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Extract screening by HPLC coupled to MS–MS, NMR, and CD: a dimeric and three monomeric naphthylisoquinoline alkaloids from *Ancistrocladus griffithii*☆

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In memoriam Prof. Tuticorin R. Govindachari, an early pioneer in the field of naphthylisoquinoline alkaloids

Abstract

Three new monomeric naphthylisoquinoline alkaloids, ancistrogriffines A, B, and C, and the first dimer of a 7,8′-coupled naphthylisoquinoline, ancistrogriffithine A, have been detected by phytochemical online screening of plant extracts of *Ancistrocladus griffithii*, using the analytical 'triad' HPLC-MS/MS, HPLC-NMR, and HPLC-CD. Ancistrogriffithine A, as well as ancistrogriffines A and C, were structurally completely assigned (including the absolute configuration) right from the extract, without previous isolation. Furthermore, two related, but known alkaloids, ancistrocladine and hamatine, were identified. Except for ancistrogriffine B, which occurs in trace quantities only, all new alkaloids were then isolated preparatively and the initial assignments were fully confirmed by conventional offline methods. Of particular interest is the constitutionally and configurationally unprecedented structure of ancistrogriffithine A, which is simultaneously the first dimeric naphthylisoquinoline alkaloid from an Asian *Ancistrocladus* species. Ancistrogriffithine A and ancistrogriffine A are active against *Plasmodium falciparum*. Furthermore, the latter compound shows good activity against *Leishmania donovani*. The results demonstrate the ability of modern online methods like HPLC-NMR, -MS/MS, and -CD to serve as powerful tools for the reliable structural elucidation of even complex structures of trace compounds in crude biological matrices.

Keywords: *Ancistrocladus griffithii*; Ancistrocladaceae; Ancistrogriffithine A; Ancistrogriffine A; Ancistrogriffine B; Ancistrogriffine C; Hyphenated analysis; Online structure elucidation; Isolation; Antimalarial activity

1. Introduction

Plants of the family Ancistrocladaceae (Bringmann et al., 1991; Gereau, 1997), which are indigenous to the tropical forests in Africa and Asia, are rewarding targets for phytochemical investigations: They are, together with the closely related Dioncophyllaceae, rich producers of naphthylisoquinoline alkaloids, a remarkable class of structurally diverse (Bringmann and Pokorny, 1995; Bringmann et al., 1998a), biosynthetically unprecedented (Bringmann et al., 2000b), and biologically active (Bringmann et al., 1998a; Hallock et al., 1998; Bringmann and Feineis, 2000; François et al., 1997, 1999; Boyd et al., 1994) secondary metabolites. In recent years, one of the West African *Ancistrocladus* species, *A. korupensis*, has received particular attention since it proved to be the as yet only known source of dimeric naphthylisoquinolines (Boyd et al., 1994; Hallock et al., 1997, 1998), among them michellamine B (**1**) (Boyd et al., 1994; Bringmann et al., 1993), both molecular halves of which are 5,8′-coupled (Fig. 1), but also including constitutionally unsymmetric, 'mixed' dimers like korundamine A (**2**), which consists of a 5,8′-coupled naphthylisoquinoline part and a 7,8′-coupled moiety (Hallock et al., 1998). Of the six dimeric naphthylisoquinoline alkaloids that have so far been identified in *A. korupensis* (Boyd et al., 1994; Hallock et al., 1997, 1998)

☆ Part 148 of the series "Acetogenic Isoquinoline Alkaloids". For part 147, see Bringmann et al. (2001a).

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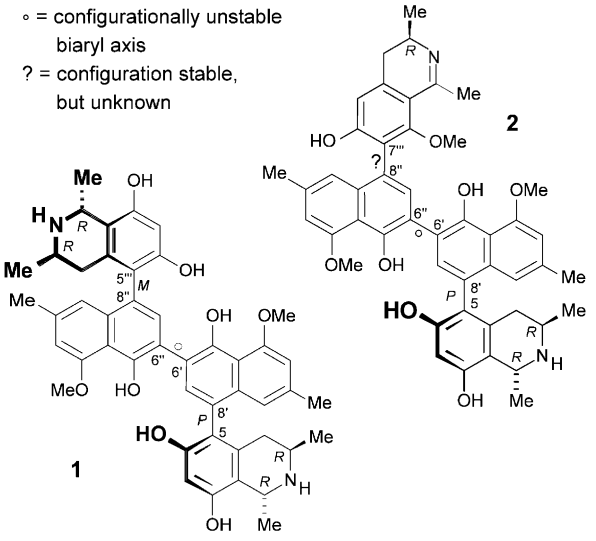

Fig. 1. Typical dimeric naphthylisoquinoline alkaloids from *A. korupensis*: michellamine B (**1**) and the constitutionally unsymmetric korundamine A (**2**).

especially michellamine B (**1**) has been in the focus of numerous investigations (De Clerq, 2000; White et al., 1999a,b), since it exhibits high anticytopathic activities against HIV-1 and -2 (Boyd et al., 1994). Although a broad series of other African and Asian Ancistrocladaceae species (as well as Dioncophyllaceae plants, which occur in West Africa) have been screened for michellamine-like naphthylisoquinoline dimers, no other dimer-producing plants have so far been identified (Cragg et al., 1994).

In order to speed up the screening process for pharmacologically interesting structures, hyphenated techniques like HPLC-MS/MS and HPLC-NMR (Albert, 1999; Wolfender et al., 1999; Lommen et al., 2000), more recently complemented by the HPLC-CD option (Bringmann et al., 1999b, 2001b), have been increasingly used during the past years. In combination, as an analytical 'triad', they permit, in favorable cases, the full structural elucidation of novel natural products right from crude plant extracts, including the constitution, but also the relative and even the absolute configuration (Bringmann et al., 1999b). Based on these techniques, we have recently succeeded in identifying a new dimeric naphthylisoquinoline alkaloid, ancistrogriffithine A (**3**) (Bringmann et al., 2001c) (Fig. 2) in the extract of the phytochemically not yet investigated Asian *Ancistrocladus* species *A. griffithii* (Planchon, 1849).

In this paper, we report on the preparative isolation of this unprecedented twofold 7,8′-coupled, constitutionally and stereochemically symmetric novel (and first Asian) bisnaphthylisoquinoline alkaloid **3** and the confirmation of its stereostructure. Furthermore, we describe the online structural investigation of three further, now monomeric alkaloids from *A. griffithii*, named ancistrogriffines A (**4**), B (**5**), and C (**6**), using the HPLC-MS/MS-NMR-CD 'triad'. Their structural elucidation, including the absolute configuration, succeeded right from the extract; solely in the case of **6**, an isolation step was necessary for completing the structural assignment. In addition, compound **4** was isolated, and all of the details of the structure previously assigned online were confirmed, by conventional offline NMR and MS methods and by chemical degradation. Furthermore, two known (Govindachari and Parthasarathy, 1971; Govindachari et al., 1975; Bringmann et al., 1998c) naphthylisoquinoline alkaloids, ancistrocladine (**7a**) and its natural atropo-diastereomer, hamatine (**7b**), were detected and identified by HPLC-MS and HPLC-UV/CD comparison with reference compounds.

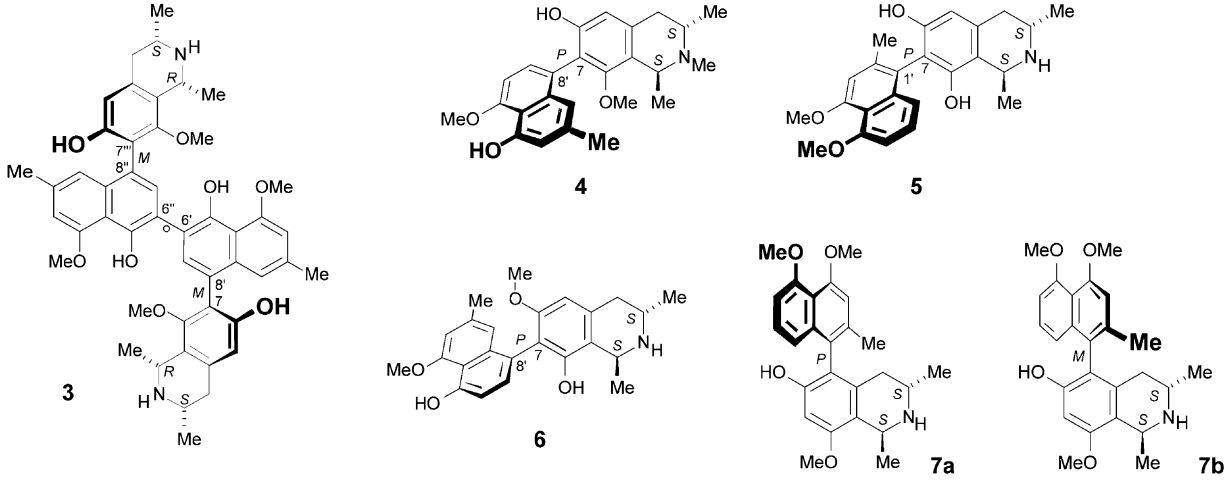

Fig. 2. New alkaloids from *Ancistrocladus griffithii*: ancistrogriffines A (**4**), B (**5**), and C (**6**), and ancistrogriffithine A (**3**); already known from Asian Ancistrocladaceae: ancistrocladine (**7a**) and hamatine (**7b**).

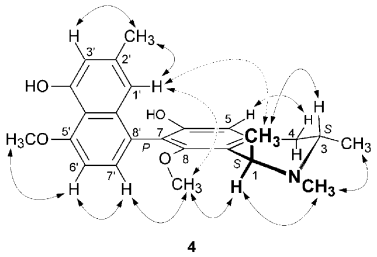

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Fig. 3. ROESY correlations of ancistrogriffine A (**4**) observed in HPLC-NMR (full-line arrows) and in offline NMR (dotted arrows: additional correlations only observed with isolated material); absolute configuration here still arbitrary.

2. Results and discussion

HPLC-UV and HPLC-ESI-MS screening of the leaf extract of *A. griffithii* showed the presence of two major components, the more slowly eluting one exhibiting a mass peak of a monoprotonated ion $[M+H]^+$ with m/z 408, which is in agreement with a tris-*O*- and/or -*N*-methylated, 6,8-dioxygenated naphthyltetrahydroisoquinoline. This was also supported by the mass spectrum obtained from a daughter ion experiment, which produced fragments of m/z 351 $[M+H-C_2H_4NCH_3]^+$, hinting at an *N*-methylated, *O*,*O*-dimethylated tetrahydroisoquinoline. To obtain more information about the structure of the compound, the peak was analyzed by stop-flow HPLC-NMR, including online ^{1}H, ROESY, and TOCSY experiments (Bringmann et al., 1999c,d) (see Fig. 3 and Table 1).

The HPLC-NMR spectrum confirmed the naphthyltetrahydroisoquinoline basic structure. Besides the two methoxy groups, whose peaks were visible at δ_H 3.17 and 4.06, a singlet at δ_H 2.73 integrating for three protons suggested the presence of an *N*-methyl group. This was confirmed by ROE correlations of *N*-Me both with H-1, and CH_3-3. CH_3-1 showed an ROE effect to H-3, revealing a relative *trans*-configuration between CH_3-3 and CH_3-1. The proton at C-1 exhibited an ROE effect to the OCH_3 group at δ_H 3.17, which was therefore assigned to be OCH_3-8. Furthermore, H-4_{eq} displayed a correlation to a singlet at d_H 6.64, which thus had to be H-5. This also revealed C-7 to be the biaryl coupling position in the isoquinoline part of **4**. The substitution pattern in the naphthalene moiety was derived by two correlation series, viz. {H-7′↔H-6″↔OCH_3-5′} and {H-3′↔CH_3-2′↔H-1′}, leaving C-8′ as the only remaining possible coupling position in the naphthalene part. Additional evidence of a 7,8′-coupled biaryl was obtained from ROE correlations between H-7′ and OCH_3-8. This is the first time that an ROE effect across a biaryl axis (Bringmann et al., 1998b, 1999b,c,d) has

Table 1
HPLC-NMR data of **3**, **4**, **5**, and **6** (600 MHz, D_2O–CH_3CN)[a]

Compound	**3**	**4**	**5**	**6**
t_R	19.7 min	23.5 min	17.6 min	21.2 min
H-atom	δ_H (mult., *J* [Hz])			
1	4.63 (*q*, 5.9)	4.58 (*q*,*br*)[b]	4.70 (*q*, 6.6)	4.75 (*q*, 6.9)
CH_3-1	1.63 (*d*, 5.9)	1.60 (*d*, 6.2)	1.54 (*d*, 6.7)	1.54 (*d*, 6.8)
NCH_3	–	2.73 (*s*)	–	–
3	3.39 (*m*)	4.06 (*m*)[c]	3.83 (*m*)	3.61 (*m*)
CH_3-3	1.41 (*d*, 6.6)	1.42 (*d*, 6.1)	1.44 (*d*, 6.2)	1.12 (*d*, 6.3)
4_{ax}	2.92 (*d*, 7.3)[c]	2.86 (*dd*, 19.2, 14.1)	2.82 (*dd*, 17.9, 11.9)	2.58 (*dd*, 17.9, 11.6)
4_{eq}	2.92 (*d*, 7.3)[c]	3.09 (*dd*, 15.1)[b]	3.10 (*dd*, 17.6, 4.1)	2.03[d]
5	6.63 (*s*)	6.64 (*s*)[c]	6.44 (*s*)	6.59 (*s*)
OCH_3-6	–	–	–	3.86 (*s*)
OCH_3-8	3.26 (*s*)	3.17 (*s*)	–	–
1′	6.81 (*s*)	6.65 (*s*)[c,e]	–	6.61 (*s*)
CH_3-2′	2.33 (*s*)	2.24 (*s*)	2.13 (*s*)	2.27 (*s*)
3′	6.90 (*s*)	6.71 (*s*)[e]	7.00 (*s*)	6.85 (*s*)
OCH_3-4′	4.04 (*s*)	–	3.94 (*s*)	4.04 (*s*)
OCH_3-5′	–	4.06 (*s*)	3.90 (*s*)	–
6′	–	6.97 (*d*, 7.7)	6.95 (*d*, 8.5)	6.84 (*d*)[c,f]
7′	7.27 (*s*)	7.27 (*d*, 8.1)	7.31 (*dd*, 8.1, 7.8)	7.12 (*d*, 7.8)
8′	–	–	6.85 (*d*, 7.8)	–

[a] All assignments based on ROESY, TOCSY, and COSY correlations, except for **6**.
[b] Coupling not fully resolved.
[c] Overlapping signals.
[d] 4-H_{eq} not visible in HPLC-^{1}H NMR due to its proximity to the solvent suppression region; shift of 4-H_{eq} determined by HPLC-TOCSY-NMR showing correlations between H-3, H-4_{ax}, CH_3-3, and 4-H_{ax}.
[e] Distinction between 1′-H and 3′-H in analogy to **3** and **6**.
[f] Assignment by offline ROESY, HMQC, and HMBC in $CDCl_3$.

been observed online, in LC-NMR coupling. Although the methoxy group at C-8 is subject to free rotation, the observed ROE effect is moreover of a certain diagnostic value for the relative configuration at the biaryl axis, because it can be assumed that the preferential conformational position of that *O*-methyl group is below the isoquinoline 'plane', and thus '*anti*' relative to the methyl group at C-1, which is above that plane. The ROE effect thus suggested H-7′ to be (at least preferentially) *syn* to that *O*-methyl group, i.e. below the plane (or *anti* to 1-CH_3), as shown in Fig. 3. This was confirmed by an ROE effect of that OCH_3-group with H-1 rather than with CH_3-1.

But since NOE or ROE effects can originate even from small conformational populations, a confirmation of this assumption, by directly establishing the *absolute* configuration at the biaryl axis, was necessary. This was achieved by stop-flow online HPLC-CD measurements and comparison of the resulting CD spectrum of **4** with that of 6-*O*-demethylancistrobrevine A (**8**, see Fig. 4), a structurally comparable, likewise 7,8′-coupled naphthylisoquinoline alkaloid previously isolated from the West African species *A. abbreviatus* (Bringmann and Pokorny, 1995). The two CD spectra showed a qualitatively very similar behavior, including the region of 200–250 nm, which is the diagnostically valuable bandwidth for $\pi\rightarrow\pi^*$ transitions arising from the axis-linked chromophores (Bringmann et al., 1999b). Therefore, **4** had to be *P*-configured.

To fully establish the absolute configuration, including the stereogenic centers at C-1 and C-3, extract portions of leaves and twigs were subjected to our ruthenium-catalyzed oxidative degradation with subsequent GC-MSD analysis of the chiral amino acids formed (Bringmann et al., 1996a). The exclusive

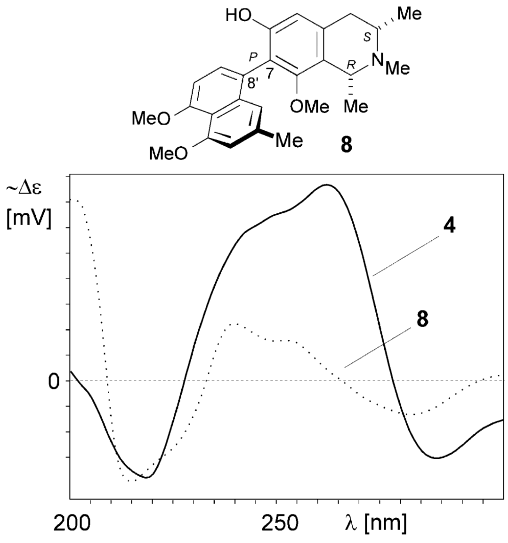

Fig. 4. Comparison of the stop-flow HPLC-CD spectrum of **4** (t_R = 23.9 min) with an offline CD-spectrum of **8** (in EtOH).

(>99:1) detection of the (*S*)-enantiomer of 3-aminobutyric acid and of 3-*N*-methylaminobutyric acid clearly revealed that only (3*S*)-configured tetrahydroisoquinoline structures were present in the extracts, which is typical of alkaloids from Asian Ancistrocladaceae species (Bringmann and Pokorny, 1995). Therefore, given the relative *trans*-orientation of Me-1 vs. Me-3 and the above CD results, the absolute configuration of ancistrogriffine A (**4**) had to be 1*S*,3*S*,7*P*, in agreement with the relative axial configuration already assumed from the above discussed ROE-interaction of H-7′ to OCH_3-8.

With the new compound **4** identified, it seemed worth the effort to isolate it for bioactivity tests. This was achieved after several chromatographic steps. Offline NMR measurements on the pure compound, including HMQC, HMBC, and ROESY experiments, confirmed the structure previously deduced online from the extract. Long-range ROE correlations (Bringmann et al., 1997) between H-1′ and CH_3-1 further corroborated the coupling type and the relative configuration at the biaryl axis, while the interactions between H-1′ and OCH_3-8, although significantly weaker than those between H-7′ and OCH_3-8, underline the problem of having a dynamic population of 'OMe_{up}-8′/'OMe_{down}-8′ conformers. This had necessitated additional 'hard core' evidence, as indeed provided by the H-1′/CH_3-1-ROE interactions and, already online, by CD.

The second main compound detected by HPLC-UV and HPLC-ESI–MS, both, in the leaf extract, but also in the twig extract, had already previously been structurally investigated online, by LC-MS/MS, -NMR, and -CD (Bringmann et al., 2001c). Accordingly, it had been found to be a novel-type dimer, named ancistrogriffithine A (**3**), a constitutionally and stereochemically symmetric (and thus C_2-symmetric) quateraryl with two stereogenic axes and four stereocenters, and with an uprecedented 7,8′,6′,6″,8″,7‴-coupling pattern. We have now isolated the compound for bioactivity testing and further structural investigations offline, by ROESY (Fig. 5), HMQC, and HMBC measurements, which fully confirmed the previous assignment. For the isoquinoline part, four 'key' correlations {H-5↔H-4_{eq}}, {H-1↔H-3}, {OCH_3-8↔CH_3-1}, and {OCH_3-8↔H-1} identified ancistrogriffithine A (**3**) to be 1,3-*cis*-configured, with a methoxy group at C-8 and the biaryl axis at C-7. Finally, the correlation sequence {OCH_3-4′↔H-3′↔CH_3-2′↔H-1′↔OCH_3-8↔H-7′} proved the monomeric part of **3** to be 7,8′-coupled, with the 'central' biaryl axis positioned between C-6′ and C-6″. An interaction between H-1′ and CH_3-1 was diagnostic to determine the relative configuration at the biaryl axis and thus correlated all of the three stereoelements in the monomeric half to each other. As the extract degradation (see above) had already excluded the occurrence of any (3*R*)-configured naphthylisoquinoline alkaloids, the absolute configuration of **3** had to be 1*R*,3*S*,7*M* in both

molecular parts, which was now confirmed offline, by an oxidative degradation of the pure compound.

During the investigation of the twig extract, the value of HPLC-NMR for the structural elucidation became evident one more time. In a rather small fraction of that extract, which had resulted from chromatography over silica gel, a now enriched, but still non-isolable new trace alkaloid, hence named ancistrogriffine B (**5**), was detected. In combination with HPLC-ESI–MS, which delivered *m*/*z* 392 for the monoprotonated species $[M+H]^+$, the HPLC-NMR spectrum suggested the presence of a 6,8-dihydroxylated tetrahydroisoquinoline alkaloid with two methoxy groups in the naphthalene part. This was evidenced by stop-flow LC-ROESY experiments (Fig. 6).

As previously for **4** (see above), the correlation of CH_3-1 with H-3 established **5** to be 1,3-*trans*-configured, while the interaction of H-5 with H-4_{eq} revealed the naphthalene part to be located at C-7. Different from the 7,8′-coupled naphthylisoquinoline **4**, however, two correlation sequences, {CH_3-2′↔H-3′↔OCH_3-4′} and {OCH_3-5′↔H-6′↔H-7′↔H-8′}, left C-1′ as the only remaining possible coupling position in the naphthalene moiety.

In this case, no ROEs indicative of the relative axial configuration were observed between the two molecular portions. Fortunately, however, the *absolute* configuration at the biaryl axis could be assigned through HPLC-CD, by comparison of the online CD spectrum of **5** with that of *trans*-1,2-dihydroancistrocladisine (**9**),

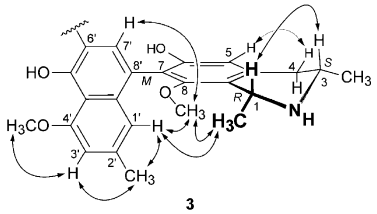

Fig. 5. Stereostructure of ancistrogriffithine A (**3**) as previously assigned online by HPLC-NMR and now confirmed offline, by ROESY measurements (and, for the absolute configuration, further complemented by oxidative degradation).

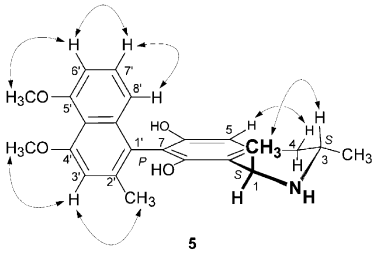

Fig. 6. Structure of ancistrogriffine B (**5**) as deduced from HPLC-NMR-ROESY experiments; absolute configuration and relative axial configuration here still assigned arbitrarily.

a structurally similar known (Bringmann et al., 1999e) naphthylisoquinoline alkaloid (Fig. 7).

The similarity of the two CD curves suggested that **5** and **9** have the same stereo array at the axis and are thus both (7*P*)-configured. Since the extract degradation had disclosed C-3 to be (*S*)-configured for the entire *ensemble* of alkaloids in *A. griffithii* and given the relative *trans*-configuration at C-1 vs. C-3 as deduced by NMR (see above), the absolute configuration of ancistrogriffine B (**5**) was deduced to be 1*S*,3*S*,7*P*.

With the discovery of the novel dimeric naphthylisoquinoline ancistrogriffithine A (**3**), a directed search for its presumable (Schlauer et al., 1998) monomeric precursor, by HPLC-MS investigation of the leaf extract, seemed rewarding. Scanning the twig extract at *m*/*z* 394 revealed the presence of a peak with the correct mass at a retention time similar to that of **3**. HPLC-NMR experiments on this substance, however, disproved the structure of a '*semi*-ancistrogriffithine', showing the — again new — monomeric naphthylisoquinoline alkaloid to be *trans*-configured at C-1 *vs*. C-3 (Fig. 8a). The ROESY correlation sequence {H-4_{eq}↔H-5↔OCH_3-6} located one of the two methoxy groups in the isoquinoline ring and excluded the possibility of the naphthalene substituent to be coupled to C-5. A similar ROESY sequence in the naphthalene part, {H-1′↔CH_3-2′↔H-3′↔OCH_3-4′}, established the second methoxy group at C-4′ and, simultaneously, excluded the isoquinoline substituent to be at C-1′. Of the two remaining possible coupling positions, 7,8′ or 7,6′, the latter seemed less probable because of the absence of any ROESY interactions of the *peri*-proton at C-1′ with a hypothetic additional *peri*-proton at C-8′ in LC-NMR. The—hence feasible—7,8′-coupling type, however, remained to be

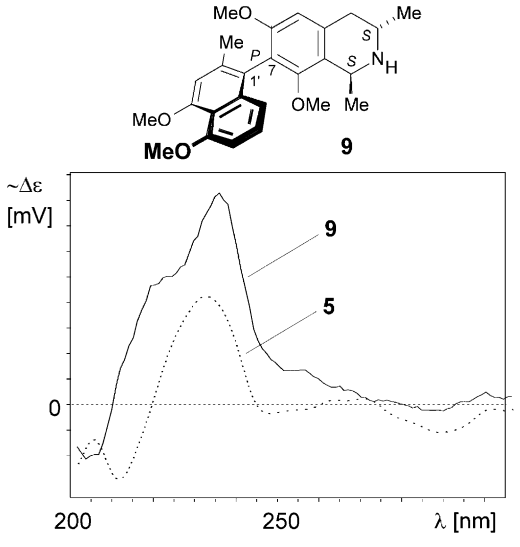

Fig. 7. Comparison of the online HPLC-CD spectrum of **5** (t_R = 15.1 min) with the offline CD spectrum of **9** (in EtOH).

verified in this case offline, after isolation of the (again new) alkaloid, now named ancistrogriffine C (**6**), giving rise to additional ROESY, HMQC, and HMBC data (see Fig. 8b) for further structural elucidation.

In contrast to the NMR spectra previously taken online (see above), however, those of the pure alkaloid **6** now showed only broad bands for all of the signals derived from the heterocyclic ring, both, in ^{1}H and in ^{13}C NMR. Furthermore, the signals of H-4_{ax} and H-4_{eq}, which sometimes, by ROESY correlations to the naphthalene protons, permit an elucidation of the axial configuration (Bringmann et al., 1999a), overlapped with CH_3-2′ at δ_H 2.38 in this case, while spectra that had been acquired right after preparative chromatography of **6** on a chiral phase showed a much better resolution of the ^{1}H and ^{13}C peaks, and H-4_{ax} and H-4_{eq} were clearly distinguishable for that sample. This useful effect was attributed to residues (ca. 1 equiv.) of triethylamine (NEt_3) in the freshly chromatographed sample; the amine had been used as a buffer in the last chromatographic step. If NEt_3 was removed in high vacuum, the signal quality degraded again. As the NEt_3 signals at δ_H 1.37 and 3.05 did not overlap with the signals of **6**, all NMR experiments were thus carried out in the presence of NEt_3. It should be noted that in no experiment any correlations between the signals of the NEt_3 residue and those of the alkaloid signals were found, thus confirming that the amine acted as a mere buffer in solution and was not chemically coordinated to **6**.

In HMBC, the 7,8′-coupling of the new alkaloid **6** was readily deduced from two ^{3}J correlations of OH-5′ to the neighboring positions C-10′ and C-6′. This left C-8′ as the only possible coupling position in the naphthalene part. The assignment was confirmed by a series of C–H correlations involving C-8′ and C-7, which by HMBC connected each of these two bridgehead carbons to both biaryl parts: C-8′ exhibited ^{3}J couplings to H-6′ and H-1′ and a ^{4}J interaction with H-5 in the isoquinoline part, while, vice versa, C-7 showed a ^{3}J interaction with H-5 and a ^{4}J correlation with H-7′ across the biaryl axis (Fig. 8b). Offline ROESY experiments produced a long-range correlation between H-1′ and 4-H_{ax}, thus corroborating the above established 7,8′-coupling and, simultaneously, evidencing a *syn*-array of these two protons, in agreement only with a *P*-configuration at the biaryl axis. By all these investigations (including offline CD and the oxidative degradation, which evidenced C-3 to be (*S*)-configured), the new alkaloid, now named ancistrogriffine C, was attributed the full absolute stereostructure **6**, as shown in Figs. 2 and 8a.

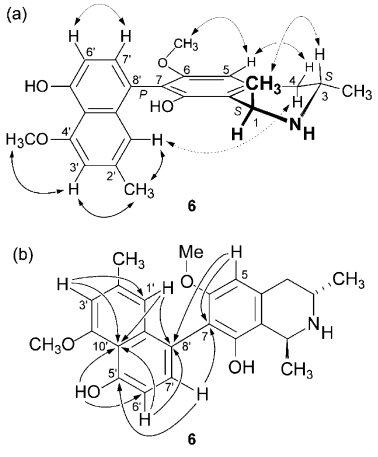

Fig. 8. (a) ROESY correlations of ancistrogriffine C (**6**) observed HPLC-NMR and in offline NMR (dotted arrows: correlation only observed with isolated material); (b) HMBC correlations of **6** (offline) evidencing a 7,8′-coupling type.

Besides the new alkaloids **3**, **4**, **5**, and **6**, HPLC-NMR of the twig extract delivered ^{1}H spectra of a tris–*O*-methylated tetrahydroisoquinoline structure at a retention time of $t_R = 19.5$ min, similar to the signal pattern of the well known (Govindachari and Parthasarathy, 1971; Bringmann et al., 1998c) 5,1′-coupled ancistrocladine (**7a**). This was also supported by HPLC-MS scanning for *m*/*z* 408. HPLC-CD experiments, however, revealed a CD spectrum virtually opposite to that of authentic **7a** isolated earlier (Bringmann et al., 1998c), with a negative sign of the CD effect at 254 nm (instead of a positive one), and the retention time of the compound was not fully in agreement with that of **7a**, either. The peak was finally identified as the likewise well-known (Govindachari and Parthasarathy, 1971; Bringmann et al., 1998c) compound hamatine (**7b**), i.e. the atropo-diastereomer of ancistrocladine (**7a**), by HPLC-MS coelution with authentic **7b** (Bringmann et al., 1998c) as a standard. But ancistrocladine (**7a**) itself does occur in the plant, too, as eventually identified as a later eluting, smaller peak, by coelution and by HPLC-MS/MS and HPLC-UV analysis.

In view of the antiprotozoal activities of some naphthylisoquinoline alkaloids (Bringmann et al., 1998a, 2000a; François et al., 1999), i.e. against *Plasmodium falciparum* (the causative agent of malaria tropica), *Trypanosoma cruzi* (the pathogen of Chagas disease), *Trypanosoma brucei rhodesiense* (causing the African sleeping sickness) or *Leishmania donovani* (causing leishmaniasis), the isolated compounds **3**, **4**, and **6** were tested in vitro against these organisms (Table 2). Compounds **3** and **4** exhibited antiplasmodial acitivities against the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine), comparable to that of chloroquine. Especially the results of **3** were surprising: Although dimeric naphthylisoquinolines rarely show considerable antiplasmodial activities (Hallock et al., 1994), it was found to be more active than the standard chloroquine by a factor of two and even more than twenty times more active than the as yet most potent natural dimer, korundamine A (**2**). Only non-natural

dimers, like jozimine A (Bringmann et al., 1996b), had so far shown lower IC_{50} values (ca. 6 ng/ml against the K1 strain). Furthermore, compound **4** showed a selective activity against *L. donovani*. Unfortunately, **3** (and, to a lesser extent, **4**) showed some cytotoxicity. The antitrypanosomal activities of compounds **3**, **4** and **6** were moderate to low.

In summary, we have described the first phytochemical investigation of the as yet unexplored Asian liana *Ancistrocladus griffithii*, using our hyphenation triad HPLC-MS/MS, HPLC-NMR, and HPLC-CD. Four new alkaloids (**3**, **4**, **5**, and **6**) and two known ones (**7a** and **7b**) were identified. Compounds **3**, **4**, and **5** were structurally elucidated solely online, without any need for isolation. Only in the case of **6**, the necessity of further C-H correlations required its additional isolation. The known alkaloids **7a** and **7b** were identified chromatographically, by HPLC-UV/CD and by HPLC-MS comparison with genuine standards.

A. griffithii is remarkable in many respects. Besides producing monomeric naphthylisoquinolines of three different coupling patterns (7,8′, 7,1′, and 5,1′), it is the as yet only Asian source of dimeric naphthylisoquinoline alkaloids (the second in total, besides the African species *A. korupensis*). Ancistrogriffithine A (**3**) is the first known 'Ancistrocladaceae type' (Bringmann and Pokorny, 1995) (i.e. with 3*S* and with an oxygen function at C-6) dimer. It is constitutionally—though not stereochemically—related to korundamine A (**2**), a 'mixed', constitutionally unsymmetric bis-naphthylisoquinoline from *A. korupensis* (Hallock et al., 1998), one 'half' of which consists of a 7,8′-coupled naphthylisoquinoline as do *both* halves in **3**. With the new dimer **3**, the coupling type series: michellamine B (**1**) and its stereoisomers (5,8′-5,8′)→korundamine A (**2**) (5,8′-7,8′)→ancistrogriffithine A (**3**) (7,8′-7,8′) has now become complete. It is noteworthy that despite the relatively high concentration of **3** and the presence of a couple of monomeric naphthylisoquinolines in the extract according to LC-MS, we have as yet not been able to detect the authentic monomeric 'half' of **3**. The search for further dimeric naphthylisoquinoline alkaloids in other Asian species is in progress.

Table 2
Bioactivities of compounds **3**, **4**, and **6**

IC_{50} (μg/ml)	**3**	**4**	**6**
P. falciparum (strain: K1)[a]	0.035	0.074	0.419
T. cruzi[b]	13.6	17.1	41.2
T. b. rhodesiense[c]	0.90	2.17	2.97
L. donovani[d]	n.d.[e]	3.1	18.3
Cytotoxicity L6[f]	5.8	14.2	35.8

[a] Chloroquine: $IC_{50} = 0.065$ μg/ml.
[b] Benznidazole: $IC_{50} = 0.3$ μg/ml.
[c] Melarsoprol $IC_{50} = 0.0063$ μg/ml.
[d] Pentostam: $IC_{50} = 47.2$ μg/ml.
[e] Not determined due to cytotoxicity towards host cells.
[f] Mefloquine: $IC_{50} = 2.1$ μg/ml.

3. Experimental

3.1. General

Offline CD spectra (25 °C, 0.2 cm cell) were acquired on a Jasco J-715 spectropolarimeter. NMR spectra were measured on Bruker DMX 600 (^{1}H 600 MHz, ^{13}C 150 MHz) and Bruker Avance 400 spectrometers (^{1}H 400 MHz, ^{13}C 100 MHz) using $CDCl_3$ (δ 7.26 and 77.01) and CD_3OD (δ 3.30 and 49.01) as the solvents. In offline NMR, proton-detected, heteronuclear correlations were measured using HMQC (optimized for $^1J_{HC} = 145$ Hz) and HMBC (optimized for $^nJ_{HC} = 7$ Hz and 3 Hz). NOE effects were recorded using standard NOESY or ROESY pulse sequences from the Bruker pulse program library. EIMS and HREIMS data were determined on Finnigan MAT 8200 and Finnigan MAT 90 instruments. ESIMS spectra were taken on a Finnigan TSQ 7000 triple quadrupole mass spectrometer. HRESIMS were acquired on a Bruker Apex III FT-ICR-MS instrument (Bruker Daltonics, Bremen) in CH_3CN–H_2O (+0.01% trifluoroacetic acid) 1:1 (v/v). The absolute configuration of the stereocenter at C-3 of the isolated naphthylisoquinolines were determined by oxidative degradation as described previously (Bringmann et al., 1996a).

3.2. Plant material

Leaves and twigs of *A. griffithii* (ex cult., originally from Thailand) were obtained from the Botanical Garden of Würzburg. A voucher specimen is deposited at the Institut für Organische Chemie (no. 49).

3.3. Extraction and isolation

Ten grams of air-dried leaves were ground and successively extracted at room temperature with 2×300 ml H_2O–CH_3CN (2:1 v/v, pH 1–2, trifluoroacetic acid). After filtration, the solution was lyophilized to yield ca. 1.5 g of crude extract, which was re-dissolved in CH_2Cl_2:MeOH 3:1 v/v and filtered over silica gel (deactivated with 7.5% aqueous NH_3, w/w) to give ca. 800 mg of a prefractionated extract. This was further fractionated by CC (CH_2Cl_2→CH_2Cl_2:MeOH 9:1 v/v, deactivated silica gel). The resulting fraction containing **4** was further purified by preparative reverse-phase HPLC (Waters Symmetry C_{18} 19×150 mm, 7 μm; flow rate 12 ml min^{-1}; UV detection 254 nm; solvent (A) CH_3CN; (B) H_2O + 0.05% v/v trifluoroacetic acid; binary gradient 0 min 5% A, 15 min 25% A, 30 min 30% A) to yield 9 mg of pure **4**.

25 g of air-dried twigs were ground and successively extracted at room temperature with 2×300 ml H_2O–CH_3CN (1:1 v/v, pH 1–2, trifluoroacetic acid). After filtration, the solution was lyophilized to yield 2.9 g of crude extract, which was prefractionated by CC (CH_2Cl_2→CH_2Cl_2:MeOH 8:2) to yield several fractions (with rising polarity) containing **6** (ca. 150 mg), **5** (ca. 4 mg) and **3** (ca. 27 mg). After filtration, these fractions were also used for hyphenated analyses. The fraction containing **6** was subjected to CC (CH_2Cl_2:*n*-hexane 8:2→CH_2Cl_2→CH_2Cl_2:MeOH 9:1 v/v) to yield ca. 30 mg of a fraction enriched in **6**. After successive semi-preparative reverse-phase HPLC [Waters Symmetry C_{18} 7.8×300 mm, 7 μm; flow rate 4 ml min^{-1}; UV detection 254 nm; solvent (A) CH_3CN; (B) H_2O + 0.05% v/v trifluoroacetic acid; binary gradient: 0 min 5% A, 15 min 25% A, 30 min 30% A] and chiral preparative normal-phase HPLC (Chiralcel OD 7.8×250 mm, 7 μm; flow rate 7 ml min^{-1}; UV detection 254 nm; solvent *n*-hexane:*i*-PrOH 1:1 + 0.1% v/v NEt_3), ca. 3 mg of **6** were isolated. The fraction containing compound **3** was subjected to preparative reverse-phase HPLC (identical to conditions described above) to yield ca. 6 mg of **3**.

3.4. Hyphenated analysis of plant material

For leaf extract analysis, 5 mg of the crude extract were redissolved in 500 μl D_2O/MeCN (8:2, v/v) and filtered through a 0.2 μm membrane filter. For twig extract analysis, the fractions containing **3**, **5**, and **6** were dissolved in D_2O/MeCN (8:2, v/v) to 3 mg/ml. All samples were filtered through a 0.2 μm membrane filter. The resulting solutions were directly used for hyphenated analyses.

All chromatographic separations were performed using Symmetry C-18 columns from Waters (5 μm, 4.6×250 mm for HPLC-NMR and HPLC-CD and 2.1×150 mm for HPLC-MS/MS). The mobile phases used were (A) CH_3CN, (B) H_2O, and (C) D_2O. Phases (B) and (C) were acidified to pH 3 with trifluoroacetic acid. Binary gradients (CH_3CN-H_2O for HPLC-MS/HPLC-CD, and CH_3CN-D_2O for HPLC-NMR) were programmed as follows: 0 min 5% A, 15 min 25% A, 30 min 30% A, and 40 min 50% A. The flow rate was set to 1.0 ml/min for HPLC-NMR, 0.2 ml/min for HPLC-MS, and 1.2 ml/min for HPLC-CD (in the case of **5** only 1.1 ml/min).

The HPLC-NMR, HPLC-ESI-MS/MS, and HPLC-CD system was set up as described earlier (Bringmann et al., 2001c).

3.5. Ancistrogriffithine A (*3*)

Light yellow powder (MeOH), mp 230 °C (decomp.): $[\alpha]_D^{25}$ +73.5° (MeOH, *c* 0.30). CD (MeOH): $\Delta\varepsilon_{214}$ −3.8, $\Delta\varepsilon_{222}$ −1.7, $\Delta\varepsilon_{245}$ +5.1, $\Delta\varepsilon_{252}$ +3.7, $\Delta\varepsilon_{265}$ 7.1$\Delta\varepsilon_{291}$ −4.6. IR ε_{max}^{KBr} cm^{-1}: 3390 (*m*), 3010 (*m*), 2970 (*m*), 2390 (*m*), 1600 (*m*), 1430 (*m*), 1375 (*m*), 1350 (*w*), 1220 (*s*), 1085 (*m*), 875 (*w*). ^1H NMR (600 MHz, CD_3OD): *d* 1.49 (3H, *d*, *J* = 6.4 Hz, CH_3-3), 1.72 (3H, *d*, *J* = 6.6 Hz, CH_3-1), 2.35 (3H, *d*, *J* = 0.6 Hz, CH_3-2′), 2.39 (2H, overlapped, H_{eq}-4 and H_{ax}-4), 3.29 (3H, *s*, OCH_3-8), 3.47 (1H, *m*, H-3), 4.08 (3H, *s*, OCH_3-4′), 4.67 (1H, *q*, *J* = 6.5 Hz, H-1), 6.58 (1H, *s*, H-5), 6.83 (1H, *d*, *J* = 1.3 Hz, H-3′), 6.83 (1H, *d*, *J* = 1.2 Hz, H-1′), 7.18 (1H, *s*, H-7′). ^{13}C NMR (150 MHz, CD_3OD): δ 18.8 (CH_3-3), 20.6 (CH_3-1), 22.2 (CH_3-2′), 35.2 (C-4), 51.1 (C-3), 52.4 (C-1), 57.0 (OCH_3-4′), 60.9 (OCH_3-8), 107.8 (C-3′), 111.8 (C-5), 109.3 (C-6′), 115.1 (C-10′), 118.4 (C-9), 120.0 (C-1′), 121.8 (C-7), 121.9 (C-8′), 134.7 (C-10), 135.1 (C-7′), 136.4 (C-9′), 137.1 (C-2′), 152.6 (C-5′), 157.3 (C-6), 157.9 (C-4′), 158.6 (C-8). ESIMS *m*/*z* (rel. int.): 393.4 $[M + 2H]^{2+}$ (100), 785.4 $[M + H]^+$ (8). HRESIMS *m*/*z*: 393.1939 $[M + 2H]^{2+}$ ($C_{48}H_{54}N_2O_8$ requires 393.1935).

3.6. Ancistrogriffine A (*4*)

Colorless solid (MeOH), mp 148 °C. $[\alpha]_D^{25}$ +26.0° (MeOH; *c* 0.40). CD (EtOH): $\Delta\varepsilon_{209}$ −3.0, $\Delta\varepsilon_{235}$ +4.4, $\Delta\varepsilon_{246}$ +1.2, $\Delta\varepsilon_{257}$ +2.0, $\Delta\varepsilon_{282}$ −1.4. IR ν_{max}^{KBr} cm^{-1}: 3394 (*m*), 2987 (*m*), 2946 (*m*), 1673 (*s*), 1588 (*m*), 1416 (*m*), 1389 (*m*), 1200 (*s*), 1132 (*s*), 1089 (*s*), 842 (*w*), 720 (*w*) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 1.40 (3H, *d*, *J* = 6.7 Hz, CH_3-3), 1.60 (3H, *d*, *J* = 6.5 Hz, CH_3-1), 2.26 (3H, *s*, CH_3-2′), 2.65 (3H, *d*, *J* = 3.1 Hz, *N*-CH_3), 2.71 (1H, overlap with *N*-CH_3, H_{ax}-4), 2.91 (1H, *dd*, *J* = 18.3, 4.8 Hz, H_{eq}-4), 3.06 (3H, *s*, OCH_3-8), 3.88 (1H, *m*, H-3), 4.02 (3H, *s*, OCH_3-5′), 4.54 (1H, *q*, *J* = 6.5 Hz, H-1), 6.55 (1H, *s*, H-5), 6.63 (1H, *s*, H-1′), 6.72 (1H, *s*, H-3′), 6.74 (1H, *d*, *J* = 8.3 Hz, H-6′), 7.21 (1H, *d*, *J* = 8.0 Hz, H-7′). ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.7 (CH_3-3), 19.6 (CH_3-1), 21.9 (CH_3-2′), 30.0 (C-4), 33.1 (*N*-CH_3), 48.0 (C-3), 56.1 (OCH_3-5′), 58.3 (C-1), 60.5 (OCH_3-8), 102.9 (C-6′), 109.7 (C-5), 113.3 (C-3′), 113.6 (C-10′), 115.6 (C-1′), 116.6 (C-8′), 118.5 (C-7), 121.9 (C-9), 129.2 (C-7′), 130.8 (C-10), 135.1 (C-9′), 139.3 (C-2′), 154.6 (C-6), 154.7 (C-4′), 156.5 (C-8), 157.0 (C-5′). EIMS *m*/*z* (rel. int.): 407 $[M]^+$ (1), 392 $[M - CH_3]^+$ (100). HREIMS *m*/*z*: 392.1862 $[M-CH_3]^+$ ($C_{24}H_{26}O_4N$ requires 392.1863).

3.7. Ancistrogriffine C (*6*)

Light yellow powder (MeOH), mp 165 °C. $[\alpha]_D^{25}$ +13.8° (MeOH, *c* 0.25). CD (EtOH): $\Delta\varepsilon_{209}$ +8.7, $\Delta\varepsilon_{214}$ +5.1, $\Delta\varepsilon_{227}$ +10.8, $\Delta\varepsilon_{238}$ −8.7. IR ν_{max}^{KBr} cm^{-1}: 3382 (*m*), 2943 (*m*), 2678 (*m*), 2471 (*m*), 1588 (*s*), 1445 (*s*), 1361 (*s*), 1333 (*s*), 1259 (*s*), 1110 (*s*), 834 (*m*) cm^{-1}. ^1H NMR (600 MHz, $CDCl_3$): δ 1.28 (3H, *d*, *J* = 4.6 Hz, CH_3-3), 1.64 (3H, *d*, *J* = 6.6 Hz, CH_3-1), 2.36 (2H, *br*, H-4_{ax} and H-4_{eq}, overlapping with CH_3-2′), 2.37 (3H, *s*, CH_3-2′), 3.40 (1H, *m*, H-3), 3.86 (3H, *s*, OCH_3-6), 4.07 (3H, *s*, OCH_3-4′), 4.68 (1H, *q*, *J* = 6.0 Hz, H-1), 6.50 (1H, *s*, H-5), 6.66 (1H, *s*, H-3′), 6.76 (1H, *s*, H-1′), 6.87 (1H, *d*, *J* = 7.8 Hz, H-6′), 7.20 (1H, *d*, *J* = 7.8 Hz, H-7′), 9.52 (1H, *s*, OH-5′).

^{13}C NMR (150 MHz, $CDCl_3$): δ 19.5 (CH_3-1), 19.8 (CH_3-3), 22.2 (CH_3-2′), 33.0 (C-4), 43.5 (C-3), 47.5 (C-1), 55.3 (OCH_3-6), 56.3 (OCH_3-4′), 96.5 (C-5), 107.4 (C-3′), 109.5 (C-6′), 113.5 (C-10′), 114.0 (C-9), 117.3 (C-7), 118.4 (C-1′), 120.5 (C-8′), 130.7 (C-7′), 133.3 (C-10), 136.0 (C-9′), 137.6 (C-2′), 154.0 (C-8), 155.5 (C-5′), 156.3 (C-4′), 156.6 (C-6). EIMS *m*/*z* (rel. int.): 393 $[M]^+$ (6), 378 $[M-CH_3]^+$ (100); HREIMS *m*/*z*: 378.1705 $[M-CH_3]^+$ ($C_{23}H_{24}O_4N$ requires 378.1700).

3.8. Biological experiments

3.8.1. Plasmodium falciparum

Antiplasmodial activity was determined using the *P. falciparum* strain K1 (resistant to chloroquine and pyrimethamine). A modification of the [^{3}H]-hypoxanthine incorporation assay (Desjardins et al., 1979) was used (Ridley et al., 1996). Briefly, infected human red blood cells were exposed to serial drug dilutions in microtiter plates for 48 h at 37 °C in a gas mixture with reduced oxygen and elevated CO_2. [^{3}H]-Hypoxanthine was added to each well and after further incubation for 24 h the wells were harvested on glass fiber filters and counted in a liquid scintillation counter. From the sigmoidal inhibition curve the IC_{50} value was calculated. The assays were run in duplicate and repeated at least once.

3.8.2. Trypanosoma cruzi

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well/100 μl in RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 h 5000 trypomastigotes of *T. cruzi* [Tulahuen strain C2C4 containing the galactosidase (Lac Z) gene] were added in 100 μl per well with a serial drug dilution. The plates were incubated at 37 °C in 5% CO_2 for 4 days. For determination of the IC_{50} the substrate CPRG/Nonidet was added to the wells. The color reaction which developed during the following 2–4 h was read photometrically at 540 nm. IC_{50} values were calculated from the sigmoidal inhibition curve. Cytotoxicity was assessed in the same assay using non-infected L-6 cells and the same serial drug dilution.

3.8.3. Trypanosoma b. rhodesiense

Minimum essential medium (50 μl) supplemented according to Baltz et al. (1985) with 2-mercaptoethanol and 15% heat-inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were added to the wells. Then 50 μl of trypanosome suspension (*T. b. rhodesiense* STIB 900) were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Alamar Blue (10 μl) was then added to each well and incubation was continued for a further 2–4 h. The plate was then read using a Millipore Cytofluor 2300 at an excitation wavelength of 530 nm and an emission wavelength of 590 nm (Räz et al., 1997). Fluorescence development was expressed as percentage of the control, and IC_{50} values were determined.

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