Artigo

# PHAEOPHYTINS FROM *Thyrsacanthus ramosissimus* Moric. WITH INHIBITORY ACTIVITY ON HUMAN DNA TOPOISOMERASE II-α[#]

**Analúcia Guedes Silveira Cabral, Fábio Henrique Tenório-Souza, Marcelo Dantas Moura, Sabrina Gondim Ribeiro Mota, Antônio Cláudio da Silva Lins, Celidarque da Silva Dias e José Maria Barbosa-Filho***
Departamento de Ciências Farmacêuticas, Universidade Federal da Paraíba, CP 5009, 58051-970 João Pessoa – PB, Brasil
**Ana Maria Giulietti**
Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, 44036-900 Feira de Santana – BA, Brasil / Royal Botanic Gardens, Kew, TW9 3AB, UK
**Tania Maria Sarmento da Silva**
Departamento de Ciências Moleculares, Universidade Federal Rural de Pernambuco, 52171-900 Recife – PE , Brasil
**Creusioni Figueredo dos Santos**
Departamento de Biologia Molecular, Universidade Federal da Paraíba, 58051-900 João Pessoa – PB, Brasil

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Our study reports the extraction and isolation of a new phaeophytin derivative 15[1]-hydroxy-(15[1]-*S*)-porphyrinolactone, designated anamariaine (**1**) herein, isolated from the chloroform fraction of aerial parts of *Thyrsacanthus ramosissimus* Moric. along with the known 15[1]-ethoxy-(15[1]-*S*)-porphyrinolactone (**2**). These compounds were identified by usual spectroscopic methods. Both compounds were subjected to *in vitro* (inhibitory activity) tests by means of supercoiled DNA relaxation techniques and were shown to display inhibitory activity against human DNA topoisomerase II-α at 50 μM. Interconversion of these two pigments under the mild conditions of the isolation techniques should be highly unlikely but cannot be entirely ruled out.

Keywords: *Thyrsacanthus ramosissimus*; phaeophytins; topoisomerase activity.

## INTRODUCTION

The family Acanthaceae comprises around 250 genera and approximately 2500 species,[1] which occur mainly in the Atlantic Forest and in the mesophilic forest formations of the Central-Western and Southeastern regions of Brazil, as well as in other types of vegetation.[2] Previous studies on the isolation of natural products from the family Acanthaceae have revealed the presence of alkaloids,[3] flavonoids,[4] terpenes,[5] coumarins,[6] lignans,[7] and quinoids.[8] These constituents have been demonstrated to have biological effects such as cytotoxicity,[7] vasorelaxant,[5] anti-inflammatory,[9] antifungal,[5] and antiviral actions,[10] as well as CNS depression and immunosuppressive activities.[9,11]

*Thyrsacanthus* Moric. is a genus with five species whose presence is restricted to South America and that occurs mainly in Brazil. Recently, the genus was restored to include the South American species traditionally assigned to *Anisacanthus* Nees. *Thyrsacanthus ramosissimus* Moric. (= *Anisacanthus brasiliensis* Lindau) is popularly known as "canudo" and is endemic to Brazil, in the states of Alagoas, Bahia, Minas Gerais, Pernambuco, and Rio Grande do Norte. It can be found principally in seasonally dry vegetation and in semideciduous and riparian forests.[12] No biological studies have been previously performed with this species and chemical and biological studies with species of this genus are also scarce.

Interest in compounds with inhibitory activity against the enzyme DNA-topoisomerase II-α has increased,[13-20] because intracellular levels of topoisomerase II-α (topo II-α) are elevated in a number of human tumors as compared to the respective normal tissues. Moreover, the development of drugs that can affect the DNA replication process by selectively interfering with the function of topo II-α continues to draw researchers' attention.[21] Topo II-α is an essential enzyme that plays a key role in DNA replication, but is also important in repair, transcription, and chromosome segregation. Topo II-α changes DNA topology by passing an intact DNA double helix through a transient double-stranded break, thereby producing another helix.[22,23] Thus, as part of our ongoing chemical investigations of this plant species,[24] in this work we evaluate the inhibitory effect of this plant on human DNA-topoisomerase II-α. The isolation of a new phaeophytin (**1**), as well as its structural elucidation by means of ESI-MS and 1D- and 2D-NMR experiments, will be reported here.

## EXPERIMENTAL

### General experimental procedures

Melting points were determined on a Koefler hot stage and are uncorrected. The infrared absorption spectra were recorded in KBr pellets, using a Bomem/MB-102 spectrophotometer operating in the 4000-400 $cm^{-1}$ range. The LC-MS spectra were obtained in the positive electrospray mode using a Quattro LC-Micromass device (Waters). Silica gel 60 was used for column chromatography, and Kieselgel 60$F_{254}$ (E. Merck) was employed for preparative TLC as precoated plates. Sephadex LH-20 (Sigma) was utilized for gel permeation chromatography. $^{1}H$ and $^{13}C$ NMR spectra were acquired on a Bruker AC 200 spectrometer (200 MHz for $^{1}H$ and 50 MHz for $^{13}C$), in $CDCl_3$.

### Plant material

The aerial parts of *Thyrsacanthus ramosissimus* Moric. were collected in the city of Rio de Contas, state of Bahia, Brazil, in March 2006. The plant was identified by Dr. A. M. Giulietti. A voucher specimen (Tombo HUEFS 59791) was deposited at the Herbarium of Universidade Estadual de Feira de Santana.

*e-mail: jbarbosa@ltf.ufpb.br
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**Extraction and isolation**

The powdered plant material (5.0 kg) was extracted successively with EtOH, to give 310.0 g dry extract. This extract was dissolved in MeOH/$H_2O$ (3:7) and successively fractionated with hexane, $CHCl_3$, and AcOEt, yielding the hexane (20.0 g), $CHCl_3$ (10.0 g), and EtOAc (5.0 g) fractions. The $CHCl_3$ fraction was successively submitted to column chromatography using silica gel and Sephadex LH-20, followed by preparative TLC on silica, to yield **1** (29.2 mg) and **2** (27.8 mg) as dark blue amorphous solids. Analysis of the spectroscopic data (IR, LC-MS and NMR spectra, including 2D NMR) led to identification of the following compounds: anamariaine ($15^1$-hydroxy-($15^1$-*S*)-porphyrinolactone, (**1**) and $15^1$-ethoxy-($15^1$-R)--porphyrinolactone (**2**).

*Anamariaine (**1**)*

Dark blue amorphous solid; mp 171.0 ºC; IR (KBr) $\nu_{max}$ 3549, 3476, 3414, 1737, 1638, 1615 $cm^{-1}$. $^1H$ NMR ($CDCl_3$, 200 MHz) (Table 1), $^{13}C$ NMR ($CDCl_3$, 50 MHz) (Table 1), ESI-MS (pos) *m*/*z* 903.99 $[M+H]^+$ ($C_{55}H_{74}N_4O_7$).

*$15^1$-Ethoxy-($15^1$-R)-porphyrinolactone (**2**)*

Dark blue amorphous solid; mp 114.0 ºC; IR (KBr) $\nu_{max}$ 3447, 2927, 1734, 1636, 1104 $cm^{-1}$, ESI-MS (pos) *m*/*z* 931.59 $[M+H]^+$ ($C_{57}H_{79}N_4O_7$).

***In vitro* assay for DNA topoisomerase II-α**

The conversion of pBR322 supercoiled plasmid DNA to the relaxed form by topo II-α was examined in the presence of phaeophytins **1** and **2**. DNA topoisomerases are enzymes that modulate the topological state of DNA and are targets for many active drugs in cancer treatment.[15,23]

Enzymatic activity was analyzed by the DNA relaxation assay

**Table 1.** $^1H$ (200 MHz) and $^{13}C$ (50 MHz) spectral data for anamariaine (**1**) obtained by heteronuclear 2D shift-correlated HSQC, HMQC, and HMBC spectra, in $CDCl_3$. Chemical shifts (δ, ppm) and coupling constants (*J* in Hz, in parenthesis)[a]

| Porphyrin moiety | | | Phytol moiety | | |
|---|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ | Position | $\delta_C$ | $\delta_H$ |
| 1 | 141.18 | | P1 | 61.45 | 4.52 (m) |
| 2 | 131.45 | | P2 | 117.70 | 5.20 (t, 7.0) |
| $2^1$ | 12.13 | 3.42 (s) | P3 | 142.86 | |
| 3 | 135.98 | | $P3^1$ | 16.27 | 1.60 (s) |
| $3^1$ | 128.93 | 8.05 (dd, 18.0; 12.0) | P4 | 39.77 | 1.93 (m) |
| $3^2$ | 122.71 | (*E*) 6.37 (dl, 18.0)<br>(*Z*) 6.24 (dl, 7.8) | P5 | 24.95 | 1.30 (m) |
| 4 | 135.98 | | P6 | 29.69 | 1.30 (m) |
| 5 | 99.59 | 9.68 (s) | P7 | 32.73 | 1.55 (m) |
| 6 | 155.71 | | $P7^1$ | 19.71 | 0.84 (d, 6.6) |
| 7 | 136.46 | | P8 | 37.36 | 1.04 (m) |
| $7^1$ | 11.24 | 3.22 (s) | P9 | 24.39 | 1.30 (m) |
| 8 | 145.51 | | P10 | 36.59 | 1.21 (m) |
| $8^1$ | 19.52 | 3.82 (m) | P11 | 32.58 | 1.35 (m) |
| $8^2$ | 17.58 | 1.68 (t, 8.0 Hz) | $P11^1$ | 19.71 | 0.81(d, 6.6) |
| 9 | 149.94 | | P12 | 37.28 | 1.24 (m) |
| 10 | 104.10 | 9.91 (s) | P13 | 24.76 | 1.30 (m) |
| 11 | 138.70 | | P14 | 39.32 | 1.48 (m) |
| 12 | 134.75 | | P15 | 27.94 | 1.52 (m) |
| $12^1$ | 12.43 | 3.87 (s) | $P15^1$ | 22.70 | 0.88 (d, 6.6) |
| 13 | 111.29 | | P16 | 22.70 | 0.88 (d, 6.6) |
| $13^1$ | 166.30 | | | | |
| 14 | 135.98 | | | | |
| 15 | 100.43 | | | | |
| $15^1$ | 101.95 | | | | |
| $15^2$ | 170.87 | | | | |
| $15^3$ | 54.15 | 3.75 (s) | | | |
| 16 | 161.06 | | | | |
| 17 | 53.68 | 4.16 (d, 8.7) | | | |
| $17^1$ | 32.13 | $17^1$ B 1.81 (m)<br>$17^2$ A 2.63 (m) | | | |
| $17^2$ | 31.29 | $17^2$ B 2.22 (m)<br>$17^2$ A 2.49 (m) | | | |
| $17^3$ | 173.30 | | | | |
| 18 | 50.12 | 4.51 (m) | | | |
| $18^1$ | 22.60 | 1.62 (d, 8.0 Hz) | | | |
| 19 | 171.12 | | | | |
| 20 | 93.87 | 8.86 (s) | | | |

[a] 2D homonuclear $^1H$-$^1H$-COSY and heteronuclear HMBC spectra were also used for these assignments. Hydrogen atoms chemical shifts obtained from $^1D$ $^1H$ NMR spectra. Carbon signals corresponding to C, CH, $CH_2$, and $CH_3$ as deduced by comparative analysis of the APT and HSQC spectra. Superimposed $^1H$ signals are described without multiplicity and chemical shifts were deduced by HSQC, HMBC, and $^1H$-$^1H$-COSY.

according to the protocol described by USB Corporation (USB Corporation, Cleveland, OH, USA). One unit of topo II-α (USB Corporation, Cleveland, OH, USA) was incubated with 0.152 μg pBR322 DNA (human recombinant from *E. coli*, Invitrogen) and with 100 or 50 μM of compound **1** or **2**, or without the test compounds, in 10 μL reaction mixture containing 10 mM Tris, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL BSA, 1 mM ATP, 10 mM $Na_2HPO_4$, and 0.2 mM DTT for 40 min, at 37 °C. The reaction was terminated by addition of 1μL stop solution consisting of 50% glycerol, 10% sodium dodecyl sulfate (SDS), and 25 % bromophenol blue. Electrophoresis was carried out on 1% agarose gel (Sigma-Aldrich) equilibrated with TAE buffer (4.84 g $L^{-1}$ Tris-base, pH 8.5, 1.14 g $L^{-1}$ glacial acetic acid and 100 mL of 0.74 g $L^{-1}$ EDTA) for 120 min at 40 V. Etoposide was used as the positive control. The gels were stained with ethidium bromide solution (5 g $L^{-1}$) after electrophoresis for 30 min, washed with water, and photographed under UV light with a digital camera.

## RESULTS AND DISCUSSION

Extensive chromatography of the $CHCl_3$ fraction of *Thyrsacanthus ramosissimus* resulted in the isolation and characterization of two phaeophytins **1** and **2** (Figure 1). Compound **1** was isolated as a dark blue amorphous solid. ESI-MS analysis in the positive ionization mode resulted in $[M+H]^+$ *m/z* 903.99, indicating 21 degrees of insaturation for a molecular formula of $C_{55}H_{74}N_4O_7$. The IR absorptions at 3549, 3476, 3414, 1737, and 1615 $cm^{-1}$ and UV data, and overall color characteristics suggested the presence of a large chromophore related to the chlorophyll derivative containing a hydroxyl group.

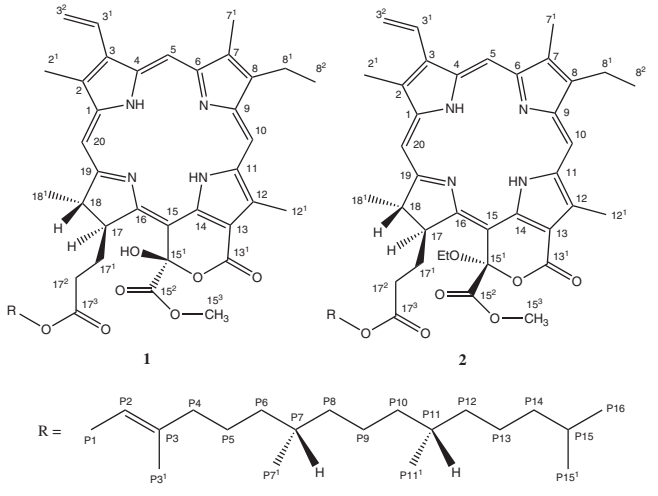


***Figure 1.*** *Structures of anamariaine (**1**) and 15¹-ethoxy-(15¹-R)-porphyrinolactone (**2**)*

The 200 MHz $^1$HNMR spectrum displayed all the resonances of phaeophytin A and its derivatives:[25] three aromatic methyl groups ($\delta_H$ 3.95, 3.48, and 3.33), a methoxyl group ($\delta_H$ 3.79), three singlet olefinic protons ($\delta_H$ 9.91, 9.68, and 8.86), vinyl substitution ($\delta_H$ 6.37, 6.24, and 8.05) with a characteristic exomethylene coupling pattern, and two singlets at $\delta_H$ 1.50 and 1.41 indicative of two pyrrole NH functionalities within the shielding ring current.[26] The attached phytol moiety was recognized by a large number of overlapping proton signals of aliphatic methylene and methyl functions. Proton resonances suggesting the phytol moiety were the carbinol resonances $CH_2$-P1 ($\delta_H$ 4.51), the olefinic proton H-P2 ($\delta_H$ 5.20) and $CH_3$-P3$^1$ ($\delta_H$ 1.6) (Figure 1).

Thirty-five carbon signals were detected in the $^{13}$C NMR spectrum for the porphyrin system of compound **1**, while the aliphatic phytol moiety afforded a partial overlapping set of signals in the aliphatic region. Overall, the $^1$H and $^{13}$C-NMR spectra were in accordance with the literature data.[27,28]

The presence of the hydroxyl group in ring E was deduced from the carbinol resonance C-15$^1$ at $\delta_C$ 101.95, with a quaternary nature confirmed by the APT and HSQS experiment. The NMR and ESI-MS data, together with the degrees of insaturation, allowed us to establish a conjugated d-lactone structure, which appeared to be formed between the C-13$^1$-C-13$^2$ bond at ring E of 13$^2$-hydroxyphaeophytin A. There was evidence of a carbonyl carbon at $\delta_C$ 166.30 instead of the resonances at $d_C$ 192.2 for the corresponding carbon.[29]

The complete analysis of the porphyrin structure was conducted on the basis of the correlations detected in the $^{2,3}J_{CH}$ long-range of the olefinic protons H-5, H-10, H-20, which provide the connectivities between the four pyrrole rings (Figure 2). The signal corresponding to H-5 ($\delta_H$ 9.68) correlated with C-7 in ring B. Similarly, H-10 ($\delta_H$ 9.91) presented connectivity with C-8 (ring B) and C-11 in ring C, while H-20 ($d_H$ 8.86) correlated with C-1 and C-2 (ring A). The signal relative to 3H-18$^1$ ($\delta_H$ 1.62) in ring D showed long-range connectivity with C-19, and H-18 ($\delta_H$ 4.51) correlated with C-16. The $^3J_{CH}$ correlation of O<u>C</u>H$_3$-15$^3$ ($d_H$ 3.75) with the carboxyl group C-15$^2$ confirmed the position of the methyl-ester. The presence of the phytol-moiety attachment to the porphyrin system was indicated by the $^2J_{CH}$ correlation of 17$^2$-B ($\delta_H$ 2.22) and 17$^2$-A (2.49) to the carboxyl function C-17$^3$ ($\delta_C$ 173.30).

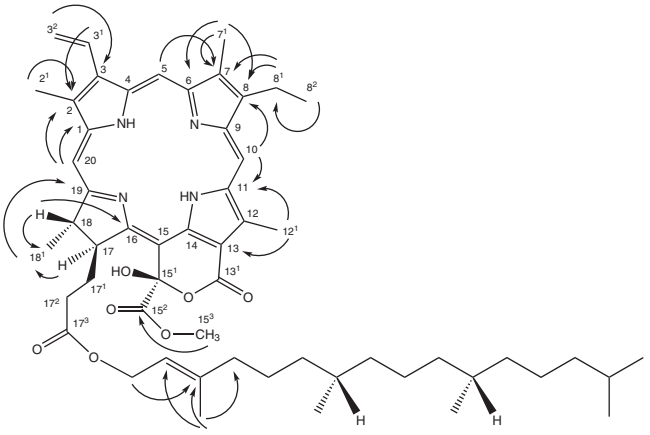

***Figure 2.*** *Structure corresponding to long-range HC-correlation signals observed in the HMBC of compound **1***

The configuration of C-15$^1$ was determined as *S* due up-field of H-17 ($d_H$ 4.16),[30] since the Nuclear Overhauser enhancements in the NOESY spectrum did not show signals referring to this chiral center. Other significant signals were observed in the porphyrin system (Figure 3). Therefore, the structure of compound **1** was identified as 15$^1$-hydroxy-(15$^1$-*S*)-porphyrinolactone (anamariaine). This is its first isolation as a natural compound.

Compound **2** displays identical signals as compared to compound **1**, except for an additional ethoxy group identified from $^1$H resonances at $d_H$ 4.36 (q, *J* = 7.1 Hz, H-2-1") and 1.50 (t, *J* = 7.10 Hz, H3-2") and the respective carbons at $d_C$ 62.44 and 15.62. The ethoxy group was deduced as being positioned at C-15$^1$ on the basis of the HMBC correlation between H2-1" and C-15$^1$ ($\delta_C$ 106.31). The spectral data of compound **2**, including the chiral center at C-15$^1$ ($\delta_H$ 4.82), agrees with those described in the literature and allows for its identification as 15$^1$-ethoxy-(15$^1$-*R*)-porphyrinolactone.[31] This compound has been previously isolated from the green alga *Cladophora fascicularis*.[31]

The isolation of phaeophytins from *Thyrsacanthus ramosissimus* raises the question as to whether this material occurs naturally or is

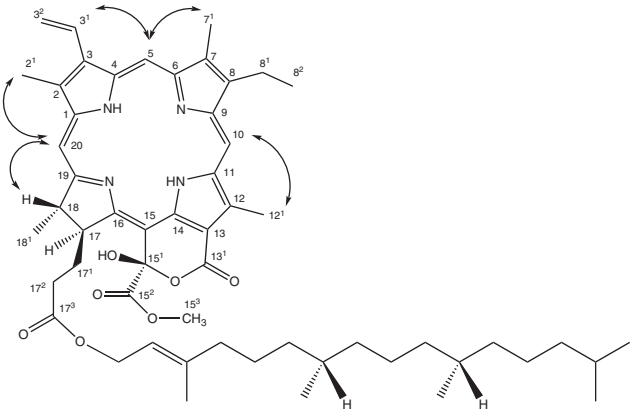

***Figure 3.*** *Structure corresponding to NOESY correlation signals observed in compound* ***1*** *showing proton-proton through-space interactions in the porphyrin-ring system*

a possible artifact produced during chromatographic separation. Our experience suggests that this rare pigment is not commonly observed in plant species.[32,33] Comparison between the structures of compounds **1** and **2** shows that an interconversion of these two pigments under the mild conditions of the isolation techniques should be highly unlikely.

The effects of the compounds on the catalytic activity of the DNA topo II-a enzyme were observed in the relaxation assays using pBR322 in the presence of ATP. Compounds **1** and **2** were evaluated at 50 μM (lanes 4 and 5, respectively) (Figure 4). The two compounds promoted significant inhibition of the catalytic activity of topo II-α as compared to etoposide, which was used as the positive control.

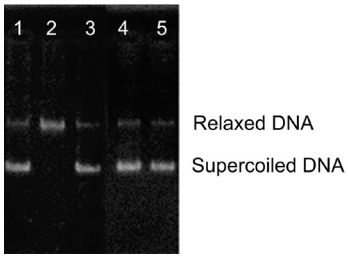


***Figure 4.*** *All lanes contain 0.152 μg DNA (pBR322) and 1.0 unit of topo II-a, with the exception of Lane 1. Lane 1: negative control (pBR322 only). Lane 2: positive control (pBR322 and topo II-a). Lane 3: etoposide (100 mM). Lane 4: compound* ***1*** *(50 μM). Lane 5: compound* ***2*** *(50 μM)*

## SUPPLEMENTARY MATERIAL

$^{1}$H and $^{13}$C NMR, HMQC, COSY, HMBC, NOESY, IR, and ESI-MS spectra of compound **1** are available at http://quimicanova.sbq.org.br, in PDF file, with free access.

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