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Antimicrobial guanidine alkaloids from the leaves of *Crotalaria bernieri* Baill. (Fabaceae)

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Abstract

A new guanidine alkaloid cyclocrotalarin together with sphaerophysin and 2''-O-α-rhamnoside vitexin have been isolated from the leaves of *Crotalaria bernieri* a Fabaceae. The structures were elucidated by spectroscopic methods including MS, 1D and 2D NMR and their antimicrobial activity examined.

Keywords: *Crotalaria*; Guanidine alkaloids; Flavonoids; Fabaceae

1. Introduction

The *Crotalaria* genus belongs to the Fabaceae family and contains about 600 species growing in tropical and subtropical areas, most of the species being found in Africa and Madagascar [1, 2]. They are annual or perennial plants displaying an economically importance as they can be used either as green manure and cover crop or in intercropping farming systems to restore soil fertility and rehabilitate degraded farmlands [3, 4, 5]. They have also been reported effective against the nematodes *Meloidogyne spp*. [6, 7].

Moreover, large numbers of *Crotalaria spp.* have been used in folk medicine to treat several diseases. The genus is known to mainly produce pyrrolizidine alkaloids responsible for the toxicity of several species [8], isoflavonoids [9, 10], chalcones [11, 12] and non-proteic aminoacids [13, 14]. They displayed a wide spectrum of biological activities such as anti-inflammatory [15], antimicrobial [16], antioxidant [17], anticancer [18, 19], cytotoxic [20], analgesic [21] and anti-HIV activities [22].

Crotalaria bernieri Baill., is one of the 53 *Crotalaria* species growing in Madagascar. It is an annual herb, found in open vegetation, grassy places and roadsides in most regions of Madagascar [23]. According our personal inquiries in the harvesting sites, the plant leaves are used in traditional medicine for the treatment of diarrhea and stomach disorders. In our previous study [16], a significant antimicrobial activity for extracts of this plant against pathogenic bacteria and molds was reported. The methanol extract of the leaves was mostly efficient against Gram (+) bacteria (*B. cereus*, *S. aureus*, *S. pneumoniae* and *S. pyogenes*) and yeast (*C. albicans* and *C. guilliermondii*). We now report the isolation from the extract of *C. bernieri* leaves and the structural elucidation by using mass spectrometry and 2D NMR, of two guanidine alkaloids, the known sphaerophysin (1) and the new cyclocrotalarin (2), together with 2''-*O*-α-rhamnoside vitexin (3). The purpose was to identify active constituents responsible of the antimicrobial activity.

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2. Material and methods

2.1. Plant material

The leaves of *C. bernieri* were harvested in Ibity, in the Vakinankaratra region, located 200 km South of Antananarivo (Madagascar). The plant was collected in April ~~2013~~ and identified by R.M. Polhill, botanist at the Royal Botanic Garden of Kew, (England). Voucher specimens (No. Herizo R.010) were deposited at the herbarium of Plant Biology and Ecology Department of the Faculty of Sciences of the University of Antananarivo.

2.2. Compound extraction and isolation

After drying in the shade, leaves were ground into a fine powder. The resulting powder (100 g) was successively extracted with a mixture of MeOH (750 ml) and 225 ml of aqueous hydrochloric acid (10%) under stirring at room temperature for 24 h. After filtration using a Buchner funnel, the filtrate was evaporated to dryness under reduced pressure at 40°C. The residue was dissolved in distilled water and extracted with CH_2Cl_2 (3 x 500 ml).

The organic (CH_2Cl_2) and aqueous phases were separately alkalinized with NH_4OH (20%) up to pH 9. The CH_2Cl_2 part was then evaporated to dryness, while the aqueous solution was extracted with CH_2Cl_2 (3 x 250 ml) to yield organic (OS) and water (WS) solutions. OS was concentrated under vacuum while WS was partitioned three times with n-butanol (v/v) in a separatory funnel.

2.3. General experimental procedures

All NMR experiments were recorded on Bruker Avance III HD 400 and 600 MHz spectrometers (Wissembourg, France) equipped with a BBFO Plus Smartprobe and a triple resonance TCI cryoprobe, respectively, operating at 400.13 and 600.19 MHz (1H), and 150.92 MHz (^{13}C). Chemical shifts (δ) were expressed in ppm, and were referenced to the residual non-deuterated solvent signals (CD_3OD or DMSO-d_6). The coupling constants (*J*) were reported in Hz. Mass spectra data were recorded in positive and negative mode on a Bruker Maxis II, a high resolution QTOF (Quadrupole time-of-flight) equipped with an electrospray interface (ESI).

Analytical thin-layer chromatographies (TLC) were performed at room temperature on precoated fluorescent silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany), which were developed in the eluent mixture butanol/water/acetic acid (6/2/2). Spots were visualized under UV light at 254 and 366 nm before spraying with either a vanillin/sulfuric acid solution in EtOH or a Dragendorff reagent followed by heating the plate at 110°C. Column chromatographies were performed on Sephadex LH-20 (25-100 µm; Pharmacia Biotech Ltd) or on silica gel RP8 (25-40 µm; LiChroprep; Merck).

3. Results

3.1. Isolation

The dried and ground leaves (100 g) of *C. bernieri* were extracted with acidified methanol at room temperature then this extract was evaporated to dryness under reduced pressure, the residue dissolved in water and extracted with CH_2Cl_2, then the two phases alkalinized with ammoniac to pH 9. The alkaline CH_2Cl_2 part was evaporated to dryness. The water part was extracted with n-butanol, concentrated to dryness under reduced pressure, yielding a total alkaloid extract (TAE) of 5.85 g. This extract was then fractionated on a Sephadex LH20 column eluted with MeOH, resulting in 51 fractions (Fr 1-Fr 51). Fractions 7-15 (327 mg) were grouped and further purified on a reversed phase (RP8) silica gel column eluted by a MeOH/H_2O gradient, yielding compound **1** (1.5 mg) and 9 sub-fractions (sFr 1-sFr 9). Purification of sFr 2 to sFr 4 (135 mg) by column chromatography on silica gel RP8 eluted with MeOH/H_2O (0 to 100 %) on reverse phase provided compound **2** (2.3 mg). Additionally, fractions 26-29, which exhibited similar TLC profiles, were combined to yield pure compound **3** (10 mg).

3.2. Structure of sphaerophysin (1)

Compound **1** was isolated as an amorphous solid. Its positive mode ESI mass spectrum showed the protonated molecular ion $[M+H]^+$ at m/z = 199.1914. Its mass M was therefore 198.1836 corresponding to the empirical formula $C_{10}H_{22}N_4$ (calc. mass: 198.1844) which involved two degrees of unsaturation.

In the 13C NMR spectrum (DMSO-d_6), the ten carbon atoms of the empirical formula were distributed into two methyls (δ_C 25.3 and 17.8), four methylenes (δ_C 25.3, 38.8, 39.0 and 40.4), one ethylenic methine at δ_C 119.3 and two sp2

quaternary carbons linked to a nitrogen at δ_C 135.8 and 155.6 (>C=N-). The two double bonds deducted from the NMR data, reflected the two degrees of unsaturation, the molecule was thus acyclic. Examination of the COSY and HSQC spectra allowed to define three structural fragments (Table 1 and Figure 1). The first structural fragment was a linear chain of four methylenes, with those at the two ends linked to a nitrogen as indicated by their chemical shifts (δ_H 3.115 and δ_C 40.4 for one and δ_H 2.707 and δ_C 39.0 the other) to form substructure **A** (Figure 1): >N-CH_2-CH_2-CH_2-CH_2-N<. As the two central -CH_2- of this di-amino-*n*-butane chain were superimposed in DMSO-d_6, the NMR spectra were recorded in CD_3OD where they were clearly distinguished (Table 1). The carbon at δ_C 155.6 was characteristic of a guanidine group: (-N-)$_2$C=NH (substructure **B**). The methylene at δ_H 3.712 (δ_C 38.8) whose chemical shifts indicated its carbon was linked to a nitrogen and its coupling allowed to define a dimethylallyl group: >N-CH_2-CH=C(CH_3)$_2$ (substructure **C**).

The assembly of these three fragments was carried out by the analysis of the HMBC spectrum (Figure 2). The carbon at δ_C 155.6 was correlated with the two methylenes at δ_H 3.712 (δ_C 38.8) and δ_H 3.15 (δ_C 40.4), via a nitrogen atom leading to the structure in Figure 3. The five exchangeable protons at δ_H 7.362 were bound to the nitrogen atoms.

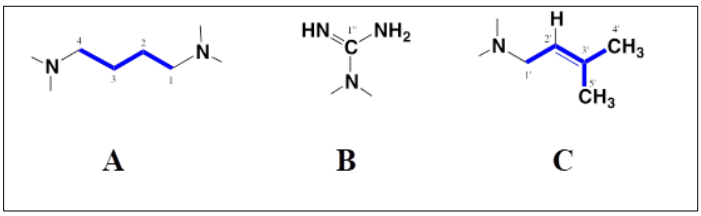

Figure 1 Fragments A, B, C deduced from the analysis of 1D NMR and 2D COSY and HSQC spectra of 1

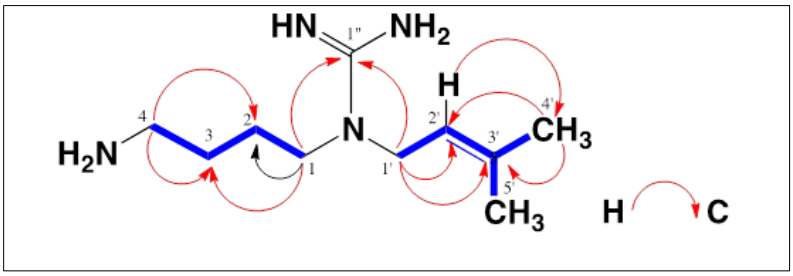

Figure 2 Assembly of substructures A, B, C from HMBC correlations

On the NOESY spectrum, the correlations of the two methyls, CH_3-4' at δ_H 1.695 with the H-2' at δ_H 5.170 and CH_3-5' at δ_H 1.634 with the methylene CH_2-1' at δ_H 3.712 made possible to assign these two methyls *E* and *Z* as regards to the methylene -CH_2-1'). All these conclusions were verified on the NMR spectra recorded in CD_3OD (Table 1). Compound **1** was therefore a derivative of guanidine formed by the substitution of one of its nitrogen atoms, both by a dimethylallyl group and by a diamino-*n*-butyric chain (Figure 3). Its biosynthesis could be explained by the alkylation by dimethylallyl-diphosphate of the nitrogen at position -6 of an arginine, followed by decarboxylation.

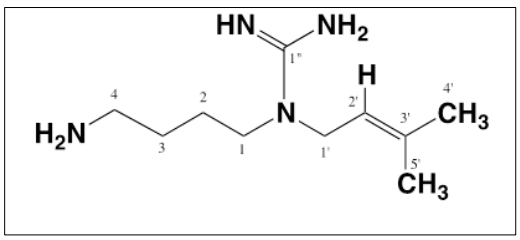

Figure 3 Structure of compound 1

Compound **1** was thus identified as *N*-(4-aminobutyl)-*N*-(3-methyl-2-buten-1-yl)-guanidine or sphaerophysin. This compound has been first isolated from *Sphaerophysa salsula*, but the structure then proposed by [24] in 1957, was revised in 1970 by [25]. Our spectral analysis confirmed the revised structure. More recently, [26] isolated related compounds from *Galega orientalis*.

3.3. Structure of cyclocrotalarin (2)

Compound **2** was obtained as an amorphous solid. Its ESI mass spectrum showed the protonated molecular ion $[M+H]^+$ at *m/z* = 553.3246. The molecular mass M, 552.3168 corresponded to the formula $C_{28}H_{40}N_8O_2$ (calc. mass: 552.3172). A doubly charged ion $[M+2H]^{++}$ was observed at *m/z* = 277.1661 and its isotopic peak due to the ^{13}C at *m/z* = 277.6665, confirmed the presence of a double charge.

The ^1H and ^{13}C NMR spectra of **2** recorded in DMSO-d_6, as well in CD_3OD (Tables 3 and 4), only showed half of the protons and carbon atoms of the molecular formula, suggesting the molecule to be symmetrical. The ^1H and ^{13}C NMR spectra of **2** (DMSO-d_6) respectively contained six carbons (Table 2) and four aromatic protons characteristic of a *para*-disubstituted benzene ring at δ_H 6.658 and 7.018, as well as of an amide function (-CO-NH-) whose proton was observed at δ_H 7.684 and carbonyl at δ_C 170.7. Two methines at δ_H 3.568 and 4.085 (δ_C 47.2 and 39.8, respectively), four methylenes (-CH_2-), two of which were bound to a nitrogen atom were also observed around δ_C 40. A quaternary carbon at δ_C 156.7 was assigned to a guanidine. Analysis of the ^1H-^1H COSY spectrum showed two vicinal methines and a linear sequence of four methylenes forming an n-butyl chain, the extremities of which were linked to a nitrogen (>N-CH_2-CH_2-CH_2-CH_2-N<). The direct connections (^1J) between protons and carbons were deduced from the correlations on the HSQC spectrum.

The HMBC spectrum showed (^2J) and (^3J) H->C correlations, made it possible to link together the substructures A-D thus defined (Figure 4). The protons at δ_H 7.018 were correlated with the carbon at δ_C 155.7 (C-7') whose chemical shift indicated it was of a phenolic carbon. The aromatic quaternary carbon at δ_C 130.5 was strongly correlated with the protons at δ_H 6.658 (^3J), 4.085 (^2J) and 3.568 (^3J). The carbonyl at δ_C 170.7 was correlated (^3J) with protons at δ_H 4.085 (CH-3'), 3.568 (CH-2'), 7.684 (NH amide) and δ_H 2.711 and 2.848 of CH_2-1. Finally, the carbon of the guanidine group at δ_C 156.7 was correlated with the protons at δ_H 7.513 (NH-5) and 2.930 (CH_2-4).

All of these considerations led to the **C_{14}** substructure (Figure 5), which used all the identified atoms on the NMR spectra, but which accounted for only half of the atoms of the molecular formula, which in addition involves 13 degrees of unsaturation. If this **C_{14}** substructure enclosing five double bonds and one ring, is doubled, it can only account for 12 degrees of unsaturation, not 13. Since there is no other observed sp2 carbons, the molecule must have an additional ring, which can be formed by linking C-2 to C-3' of a second **C_{14}** substructure, to form a cyclobutane ring. The HMBC spectrum showed that the carbon at δ_C 39.8 (CH-3') was correlated ^2J (or ^3J) with the proton to which it is directly bound (^2J), and it is the same with the carbon at δ_C 47, 2 (CH-2') in agreement of the presence of such a ring and a symmetry in the molecule. The chemical shifts of the protons and carbon atoms of this cyclobutane in Tables 3 and 4 were consistent with those observed in four-membered rings of a cyclobutane [27, 28]. But there are two ways to form this 4 members ring from the C_{14} substructure, either in a parallel (**A**) or an antiparallel (**B**) mode (Figure 6).

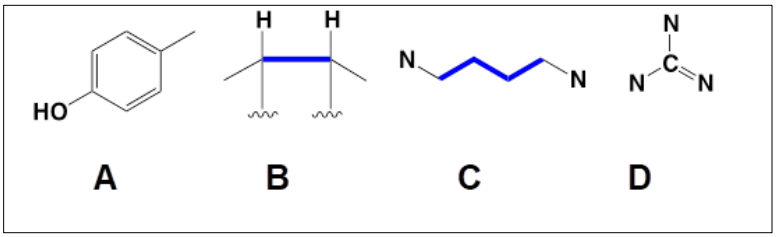

Figure 4 Elements of structure A, B, C and D deduced from the COSY and HSQC spectra of 2

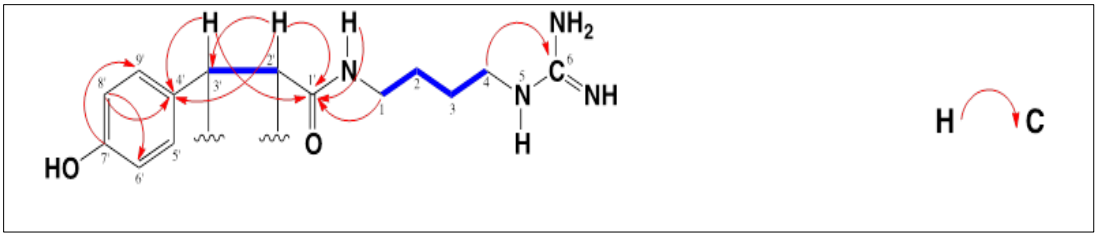

Figure 5 Assembly of structural elements A, B, C and D thanks to HMBC correlations to form the C_{14} substructure

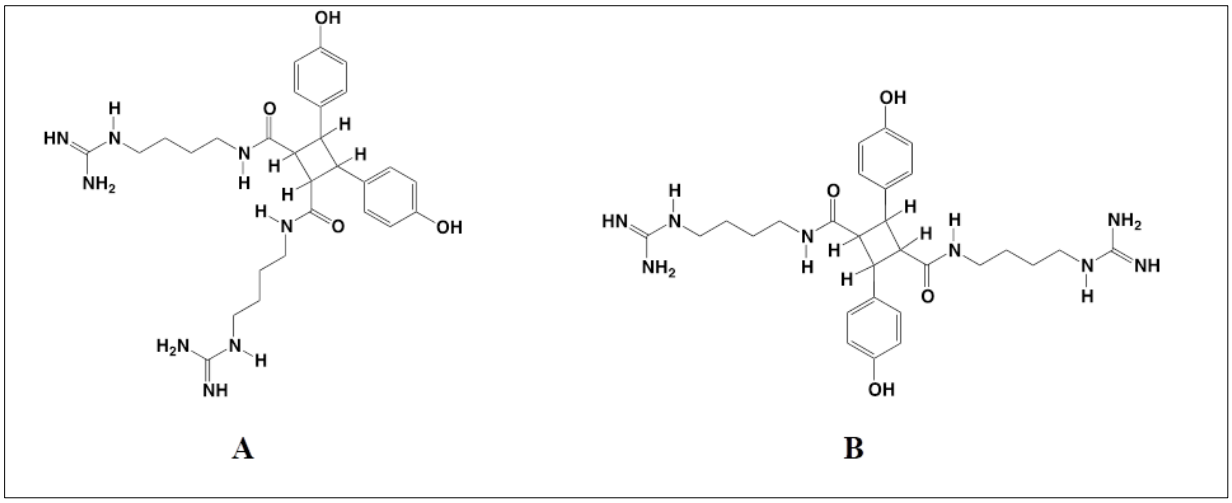

Figure 6 Parallel (A) and antiparallel modes (B) for assembly of the C_{14} substructure

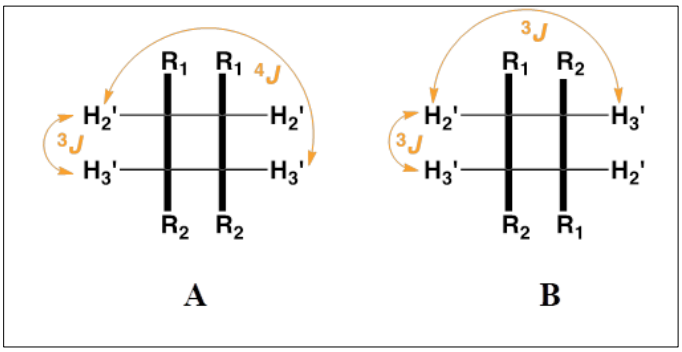

Figure 7 Coupling between the H-2'and H-3' protons in parallel A and antiparallel B structures

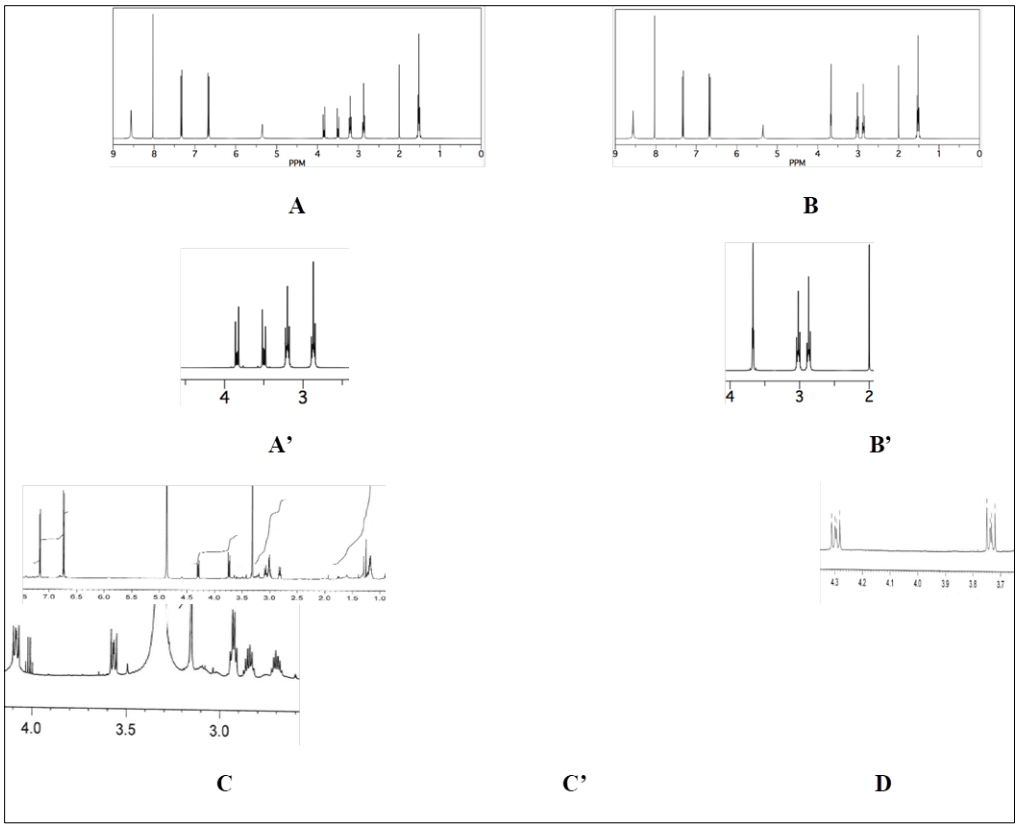

Figure 8 1H NMR calculated spectra for the assemblies: parallel A and antiparallel B; A' and B': enlargement of the portion 3-4 ppm. C: experimental spectrum (CD_3OD); C': enlargement of the portion 2.7-4.3 ppm. D: Experimental spectrum (DMSO-*d6*)

Calculating the coupling constants between (CH-2') and (CH-3') allowed to discriminate between these two hypotheses. The observed constants are shown in ~~the~~ Figure 7. We calculated them in both configurations and see the one that corresponded to the experimental spectrum. In the parallel structure (**A**), H-2' was both at 3 and 4 bonds (^3J and ^4J) of H-3' and thus gave two coupling constants with it (J = 7.2 and 10.2 Hz) and reciprocally H-3' at both 2 and 3 H-2' bonds gave the same couplings. In the antiparallel structure (**B**) H-2' was vicinal (3 bonds, or ^3J) of H-3' and will formed a triplet with a single coupling constant (Figures 7 and 8). The experimental results showed that compound **2** was a dimer of the **C_{14}** substructure (Figure 5) where the two **C_{14}** substructures were parallel, as shown in Figure 9.

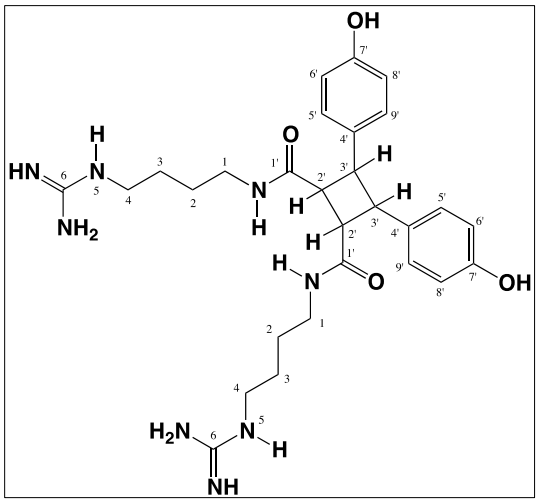

Figure 9 Structure of compound 2

A second question was to determine if compound **2** had either a planar or an axial symmetry. In the first case no optical rotation could be observed and in the second it would be optically active.

The structure of **2** was original. Natural products related to the above C_{14} substructure had previously been isolated from *Albizia julibrissin*, where they play a role in leaf closure caused by darkness or nystinastic movement [29, 30] and also as antifungal factor in barley seedlings [31, 32]. A number of cyclobutane dimers have been isolated from different plant families such as Asteraceae [33], Ginkgoaceae [34], Moraceae [28], Compositae [27], and also from marine sponges such as sceptrine from *Agelas sceptrum* [35].

3.4. Structure of compound 3

The positive mode ESI mass spectrum of compound **3** showed the $[M+H]^+$ ion at *m/z* = 579.1685 and its negative mode ESIMS the $[M-H]^-$ ion at m/z = 577.1553, in agreement with the molecular formula $C_{27}H_{30}O_{14}$ (calc.: 578.1635). The structure was determined from 2D NMR studies and **3** was identified as apigenin-8-C-α-rhamnopyranosyl-(1->2)-β-glucopyranoside or 2''-*O*-α-rhamnoside vitexin, in agreement with previous phytochemical studies[36, 37], and as shown in Figure 10.

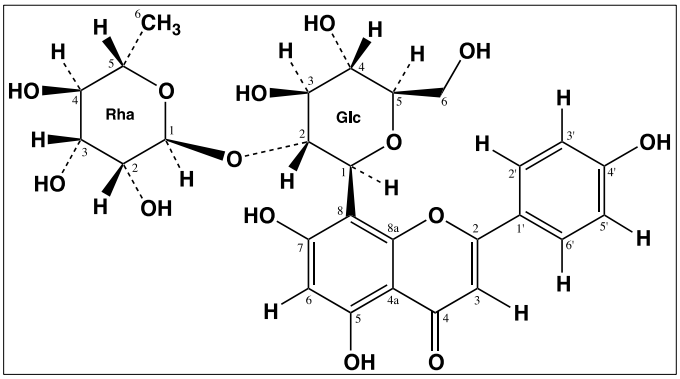

Figure 10 Structure of compound 3

3.4.1. Sphaerophysin (1)

HRMS *m/z* (%): 199.1914 (100) $[M+H]^+$; 182.1654 (2); 160.1084 (2); 131.1290 (2); 128.1184 (3); 114.1043 (13); 97.0762 (16); 89.1073 (32); 86.0964 (4); 72.0803 (7).

^1H and ^{13}C NMR: see Table 1.

3.4.2. Cyclocrotalarin (2)

HRMS *m/z* (%): 553.3213 (100) $[M+H]^+$; 536.2948 (26); 519.2684 (3); 511.2996 (19); 494.2734 (5); 277.1643 (49); 260.1377 (29); 235.1426 (10); 218.1161 (26); 147.0429 (9).

^1H and ^{13}C NMR: see Table 2.

3.4.3. 2''-O- α -rhamnoside vitexin (3)

MS *m/z* (%): 579 (45) $[M+H]^+$; 433 (65); 415 (50); 397 (60); 379 (22); 367 (27); 337 (30); 313 (100); 283 (37). MS *m/z* (+): 579.1699 $[M+H]^+$. MS *m/z* (-): 577.1553 $[M-H]^-$.

^1H NMR (DMSO-d_6, 298 K, ^1H 600.19 MHz): 6.757 *s* (3); 6.199 *s* (6); 8.025 *m* 8.8 (2',6'); 6.882 *m* 8.8 (3',5'); 4.764 *d* 10.0 (1"); 4.045 *dd* 10.0, 8.6 (2"); 3.422 *dd* 8.7, 8.6 (3"); 3.376 *dd* 9.2, 8.7 (4"); 3.225 *ddd* 9.2, 5.7, 1.9 (5"); 3.753 *dl* 11.5 and 3.510 *dd* 11.5, 5.7 (6"); 4.967 *d* 1.3 (1'"); 3.563 *sl* (2'"); 3.084 *dd* 9.4, 2.9 (3'"); 2.900 *dd* 9.4, 9.2 (4'"); 2.136 *dq* 9.2, 6.2 (5'"); 0.465 *d* 6.2 (6'"); 13.125 *sl* (OH-5); 10.566 *sl* (OH-7); 10.566 *sl* (OH-4'); 5.226 *sl* (OH-3"); 5.095 *sl* (OH-4"); 4.312 *sl* (OH-6"); 4.396 *sl* (OH-2'"); 4.622 *sl* (OH-3'"); 4.396 *sl* (OH-4'").

^{13}C NMR (DMSO-d_6, 298 K, ^{13}C 150.92 MHz): 163.8 (2), 102.3 (3), 181.9 (4), 103.7 (4a),160.6 (5), 98.4 (6),155.8 (7), 104.5 (8), 155.8 (8a), 121.6 (1'),128.9 (2',6'), 115.8 (3',5'), 161.2 (4'), 71.7 (1"), 75.0 (2"), 79.9 (3"), 70.6 (4"), 81.8 (5"), 61.1 (6"), 100.3 (1'"), 70.4 (2'"), 70.2 (3'"), 71.5 (4'"), 68.2 (5'"), 17.7 (6'").

Table 1 ^{1}H and ^{13}C NMR data for (1) (DMSO-d_6 or CD_3OD)

Compound 1		**DMSO-d_6**		**CD_3OD**
Position	**δ_C**	**δ_H *mult. J* (Hz)**	**δ_C**	**δ_H *mult. J* (Hz)**
CH_2-1	40.4	3.115 br*t* 6.6	42.0	3.256 *t* 6.8
CH_2-2	25.6	1.499 *m*	27.0	1.680 *m*
CH_2-3	25.6	1.499 *m*	26.0	1.716 *m*
CH_2-4	39.0	2.707 br*t* 6.6	40.5	3.801 *dh* 6.6; 0.6
CH_2-1'	38.8	3.711 br*d* 6.6	40.4	2.962 *t* 7.2
CH-2'	119.3	5.170 *th* 6.6; 1.4	119.3	5.258 *th* 6.6; 0.6
C-3'	135.8	-	139.2	-
CH_3-4'	25.3	1.695 br*d* 1.0	25.7	1.778 br*qt* 1.2; 0.6
CH_3-5'	17.8	1.634 br*d* 1.0	18.0	1.727 br*qt* 1.2; 0.6
C-1''	155.6	-	157.4	-
5 NH	-	7.362 br*s*	-	-

Table 2 ^{1}H and ^{13}C NMR data for 2 (DMSO-d_6 or CD_3OD)

Compound 2	**DMSO-d_6**		**CD_3OD**	
Position	**δ_C**	**δ_H *mult. J* (Hz)**	**δ_C**	**δ_H *mult. J* (Hz)**
CH_2-1	37.6	2.848 *m*	39.3	3.078 *ddd* 13.6; 6.2; 6.2
	-	2.711 *m*	-	2.818 *ddd* 13.6; 6.1; 6.1
CH_2-2	26.2	1.068 *m*	27.7	1.174 *m*
CH_2-3	25.7	1.127 *m*	26.7	1.198 *m*
CH_2-4	40.3	2.930 *dt* 6.1; 6.8	42.1	3.011 *dt* 6.1; 6.8
C-6	156.7	-	158.5	-
CO-1'	170.7	-	174.0	-
CH-2'	47.2	3.568 *dd* 10.2; 7.2	49.6	3.735 *dd* 10.3; 7.2
CH-3'	39.8	4.085 *dd* 10.2; 7.2	41.8	4.296 *dd* 10.3; 7.2
C-4'	130.5	-	131.8	-
CH-5',9'	128.7	7.018 *m* 8.6	130.2	7.166 *m* 8.6
CH-6',8'	114.6	6.658 *m* 8.6-	116.0	6.737 *m* 8.6
C-7'	155.7	-	157.3	-
OH (7')	-	9.200 *s*	-	-
NH-CO	-	7.684 *dd* 5.8; 5.8	-	-
NH-5	-	7.513 *dd* 5.6; 5.6	-	-

4. Conclusion

The results obtained in the present work provided new data on the chemistry of *C. bernieri*. The in-depth chemical study led to isolate and characterize three chemical components. Two of them are already known, the flavonoid 2''-O-α-rhamnoside vitexine and the alkaloid sphaerophysine, but not yet found in the genus *Crotalaria*, and the third, a new alkaloid that we had named cyclocrotalarin.

These new data have contributed to a better knowledge of Malagasy endemic plants, in particular the *Crotalaria* genus.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interests.

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