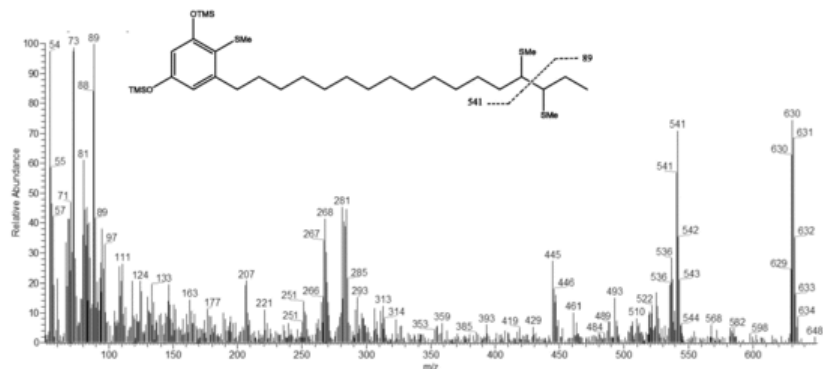


Fig. 7 EI mass spectra including key fragmentation of compound (30) (dimethylsulfoxide-trimethylsilyl derivative).

Table 5 Percentage composition of identified alk(en)ylresorcinols (23–31) in Cameroonian propolis.^a

Compound name	Range (%)	Mean \pm SD (%)
5-Pentadecylresorcinol (23)	6.89–9.03	7.64 \pm 1.20
5-Hexadecylresorcinol (24)	0.53–0.86	0.67 \pm 0.17
5-Heptadecylresorcinol (25)	13.25–14.66	13.86 \pm 0.73
5-(10'Z-Pentadecenyl)-resorcinol (26)	13.86–14.1	13.99 \pm 0.12
5-(8'Z-Heptadecenyl)-resorcinol (27)	1–1.17	1.08 \pm 0.08
5-(11'Z-Heptadecenyl)-resorcinol (28)	0.4–0.64	0.52 \pm 0.12
5-(12'Z-Heptadecenyl)-resorcinol (29)	55.89–59.1	57.97 \pm 1.81
5-(14'Z-Heptadecenyl)-resorcinol (30)	1.46–1.68	1.58 \pm 0.11
5-(14'Z-Nonadecenyl)-resorcinol (31)	2.45–2.98	2.69 \pm 0.27

^a Determined on the basis of peak areas obtained by GC–MS (three replicates).

All phenolic lipids isolated in this study, except for (23), (25), (27) and (28) which are present in Indonesian propolis (Trusheva et al., 2011), are reported for the first time in propolis. It is noteworthy to mention that Brazilian, Thai and Omani propolis are also known to contain alk(en)ylphenols and alk(en)ylresorcinols (Silva et al., 2008; Teerasripreecha et al., 2012; Popova et al., 2013), but that these compounds have never been reported in African propolis. Except for (8), (12) and (13) which are new natural products, the alk(en)ylphenols characterised in this study are constituents of *Rhus thyrsoiflora*, *Pistacio vera*, and other plants belonging to the Anacardiaceae family (Saitta et al., 2009; Franke et al., 2001). Interestingly, *Rhus javanica* var. *chinensis* has been identified as a plant origin of Japanese propolis (Murase et al., 2008). The relative proportion of alk(en)ylphenols in Cameroonian propolis differs from the composition reported for *Rhus thyrsoiflora* which contains mainly compound (3) (~48%), (5) (~19%) and (9) (~17%) but only minor amounts of (7) (0.8%) and (10) (2.4%) (Franke et al., 2001). Both mixtures of 5-alk(en)ylresorcinols and 3-alk(en)ylphenols contained similar proportions of four main groups which included C15:0, C17:0, 10'Z-C15:1, 12'Z-C17:1 derivatives and 14'Z-C19:1 derivatives as minor constituents. Among the major alk(en)ylresorcinols, compounds (23) and (25) have previously been reported in cereal grains (Kulawinek et al., 2008) and in mango (*Mangifera indica*) (Kienzle et al., 2014). Compound (26) has previously been isolated from *Grevillea* species (Wang et al., 2009). Compound (29) is also a constituent of mango (Cojocararu et al., 1986).

Triterpenes were predominant in the hexanic and EtOAc phases (see S1 in Supplementary Information). Among the identified compounds, (14–17), (19) and (21) are found in Brazilian propolis (Da Silva et al., 2005; Silva et al., 2008). Mangiferonic acid (18), mangiferolic acid (20) and isomangiferolic acid (22) have been identified in propolis from Myanmar (Li et al., 2009) and from Brazil (Da Silva et al., 2005). Mangiferolic acid (20) and isomangiferolic acid (22) are found in Indonesian propolis (Trusheva et al., 2011) To the best of our knowledge, this is the first report of the presence of all the isolated triterpenes, except for (16) (Talla et al., 2013), in African propolis. Interestingly, the studied Cameroonian propolis

sample showed a distinct chemical composition in comparison to other African samples (Zhang et al., 2014). The characterised triterpenes (**14–22**) and alk(en)ylresorcinols (**23**), (**25**), (**28**) and (**29**) are known constituents of mango (*Mangifera indica*, Anacardiaceae) (Anjaneyulu and Radhika, 2000; Cojocarú et al., 1986; Escobedo-Martinez et al., 2012; Knödler et al., 2008; Kienzle et al., 2014), a resin-producing plant widely used in honey production in Cameroon and throughout tropical Africa (Nguemo Dongock et al., 2004; Focho et al., 2009). The hypothesis that mango is used as a botanical source of phytochemicals for our sample needs to be confirmed with further field studies (i.e., observation of bees foraging) and comparative analyses of the chemical composition, if collected, of mango resin and of propolis used in local hives.

3 Experimental

3.1 General experimental procedures

The GC–MS trace for the *n*-hexane extract was analysed on a Thermo Scientific Focus GC DSO™ II single quadrupole system equipped with a splitless-split injector and an InertCap GC column (30 m, 0.25 mm I.D., 0.25 µm film thickness). The initial oven temperature was programmed at 50 °C for 1 min, then from 50 to 250 °C (10 °C/min), held for 2 min at 250 °C, then raised to 320 °C (2 °C/min), and isothermal for 10 min. The injection was performed in splitless mode with He as carrier gas (1.5 mL/min, constant rate). The ion source temperature was set at 250 °C. The mass spectrometer was used in EI mode (70 eV) and operated from 50 to 800 amu with full scan detection each second.

The phenolic acid derivatives were analysed on the same system using a InertCap GC column (30 m, 0.25 mm I.D., 0.25 µm film thickness). The following analysis conditions were used for the dimethyl disulfide trimethyl silyl derivatives of alk(en)ylphenols; the initial oven temperature was programmed at 60 °C for 3 min, then from 60 to 150 °C (15 °C/min), from 150 to 275 °C (5 °C/min), and maintained at 275 °C for 24 min. For the dimethyl disulfide trimethyl silyl derivatives of alk(en)ylresorcinols; the initial oven temperature was set at 50 °C for 2 min, then raised to 200 °C (40 °C/min), held for 2 min at 200 °C, then raised to 320 °C (3 °C/min), and isothermal for 30 min. For the dimethyl disulfide derivatives of orcinol and resorcinol; the initial oven temperature was programmed at 140 °C for 3 min, then raised to 320 °C (40 °C/min) and maintained at 320 °C for 40 min. All injections were performed in splitless mode with He as carrier gas (1.5 mL/min, constant rate). The ion source temperature was set at 250 °C. The mass spectrometer was used in EI mode (70 eV) and operated from 50 to 600 amu (alk(en)ylphenols) and 50 to 800 amu (alk(en)ylresorcinols, orcinol and resorcinol) with full scan detection each second.

MS data (high resolution) for triterpenes (**18–22**) were recorded using electrospray ionisation (ESI) on an Exactive® Orbitrap mass spectrometer operating in a positive and negative switching mode. Triterpenes (**14–17**) were analysed using high resolution electron impact (HREI) MS on a JEOL 505HA spectrometer at 70 eV. All MS data were processed using Xcalibur® software version 2.2 (Thermo Scientific, UK). NMR spectra were recorded in CDCl₃ on a JEOL Lambda Delta 400 spectrometer. All spectra were referenced on the residual solvent peaks and processed using Mestre Nova® (MNOVA) software version 8.0.0 (Mestrelab Research SL, Spain).

Dimethyl disulfide (DMDS), sodium thiosulphite (Na₂S₂O₃) 0.01 N, bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (BSTFA:TMCS 99:1), Sephadex® LH 20–100 for gel filtration, *p*-anisaldehyde, α- and β-amyrin, cycloartenol lupeol, orcinol, resorcinol, 3-pentadecylphenol, 5-pentadecylresorcinol and 5-heptadecylresorcinol were purchased from Sigma–Aldrich Ltd (Gillingham, UK). Silica gel 60 (0.063–0.200 mm) for open column chromatography (CC), silica gel 60H for vacuum liquid chromatography (VLC) and silica gel 60 PF₂₅₄ pre-coated plates for TLC analysis were obtained from VWR International Ltd (Lutterworth, UK). Detection of spots on TLC was carried out under short (λ = 254 nm) UV light followed by spraying with *p*-anisaldehyde sulphuric acid reagent and heating until colours appeared.

3.2 Propolis material

The sample of propolis originating from Sel and Fio C.G beekeepers, Nguabum-Konene (Menchum division, North-West region, Cameroon) was supplied by the Apiceutical Research Centre.

3.3 Extraction and isolation

An ethanolic extract of propolis was prepared by sonicating the sample (52.8 g) with 70% ethanol (10 × 500 mL) at 60 °C for 1 h. Following removal of insoluble material, the extract was concentrated to dryness under reduced pressure at <40 °C, suspended in water (250 mL) and further partitioned between *n*-hexane (3 × 250 mL), ethyl acetate (3 × 250 mL) and *n*-BuOH (3 × 250 mL). All organic phases were concentrated to dryness under reduced pressure and stored at –20 °C prior to analysis.

A portion (5.8 g) of the *n*-hexane extract (15.5 g) was subjected to VLC eluting with *n*-hexane:EtOAc mixtures and then EtOAc:MeOH mixtures of increasing polarity to afford eleven fractions (F1–F11). Fraction 5 (964.1 mg), eluted with 10% EtOAc in hexane, was further fractionated by gel filtration. Elution with 5% *n*-hexane in CH₂Cl₂ afforded F5-A (**1–13**) as a yellow-coloured oil (29.5 mg). Fraction 6 (654.8 mg), eluted with 15% EtOAc in hexane was subjected to CC eluting with hexane:EtOAc mixtures of increasing polarity to afford an inseparable mixture (13.9 mg) of (**14**), (**15**), (**16**) and (**17**). Fractions F8 and F9, eluted with 25% and 30% EtOAc in *n*-hexane, respectively, were combined (829 mg) and subjected to gel filtration. Elution with 5% *n*-hexane in CH₂Cl₂ afforded an inseparable mixture (26.8 mg) of (**18**) and (**19**) (2:1 ratio). Fractions F10 and F11, eluted with 40 and 50% EtOAc in hexane, respectively, were combined (726 mg) and subjected to gel filtration (5% *n*-hexane in CH₂Cl₂) followed by CC, eluting with hexane:EtOAc mixtures of increasing polarity, to afford a (1:1) mixture (6.7 mg) of (**20**) and (**21**).

A portion (6 g) of the EtOAc extract (13.4 g) was subjected to VLC eluting with *n*-hexane:EtOAc mixtures and then EtOAc:MeOH mixtures of increasing polarity to afford twelve fractions (F1–F12). Fraction 6 (1.64 g), eluted with 40% EtOAc in hexane, was further fractionated by gel filtration eluting with 5% *n*-hexane in CH₂Cl₂ followed by 100% CH₂Cl₂ to yield a mixture (7.2 mg) of (**21**) and (**22**), along with (**18**) (13.1 mg) and (**20**) (15.5 mg) as pure compounds.

The BuOH extract (10.4 g) was subjected to VLC eluting with *n*-hexane:EtOAc and then EtOAc:MeOH mixtures of increasing polarity to afford 15 fractions F1–F15. Fractions 7 and 8, eluted with 25% and 30% EtOAc in hexane, respectively, were combined (220 mg), and further fractionated by gel filtration. Elution with CH₂Cl₂ and later 10% EtOAc in CH₂Cl₂ afforded F7/8-A (**23–31**) as a yellow-coloured oil (40 mg).

3.3.1 Compound 14 (α -amyrin)

White amorphous solid; R_f 0.40 in hexane–EtOAc, 8:2; HR-EIMS *m/z* (% rel. int.): 426.3818 [M]⁺ (1) (calculated for C₃₀H₅₀O 426.3862), 218 (100), 203 (80), 189 (30), 109 (40), 95 (50), 69 (60); ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S2 in Supplementary Information.

3.3.2 Compound 15 (β -amyrin)

White amorphous solid; R_f 0.40 in hexane–EtOAc, 8:2; HR-EIMS *m/z* (% rel. int.): 426.3818 [M]⁺ (1) (calculated for C₃₀H₅₀O 426.3862), 218 (100), 203 (80), 189 (30), 109 (40), 95 (50), 69 (60); ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S2 in Supplementary Information.

3.3.3 Compound 16 (*lupeol*)

White amorphous solid; R_f 0.40 in hexane–EtOAc, 8:2; HR-EIMS *m/z* (% rel. int.): 426.3848 [M]⁺ (2) (calculated for C₃₀H₅₀O 426.3862), 207 (45), 189 (60), 147 (30), 121 (80), 95 (100); ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S2 in Supplementary Information.

3.3.4 Compound 17 (*cycloartenol*)

White amorphous solid; R_f 0.40 in hexane–EtOAc, 8:2; HR-EIMS *m/z* (% rel. int.): 426.3838 [M]⁺ (2) (calculated for C₃₀H₅₀O 426.3862), 218 (45), 189 (45), 147 (45), 135 (70), 109 (70), 95 (95), 81 (100), 69 (95), 55 (80); ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S3 in Supplementary Information.

3.3.5 Compound 18 (*mangiferonic acid*)

White amorphous solid; R_f 0.23 in hexane–EtOAc, 8:2; HR-ESIMS: [M+H]⁺ *m/z* 455.3523 (calculated for C₃₀H₄₇O₃ 455.3525). ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S3 in Supplementary Information.

3.3.6 Compound 19 (*ambonic acid*)

White amorphous solid; R_f 0.23 in hexane–EtOAc, 8:2; HR-ESIMS: [M+H]⁺ *m/z* 469.3678 (calculated for C₃₁H₄₉O₃ 469.3682). ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S3 in Supplementary Information.

3.3.7 Compound 20 (*mangiferolic acid*)

White amorphous solid; R_f 0.40 in hexane–EtOAc, 7:3; HR-ESIMS [M+H]⁺ *m/z* 457.3674 (calculated for C₃₀H₄₉O₃ 457.3682); ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S4 in Supplementary Information.

3.3.8 Compound 21 (*ambolic acid*)

White amorphous solid; R_f 0.40 in hexane–EtOAc, 7:3; HR-ESIMS: [M–H][–] *m/z* 471.3833 (calculated for C₃₁H₅₁O₃ 471.3838). ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S4 in Supplementary Information.

3.3.9 Compound 22 (*isomangiferolic acid*)

White amorphous solid; R_f 0.40 in hexane–EtOAc, 7:3; HR-ESIMS: [M+H]⁺ *m/z* 457.3676 (calculated for C₃₀H₄₉O₃ 457.3682). ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz), see S4 in Supplementary Information.

3.4 Preparation of dimethyl disulfide and dimethyl disulfide trimethylsilyl derivatives

Dimethyl disulfide derivatives of alk(en)ylphenols and alk(en)ylresorcinols were prepared following a previously reported method (Saitta et al., 2009) with some modifications. Briefly, F5A or F7.8A (2 mg) was mixed with DMDS (0.5 mL) in 0.1 mL of a solution of iodine-diethyl-ether (60 mg/mL). The mixture was stirred at room temperature for 24 h and then *n*-hexane (5 mL) was added. The excess iodine was removed by washing with Na₂S₂O₃ 0.01 N (2 × 5 mL). The organic phase was recovered and evaporated to dryness under a stream of nitrogen. The residue was further used to produce trimethylsilyl derivatives following stirring with BSTFA-TMCS (99:1) (0.2 mL) at room temperature for 30 min (alk(en)ylphenols) and 2 h (alk(en)ylresorcinols). The dimethyl

disulphide trimethylsilyl derivatives obtained were dissolved in CH₂Cl₂ and analysed by GC–MS.

Orcinol (5 mg) and resorcinol (5 mg) were derivatised with DMDS (5 mL) in 1 mL of a solution of iodine-diethyl-ether (60 mg/mL). The mixture was stirred at room temperature for 24 h and then *n*-hexane (25 mL) was added. The excess iodine was removed by washing with Na₂S₂O₃ 0.01 N (2 × 25 mL). The organic phase was recovered and evaporated to dryness under a stream of nitrogen. The resulting dimethyl disulphide derivatives were dissolved in CH₂Cl₂ and analysed by GC–MS.

Uncited references

~~Petrova et al. (2010), Snewin et al. (1999) and Stasiuk and Kozubeck (2010).~~

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phytochem.2014.07.016>.

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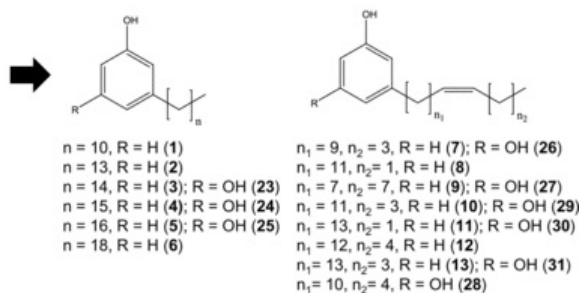
Appendix A. Supplementary data

[Multimedia Component 1](#)

Supplementary data 1

Graphical abstract

Chemical investigation of Cameroonian propolis led to the isolation of twenty-two phenolic lipids (including four **new** structures) along with nine triterpenes.



Highlights

- Twenty-two phenolic lipids and nine triterpenes were found in Cameroonian propolis.
 - Four new phenolic lipids were characterised by GC–MS analysis.
 - The most abundant phenolic lipids were identified as 3-(12'Z-heptadecenyl)-phenol and 5-(12'Z-heptadecenyl)-resorcinol.
 - Eighteen compounds are reported for the first time in propolis.
-

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