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Aggregation Behavior of 6-Isocassine and *N*-Methyl-6-Isocassine: Insights into the Biological Mode of Action of Lipid Alkaloids

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Dedicated to the memory of our esteemed colleague and friend Eduardo Alonso Paz.

The aggregation behavior of 6-isocassine and *N*-methyl-6-isocassine, two piperidin-3-ol alkaloids isolated respectively from the barks of *Prosopis nigra* and *P. affinis*, was investigated using a combination of NOE experiments and diffusion measurements in solvents of varying polarity and hydrogen bonding capacity. While the NOE enhancements for *N*-methyl-6-isocassine are positive, regardless of the solvent, those for 6-isocassine shift from negative to positive when going from chloroform-*d* to methanol-d_4 solution. In addition, despite the self-diffusion coefficients of both compounds being virtually identical in methanol-d_4, *N*-methyl-6-isocassine diffuses nearly twice as fast as the non-methylated alkaloid in chloroform-*d*. The changes in rotational and translational dynamics observed between solvents for 6-isocassine suggest that the molecule forms dimeric head-to-head aggregates in non-polar aprotic environments, a behavior that could help explain the biological mode of action that has been proposed for this type of alkaloids.

Keywords: Aggregation, Diffusion, Hydrogen bonding, Piperidine alkaloids, NOE enhancements.

The piperidine alkaloids are a vast class of compounds derived biosynthetically from lysine [1], which include piperine, lobeline, and carpaine [2]. The last is one of the most relevant members of the "lipid alkaloids," a family that also comprises prosopine, juliprosopine, cassine, and carnavaline [3-7]. The biological activities of these compounds range from antifungal to antimicrobial [8-11], and reports also point to their anti-inflammatory properties and propose them as candidates for use in Alzheimer's disease therapy [12,13]. Several studies suggest that the mode of action of these molecules is related to their ability to interact with ion channels and transport mechanisms [12-16].

The *Prosopis* genus, composed by more than 40 species of shrubs and trees distributed throughout Asia, Africa, and the Americas [17], is an important source of piperidine alkaloids. For example, prosopine and prosopinine have been obtained from the leaves of *P. africana* [4], the barks of *P. glandulosa*, *P. nigra*, *P. ruscifolia*, and *P. vinalillo* have yielded several cassine and *N*-methylcassine isomers [9,18], and juliprosopin and many related compounds have been isolated from the leaves of *P. juliflora* and *P. glandulosa* [5,8-11,19]. More recently, we described the isolation and structural characterization of three new *N*-methylated piperidin-3-ols from the bark of *P. affinis*, including *N*-methyl-2-isocassine, *N*-methyl-6-isocassine, and *N*-methyl-6-isocarnavaline [20]. In continuation of our bioprospection program for the valorization of Uruguayan native woodlands [21], we revisited the phytochemical analysis of *P. nigra*, a species which is widespread in the region.

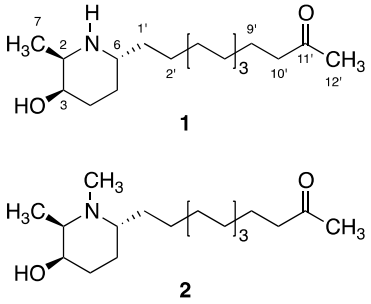

Figure 1: Chemical structure and numbering of 6-isocassine (**1**) and *N*-methyl-6-isocassine (**2**).

A standard alkaloid extraction of the powdered bark of *P. nigra* followed by purification afforded a compound with a $[M+H]^+$ quasimolecular ion peak at *m/z* 298.2719 in the HR-ESIMS consistent with a molecular formula $C_{18}H_{35}NO_2$. Together with the IR absorption spectrum and EIMS fragmentation patterns, careful inspection of the ^1H and ^{13}C NMR data obtained from 1D and 2D experiments, summarized in Table 1, allowed us to identify this compound as an isomer of cassine [6]. As reported by us and others [20,22], the piperidine ring in these systems exist as a rapid equilibrium of chair conformers in solution. Thus, the relative configurations of the C-2, C-3, and C-6 stereocenters were determined through detailed analysis of conformationally averaged coupling constants following our recently reported approach [20].

Briefly, molecular models of the two possible chair conformers of all distinct cassine diastereomers were first built and optimized, and vicinal coupling constants between protons on positions C-2 and C-3 ($^3J_{H2-H3}$), C-3 and C-4 ($^3J_{H3-H4\square}$ and $^3J_{H3-H4\square}$), and C-5 and C-6 ($^3J_{H5\square-H6}$ and $^3J_{H5\square-H6}$) for each pair of conformers were then computed from the measured <H-C-C-H> dihedral angles using a generalized Karplus equation [23]. The weighted averages of these calculated $^3J_{HH}$ couplings were compared with experimental values in order to assign the correct diastereomer and determine the conformer ratio. Following this protocol we concluded that compound **1** has a 2*R*,3*R*,6*R* relative stereochemistry that corresponds to 6-isocassine, and exists as a 60:40 and 50:50 mixture of conformers in chloroform and methanol solution, respectively (Figure 2). Although this is the first report of this piperidin-3-ol in a *Prosopis* species, the alkaloid was previously isolated from *Senna spectabilis* and *Cassia spectabilis* [15,22].

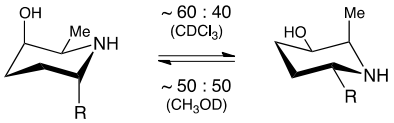

Figure 2: Conformational equilibria in chloroform and methanol solution proposed for compound **1** (R = -$(CH_2)_{10}C(O)CH_3$).

Table 1: ^{1}H (500 MHz) and ^{13}C (100 MHz) NMR data for 6-isocassine (**1**) in $CDCl_3$ and CD_3OD (δ in ppm, *J* in Hz).

Position	$CDCl_3$		CD_3OD	
	δ_H	δ_C	δ_H	δ_C
2	3.38 (dd, 6.6, 2.8)	50.6	3.36 (dd, 6.7, 3.4)	52.3
3	3.87 (ddd, 7.4, 4.2, 2.8)	67.1	3.80 (ddd, 7.7, 3.6, 3.4)	67.7
4	1.76 (dddd, 13.6, 8.6, 5.8, 4.2) 1.70 (dddd, 13.6, 7.4, 6.4, 4.4)	26.3	1.80 (dddd, 13.6, 8.6, 4.4, 3.6) 1.68 (dddd, 13.6, 7.9, 7.7, 4.4)	26.8
5	2.03 (dddd, 13.6, 8.6, 4.4, 3.6) 1.44 (dddd, 13.6, 6.4, 5.8, 5.6)	23.7	2.01 (dddd, 13.6, 8.6, 4.4, 4.2) 1.41 (dddd, 13.6, 7.9, 4.4, 6.9)	25.2
6	3.10 (dddd, 10.4, 5.6, 3.6, 2.6)	50.9	3.11 (dddd, 9.8, 6.9, 4.2, 3.0)	52.1
7	1.30 (bd, 6.6)	13.8	1.26 (bd, 6.7)	11.9
1’	1.73 (m) 1.56 (m)	31.0	1.60 (m) 1.56 (m)	32.5
2’	1.30 (m)	26.3	1.34 (m)	27.2
3’-8’	1.27 (m)	29.7-29.2	1.30 (m)	30.6-30.2
9’	1.57 (tt, 7.4, 7.2)	23.8	1.54 (tt, 7.3, 7.2)	24.8
10’	2.42 (t, 7.4)	43.8	2.47 (t, 7.3)	43.3
11’	-	209.5	-	212.2
12’	2.14 (s)	29.9	2.12 (s)	29.8

While NOE data were not employed in the determination of relative configurations of the piperidine ring asymmetric centers, we were surprised to observe that the 1D-NOESY spectrum of compound **1** recorded in chloroform-*d* solution displayed weak negative enhancements (Figure 3a). On the other hand, and as normally expected for molecules of this size, positive NOE enhancements were observed when the experiments were carried out in methanol-d_4 (Figure 3b). This behavior is indicative of a shift from the diffusion limit to the extreme narrowing relaxation regimes [24], and directly related to a decrease in correlation times and apparent molecular size. It can be explained qualitatively by considering that the molecule, which bears two strong H-bond donor/acceptor groups in the piperidine ring, forms intermolecular hydrogen bonds (H-bonds) in non-polar aprotic environments, which are broken in polar protic solvents. Similar changes in NMR relaxation behavior upon aggregation have been reported in a variety of systems, including pharmaceuticals and supramolecular gels [25,26].

To corroborate this hypothesis we carried out an analogous study with *N*-methyl-6-isocassine (**2**), the *N*-methylated derivative of **1**

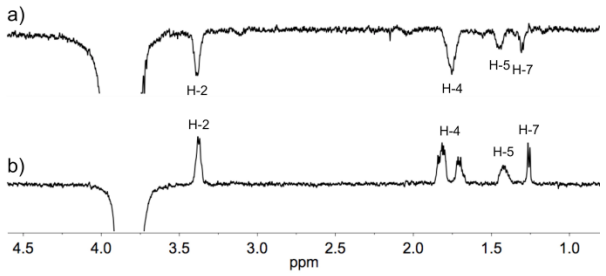

Figure 3: 1D-NOESY spectra of **1** obtained by selective inversion of the H-3 proton in $CDCl_3$ (a) and CD_3OD (b) solution.

Table 2: ^{1}H (500 MHz) and ^{13}C (100 MHz) NMR data for *N*-methyl-6-isocassine (**2**) in $CDCl_3$ and CD_3OD (δ in ppm, *J* in Hz).

Position	$CDCl_3$		CD_3OD	
	δ_H	δ_C	δ_H	δ_C
2	2.93 (qd, 6.7, 2.8)	57.8	3.08 (qd, 6.7, 4.4)	61.6
3	3.73 (ddd, 7.0, 3.6, 2.8)	68.0	3.80 (ddd, 10.0, 4.4, 4.2)	69.7
4	1.69 (dddd, 13.2, 8.6, 4.4, 3.6) 1.61 (dddd, 13.2, 7.6, 7.0, 4.4)	27.0	1.71 (dddd, 13.4, 4.4, 4.4, 4.2) 1.57 (dddd, 13.4, 10.2, 10.0, 4.4)	27.5
5	1.87 (dddd, 13.4, 8.6, 4.4, 4.2) 1.40 (dddd, 13.4, 6.5, 7.6, 4.4)	25.0	1.85 (dddd, 13.6, 4.4, 4.4, 3.8) 1.33 (dddd, 13.6, 10.6, 10.2, 4.4)	27.9
6	2.58 (dddd, 9.5, 6.5, 4.2, 3.2)	57.8	2.56 (m)	56.9
7	1.06 (d, 6.7)	10.8	1.05 (d, 6.7)	6.7
1’	1.51 (m) 1.37 (m)	26.7	1.62 (m) 1.31 (m)	31.9
2’	1.28 (m) 1.15 (m)	26.7	1.38 (m) 1.24 (m)	27.1
3’-8’	1.26 (m)	29.9-29.1	1.30 (m)	31.0-30.2
9’	1.55 (tt, 7.5, 7.2)	23.8	1.54 (tt, 7.4, 7.2)	24.8
10’	2.40 (t, 7.5)	43.8	2.47 (t, 7.4)	44.3
11’	-	209.4	-	214.6
12’	2.12 (s)	29.9	2.13 (s)	29.8
N-CH_3	2.34 (s)	39.4	2.40 (s)	39.8

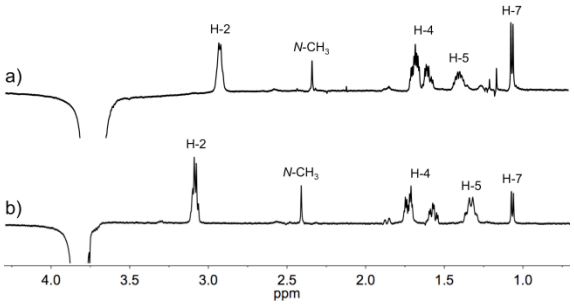

Figure 4: 1D-NOESY spectra of **2** obtained by selective inversion of the H-3 proton in $CDCl_3$ (a) and CD_3OD (b) solution.

that we have previously isolated from the bark of *P. affinis* and for which ^{1}H and ^{13}C assignments are presented in Table 2 [20]. In this case, the NOE enhancements were positive regardless of the solvent (Figure 4). This is consistent with the lower H-bonding capacity of compound **2**, which precludes the formation of intermolecularly H-bonded aggregates, even in non-polar aprotic solvents.

Similar conclusions can be reached by measuring the self-diffusion coefficient (*D*) of the compounds in both solvents using pulse-field gradients [27,28]. For **1**, the *D* changes from 4.46 ± 0.07 × 10^{-10} m^2/s in chloroform-*d* to 5.62 ± 0.08 × 10^{-10} m^2/s in methanol-d_4, indicating a decrease in the apparent molecular radius of ~25% if a Stokes-Einstein diffusion model is assumed [27]. On the other hand, the *D* for compound **2** goes from 6.45 ± 0.05 × 10^{-10} m^2/s to 5.75 ± 0.03 × 10^{-10} m^2/s when going from non-polar aprotic to polar protic media, consistent with an increase in the Stokes radius of ~10% in

this case. Furthermore, the apparent molecular radius of **1** in chloroform-*d* is nearly ~50% larger than that of **2** in the same solvent. These data are in line with the changes observed with NOE experiments, and provides quantitative evidence that strongly suggests the presence of 6-isocassine aggregates in non-polar media. Taking into account the H-bonding acceptor and donor groups present in the piperidine ring and the changes observed in apparent molecular radii, the formation of head-to-head dimers involving interactions between the secondary NH of one monomer and OH of the other is likely (Figure 5).

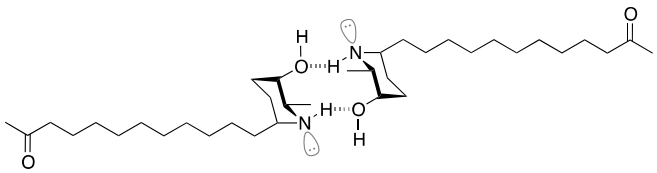

Figure 5: Proposed model of the 6-isocassine H-bonded head-to-head dimeric aggregates formed in apolar non-protic environments.

This behavior could help explain the biological mode of action of this type of piperidine alkaloids. As stated earlier, the ability of these molecules to affect ion transport mechanisms has been associated with their activity [12-16]. Based on the structural model portrayed above, these compounds would have a tendency to form ionophore-like aggregates spanning the width of a hydrophobic lipid bilayer with the potential of disrupting ion transport across membranes [29]. A number of studies should be carried out to validate these hypotheses. First, the existence of H-bonded aggregates in hydrophobic media could be further corroborated using IR experiments [30]. In addition, the effect of this class of compounds on membrane potentials will be investigated in an effort to understand better their biological activity.

Experimental

General: Flash column chromatography was carried out using 230-450 mesh Macherey-Nagel 60 silica gel. IR spectra were recorded using NaCl plates on a Shimadzu IRPrestige-21 FT-IR spectrophotometer. Optical rotations were measured on a Labotec WZZ-2B digital polarimeter at ambient temperature. EIMS were collected on a Shimadzu GCMS-QP2010 Ultra single quadrupole mass spectrometer using a 70 eV ionization voltage, while HR-ESIMS were recorded on a Bruker Daltonics micrOTOF-Q mass spectrometer. NMR experiments were performed at 25ºC on Bruker AVANCE III 500 and 400 spectrometers operating at ^{1}H frequencies of 500.13 and 400.13 MHz, and ^{13}C frequencies of 125.76 and 100.62 MHz, respectively, using tetramethylsilane (TMS) as internal standard. Resonance assignments were in many cases corroborated with 1D-TOCSY data obtained at varying mixing times (120, 80, and 40 ms), and ^{1}H homonuclear decoupling experiments were employed to determine coupling constants in complex signals. COSY, HSQC, and HMBC spectra were acquired using standard pulse sequences. Complex multiplets and coupling constants were analyzed using the routines available in MNova 10.0 (Mestrelabs Research, S. L., Santiago de Compostela, Spain). Molecular models of the different *N*-methylcassine and *N*-methylcarnavaline diastereomers employed in the calculation of $^{3}J_{HH}$ vicinal couplings were built and optimized using Avogadro 1.0 and the MMFF94 force field [31,32].

Experimental

Plant material: *P. nigra* bark was collected in March 2014 from the trunk of an individual tree found in a native woodland near Nuevo Berlín, Rio Negro department, Uruguay (S 32° 52' 58" W 58° 02' 39"). A voucher specimen was authenticated by Prof. Eduardo Alonso Paz and deposited in the herbarium of the Cátedra de Botánica, Facultad de Química, UdelaR, with ID number 4423 MVFQ.

Extraction and isolation: The air-dried bark of *P. nigra* (40 g) was finely powdered, soaked in 0.25% ammonia solution (10 mL), and extracted with CH_2Cl_2 (2 × 150 mL). The organic fractions were combined, their volume reduced to half under vacuum, and then extracted with 1% HCl (3 × 50 mL). The resulting aqueous extracts were pooled, basified using concentrated ammonia, and extracted with CH_2Cl_2 (3 × 50 mL). The organic fractions were combined, dried with Na_2SO_4, and the solvent was then removed under reduced pressure. The crude extract was subjected to flash column chromatography using CH_2Cl_2-EtOAc-diethylamine (7:2:1) as eluting solvent to yield compound **1** (37 mg, 0.1%).

6-Isocassine (1)
Pale yellow gum.
Rf: 0.35 (CH_2Cl_2-EtOAc-diethylamine, 7:2:1).
$[\alpha]_D^{23}$: +2.7 (*c* 0.15, $CHCl_3$).
IR (film) ν_{max}: 3356, 2926, 1712, 1464, 1366, 1165, 1082 cm^{-1}.
^{1}H NMR and ^{13}C NMR: Table 1.
EIMS *m*/*z* (%): 43 (6.4), 44 (7.1), 55 (2.9), 70 (2.2), 96 (10.3), 114 (100.0), 115 (7.7), 240 (11.3), 282 (1.7), 297 ([M]$^+$, 0.7).
HR-ESIMS *m*/*z*: 298.2719 ([M+H]$^+$, calcd. for $C_{18}H_{36}NO_2$: 298.2740).

***N*-Methyl-6-isocassine (2)** [20]
Pale yellow gum.
Rf: 0.54 (CH_2Cl_2-EtOAc-diethylamine, 7:2:1).
$[\alpha]_D^{23}$: -6.3 (*c* 0.35, $CHCl_3$).
IR (film) ν_{max}: 3385, 2929, 2855, 1717, 1458, 1366, 1063 cm^{-1}.
^{1}H NMR and ^{13}C NMR: Table 2.
EIMS *m*/*z* (%): 43 (4.5), 57 (3.2), 58 (6.3), 110 (6.7), 128 (100.0), 129 (8.3), 236 (1.0), 254 (5.4), 296 (1.8), 311 ([M]$^+$, 0.4).
HR-ESIMS *m*/*z*: 312.2941 ([M+H]$^+$, calcd. for $C_{19}H_{38}NO_2$: 312.2903).

NOE and diffusion experiments: 1D-NOESY spectra were recorded at 500 MHz using the DPFGSE-NOE pulse sequence and a mixing time of 300 ms [33]. Selective inversion of specific protons was achieved with Gaussian shaped pulses, the widths of which were calculated from the desired excitation regions. Self-diffusion coefficients (*D*) were also obtained at 500 MHz using a ^{1}H-detected PFG-STE sequence [28]. *D* values were estimated by fitting the ^{1}H signal variation versus gradient strength to mono-Gaussian curves according to Pelta and coworkers [28]. The self-diffusion coefficient for each compound was computed as the average of the *D* values obtained from five of its ^{1}H signals.

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References

[1] Robinson T. (**1981**) Pyrrolidine, piperidine, and pyridine alkaloids. In *The Biochemistry of Alkaloids*, Springer-Verlag, Berlin, 35-57.

[2] Harborne JB, Baxter H, Moss GP. (**1999**) Pyrrolidine and piperidine alkaloids. In *Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plants*, Taylor & Francis, London, 274-287.
[3] Burdick EM. (**1971**) Carpaine: an alkaloid of *Carica papaya*: its chemistry and pharmacology. *Economic Botany*, ***25***, 363-365.
[4] Khuong-Huu Q, Ratle G, Monseur X, Goutarel R. (**1972**) Alcaloïdes pipéridiniques. II. Structures de la prosopine et de la prosopinine, alcaloïdes du *Prosopis africana* (Guill. et Perr.) Taub. *Bulletin des Sociétés Chimiques Belges*, ***81***, 425-442.
[5] Ott-Longoni R, Viswanathan N, Hesse M. (**1980**) Die konstitution des alkaloides juliprosopin aus *Prosopis juliflora* A. DC. *Helvetica Chimica Acta*, ***63***, 2119-2129.
[6] Highet RJ. (**1964**) Alkaloids of *Cassia* species. I. Cassine. *Journal of Organic Chemistry*, ***29***, 471-474.
[7] Lythgoe D, Vernengo, MJ. (**1967**) Alkaloids from *Cassia carnaval* Speg.: cassine and carnavaline. *Tetrahedron Letters*, ***8***, 1133-1137.
[8] Ahmad VU, Sultana A, Qazi S. (**1989**) Alkaloids from the leaves of *Prosopis juliflora*. *Journal of Natural Products*, ***52***, 497-501.
[9] Samoylenko V, Dunbar DC, Jacob MR, Joshi VC, Ashfaq MJ, Muhammad I. (**2008**) Two new alkylated piperidine alkaloids from western honey mesquite: *Prosopis glandulosa* Torr. var. *torreyana*. *Natural Product Communications*, ***3***, 35-40.
[10] Samoylenko V, Ashfaq MK, Jacob MR, Tekwani BL, Khan SI, Manly SP, Joshi VC, Walker LA, Muhammad I. (**2009**) Indolizidine, antiinfective and antiparasitic compounds from *Prosopis glandulosa* Torr. var. *glandulosa*. *Journal of Natural Products*, ***72***, 92-98.
[11] Rahman AA, Samoylenko V, Jacob MR, Sahu R, Jain SK, Khan SI, Tekwani BL, Muhammad I. (**2011**) Antiparasitic and antimicrobial indolizidines from the leaves of *Prosopis glandulosa* var. *glandulosa*. *Planta Medica*, ***77***, 1639-1643.
[12] Choudhary MI, Nawaz SA, Zaheer-ul-Haq, Azim MK, Ghayur MN, Lodhi MA, Jalil S, Khalid A, Ahmed A, Rode BM, Atta-ur-Rahman, Gilanib AH, Ahmad VU. (**2005**) Juliflorine: a potent natural peripheral anionic-site-binding inhibitor of acetylcholinesterase with calcium-channel blocking potential, a leading candidate for Alzheimer's disease therapy. *Biochemical and Biophysical Research Communications*, **332**, 1171-1179.
[13] Freitas RM, Silva FO, Silva MGV, Feng D. (**2011**) Antioxidant mechanisms of iso-6-cassine in suppressing seizures induced by pilocarpine. *Brazilian Journal of Pharmacognosy*, ***21***, 437-443.
[14] Maioli MA, Lemos DECV, Guelfi M, Medeiros HCD, Riet-Correa F, Medeiros RMT, Barbosa-Filho JM, Mingatto FE. (**2012**) Mechanism for the uncoupling of oxidative phosphorylation by juliprosopine on rat brain mitochondria. *Toxicon*, ***60***, 1355-1362.
[15] Silva FO, Silva MGV, Feng D, Freitas RM. (**2011**) Evaluation of central nervous system effects of iso-6-cassine isolated from *Senna spectabilis* var. *excelsa* (Schrad) in mice. *Fitoterapia*, ***82***, 255-259.
[16] da Silva KABS, Manjavachi MN, Paszcuk AF, Pivatto M, Viegas Jr. C, Bolzani VS, Calixto JB. (**2012**) Plant derived alkaloid (-)-cassine induces anti-inflammatory and anti-hyperanalgesic effects in both acute and chronic inflammatory and neuropathic pain models. *Neuropharmacology*, ***62***, 967-977.
[17] Burkart A. (**1976**) A monograph of the genus *Prosopis* (Leguminosae subfam. Mimosoideae). *Journal of the Arnold Arboretum*, ***57***, 219-249.
[18] Gianinetto IB, Cabrera JL, Juliani HR. (**1980**) Isolation of *N*-methylcassine from species of the genus *Prosopis*. *Journal of Natural Products*, ***43***, 632-633.
[19] Dätwyler P, Ott-Longoni R, Schöpp E, Hesse M. (**1981**) Über juliprosin, ein weitres alkaloid aus *Prosopis juliflora* A. DC. *Helvetica Chimica Acta*, ***64***, 1959-1963.
[20] Reina L, Bennadji Z, Vinciguerra V, Ferreira F, Moyna G, Menendez P. (**2015**) Isolation and structural characterization of new piperidine alkaloids from *Prosopis affinis*. *Phytochemistry Letters*, ***14***, 265-269.
[21] Benadji Z, Ferreira F, Castillo D, Alfonso M. (**2014**) Desarrollo de estrategias para la valorización de la flora arbórea nativa del Uruguay: de la bioprospección a la biorefinería. *Revista INIA*, ***39***, 57-61.
[22] Christofidis I, Welter A, Jadot J. (**1977**) Spectaline and iso-6-cassine, two new piperidin-3-ol alkaloids from the leaves of *Cassia spectabilis*. *Tetrahedron*, ***33***, 977-979.
[23] Haasnoot CAG, de Leeuw FAAM, Altona C. (**1980**) The relationship between proton-proton coupling constants and substituent electronegativities - I. *Tetrahedron*, ***36***, 2783-2792.
[24] Claridge TDW. (**2009**) Correlations through space: the nuclear Overhauser effect. In *High-Resolution NMR Techniques in Organic Chemistry*, Elsevier, Amsterdam, 247-301.
[25] Ding X, Stringfellow TC, Robinson JR. (**2003**) Self-association of cromolyn sodium in aqueous solution characterized by nuclear magnetic resonance spectroscopy. *Journal of Pharmaceutical Sciences*, ***93***, 1351-1358.
[26] Rodríguez-Llansola F, Miravet JF, Escuder B. (**2009**) Supramolecular gel formation and self-correction induced by aggregation-driven conformational changes. *Chemical Communications*, 209-211.
[27] Brand T, Cabrita EJ, Berger S. (**2008**) Theory and application of NMR diffusion studies. In *Modern Magnetic Resonance: Part I: Applications in Chemistry, Biological and Marine Sciences*, Springer, Dordrecht, 135-143.
[28] Pelta MD, Barjat H, Morris GA, Davis AL, Hammond SJ. (**1998**) Pulse sequences for high-resolution diffusion-ordered spectroscopy (HR-DOSY). *Magnetic Resonance in Chemistry*, ***36***, 706-714.
[29] Pressman BC. (**1976**) Biological applications of ionophores. *Annual Reviews of Biochemistry*, ***45***, 501-530.
[30] Coggeshall ND, Saier AL. (**1951**) Infrared absorption study of hydrogen bonding equilibria. *Journal of the American Chemical Society*, ***73***, 5414-5418.
[31] Hanwell MD, Curtis DE, Lonie DC, Vandermeersch T, Zurek E, Hutchison GR. (**2012**) Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. *Journal of Cheminformatics*, ***4***, 17.
[32] Halgren TA. (**1996**) Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94. *Journal of Computational Chemistry*, ***17***, 490-519.
[33] Stott K, Keeler J, Van QN, Shaka AJ. (**1997**) One-dimensional NMR experiments using pulse field gradients. *Journal of Magnetic Resonance*, ***125***, 302-324.