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TETRAHEDRON

Calothrixins A and B, Novel Pentacyclic Metabolites from *Calothrix* Cyanobacteria with Potent Activity against Malaria Parasites and Human Cancer Cells

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Abstract: Cell extracts from photoautrophic cultures of two cyanobacterial *Calothrix* isolates inhibited the growth *in vitro* of a chloroquine-resistant strain of the malaria parasite, *Plasmodium falciparum*, and of human HeLa cancer cells, in a dose-dependent manner. Bioassay-directed fractionation of the extracts led to the isolation and structural characterization of calothrixins A (**1**) and B (**2**), pentacyclic metabolites with an indolo[3,2-*j*]phenanthridine ring system unique amongst natural products, which exert their growth-inhibitory effects at nanomolar concentrations. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: anticancer; antiplasmodial; cyanobacteria; polycyclic heterocyclic compounds.

INTRODUCTION

Bioactive metabolites with pharmaceutical potential have been isolated from a variety of cyanobacteria.[1,2] These include several compounds with anticancer activity,[3] the depsipeptide cryptophycins being the most promising at this time.[1,4]

In an earlier screening of a range of cyanobacterial genera, we found twenty isolates that had bioactivity against green algae and other cyanobacteria.[5] The twenty were all *Fischerella*, *Nostoc* or *Calothrix* species. Members of the first two genera have been shown to be rich sources of bioactive metabolites, with *Calothrix* species less so, although *C. fusca* has been shown to produce calophycin, a fungicidal cyclic decapeptide.[6]

Malaria, an infectious disease estimated to cause the death of up to 3 million people each year, is caused by protozoan parasites of the genus *Plasmodium*. With the emergence and spread of malaria parasite strains that

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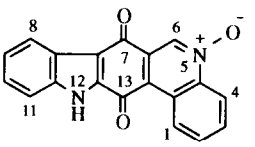
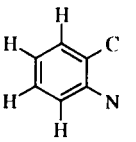
are resistant to many of the antimalarial drugs presently available (*e.g.*, chloroquine) there is an urgent need for the identification of new antimalarial agents.[7,8] Plasmodia and other apicomplexans have recently been shown to have a plastid-like organelle, thought to be a relic of endosymbiosis with an ancient green alga[9] or cyanobacterium. Furthermore, there is evidence that this 'apicoplast' offers a potential antimalarial drug target.[10] This prompted us to test dimethylsulfoxide extracts of lyophilized cells from photoautotrophic, nitrogen-fixing cultures of the twenty algicidal cyanobacteria for their effect on the growth *in vitro* of a chloroquine-resistant strain of *Plasmodium falciparum*, the most virulent of the four strains of plasmodia that are infectious to humans.

In preliminary experiments it was found that the extracts from two of the *Calothrix* isolates inhibited *P. falciparum* growth in a dose-dependent manner. The third *Calothrix* isolate of the twenty selected organisms showed no activity, nor did six other *Calothrix* isolates in the original collection. Extracts of the active two *Calothrix* strains also inhibited the growth *in vitro* of human HeLa cancer cells. We report here the isolation from the *Calothrix* extracts, and the structural characterisation, of the two compounds responsible for the anti-plasmodial and anticancer cell activity. Calothrixins A (**1**) and B (**2**) are novel indolo[3,2-*j*]phenanthridine alkaloids which exert their growth-inhibitory effects at nanomolar concentrations.



1
2 N5-deoxy



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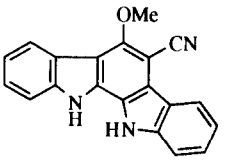

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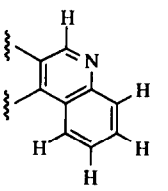

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RESULTS AND DISCUSSION

Isolation and Structure Determination of Calothrixins A and B

Lyophilized cells of the two bioactive *Calothrix* strains yielded active extracts with dimethylsulfoxide, large volumes of ethyl acetate, or most conveniently with the latter solvent under Soxhlet conditions. Bioassay-directed fractionation of the extracts, using a combination of differential solubility and chromatography, led to the isolation of the relatively insoluble calothrixin A (**1**) and its more soluble co-metabolite calothrixin B (**2**).

The electronic spectrum of the wine-red calothrixin A showed multiple maxima, unaffected by acid but shifted reversibly to longer wavelength upon the addition of aqueous sodium hydroxide, indicating the presence of an ionisable acidic group on the chromophore. Electron impact mass spectra (EIMS) of calothrixin A showed a base peak at *m/z* 298, with strong peaks at 270, 242 and 214 confirmed as daughter ions of 298 by linked scan spectrometry. High resolution measurement established the ion compositions $C_{19}H_{10}N_2O_2$, $C_{18}H_{10}N_2O$, $C_{17}H_{10}N_2$ and $C_{16}H_8N$, respectively, indicating a highly unsaturated polycyclic system fragmenting by sequential losses of CO, CO, and CH_2N units. A weak variable intensity ion at *m/z* 314, $C_{19}H_{10}N_2O_3$, carrying an extra O atom relative to *m/z* 298, was initially attributed to an oxidised impurity but later shown to be the true molecular ion.

The ^{13}C NMR spectrum of calothrixin A, recorded for a super-saturated solution in DMSO-d_6 over 11 days due to its insolubility, showed only 18 signals, one of which (later shown to be that at δ 122.1) must contain two superimposed resonances (Table 1). Two low-field oxygenated resonances were apparent at δ 178.4 and 177.9, the remaining 17 unsaturated carbons resonating between δ 143 and 114.

Table 1. ^{13}C and ^{1}H NMR data for calothrixins A (1) and B (2).[a]

	Calothrixin A (**1**)		Calothrixin B (**2**)	
Position	^{13}C[b]	^{1}H[c]	^{13}C[b]	^{1}H[c]
CH-1	128.2	9.68 (bd)	127.2	9.57 (d)
CH-2	132.0	7.98 (m)	130.2	7.87 (t)
CH-3	132.1	7.96 (m)	131.5	7.94 (t)
CH-4	119.2	8.60 (bd)	129.8	8.16 (bd)
C-4a	143.1		151.2	
CH-6	131.9	8.88 (s)	147.6	9.61 (s)
C-6a	130.0		132.7	
C-7	178.4		180.2	
C-7a	115.2		115.5	
C-7b	123.6		123.6	
CH-8	122.1	8.11 (bd)	122.2	8.16 (bd)
CH-9	124.6	7.37 (bt)	124.3	7.36 (t)
CH-10	127.1	7.44 (dt)	127.0	7.44 (t)
CH-11	114.2	7.60 (bd)	114.3	7.61 (d)
C-11a	138.4[d]		139.0[d]	
C-12a	139.0[d]		138.7[d]	
C-13	177.9		181.1	
C-13a	122.1		125.0	
C-13b	126.9		122.6	
NH		13.2 (b)		N.d.

[a] Spectra recorded for solutions in DMSO-d_6, referenced to solvent, at 500 or 600 MHz for ^{1}H and 125.75 or 150.89 MHz for ^{13}C.

[b] Derived from direct APT ^{13}C observation data, and assigned from HMQC and HMBC data.

[c] b broad, d doublet, m multiplet, s singlet, t triplet.

[d] Assignments may be interchanged.

N.d. Not detected.

^{1}H NMR spectroscopy (including spin-decoupling and $^{1}H,^{1}H$ correlation spectroscopy) of calothrixin A (in DMSO-d_6) confirmed its homogeneity and established the presence between δ 13.2 and 7.3 of a broad exchangeable resonance presumably due to the ionisable proton, an isolated aromatic proton singlet, and two spin systems each containing four adjacent aromatic protons (Table 1). Short and long range $^{1}H,^{13}C$ correlation

spectroscopy defined the aromatic systems as *ortho*-disubstituted benzenoid rings, each with only one carbon substituent. The second substituent on each ring was nitrogen, as indicated by the deshielding (δ 138.4 or 139.0, and 143.1) of the *N*-substituted ring carbons, leading in each case to a partial structure of the type (**3**) in which all the expected 1- and 3-bond (and some 2-bond) heteronuclear couplings were observed. The ^{1}H and ^{13}C shifts of the first of these segments (**3**) (C7a-CH8-CH11-N12) closely resembled those of the indole rings of the indolo[2,3-*a*]carbazole alkaloids from soil microorganisms, slime moulds and cyanobacteria,[11] such as the 6-cyano-5-methoxyindolo[2,3-*a*]carbazole (**4**) from the cyanobacterium *Nostoc sphaericum*,[12] indicating a similar indole system in calothrixin A. The ^{1}H and ^{13}C resonances of the second type (**3**) segment (C13a-CH1-CH4-N5, Table 1), however, were all shifted downfield by 0.5-1.6 and 3-7 ppm respectively, necessitating a substantially different environment. The $^{1}J_{H,C}$ of the isolated CH proton was unusually large (190 Hz), indicating that it was adjacent to an electronegative nitrogen atom in a heteroaromatic ring,[13] necessarily the remaining N5. In agreement, this isolated proton showed a long range, probable 3-bond correlation across N5 to C4a, in addition to other correlations to the carbonyl carbon at δ 178.4 and to resonances at δ 130.0 and 122.1. These data suggested the extension of this second segment (**3**) to the quinoline structure (**5**), although possible ambiguity resulted from the apparent superimposition of two resonances at δ 122.1. Significant discrepancies between the ^{1}H and ^{13}C chemical shifts observed for this segment and those expected for a disubstituted quinoline of the type (**5**),[14] however, raised serious doubts about such a structure.

Calothrixin A was clearly a fused pentacyclic ring system with indole and possibly quinoline systems as the terminal rings. The absence of any $^{1}H,^{1}H$ nuclear Overhauser enhancements between these systems, coupled with the paucity of long range $^{1}H,^{13}C$ correlations and the impossibility of recording $^{13}C,^{13}C$ correlations given its extreme insolubility, necessitated definition of the central rings by X-ray diffraction. Fortunately, calothrixin A on long standing in supersaturated DMSO solutions crystallised as very long, thin red needles, from which a segment could be cut which was suitable for single-crystal analysis. The resulting thermal ellipsoid diagram obtained from data collected at 25°C is shown in Fig. 1.

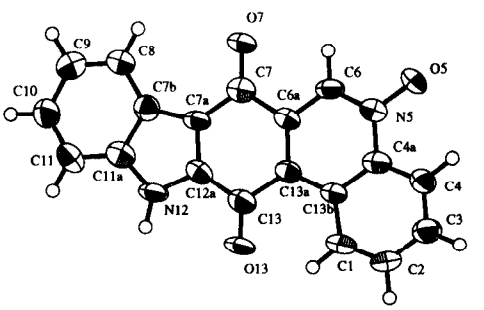


Fig. 1. Thermal ellipsoid diagram of calothrixin A (**1**) with atom labelling. Ellipsoids enclose 50% probability levels. Hydrogen atoms are drawn as circles with small radii.

This structure (**1**) for calothrixin A is in full agreement with the NMR data, which is now assigned as in Table 1. Furthermore, it reveals the presence of an *N*-oxide group, thus establishing the variable intensity EIMS ion *m/z* 314, $C_{19}H_{10}N_2O_3$, as the molecular ion of calothrixin A. Heteroaromatic *N*-oxides are reported to show

variable loss of O on EIMS, due to thermal decomposition in the ion source before ionization.[15] Linked scan EIMS established that this was the case with calothrixin A, the molecular ion *m/z* 314 affording daughter ions at *m/z* 297, 286 and 258 by loss of OH and two CO units. The previously observed ion series at *m/z* 298, 270, 242 and 214 was thus derived from the thermal deoxygenation product rather than from calothrixin A itself. In agreement, electrospray MS of calothrixin A showed MH^+ and MNa^+ ions at *m/z* 315 and 337 in positive mode, and an M - H^- ion at 313 in negative mode.

The EIMS of calothrixin B showed a molecular ion at *m/z* 298 with the composition $C_{19}H_{10}N_2O_2$ and fragmentation pattern identical to the *m/z* 298 ion observed in the calothrixin A spectrum. A strong protonated molecular ion at *m/z* 299 in the chemical ionisation mass spectrum confirmed the molecular weight. The structure of calothrixin B as the N5-deoxy analogue (**2**) of calothrixin A followed from comparison of their NMR data, the only significant differences occurring in the chemical shifts of ^{13}C and ^1H nuclei near N5 (Table 1). Thus the C4, C4a and C6 resonances of calothrixin B were 8-15 ppm downfield of those in calothrixin A, and the H4 and H6 resonances of calothrixin B were respectively 0.44 ppm upfield and 0.73 ppm downfield of those in A, paralleling the shifts observed for the corresponding nuclei in quinoline and quinoline *N*-oxide.[14]

The structures (**1**) and (**2**) also explain the pH dependency of the electronic spectra of calothrixins A and B. Both compounds are ionised by alkali due to the acidifying effect of the two neighbouring carbonyl groups on the indolic N12 proton,[16] whilst the same groups reduce the basicity of the quinolinoid N5 nitrogen of calothrixin B and prevent its protonation by acids.

Activity of Calothrixins A and B against P. falciparum *and HeLa Cells* in vitro

The dose response curves for the effects of pure calothrixin A (**1**) and B (**2**) on the growth *in vitro* of the chloroquine-resistant strain of *P. falciparum* and of HeLa cells are presented in Fig. 2, which shows that they are active at nanomolar concentrations.

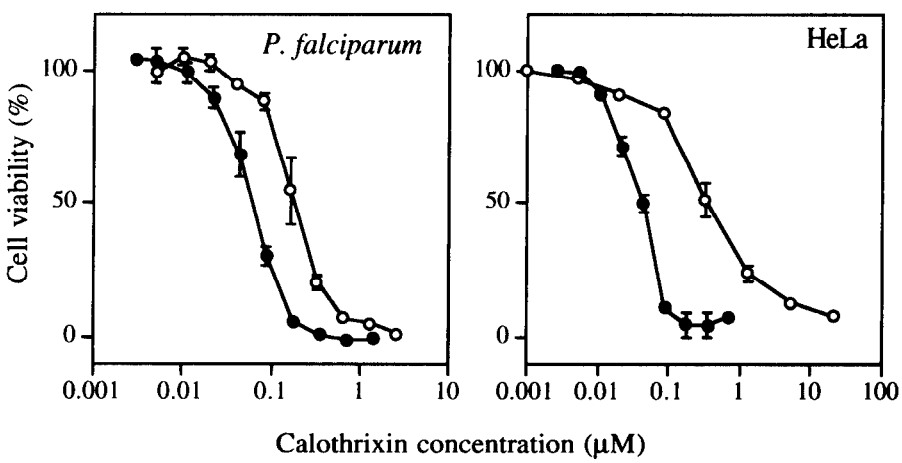
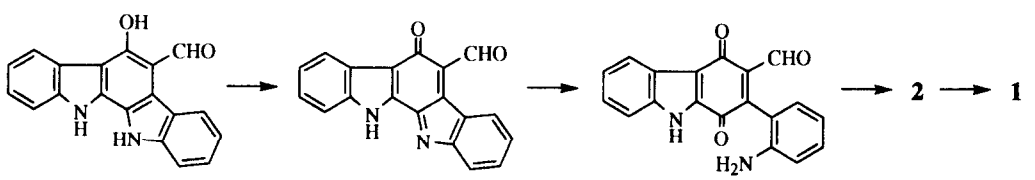


Fig. 2. Dose-response curves for calothrixins A (●) and B (○) against cultures of the malaria parasite *P. falciparum* and HeLa cells. The error bars represent standard deviations and, where not shown, fall within the symbol.

The IC_{50} values of calothrixins A and B against *P. falciparum* were 58 ± 8 s.d. nM and 180 ± 44 s.d. nM, respectively, compared with a value of 83 ± 17 s.d. nM for the widely used antimalarial chloroquine in the same assay; the chloroquine-resistance of this strain is reflected by the chloroquine IC_{50} being greater than 70 nM.[17] The corresponding IC_{50} values against the human cervical cancer cell line, HeLa, were 40 ± 9 s.d. nM and 350 ± 82 s.d. nM, respectively. The similar IC_{50} values obtained for the two different rapidly proliferating cell types are consistent with the calothrixins inhibiting growth of both via a common mechanism. It is therefore unlikely that their antiplasmodial activity is due to a specific effect on the apicoplast.

CONCLUSION

The indolo[3,2-*j*]phenanthridine ring system of the calothrixins is unique amongst natural products. It may be derived biosynthetically from a hypothetical metabolite (**6**) of the relatively common indolo[2,3-*a*]carbazole type,[11] closely related to the known 6-cyano-5-methoxyindolo[2,3-*a*]carbazole (**4**).[12] Oxidation of the *p*-aminophenol (**6**) and hydrolytic cleavage of the resulting quinone imine (**7**) would afford an *o*-aminophenyl-substituted quinone (**8**). Rotation around the biaryl bond and condensation of the amine with the formyl group or its equivalent would close the quinoline ring of calothrixin B (**2**). *N*-Oxidation would then afford calothrixin A (**1**). Labelling studies with L-tryptophan, the established precursor of several indolo[2,3-*a*]carbazole metabolites,[11] would be informative in this regard.



6 **7** **8**

In conclusion, the calothrixins are novel cyanobacterial metabolites that exert potent inhibitory effects on the *in vitro* growth of both the human malaria parasite and human cancer cells. The biomedical potential of these agents is under study.

EXPERIMENTAL

P. falciparum in vitro *growth assay*

Parasites (strain FAF6) were cultured in standard media[18] with modifications.[19] After incubation in the presence of extracted material or pure compounds for 48h, parasite viability *in vitro* was measured as parasite-derived lactate dehydrogenase activity.[20]

HeLa cell in vitro *growth assay*

The cytotoxicity of cell extracts and of the calothrixins was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colourimetric assay,[21] with appropriate buffering.[22]

Production and isolation of the calothrixins

The two bioactive *Calothrix* strains, CAN 95/2 and CAN 95/3, were collected in the Australian Capital Territory and grown photoautotrophically under nitrogen-fixing conditions.[5] Lyophilised cells (2.6 g) of strain CAN 95/2 were extracted with ethyl acetate under Soxhlet conditions. A red precipitate formed in the solvent on standing, which was collected by centrifugation and washed with small volumes of hexane and acetone, leaving calothrixin A (42 mg) as an amorphous, wine-red powder. Evaporation of the ethyl acetate afforded an orange residue which was washed with hexane to remove chlorophyll and lipids. Extraction of this residue with several small portions of acetone then preferentially removed the more soluble orange calothrixin B (12 mg), leaving additional calothrixin A (5 mg). Pure calothrixin B was obtained by absorption of the acetone-extracted material from DMSO on to silica gel and elution with 1% acetic acid in ethyl acetate. Calothrixin A (**1**) formed long wine-red needles from DMSO, dec. 280°C, λ_{max} (EtOH or EtOH + HCl) 292, 362 and 413 nm (ε 19000, 4260, 3100), λ_{max} (EtOH + NaOH) 291, 310 (sh), 357 (sh), 367 and 484 nm (ε 14900, 12100, 7820, 9680, 2050); HREIMS *m/z* 314.0695, 298.0747, 270.0790, 242.0841 and 214.0658 ($C_{19}H_{10}N_2O_3$, $C_{19}H_{10}N_2O_2$, $C_{18}H_{10}N_2O$, $C_{17}H_{10}N_2$ and $C_{16}H_8N$ require respectively *m/z* 314.0691, 298.0742, 270.0793, 242.0840, 214.0657). Calothrixin B (**2**), an amorphous orange-red solid, showed λ_{max} (EtOH or EtOH + HCl) 283, 352 and 405 nm (ε 15000, 2900, 1710), λ_{max} (EtOH + NaOH) 290, 330 (sh) and 469 nm (ε 12500, 8080, 1520); EIMS *m/z* 298.0744 ($C_{19}H_{10}N_2O_2$ requires *m/z* 298.0742), 270, 242 and 214.

The extraction of strain CAN 95/3 proceeded similarly to that described for CAN 95/2.

Single-crystal X-ray analysis

Calothrixin A crystals were obtained as very long extremely thin needles, from which a fragment of size 0.37 x 0.03 x 0.01mm was cleaved for X-ray study. Data were collected on a Rigaku AFC6R diffractometer using Cu Kα radiation from a rotating anode X-ray generator. Crystal data: $C_{19}H_{10}N_2O_3$, monoclinic space group $P2_1/c$, a = 6.887(4) Å, b = 27.444(3) Å, c = 7.240(3) Å, β = 92.33(4)°, V = 1367(1) Å^3, Z = 4, D_{calcd} = 1.527 g cm^{-1}, $F(000)$ = 648. The structure was solved by direct methods and refined on F to a final R = 0.055 on 827 reflections with $I > 2\sigma(I)$.

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