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Leishmanicidal, Antiplasmodial and Cytotoxic Activity of Indole Alkaloids from Corynanthe pachyceras

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Abstract: Five indole alkaloids, corynantheidine, corynantheine, dihydrocorynantheine, α-yohimbine and corynanthine were isolated from bark of Corynanthe pachyceras K. Schum. (Rubiaceae). The structures were established by spectroscopic methods, including previously unreported assignment of all 1H-NMR resonances by COSY and NOESY experiments. These and related alkaloids showed pronounced activity against Leishmania major promastigotes (IC₅₀ at the micromolar level) but no significant in vitro antimalarial activity (against chloroquine-sensitive Plasmodium falciparum). Cytotoxicity assessed with drug sensitive KB-3-1 and multidrug-resistant KB-V1 cell lines was low; the alkaloids are apparently not substrates for the P-glycoprotein (P-170) efflux pump.

Key words: Indole alkaloids, Plasmodium, Leishmania, multidrug resistance, NMR.

Introduction

Corynanthe pachyceras K. Schum. [syn. Pausinystalia pachyceras (K. Schum.) De Wild., Pseudocinchona pachyceras (K. Schum.) A. Chev., Pseudocinchona africana A. Chev.] is a lower storey forest tree growing in tropical West Africa. In Ghana, the plant is used as intoxicant, local anaesthetic, and a febrifuge (1). This and related Rubiaceae species, such as C. johimbe K. Schum., are the classical sources of indole alkaloids of the yohimbine- and corynantheine-type. In a screening program for antimalarial and leishmanicidal plants conducted at this laboratory, crude extracts of bark of C. pachyceras exhibited a high activity. Isolation and characterization of active constituents responsible for this activity is described below.

Materials and Methods

General procedures

NMR spectra were recorded at 25 °C on a Varian Gemini 2000 or a Bruker AMX 400 spectrometer (proton frequency 300.07 and 400.13 MHz, respectively), with CDCl₃ as solvent and TMS as internal standard, using standard library pulse sequences.

NOESY spectra were obtained with mixing times of 500-900 ms. HMBC spectra were optimized for 3J_C,H of 4–11 Hz. Mass spectra were obtained on a JEOI JMS-AX505W double focussing spectrometer with EI or FAB ionization (positive ion mode). Column chromatography was performed on silica gel 60 (Merck, 0.063 - 0.2 mm). Fractions were monitored by TLC (Merck precoated silica gel 60 F₂₅₄, plates), using UV light and Dragendorff reagent to visualize the spots. Preparative HPLC was carried out on a 250 × 16 mm Knauer column packed with LiChrospher 100 RP18, 5 μm, using a Waters system consisting of a model 590 pump and a model 481 UV spectrophotometer operating at 225 nm. Compounds 6–8 were obtained from commercial sources, whereas 9 and 10 were synthetic, racemic compounds (14); the identity and purity of all materials was confirmed by 1H-NMR spectroscopy.

Plant material

C. pachyceras K. Schum. was identified and its bark collected by Mr. D. K. Abbiw, Department of Botany, University of Ghana, near Agriculture Research Station, Kede, South Ghana. A voucher specimen was deposited in Ghana Herbarium (GC 47529).

Extraction and isolation

Powdered stem bark (250 g) was macerated three times with 1 l of CH₃Cl-MeOH (1:1) to give a total of 47 g of raw extract. The extract was partitioned between light petroleum, EtOAc and H₂O, and the fractions tested for antileishmanial and antimalarial activity. The activity was confined to the EtOAc fraction, which was subjected to open column chromatography (silica gel, stepwise gradient elution from CH₃Cl to EtOAc to MeOH). The activity was present in alkaloid-containing fractions (Dragendorff reagent). A portion (182 mg) of the combined alkaloid fraction (6.34 g) was subjected to preparative HPLC (6 ml/min of 70% MeOH in 0.01 M aq. AcONa, pHi 8.04), to give, in the order of elution, 16.6 mg of 1, 2.1 mg of 2, 10.3 mg of 3, 6.3 mg of 4, and 9.2 mg of 5.

Corynanthe (1): Colorless oil: [α]D° -76.5° (c 0.38, pyridine). lit. (2) -73° (pyridine); HREIMS: m/z = 353.1875 ([M - H]⁻), C₂₁H₂₂NO₂ requires 353.1865. Content in the bark 0.23 %.

α-Yohimbine (2): Colorless oil: [α]D° +17° (c 1.38, pyridine). lit. (3) -18° (pyridine); HRFABMS: m/z = 355.2044 ([M + H]⁺), C₂₁H₂₂NO₂ requires 355.2022. Content in the bark 0.03 %.

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Dihydrocorynantheine (3): Colorless oil; [α]D20 +25° (c 0.67, MeOH), lit. (4) +36.2° (MeOH); HRFABMS: m/z = 369.2155 ([M + H]+). C20H32N2O2 requires 369.2178. Content in the bark 0.15%.

Corynantheine (4): Colorless oil; [α]D50 +32° (c 1.10, MeOH), lit. (5) +28.5° (MeOH); HRFABMS: m/z = 367.1973 ([M + H]+). C20H32N2O2 requires 367.2022. Content in the bark 0.09%.

Corynantheidine (5): Colorless oil; [α]D50 −181° (c 7.41, MeOH), lit. (6) −171° (MeOH); HRFABMS: m/z = 369.2137 ([M + H]+). C20H32N2O2 requires 369.2178. Content in the bark 0.13%.

Assay for antiplasmodial activity

A modification of Desjardins’ radioisotope method (7) for measuring growth of a chloroquine sensitive strain of Plasmodium falciparum (3D7) was adopted, using uptake of [3H]phenylalanine as an index of growth. Thus, 50 μl of the growth medium (RPMI 1640 added 5% Albumax, 5.95 g/l of HEPES, 31 ml/l of 7.5% sodium bicarbonate, and 500 ml/g of glucose) containing test substances added from a DMSO stock were mixed with a suspension of parasitic erythrocytes (5% hematocrit, 2-3% parasitemia) in 96-well microtiter plates. The maximal final DMSO concentration was 0.5%. Each concentration of the test substance was tested in triplicate. The plates were incubated at 37°C for 24 hours before the addition of [3H]phenylalanine. After an additional 24 hours incubation period, the parasites were harvested and incorporation of radioactivity determined by liquid scintillation counting.

Assay for leishmanicidal activity

Promastigotes from a WHTO reference vaccine strain of Leishmania major were maintained at 26°C in RPMI 1640 medium containing 25 mM HEPES, 4 mM L-glutamine, 0.02 mg/ml of gentamicin, and 10% of heat-inactivated fetal calf serum. The effect of plant extract and pure compounds on the growth of promastigotes was assessed by monitoring inhibition of [3H]thymidine uptake similarly as previously described (8). The compounds for testing were dissolved in DMSO, the stock solution diluted appropriately with the growth medium, and aliquots incubated in 96-well microtiter plates with promastigotes (1 × 10⁴ per ml, 180 μl well) for 2 hours. After addition of [3H]thymidine the plates were incubated for 18 hours, the cells harvested, and the incorporation of radioactivity determined by liquid scintillation counting.

Assay for cytostatic activity

Myeloma-free carcinoma cell lines KB-3-1 (a HeLa subclone) and KB-V1, selected for resistance with vinblastine from the KB-3-1 cells (9), were obtained from the Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA. The cells were maintained in monolayers at 37°C in an atmosphere containing 5% CO2 (humidity 98%), using Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum, glucose (4.5 g/l), L-glutamine (0.58 g/l), sodium pyruvate (1 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). The KB-V1 cells were grown in the absence of cytostatic pressure from vinblastine, but the cells from passage number 5-15 preserved unchanged degree of resistance to rhodamine 123 (10), and were used in the assay. For the cytotoxicity assays, the KB-3-1 cells (70–80% confluency) or the KB-V1 cells (60–70% confluency) were harvested by trypsinization, applied into 96-well plates (4 × 10⁴ of KB-3-1 cells or 7 × 10⁴ of KB-V1 cells per well) in 75 μl of the culture medium, and grown for 24 hours. Test substances were applied in 75 μl of a solution prepared by mixing 50 μl of a DMSO stock with 0.95 ml of the medium and appropriately diluted with the medium to required concentrations; thus, no well contained more than 0.25% of DMSO, which was also present in the control wells. Six wells were used for each concentration of the test substance. Time of incubation and the amounts of the cells used were such that the cells in the control wells reached 70% (KB-3-1) or 60–70% (KB-V1) confluence at the end of the 72 hours incubation period. After the incubation the medium was removed and the amount of cells was determined using the CellTiter 96 aqueous cell proliferation assay kit from Promega Corporation. The MTS/PMS reagent (11) was freshly prepared by mixing 2.0 ml MTS solution (2 mg/ml) with 95.4 μl PMS solution (0.32 mg/ml) and 10.4 ml of growth medium (without phenol red and serum). After addition of 120 μl of the reagent mixture to each well, the plate was incubated for 50 min and the absorbance determined at 490 nm. The reported IC50 values are the result of three separate determinations with different passages of the cells.

Results and Discussion

Crude ethanolic extracts of C. pachyceras bark strongly inhibited growth of Plasmodium falciparum and of Leishmania major promastigotes. Fractionation of the extract on silica gel showed that the activity was confined to fractions which gave a positive reaction with the Dragendorff reagent. Individual constituents of the alkaloid fraction were separated by preparative, reversed-phase HPLC using a mixture of methanol and ammonium acetate buffer. Optimization of the HPLC system showed that the separation was improved with increasing pH, and baseline separation was achieved at pH 8.04. Five indole alkaloids were isolated in amounts corresponding to the total alkaloid content in the plant material of 0.63%.

Compounds 1 and 2 (Fig. 1) were isomers with the molecular formula C20H32N2O2 as determined by HRMS. 13C-NMR spectra indicated the presence of a yohimboid skeleton (12). Analysis of coupling patterns, supported by COSY and NOESY experiments, led to identification of the stereochemistry as 3α,15α,20β (normal configuration) for 1 and as 3α,15α,20α (allo configuration) for 2. Furthermore, both compounds have H-16 in the α-configuration and H-17 in the β-configuration. The compounds 1 and 2 thus correspond to corynanthe and α-yohimbine, respectively. Compounds 3 and 5 were isomers with the molecular formula C20H32N2O2 (HRMS). 13C-NMR spectra showed the presence of a corynantheine-type structure (13). The stereochemistry of the coryneine skeleton in 5 was established as 3α,15α,20αβ. 5 is thus corynantheine. Compound 3 showed broadened resonances in the 400 MHz 1H-NMR spectrum, indicating the presence of several conformers in slow exchange on the NMR time scale, but the alkaloid could be unambiguously identified as dihydrocorynantheine by comparison of its 1H- and 13C-NMR spectra with those of an original sample of synthetic (14), racemic dihydrocorynantheine. Compound 4 was identified as an unsaturated analogue of 3, corynantheine (13).
Fig. 1 Structures of indole alkaloids isolated from *Corynanthe pachyeras*: 1. corynanthe; 2. α-yohimbine; 3. dihydrocorynanthe; 4. corynanthe; 5. corynantheidine.

The $^{13}$C-NMR data for 1–5 (Fig. 1) are given in Table 1. On the basis of $^{1}$H, $^{13}$C chemical shift correlation experiments, the assignments of C-10 and C-11 in 3–5 were reversed as compared to the earlier reports (13, 15). The original literature assignments were apparently based on a previous (16), erroneous assignment of C-5 and C-6 in indole, which persisted in the literature for a long time before being corrected (17, 18). The assignments in Table 1 are based on HMOC experiments and are in agreement with those recently published for synthetic rac-5 (19). The fully assigned $^{1}$H-NMR data for 1–5, not reported previously, are shown in Table 2.

Pure indole alkaloids 1–5 were tested in vitro for antileishmanial, antimalarial and cytotoxic activity. The results are shown in Table 3. The leishmanicidal activity was assessed using *L. major* promastigote cultures and compared with that of antimony (V) sodium gluconate (sodium stibogluconate, Pentostam), which is the recommended drug for the treatment of leishmaniasis, but is rather inactive in this in vitro test. The antimalarial activity was assessed with a chloroquine-sensitive strain of *P. falciparum*. Assay for cytotoxicity included growth inhibition of two carcinoma cell lines, a drug sensitive KB-3-1 cell line and a multidrug resistant KB-V1 cell line. The KB-V1 cells display the complete multidrug-resistance phenotype including the expression of the P-170 glycoprotein (9), (20), (21).

Since no previous reports about leishmanicidal activity of yohimbine- and corynantheine-type alkaloids exist, several structurally related alkaloids were included in the test. These include reserpine (6), ajmalicine (7), ajmaline (8), as well as two synthetic, racemic compounds rac-9 and rac-10 (Fig. 2) (14). The leishmanicidal activity of 3–5, 7, rac-9 and rac-10 corresponded to IC$_{50}$ values below 3 μM (Table 3). Interestingly, ajmaline (8) was inactive against *L major* promastigotes, suggesting that the active alkaloids should contain a relatively planar tetracyclic structure.

The antimalarial activity exhibited by the compounds tested was rather low (Table 3). The most active of the alkaloids tested was reserpine (6). However, it should be noted that the three alkaloids with the corynene skeleton 3–5 were significantly more toxic to *P. falciparum* than the yohimbine-type alkaloids 1 and 2. The activity of rac-9 and rac-10 was also higher than that of 1 and 2. There was no difference in the antiparasoidal activity of 3 and rac-3, showing that the eudismic index for dihydrocorynanthe is close to unity. On the other hand, a small difference in the IC$_{50}$ values for the leishmanicidal activity between 3 and rac-3 suggests that the natural, dextrorotatory 3 is somewhat more potent than its enantiomer.

None of the alkaloids isolated from *C pachyeras* exhibited significant cytotoxicity (Table 3). This confirms that the leishmanicidal activity exhibited by these alkaloids is not due to a general antiproliferative effect. The toxicity of 1–5 to the drug-sensitive KB-3-1 cells and the multidrug-resistant KB-V1 cells was identical. The resistance of the KB-V1 cells relative to the KB-3-1 cells is 210-fold for vinblastine and even

<table>
<thead>
<tr>
<th>Table 1</th>
<th>$^{13}$C-NMR data (CDCl$_3$) for <em>C. pachyeras</em> alkaloids.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>C-2</td>
<td>134.6</td>
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<td>C-3</td>
<td>60.4</td>
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<tr>
<td>C-6</td>
<td>21.5</td>
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<tr>
<td>C-7</td>
<td>108.0</td>
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<td>C-8</td>
<td>127.4</td>
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<tr>
<td>C-21</td>
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<tr>
<td>C-22</td>
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<tr>
<td>C-23</td>
<td>172.5</td>
</tr>
<tr>
<td>C-24</td>
<td>61.0</td>
</tr>
</tbody>
</table>

*Not detectable due to exchange-broadening.*
higher for other cytotoxic drugs (9), (10). This indicates that the Corynanthe alkaloids, unlike many other alkaloids including Catharanthus alkaloids, are not substrates for the P-170 efflux pump.

Since pentavalent antimony complexes are the only antileishmanial agents with a clearly favorable therapeutic index, there is a high interest in identification of alternative chemotherapeutic leads. The alkaloids identified in this work as antileishmanial compounds with their IC_{50} values of 1 μM or below (Table 3) belong to the most potent natural products showing leishmanicidal activity in similar in vitro assays (22–26). At present, there is no basis for a conclusion about the mechanism of the leishmanicidal effects of these alkaloids.
Table 3  Antileishmanial, antimalarial and cytotoxic activity of C. pachyceras alkaloids and related compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>L. major IC₅₀ [µM]</th>
<th>P. falciparum IC₅₀ [µM]</th>
<th>KB-3-1 IC₅₀ [µM]</th>
<th>KB-VI IC₅₀ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>corinnamine (1)</td>
<td>23.4 ± 5.4</td>
<td>&gt;200</td>
<td>186 ± 1</td>
<td>214 ± 26</td>
</tr>
<tr>
<td>α-yohimbine (2)</td>
<td>23.8 ± 2.6</td>
<td>&gt;200</td>
<td>200 ± 23</td>
<td>263 ± 12</td>
</tr>
<tr>
<td>dihydrocorinnamine (3)</td>
<td>1.65 ± 0.3</td>
<td>66.4 ± 6.5</td>
<td>161 ± 19</td>
<td>158 ± 15</td>
</tr>
<tr>
<td>corinnamine (4)</td>
<td>1.12 ± 0.4</td>
<td>81.1 ± 1.6</td>
<td>140 ± 11</td>
<td>144 ± 4</td>
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<tr>
<td>corinnamine (5)</td>
<td>2.81 ± 0.4</td>
<td>41.1 ± 2.5</td>
<td>80 ± 8</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>reserpine (6)</td>
<td>16.4 ± 2.3</td>
<td>8.1 ± 0.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ajmalicine (7)</td>
<td>0.57 ± 0.1</td>
<td>&gt;200</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ajmaline (8)</td>
<td>&gt;300</td>
<td>121 ± 9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>rac-9</td>
<td>0.71 ± 0.2</td>
<td>132 ± 1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>rac-10</td>
<td>1.51 ± 0.1</td>
<td>68 ± 12</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>rac-3</td>
<td>2.80 ± 0.43</td>
<td>77.2 ± 4.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pentostam</td>
<td>219 ± 25</td>
<td>–</td>
<td>0.00130 ± 0.00005</td>
<td>–</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>–</td>
<td>–</td>
<td>1.0 ± 0.3</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

Fig. 2 Structures of indole alkaloids tested for leishmanicidal and antiplasmodial activity: 6, reserpine; 7, ajmalicine; 8, ajmaline; 9 and 10, synthetic compounds.

However, it should be noted that ajmalicine (7) is an extremely potent inhibitor of the 2D6 subfamily of cytochrome P450 (27). Whether or not the observed high activity of 7 and the isolated C. pachyceras alkaloids (Table 3) is related to an inhibition of the respiratory chain of L. major has yet to be determined.

Acknowledgements

We are indebted to Mr. Daniel K. Abiw, Department of Botany, University of Ghana, for collection and identification of the plant material used in this work. We also thank Dr. Istvan Toth, School of Pharmacy, University of London, for samples of synthetic 3, 9 and 10.

References


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