

Fluorescence, circular dichroism, NMR, and docking studies of the interaction of the alkaloid malbrancheamide with calmodulin

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Abstract:

A new malbrancheamide analogue, isomalbrancheamide B (**3**), along with three known compounds, malbrancheamide (**1**), isomalbrancheamide (**2**), and premalbrancheamide (**4**), were isolated in higher yields from the alkaloid fraction of the fungus *Malbranchea aurantiaca*. The interaction of the alkaloids **1–4** with calmodulin (CaM) was analyzed using different enzymatic, fluorescence, spectroscopic, nuclear magnetic resonance (NMR), and molecular modelling techniques. On the basis of the enzymatic and fluorescence experiments, malbrancheamides **1–3** are classical CaM inhibitors. Compound **4**, however, did not quench the extrinsic fluorescence of the CaM biosensor indicating that it could be a functional inhibitor. Circular dichroism, NMR, and molecular modelling studies revealed that **1** binds to CaM in the same hydrophobic pocket than the chlorpromazine and trifluoperazine, two classical CaM inhibitors. Thus, malbrancheamide and related monochlorinated analogues are compounds with a high potential for the development of new therapeutic agents, involving CaM as their molecular target.

Keywords: *Malbranchea aurantiaca* | calmodulin | fluorescence | circular dichroism | NMR titration | docking

Article:

Introduction

Calmodulin (CaM), the primary transducer of Ca²⁺-mediated signals in eukaryotes, is a small multifunctional protein of 148 amino acids, highly conserved through evolution. The crystal structure of this protein revealed a dumbbell-shaped molecule with two globular domains connected by a flexible linker^{1–3}. Each domain consists of two-loop-helix Ca²⁺-binding regions referred to as EF-hand structures, which expose hydrophobic surfaces. These hydrophobic regions interact with a large number of regulatory proteins including phosphatases, kinases, and

ion channels. As a consequence, CaM plays an important role in muscle contraction, intracellular movement, DNA repair, cell proliferation, apoptosis, and short- and long-term memory, among other important physiological events⁴⁻⁶.

The regulatory properties of CaM can be inhibited by a wide range of drugs and peptides, which modify its function by blocking its interaction with other proteins. Such is the case of the antipsychotics phenothiazines trifluoperazine (TFP) and chlorpromazine (CPZ), which bind to CaM through hydrophobic interactions with high affinity and known stoichiometry⁷⁻⁹. Other compounds, however, bind to CaM with lower specificity and uncertain stoichiometry^{10,11}. In any case, CaM inhibitors might have important pharmacological repercussions thus representing important leads for the development of new drugs; on the other hand, the analysis of such events are important for the study of protein interactions and could help to understand better the physiological role of CaM.

As part of our efforts to search for new potent and specific CaM inhibitors, we previously reported the isolation and structure elucidation of two novel indole alkaloids, namely malbrancheamide (**1**) and malbrancheamide B (**2**), from both the mycelium and culture broth of the fungus *Malbranchea aurantiaca* Sigler & Carmichael (*Myxotrichaceae*)^{12,13}. Compounds **1** and **2** inhibited the activation of the CaM-deficient-CaM-dependent cyclic adenosine monophosphate (*c*AMP) phosphodiesterase (PDE1), such as CPZ. According to a kinetic analysis, alkaloid **1** competitively inhibits the regulation of PDE1 by CaM in a concentration dependent manner. Furthermore, several structural analogues of the malbrancheamides, prepared while attempting the synthesis of **1** and **2**, were functionally tested for their ability to inhibit PDE1¹⁴. The relative stereochemistry at C-12a, the bicyclo[2.2.2]diazaoctane core oxidation state, and the indole chlorination of these analogues were found to have an important impact on the enzymatic activity of the complex CaM-PDE1¹⁴. However, none of the tested compounds was more active than **1**.

Since **1** is a potent inhibitor of the complex CaM-PDE1, the main goal of the present investigation was to determine the nature of its inhibition (classical vs. functional), in solution, using fluorescence, circular dichroism (CD), and nuclear magnetic resonance (NMR) titration experiments, as well as molecular modelling studies.

Materials and methods

General experimental procedures

Infrared (IR) spectra were taken on a Perkin-Elmer 599B spectrophotometer in KBr disks. CD was recorded on a JASCO J-715 spectrometer equipped with a thermostated cell-holder under N₂ atmosphere at 37°C. 1D and 2D NMR spectra were recorded with a Varian Innova 500 or on a Varian Unity plus 400 spectrometers at 500 MHz (¹H) or 125 MHz (¹³C), and 400 MHz (¹H) or 100 MHz (¹³C), respectively. High resolution mass spectra by fast atom bombardment (HRMS-FAB⁺) were measured on a JEOL JMSAX505HA mass spectrometer. Melting points were obtained with a Fisher–Johns melting point apparatus and are reported uncorrected. Semi-preparative high-performance liquid chromatography (HPLC) was performed using a Symmetry C₁₈ (7 μm, 7.8 × 300 mm; Waters, Milford, MA, USA), a Nucleosil C₁₈ (5 μm, 10 × 250 mm;

Phenomenex, Torrance, CA, USA), or a Purospher STAR RP-18e (5 μm , 10 \times 250 mm; Merck, Darmstadt, Germany) columns. Control of the equipment, data acquisition, processing, and management were performed by the Empower 2 software (Waters). Open column chromatography (CC) was carried out on Si-gel 60 (0.063–0.200 mm), 70-230 Mesh (Merck, Darmstadt, Germany).

Fungal material

M. aurantiaca Sigler & Carmichael (*Myxotrichaceae*) was collected from bat detritus in the Juxtlahuaca Caves, Guerrero, Mexico, in December 2002. A voucher specimen (# 24428) of *M. aurantiaca* was deposited in the mycological collection of the Laboratorio de Micopatología, Instituto de Biología, UNAM.

Extraction and isolation

Forty litres of potato dextrose broth (PDB; Difco, Detroit, MI, USA) were inoculated with 10 cm² agar plugs, taken from a stock culture of *M. aurantiaca* maintained at 4°C on potato dextrose agar (Difco). Culture broth was incubated at room temperature for 30 days. After incubation, the fermented whole broth (40 L) was filtered through cheesecloth to separate into supernatant and mycelia. The former was extracted exhaustively with CH₂Cl₂ (3 \times 40 L). The combined organic phase was dried over anhydrous Na₂SO₄ and concentrated *in vacuo* to give a dark brown solid (9 g). The mycelium was macerated with CH₂Cl₂ (3 \times 10 L). After evaporating the solvent *in vacuo*, 12.0 g of combined mycelia and culture extract were obtained. Six grams of the extract were subjected to Si-gel CC with a hexane-CH₂Cl₂-MeOH gradient to yield 19 primary fractions (F₁-F₁₉). Fraction F₁₄ (312 mg) was further resolved by preparative HPLC (Symmetry C₁₈, MeCN, flow rate 2.0 mL/min) to yield compounds **1** (93.4 mg; *R_T* 6.1 min), **4** (8.0 mg; *R_T* 5.0 min), and a mixture of **2** and **3** (47.6 mg). The latter was separated on a Nucleosil C₁₈ column eluting with MeOH (flow rate 3.5 mL/min) to afford pure compounds **2** (30.0 mg; *R_T* 4.9 min) and **3** (5.0 mg; *R_T* 5.3 min). The remaining part of the crude extract (6 g) was submitted to an acid-base treatment as follows: the extract was dissolved in CH₂Cl₂ (2 L) and extensively partitioned with HCl 1N (4 \times 2 L); then, the acid extracts were basified with NH₄OH 1N until pH 9, and extracted with CH₂Cl₂ (4 \times 2 L). The resulting organic fractions were dried over Na₂SO₄ and concentrated *in vacuo* to yield 591 mg of crude alkaloidal fraction (FA). FA was resolved on a reverse phase Purospher STAR column eluting with MeCN (flow rate 2.0 mL/min) to yield compounds **1** (199.2 mg; *R_T* 7.4 min), **4** (12.4 mg; *R_T* 5.7 min), and a mixture of **2** and **3** (71.1 mg). The latter was resolved using a Nucleosil C₁₈ column eluting with MeOH (flow rate 2.0 mL/min) to give **2** (19.2 mg; *R_T* 7.05 min) and **3** (40.6 mg; *R_T* 7.6 min).

Isomalbrancheamide B (**3**). White solid; m.p. 319–321°C; ultraviolet (UV)/vis (MeOH): λ_{max} (log ϵ) = 233 (3.90), 284 (4.70) nm; IR (KBr): ν_{max} = 3296, 2954, 2920, 1737, 1657, 1460, 1313, and 1240 cm⁻¹; ¹H-NMR (400 MHz, MeOH-*d*₄) δ_{H} : 7.32 (d, *J* = 2 Hz; H-7), 7.22 (d, *J* = 8.6 Hz; H-10), 7.00 (dd, *J* = 2.6, 8.6 Hz; H-9), 3.42 (d, *J* = 10.0 Hz; H-5_B), 3.05 (m; H-3_A), 2.86 (m; H-6_A), 2.85 (m; H-6_B), 2.51 (m; H-1_A), 2.26 (dd, *J* = 2.0, 10.0 Hz; H-5_A), 2.15 (q, *J* = 2.0, 5.0 Hz; H-3_B), 2.14 (m; H-12_A), 1.99 (m; H-13_A), 1.94 (m; H-13_B), 1.86 (m; H-2), 1.46 (m; H-1_B), 1.42 (s; H-17), 1.32 (s; H-16); ¹³C-NMR (100 MHz; MeOH-*d*₄) δ_{C} : 23.6, 24.2, 27.8, 28.2, 30.3, 30.8,

32.6, 49.8, 52.9, 55.3, 59.5, 66.1, 104.9, 111.6, 117.9, 120.0, 123.0, 125.3, 135.6, 146.7, 176.7; HRMS-FAB⁺: m/z 369.1589, calculated for C₂₁H₂₄N₃OCl: 369.1608.

Protein purification

Unlabelled and fluorescent-engineered human calmodulin (*hCaM*) were modified, over-expressed, and purified from *Escherichia coli* strain BL21-A1, as previously described by González-Andrade et al.¹⁵. Uniformly ¹⁵N and ¹³C labelled *hCaM* were prepared in a salts minimal M9 medium containing ¹⁵NH₄Cl (0.5 g/L) and ¹³C₆-glucose (1.5 g/L), following the same procedure as for the unlabelled protein.

PDE1 activity assay

PDE1 activity was measured according to the method described by Rivero et al. with some modifications¹⁶. Briefly, CaM (0.08 μg) was incubated with 0.015 units of PDE1 from bovine brain (Sigma, St. Louis, MO, USA), during 30 min in 40 μL of assay solution containing: 0.063 units of 5'-nucleotidase (*Crotalus atrox* venom; Sigma), 45 mM Tris-HCl, 5.6 mM magnesium acetate, 45 mM imidazole, 2.5 mM calcium chloride, and 10 mM bovine serum albumin, pH 7.0. Tested compounds were then added to the assay medium at 0.5, 1, 2, 3, 4, 7, 13, 20, 32, 50, and 65 mM in MeCN-water (1:1), and the samples incubated during 30 min; thereafter 10 μL of 10.8 mM *c*AMP were added to start the assay. After 15 min, the assay was stopped by the addition of 190 μL of malachite green solution. The amount of inorganic phosphate released was measured at 700 nm, and correlated with the activity of the PDE1. CPZ was used as positive control. All the results are expressed as the mean of at least six experiments ± SEM. The IC₅₀ (concentration inhibiting by 50% the activity of the enzyme) values were determined by non-linear regression analysis by fitting to hyperbolic inhibition.

Steady-state fluorescence

All experiments were performed on an ISS-PC1 spectrofluorophotometer (ISS, Champaign, IL). Protein-specific intrinsic fluorescence of CaM (5 μM) in phosphate buffer (100 mM, pH 5.1) with CaCl₂ (10 mM) was scanned between 400 and 550 nm at 381 nm of excitation. Each sample was stirred and titrated with compounds 1–4 to a different final protein/inhibitor ratio. The samples were contained in a 2 mL thermostat container with excitation and emission path lengths of 1 nm, at 37°C. Fluorescence emission spectra were acquired with excitation and emission slit widths of 4 and 8 nm, respectively. The apparent dissociation constant (K_d) and fractional degree of saturated CaM with ligand (v_0) values were determined by fitting to the following second order equation:

$$v_0 = \frac{(1 + k_d/P_0 + L_0/P_0) - \sqrt{(1 + k_d/P_0 + L_0/P_0)^2 - 4L_0/P_0}}{2}$$

where P_0 and L_0 are the total concentrations of the protein and inhibitor, respectively, and K_d is the apparent dissociation constant for the ligand. CPZ was used as positive control. All data were analyzed using the Origin version 8.0 program (OriginLab, Northampton, MA).

Circular dichroism

CD was performed with samples of pure protein and complexed with the inhibitor. The concentration of protein used was 5 μM and ligands at saturation (100 μM), in 100 mM of potassium acetate (pH 5.1) at 37°C. The data were acquired with the following specifications: range of 200–260 nm, resolution of 1.0 nm, band width 1.0 nm, sensitivity of 100 mdeg, and speed of 8 nm min⁻¹. The α -helix/ β -sheet structural content of CaM, in the presence of Ca²⁺ and the inhibitors, was calculated using the K2D2 software¹⁷.

NMR titration experiments

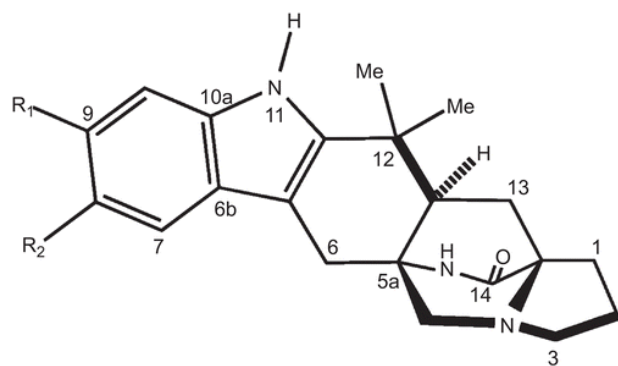
All experiments were run in the Varian Innova 500 instrument equipped with a ¹H, ¹³C, ¹⁵N TXI probe, by using sample tubes with a 5 mm outer diameter. Two-dimensional NMR spectra were recorded in the phase-sensitive mode using the States-TPPI method, and were carried out using the WATERGATE pulse sequence or excitation sculpting for water suppression to eliminate solvent signal in H₂O/²H₂O (95:5) solution. CaM solution (1.5 mM) was prepared in a 5% ²H₂O solution containing 50 mM TRIS-HCl, and 10.6 mM CaCl₂, and was titrated with **1** at final concentrations between 0 and 6 mM. ¹H-¹³C and ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) experiments were acquired at 308 K and processed using vNMRj software (Varian Inc., Palo Alto, CA).

Docking simulations

To generate a more accurate and physically realistic model of CaM (PDB code: 1A29), several iterations of rebuilding and refinement, using the idealization application of Rosetta 3.1 software, were performed. Docking simulations of the final all-atom refinement of CaM and compound **1** were carried out using AutoDock 4.0^{18,19}. Default parameters were used except for the number of GA runs (100). The protein was held rigid during the docking process while the ligand was allowed to be flexible. The grid box size was 60 Å × 60 Å × 60 Å in the x, y, and z dimensions, with the centre of the grid corresponding to the protein. FlexDock analyses were carried out using the residues found to 9 Å from the centre of the complex Ca²⁺-CaM-**1** (Glu14, Phe16, Leu18, Phe19, Leu32, Val35, Met36, Ser38, Leu39, Met51, Phe68, Met71, Met72, Met76, Val91, Phe92, Leu105, Val108, Met109, Thr110, Asn111, Leu112, Glu114, Met124, Phe141, Met144, and Met145). The docked inhibitor-enzyme complexes were ranked according to the predicted binding energies and on the basis of the ideal conformation of the docked structures. The root-mean-square deviation (RMSD) values for each residue involved in the flexible docking were calculated in the RMSD software implemented in VMD 1.8.6 program.

Results and discussion

Reinvestigation of the extract from the mycelium and culture medium of *M. aurantiaca* led to the isolation of isomalbrancheamide B (**3**) [(5a*S*,12a*S*,13a*S*)-9-chloro-12,12-dimethyl-2,3,11,12,12a,13-hexahydro-1*H*,6*H* 5a,13a-(epiminomethano) indolizino[7,6*b*]carbazol-14-one (**3**)], along with the known compounds malbrancheamide (**1**), malbrancheamide B (**2**), and premalbrancheamide (**4**) (Figure 1)^{13,20}. Compound **4** was recently detected in the fungus culture by Ding et al.²¹ while studying the biosynthesis of **1** and **2**.



Malbrancheamide (1)	R ₁ = Cl	R ₂ = Cl
Malbrancheamide B (2)	R ₁ = Cl	R ₂ = H
Isomalbrancheamide B (3)	R ₁ = H	R ₂ = Cl
Premalbrancheamide (4)	R ₁ = H	R ₂ = H

Figure 1. Structures of malbrancheamide analogues.

For the isolation of the malbrancheamide series, the initial extract was split in two equal parts: from the first one, compounds **1–4** were isolated by CC and HPLC procedures; the second, however, was subjected to an acid-base partition process to yield a crude FA. HPLC separation of FA led to the isolation of compounds **1–4** (see Supplementary Figure 1). Comparison between the two isolation strategies showed that the yield of **1** was significantly higher with the second method (5.19 vs 9.82%, respectively).

Compounds **1** and **2** were characterized by comparison with authentic samples previously isolated from *M. aurantiaca*^{12–14}, and the NMR data of **4** was consistent with the synthetic premalbrancheamide described by Ding et al.²¹. Compound **3** is a new natural product and its complete structural characterization was achieved using a combination of IR, CD, UV, as well as 1D (¹H and ¹³C-NMR) and 2D (HSQC and HMBC) NMR analyses. The molecular formula calculated from HRMS data was identical to that of **2** suggesting that both were isomers. The NMR spectra of **3** were almost identical to those of **2**. The most important differences were observed in the ¹H spectra, which in both cases displayed an ABX system for the aromatic protons, but as expected, their chemical shifts were different. Thus, the signals at δ_{H} 7.31 (d, $J = 8.7$ Hz, H-7), 6.94 (dd, $J = 8.4, 1.8$ Hz, H-8), and 7.26 (d, $J = 1.7$ Hz, H-10) in **2**, were replaced by signals at δ_{H} 7.32 (d, $J = 2$ Hz; H-7), 7.22 (d, $J = 8.6$ Hz; H-10), and 7.00 (dd, $J = 8.6, 2.6$ Hz; H-9) in **3**. The ¹³C-NMR spectrum (see experimental) displayed also signals for the bicyclo[2.2.2]diazaoctane ring system including the lactam functionality and for a monohalogenated indole moiety. Altogether, this information suggested that **3** was the C-9 monochlorinated isomer of malbrancheamide B (**2**).

Next, in order to determine if compounds **1–4** are classical or functional CaM inhibitors, additional experiments involving quenching of the fluorescence and CD were performed. In the first case, fluorescence titration curves for CaM with alkaloids **1–4** were constructed using a fluorescent-engineered *hCaM* (*hCaM*-M124C-mBBr) biosensor, according to the protocol previously described by González-Andrade et al.¹⁵. For this, Ca²⁺-CaM was titrated with different amounts of compounds **1–4** and the fluorescence changes were monitored in the range

between 400 and 550 nm, being the maximum response at 470 nm. As shown in Figure 2, the fluorescence intensity of the protein changed with increasing concentrations of compounds **1–3**, however, no significant effect was observed with **4**. Thus, alkaloid **1** displayed the highest affinity while **4** the least. The spectral changes produced by **1–3** were attributed to the formation of CaM-ligand complexes; the calculated K_d values were 1.11 ± 0.08 , 4.82 ± 0.30 , and 4.82 ± 0.09 μM , respectively, against the 1.43 ± 0.09 μM value observed for CPZ. The stoichiometry for the three alkaloids was 1:2, while for CPZ was 1:1 (Table 1). These results are in agreement with the enzymatic experiments carried out with Ca^{2+} -CaM-PDE1 complex¹⁶, since compound **1** showed a concentration-dependent inhibitory activity of the Ca^{2+} -CaM-PDE1 complex (IC_{50} 19.33 ± 1.40), whereas **2–4** displayed marginal effect (IC_{50} 183.28 ± 37.58 , 41.56 ± 4.64 , and 35.73 ± 3.01 μM , respectively). The effect of **1** was comparable with that of the CPZ (positive control; IC_{50} 16.78 ± 3.99 μM).

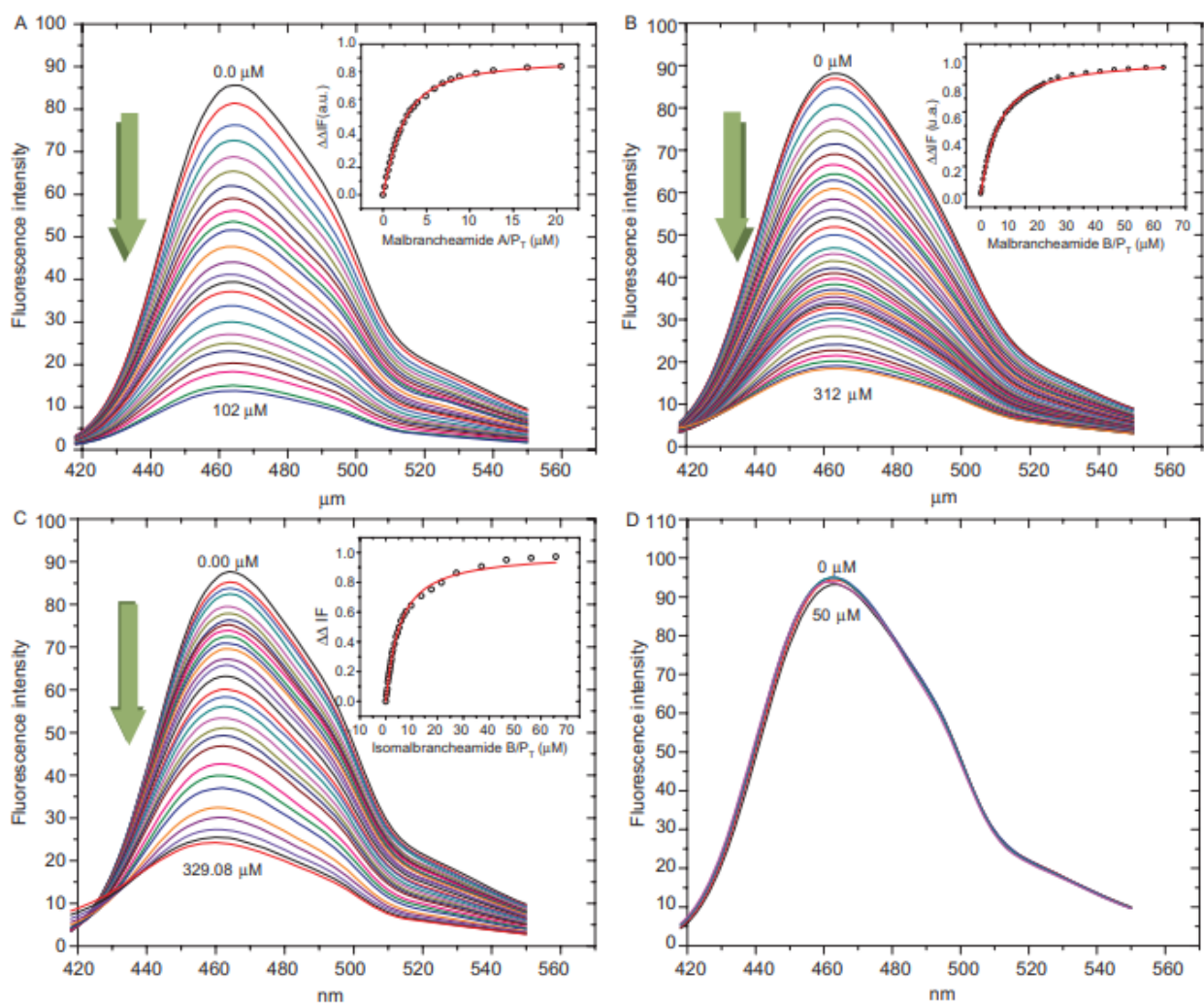


Figure 2. Fluorescence spectra and titration curves of Ca^{2+} -*h*CaM-M124C-mBBr in the presence of **1** (A), **2** (B), **3** (C), and **4** (D). Buffer was 100 mM of potassium acetate (pH 5.1) at 37°C and 1 mM CaCl_2 . Samples were excited at 381 nm, and emission spectra recorded for light scattering effects from 400 to 550 nm. The absolute changes of maximal fluorescence emission were plotted against the ration alkaloids/protein total and fitted to the binding equation model to obtain the K_d and stoichiometric ration.

Table 1. Activity and binding properties of malbrancheamides analogues on the Ca²⁺-CaM complex.

Compound	IC ₅₀ (μM) ^a	IC ₅₀ CPZ (μM) ^b	Potency ^c	K _d (μM) ^d	Stoichiometry ^d
1	19.33 ± 1.40	16.78 ± 3.99	1.1	1.11 ± 0.08	2.08 ± 0.19
2	183.28 ± 37.58	14.11 ± 1.75	0.1	4.82 ± 0.30	2.04 ± 0.63
3	41.56 ± 4.64	14.11 ± 1.75	0.3	4.82 ± 0.09	2.37 ± 0.21
4	35.73 ± 3.01	16.78 ± 3.99	0.5	NA	NA

CPZ, chlorpromazine; NA, not determined.

^aEnzymatic assay.

^bCPZ positive control: K_d = 1.43 ± 0.09 μM; stoichiometry = 0.69 ± 0.09.

^cPotency was obtained by the formula: IC₅₀ (CPZ)/IC₅₀ (compound), assuming a value of 1.00 for CPZ.

^dFluorescence experiments.

Concerning the CD experiments, the folding stability of CaM in the presence of **1–4** was assessed measuring their far-UV CD spectra (Figure 3). According to previous analyses, the complex Ca²⁺-CaM possesses a high content of α-helix (84.2%)^{22,23}; after addition of saturating concentrations of CPZ, the α-helice content decreased almost by 23% (61.3% of α-helix), bringing out significant changes in the secondary structure of Ca²⁺-CaM. The same trend of effects was observed after saturating the protein with **1** (68.1% of α-helix). Compounds **2** and **3** (75.45 and 78.33% of α-helix, respectively) were less effective, and **4** did not induce any change (84.2% of α-helix).

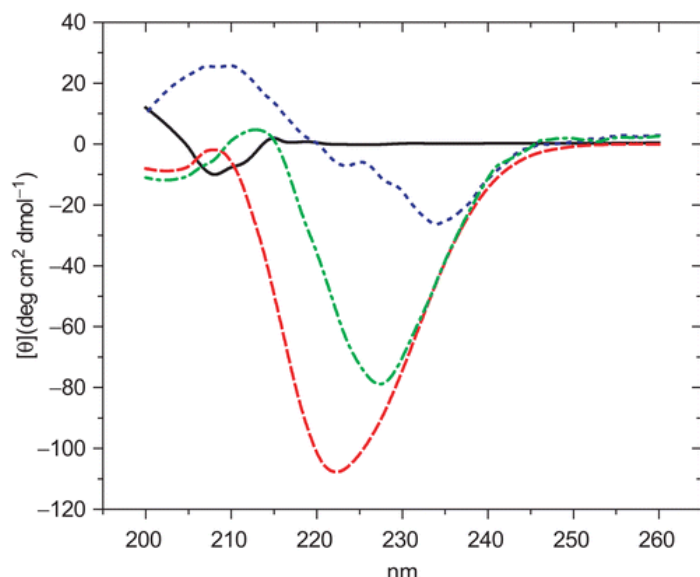


Figure 3. Far-UV circular dichroism of *h*CaM M124C-mBBr. in presence of 1 mM CaCl₂. Buffer (—); Ca²⁺-*h*CaM M124C-mBBr (—); Ca²⁺-*h*CaM M124C-mBBr-**1** (---); and Ca²⁺-*h*CaM M124C-mBBr-**4** (-.-). The estimation of secondary structures from circular dichroism spectral in presence of all ligands was determined using K2D2 program.

Altogether, these results revealed that **1–3** behave as classical CaM inhibitors while **4** as functional inhibitor²⁴. The latter compound did not quench the extrinsic fluorescence of the CaM biosensor nor modify the secondary structure of CaM according to the CD analysis.

On the basis of the fluorescence and CD studies, compound **1** was selected to assess its binding mode to CaM. For this purpose the changes in the ¹H-¹³C HSQC spectra upon addition of unlabelled **1** to uniformly ¹⁵N-¹³C-labelled CaM were analyzed. Compound **1** was titrated up to

four equivalents of Ca^{2+} -CaM. Most of the HSQC peaks of CaM were gradually shifted with little changes in their intensities (Figure 4).

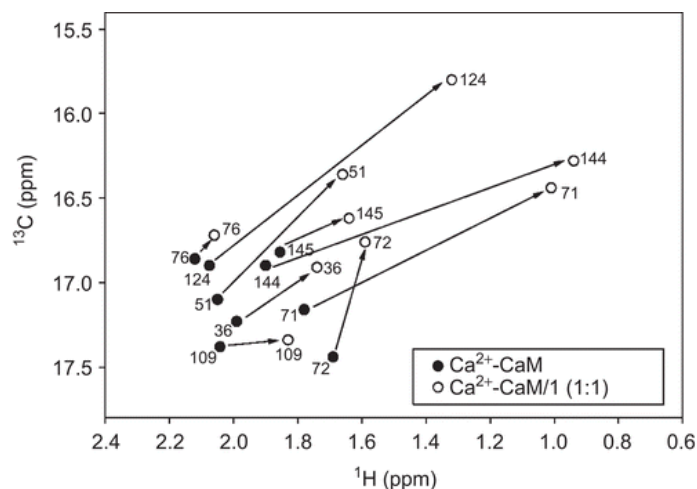


Figure 4. Schematic ^1H - ^{13}C nuclear magnetic resonance spectra of the methyl groups of the methionine residues in (●) Ca^{2+} -CaM and (○) Ca^{2+} -CaM-**1** complex. Chemical shift differences of methionines at the C-terminal (Met109, Met124, Met144, Met145) and N-terminal (Met36, Met51, Met71, Met72) domains of CaM, as well as the Met76 of the flexible linker region, are indicated with solid arrows.

The resonances of Ca^{2+} -CaM affected by **1** were situated in the methionine methyl region, suggesting that **1** binds to the hydrophobic pockets of the protein rich in methionines, and localized at the interdomain sites of CaM, regarded as T2 and T3^{24,25}. All the chemical shifts changes for the methionines residues 36, 51, 71, 72, 76, 109, 124, 144, and 145 were recorded and summarized in Table 2.

Table 2. NMR and flexible docking results of the Ca^{2+} -CaM-**1** complex formation.

Residue	NMR ^1H $\Delta\delta$ / ^{13}C $\Delta\delta$ (ppm)	Autodock RMSD (Å)
Met 36	0.239/0.283	0.177
Met 51	0.342/0.650	0.606
Met 71	0.704/0.663	0.788
Met 72	0.079/0.649	0.857
Met 76	0.069/0.136	1.251
Met 109	0.226/0.070	1.263
Met 124	0.708/0.997	0.110
Met 144	0.919/0.585	0.996
Met 145	0.212/0.208	0.823

NMR, nuclear magnetic resonance; RMSD, root-mean-square deviation.

Next, in order to predict the location where compound **1** binds to CaM, ligand-receptor docking analyses into the preferred CaM-binding pocket of TFP were performed using AutoDock 4.0, and the reported X-ray structure of the TFP-CaM complex (PDB code: 1A29) as template. The lowest free energy of binding (EFEB) of the complex **1**-CaM was -9.04 kcal/mol, and the calculated inhibition constant (K_i) was 0.23 μM . These values are in agreement with the parameters of binding and activity obtained by steady-state fluorescence and enzymatic tests (Table 3). The refined flexible docking analysis revealed that the binding of **1** to CaM involved several hydrophobic interactions with the residues Glu11, Phe92, Ile100, Leu105, Met124,

Ile125, Ala128, Val136, Phe141, and Met144, located at T2 and T3 hydrophobic pockets (Figure 5). These residues are involved in the binding of most drugs with CaM and are located between the globular domains²⁵, being their aromatic ring inside the hydrophobic area and their aliphatic side chains projecting towards the outside, so that the positive charge is in the acid patches.

Table 3. Binding free energy (EFEB) and inhibition constant (K_i) values of compounds 1–4, obtained from the molecular docking analyses (AutoDock 4.0).

Compound	EFEB (Kcal/mol)	K_i (μM)
CPZ ^a	-7.82	1.85
1	-9.04	0.23
2	-8.58	0.51
3	-8.56	0.53
4	-8.04	1.27

CPZ, chlorpromazine.

^aPositive control.

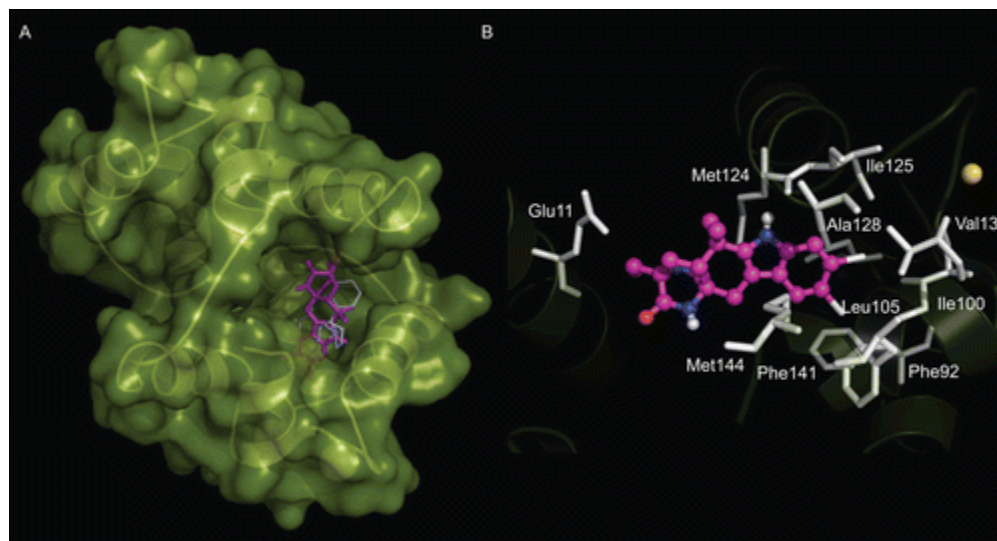


Figure 5. Flexible docking model of the complex Ca^{2+} -CaM-1. CaM is represented in green surface, 1 is depicted in purple sticks, and CPZ is shown in white lines. (A) Lowest energy AutoDock conformation of 1; (B) Residues of CaM (white sticks; Glu11, Phe92, Ile100, Leu105, Met124, Ile125, Ala128, Val136, Phe141, and Met144) involved in the complex formation. Ca^{2+} ions are showed in yellow balls.

Conclusions

Steady-state fluorescence and CD experiments, along with the enzymatic assay using CaM sensitive PDE1, revealed that all the alkaloids but 4 bind to Ca^{2+} -CaM. Thus, compounds 1–3 are classical inhibitors, while compound 4 behaves as functional inhibitor. According to the NMR and docking analyses compound 1 binds to the same hydrophobic pocket than CPZ, W-7, J-8, and TFP. Furthermore, the docking study also indicated that the binding of 1 with the protein involved hydrophobic and electrostatic interactions. These observations are consistent with the highest affinity of compound 1 to CaM in comparison to that of 2 and 3, with one halogen in their structures, or 4, with no halogen in its molecule, and with not affinity at all for the protein. Finally, the anti-CaM properties exhibited by compound 1 are comparable to those of classical CaM inhibitors, and make this product a lead for the development of new drugs and a valuable tool for the study of the physiological role of CaM.

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Declaration of interest

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