Isoneocryptolepine, a Synthetic Indoloquinoline Alkaloid, as an Antiplasmodial Lead Compound

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The antiprotozoal activities of three naturally occurring isomeric indoloquinoline alkaloids, i.e., cryptolepine (**1**), neocryptolepine (**2**), and isocryptolepine (**3**), and two dimeric indoloquinoline alkaloids, cryptoquindoline (**6**) and biscryptolepine (**7**), originally obtained from the plant *Cryptolepis sanguinolenta*, were compared with those of a new synthetic indoloquinoline isomer, isoneocryptolepine (**4**), and a quaternary derivative, *N*-methyl-isocryptolepinium iodide (**5**). The latter compounds showed a high antiplasmodial activity against the chloroquine-resistant *Plasmodium falciparum* strain K1 (IC_{50} of 0.23 ± 0.04 and 0.017 ± 0.004 μM, respectively), while the cytotoxicity (L6 cells) was 4.32 ± 0.04 and 12.7 ± 2.0 μM, respectively. Isoneocryptolepine (**4**) was found to act as an inhibitor of β-hematin formation and as a DNA-intercalating agent.

Malaria is now one of the leading infectious diseases on a global scale, occurring not only in the tropics but also in more temperate regions. It is caused by parasites of the genus *Plasmodium*, with the most serious infections being those due to *Plasmodium falciparum*. Because of the widespread and still increasing resistance against the existing antimalarial drugs, there is a continuing need for new therapeutic agents against malaria.[1] Isolation of new lead compounds from plants is one of the strategies that can be followed in the search for new drugs.[2] *Cryptolepis sanguinolenta* (Lindl.) Schltr. (Asclepiadaceae), a plant used in traditional medicine in West and Central Africa against malaria, has already yielded several isomeric indoloquinoline alkaloids with antiplasmodial properties. The in vitro antiplasmodial activity of cryptolepine (**1**), the major alkaloid, which is an indolo[3,2-*b*]quinoline or a benzo-δ-carboline, has already been reported.[3] Against the multidrug-resistant K1 strain of *P. falciparum* an IC_{50} value of 0.031 μg/mL (or 0.134 μM) was obtained. However, no significant reduction in parasitemia in *P. berghei*-infected mice treated subcutaneously with cryptolepine (7−113 mg/kg body weight daily for 4 days) was observed when compared with untreated controls. Cimanga et al. reported an in vitro IC_{50} value of cryptolepine against the same *P. falciparum* strain (K1) of 0.033 μg/mL (or 0.14 μM), but also in vivo antiplasmodial activity (significant reduction of parasitemia) in the same model when cryptolepine or its hydrochloride were administered orally (dissolved in 2.5% Tween 80) at a dose of 50 mg/kg body weight daily for 4 days.[4] Also Grellier et al. confirmed the antiplasmodial activity of cryptolepine in vivo in a similar 4-day suppressive test in mice infected with *P. vinckei petteri* or *P. berghei* (intraperitoneal treatment with 1.25−10 mg/kg body weight).[5] The negative result obtained by Kirby et al. may have been due to low bioavailability of the test compound, related to the poor water solubility of cryptolepine base. Cryptolepine has been used as a lead compound for synthetic antiplasmodial agents,[6,7] as well as the minor alkaloid neocryptolepine (**2**), being an indolo[2,3-*b*]quinoline or a benzo-α-carboline.[8,9] Although initially neocryptolepine (also referred to as cryptotackieine)[10] was reported to show an activity comparable to cryptolepine (IC_{50} value 0.051 μg/mL or 0.22 μM, K1 strain),[4] more recent investigations have shown that it was about 7 times less active against the chloroquine-resistant *P. falciparum* Ghana-strain.[9] Another minor alkaloid from the same plant showing an indolo[3,2-*c*]quinoline (or a benzo-γ-carboline) structure, which was named isocryptolepine (**3**),[11] but which is also referred to as cryptosanguinolentine,[10] also showed antiplasmodial properties. Isocryptolepine also showed in vitro antiplasmodial activity.[5] Against various strains of chloroquine-sensitive as well as chloroquine-resistant strains of *P. falciparum*, IC_{50} values in the range of 0.2−0.6 μM were observed for cryptolepine and of about 0.8 μM for isocryptolepine.

Because of the considerable interest that has been paid to these antiplasmodial indoloquinolines, we have synthesized the missing isomer in this quartet of indoloquinolines, i.e., the corresponding benzo-β-carboline, being an indolo-[2,3-*c*]quinoline derivative, to compare its biological activities with the naturally occurring isomers. This compound, for which we have adopted the name isoneocryptolepine (**4**), has not been reported from nature.[12] Obviously the comparison of the antiplasmodial activity of the cryptolepine isomers is hampered by the fact that until now only IC_{50} values obtained in different laboratories against different *Plasmodium* strains are available. In the present work the antiprotozoal activity of isoneocryptolepine (**4**) against chloroquine-resistant *P. falciparum* strain K1, *Trypanosoma brucei rhodesiense*, *T. cruzi*, *Leishmania donovani*, and its cytotoxicity on mammalian L6 cells, is reported and compared with the three naturally occurring indoloquinolines **1**−**3**, a synthetic dimethylated indoloquinoline derivative, *N*-methyl-isocryptolepinium iodide (**5**), and two natural dimeric indoloquinoline alkaloids, cryptoquindoline (**6**) and biscryptolepine (**7**).[8,13] The same compounds are also evaluated in some functional assays related to possible mechanisms of action or cytotoxicity reported

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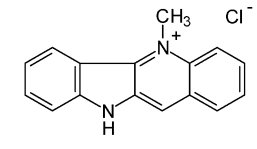
Table 1. Antiprotozoal Activity and Cytotoxicity of Compounds **1**−**7** (IC_{50}, μM)

compound	*Trypanosoma brucei rhod.*	*Trypanosoma cruzi*	*Leishmania donovani*	*Plasmodium falciparum*	cytotoxicity (L6 cells)
1	0.60 ± 0.07	0.22 ± 0.07	2.68 ± 0.89	0.12 ± 0.02	1.12 ± 0.07
2	2.23 ± 0.82	2.01 ± 1.30	49.5 ± 3.7	2.61 ± 0.67	3.24 ± 0.04
3	0.52 ± 0.11	1.27 ± 0.78	39.1 ± 11.5	0.78 ± 0.30	1.19 ± 0.26
4	6.48 ± 0.67	21.0 ± 0.2	75.4 ± 2.4	0.23 ± 0.04	4.32 ± 0.04
5	0.37 ± 0.21	236.10	1.15	0.017 ± 0.004	12.7 ± 2.0
6	96.4 ± 31.0	84.4 ± 35.9	>65	>10	165.4 ± 42.6
7	2.57 ± 0.47	3.48 ± 1.43	8.93	0.27 ± 0.02	13.62
melarsoprol[a]	0.0038 ± 0.0023				
benznidazole[a]		1.50 ± 0.58			
miltefosine[a]			0.56 ± 0.07		
chloroquine[a]				0.17 ± 0.06	
artemisinin[a]				0.042 ± 0.002	
podophyllotoxin[a]					0.018

[a] Positive control.

for cryptolepine and neocryptolepine, i.e., inhibition of β-hematin formation and DNA intercalation.[6,7,9]



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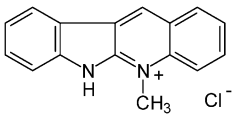

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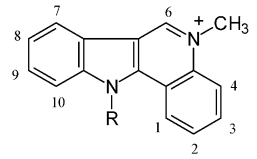
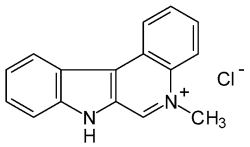


3 R = H (Cl⁻)
5 R = CH_3 (I⁻)



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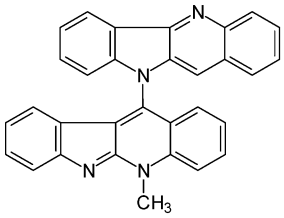

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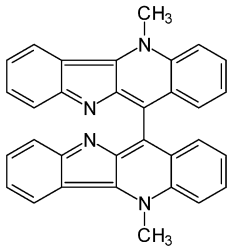

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Results and Discussion

Results of the antiparasitic screening are summarized in Table 1. In the series of the cryptolepine isomers **1**−**4**, cryptolepine itself (**1**) showed the highest antiplasmodial activity; isoneocryptolepine (**4**) was about 2 times less active, isocryptolepine (**3**) about 7 times, and neocryptolepine (**2**) about 20 times. Cryptolepine (**1**) and isocryptolepine (**3**) showed about the same cytotoxicity toward L6 cells (about 1 μM), whereas neocryptolepine (**2**) was about 3 times and isoneocryptolepine (**4**) about 4 times less cytotoxic. Against *Leishmania donovani* (axenic amastigotes) and *T. cruzi*, cryptolepine (**1**) was roughly an order of magnitude more active than neocryptolepine (**2**) or isocryptolepine (**3**), with isoneocryptolepine (**4**) being even less active.

Both dimeric alkaloids, cryptoquindoline (**6**) and biscryptolepine (**7**), were far less active than cryptolepine. With regard to *P. falciparum*, biscryptolepine was more active than neocryptolepine and isocryptolepine, but less active than cryptolepine and isoneocryptolepine. However, it was also less cytotoxic. Nevertheless, comparison of the results obtained for cryptolepine (**1**) and biscryptolepine (**7**) demonstrated that dimerization does not have a positive effect on the biological activity. This is in contrast with chloroquine, where it was observed that bulky bisquinolines inhibited the growth of both chloroquine-sensitive and chloroquine-resistant *Plasmodium* parasites with similar efficacy.[14]

The most antiplasmodially active and most selective compound was the quaternary alkaloid *N*-methyl-isocryptolepinium iodide (**5**): it was active in the nanomolar range and showed a good selectivity index (SI) with regard to the cytotoxicity on L6 cells (SI > 700). All monomethylated indoloquinolines (**1**−**4**) suffered from a lack of selectivity, with the new synthetic isomer isoneocryptolepine (**4**) being the most favorable one (cytotoxicity/antiplasmodial activity SI of about 20). Compounds **4** and **5** were evaluated in vivo in mice infected with *P. berghei*, but failed to show significant in vivo activity. Compound **4** suppressed parasitemia by only 10.3%, and compound **5** by 38.9% (50 mg/kg s.c. for 4 days), whereas chloroquine treatment resulted in a 99.9% reduction of parasitemia. For compound **5** a possible explanation may be that it was not sufficiently absorbed due to its quaternary nature. In a second experiment compound **5** was administered intraperitoneally twice daily at 50 mg/kg. This treatment protocol proved to be toxic, leading to the death of the animals on day 1 or day 2 post-infection.

Two functional assays related to the inhibition of the heme detoxification process in the acid food vacuole of the *Plasmodium* parasite were carried out. Both assays are based upon the inhibition of the in vitro conversion of hemin to β-hematin, a process that corresponds to the in vivo conversion of heme to hemozoin or malaria pigment. Both the original assay developed by Egan et al.[15] and a related assay producing quantitative results, developed by Parapini et al.,[16] were used. Results are summarized in Table 2. Cryptolepine (**1**) was 3 times more active than isoneocryptolepine (**4**); the IC_{50} values for neocryptolepine (**2**), isocryptolepine (**3**), and isoneocryptolepine (**4**) were in the same range, with isocryptolepine being slightly less active. Nevertheless, all four isomers were capable of inhibiting β-hematin formation. In this functional assay, their activity was in the same range as observed for known antimalarials such as chloroquine or quinine, and they can be considered to be antiplasmodial lead compounds. The dimethylated quaternary indoloquinoline **5** failed to show inhibition of β-hematin formation in both assays, in contrast to the isomeric compounds **1**−**4**, indicating that its antiplasmodial activity is due to a different mechanism of action, unknown as yet.

Table 2. Heme-Binding Activity and DNA-Intercalation of Compounds **1**−**5**

test compound	inhibition of β-hematin formation[a]	BHIA IC_{50}[b]	DNA interaction IC_{50}[c]
1	+	1.72 ± 0.36	65 ± 3
2	+	5.97 ± 0.22	93 ± 10
3	+	7.59 ± 0.34	119 ± 25
4	+	5.24 ± 0.23	124 ± 19
5	−	>13[d]	not tested
chloroquine	+	2.56 ± 0.31	not tested
quinine	+	7.40 ± 0.72	>360

[a] + inhibition of β-hematin formation; − no inhibition. [b] IC_{50} represents the molar equivalents of test compounds, relative to hemin, required to inhibit β-hematin formation by 50%. [c] IC_{50} expressed in M. [d] No inhibition observed at the highest test concentration (13 Meq).

The lack of selectivity of compounds **1**−**4**, observed in the in vitro antiprotozoal and cytotoxic evaluation, was confirmed in the functional assays. Cryptolepine (**1**) has already been characterized as a potent DNA-intercalator, topoisomerase II inhibitor, and cytotoxic agent.[17,18] These properties are much less pronounced for neocryptolepine.[19] The antiplasmodial activity of cryptolepine and neocryptolepine has been attributed to a combination of their DNA-intercalating properties and their ability to inhibit the heme detoxification process.[6,7,9,20] Therefore in this work test compounds were evaluated not only as inhibitors of β-hematin formation but also as DNA-interacting agents using the DNA−methyl green assay, a relatively simple assay based upon the displacement of the dye methyl green from a DNA−methyl green complex. All four isomers **1**−**4** showed a pronounced activity in the DNA−methyl green assay, indicative of their DNA-intercalating properties, which is associated with cytotoxicity.

The antiplasmodial activity of compounds **1**−**4** is due to a combination of at least two mechanisms of action. Inhibition of the heme detoxification process is a selective mechanism, whereas DNA-intercalation, a nonselective mechanism, is responsible for the cytotoxicity and probably also for the activity against the other parasites tested. Substituted cryptolepine and neocryptolepine derivatives have already been reported with a higher and/or more selective antiplasmodial activity than the parent compound. In the cryptolepine series, the most potent derivative was 2,7-dibromocryptolepine, the activity of which was confirmed in vivo in mice. Despite a relativaly high in vitro cytotoxicity, no systemic toxicity was observed in the in vivo experiments.[7] In the neocryptolepine series, 2-bromoneocryptolepine showed a higher and more selective antiplasmodial activity than neocryptolepine, in the absence of DNA-intercalating properties and obvious cytotoxicity.[9,20] With this in mind, it may be a promising strategy to prepare substituted derivatives of isocryptolepine and isoneocryptolepine as well, to obtain more active and more selective antiplasmodial agents, which might show in vivo activity.

In conclusion, it can be stated that both isoneocryptolepine (**4**) and *N*-methyl-isocryptolepinium iodide (**5**) showed promising in vitro antiplasmodial properties. Whereas further development of *N*-methyl-isocryptolepinium iodide (**5**) may be hampered by its quaternary nature and its lack of in vivo activity, isoneocryptolepine (**4**) can be considered as a new synthetic antiplasmodial lead compound, in addition to the existing naturally occurring indoloquinoline alkaloids cryptolepine, neocryptolepine, and isocryptolepine.

Experimental Section

General Experimental Procedures. Melting points were determined on a Büchi apparatus and are uncorrected. NMR spectra were recorded in DMSO-d_6 (99.9 atom % D) at 30 °C on a Bruker DRX-400 instrument operating at 400 MHz for ^{1}H. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane using the solvent signal (δ_H 2.49 ppm, δ_C 39.50 ppm) as the internal standard. ^{1}H, ^{13}C NMR, DEPT-135, and DEPT-90 (to determine carbon multiplicities) spectra were recorded, as well as two-dimensional NMR spectra: COSY (^{1}H−^{1}H correlations), HSQC (one-bond ^{1}H−^{13}C correlations), and HMBC (long-range ^{1}H−^{13}C correlations, optimized for a long-range coupling constant of 8.3 Hz). Two-dimensional NMR experiments were carried out using pulsed field gradients, and for all NMR experiments standard Bruker software was used. Accurate mass data were acquired on a quadrupole-time-of-flight mass spectrometer (Q-Tof-II, Micromass), equipped with a standard electrospray ionization (ESI) interface.

Test Compounds. Cryptolepine (**1**), cryptoquindoline (**6**), and biscryptolepine (**7**) were isolated from *Cryptolepis sanguinolenta*, as described before.[8,21] Neocryptolepine (**2**), isocryptolepine (**3**), and isoneocryptolepine (**4**) were obtained by organic synthesis.[9,12,22] All compounds except **5** were evaluated as their hydrochloride salts. *N*-Methyl-isocryptolepinium iodide (**5**) was obtained from 11*H*-indolo[3,2-*c*]quinoline via a double methylation reaction using a large excess of MeI reagent in the presence of carbonate base (T. Jonckers et al., unpublished data).

***N*-Methyl-isocryptolepinium iodide (5):** mp > 300 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.15 (1H, s, H-6), 9.05 (1H, dd, J = 8.5, 1.0 Hz, H-1), 8.46 (1H, br d, J = 8.5 Hz, H-4), 8.35 (1H, br d, J = 7.7 Hz, H-7), 8.18 (1H, ddd, J = 8.5, 7.1, 1.0 Hz, H-3), 8.06 (1H, br dd, J = 8.5, 7.1 Hz, H-2), 8.03 (1H, br d, J = 7.2 Hz, H-10), 7.73 (1H, ddd, J = 8.2, 7.2, 0.9 Hz, H-9), 7.57 (1H, br dd, J = 8.2, 7.7 Hz, H-8), 4.53 (3H, s, N-5 methyl), 4.48 (3H, s, N-11 methyl); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 143.2 (CH, C-6), 142.3 (C, C-11a), 141.9 (C, C-10a), 136.6 (C, C-4a), 132.3 (CH, C-3), 128.44 (CH, C-2), 128.36 (CH, C-9), 125.3 (CH, C-1), 123.9 (CH, C-8), 121.0 (C, C-6b), 120.6 (CH, C-7), 119.7 (CH, C-4), 117.2 (C, C-11b), 113.7 (C, C-6a), 112.0 (C, C-10), 44.8 (CH_3, N-5 methyl), 34.4 (CH_3, N-11 methyl); HRESIMS *m/z* 247.1230 (calcd for $C_{17}H_{15}N_2^+$, 247.1235).

Antiprotozoal Evaluation and Cytotoxicity. Antiplasmodial activity was determined against the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine), using a modified [^{3}H]-hypoxanthine incorporation assay as described before.[23] Chloroquine and artemisinin were used as positive controls. Activity against *Trypanosoma brucei rhodesiense*, *T. cruzi*, and *Leishmania donovani* and cytotoxicity against rat skeletal myoblasts (L-6 cells) were evaluated as described before.[23] Melarsoprol was used as positive control against *T. b. rhodesiense*, benznidazole against *T. cruzi*, miltefosine against *L. donovani*, and podophyllotoxin for cytotoxicity. Results are expressed as IC_{50} in μM ± standard deviation (SD) (n = 3).

In vivo antiplasmodial activity was evaluated in the *Plasmodium berghei* (ANKA S) mouse model in female NMRI mice. Animals were infected on day 0 and treated at 50 mg/kg s.c. for 4 days (days 0, 1, 2, and 3). Parasitemia was determined 24 h after the last treatment and expressed as percent reduction of parasitaemia versus the untreated control. In a second experiment the test compound was administered intraperitoneally twice a day at the same dose for 4 days. Chloroquine (1 × 10 mg/kg) was used as positive control.

Inhibition of β-Hematin Formation. An in vitro method to measure the inhibition of β-hematin formation, based on the original method described by Egan et al.[15] and modified by Wright et al.,[7] was used, as described before.[20] Parapini et al. developed a microassay [β-hematin inhibitory activity assay (BHIA)] to investigate quantitatively the ability of compounds to inhibit β-hematin formation, and it was implemented as described before.[16,20] IC_{50} values were calculated, representing

the molar equivalents (Meq) of test compound, relative to hemin, required to inhibit β-hematin formation by 50%. Results are expressed as the mean ± SD from three independent experiments. Chloroquine and quinine were used as positive controls.

DNA−Methyl Green Assay. Agents that displace methyl green from a DNA−methyl green complex were detected spectrophotometrically (Labsystems Multiscan MCC/340) by a decrease in absorbance at 620 nm.[24,25] IC_{50} values represent the concentration of test compound leading to a 50% decrease of absorbance, corresponding to 50% displacement of dye from the DNA complex. IC_{50} values were calculated from three independent experiments in triplicate (mean ± SD). Quinine was used as negative control and cryptolepine (**1**) as positive control.[20]

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