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# A new benzophenanthridine alkaloid and other bioactive constituents from the stem bark of *Zanthoxylum heitzii*

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ABSTRACT

Heitziquinone (**7**), a new benzophenanthridine alkaloid, together with five known compounds; isoarnottianamide (**5**), rhoifoline B (**6**), isobauerenol (**8**), 6-hydroxypellitorine (**9**) and sylvamide (**10**), were isolated as minor compounds from the hexane extract of stem bark from *Zanthoxylum heitzii*. Four previously reported compounds (**1**–**4**) were found, as well. Compounds **5** and **7** were both found to exist as 4:1 mixtures of two atropisomers. The structures were elucidated by 1D and 2D NMR spectroscopy and by mass spectrometry. Compounds **5**–**10** were identified for the first time in this species, and they are all rare natural compounds. Pellitorine (**4**), one of the main compounds from the hexane bark extract, was found to be responsible for the brine shrimp larvae toxicity ($LC_{50}$ 37 μM, 8 μg/ml) of the crude extract ($LC_{50}$ 24 μg/ml). Low cytotoxicity against a macrophage cell line was observed.



## 1. Introduction

The genus *Zanthoxylum* belongs to the Rutaceae family. It comprises ca 550 species, and secondary metabolites that are often reported from *Zanthoxylum* species are isoquinoline (e.g. benzophenanthridine) and quinolone alkaloids, lignans, coumarins and amides. Many *Zanthoxylum* species are known to be valuable medicinal agents, and their medicinal properties have been attributed to their content of secondary metabolites [1]. *Zanthoxylum heitzii* (Aubrév. & Pellegr.) P. G. Waterman, syn. *Fagara heitzii* Aubrév. & Pellegr., is a West African tree found in forests from Congo to Cameroon [2]. The tree is reported to be used in traditional medicine against jaundice, toothache, gonorrhoea, rheumatic ailments, impotence and malaria [2–5]. It has also been used as a fish poison [2]. A few studies have investigated the chemical constituents of this plant. We have recently found that the main compounds in the hexane extract of its bark are the sesquiterpenoid caryophyllene oxide (**1**), the benzophenanthridine alkaloid dihydronitidin (**2**), the lignan sesamin (**3**) and the amide pellitorine (**4**) [6]. Previously, alkaloids, amides, lignans, triterpenoids, sterols and fatty acids have been reported [7–9]. We have also found that the hexane extract of *Z. heitzii* bark has insecticidal activity against the malaria vector *Anopheles gambiae* [10] which could be attributed to compound **4** [6]. In our continuation of work with *Z. heitzii* we have identified minor constituents of the hexane bark extract, including a new alkaloid, and evaluated the cytotoxic effects and brine shrimp lethality of the main and minor constituents from the bark.

## 2. Experimental

### 2.1. General experimental procedures

Column chromatographic separation was done on pre-packed Versapak normal phase Si gel columns (Supelco, Bellefonte, PA, USA) and preparative centrifugally accelerated thin-layer chromatography (CA-TLC) on a Chromatotron model 7924 T (Harrison Research, Palo Alto, CA, USA), using 1 or 2 mm layers of Si gel $60PF_{254}$ containing gypsum (Merck, Darmstadt, Germany). Preparative HPLC was performed on a ProStar Polaris system (Varian, Palo Alto, CA, USA) connected to a UV/VIS detector (Varian). Analytical TLC was carried out on 0.2 mm Si gel $60F_{254}$ plates (Merck). Spots were visualized by irradiation with short-wave (254 nm) and long-wave (366 nm) and by spraying with a 1% solution of $Ce(SO_4)_2$ in 10% aqueous $H_2SO_4$ followed by heating to 105 °C for 5 min. One- and two-dimensional NMR spectra were recorded in $CDCl_3$ solution on a Bruker DPX300 instrument (300 MHz for $^1H$/75 MHz for $^{13}C$), a Bruker AVII400 instrument (400 MHz for $^1H$/100 MHz for $^{13}C$) or a Bruker AVI600 instrument (600 MHz for $^1H$/150 MHz for $^{13}C$) (Bruker, Rheinstetten, Germany). LC-HRMS analysis was performed using a Q Exactive mass spectrometer (Thermo

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Scientific, Bremen, Germany) as detector, with spray voltage 3.5 kV, capillary temperature 350 °C, probe heater 300 °C, S-lens RF level 50, with sheath and auxiliary gas 35 and 10, respectively. Chromatographic separation was performed on a Kinetex C18 core-shell column (2.6 μm, 75 × 2.1 mm i.d.; Phenomenex, Torrance, CA, USA) with a Waters Acquity UPLC pump and autosampler, and a linear gradient (0.3 ml/min) of water (A) and acetonitrile (B), each containing 5 mM ammonium formate and 0.01% formic acid. The gradient was from 35 to 100% B over 10 min, with a 5 min hold and return to starting conditions over 1 min (4 min hold). The spectrometer was operated in all-ion-fragmentation (AIF) mode (full scan: scanned *m/z* 300–600, AGC target $5 \times 10^6$, resolution 70,000, and max IT 200 ms; AIF scanned *m/z* 110–1050, AGC target $3 \times 10^6$, resolution 35,000, max IT 200 ms, and normalized collision energy 50). Bioassay data were analysed with GraphPad Prism 6.2.

### 2.2. Plant material

Stem bark of *Z. heitzii* was collected in Douakani, Republic of Congo, in November 2011. The plant name is in accordance with www.theplantlist.org (checked 22.10.2015). A voucher sample of the bark is kept in the Section of Pharmacognosy, School of Pharmacy, University of Oslo (registry number ZH-B-111,202).

### 2.3. Extraction and isolation of compounds

The bark was air-dried and milled in a knife mill (Brabender, Duisburg, Germany; 4 mm sieve). Aliquots of the powdered bark (ca 300 g) were extracted with 3 l portions of hexane in a Soxhlet for 10 h. The solvent was removed on a rotary evaporator. Average yield of extract was ca 1.9%. The hexane extract (ca 21 g) was dissolved in 100 ml dichloromethane (DCM) and chromatographed on a VersaPak Si-gel column (110 × 300 mm), eluting with hexane-DCM (1:1; 2.5 l), DCM (1 l), and a gradient containing increasing amounts of EtOAc (5–80%) in DCM (5 l), EtOAc (1.5 l) and acetone (1 l) to give fractions Fr1–Fr12. The process for isolation and identification of compound **1**–**4** is previously described [6]. Other constituents were obtained as follows: Fr 3 (1.4 g) was dissolved in 8 ml DCM and applied to a VersaPak Si gel column (40 × 150 mm) eluting with DCM (1 l), DCM containing increasing amounts of EtOAc (5%, 0.2 l; 10%, 0.2 l; 15%, 0.2 l; 20%, 0.2 l; 20%, 0.2 l; 40%, 0.2 l), and EtOAc (0.4 l). According to TLC analysis and patterns in NMR spectra the 42 fractions collected were combined into fractions Fr3V1–Fr3V13. Fr3V11 (90 mg) was identified as isoarnottianamide (**5**). The atropisomers **5a** and **5b** were purified by preparative HPLC using a Microsorb 60-8 $C_{18}$ (250 × 21.4 mm) column (Varian), flow rate 20 ml/min, and a mobile phase gradient of water (A) and acetonitrile (B): 40% B, 0–2 min; 40–95% B, 2–15 min; 95% B, 15–25 min, wavelength 237 nm, to yield **5a** (5–6 min) and **5b** (8–9 min). Fr3V2 (70 mg) was rechromatographed by CA-TLC over 1 mm silica gel in an $N_2$-atmosphere. DCM (60 ml) followed by DCM-EtOAc (4:1; 50 ml) and DCM-EtOAc (3:2; 35 ml) were used as mobile phases. Fractions of 12 ml were collected and combined into six fractions (V3V2C1-C6) as indicated by $^1$H NMR and analytical TLC. Fr3V2C3 (28 mg) was identified as rhoifoline B (**6**). Fr3V9 (8 mg) and Fr3V10 (15 mg) were combined and rechromatographed by preparative HPLC using the same gradient as above. Heitziquinone (**7**) (1.9 mg) eluted at 9–10 min. Fr5 (2 g) was applied to a VersaPak Si gel column (40 × 150 mm) and eluted with DCM (0.3 l), DCM-EtOAc (4:1; 0.2 l) and EtOAc (0.2 l). 35 fractions with volumes of 15 ml were collected and combined into nine subfractions (Fr5V1–V9) as indicated by TLC analysis and NMR spectroscopy. Fr5V2 (1.2 g) was rechromatographed similarly, eluting with DCM-EtOAc (1:20; 0.6 l) and DCM-EtOAc (1:10; 0.6 l) yielding Fr5V2V1–8. Fr5V2V4 was chosen for further purification by CA-TLC (2 mm layer) using a gradient containing increasing amounts of EtOAc (5–20%) in hexane (285 ml) as eluent to yield isobauerenol (**8**) (27 mg). Fr5V2V5 was purified by CA-TLC using the same gradient as above to yield **8** (88 mg). Fr11 (0.72 g) was applied to a VersaPak Si gel column (40 × 150 mm) eluting with DCM (150 ml), DCM-EtOAc (9:1; 250 ml), DCM-EtOAc (4:1; 250 ml), DCM-EtOAc (7:3; 250 ml), DCM-EtOAc (3:2; 250 ml) ), DCM-EtOAc (1:1; 250 ml) and EtOAc (400 ml). According to TLC analysis and NMR spectroscopy the 42 fractions collected were combined into Fr11V1–Fr11V11. Fr11V11 (70 mg) was rechromatographed using CA-TLC (1 mm) eluting with DCM-EtOAc (4:1; 50 ml), DCM-EtOAc (3:1; 100 ml), DCM-EtOAc (7:3; 50 ml), DCM-EtOAc (3:2; 50 ml), EtOAc (50 ml) to yield 6-hydroxypellitorine (**9**) (5 mg). Finally, Fr12 (1.5 g) was fractionated (VersaPak Si-gel, 40 × 150 mm) eluting with DCM-EtOAc (4:1; 200 ml), DCM-EtOAc (7:3; 280 ml), DCM-EtOAc (3:2; 240 ml) ), DCM-EtOAc (1:1; 340 ml), DCM-EtOAc (3:7; 280 ml) and EtOAc (250 ml) giving 35 fractions (55 ml each) that were combined into 10. Fr12V9 (400 mg) was rechromatographed by CA-TLC (2 mm) using DCM-EtOAc (2:1; 15 ml), DCM-EtOAc (3:2; 25 ml), DCM-EtOAc (1:1; 50 ml), DCM-EtOAc (2:3; 50 ml) ), DCM-EtOAc (3:7; 50 ml), DCM-EtOAc (1:4; 50 ml) and EtOAc (50 ml) as mobile phases. Fr12V9C4 (37 mg) (eluted with DCM-EtOAc, 1:1) was purified by preparative HPLC on a LichroCART C18 column, 250 × 10 mm (Merck, Darmstadt, Germany), flow rate 10 ml/min, using a gradient of water (A) and acetonitrile (B) as mobile phase: 30% B, 0–2 min; 30–80% B, 2–25 min, 230 nm was used for detection. Sylvamide (**10**) (0.6 mg) was obtained after 6–7 min.

#### 2.3.1. Isoarnottianamide (*5*)

**5a**: White solid. $^1$H and $^{13}$C NMR data, see Table 1; LC–MS $t_R$ 3.95 min,*m/z* = 382.1280 $[M + H]^+$, calc. 382.1285.

**5b**: White solid. $^1$H and $^{13}$C NMR data, see Table 1; LC–MS $t_R$ 3.42 min, *m/z* = 382.1283 $[M + H]^+$, calc. 382.1285.

#### 2.3.2. Heitziquinone (*7*)

**7**: Orange solid. UV (MeOH) $\lambda_{max}$ 239, 329 nm; $^1$H NMR and $^{13}$C NMR, see Table 1; LC–MS: **7a**, $t_R$ 4.05 min, *m/z* = 366.0975 $[M + H]^+$ (calc. 366.0972); **7b**, $t_R$ 3.58 min, *m/z* = 366.0972 $[M + H]^+$ (calc. 366.0972).

**Table 1**
$^1$H and $^{13}$C NMR data for compound **7** and the atropisomers **5a** and **5b**.

| | 7[a] | | 5a[b] | | 5b[c] |
|---|---|---|---|---|---|
| | $\delta_H$ (*J* in Hz)[d] | $\delta_C$[d] | $\delta_H$ (*J* in Hz)[d] | $\delta_C$[d] | $\delta_H$ (*J* in Hz)[d] |
| 1 | 7.20 (s) | 104.5 | 7.20 (s) | 104.4 | 7.22 (s) |
| 2 | | 149.9 | | 148.3 | |
| 3 | | 150.2 | | 149.5 | |
| 4 | 7.02 (s) | 99.2 | 7.06 (s) | 99.0 | 6.98 (s) |
| 4a | | 128.5 | | 128.8 | |
| 4b | | 135.6 | | 135.9 | |
| 6 | | 185.6 | | 147.5 | |
| 7 | 6.03 (s) | 107.9 | 6.56 (s) | 100.6 | 6.63 (s) |
| 8 | | 159.3 | | 150.0 | |
| 9 | | 181.4 | | 142.7 | |
| 10 | 6.71 (s) | 133.2 | 6.61 (s) | 113.6 | 6.11 (s) |
| 10a | | 147.3 | | 115.7 | |
| 10b | | 129.0 | | 133.5 | |
| 11 | 7.16 (d, J = 8.4) | 125.2 | 7.33 (d, J = 8.3) | 127.2 | 7.27[e] |
| 12 | 7.74 (d = 8.4) | 128.0 | 7.76 (d, J = 8.2) | 128.1 | 7.76 (d, J = 8.2) |
| 12a | | 132.7 | | 131.3 | |
| N-$CH_3$ | 3.16 (s)**7a**/ (3.19 (s) **7b**)[f] | 34.1 | 3.01 (s) | 33.1 | 3.12 (br s) |
| N-CHO | 8.08 (s) **7a**/ (8.37 (s) **7b**)[f] | 164.1 | 8.18 (s) | 165.2 | 8.32 (s) |
| 8-$OCH_3$ | 3.88 (s) | 56.4 | 3.84 (s) | 55.8 | 3.90 (s) |
| 9-$OCH_3$ | – | – | 3.81 (s) | 56.7 | 3.80 (s) |
| O-$CH_2$-O | 6.11 (s) | 101.8 | 6.08 (s) | 101.6 | 6.07, 6.10 (2 × d, J = 1.1 ) |

[a] 400 MHz instrument.
[b] 600 MHz instrument.
[c] 300 MHz instrument.
[d] NMR data recorded in $CDCl_3$.
[e] Obscured by $CHCl_3$ signal, ppm values detected in HSQC spectrum.
[f] Individual signals for each atropisomer **7a** (major) and **7b** (minor).