Isolation and characterisation of the monoterpenoid indole alkaloids of *Aspidosperma pyrifolium*

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Abstract Malaria is a major parasitic infection in many tropical and subtropical regions with the most severe forms of the disease being caused by *Plasmodium falciparum*. Dramatic increases in the resistance of this mosquito-transmitted parasite to classical treatments have been observed in recent years, and much research effort is now aimed at the discovery of novel natural products with antiplasmodial activities. On the basis of its use in popular medicine, *Aspidosperma pyrifolium* Mart. (Apocynaceae), a tree popularly known as "pereiro-do-sertão," was selected for detailed study. A phytochemical investigation of the aqueous extract of its stem bark revealed the presence of two known monoterpenoid indole alkaloids, 15-demethoxypyrifoline and aspidofractinine, together with the novel compound *N*-formylaspidofractinine. The structures of these compounds were established from UV, IR, MS and NMR data, and their ^{1}H- and ^{13}C-NMR spectra have been unambiguously assigned for the first time.

Keywords Apocynaceae · *Aspidosperma pyrifolium* · Malaria · Monoterpenoid indole alkaloids · *Plasmodium falciparum*

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Introduction

For thousands of years, plants and plant-derived preparations have been employed by man in the treatment of numerous and diverse medical conditions. Plants remain to this day one of the most important sources of biologically active compounds and continue to provide many of the lead molecules that facilitate the development of new drugs.

It is believed that more than half of the 500,000 plant species estimated to exist worldwide are located in tropical forests, but that less than 1% of such species have been analysed for potential

biological activity (Conte 1996). Moreover, tropical plants are typically three to four times more prolific in their production of biologically active molecules than their temperate counterparts (Rodriguez and West 1995). However, as a result of extensive deforestation occasioned by increasing agricultural activity and cattle farming, the irreplaceable tropical botanical resource is under severe threat, and there is an urgent need for the collection, conservation, documentation and analysis of the endangered species before access to this knowledge base is lost forever.

Amongst diseases that give rise to major health concern worldwide, those mediated by parasitic protozoa and helminths, including malaria, African trypanosomiasis, amebiasis, leishmaniasis, schistosomiasis and lymphatic filariasis, are particularly problematic (Nyame et al. 2004). Malaria, which is responsible for more deaths than any other communicable diseases apart from tuberculosis, is a public health problem in more than 90 countries inhabited by some 2,400 million people, i.e. 40% of the world's population. An estimated 300–500 million new cases of malarial infection occur each year resulting in the death of over 1 million individuals (Breman 2001; Greenwood et al. 2005), most of whom are African children under the age of 5 years (Winstanley 2000).

There is presently no effective vaccine available with which to treat malaria, and therapy relies mainly on two classes of antimalarial, both of which were originally based on plant secondary metabolites. The alkaloid quinine, isolated from *Cinchona* trees (Foley and Tilley 1997), was the lead molecule for the synthetic compounds chloroquine and mefloquine (Wright 2005), whilst the sesquiterpene endoperoxide artemisinin from *Artemisia annua* was the natural prototype for the development of a number of potent antimalarial drugs including artesunate (Olliaro et al. 2001) and artemether (Pittler and Ernst 1999; Fig. 1). However, the most deadly of the *Plasmodium* species that causes human malaria, *Plasmodium falciparum*, is becoming increasingly resistant to the currently available antimalarial agents. Moreover, few new antimalarial drugs are under clinical trials at this time and, hence, the identification of new compounds with activity against *P. falciparum* is of considerable
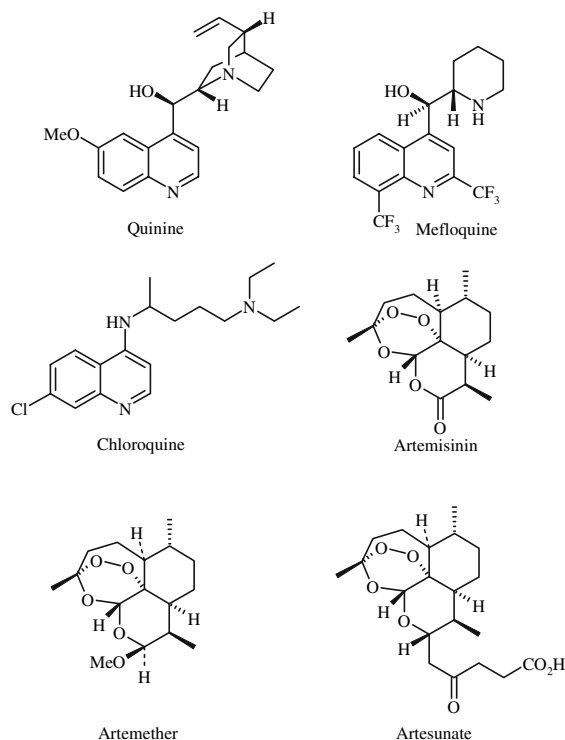

Fig. 1 Structures of some antimalarial natural products and synthetic derivatives

importance (Ridley 2002). More specifically, there is an urgent need for the development of therapies based on novel mechanisms of action (Olliaro and Yuthavong 1999; Berry 2000).

As part of our continuing research programme concerning medicinal plants used for treatment of tropical diseases, we have carried out ethnopharmacological and chemosystematic surveys of a wide variety of Brazilian species in an attempt to discover new sources of antimalarial compounds. We here report on a study of the alkaloids from *Aspidosperma pyrifolium* Mart. (family Apocynaceae), a tree that is widely distributed in the Northeast of Brazil (Craveiro et al. 1983) and popularly known as "pereiro-preto" or "pereiro-do-sertão."

The family Apocynaceae is of considerable economic and medicinal importance. Although most of the species are distributed within the tropics, a few are to be found in temperate regions, and a number are cultivated on a worldwide basis as house (e.g. *Nerium oleander* L.), garden (e.g. species of *Catharanthus*) or

greenhouse (e.g. species of *Allemanda*) plants. Whilst some tree species of this family are prized for their timber, many more are of therapeutic value by virtue of their accumulation of cardiac glycosides and alkaloids (Botanical Dermatology Database 2006). Along with several other families of the order Gentiales, including Loganiaceae and Rubiaceae, the Apocynaceae is particularly rich in monoterpenoid indole alkaloids, a chemically diverse group of compounds accounting for ca 25% of the 12,000 or so alkaloids that have been identified. The indole alkaloids are formed by the condensation of tryptophan with secologanin to yield strictosidine, and the latter may be further elaborated to produce an impressive array of structural variants including such sub-groups as the kopsane, quebrachamine, strychnos, cinchona, ajmaline, yohimbine, oxindole, aspidospermine and ajmalicine alkaloids (De Luca and St Pierre 2000; Lorence and Nessler 2004).

Amongst the genera of the Apocynaceae that have been found to accumulate indole alkaloids, the most important are *Catharanthus*, *Rauwolfia*, *Alstonia*, *Kopsia* and *Aspidosperma*. Several species of *Alstonia* are used in traditional medicine throughout Southeast Asia for the treatment of malaria and dysentery (Kam et al. 1999), whilst a number of indole alkaloids have been isolated from the root bark of *Alstonia scholaris* (L.) R. Brown (Makabeo et al. 2005).

The genus *Aspidosperma* is known to accumulate a wide variety of indole alkaloids including those with quebrachamine and aspidospermane skeletons (Deutsch et al. 1994), the latter being particularly prolific in *A. pyrifolium* Mart. and *Aspidosperma megalocarpon* Mull. Arg. (Craveiro et al. 1983; Mitaine et al. 1996, 1998). A number of species of *Aspidosperma* are used in folk medicine to treat fever in general, for example, *A. album* (Grenand et al. 1987), *A. cuspa* (Pittier 1978), *A. excelsium* (Brandão et al. 1985) and *A. vargasii* (Rutter 1990), whilst others are used specifically against malaria, for example, *A. quebracho-blanco* (Dominguez 1932; Loizaga and Sagastume 1935) and, in Brazil, *A. nitidum* (Brandão et al. 1992). The antiplasmodial properties of the aspidospermane indole alkaloids isolated from Bolivian and Colombian species of *Aspidosperma* have been reported by Mitaine

et al. (1996, 1998), and the same authors have determined the in vitro activities of 12 such indole alkaloids against chloroquine-resistant and sensitive strains of *P. falciparum* (Mitaine-Offer et al. 2002).

Materials and methods

Aspidosperma pyrifolium was collected in October 2001 in São José da Tapera, AL, Brazil, and identified by Dr J.E. de Paula (Universidade de Brasília, Brasília, DF, Brazil). A voucher specimen [number JEP3686 (UB)] was deposited in the herbarium at the Universidade de Brasília.

The dried and powdered stem bark of *A. pyrifolium* (3 kg) was extracted exhaustively with ethanol (5.5 l) in a Soxhlet apparatus for 72 h, and the methanolic solution was concentrated under reduced pressure to yield 150 g of a brownish viscous liquid. This crude methanol extract was dissolved in methanol (300 ml), water (450 ml) was added, and the resulting mixture partitioned against ethyl acetate (5 × 350 ml). The ethyl acetate layer was concentrated under reduced pressure to yield 91 g of a residue that was retained for further studies, whilst the hydromethanolic phase was lyophilised to furnish 55 g of an alkaloid-rich extract. An aliquot (2.0 g) of this extract was submitted to column chromatography of Sephadex LH-20 and eluted with methanol to afford 43 fractions of 10 ml each. The bulk of the alkaloids were present in fractions 8–14 (as determined by TLC analysis with detection by Draggendorf-reagent), and these were combined and concentrated under vacuum to yield 1.0 g of a crude alkaloid mixture. The alkaloids were separated using a Biotage SP1 Automated Flash Chromatography system (TLC-to-gradient method) employing a Flash40^{TM} + M cartridge eluted with a gradient from dichloromethane to dichloromethane:methanol (1:1) at a flow rate of 40 ml/min. The collected fractions (75 × 19 ml) were analysed by TLC and those containing a single component were combined and concentrated under reduced pressure as follows: fractions 15–25 yielded 100 mg of 2 (viscous mass), fractions 39–47 yielded 150 mg of 3 (viscous

mass) and fractions 61–74 yielded 200 mg of 1 (viscous mass).

The structures of compounds 1–3 (Fig. 2) were elucidated from UV (Cary 100 Bio UV–Vis spectrophotometer), IR (Nicolet 380 with ATR), electrospray ionisation MS (Mariner Perspective Biosystems) and HRMS (Micro-TOF, Bruker) data, in conjunction with ^{1}H- and ^{13}C-NMR spectral information determined using Bruker Avance 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) and Avance 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) instruments equipped with a 2.5-mm dual or a 5-mm BBi probe head, as appropriate. One- and two-dimensional NMR experiments were performed at a constant temperature of 25 °C with the aid of Bruker software library and modified pulse programs. Analytes were dissolved in 0.5 or 0.2 ml of deuterochloroform, and spectra were

calibrated using chloroform (residual non-deuterated solvent) as internal standard.

Results and discussion

The ^{1}H- and ^{13}C-NMR data for compounds 1–3, derived from one- and two-dimensional (DEPT, HSQC, TOCSY and ROESY) experiments, are presented in Table 1. On the basis of this information, compounds 1 and 3 could be identified as the known monoterpenoid indole alkaloids aspidofractinine and 15-demethoxypyrifoline, respectively (Fig. 2). Although the NMR spectra of these compounds have been published previously (Craveiro et al. 1983; Cartier et al. 1989; Dufour et al. 1990), these data were obtained using low-resolution instruments and hence the chemical

**Table 1** ^{1}H (500 MHz, CDCl$_3$) and ^{13}C NMR (125 MHz, CDCl$_3$) of compounds 1–3

Position	Aspidofractinine (1)		N-Formylaspidofractinine (2)		15-Demethoxypyrifoline (3)	
	^{13}C (δ)[a]	^{1}H (δ)[a] (multiplicity, J in Hz)[b]	^{13}C (δ)[a]	^{1}H (δ)[a] (multiplicity, J in Hz)[b]	^{13}C (δ)[a]	^{1}H (δ)[a] (multiplicity, J in Hz)[b]
2	64.9		66.0		71.9	
3	48.0	3.10 (m);[c] 3.00 (dt, 3.5, 14.0)	46.9	3.03 (m)[c]	47.4	3.07 (br td, 4.0, 14.0)[c,d]
5	51.0	3.24 (q, 8.6); 3.08 (m)[c]	50.1	3.24 (m);[c] 3.02 (m)[c]	50.8	3.27 (t 8.6)
6	34.4	2.72 (ddd, 3.3, 8.6, 14.0); 1.67 (br dd)[c]	34.8	2.50 (ddd, 3.0, 8.6, 14.0); 1.63 (m)[c]	34.3	2.66 (br s);[c,d] 1.86 (m)[c]
7	57.4		57.4		60.4	
8	139.6		141.2		145.9	
9	122.3	7.36 (d, 7.3)	121.7	7.41 (d, 7.6)	115.1	7.15 (d, 7.5)
10	119.9	6.78 (t, 7.3)	125.6	7.09 (dd, 7.6, 8.1)	126.7	7.09 (dd, 7.5, 8.3)
11	126.8	7.00 (t, 7.3)	127.6	7.19 (dd, 8.1, 8.4)	111.3	6.78 (d, 8.3)
12	110.8	6.64 (d, 7.3)	117.2	7.90 (d, 8.4)	149.5	
13	149.8		139.7		130.8	
14	16.8	1.83; 1.31 (m)[c]	15.7	1.83; 1.35 (m)[c]	16.9	1.82; 1.36 (m)[c]
15	35.1	1.42; 1.28 (m)[c]	34.0	1.42; 1.22 (m)[c]	29.0	1.85; 1.46 (m)[c]
16	29.5	2.21; 1.28 (m)[c]	24.9	2.24; 2.09 (m)[c]	26.1	2.72 (br s);[c,d] 2.27 (m)[c]
17	26.4	2.19; 1.73 (m)[c]	24.6	1.55 (m)[c]	25.8	1.65 (m)[c]
18	31.3	1.82; 1.28 (m)[c]	34.5	2.22 (m)[c]	36.3	2.27 (m)[c]
19	35.7	1.52; 1.28 (m)[c]	35.0	1.53; 1.28 (m)[c]	35.7	1.50; 1.27 (m)[c]
20	31.2		31.7		31.2	
21	69.0	3.12 (s)	67.7	3.14 (s)	68.1	3.13 (s)
NH		3.40 (br s)[d]				
NCHO			158.1	8.53 (s)		
NCOCH$_3$					171.0	
NCOCH$_3$					25.1	2.15 (s)
OCH$_3$					55.8	3.83 (s)

[a] Chemical shifts in ppm

[b] For each methylene carbon, the HSQC spectrum showed correlations for two protons

[c] Deformed/superimposed signals

[d] Deformed/broadened signals
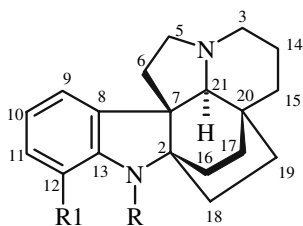
R = R$_1$ = H   Aspidofractinine (**1**)

R = CHO, R$_1$ = H   *N*-Formylaspidofractinine (**2**)

R = COCH$_3$, R$_1$ = OCH$_3$ 15-Demethoxypyrifoline (**3**)

Fig. 2 Structures of aspidofractinine (1), *N*-formylaspido-fractinine (2) and 15-demethoxypyrifoline (3)

shift values for the methylene protons and for the carbon atoms could not be assigned unequivocally.

Compound 2 (Fig. 2) presented a molecular formula of $C_{20}H_{25}N_2O$ (M + 1) cal.: 309.1961, Ob.: 309.1959 by HRMS and a UV spectrum that was quite similar to those of 1 and 3. The IR spectra of 1–3 were comparable, except that those of 2 and 3 exhibited signals at 1,678 and 1,674 cm^{-1}, respectively, associated with carbonyl groups. The ^{1}H and ^{13}C-NMR spectra of 2 were very similar to those of 1 except for the proton and carbon signals at δ 8.53 s and δ 158.1, respectively (Table 1). Compound 2 was identified as *N*-formylaspidofractinine. The occurrence of formyl derivatives in this class of alkaloids is already known (Bolzani et al. 1987; Djerassi et al. 1963; Gilbert et al. 1962).

Conclusion

A phytochemical investigation of the alkaloids of *A. pyrifolium* furnished aspidofractinine (1), 15-demethoxypyrifoline (3) and the new derivative *N*-formylaspidofractinine (2). Although the NMR data for 1 and 3 have been previously reported, the ^{1}H and ^{13}C have been unambiguously assigned for the first time in the present work. The antiplasmodial activities of these compounds are currently being determined in our laboratories and the results will be presented soon.

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