Full Papers

Isolation and in Vitro Antiplasmodial Activities of Alkaloids from *Teclea trichocarpa*: In Vivo Antimalarial Activity and X-ray Crystal Structure of Normelicopicine

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Seven alkaloids have been isolated from *Teclea trichocarpa* including four, normelicopicine (**1**), arborinine (**2**), skimmianine (**6**), and dictamnine (**7**), that are reported for the first time in addition to the previously reported alkaloids melicopicine (**3**), tecleanthine (**4**), and 6-methoxytecleanthine (**5**). The structure of **1** was confirmed by single-crystal X-ray crystallography. Two alkaloids, **1** and **2**, displayed limited in vitro activities against *Plasmodium falciparum* strains HB3 and K1, but there appeared to be little cross-resistance with chloroquine. Alkaloid **1** was found to have some activity against *P. berghei* in mice (32% suppression of parasitaemia at a dose of 25 mg kg^{-1} day^{-1}), but unlike chloroquine it did not inhibit β-haematin formation in a cell-free system; **1** was found to have low in vitro cytotoxicity to KB cells (IC_{50} > 328 μM).

The treatment of malaria in Africa is becoming increasingly difficult due to the rising prevalence of *Plasmodium falciparum* resistant to chloroquine and to other antimalarial drugs.[1] With an estimated 1 million children under the age of 5 years dying of malaria each year there is an urgent need to evaluate the many traditional remedies that are used for the treatment of this disease. This is necessary to determine the efficacy and safety of locally used preparations as well as to investigate their potential to provide novel compounds as leads to new antimalarial agents.

Teclea trichocarpa Enge. (Rutaceae), a species that grows in tropical and warm temperate climates, is used in Kenyan traditional medicine for a variety of purposes. Traditional healers belonging to the Akamba tribe have reported that this tribe uses the roots for malaria treatment and as an anthelmintic, while the hunters of the Giriama tribe steam the leaves and inhale the vapor as a cure for fever and also place the leaves in hunting dogs' noses to improve their power to follow a scent.[2] Some other species of *Teclea* are also used in Africa including *T. nobilis*, the bark of which is used in South Africa as a remedy for gonorrhea, while in Ethiopia the bark and leaves of this species are used as analgesics.[2] In North Cameroon *T. ouabanguiensis* is used as a remedy for coughs and asthma.[2]

In this paper we report the isolation of some alkaloids from *T. trichocarpa* and their in vitro activities against *P. falciparum*; these were assessed against both chloroquine-sensitive (HB3) and chloroquine-resistant (K1) strains of *P. falciparum*. In addition, the major alkaloid isolated, normelicopicine (**1**), was assessed for in vivo antimalarial activity against *P. berghei* in mice, for in vitro cytotoxicity to KB cells, and for its ability to inhibit β-haematin formation to provide information concerning the mode of action of this compound. The stucture of **1** has been confirmed by means of X-ray crystallography.

Results and Discussion

Phytochemistry. Seven alkaloids were isolated from the methanolic extract of *T. trichocarpa* leaves. The acridone alkaloid normelicopicine (**1**) was the major alkaloid present (0.06% of the dried leaves). The mass spectrum of this compound showed a molecular ion of *m*/*z* 315, 14 mass units less than that of melicopicine, an alkaloid previously reported to be present in *T. trichocarpa*.[3] The ^{1}H NMR spectrum resembled that reported for melicopicine[3] except that one three-proton singlet was replaced by a one-proton singlet at δ 14.5, indicating the presence of a phenolic proton adjacent to the keto group, suggesting that **1** is 1-hydroxy-2,3,4-methoxy-*N*-methylacridone. Although this is the first report of **1** as a constituent of *T. trichocarpa*, it has previously been isolated from *Achronychia baueri* leaves and bark.[4] The structure of **1** was confirmed by means of X-ray crystallography (see below).

The ^{1}H NMR spectrum of alkaloid **2** (0.01% dried leaves) resembled that of **1** except that the signal for one of the methoxy groups was replaced by a one-proton singlet and the mass spectrum of **2** showed a molecular ion of *m*/*z* 285,

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Table 1. Activities of Alkaloids Isolated from *T. trichocarpa* and Chloroquine Diphosphate against *P. falciparum* Strains K1 and HB3 in Vitro

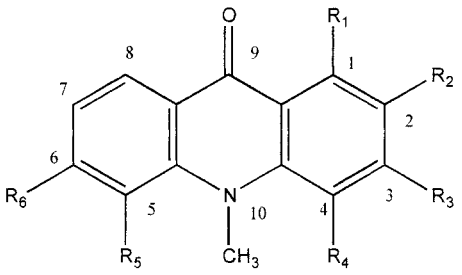
	activity against *P. falciparum*, IC_{50} μM ± S.D.; $n = 3$	
alkaloid	strain HB3	strain K1
normelicopicine **1**	8.25 ± 0.12	14.7 ± 0.26
arborinine **2**	3.85 ± 0.11	9.34 ± 0.37
melicopicine **3**	>100	>100
tecleanthine **4**	23.2 ± 2.6	53.0 ± 0.64
6-methoxytecleanthine **5**	32.3 ± 1.9	56.9 ± 1.75
skimmianine **6**	47.5 ± 0.42	59.0 ± 0.32
chloroquine diphosphate	0.028 ± 0.00043	0.63 ± 0.063

again indicating that **2** differs from **1** by the loss of a methoxy group. The two methoxy groups in **2** could have been in the 2,3-, 2,4-, or 3,4-positions, but this question was resolved by examining the ^{13}C NMR spectrum, in which the resonance of the *N*-methyl carbon was found to occur at δ 34.2 ppm. The latter is found only in acridone derivatives in which the positions adjacent to the *N*-methyl group (C-4 and C-5) are unsubstituted,[5] and therefore **2** was identified as 1-hydroxy-2,3-dimethoxy-*N*-methylacridone, known as arborinine.[6] The identity of **2** as arborinine was further confirmed by comparison of the ^{13}C NMR spectrum with published data.[7] Arborinine (**2**) has been isolated from the leaves *A. haplophylla*[4] as well as from the bark of *T. natalensis*.[8] In addition, three acridone alkaloids, melicopicine (**3**), tecleanthine (**4**), and 6-methoxytecleanthine (**5**), which have previously been reported as constituents of *T. trichocarpa*,[3] were isolated (yields 0.02, 0.01, and 0.02% of the dried leaves, respectively). Two furanoquinoline alkaloids, skimmianine **6** and dictamnine **7** (0.04 and 0.001% of the dried leaves, respectively), were also isolated. Compound **6** was identified by comparison of the spectral data with published values[9] and is a constituent of the related species *T. simplicifolia*,[10] *T. nobilis*, and also *A. baueri*.[4] The identity of **7**, a constituent of a number of species of the Rutaceae, was confirmed by comparison of the spectral data with published values.[11]

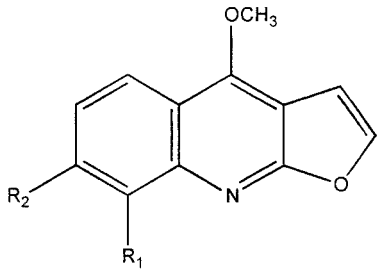
Antiplasmodial Activities. Antiplasmodial activities of alkaloids **1**−**6** together with that of the control drug chloroquine diphosphate against chloroquine-sensitive *P. falciparum* strain HB3 and against chloroquine-resistant strain K1 are shown in Table 1. Two alkaloids, **1** and **2**, displayed limited antiplasmodial activities against both strains of the parasite. Arborinine (**2**), the most active, was about twice as potent as **1**, and both compounds were about twice as potent against strain HB3 than against chloroquine-resistant strain K1. However, chloroquine was approximately 20-fold less active against the chloroquine-resistant strain, suggesting that **1** and **2** did not show cross-resistance with chloroquine. Nevertheless, the activities of these compounds were relatively weak compared to chloroquine; **2** was found to be 15-fold less active than chloroquine against strain K1 and 140-fold less active than chloroquine against strain HB3. The other acridone alkaloids, **3**, **4**, and **5**, did not display significant activities against either strain of *P. falciparum*, although they have been reported to have antifungal activities against *Cladosporium curcumerinum* and antibacterial effects against *Bacillus subtilis*.[3] Methylation of the hydroxy group of **1** as in compound **3** results in a marked loss of activity and suggests that in these compounds a 1-hydroxyl group is essential for antiplasmodial activity. However, Basco et al. (1994)[12] reported that the related alkaloid normelicopidine (1-hydroxy-2,3-methylenedioxy-4-methoxy-*N*-methylacridone) was inactive against *P. falciparum* in vitro, whereas

Table 2. Activities of Normelicopicine (**1**) and Chloroquine Diphosphate against *P. berghei* (ANKA) in Mice

group	dose schedule, mg kg^{-1} day^{-1}	mean parasitaemia, % ± SD	suppression of parasitaemia, %
control		12.8 ± 2.0	
normelicopicine **1**	25	8.7 ± 4.4	32.1
chloroquine diphosphate	10	0.0	100



1 R_1 = OH; R_2, R_3, R_4 = OCH_3; R_5, R_6 = H
2 R_1 = OH; R_2, R_3 = OCH_3; R_4, R_5, R_6 = H
3 R_1, R_2, R_3, R_4 = OCH_3; R_5, R_6 = H
4 R_1 = OCH_3; R_2, R_3 = -OCH_2O-; R_4, R_6 = H; R_5 = OCH_3
5 R_1 = OCH_3; R_2, R_3 = -OCH_2O-; R_4 = H; R_5, R_6 = OCH_3



6 R_1, R_2 = OCH_3
7 R_1, R_2 = H

Figure 1. Structures of alkaloids isolated from *T. trichocarpa*.

the 1-methoxy analogue melicopidine was moderately active, indicating that structure−activity relationships in this group of compounds are more complex.

Further Studies on Alkaloid 1. The effect of **1** against *P. berghei* ANKA in mice is shown in Table 2. A daily dose of 25 mg kg^{-1} i.p. suppressed parasitaemia by 32% compared to that in untreated infected control animals. However, **1** was less potent than chloroquine diphosphate, which completely suppressed parasitaemia at a daily dose of 10 mg kg^{-1}. To gain information concerning the possible antiplasmodial mode of action of this alkaloid, **1** was tested for its ability to inhibit the formation of β-haematin. In red blood cells infected with malaria parasites haemoglobin is digested, leaving behind haem which is toxic to the parasite. This is detoxified by conversion to haemozoin (malaria pigment), which appears to be identical to β-haematin.[13] Chloroquine and related 4-aminoquinoline antimalarials appear to act primarily by binding to haem and preventing its detoxification.[14] Interestingly, **1** failed to inhibit β-haematin formation, suggesting that it does not have a chloroquine-like mode of action. When tested for in

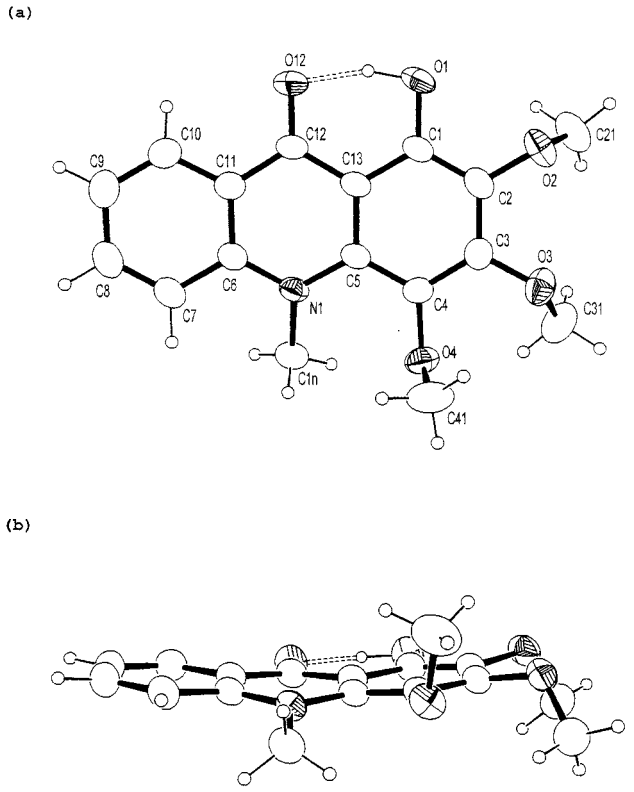


Figure 2. ORTEP[1] plots of the crystal structure of **1** showing (a) the intramolecular hydrogen bond between the hydroxy and carbonyl functions of the molecule; (b) a side-on view of **1** showing the distribution of the substituents with respect to the molecular plane and the deviation of the acridone from planarity.

vitro cytotoxicity against KB (human mouth cancer) cells, **1** was found to have little activity (IC_{50} > 328 μM), while podophyllotoxin as a positive control was highly cytotoxic (IC_{50} = 0.0048 μM).

X-ray Crystallography. The crystal data obtained[15] confirmed the structure of **1**. A view of the structure with the numbering scheme adopted is given in Figure 2. Bond lengths of the *N*-methylacridone unit in the molecule are unremarkable, lying within the ranges observed for analogous *N*-substituted acridones.[16] The tricycle deviates significantly from the plane, showing a clear "saddle" conformation that appears to originate in the slight boat conformation of the central six-membered ring of the acridone (Figure 2b). Within the boat, N(1) and C(12) lie significantly below the mean plane of the ring and, as a result, the *N*-methyl group lies ca. 0.5 Å below the tricycle mean plane. In addition, the methyl groups of the methoxy substituents lie out of the plane, with those at C(2) and C(3) on the same side and that at C(4) on the opposite side. A short intramolecular hydrogen bond between the hydroxyl and carbonyl groups {*d*[H(1)···O(12)] 1.518, *d*[O(1)···O(12)] 2.494 Å} is apparent, but no significant intermolecular H-bonding is apparent to influence crystal packing. The most noticeable feature of the crystal packing is the inversion-related face-to-face stacks of molecules running parallel to the *b*-axis. The separation of ca. 3.5 Å between the aromatic units and the alternating arrangement of the molecules within these stacks indicates that attractive electrostatic $\pi-\pi$ interactions may play a significant role in crystal formation.

Conclusion

This study has shown that *T. trichocarpa* contains both acridone and furoquinoline alkaloids. Four alkaloids, **1**, **2**, **6**, and **7**, have been shown to be present in this species for the first time in addition to the previously reported alkaloids **3**, **4**, and **5**[3]. Two alkaloids, **1** and **2**, displayed limited in vitro activities against *P. falciparum*, and there appeared to be little cross-resistance with chloroquine. Alkaloid **1** was found to have low-grade activity against *P. berghei* in mice but unlike chloroquine did not inhibit β-haematin formation. These results lend some support to the use of *T. trichocarpa* in African traditional medicine for the treatment of malaria, but further studies are needed to evaluate this. Although the activities of **1** and **2** are modest compared to chloroquine, compounds of this type may be worthy of further study, as they did not show cross-resistance with chloroquine and appear to have a mode of action different from that of the quinoline antimalarials. In addition, a comparison of the cytotoxicity of **1** against KB cells with its antiplasmodial activities indicates selective toxicity to malaria parasites (selectivity indices 40 and 22 for the chloroquine-sensitive and chloroquine-resistant strains, respectively).

Experimental Section

Isolation of Alkaloids. Leaves of *T. trichocarpa* were collected in the area around Nairobi and dried in the shade. Authentication was carried out by Mr. Mutisho and Mr. Mathenge of the University of Nairobi Herbarium, where a voucher specimen, number 2001/1, is deposited. Powdered leaves (250 g) were extracted three times by shaking with methanol, and the combined extracts were concentrated by evaporation under reduced pressure at 40 °C and dried under vacuum to yield 15 g of gummy material. Ten grams of the latter was dissolved in 100 mL of methanol, and then 300 mL of ethyl acetate was added. After filtering, the extract was dried as above, and the residue (4 g) was fractionated using positive pressure column chromatography over TLC grade silica gel G (Merck) eluting with hexane followed by hexane containing increasing amounts of ethyl acetate. Fractions that appeared similar on TLC (silica gel G_{F254}, hexane/ethyl acetate 7.5:2.5) sprayed with anisaldehyde/sulfuric acid spray reagent were combined, dried, and tested for in vitro antiplasmodial activities as described below. Repetition of the above led to the isolation of seven alkaloids, which were identified using spectroscopic methods. Mass spectra were recorded on a Finnigan MAT 95 spectrometer at 70 eV. NMR spectra were recorded on a Bruker DMX-600 NMR spectrometer.

Antiplasmodial Assay. Malaria parasites were maintained in human A^+ erythrocytes suspended in RPMI 1640 medium supplemented with A^+ serum and D-glucose according to previously published methods.[17,18] Cultures containing predominantly early ring stages were used for testing. Compounds were dissolved or micronized in DMSO and further diluted with RPMI 1640 medium (the final DMSO concentration did not exceed 0.5%, which did not affect parasite growth). Twofold serial dilutions were made in 96-well microtiter plates in duplicate, and infected erythrocytes were added to give a final volume of 100 μL with haematocrit 2.5% and 1% parasitaemia. Chloroquine diphosphate was used as a positive control, and uninfected and infected erythrocytes without compounds were included in each test. Plates were placed into a modular incubator gassed with 93% nitrogen, 3% oxygen, and 4% carbon dioxide and incubated at 37 °C for 48 h. Parasite growth was assessed by measuring lactate dehydrogenase activity.[19] The reagent used contained the following in each milliliter: 0.74 mg of acetylpyridine adenine dinucleotide (APAD), 19.2 mg of lithium lactate, 0.1 mg of diaphorase, 2 μL of Triton X-100, 1 mg of nitroblue tetrazolium, and 0.5 mg of phenazine ethosulfate. Fifty microliters of this reagent was added to each well and mixed, and plates were incubated for 15 min at 37 °C. Optical densities were read at 550 nm using a Dynatech Laboratories MRX microplate reader, and percent inhibition of growth was calculated by comparison with control values.

IC_{50} values were determined using linear regression analysis (Microsoft Excel). A minimum of three separate determinations was carried out for each compound.

In Vivo Antimalarial Test. This was carried out using Peters' 4-day suppressive test against *P. berghei* infection in mice.[20] Female BALB/C mice, weight 18−20 g, were inoculated with *P. berghei* (ANKA); each mouse received 1×10^7 infected erythrocytes by i.v. injection. Drugs were administered to mice by intraperitoneal injection in 0.2 mL inoculum daily for 4 consecutive days. Control and test groups all contained 5 mice. On day 5 of the test a blood smear was taken and the percent supression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected controls with those of dosed mice. Chloroquine diphosphate was used as a positive control.

Inhibition of β-Haematin Formation. The methodology used was adapted from that of Egan et al. (1994).[21] Haemin (7.5 mg) was dissolved in 1.25 mL of 0.1 M NaOH, and then 0.125 mL of 1 M HCl was added. Three molar equiv of the compound under test (with respect to haemin) was then added, followed by 1.15 mL of 9.78 M acetate buffer at pH 5.0, preincubated at 60 °C. The pH was adjusted to 5.0 by the addition of glacial acetic acid, and the mixture was then stirred for 30 min at 60 °C. After cooling on ice, the precipitate was filtered using a 5 μM membrane filter and washed with water. After drying under vaccuum for 24 h the Fourier transformed infrared spectrum of the product was recorded on a Mattson Galaxy 6020 FTIR spectrometer. A control in which no compound was added was also carried out. The formation of β-haematin was detected by the presence of peaks at 1660 and 1210 cm^{-1}.

Cytotoxicity Test. Cytotoxicity against KB cells was carried out as previously described using the method of Pham and Huff (1999)[22] in which cell viability is assessed by measuring the reduction of AlamarBlue to a fluorophore. Podophyllotoxin was used as a positive control.

X-ray Crystallography. Intensity data for **1** were collected from an orange block ($0.516 \times 0.316 \times 0.213$ mm^3) using a Stoe STADI-4 diffractometer with Cu Kα radiation in the θ range 4.56−60.25°. The structure was solved by direct methods[23] and refined[24] on F^2 to convergence with all non-H atoms anisotropic. All H atoms were located from difference Fourier synthesis and refined with isotropic displacement parameters. For the final model $wR_2 = 0.1151$ (all data) and $R_1 = 0.0394$ [$I_{hkl} > 2\sigma(I)$].

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Supporting Information Available: Tables containing full details of the crystal data obtained for normelicopicine **1** are available free of charge via the Internet at http://pubs.acs.org.

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(15) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).
(16) CSD version 5.22. A search for N-aryl- or alkylacridones yielded 24 hits with the following CDS codes: BHACRY, COTCOD, COTCUJ, CUDVOM, CUXYOJ, EACDRO, GURBOK, HOYJEK, NMACRO10, NMEQAC, NMEQAC01, PHACRD, PIVRIV, SULTUO, TIQFEE, VOBXIT, VOBXOZ, WIDPII10, XAGVUW, YOYXIT, ZIPWIE, ZOVGEW, ZUZSAO, ZUZSES.
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