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Lupane Type Triterpene Isolated from the Leaves of Thecacoris Annobonea (Euphorbiaceae)

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ABSTRAT

A new lupane type triterpene (**1**), together with betulinic acid (**2**), friedelin (**3**), aristolochic acid I (**4**), alpinumisoflavone (**5**) and 4'-*O*-methylepinumisoflavone (**6**) have been isolated from the leaves of *Thecacoris annobonea*. The structure of the new compound was elucidated on the basis of 1 and 2D NMR experiments. The isolated compounds were evaluated for their phytotoxicity and antimicrobial activity. **1** exhibited significant antimicrobial activity at 30 *µ*g/ml and compounds **1**, **2**, **3**, **4**, **5** and **6** inhibited root growth lettuce at 100 *µ*g/ml.

Indexing terms/Keywords

Thecacoris annobonea; lupane; phytotoxic; antimicrobial.

Academic Discipline and Sub-Disciplines

Organic Chemistry

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Triterpene and flavonoid compounds

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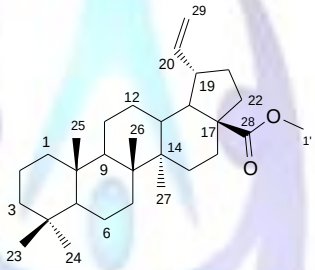
INTRODUCTION

Thecacoris annobonea (Euphorbiacea) is a small tree or shrub growing in South and South-West provinces of Cameroon. It is widely used in traditional Cameroonian folk medicine. The leaf decoction of *T. batesii* is used as purgative and anti-rheumatic remedies in the medicinal plant therapy in Cameroon [1]. In our previous study on the genus *Thecacoris*, aristolochic acid, vanillic acid and friedelin which showed good antimicrobial activities were isolated from the stem bark of *T. annobonea* [2] and, diterpenoids and triterpenoids were isolated from the twigs of *T. batesii* [1]. As a continuation of our search for biologically active compounds in the genus *Thecacoris*, one new compound together with five known compounds were isolated from the leaves of *T. annobonea*. The known compounds were identified as betulinic acid (**2**), friedelin (**3**), aristolochic acid I (**4**), alpinumisoflavone (**5**) and 4'-*O*-methylepinumisoflavone (**6**). The present paper deals with the isolation and structural elucidation of one new lupane type triterpene and also demonstrates its phytotoxicity and antimicrobial activity together with the other isolated known compounds.

RESULTS AND DISCUSSION

The leaves of *T. annobonea* were extracted with $CHCl_3$/acetone (1:1) at room temperature during 24 hours. The extract was submitted to repeated column chromatography and preparative TLC to afford betulinic acid, friedelin, aristolochic acid I, alpinumisoflavone, 4'-*O*-methylepinumisoflavone as well as one new lupane type triterpene (**1**). The ^1H and ^{13}C NMR, and MS of the known compounds were consistent with those reported in the literature.

Compound **1** was obtained as a white powder in fraction B, with the optical rotation $[\alpha]_D^{23}$ +34.2 (*c* 0.7, $CHCl_3$). The empirical formula was deduced as $C_{30}H_{49}O_2$ by ESI MS ($[M+H]^+$ *m/z* 441), and confirmed by the HRESI MS (found 441.3731, calcd. 441.3732 for $C_{30}H_{49}O_2$). The IR spectrum showed an ester [3] absorption bands at υ 1719 cm^{-1} (C=O), 1250 cm^{-1} (C-O) and three absorption bands at *υ* 2937, 1639 and 878 cm^{-1} indicating the presence of a vinylidene group (=CH_2), characteristic of lup-20(29)-ene [4, 5].



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The ^1H NMR and DEPT spectra (Table 1) indicated that compound **1** is a pentacyclic triterpenoid [6] with six methyl groups at δ 0.86, 0.89, 0.92, 0.99, 1.00 and 3.63 (3H each, s), six methine and twelve methylene groups. The signal at δ 3.63 (3H, s) was attributed to a methyl connected to an oxygen which showed a correlation with the carbon C-28 (δ 174.1). The olefinic methyl which normally appears in lupeol/lupane type triterpene at δ 1.70 was absent suggesting the absence of C-30 in the molecule. The methine signals at δ 2.30 (2H) were attributed to the protons at positions C-18 and C-19 according to the ^1J (^1H-^{13}C) correlation in HSQC spectrum. Three olefinic signals appeared at δ 4.90 (2H, ddd, *J* = 14.2, 9.0, 1.3 Hz, H-29) and 5.61 (1H, ddd, *J* = 14.2, 9.0, 7.3 Hz, H-20) indicating an exomethylene and an exomethine group, respectively.

The ^{13}C NMR spectrum (Table 1) of compound **1** revealed the presence of 30 carbon atoms, which were in accordance with the proton data, in addition to a carboxylic ester signal at δ 174.1. The carbon atoms at δ 150.9 and 110.8 are characteristic for the carbons 20 and 29 of lup-20(29)-ene [7]. The ^1J (^1H-^{13}C) correlation of one proton with the carbon at δ 150.9 in HSQC spectrum, confirmed the absence of carbon C-30 in compound **1**. These data indicated that compound **1** might be a lupan-type triterpene. The fragment at *m/z* 191 supported the presence of 30-lup-20(29)-ene [7]. The two main fragments at *m/z* 191 and *m/z* 249 in EI mass spectrum came from the breaking of alkane carbons C8-C14 and C9-C11 (Figure 1).

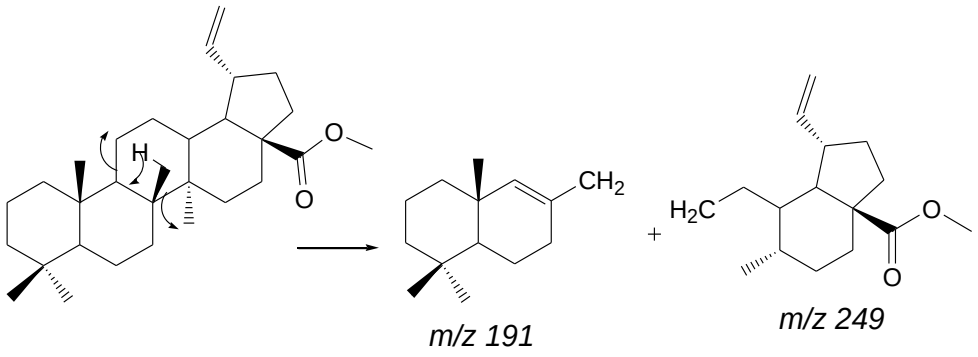

Fig 1: EI MS fragments justifying the structure of compound 1.

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In the HMBC spectrum (Figure 2), correlations between the proton H-18/H-19 (δ 2.30) signals and carbons 12, 13, 14, 17, 18, 20, 21, 22, 28 and 29 indicated that compound **1** was 30-norlup-20-en-28-oic acid methyl ester, which is described here for the first time.

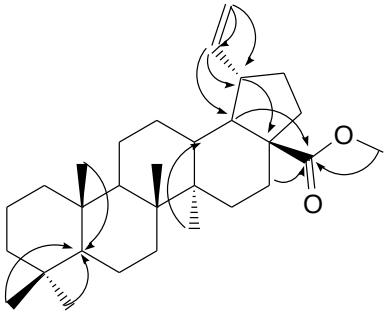

Fig 2: Selected HMBC correlation in compound 1

Table 1: ^{1}H (400 MHz), ^{13}C (100 MHz) NMR spectral data of 30-norlup-20-en-28-oic acid methyl ester (1) in $CDCl_3$. Multiplicities and coupling constant in Hz are given in parentheses.

C/H	30-norlup-20-en-28-oic acid methyl ester (1)		
	δ_H	δ_C, DEPT	HMBC (^{1}H–^{13}C)
1	0.84 (2H, m)	37.2, CH_2	C-2, C-3, C-5, C-10, C-25
2	1.15 (2H, m)	29.9, CH_2	C-1, C-4, C-10
3	1.17 (2H, m)	35.2, CH_2	C-1, C-4, C-23, C-24
4	-	39.6, C	-
5	1.09 (1H, m)	51.3, CH	C-7, C-10, C-24
6	1.11 (2H, m)	18.0, CH_2	C-4, C-8
7	1.18 (2H, m)	35.9, CH_2	C-5, C-6, C-8, C-9
8	-	39.8, C	-
9	1.25 (1H, m)	41.4, CH	C-10, C-25, C-26
10	-	38.6, C	-
11	1.87 (2H, m)	30.2, CH_2	C-8, C-12, C-13
12	1.39 (2H, m)	31.8, CH_2	C-9, C-11, C-13, C-14
13	1.63 (1H, m)	35.1, CH	C-8, C-14, C-17
14	-	38.3, C	-
15	1.83 (1H, m)	34.9, CH_2	C-13, C-14, C-16, C-17, C-27
	1.24 (1H, m)		
16	2.19 (1H, m)	32.2, CH_2	C-14, C-15, C-17, C-18, C-28
	1.04 (1H, m)		
17	-	58.2, C	-
18	2.30 (1H, m)	42.0, CH	C-12, C-13, C-14, C-17, C-18, C-20, C-21, C-22, C-28, C-29
19	2.30 (1H, m)	42.7, CH	C-12, C-13, C-14, C-17, C-18, C-20, C-21, C-22, C-28, C-29
20	5.61 (1H, ddd, 14.2, 9.0,7.3)	150.9, CH	C-18, C-19, C-21, C-29
21	1.99 (1H, m)	32.1, CH_2	C-17, C-18, C-19, C-20, C-22

	1.46 (1H, m)		
22	1.54 (2H, m)	32.8, CH_2	C-18, C-19, C-28
23	0.92 (3H, s)	28.1, CH_3	C-3, C-4, C-24
24	0.86 (3H, s)	17.9, CH_3	C-3, C-5, C-23
25	0.89 (3H, s)	18.7, CH_3	C-1, C-5, C-9
26	1.00 (3H, s)	18.1, CH_3	C-7, C-9, C-14
27	0.99 (3H, s)	21.3, CH_3	C-8, C-14, C-15
28	-	174.1, C	-
29	4.90 (2H, ddd, 14.2, 9.0, 1.3)	110.8, CH_2	C-19, C-20
1’	3.63 (3H, s)	52.9, CH_3	C-28

Biological Activities

The antifungal and antibacterial activities of **1**, **2**, **3**, **4**, **5** and **6** were determined using the agar diffusion method with 8 mm paper disks loaded with 30 *µ*g of each compound isolated from this plant. 30-Norlup-20-en-28-oic acid methyl ester (**1**) showed some activities against *Bacillus subtilis* (16 mm inhibition diameter), *Staphylococcus aureus* (13 mm), *Escherichia coli* (14 mm), *Streptomyces viridochromogenes* (Tü 57) (15 mm), *Mucor miehei* (10 mm), *Chlorella vulgaris* (10 mm) and *Scenedesmus subspicatus* (14 mm). Compounds **2**, **3** and **4** showed weak activities against *Bacillus subtilis* (11, 10, 13 mm); *Escherichia coli* (11, 9, 13 mm), and *Candida albicans* (12, 11, 14 mm), respectively. Compounds **5** and **6** showed some activities against *Streptomyces viridochromogenes* (Tü 57) (15, 13 mm), *Bacillus subtilis* (11, 13 mm), *Escherichia coli* (9, 12 mm) and *Staphylococcus aureus* (15, 15 mm), respectively. Nystatin was used as the reference and the test was repeated three times.

The plant growth inhibitory activities of compounds **1**, **2**, **3**, **4**, **5** and **6** (Figure 3) was determined using seedling growth test on lettuce. Compounds **1**, **2**, **3**, **4**, **5** and **6** at a concentration of 100 *µ*g/mL inhibited lettuce root growth by 10%, 25%, 55%, 20%, 15% and 17% respectively, of that of the control. Betulinic acid (**2**) is reported to have a good activity against cancer cells of neuroectodermal origin and leukaemias [8] and, it is highly selective inhibitors of HIV-1 [9].

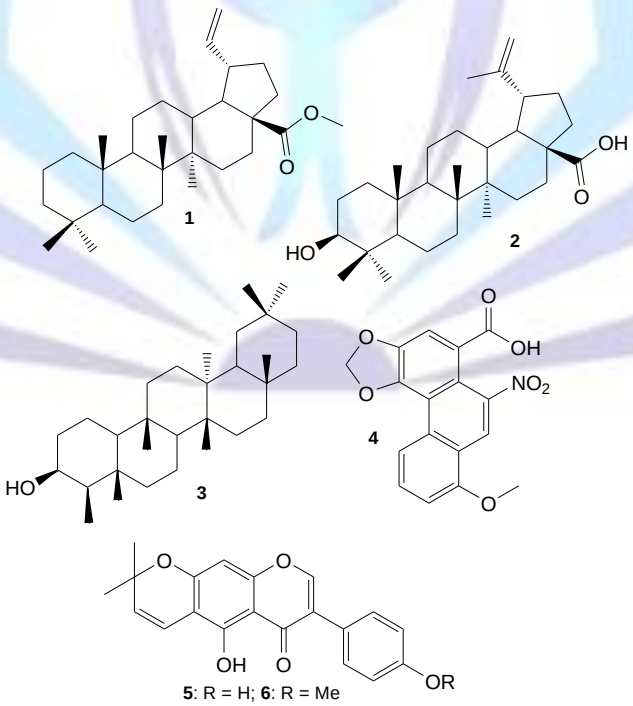

Fig 3: Structure of compounds 1, 2, 3, 4, 5 and 6

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Experimental

General

ESI mass spectra were recorded on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument). HRESI mass spectra were recorded on a Bruker FTICR 4.7 T mass spectrometer. EI mass spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorkerosene as a reference substance for HREI-MS.

The ^{1}H and ^{13}C NMR spectra were acquired with a Jeol EX-400 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Melting point is uncorrected and was obtained with a micro melting point apparatus (Yanaco, Tokyo-Japan). Optical rotation values were measured with a Horiba SEPA-300 polarimeter, and IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer from films. Column chromatography was conducted on silica gel 60 (Kanto Chemical Co., Inc., Japan) and Sephadex LH-20 (Pharmacia, Sweden). TLC analysis was carried out using precoated silica gel plates (Merck), and spots were detected by spraying with H_2SO_4/10% vanillin and then heating. Flash chromatography was carried out on silica gel (230-400 mesh). R_f values were measured on Polygram SIL G/F_{254} (Macherey-Nagel & Co.) and melting and decomposition points were measured by the melting point apparatus of Electrothermal and were not corrected.

Plant Material

The leaves of *T. annobonea* were collected in April 2008 from Kumba, South-West Cameroon and were identified by Mr. Victor Nana of the Cameroon National Herbarium (Yaoundé), where a voucher specimen was deposited (Ref. N° 38569/HNC).

Extraction and Isolation

The leaf powder of *T. annobonea* (500 g) was extracted with $CHCl_3$/acetone (1:1) at room temperature for 24 hours. After removing the solvents by evaporation under reduced pressure, the crude extract (40 g) was chromatographed on silica gel. Using hexane/ethyl acetate of increasing polarity, a total of 105 sub-fractions (ca. 250 ml each) were collected and combined on the basis of TLC analysis leading to two main fractions A and B.

Fraction A (3.0 g) was applied on a silica gel column chromatography and eluted with hexane/ethyl acetate (6:1) to achieve friedelin (**3**, 11.3 mg) [2, 10] and aristolochic acid I (**4**, 7.9 mg) [2, 11].

Fraction B (13.0 g) was chromatographed on silica gel and eluted with a mixture of hexane/ethyl acetate of increasing polarity to give two main fractions (I and II). Fraction I (2.0 g) was purified with a small Sephadex LH-20 column with $CHCl_3$/3%MeOH as solvent to yield betulinic acid (**2**, 23.0 mg) [12, 13] and 30-norlup-20-en-28-oic acid methyl ester (**1**, 127.8 mg). Fraction II (500 mg) produced in the same way alpinumisoflavone (**5**, 4.9 mg) and 4'-*O*-methylepinumisoflavone (**6**, 7.3 mg) [14].

30-Norlup-20-en-28-oic acid methyl ester (1)

White powder

MP: 138-139 °C

R_f = 0.73 ($CHCl_3$)

$[\alpha]_D^{23}$: +34.2 (*c* 0.7, $CHCl_3$)

IR (film) (ν/cm^{-1}): 2937, 2874, 1719, 1639, 1453, 1374, 1250, 1182, 1026, 985, 964, 920, 878; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): See Table 1

^{13}C NMR (100 MHz, $CDCl_3$): See Table 1

EIMS (70 eV): *m/z* (rel. Int.) 440 (M^+, 18), 425 (22), 410 (78), 387 (10), 332 (14), 313 (12), 274 (43), 249 (51), 208 (28), 205 (100), 191 (64), 177 (62), 166 (43), 151 (34), 126 (12), 109 (54), 86 (80), 72 (21), 43 (40)

(+) – HRESI MS calcd $C_{30}H_{49}O_2$ 441.3732 $[M+H]^+$, found *m/z* 441.3731.

Phytotoxic Assay

Lettuce seeds (*Lactuca sativa* L.) were used for the bioassay. Nine seeds were deposited on filter paper containing a defined concentration of the test compound in a Petri dish (4 cm id.). Distilled water (1 ml, containing 100 ppm (w/v) Tween 80) was added to the Petri dish, and incubation was done at 25 °C under continuous light for 7 days. The control experiments were conducted with distilled water alone. The elongation of the roots and shoots was measured and compared with those of the control.

Antimicrobial assay

Agar diffusion tests were performed in the usual manner [15] with *Bacillus subtilis* and *Escherichia coli* (on peptone agar), *Staphylococcus aureus* (Bacto nutrient broth), *Streptomyces viridochromogenes* (M Test agar), the fungi *Mucor miehei* and *Candida albicans* (Sabouraud agar), and three microalgae (*Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus*).

Compounds were dissolved in an azeotrope chloroform/MeOH (87:13) and 30 μg pro paper disks (Ø 8 mm) were impregnated with each using a 100 μl syringe, dried for 1 h under sterile conditions and placed on the pre-made agar test plates. Bacteria and fungi plates were kept in an incubator at 37 °C for 12 h, micro algae plates for three days at room temperature in a day light incubator. The diameter of inhibition zones was measured.

Conclusion

In this study, we focused on the secondary metabolites of $CHCl_3$/acetone (1:1) extract. One new compound, 30-norlup-20-en-28-oic acid methyl ester (**1**) with five known compounds, betulinic acid (**2**), friedelin (**3**), aristolochic acid I (**4**), alpinumisoflavone (**5**) and 4'-*O*-methylepinumisoflavone (**6**) were isolated from the leaves of *T. annobonea*. Using 1 and 2D NMR, the new compound was fully characterized. All the isolated compounds were evaluated for their phytotoxic and antimicrobial activity. At a concentration of 100 μg/mL and 30 μg/mL, the new isolated (**1**) was the most active compound compare to the control for phytotoxicity and antimicrobial activity, respectively. Friedelin (**3**) and aristolochic acid I (**4**) were also isolated from the stem bark of *T. annobonea*.[2] It still difficult to predict the biological activities as well as the chemical constituents from *Thecacoris* species. The few differences between the secondary metabolites isolated from the leaves of *T. annobonea* and other *Thecacoris* species [1, 2] are may be related to the real specific differences or, more probably to a geographic or environmental influence on biosynthesis [16]. In conclusion, more studies are required to determine properly the chemical constituents of the *Thecacoris* species as well as a relationship between their biological activities.

Acknowledgments

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