Two New Triterpenoids from *Acacia mellifera* (Vahl) Benth

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Abstract

Two new pentacyclic triterpenoids, (20R) 3-oxolupane-30al **(1)**, and (20S) 3-oxolupane-30al **(2)**, along with six known metabolites; 30-hydroxylup-20 (29)-en-3-one **(3)**, 30-hydroxylup-20 (29)-en-3ß-ol **(4)**, Atranorin **(5)**, Methyl-2hyndroxy-4-hydroxy-3, 6 dimethy benzoate **(6)**, ß-stosterol-3 ß-O-glucoside **(7)**, and linoleic acid **(8)**, have been isolated from the dichloromethane extract of a Kenyan *Acacia mellifera*. The structures of new metabolites **(1-2)** were elucidated on the basis of extensive spectroscopic analyses and their relative stereochemistries were determined by NOESY experiments. The new metabolite **(1)** has been shown to exhibit significant cytotoxic activity against the NSCLC-N6 cell line, derived from a human non-small-cell bronchopulmonary carcinoma.

Author Keywords: *Acacia mellifera*; (20R) 3-oxolupane-30al **(1)**, and (20S) 3-oxolupane-30 al **(2)**.

1. Introduction

In recent years, there have been studies on the chemical components of several *Acacia* species (Leguminosae) [Lee *et al*., 2000; Readel *et al*., 2001; Seo *et al*., 2002]. *Acacia* is the second largest genus in the family Leguminosae comprising more than 1200 species [Harden, 1991] and occurs in almost all habitat types. Of the 1200 *Acacia* species worldwide, 700-800 occurs in Australia [Doran *et al*., 1983], and 129 in Africa (Ross 1979], a small number in Asia, and the remainder (about 200 species) in the rest of the world. *Acacia mellifera* is widely used in African traditional medicine and particularly in Kenya [Kokwaro, 1976]. During the course of our investigation on the bioactive metabolites of *Acacia mellifera* nine triterpenoids **1-8** were isolated. Also, *Acacia mellifera*, which is investigated for the second time, afforded (20R) 3-oxolupane-30al **(1)**, and its related isomers (20S) 3-oxolupane-30al **(2)**. Several related triterpenoids have also been discovered during the investigations of other species; namely *Gymnosporia wallichiana* and *Pseudocyphellaria rubella* [Kulshreshtha, 1977; Corbett *et al* 1987]. The molecular structures, including the relative configuration of the new triterpenoids, were elucidated by the extensive spectral analyses. Cytotoxicity of metabolite **(1)** against the NSCLC-N6 cell line, derived from a human non-small-cell bronchopulmonary carcinoma was observed.

2. Results and discussion

The stem bark of *Acacia mellifera* was pulverized, extracted and filtered. The extracts were evaporated under vacuum to afford residue. The dichloromethane extract was subjected to a series of chromatographic separation and purification, including HPLC, to afford metabolites **1-8**.

Metabolite **(1)** was obtained as colourless gum. Its HRFABMS spectrum exhibited an ion peak at *m/z* 439.3596, $[M-H]^+$), consistent with the molecular formula $C_{30}H_{47}O_2$. The IR spectrum of **1** suggested the presence of aldehyde, and keto-carbonyl functionalities. The ^{13}C NMR spectrum of **1** measured in $CDCl_3$, showed signals of 30 carbon atoms, which were identified by the assistance of DEPT spectrum as seven methyls, ten methylenes, and six quaternary carbons (Table 1). The signals appearing at δ 206.7, and 218.0 were attributable to carbons of an aldehyde and a normal ketone, respectively. Furthermore, the five carbon signals appearing at δ_c 42.7 (d), 49.0 (s), and 49.2 (t) designate the presence of one saturate carbon-carbon bond in **1**. From the above findings, **1** was thus suggested to be saturated triterpenoid possessing one saturate olefinic bond, one group keto, and one carbonyl group. By comparison of the ^{13}C NMR spectral data of **1** with those of 28-hydroxy-3-oxo-lup-20-(29)-en-30-al, a known metabolite also isolated in the present study, it was suggested that 1 has the same lupane carbon skeleton as that of 28-hydroxy-3-oxo-lup-20- (29)-en-30-al but with only a difference in E-ring carbons due to addition saturated group at C-19 (δ_c 14.4, d and 49.0, s). The location of this saturated bond was established at C-29 and C-20 due to the downfield shift of H-19 (δ_H 1.90, H, m) and the appearance of an additional vinylic proton at δ_H 2.57 (1H,s, H-20) in the ^1H NMR spectrum of 1 (Table 2). This was further supported by the $^1H/^{13}C$ long-range correlation observed in the HMBC spectrum between H-30 and both C-29 (δ_c 14.4, s) and C-19 (δ_c 42.9, d), and between H-29 and the C-19 (δ_c 42.7, s). The position of the quaternary carbon signal at δ_c 218.0 was readily assigned to C-3 on the basis of long-range connectivities of 3H-24. On the basis of the above observations, and by the assistance of a series of 2D NMR (^1H-^1H COSY, HMQC and HMBC) experiments, it was possible to establish the structure of 1, as illustrated above.

The relative stereochemistry of the one chiral center at C-20 in (1) was determined on the basis of the NOE correlations observed in a NOESY spectrum, in addition to the chemical shifts and coupling constants of the concerned protons. The R-geometry of the C-20 bond was established by the NOE interaction between H-20 (δ_H 2.57,m) and H-18 (δ_H 1.64, m). One proton attaching at C-19 and resonating at δ_H 1.91 (m) was found to show NOE interactions with H-28 (δ_H a, 3.26, d, *J*=10.9 Hz; b, 3.76, d, *J*=10.9 Hz) and was assigned as H-19β. Thus, the isopropenyl group located at C-19 should be α-oriented. The other proton attaching at C-28, H-28ß (δ_H a, 3.26, d, *J*=10.9 Hz; b, 3.76, d, *J*=10.9 Hz), showed NOE interactions with the proton H-13 (δ_H 1.70, m), confirming the upward orientation of H-13. The significant NOE interactions shown between methine H-20 and proton H-21β revealed the parallel orientation of C_{20}-H and $C_{21\beta}$-H, and hence, the R^* configuration at C-20 (above the plane)[Chakrabartty et al., 1982]. One H-30 (δ_H 9.83, 2.0, Hz) showed lower coupling with H-20 and this further supported the assignment of H-20 as R-epimer [Kulshreshtha, 1977]. Kulshreshtha *et al*, [1977] showed that the 20R epimer exhibits the C-20 methyl signal at higher field and the coupling constant between the C-20 proton and C-19β proton has a lower value than those of 20S epimer. Therefore, the low value of coupling constant disclosed between both H-20 and H-19β, and high value for C-20 methyl suggest in our case revealed that H-20 is positioned on the upward face of the pentacyclic ring (R-epimer). On the basis of the above findings and other key NOE interactions observed, the structure of metabolite **1** was established as (20R) 3-oxolupane-30al

The new metabolite, (20R) 3-oxolupane-30al **(2)**, also isolated, was obtained as colourless gum. On the basis of its HRFABMS (*m/z* 439.3596, $[M-H]^+$), along with the ^1H and ^{13}C NMR spectral data, the molecular formula of metabolite **2** was established as $C_{30}H_{47}O_2$. As in the case of **1, 2** also revealed the presence of a carbonyl group and the aldehyde group.

Furthermore, it was found that the ^{1}H and ^{13}C NMR spectral data of **2** were very similar to those of **1** (Table 1 and Table 2), suggesting that **2** have the same R-epimer as of **1**. By the assistance of 2D NMR spectra, including COSY, HMQC, and HMBC, **2** was shown to posses the same molecular framework as that of **1**. However, the significant downfield shifts for C-22 ($\Delta\delta_c$ + 6.0 ppm) and C-16 ($\Delta\delta_c$ + 6.0 ppm) in comparison with those of **1** (Table 1), suggesting that **2** might be the C-28 oxidized of 1. By careful investigation on the NOESY spectrum of **2**, it was found that 3H-19β showed significant NOE interactions with H-28 and H-13β (δ_H 1.74, m), revealing the α-orientation of isopropenyl group. Further analyses on other NOE interactions revealed that **2** possessed the same relative configurations at C-20, C-29, and C-30, as those of **1**. Therefore, metabolite could be established as (20R) 3-oxolupane-30al described by molecular formula above.

The second related natural product lupane isolated, metabolite **(2)**, has the same molecular formula $C_{30}H_{49}O_2$ and was considered to be an isomer of **1** on the basis of HRFABMS and NMR spectral data (Table 1 and 2). Also, spectral data revealed the presence of a carbonyl group and an aldehyde group in **2**. In general, the ^{1}H and ^{13}C NMR data of **2** were found to be similar to that of **1**. Nevertheless, δ_c of ring A, B, C, D, and E were more or less close to those above (Table 1), suggesting that the relative configuration is possibly the same as that of **1**. Moreover, the upfield and down shifts observed for C-12 ($\Delta\delta_c$ -0.9 ppm), C-18 ($\Delta\delta_c$ -1.9 ppm), C-19 ($\Delta\delta_c$ -5.3 ppm), C-20 ($\Delta\delta_c$ + 1.2 ppm), 22 ($\Delta\delta_c$ + 0.7 ppm), and 28 ($\Delta\delta_c$ + 0.4 ppm) in comparison with those of **1** (Table 1), suggested that **2** could posses different configuration for the 20-carbon, in contrast to those of **1**. According to the NOESY spectrum of **2**, H-20 did not show NOE response with H-21β, possibly suggesting the S-geometry of H-20. The significant interaction exhibited between methine H-19 and olefinic proton H-20 again revealed the S^* configuration at C-20, not the same as that in **1**. By consideration of the above findings, along with other NOE responses observed above, the structure of compound **2** was established as (20S) 3-oxolupane-30al.

Although **2** was the first structure of saturated 20(29)-lupane to be synthesized the R and S configuration was not distinguished, [Fang *et al*., 1984] as confirmed latter through chemical shift difference in related acetylated structures, [Vystrcil *et al*., 1973] it was misleadingly configured occasionally as a 20R and 20S-epimer could not be separated and therefore they were isolated as a mixture [Corbett *et al*., 1987]. In addition, due to slight differences in proton NMR, some groups even consider the mixtures as one compound [Fang *et al*., 1984]. This contradiction prompted us to investigate the ^{1}H and ^{13}C NMR spectral data of **2**, which lacked full assignment of both NMRs, which appear similar and even showed the same molecular weight with **1**. It was found that the spectral data in $CDCl_3$ are relatively in full agreement with those published by Corbett *et al*. [1985], which represented acetylated form of related lupans with 20S-configuration. Thus, the relative configuration of this metabolite should also be mentioned as that of 20S. On the basis of all above findings, we can conclude that metabolite **1** has not been isolated yet.

Other known compounds **3-8,** which were also isolated, were found to be identical with the previously reported 30-hydroxylup-20 (29)-en-3-ol [Burns *et al* 2000; Betancor *et al* 1980] isolated from *catha cassinoides* and *cassine papillosa* respectively. Atranorin, and methyl-2-hydroxy-4-hydroxy-3, 6 dimethy benzoate isolated from *S. scabra*, ineleganolide [Mario *et al* 2000; carvallo, 2000), ß-sitosterol-3ß-O-glucoside [Alam *et al*., 1995], and linoleic acid [Grindley, 1945].

Our previous study revealed some cytotoxic activity for the lupane types triterpinoids activity against the NSCLC-N6 cell line, derived from a human non-small-cell bronchopulmonary carcinom (CI_{50} 15-30 g/mL) (Mutai *et al*., 2004). This result prompted us to extend our study on biological activity of the related lupanes. The cytotoxicity of metabolites **1-8** was thus evaluated on the same cell lines. It was found that the new metabolites **2** and **3** and the known compounds **4-8** were inactive against the growth of cells, while one new lupane **1** exhibited some activity against activity against the NSCLC-N6 cell line with CI_{50} of 30 g/mL,

Table 1. ^{13}C NMR spectral data of compounds **1-2**

Carbons	**1** **CMR**	**2** **CMR**
1	39.4	39.5
2	34.0	34.1
3	218.5	217.8
4	47.3	47.3
5	54.7	54.8
6	19.6	19.6
7	33.4	33.6
8	40.6	40.8
9	49.2	49.3
10	36.7	36.8
11	21.3	21.2
12	27.4	26.5
13	37.8	37.8
14	42.9	43.1
15	27.1	27.2
16	35.1	35.2
17	43.0	42.9
18	48.9	47.0
19	42.6	37.3
20	48.9	49.7
21	25.0	23.6
22	39.9	40.4
23	26.6	26.6
24	21.0	21.1
25	15.7	15.9
26	15.7	15.8
27	14.2	14.3
28	17.5	17.9
29	14.4	7.4
30	207.1	205.1

Spectra recorded at 50.3 MHz in $CDCl_3$ at 25^0C.

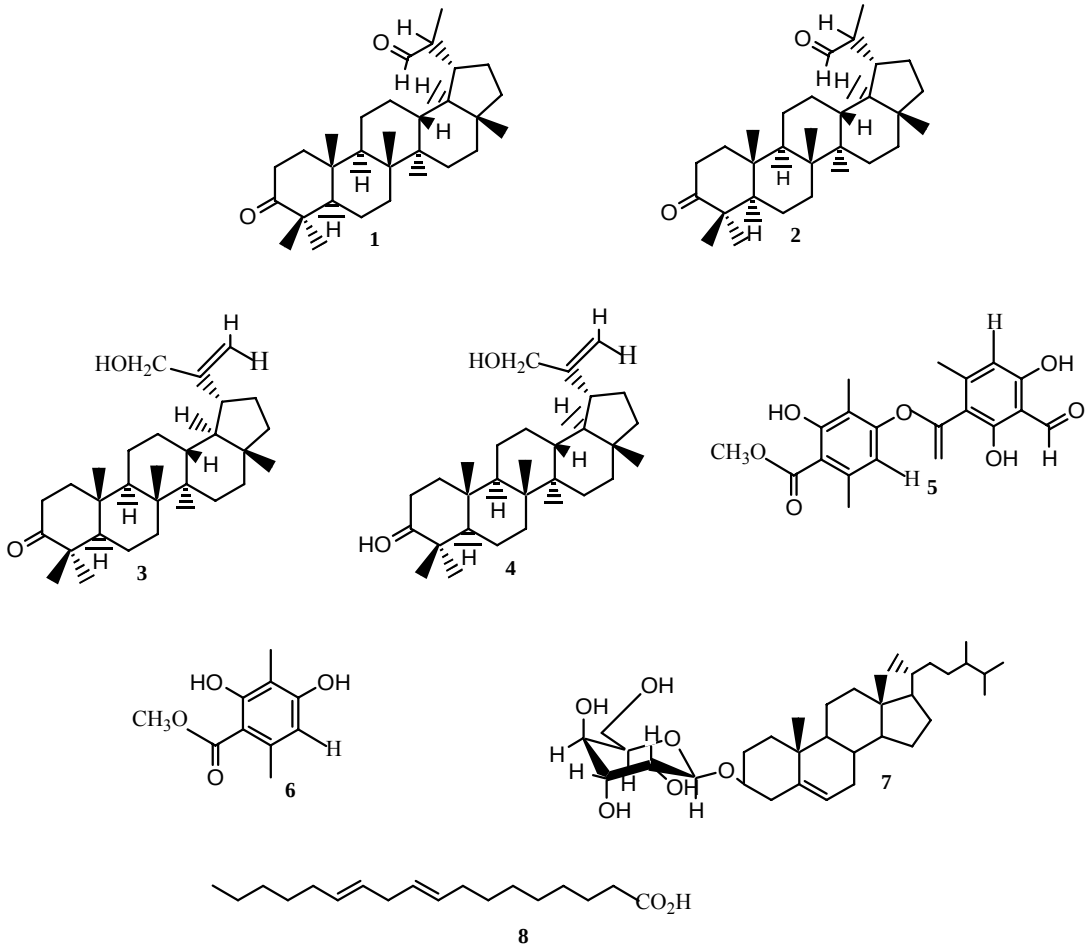
Table 2. [1] H NMR spectral data of compounds **1-2**

Carbons	**1**[b] **PMR**	**2**[b] **PMR**
1	α, 1.40 (m), ß, 1.91 (m)	α, 1.90 (ddd, 4.41, 7.64, 13.24 ß, 1.38 (m)
2	α, 2.40 (ddd, 4.76, 7.84, 15.68), ß, 2.47 (ddd, 7.52, 9.56, 15.68)	α, 2.40 (m) ß, 2.47 (ddd, 7.35, 9.70, 15.88)
3	-	
4	-	
5	1.33 (m)	1.32 (m)
6	α, 1.44 (m), ß, 1.56 (m)	1.41-1.53 (m)
7	1.44 (m)	1.45 (m)
8	-	
9	1.38 (m)	1.38 (m)
10	-	
11	ß, 1.40 (m) α, 1.55 (m)	1.06 (m), 1.52 (m)
12	α, 1.38 (m) ß, 1.56 (m)	1.35 (m), 1.65 (m)
13	1.74 (m)	1.79 (ddd, 4.12, 11.75, 11.75)
14	-	
15	α,1.05 (m) ß, 1.69 (m)	1.03 (m), 1.69 (m)
16	2H, 1.47 (m)	1.6 (m), 1.53 (m)
17	-	
18	1.43 (m)	1.26 (m)
19	1.89 (m)	2.35 (m)
20	2.60 (m)	2.63 (dq, 2.94, 6.76)
21	α, 1.51 (m) ß, 1.88 (m)	1.5 (m), 1.68 (m)
22	α, 1.36 (m) ß, 1.47 (m)	1.39 (m), 1.45 (m)
23	1.06 (s)	1.06 (s)
24	1.01 (s)	1.01 (s)
25	0.91 (s)	0.93 (s)
26	1.06 (s)	1.07 (s)
27	0.92 (s)	0.93 (s)
28	0.75 (s)	0.78 (s)
29	1.07 (d, 7.17 Hz)	1.01 (d, 6.76)
30	9.84 (d, 2.04 Hz)	9.60 (s)

a Spectra recorded at 500 MHz in $CDCL_3$ at 25^0C
b Spectra recorded at 400 MHz in $CDCL_3$ at 25^0C

The *J* values are in Hz in parentheses.

Figure 1. Structures of new metabolites **1-2** and known compounds **3-8** of *Acacia mellifera.*



3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer model 341 polarimeter with a 10 cm cell. IR spectra were recorded on a Paragon 500 Perkin-Elmer infrared spectrophometer. EIMS data was obtained with a Hewlett Packard 5973 GC/MS spectrometer. The NMR spectra were recorded on a Bruker DRX 400 for ^{1}H and Bruker AC 200, respectively, in $CDCL_3$. Chemical shifts are given in δ (ppm) scale using TMS as internal standard. The 2D experiments (^{1}H-^{1}H COSY, HMQC, HSQC, HMBC) were performed using standard Bruker microprograms. EIMS data were recorded on a Hewlett Packard 5973 Mass Selective Detector. Column chromatography was performed with Kiesegel 60 (Merck), HPLC was conducted on an Agilent 1100 series with refractive index dectector, with Kromasil Sil 100, 5 um, 280x8mm column. TLC were performed with Kiesegel 60 F-254 (Merck aluminum support plates.

3.2. Plant material

A. mellifera was collected by Mr. Onesmus Mwangangi at Machakos, Kenya in January 2000, and stored in a freezer until extraction. A voucher specimen (ChM-1) was deposited at the East Africa Herbarium-Nations Museums of Kenya, Nairobi.

3.3. Extraction and isolation

The air-dried powdered stem bark of *A. mellifera* (2.25kg) was extracted with CH_2Cl_2 (100%) and MeOH (100%). The resulting extracts were filtered and concentrated under vaccum to afford a dark brown residue. The CH_2Cl_2 residue was subjected to column chromatography on silica gel, using cyclo-hexane, cyclo-hexane and EtOAc mixture of increasing polarity, and finally pure EtOAc, to yield 61 fractions. These fractions were further combined to fractions **I-IX** on the basis of TLC. Fraction **III** was eluted with cyclo-hexane-EtOAc (gradient, 98:2), were further purified on silica gel using cyclo-hexane EtOAc (9:1) to yield **1** (3.5 mg) and **2** (2.7 mg) from fraction **IIId**. Fractions **VI** eluted with cyclo-hexane-EtOAc (gradient, 75:25 – 70:30), was purified separately by normal phase HPLC using cyclo-hexane-EtOAc (4:1) to afford **3** (3 mg) and **5** (3.4) from fraction **VI14**, respectively. Fraction **V** eluted with cyclo-hexane-EtOAc (4:1), was further chromatographed over silica gel using *n*-hexane-EtOAc to yield **4** (32 mg) and others, which was further purified by normal phase HPLC using *n*-hexane-EtOAc (4:1) to afford **6** (2 mg) and **7** (1.2 mg), respectively. Fractions **IV** was further chromatographed over silica gel and purified by normal phase HPLC, using cyclo-hexane-EtOAc (5:11) to yield **8** (5 mg).

3.4. Cytotoxicity evaluation

The NSCLC-N6 cell line (Roussakis *et al*., 1991), derived from a human non-small-cell broncho-pulmonary carcicona (moderately differentiated, rarely keratinizing, classified as T2N0M0) was used for all experiments. The cell were cultured in RPMI 1640 medium with 5% fetal calf serum, to which were added 100 IU penicillin.ml^{-1}, 100 ug streptomycin.ml^{-1} and 2 mM glutamine, at 37^0C in an air/carbon dioxide (95:5, v/v) atmosphere. In these conditions, cell-doubling time was 48 h. Cell used in the experiment never exceeded 35 passage.

Experiments were performed in 96 wells microtiter plates (2 x 10^5 cells.ml^{-1}). Cell growth was estimated by calorimetric assay based on the conversion of tetrazelium dye (MTT) to blue formazan product by live mitochondria (Mossmann, 1983). Eight repeats were performed for each concentration. Control growth was estimated from 16 determinations. Optical density at 570nm corresponding to solubility formazan was read for each well on titertek mutiskan MKII.

3.6. (20R) 3-oxolupane-30al (1)

Colourless gum, $[\alpha]_D^{20}$=-3.14 (*c* 0.64, $CHCL_3$); IR ($CHCl_3$) ν_{max} 2942, 2348, 1704, 1526, 1234 cm^{-1}; ^1H NMR ($CDCL_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 50.3 MHz), see Table 1 and Table 2, respectively; EIMS 70eV, *m/z* (rel. int.%): 382 (100), 163 (40), 205 (28), 81 (22), 55 (21), 107 (18), 189 (12), 135 (12), 425 (4), 273 (4); HRFAB-MS *(m/z)*: 439.3596 $[M-H]^+$ (calcd. for $C_{30}H_{47}O_2$, $[M-H]^+$, 439.35781).

3.7. (20S) 3-oxolupane-30al (2)

Colourless gum, $[\alpha]_D^{20}$=5.5 (*c* 0.18, $CHCl_3$); IR ($CHCl_3$) ν_{max} 2942, 2348, 1704, 1526, 1234 cm^{-1}; EIMS 70eV, *m/z* (rel.int. %): 382 (100), 163 (40), 205 (28), 81 (22), 55 (21), 107 (18), 189 (12), 135 (12), 425 (4), 273 (4); HRFAB-MS *(m/z)*: 441.3735 $[M+H]^+$ (calcd. for $C_{30}H_{49}O_2$, $[M+H]^+$: 441.37347).

Acknowledgements

This work was supported by state scholarships foundation of Greece awarded to C. Mutai. I wish to thank the Director of Kenya Medical Research Institute for granting me study leave

and Dr. Leonard Hagmann (Syngenta Agro, Basel, Switzerland) for the performance of some NMR experiments.

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