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In vitro inhibition of liver forms of the rodent malaria parasite *Plasmodium berghei* by naphthylisoquinoline alkaloids – structure-activity relationships of dioncophyllines A and C and ancistrocladine

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Abstract Naphthylisoquinoline alkaloids are derived from Dioncophyllaceae and Ancistrocladaceae species and comprise a new class of promising antimalarials with a demonstrated potential against asexual erythrocytic *Plasmodium falciparum* and *P. berghei* stages in vitro. We report herein the pronounced activity of pure naphthylisoquinoline alkaloids against exoerythrocytic malaria parasites. *P. berghei*-infected human hepatoma cells (Hep G2) were incubated with culture medium containing selected alkaloids at 10 μg/ml. The most active compounds, showing inhibitory activity of more than 40%, were dioncophylline A (compound **1**), dioncophyllacine A (compound **6**), and ancistrobarterine A (compound **12**). For structure-activity investigations of dioncophyllines A (compound **1**) and C (compound **3**) and ancistrocladine (compound **7**) a selection of their analogs from natural or synthetic sources was examined. Dioncophylleine A (compound **16**), 5′-*O*-demethyl-8-*O*-methyl-7-*epi*-dioncophylline A (compound **17**), *N*-formyl-8-*O*-methyl-dioncophylline C (compound **21**), and *N*-formyl-8-*O*-benzoyldioncophylline C (compound **24**) were found to display high levels of activity as well, although the former two compounds caused damage to the host-cell monolayers. As naphthylisoquinoline alkaloids are also highly active against blood forms of *Plasmodium* spp., they should be regarded as lead compounds for further development as drugs against erythrocytic and exoerythrocytic stages of *Plasmodium* spp.

Introduction

Shortly after a vertebrate host has been bitten by a *Plasmodium* sp.-infected mosquito of the genus *Anopheles*, mobile sporozoites penetrate the hepatocytes and develop into mature exoerythrocytic forms prior to the onset of the erythrocytic cycle (Wyler 1993). Malaria liver forms are not responsible for any of the clinical symptoms and, hence, most of the attention is focused on the chemotherapy of blood forms (Peters 1993). However, the elimination of exoerythrocytic stages remains important since several species, such as *P. vivax* and *P. ovale*, develop hypnozoites, leading to relapses that often occur a long time after the infective bite (Karbwang and Harinasuta 1992; Karbwang and Na Bangchang 1992). The list of effective and widely applied drugs against the liver forms is limited and its main representative is primaquine, an 8-aminoquinoline (World Health Organization 1986; McChesney et al. 1987).

In vivo assessment of causal prophylactic activity is complex, since possible residual drug action against emerging erythrocytic forms has to be taken into account (Gregory and Peters 1970; Peters 1975). The activity of experimental drugs against exoerythrocytic forms can best be examined in vitro (Landau et al. 1987) by use of monolayers of human hepatoma cells infected with *P. berghei* sporozoites (Hollingdale et al. 1983a; François et al. 1991; Davies et al. 1993). Developing intracellular schizonts can be revealed by classic Giemsa-staining methods or by indirect immunofluorescence antibody assays (Hollingdale et al. 1983b), which allow the detection of younger liver stages as well.

Species belonging to the Dioncophyllaceae and the Ancistrocladaceae, two tropical plant families, have

This work represents part 8 of the series, "Antiprotozoal activity of naphthylisoquinoline alkaloids" (for part 7, see Bringmann et al. 1996a), and part 85 of the series, "Acetogenic isoquinoline alkaloids" (for part 84, see Bringmann 1996)

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traditionally been used against several tropical diseases. For example, *Dioncophyllum thollonii* has been described for its activity against leprous skin diseases (Lavault and Bruneton 1980); *Ancistrocladus abbreviatus*, for its activity against measles and fever (Iwu 1993); and *A. tectorius*, for its activity against dysentery and malaria (Ruangrungsi et al. 1985). All these species contain representatives of the naphthylisoquinoline alkaloids, a structurally and biosynthetically remarkable young class of natural products. Their naphthalene and isoquinoline moieties are linked by a so-called biaryl axis, giving rise to the phenomenon of rotational isomerism and, thus, to axial chirality (Bringmann and Pokorny 1995).

Naphthylisoquinoline alkaloids display a wide range of biological activity, such as fungicidal (Bringmann et al. 1992a), molluscicidal (Bringmann et al. 1996b), larvicidal (François et al. 1996b), insect antifeedant and growth-retarding (Bringmann et al. 1992b), and anti-human immunodeficiency virus (Boyd et al. 1994) activity. Interactions with herbal parasites have been described as well (Bringmann and Pokorny 1995). One of the most promising properties of this intriguing class of natural products is their potential against asexual erythrocytic forms of human (*P. falciparum*; François et al. 1994a,b, 1996a; Boyd et al. 1995) and rodent (*P. berghei*; François et al. 1994b, 1995, 1996a; Boyd et al. 1995) malaria species in vitro.

Recently, pronounced exoerythrocytic schizonticidal effects of naphthylisoquinoline alkaloid-containing extracts from several Dioncophyllaceae and Ancistrocladaceae species have been demonstrated in vitro (François et al. 1997). Therefore, it seemed worthwhile to investigate the active principles. In this paper we present the activity of a selection of purified

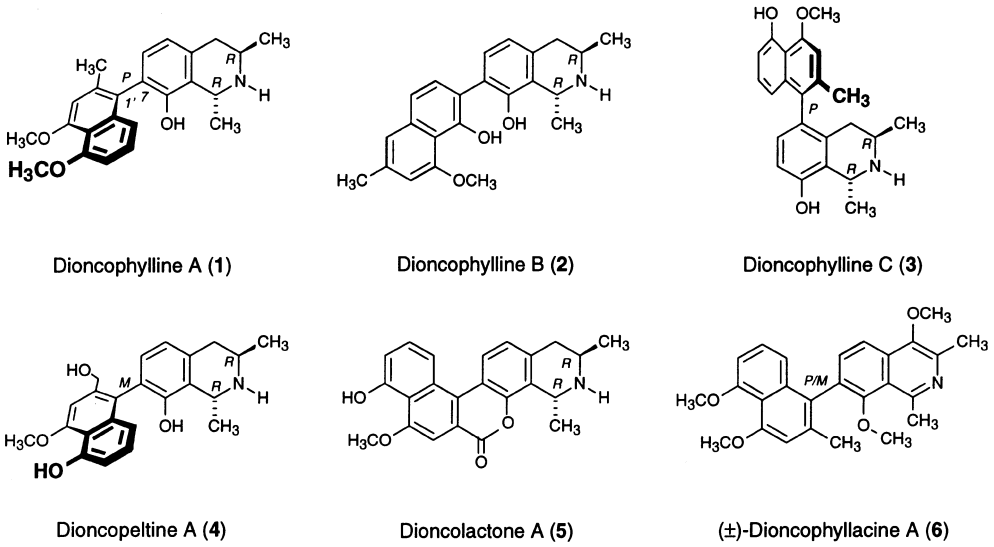


Fig. 1 Structures of selected naturally occurring naphthylisoquinoline alkaloids from *Triphyophyllum peltatum* (Dioncophyllaceae)

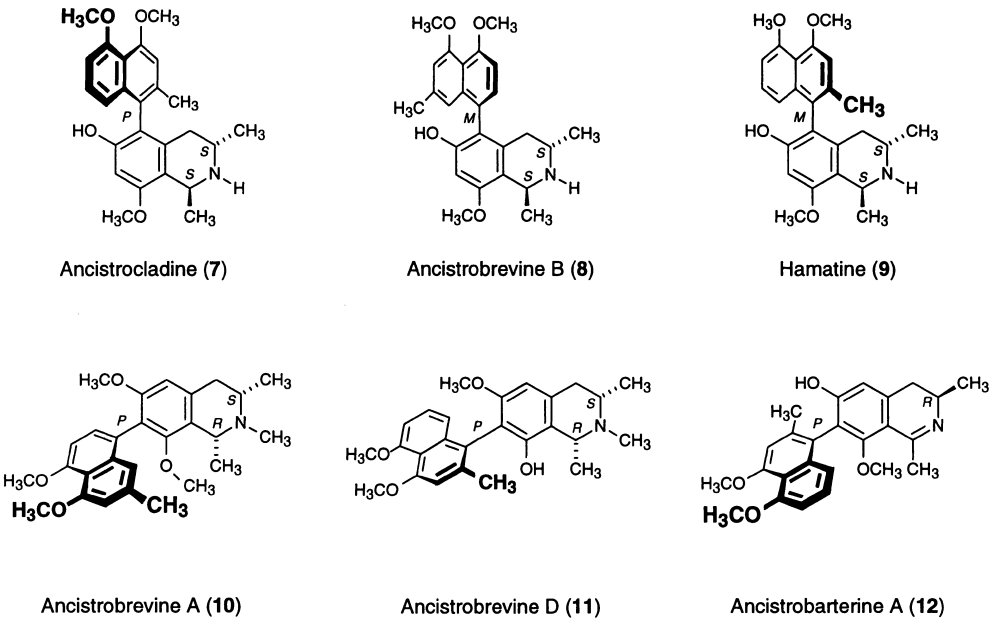


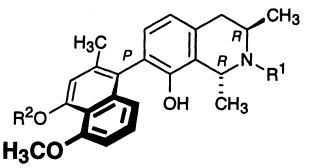
Fig. 2 Structures of selected naturally occurring naphthylisoquinoline alkaloids from *Ancistrocladus* spp. (Ancistrocladaceae)

naphthylisoquinoline alkaloids against developing *P. berghei* liver stages in human hepatoma cells.

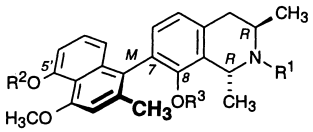
Materials and methods

Alkaloids

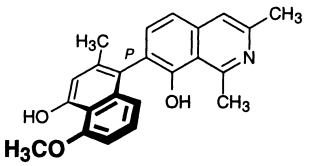
The following naturally occurring alkaloids (Figs. 1–3) were isolated and characterized as described previously: dioncophylline A (compound **1**; Bringmann et al. 1990), dioncophylline B (compound **2**; Bringmann et al. 1991c), dioncophylline C (compound **3**; Bringmann et al. 1992d), dioncopeltine A (compound **4**; Bringmann et al. 1991d), dioncolactone A (compound **5**; Bringmann et al. 1991d), (±)-dioncophyllacine A (compound **6**; Bringmann et al. 1991b, 1992c), ancistrocladine (compound **7**; Bringmann et al. 1992f), ancistrobrevine B (compound **8**; Bringmann et al. 1992f), hamatine (compound **9**; Bringmann et al. 1992f), ancistrobrevine A (compound **10**; Bringmann et al., unpublished observations), ancistrobrevine D (compound **11**; Bringmann et al. 1992e), ancistrobarterine A (compound **12**; Bringmann et al. 1993), 7-*epi*-dioncophylline A (compound **13**; Bringmann and Pokorny 1995), *N*-methyldioncophylline A (a 1:1 mixture of atropisomers **14** and **15**; Bringmann et al. 1991a), and 5′-*O*-demethyl-8-*O*-methyl-7-*epi*-dioncophylline A (compound **17**; Bringmann and Pokorny 1995).



	R^1	R^2	name
1	H	CH_3	Dioncophylline A
14	CH_3	CH_3	*N*-Methyldioncophylline A



	R^1	R^2	R^3	name
13	H	CH_3	H	7-*epi*-Dioncophylline A
15	CH_3	CH_3	H	7-*epi*-*N*-Methyldioncophylline A
17	H	H	CH_3	5′-*O*-Demethyl-8-*O*-methyl-7-*epi*-dioncophylline A



Dioncophylleine A (**16**)

Fig. 3 Structures of dioncophylline A (compound **1**) and selected derivatives from natural and synthetic sources

Synthetic derivatives of naphthylisoquinoline alkaloids (compounds **18–28**; Figs. 4, 5) were obtained using established, highly selective derivatizing reactions. Dioncophylleine A (compound **16**), a derivative of compound **1**, was synthesized as described by Fleischhauer et al. (1993).

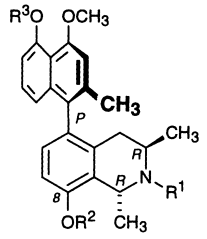
All alkaloids were first dissolved in dimethylsulfoxide (DMSO, Merck) at a concentration of 1 mg/20 µl and diluted with physiological saline until a concentration of 500 µg/ml was reached. This stock solution was further diluted with complete MEM Rega 3 medium (GibcoBRL; final concentration 10 µg/ml).

Mosquitoes

Anopheles stephensi mosquitoes were reared in the insectary of the Prins Leopold Instituut voor Tropische Geneeskunde, Antwerpen (François et al. 1991). Their full development was completed in about 3 weeks under standard conditions of 25 °C, 85% relative air humidity, and a cycle of 12 h illumination (Sylvania Gro-Lux Lamps, 36 W) and 12 h darkness. Younger larvae were fed with a suspension of algae (*Chlorella emersonii*) and 10% baker's yeast. The more developed stages received a mixture of cereals (Bambix, Nutricia), wheat germ (Supergermes, Gayelord Hauser), and vitamins supplemented with minerals (Supradyn, Roche).

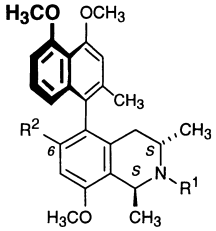
Plasmodium berghei sporozoites

Adult mosquitoes were fed with blood from mechanically infected (*P. berghei*, Anka strain) OF1 mice, after which the females were separated from the males. The cages (30 cm^3) with mosquitoes were kept at 21 °C and a batch of insects (80–120 female mosquitoes per experiment) was aseptically dissected at 21–28 days after the feeding. The salivary glands were suspended in a minimal volume of RPMI 1640 medium (GibcoBRL), cautiously disrupted in a glass tissue grinder, and further diluted with RPMI (final concentration 10,000 sporozoites/50 µl). Each suspension was kept at 4 °C prior to the experiment.



	R^1	R^2	R^3
3	H	H	H
18	CH_3	H	H
19	CHO	H	H
20	H	CH_3	H
21	CHO	CH_3	H
22	CHO	$COCH_3$	H
23	CHO	CO*t*Bu	H
24	CHO	COPh	H
25	CHO	CH_3	CH_3

Fig. 4 Structures of dioncophylline C (compound **3**) and selected synthetic derivatives



	R^1	R^2
7	H	OH
26	CH_3	OH
27	CHO	OH
28	CH_3	H

Fig. 5 Structures of ancistrocladine (compound **7**) and selected synthetic derivatives

Hepatoma cells

Continuous cultures of a human hepatoma cell line (Hep G2; Aden et al. 1979) were grown in 25-cm^2 sterile culture flasks (Nunc) containing 5 ml MEM Rega 3 (GibcoBRL), 10% heat-inactivated fetal calf serum (FCS; Bio-Lab), 2 m*M* L-glutamine (GibcoBRL), 100 U penicillin/ml (Sigma), 100 U streptomycin/ml (Sigma), and 10 µg fungizone/ml (GibcoBRL). The monolayers were subinoculated (1:5) at near confluence and were allowed to grow in an incubator (5% CO_2, LEEC GA3N) at 37 °C and a relative humidity of 99% (François et al. 1993).

P. berghei exoerythrocytic stages

Hep G2 cells were subinoculated (1:4) in sterile chambers (Lab-Tek Chamber Slides, Nunc, 8 chambers/slide) in 200 µl complete MEM Rega 3 and were incubated at 37 °C in a CO_2 incubator. After 4 days the supernatant was removed and the monolayers were washed. Subsequently the cells were infected with *P. berghei* (Hollingdale et al. 1983a; Aikawa et al. 1984). To each chamber, 50 µl (10,000 sporozoites) of the sporozoite suspension was added. The cells were incubated for a further 2 h at 37 °C in the CO_2-containing incubator (Zavala et al. 1985). The supernatant was removed and the cells were washed three times with MEM Rega 3. Control chambers then received 200 µl complete MEM Rega 3, whereas the experimental chambers received 200 µl complete MEM Rega 3 containing a 10-µg/ml concentration of the corresponding alkaloid. The cells were further incubated for 66 h in 5% CO_2.

Immunofluorescence antibody test

A polyclonal antiserum was obtained from 6-week-old OF1 mice (Iffa Credo), that had been immunized i.p. with 10^6 *P. berghei* (Anka, chronic line) erythrocytic forms at day 0. At day 24 the blood was taken by heart puncture and stored at 4 °C for 24 h. The supernatant was taken and stored at –24 °C. Sheep anti-mouse IgG (heavy plus light chains) conjugated with fluorescein isothiocyanate (FITC, Institut Pasteur) was diluted (1:100) in phosphate-buffered saline (PBS, pH 7.2–7.4) containing 0.01% Evans' blue.

After 66 h of incubation, *P. berghei*-infected Hep G2 cells were fixed with methanol (Merck) and washed three times with PBS. The plastic chambers were removed from the slides and 50 µl antiserum was added to each remaining well (0.81 mm^2). After 30 min the slides were washed two times in PBS. Then the cells were incubated for 30 min with conjugate at 50 µl/well and were washed two times with PBS. Finally, the slides were observed under a fluorescence microscope (Leitz 470, 500×).

Statistical analysis

The schizonts developing per chamber were counted and logarithmically transformed (Lison 1968) to facilitate the interpretation of the growth inhibition caused by the alkaloids. The average logarithms derived from four experiments and the corresponding 95% confidence intervals were calculated, and the degree of significance of the differences from the control values was determined with the aid of one-way analysis of variance (Statgraphics, Graphic Software Systems).

Results

The inhibitory activities of a series of naturally occurring naphthylisoquinoline alkaloids from *Triphyophyllum peltatum* (Dioncophyllaceae) are presented in Table 1. Among these compounds, especially dioncophylline A (compound **1**) and dioncophyllacine A (compound **6**) displayed excellent activity (logarithmic values 0.81 and 0.91, or 67.5% and 41.9% inhibition, respectively).

In Table 2 the growth-inhibitory activity of a range of naturally occurring naphthylisoquinoline alkaloids isolated from *Ancistrocladus* spp. (Ancistrocladaceae) are given. Whereas most of them were not active at all, only ancistrobarterine A (compound **12**) showed strong inhibitory effects (logarithmic value 0.85, or 51.6% inhibition).

In a first attempt to elucidate the structure-activity relationships of dioncophylline A (compound **1**), thus far the most active alkaloid (Table 1), a selection of structurally related alkaloids (Table 3) was examined. Besides the lead compound itself, dioncophylleine A (compound **16**) and 5′-*O*-demethyl-8-*O*-methyl-7-*epi*-dioncophylline A (compound **17**) effected highly significant inhibitory activity (logarithmic values 0.88 and 0.86, or 48.8% and 56.1% inhibition, respectively). The latter compound, however, apparently damaged the hepatoma-cell monolayer.

Although dioncophylline C (compound **3**) was among the inactive naphthylisoquinoline alkaloids derived from Dioncophyllaceae species (Table 1), it seemed reasonable to investigate a broad series of its derivatives, since dioncophylline C (compound **3**) has shown a high level of in vitro activity against blood forms of *Plasmodium* spp. (François et al. 1994a,b, 1995, 1996a; Boyd et al. 1995), and the derivatives **18–25** were available from these former investigations. The corresponding levels of activity against exoerythrocytic stages of *P. berghei* are given in Table 4. Two derivatives, *N*-formyl-8-*O*-methyldioncophylline C (compound **21**) and *N*-formyl-8-*O*-benzoyldioncophylline C (compound **24**), inhibited the growth of the liver stages significantly

Table 1 Inhibitory activity of selected naturally occurring naphthylisoquinoline alkaloids from *Triphyophyllum peltatum* (Dioncophyllaceae) against the development of exoerythrocytic stages of the *Plasmodium berghei* Anka strain in Hep G2 cells (*NS* Not significant)

Naphthylisoquinoline alkaloid (10 µg/ml)	Log number of schizonts[a]	Significance level (P)[b]
Dioncophylline A (**1**)	0.81 (0.78–0.84)	< 0.01
Dioncophylline B (**2**)	1.00 (0.99–1.01)	NS
Dioncophylline C (**3**)	1.02 (1.00–1.04)	NS
Dioncopeltine A (**4**)	1.00 (0.94–1.05)	NS
Dioncolactone (**5**)	0.94 (0.84–1.05)	NS
Dioncophyllacine A (**6**)	0.91 (0.86–0.95)	< 0.05

[a] Relative to the logarithm of the control values. Data represent mean values ($n = 4$) and 95% confidence intervals

[b] Calculated with the aid of one-way analysis of variance

Table 2 Inhibitory activity of selected naturally occurring naphthylisoquinoline alkaloids isolated from *Ancistrocladus* spp. (Ancistrocladaceae) against the development of exoerythrocytic stages of the *P. berghei* Anka strain in Hep G2 cells (*NS* Not significant)

Naphthylisoquinoline alkaloid (10 μg/ml)	Log number of schizonts[a]	Significance level (P)[b]
Ancistrocladine (**7**)	0.99 (0.98–1.00)	NS
Ancistrobrevine B (**8**)	1.00 (0.88–1.13)	NS
Hamatine (**9**)/Ancistrocladine (**7**)	1.00 (0.98–1.02)	NS
Ancistrobrevine A (**10**)	0.90 (0.73–1.08)	NS
Ancistrobrevine D (**11**)	1.00 (0.94–1.06)	NS
Ancistrobarterine A (**12**)	0.85 (0.78–0.92)	<0.01

[a] Relative to the logarithm of the control values. Data represent mean values ($n = 4$) and 95% confidence intervals
[b] Calculated with the aid of one-way analysis of variance

Table 3 Structure-activity relationships of dioncophylline A: inhibitory activity against the development of exoerythrocytic stages of the *P. berghei* Anka strain in Hep G2 cells (*NS* Not significant)

Naphthylisoquinoline alkaloid (10 μg/ml)	Log number of schizonts[a]	Significance level (P)[b]
Dioncophylline A (**1**)	0.81 (0.78–0.84)	<0.01
7-*epi*-Dioncophylline A (**13**)	0.98 (0.95–1.01)	NS
N-Methyl-dioncophylline A (**14**)	1.00 (0.99–1.01)	NS
N-Methyl-dioncophylline A (**14**, **15**) (1:1 mixture of atropisomers)	1.03 (0.97–1.08)	NS
Dioncophylleine A (**16**)	0.88 (0.82–0.94)	<0.01
5′-*O*-Demethyl-8-*O*-methyl-7-*epi*-dioncophylline A (**17**)[c]	0.86 (0.79–0.92)	<0.01

[a] Relative to the logarithm of the control values. Data represent mean values ($n = 4$) and 95% confidence intervals

Table 4 Structure-activity relationships of dioncophylline C: inhibitory activity against the development of exoerythrocytic stages of the *P. berghei* Anka strain in Hep G2 cells (*NS* Not significant)

Naphthylisoquinoline alkaloid (10 μg/ml)	Log number of schizonts[a]	Significance level (P)[b]
Dioncophylline C (**3**)	1.02 (1.00–1.04)	NS
N-Methyl-dioncophylline C (**18**)	0.97 (0.92–1.03)	NS
N-Formyl-dioncophylline C (**19**)	0.99 (0.92–1.05)	NS
8-*O*-Methyl-dioncophylline C (**20**)	0.96 (0.90–1.02)	NS
N-Formyl-8-*O*-methyl-dioncophylline C (**21**)[c]	0.27 (0.15–0.39)[c]	<0.01
N-Formyl-8-*O*-acetyl-dioncophylline C (**22**)	0.93 (0.80–1.06)	NS
N-Formyl-8-*O*-pivalyl-dioncophylline C (**23**)	0.96 (0.92–1.00)	NS
N-Formyl-8-*O*-benzoyl-dioncophylline C (**24**)	0.86 (0.66–1.05)	<0.05
N-Formyl-*O*,*O*-dimethyl-dioncophylline C (**25**)	0.98 (0.92–1.03)	NS

[a] Relative to the logarithm of the control values. Data represent mean values ($n = 4$) and 95% confidence intervals
[b] Calculated with the aid of one-way analysis of variance
[c] Monolayer damaged

Table 5 Structure-activity relationships of ancistrocladine: inhibitory activity against the development of exoerythrocytic stages of the *P. berghei* Anka strain in Hep G2 cells (*NS* Not significant)

Naphthylisoquinoline alkaloid (10 μg/ml)	Log number of schizonts[a]	Significance level (P)[b]
Ancistrocladine (**7**)	0.99 (0.98–1.00)	NS
N-Formyl-ancistrocladine (**26**)	0.97 (0.94–1.00)	NS
N-Methyl-ancistrocladine (**27**)	0.98 (0.96–0.99)	NS
6-Deoxy-*N*-methyl-ancistrocladine (**28**)	0.97 (0.94–1.01)	NS

[a] Relative to the logarithm of the control values. Data represent mean values ($n = 4$) and 95% confidence intervals
[b] Calculated with the aid of one-way analysis of variance

(logarithmic values 0.27 and 0.86, or 98.3% and 52.7% inhibition, respectively), even though the first compound damaged the host cells.

Encouraged by the dramatic increase obtained in the activity of dioncophylline C (compound **3**) via derivatization, we likewise tried to improve the activity of the readily available ancistrocladine (compound **7**) by chemical modification. None of the investigated derivatives (Table 5) showed significant activity against *P. berghei* exoerythrocytic stages.

Discussion

In former investigations (François et al. 1997) we have demonstrated the high potential of naphthylisoquinoline

alkaloid-containing extracts from species belonging to the Dioncophyllaceae and the Ancistrocladaceae against exoerythrocytic forms of *Plasmodium berghei*. Especially the extracts from *Triphyophyllum peltatum* were shown to possess high levels of growth-inhibitory activity. This could be explained by the predominant presence of dioncophylline A (compound **1**) and dioncophyllacine A (compound **6**), two very active constituents (Table 1) of these extracts. These alkaloids do not cause any visible damage to the monolayers and display higher levels of activity than, e.g., primaquine within the same test system. This reference drug was active (62.1%) only at 25 μg/ml (François et al. 1997).

Interestingly, parallel to our findings concerning the blood schizonticidal activity of the naphthylisoquinolines (François et al. 1994a, 1995, 1996a), most alkaloids isolated from *Ancistrocladus* spp. were inactive (Table 2). Only ancistrobarterine A (compound **12**) was a potent inhibitor of *Plasmodium* exoerythrocytic forms. Ancistrobarterine A (compound **12**) is structurally closely related to the ''Dioncophyllaceae'' alkaloids; thus, it might be interesting to investigate further the relationship between their common features and their remarkable activity against *P. berghei* liver stages. It would seem reasonable to assume that a dioncophylline A (compound **1**)-like biaryl-coupling type (i.e., with the axis between C-7 and C-1') would favorably influence the activity.

On the basis of the results of these investigations a series of derivatives of three selected alkaloids was evaluated in the same test system. Dioncophylline A (compound **16**) and 5′-*O*-demethyl-8-*O*-methyl-7-*epi*-dioncophylline A (compound **17**), two structurally related derivatives of dioncophylline A (compound **1**), thus far the most interesting lead compound, displayed levels of activity in the same range as that of the original compound (Table 3). In the case of dioncophylline C (compound **3**), however, two derivatives, *N*-formyl-8-*O*-methyldioncophylline C (compound **21**) and *N*-formyl-8-*O*-benzoyldioncophylline C (compound **24**), were shown to exceed dramatically the effect of the parent compound (Table 4), although compound **21** was toxic to the host cells. Finally, similar to ancistrocladine (compound **7**) itself, none of its derivatives was active (Table 5). The examples illustrate the possibility of optimizing the activity of naphthylisoquinoline alkaloids significantly in certain cases, starting not only from highly active lead compounds but also from inactive parent alkaloids. This strongly underlines the potential of structure-activity investigations of naphthylisoquinoline alkaloids as inhibitors of the development of *P. berghei* liver forms.

Preliminary experiments wherein single oral doses (50–200 mg/kg) of a number of naphthylisoquinoline alkaloids were given to female outbred OF1 mice revealed absolutely no sign of acute or subchronic in vivo toxicity (François et al. 1996b).

The selected naphthylisoquinoline alkaloids are promising lead compounds for further development as drugs against exoerythrocytic stages of *Plasmodium* spp. The broad possibilities of structural variation within this group and the knowledge that derivatization potentially leads to increased levels of activity warrant the continued search for related structures with even higher levels of activity and their development as causal prophylactic antimalarials.

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