



UNIVERSITAT DE
BARCELONA

Estudio de los alcaloides de las Amaryllidaceae como fuente de nuevas moléculas bioactivas

Luciana Ruschel Tallini

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UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

**ESTUDIO DE LOS ALCALOIDES DE LAS AMARYLLIDACEAE COMO
FUENTE DE NUEVAS MOLÉCULAS BIOACTIVAS**

LUCIANA RUSCHEL TALLINI

2018

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FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

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**ESTUDIO DE LOS ALCALOIDES DE LAS AMARYLLIDACEAE COMO
FUENTE DE NUEVAS MOLÉCULAS BIOACTIVAS**

Memòria presentada per Luciana Ruschel Tallini per optar al títol de doctor per la
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Listado de abreviaturas

AC	Acetilcolina
ACE	Acetilcolinesterasa
BuCE	Butirilcolinesterasa
Calcd	Calculado
CG	Cromatografía de Gases
CG-EM	Cromatografía de Gases acoplada a Espectrometría de Masas
COSY	Espectroscopía de Correlación ^1H - ^1H
DC	Dicroísmo Circular
EC ₅₀	Concentración Efectiva de 50%
EA	Enfermedad de Alzheimer
EI	Ionización por Impacto de Electrones
EIMS	Espectrometría de Masas por Impacto Electrónico
EM	Espectrometría de Masas
ERA	Extracto Rico en Alcaloides
EtOAc	Acetato de etilo
HMBC	Correlación Heteronuclear a Multiple Enlace ^1H - ^{13}C
HR-ESI-MS	Espectrometría de Masas – Electrospray – Alta Resolución
HRMS	Espectrometría de Masas de Alta Resolución
HSQC	Correlación Heteronuclear de Simple Cuanto (^1H - ^{13}C)
Hz	Hertz
IC ₅₀	Concentración inhibidora del 50%
IR	Infrarrojo
<i>J</i>	Constante de acoplamiento
MeOH	Metanol
MHz	Megahertz
<i>m/z</i>	masa/carga
<i>n</i> -Hex	<i>n</i> -Hexano
NOESY	Espectroscopía de Efecto Nuclear Overhauser
RMN	Resonancia Magnética Nuclear
^1H -RMN	Resonancia Magnética Nuclear de Protón
^{13}C -RMN	Resonancia Magnética Nuclear de Carbono 13
1D-RMN	Resonancia Magnética Nuclear monodimensional
2D-RMN	Resonancia Magnética Nuclear bidimensional
sp	Especies
TLC	Cromatografía en capa fina
UV	Ultravioleta
VLC	Cromatografía Líquida en Vacío
α_{D}	Rotación óptica
δ	Desplazamiento químico

Con la finalidad de uniformizar los nombres de los compuestos aislados o identificados, se ha optado por no llevar a cabo su traducción al español.

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1. INTRODUCCIÓN

1. INTRODUCCIÓN

1.1 Los productos naturales y el desarrollo de nuevos fármacos

Los productos naturales representan uno de los pilares básicos en el desarrollo de nuevos fármacos debido a su diversidad estructural y potencial biológico (Feher y Schmidt, 2003; Newman y Cragg, 2016). Entre 1940 y 2014, alrededor del 50% de las pequeñas moléculas aprobadas para el tratamiento del cáncer se desarrollaron directa o indirectamente a partir de productos naturales (Newman y Cragg, 2016), siendo la riqueza estructural de estos compuestos una guía inestimable en la investigación de nuevos medicamentos (Lee y Schneider, 2001).

Las plantas poseen una amplia influencia en la medicina tradicional y, en consecuencia, la búsqueda de especies utilizadas popularmente para el tratamiento de alguna enfermedad constituye una de las líneas más importantes para el desarrollo de nuevos fármacos (Harvey, 2000). Los alcaloides, metabolitos secundarios encontrados principalmente en las plantas, juegan un papel crucial en el descubrimiento de nuevos fármacos debido a su diversidad estructural y su complejidad espacial (Rodrigues et al., 2016; Stratton et al.; 2015; Newman y Cragg, 2016). En la medicina actual, diversos fármacos se desarrollaron en base a sus usos en la medicina popular, como por ejemplo la atropina, morfina y quinina (Verpoorte, 1998).

1.2 La familia Amaryllidaceae

Las Amaryllidaceae constituyen una familia de plantas perteneciente a las Monocotiledóneas del orden Asparagales y que actualmente engloba tres subfamilias: Amaryllidoideae, Agapanthoideae y Allioideae, aunque hasta hace poco se consideraban familias separadas: Amaryllidaceae, Agapanthaceae y Alliaceae, respectivamente (APG III, 2009). En este trabajo se utilizará la denominación “alcaloides de las Amaryllidaceae” la cual corresponderá, única y exclusivamente, a los alcaloides sintetizados por plantas de la subfamilia Amaryllidoideae.

La subfamilia Amaryllidoideae comprende 59 géneros y cerca de 850 especies que se distribuyen preferencialmente en regiones tropicales y subtropicales, pero también en zonas templadas. Estas plantas presentan una gran capacidad de adaptación y sus principales centros de diversidad son Sudáfrica, Sudamérica y la región Mediterránea (Rønsted et al., 2012). Actualmente, se cultivan como ornamentales debido a la belleza de sus flores, sin embargo, desde hace siglos vienen recibiendo una especial atención

debido a sus propiedades terapéuticas (Bastida et al., 2006; Ingrassia et al., 2008; Goietsenoven et al., 2010). Hipócrates de Cos (a. C. 460-370), considerado el padre de la medicina, recomendaba el aceite de especies de *Narcissus* (Amaryllidaceae) para el tratamiento de lo que hoy conocemos como tumores de útero (Goietsenoven et al., 2013).

1.2.1 Algunos géneros representativos de las Amaryllidaceae de Sudamérica

El género *Amaryllis* L. comprende apenas dos especies, *A. acuminata* y *A. belladonna*, ambas nativas de Sudáfrica y utilizadas en medicina tradicional por las etnias Sotho, Xhosa, Zulu y en Java para el tratamiento del “swelling”, sinónimo de cáncer (Petit et al., 1984; Nair et al., 2016). Sin embargo, es posible encontrar la especie *A. belladonna* en regiones de Sudamérica, como Argentina y el sur de Brasil.

El género *Clinanthus* Herb. representa cerca de 30 especies y tiene una amplia incidencia en Perú. Normalmente está localizado en regiones de montaña de más de 2000 metros de altitud, pero también es posible encontrarlo en la región costera (León et al., 2006; Amaral-Lopes y Cavalcanti, 2015). El *Crinum* L. es el único género pantropical de Amaryllidaceae, lo cual presenta un gran interés económico y medicinal debido a sus usos como planta ornamental y en la medicina tradicional (Fennell y Staden, 2001; Tram et al., 2002; Refaat et al., 2012). Este género contiene cerca de 130 especies, muchas popularmente utilizadas como vermífugos y purgantes, pero también para el tratamiento de problemas urinarios, mordeduras de serpiente, para la tos crónica y el asma. (Amos et al., 2003). El género *Eucrosia* Ker. Gawl. es poco conocido y limitado a la región de los Andes. Este género está representado por apenas 7 especies, reportadas únicamente en Ecuador y Perú (Meerow, 1987).

Los géneros *Habranthus* Herb. e *Hippeastrum* Herb. son nativos de Sudamérica y comprenden cerca de 40 y 60 especies, respectivamente. La mitad de las especies de ambos géneros se encuentran localizadas en territorio brasileño (Oliveira y Sano, 2009; Amaral-Lopes y Cavalcanti, 2015). Algunas plantas del género *Hippeastrum* han sido utilizadas en medicina popular de Sudamérica para la cicatrización de heridas y la curación de tumores (Deepa y Kuriakose, 2014).

El género *Phaedranassa* Herb. comprende unas 10 especies, de las cuales la mayoría son endémicas de Ecuador (Minga et al., 2015). Muchas son conocidas únicamente en localidades específicas, formando poblaciones muy localizadas pero

amplias, normalmente por encima de los 2000 metros de altitud (Meerow y Snijman, 1998). *Phycella* Lindl. es un género originario de Argentina y Chile que incluye seis especies, entre las cuales *P. australis* es muy utilizada como ornamental en Chile (Baeza et al., 2012; Flores et al., 2013; Zuloaga y Belgrano, 2015).

El género *Rhodophiala* C. Presl. es nativo de Sudamérica y se encuentra representado por aproximadamente 40 especies distribuidas entre Argentina, Bolivia y sur de Brasil, (Baeza et al., 2012). El género *Stenomesson* Herb. comprende cerca de 15 especies las cuales usualmente se encuentran localizadas en regiones secas o en las zonas limítrofes de los bosques por encima de 2000 metros de altitud de regiones de Perú y de Ecuador (Meerow et al., 2015). Finalmente, el género *Zephyranthes* Herb. se encuentra representado por aproximadamente 50 especies de las cuales la especie *Z. candida* es reconocida dentro de la medicina tradicional china para el tratamiento de convulsiones, epilepsia y tétanos (Zhang et al., 2016), mientras que la especie *Z. parulla* es popularmente reportada en Perú para el tratamiento de tumores (Herrera et al., 2001).

1.2.2 La biosíntesis de los alcaloides de las Amaryllidaceae

La familia de las Amaryllidaceae contiene un grupo exclusivo de alcaloides los cuales reciben considerable atención en vista de sus características estructurales y del potencial biológico que presentan (Bastida et al., 2006). Actualmente, ya se han aislado y caracterizado cerca de 500 alcaloides a partir de diferentes especies de Amaryllidaceae (Rønsted et al., 2012). La diversidad estructural que presentan estos compuestos hace que sean considerados como una fuente importante para el desarrollo de nuevos fármacos (Cimmino et al., 2017).

Ya en 1877, la lycorine fue el primer alcaloide de las Amaryllidaceae aislado y elucidado estructuralmente, previsiblemente a partir de una especie del género *Lycoris* (Bastida et al., 2006), aunque hay datos discordantes. Este compuesto es uno de los alcaloides de las Amaryllidaceae de mayor distribución entre las distintas especies de esta familia y ha sido muy reportado en la literatura debido a su potencial biológico (Guo et al., 2014a). Sin embargo, la galanthamine, aislada de *Galanthus woronovii* en la década de los 50 es, hasta el momento, el único alcaloide aprobado por la FDA (Food and Drug Administration) para ser comercializado como fármaco para el tratamiento paliativo de la enfermedad de Alzheimer, en los estadios de leve a moderado (Maelicke et al., 2001). A partir de este momento, el interés por los alcaloides de las

Amaryllidaceae como fuente de nuevos medicamentos se vió incrementado notablemente (Kornienko y Evidente, 2008).

En general, los alcaloides de las Amaryllidaceae están clasificados en nueve grupos distintos representados por norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, tazettine, montanine y galanthamine (**Figura 1**) (Bastida et al., 2011). Todas estas estructuras se originan a partir de un precursor común conocido como *O*-methylnorbelladine, que deriva de la combinación de los aminoácidos aromáticos fenilalanina (L-Phe) y tirosina (L-Tyr) (Battersby et al., 1961).

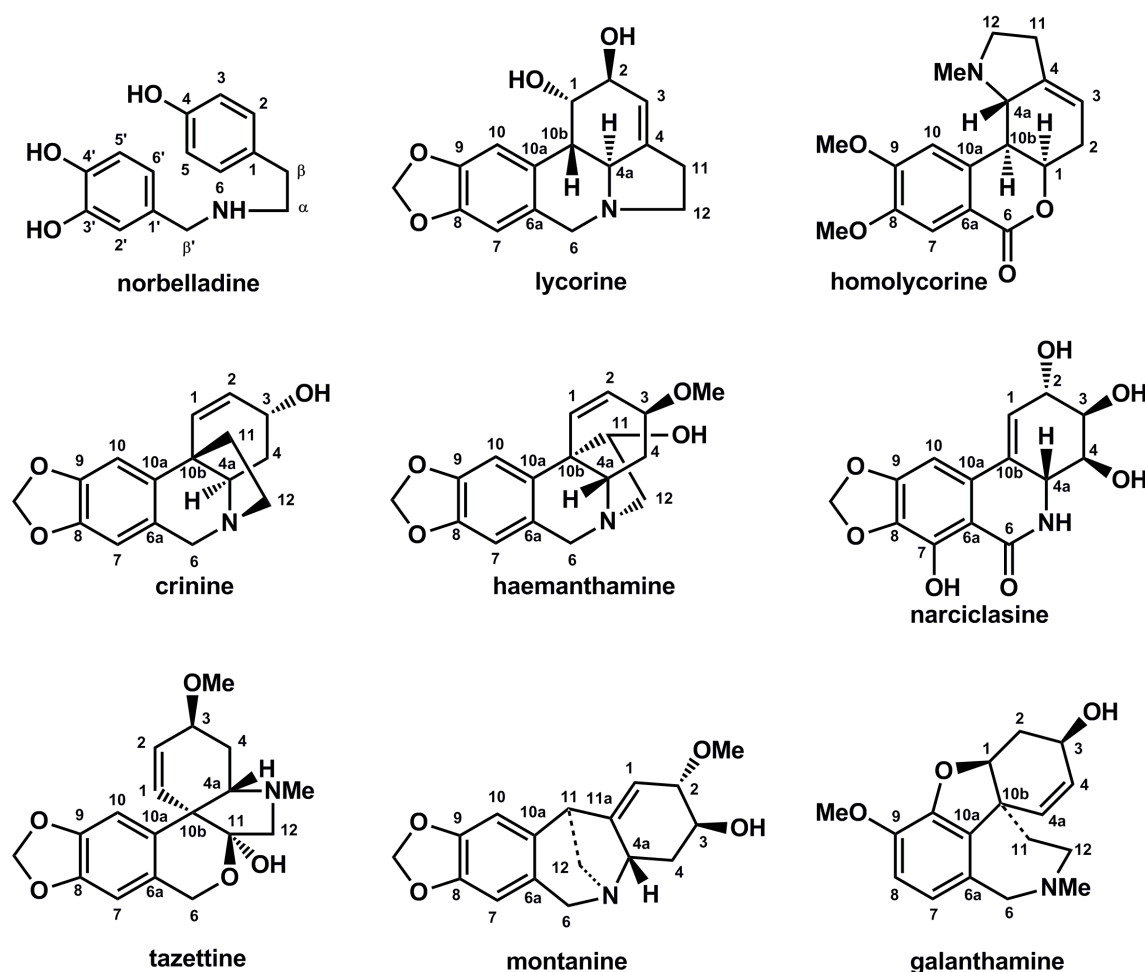


Figura 1. Alcaloides de Amaryllidaceae representativos de los 9 grupos. La numeración de las estructuras se corresponde con la propuesta por Ghosal et al. (1985).

En la biosíntesis de los alcaloides de las Amaryllidaceae, L-Phe es el precursor primario del fragmento C₆-C₁, que corresponde al anillo A y a la posición bencílica, mientras que L-Tyr es el precursor del anillo C, de la cadena lateral de los carbonos y del nitrógeno, fragmento C₆-C₂-N (Battersby et al., 1961).

A través de la vía de los ácidos cinámicos, la L-Phe se convierte en protocatechuic aldehyde en presencia de la enzima fenilalanina amonio liasa (PAL) para formar la porción C₆-C₁. Por su parte, L-Tyr por acción de la enzima tyr-descarboxilasa da lugar a la tyramine para formar la porción C₆-C₂-N (Bastida et al., 2006). La unión entre protocatechuic aldehyde y tyramine da origen a norbelladine por medio de la formación de una base de Schiff y reducción. Finalmente, la metilación de norbelladine en la posición 4' del anillo A, da lugar a la *O*-methylnorbelladine (Figura 2) (Bastida et al., 2006).

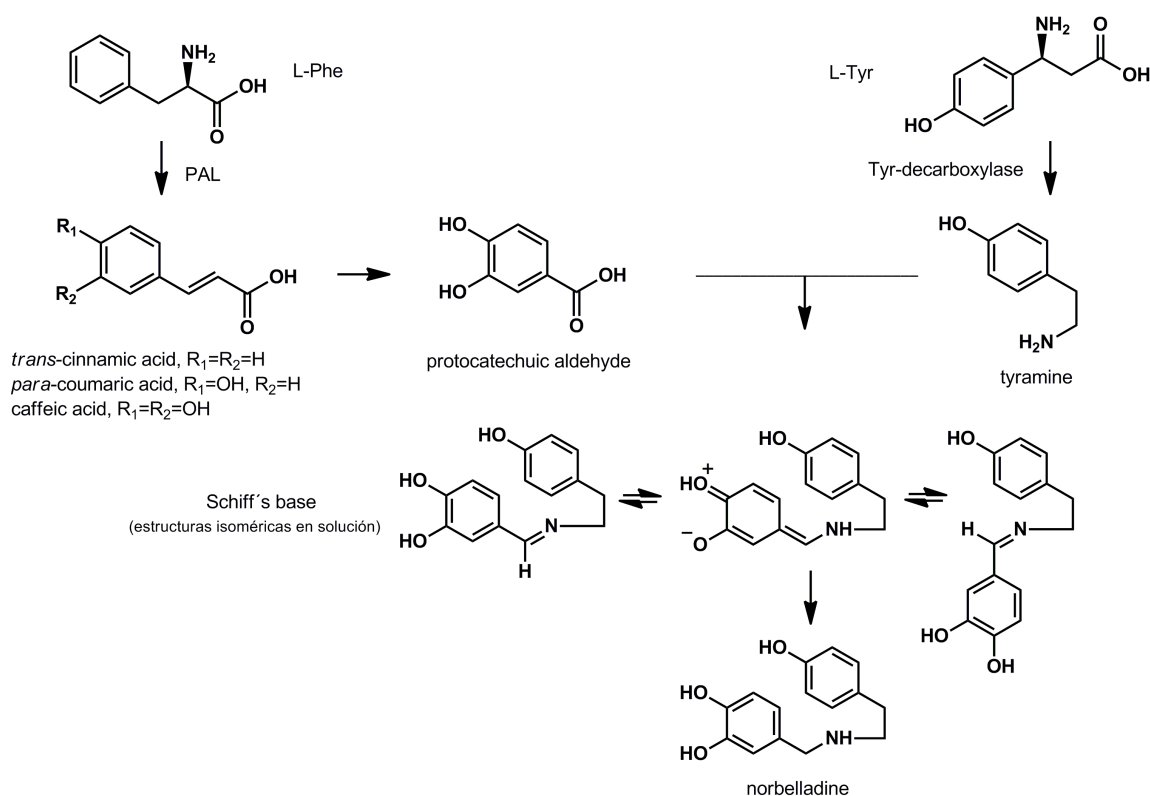


Figura 2. Ruta biosintética de la norbelladine.

El paso siguiente consiste en una ciclación secundaria producida por acoplamiento fenol oxidativo intramolecular de *O*-methylnorbelladine, lo cual puede transcurrir a través de tres vías diferentes para dar lugar a los distintos tipos estructurales (Figura 3). Del acoplamiento fenol oxidativo *orto-para'* resulta la formación de los esqueletos tipo lycorine y homolycorine. Por otro lado, la ciclación secundaria *para-para'* produce los alcaloides con estructuras del tipo crinine, haemanthamine, tazettine, narciclasine y montanine. Finalmente, los alcaloides del tipo

galanthamine derivan de un acoplamiento fenol oxidativo *para-orto'* (Bastida et al., 2006).

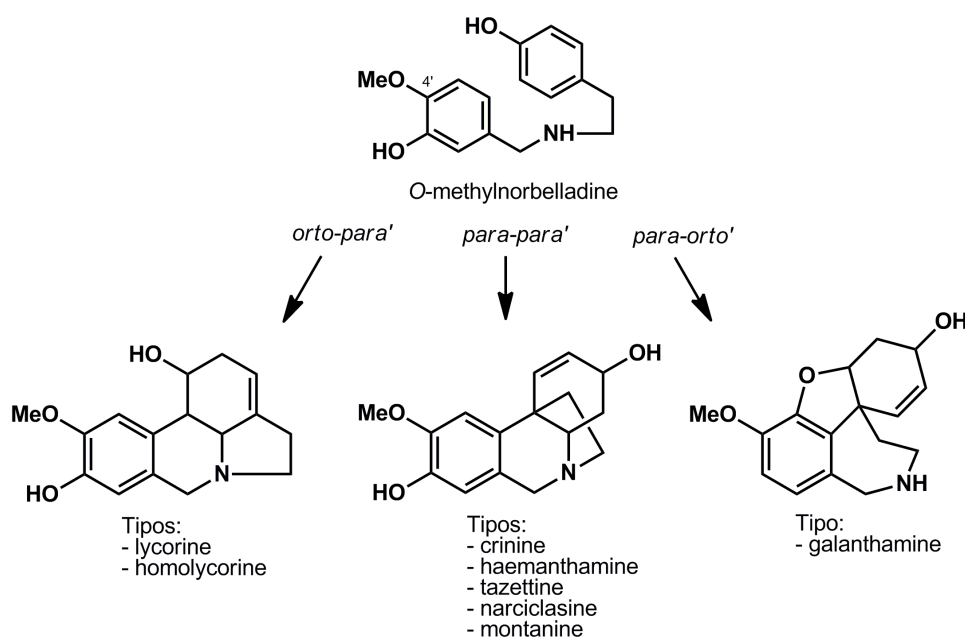


Figura 3. Vías alternativas del acoplamiento fenol-oxidativo de la *O*-methylnorbelladine.

1.2.3 Actividades biológicas de los alcaloides de las Amaryllidaceae

Los estudios de una planta muchas veces empiezan por la observación de su uso en la medicina tradicional, seguido de un *screening* para verificar el potencial biológico del extracto de esta especie frente a una determinada enfermedad y del fraccionamiento de los extractos hasta obtener el compuesto activo aislado. Un gran número de extractos vegetales han sido evaluados demostrando un amplio rango de actividades biológicas. El aislamiento de los alcaloides puros ha permitido establecer ciertas actividades para los diversos tipos de estructuras de alcaloides de Amaryllidaceae.

El compuesto norbelladine (**Figura 4**), perteneciente a los alcaloides del tipo homónimo, presentó una actividad antioxidante de 31 y 33% mediante los métodos del DPPH¹ y del radical superóxido, respectivamente, a una concentración de 10 µM. Además, este compuesto demostró la capacidad de inhibir tres enzimas involucradas en

¹ Este estudio evalúa la actividad antioxidante utilizando el método del DPPH (2,2-difenil-1-picrilhidracilo).

procesos inflamatorios: 51% de la COX-1² y 25% de la COX-2², a concentraciones de 0.25 μ M; y 23% de la NF- κ B² a una concentración de 10 μ M (Park, 2014).

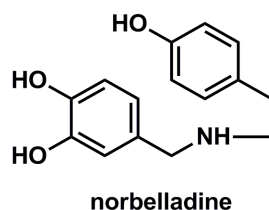


Figura 4. Estructura del tipo norbelladine.

Los alcaloides del tipo lycorine, probablemente los más conocidos de la familia Amaryllidaceae, poseen una amplia gama de actividades biológicas. Recientemente, un estudio reveló que lycorine (**Figura 5**) presenta una fuerte actividad frente al virus de la influenza A (H5N1)³, con EC₉₀⁴ de 0.52 μ M. Se sugiere que este compuesto estaría inhibiendo el transporte del complejo ribonucleoproteína del núcleo hacia el citoplasma durante la replicación del virus caracterizándolo como un posible candidato como fármaco anti-influenza (He et al., 2013).

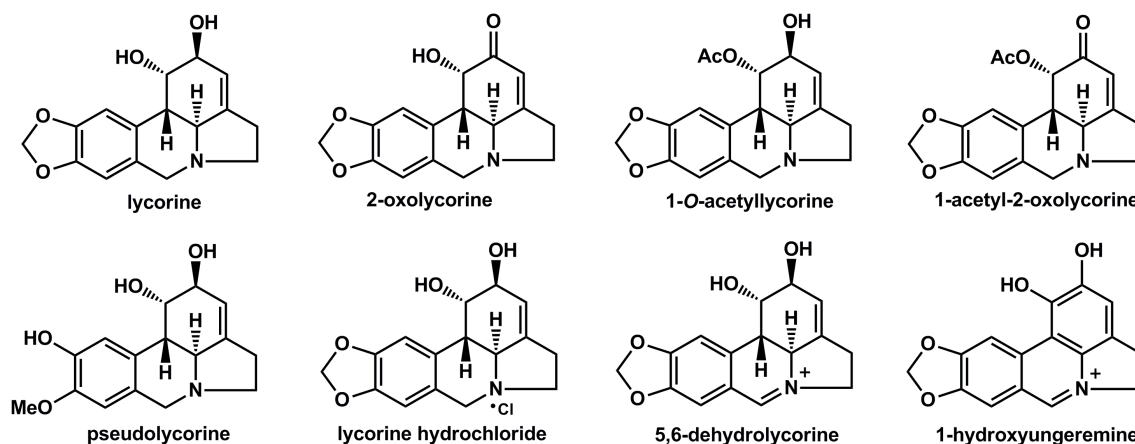


Figura 5. Estructuras del tipo lycorine.

Lycorine también mostró actividad inhibidora del virus de la polio, con una EC₅₀ de 0.06 μ g ml⁻¹ y un índice de selectividad de 24.9 (Oluyemisi et al., 2015), así como en la replicación del virus de la hepatitis C, con una EC₅₀ de 0.316 μ M y un índice de

² COX-1 (ciclooxigenasa-1), COX-2 (ciclooxigenasa-2) y NF- κ B (factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas) son marcadores antiinflamatorios.

³ Cepa altamente patógena causante de enfermedades respiratorias graves y muerte en aves.

⁴ Concentración de fármaco que induce 90% del efecto máximo.

selectividad de 19 (Chen et al., 2015). También se han sintetizado derivados de lycorine, logrando la pérdida de su citotoxicidad en distintos grados, con un elevado potencial frente al virus de la hepatitis C. Los resultados experimentales sugieren que una cetona en la posición C-2 mejora la actividad de este compuesto frente al mencionado virus, así como su índice de selectividad (Chen et al., 2015).

El compuesto 1-*O*-acetyllycorine (**Figura 5**) también inhibió el virus de la hepatitis C (VHC), con EC_{50} de 2.1 μ M, y suprimió la proliferación de múltiples cepas celulares del enterovirus 71 (EV71)⁵, con valores de EC_{50} entre 0.45 y 1.83 μ M y con índices de selectividad superiores a 450 (Guo et al., 2016). Diversos análogos de lycorine han sido sintetizados y evaluados frente al virus del dengue⁶. 1-*O*-Acetyllycorine exhibió la mejor capacidad de inhibición de este virus, con una EC_{50} de 0.4 μ M, y un índice de selectividad de 750. 2-Oxolycorine y 1-acetyl-2-oxolycorine (**Figura 5**) también mostraron una excelente actividad frente al virus del dengue, con una EC_{50} de 0.5 y de 1.8 μ M, respectivamente, y baja citotoxicidad (Wang et al., 2014).

Algunos alcaloides del tipo lycorine presentan actividad antitumoral. Un estudio reciente investigó la utilidad del compuesto lycorine para tratar el cáncer de páncreas utilizando la línea celular PANC-1 *in vitro* y ratones BALB/c “athymic nude” *in vivo*. Los resultados demostraron que este alcaloide induce la apoptosis *in vitro* e inhibe de manera eficaz el crecimiento tumoral en este modelo animal, sugiriendo que lycorine podría ser una opción prometedora para el tratamiento del cáncer de páncreas (Liu et al., 2016).

Además, se demostró que lycorine presenta una potente actividad frente a células del adenocarcinoma colorrectal con una mutación del gen p-53⁷ (Caco-2 y HT-29), con valores de EC_{50} de 0.99 y 1.2 μ M, respectivamente (Dorskocil et al., 2015), y frente a cuatro diferentes líneas celulares de cáncer de próstata (PC-3M, DU145, LNCaP y 22RV1), observándose valores de EC_{50} entre 5 y 10 μ M (Hu et al., 2015). Lycorine y pseudolycorine (**Figura 5**) presentaron actividad citotóxica frente al cáncer de pulmón (A549), con valores de EC_{50} de 11.36 y 4.48 μ M, respectivamente, y de colon (LoVo), con valores de EC_{50} de 17.37 y 9.02 μ M, respectivamente (Ang et al., 2015).

⁵ Produce una meningitis aséptica grave, encefalitis, miocarditis, parálisis flácida aguda y edema pulmonar.

⁶ Agente causal de la enfermedad de dicho nombre, transmitida por la hembra del mosquito *Aedes aegypti*.

⁷ Proteína supresora de tumores.

Los resultados sugieren que lycorine hydrochloride (**Figura 5**) inhibe selectivamente la proliferación de una línea celular de cáncer de ovario altamente invasiva (Hey1B), con un EC_{50} de 1.2 μM (Cao et al., 2013). Además, los alcaloides 5,6-dehydrolycorine y 1-hydroxyungeremine (**Figura 5**) mostraron una potente actividad citotóxica frente a líneas celulares de astrocitoma (CCF-STTG1) y glioma (CHG-5, SHG-44, y U251), así como, frente a las de leucemia mielítica humana (HL-60), hepatocarcinoma (SMMC-7721) y cáncer de colon (W480), con valores de EC_{50} entre 9.4 y 11.6 μM y entre 9.4 y 11.8 μM , respectivamente (Hao et al., 2013; Liu et al., 2015). Finalmente, hay estudios que revelan la existencia de una interesante correlación entre la lipofilia del alcaloide lycorine y su actividad antitumoral, lo que indica que la permeabilidad celular es un factor importante en la propuesta de agentes antitumorales derivados de lycorine (Dasari et al., 2014).

Además de la actividad antitumoral, 1-hydroxyungeremine (**Figura 5**) también presentó una marcada capacidad de inhibir selectivamente la COX-2, mostrándose como un posible agente antiinflamatorio, comparándolo con la sustancia de referencia NS-398 (>90%) (Liu et al., 2015). Otra actividad destacable de los alcaloides del tipo lycorine es la antiparasitaria. El compuesto 5,6-dehydrolycorine (**Figura 5**) exhibió una notable actividad antimalárica frente a cepas fármaco-resistentes (D-6) y fármaco-sensibles (W-2) de *Plasmodium falciparum*, con EC_{50} de 2.3 y 1.9 μM , respectivamente (Hao et al., 2013), mientras que la lycorine presentó actividad frente *Trichomonas vaginalis* a través de un mecanismo conocido como paraptosis⁸ (Giordani et al., 2011).

El alcaloide hippeastrine (**Figura 6**), del tipo homolycorine, mostró una alta capacidad de inhibición de la enzima topoisomerasa tipo I⁹ *in vitro*, con una EC_{50} de 7.25 $\mu g\ ml^{-1}$, mientras que el control positivo (camptothecine) presentó una EC_{50} de 6.72 $\mu g\ ml^{-1}$. Además, este alcaloide inhibió el crecimiento de líneas celulares de adenocarcinoma colorrectal (HT-29) y hepatocarcinoma humano (Hep-G2), con unos valores de EC_{50} de 3.98 y 11.85 $\mu g\ ml^{-1}$, respectivamente, sugiriéndose que esta estructura podría ser de alto interés para el desarrollo de nuevos antitumorales (Chen et al., 2016). Tanto hippeastrine como los dímeros de este compuesto (**Figura 6**) presentaron una buena actividad antimalárica frente a la cepa fármaco-sensible (F-32 Tanzania) de *Plasmodium falciparum*, con una EC_{50} de 12.7 y 1.3 μM , respectivamente (Cedron et al., 2013).

⁸ Forma no apoptótica de muerte celular programada.

⁹ Enzima clave para el desarrollo de posibles agentes quimioterápicos.

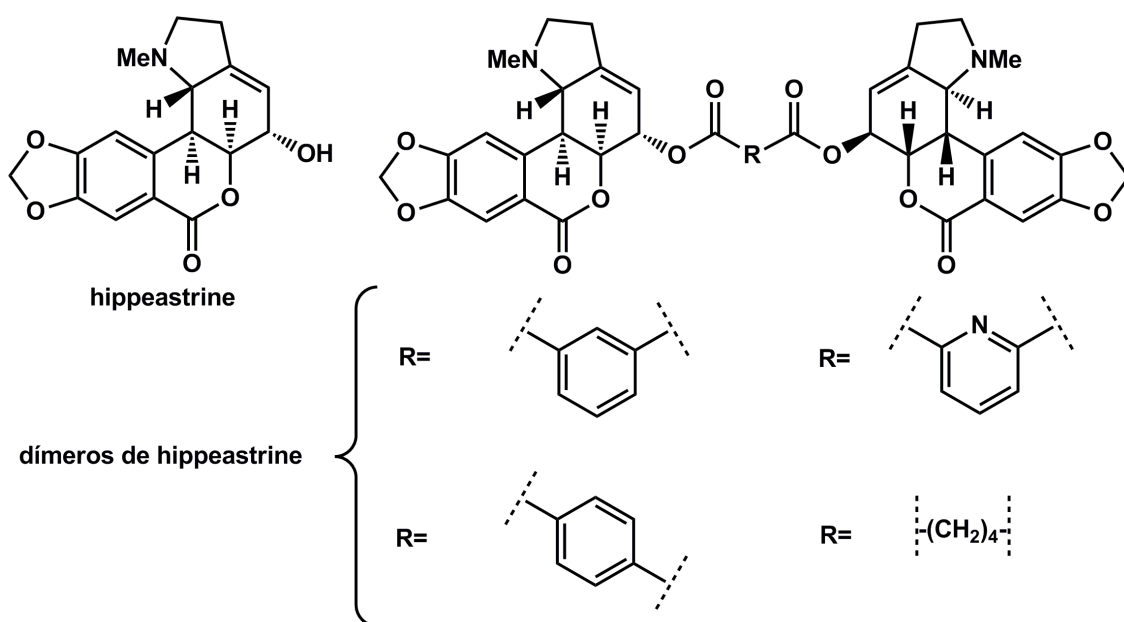


Figura 6. Estructuras del tipo homolycorine.

Varios alcaloides de la serie haemanthamine exhiben efectos inhibitorios significativos frente a una gran variedad de células tumorales (Bastida et al., 2006). Haemanthamine y haemanthidine (**Figura 7**) presentaron buena actividad anti-proliferativa frente a líneas celulares de cáncer de ovario (A2780), con una EC_{50} de 0.68 y 1.5 μM ; de pulmón (SW1573), con una EC_{50} de 2.1 y 2.0 μM ; de mama (T47-D), con una EC_{50} de 0.87 y 1.8 μM ; y de colon (WiDr), con una EC_{50} de 1.2 y 2.7 μM , respectivamente (Cedrón et al., 2015). Además, se demostró que haemanthamine presenta actividad frente a células de adenocarcinoma colorrectal con una mutación en el gene p-53¹⁰ (Caco-2 y HT-29), con una EC_{50} de 0.99 y 0.59 μM , respectivamente (Dorskocil et al., 2015).

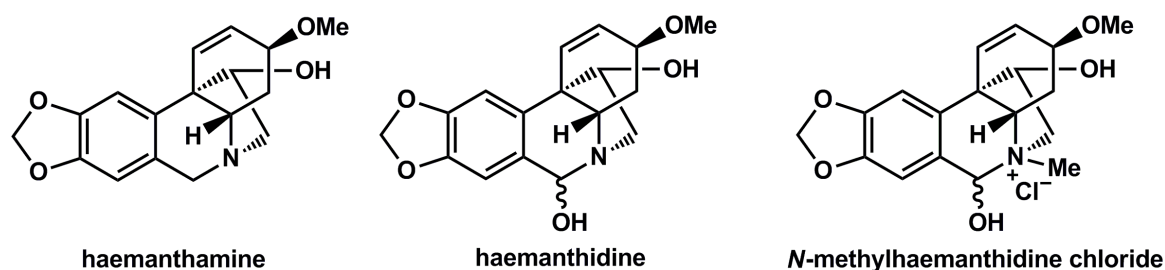


Figura 7. Estructuras del tipo haemanthamine.

¹⁰ La activación anormal de esta vía conduce a una respuesta proliferativa y antiapoptótica que se relaciona con el desarrollo de múltiples tipos de cáncer, y su estudio es parte crucial para el conocimiento de los procesos de carcinogénesis

De todos los compuestos del tipo haemanthamine, cabe destacar el compuesto *N*-methylhaemanthidine chloride (**Figura 7**) como un potente agente quimioterápico para el tratamiento del cáncer de páncreas al detener el ciclo celular, inducir apoptosis, inhibir el metabolismo de la glucosa y reprimir la migración celular, probablemente ejerciendo su actividad antineoplásica a través de la inhibición de la vía AKT¹⁰ (Guo et al., 2014b). Resultados experimentales demuestran la capacidad de los compuestos haemanthamine y haemanthidine en inhibir la proliferación celular, aumentar la apoptosis por activación de las caspasas, disminuir el potencial de la membrana mitocondrial y detener el ciclo celular en células Jurkat de leucemia (Havelek et al., 2014).

Al igual que ocurre con lycorine, haemanthamine (**Figura 7**) también exhibió una fuerte actividad antiviral frente al virus de la influenza A (H5N1), con una EC₉₀ de 4.15 μ M, lo que también induciría a pensar que este alcaloide pueda ser un candidato válido como fármaco anti-influenza (He et al., 2013). Otra actividad destacada para este tipo de estructuras es la antimalárica. Algunos resultados sugieren que la presencia de un grupo metilendioxo y un nitrógeno terciario no metilado, contribuirían a su actividad antiparasitaria (Osorio et al., 2008).

Con respecto a los alcaloides tipo crinine, con su característico puente 5,10b-etano en β , un estudio reciente sugiere que crinsarnine (**Figura 8**) tendría un notable potencial como insecticida frente al mosquito *Aedes aegypti* en su fase adulta, agente transmisor del dengue, con un 97% de mortalidad a una dosis de 5 μ g por mosquito y EC₅₀ de 2.29 μ g por mosquito (Masi et al., 2016). La buphanisine (**Figura 8**) mostró una interesante actividad citotóxica frente a dos líneas de células de adenocarcinoma colorrectal (Caco-2 y HT-29), con valores de EC₅₀ de 8.59 y 5.32 μ M, respectivamente (Vaněčková et al., 2016). El alcaloide undulatine (**Figura 8**), también del tipo crinine, presentó un efecto promisorio en la inhibición de la enzima ACE (EC₅₀ de 7.4 μ M), siendo capaz de cruzar la barrera hematoencefálica por permeación pasiva. Además de esto, la undulatine también exhibió actividad inhibitoria de la prolil-oligopeptidasa, enzima clave involucrada en funciones fisiológicas, cuya inhibición podría representar un importante apoyo en el tratamiento de la enfermedad de Alzheimer (Cahlíková et al., 2015).

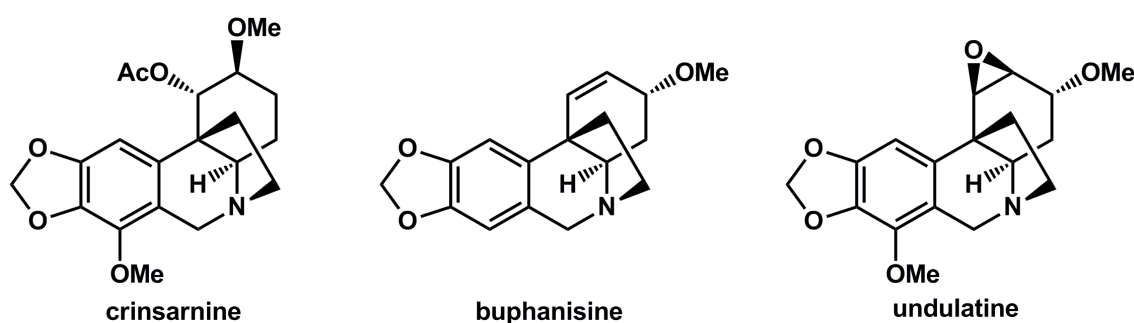


Figura 8. Estructuras del tipo crinine.

Los alcaloides del tipo narciclasine siguen siendo foco de numerosos estudios debido a su potencial antitumoral (Bastida et al., 2006). Investigaciones relacionadas con el compuesto narciclasine (**Figura 9**) lo revelan como un agente promisorio para el tratamiento de diversos tipos de cáncer cerebral primario y metástasis cerebrales (Goietsenoven et al., 2013). Además, hay estudios que indican el potencial del alcaloide pancratistatine (**Figura 9**) frente a diferentes tipos de cáncer, incluyendo colon, próstata, mama, leucemia, melanoma y glioblastoma, siendo mínima la toxicidad ejercida sobre las células no cancerígenas (Griffin, 2012). Recientemente, una nueva estructura del tipo narciclasine, la zephycandidine A, (**Figura 9**) exhibió una actividad citotóxica significativa frente a líneas de cáncer hematológico (HL-60), de pulmón (A549), de mama (MCF-7), de colon (SW480) y hepático (SMMC-7721), con valores de EC_{50} entre 1.98 y 7.03 μM y con índices de selectividad superiores a 10 (Zhan et al., 2017).

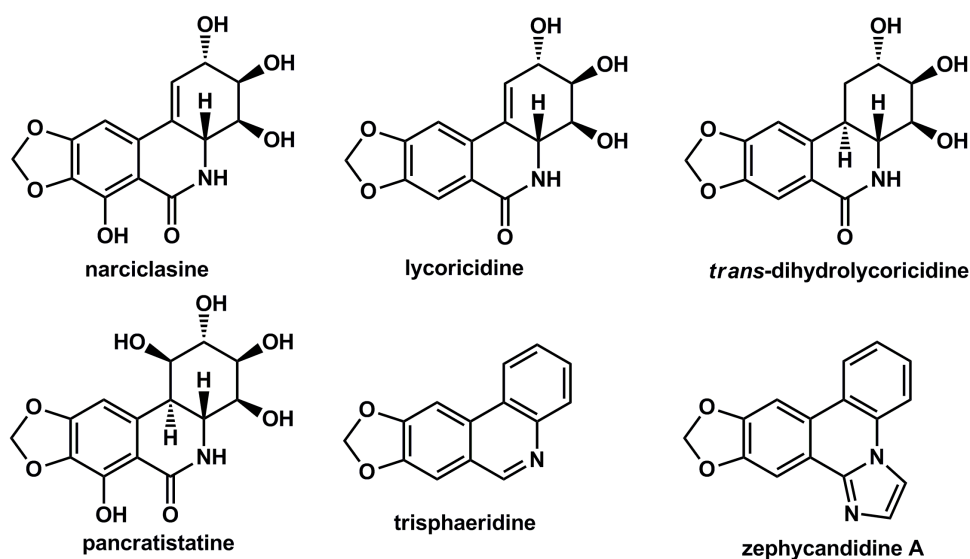


Figura 9. Estructuras del tipo narciclasine.

Investigaciones recientes sugieren que modificaciones en ciertos grupos funcionales de la lycoricidine (**Figura 9**) pueden reducir la citotoxicidad de esta estructura para mejorar su aplicación frente al virus de la hepatitis C, una vez que ésta presentó una EC_{50} de $0.55 \text{ nmol ml}^{-1}$ y un índice de selectividad de 12.7 (Chen et al., 2013). El compuesto *trans*-dihydrolycoricidine, (**Figura 9**) obtenido por síntesis, presentó una importante actividad antiviral, incluso mejor que el acyclovir, frente al virus del herpes tipo 1 (VHS-1), e inhibió sustancialmente la infección lítica del virus de la varicela-zóster (VVZ) (McNulty y Zepeda-Velázquez, 2014). Por último, es interesante mencionar que la trisphaeridine (**Figura 9**), alcaloide presente en diversas especies de Amaryllidaceae, posee actividad inhibitoria del virus de la polio, con una EC_{50} de $0.14 \text{ } \mu\text{g ml}^{-1}$ (Oluyemisi et al., 2015).

Desde inicios de este siglo hasta este momento, la galanthamine (**Figura 10**) es el alcaloide más importante de las Amaryllidaceae. Su estructura fue aislada por primera vez a partir de la planta *Galanthus woronowii* en la década de los 50 y fue aprobada por la FDA en el 2001 para el tratamiento paliativo de la EA en sus grados de leve a moderado (Heinrich y Teoh, 2004; Maelicke et al., 2001). De manera general, se ha descrito la presencia de galanthamine en plantas de diversos géneros de la familia Amaryllidaceae, incluyendo *Haemanthus*, *Lycoris*, *Hippeastrum*, *Hymenocallis*, *Narcissus* y *Leucojum* (Berkov et al., 2009). A pesar de que se conoce su síntesis química, Janssen-Cilag - la empresa propietaria de la patente - siguió utilizando el producto natural para sus formas farmacéuticas y su demanda ha provocado un gran interés en la búsqueda de nuevas especies que sean altamente productoras de galanthamine.

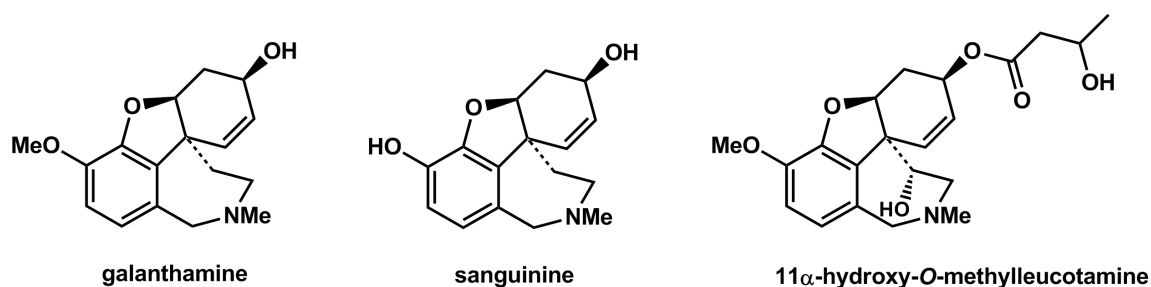


Figura 10. Estructuras del tipo galanthamine.

Curiosamente, una investigación publicada en 1996, reveló que el alcaloide sanguinine (9-*O*-demethylgalanthamine) (**Figura 10**) presenta una actividad inhibitoria

de la ACE cerca de 10 veces mayor que galanthamine, sin embargo, también expusieron que galanthamine presenta una mejor permeabilidad para atravesar la barrera hematoencefálica que sanguinine (Bores et al., 1996). En estudios recientes, se ha mostrado que el compuesto 11 α -hydroxy-*O*-methyllleucotamine (**Figura 10**) presenta una concentración de inhibición de la ACE (EC_{50} de 3.5 μ M) muy próxima a la galanthamine (EC_{50} de 1.5 μ M), aunque se desconoce su nivel de permeabilidad para pasar la barrera hematoencefálica (Iannello et al., 2014).

Hasta el momento, existe poca información acerca de los alcaloides de Amaryllidaceae con estructuras del tipo montanine (Bastida et al., 2006). El compuesto montanine presenta actividades psicofarmacológicas, incluyendo efectos ansiolíticos, antidepresivos y anticonvulsivos, en ratones (Silva et al., 2006). Se ha reportado que tanto montanine, como coccinine (**Figura 11**) tienen una baja actividad sobre las proteínas transportadoras de serotonina, con una EC_{50} de 121.3 y 196.3 μ M, respectivamente, las cuales pueden ser consideradas dianas para el desarrollo de diversos fármacos antidepresivos. Además, ambos alcaloides presentan baja afinidad por la glicoproteína-P¹¹ lo que sugiere que ésta no sería un factor limitante para el paso de los alcaloides por la barrera hematoencefálica (Stafford et al., 2013).

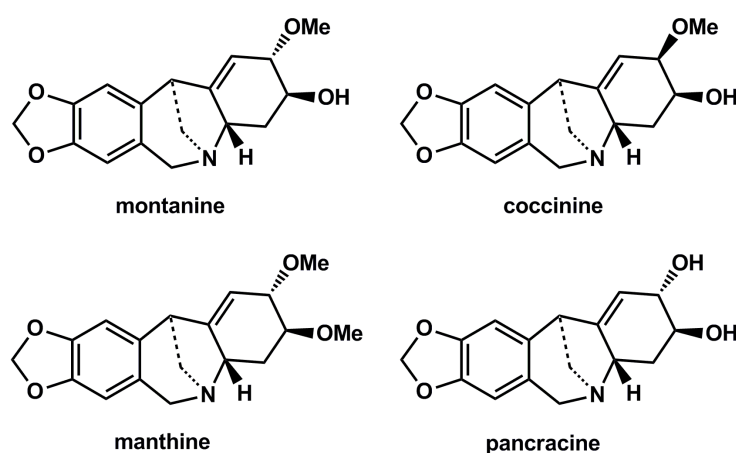


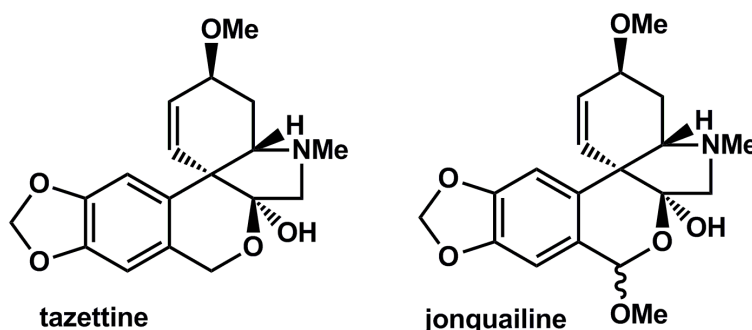
Figura 11. Estructuras del tipo montanine.

Trabajos recientes sugieren que el alcaloide montanine (**Figura 11**) tiene potencial para convertirse en un fármaco para el tratamiento de enfermedades autoinmunes, como la artritis (Farinon et al., 2017; De Oliveira et al, 2015). Además, derivados sintéticos de montanine podrían ser un punto de partida para el desarrollo de

¹¹ Actúa como una bomba expulsora de drogas. La sobreexpresión de esta proteína es la responsable de la disminución en el acúmulo intracelular de drogas antineoplásicas observado en células multirresistentes.

agentes para combatir tumores con resistencia a la apoptosis, como las células de cáncer de pulmón (A549), glioblastoma (U373) y melanoma (SKMEL-28), donde el alcaloide manthine (**Figura 11**) se mostró el más activo, con valores de EC_{50} entre 3 y 5 μM frente a estas líneas celulares (Govindaraju et al., 2018). Por otro lado, pancracine (**Figura 11**), alcaloide también del tipo montanine, presentó notable actividad antiproliferativa frente a líneas celulares de cáncer de ovario (A2780), de pulmón (SW1573), de mama (T47-D) y de colon (WiDr), con valores de EC_{50} de 8.3, 4.3, 6.5 y 9.1 μM , respectivamente (Cedrón et al., 2015).

Estudios previos relatan que los alcaloides del tipo tazettine (**Figura 12**) presentan actividad frente a ciertas líneas de tumores celulares (Bastida et al., 2006). Estudios recientes demuestran que el compuesto jonquailine (**Figura 12**) presenta actividad biológica frente a las células de cáncer fármaco-resistentes de glioma (U87, U373 y Hs683), melanoma (SKMEL-28), carcinoma pulmonar (A549, H1993 y H2073) y sarcoma uterino (MES-SA y MES-SA/Dx5), con valores de EC_{50} entre 1 y 85 μM . Este compuesto también mostró sinergismo con paclitaxel, indicando su uso como co-adyuvante en la quimioterapia convencional (Masi et al., 2015).



también del tipo mesembrane, presentó una potencial actividad como insecticida frente al mosquito *Aedes aegypti* en su fase adulta, con 93.3% de mortalidad a una dosis de 5 μg por mosquito y EC_{50} de 1.38 μg por mosquito (Masi et al., 2017).

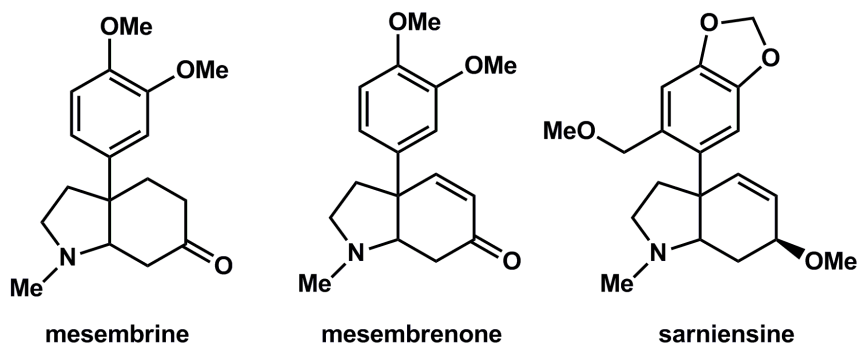


Figura 13. Estructuras del tipo mesembrane

1.3 La enfermedad de Alzheimer

La enfermedad de Alzheimer (EA), también conocida como demencia senil del tipo Alzheimer, fue identificada primeramente por el psiquiatra y neuropatólogo alemán Alois Alzheimer en 1906 (Wu et al., 2017). Esta enfermedad se caracteriza por una degeneración progresiva del sistema nervioso central, con pérdida de memoria y reducción de la habilidad de realizar actividades cotidianas básicas (Craig et al., 2011). Se la considera la forma más común de demencia y tiene prevalencia normalmente en personas mayores de 65 años (Querfurth y LeFerla, 2010). Actualmente se calcula que 47 millones de personas viven con demencia en el mundo, lo que resulta en un impacto económico estimado de 818 billones de dólares a nivel global (Alzheimer's Disease International, 2016).

Aún no se encuentra bien detallada la etiología precisa de la EA, considerándose una enfermedad multifactorial. Existen algunos factores conocidos involucrados en el desarrollo de la EA, incluyendo una reducción de los niveles de neurotransmisores colinérgicos, estrés oxidativo y agregación del péptido β -amiloide y de la proteína tau (τ) (Selkoe, 2001). El primero trata acerca del restablecimiento de los niveles de los neurotransmisores con inhibición de las enzimas acetilcolinesterasa (ACE), butirilcolinesterasa (BuCE) y monoaminoxidasa (MAO). El segundo se refiere a los efectos neuroprotectores, donde se considera que el estrés oxidativo puede presentarse como un efecto inicial de la enfermedad, lo que sugiere el posible uso de antioxidantes para evitar radicales libres en las células nerviosas. El último trata aspectos específicos

relacionados con la EA, incluyendo la disminución de la producción o agregación de los péptidos β -amiloide e inhibición de las enzimas γ - y β -secretasa, que poseen un importante papel en la vía amiloidogénica y de la proteína tau (Konrath et al., 2013).

En cuanto a la hipótesis colinérgica, tanto la ACE como la BuCE se encuentran involucradas en la hidrólisis del neurotransmisor acetilcolina (AC) (Basiri et al., 2013). La enzima ACE presenta alta selectividad por el proceso de hidrólisis de la AC y, por otro lado, la enzima BuCE es capaz de metabolizar diferentes sustratos, incluyendo varios péptidos neuroactivos (Greig et al., 2002). En el cerebro de pacientes con EA, la actividad de la ACE tiende a disminuir, mientras que la BuCE tiende a aumentar (Giacobini, 2004; Basiri et al., 2013). Consecuentemente, los inhibidores de las colinesterasas que tienen la capacidad de suprimir ambas enzimas a la vez probablemente generarían mejor respuesta terapéutica que agentes selectivos de la ACE (Ballard, 2002).

1.3.1 Docking molecular

Técnicas de cristalografía y resonancia magnética nuclear han contribuido de manera crucial a la elucidación estructural de proteínas y al conocimiento de las interacciones entre los complejos ligando-proteína (Meng et al., 2011). Estos avances permiten que estrategias multidisciplinarias, como la bioinformática y la química computacional, ayuden a impulsar el desarrollo de nuevos fármacos (Jorgensen, 2004).

El docking molecular es una herramienta cada día más importante en el ámbito de la investigación farmacéutica (Meng et al., 2011). Su objetivo es proporcionar información relacionada con las conformaciones y las interacciones más estables entre una molécula y el sitio activo de la enzima (McConkey et al., 2002). Existe una variedad muy grande de programas computacionales que calculan los posibles modos de interacción entre ligando-proteína, entre ellos se puede citar el Dock, el AutoDock, el Glide y el GOLD (Meng et al., 2011).

Los análisis cristalográficos de las colinesterasas han establecido que el centro catalítico de estas enzimas está localizado dentro de una cavidad estrecha y de unos 20Å de profundidad, denominada garganta la cual contiene una tríada catalítica representada por los residuos de aminoácidos serina, histidina y glutámico, conocida como sitio-A (o de acilación) y hallada en el fondo de la garganta. La región cerca del borde de la garganta fue denominada sitio-P (periférico). Los estudios comparativos muestran que

la diferencia en la especificidad de unión del ligando con las enzimas ACE y BuCE está en función del número de residuos aromáticos que hay en la garganta del centro catalítico de cada cual, pues mientras en la *hACE*¹² hay diez residuos interactuando con los ligandos, en la *hBuCE*¹³ permanecen apenas cuatro de ellos (Rosenberry et al., 2017).

1.3.2 Alkaloides de las Amaryllidaceae con actividad inhibitoria de las colinesterasa *in silico*

Haciendo uso del software GOLD v.5.1, se han realizado investigaciones utilizando el docking molecular con la finalidad de evaluar el potencial inhibitorio de 41 alcaloides derivados de la galanthamine sobre la ACE (*TcACE*¹⁴). A continuación, se sintetizaron y evaluaron *in vitro* las cuatro estructuras que presentaron mejores resultados *in silico* (**Figura 14**) y como resultado, se observó que estos compuestos presentaron *in vitro* una actividad entre 11 y 95 veces más alta que la galanthamine, con valores de EC_{50} entre 0.011 y 0.094 μ M. Los autores sugieren que los compuestos *N*-sustituídos de cadena larga inhiben la ACE (*hACE*, estructura: 4EY6) gracias a su interacción con los residuos de aminoácidos del sitio-P aniónico (Tyr72, Asp74, Tyr124, Trp286 y Tyr341) (Atanasova et al., 2015).

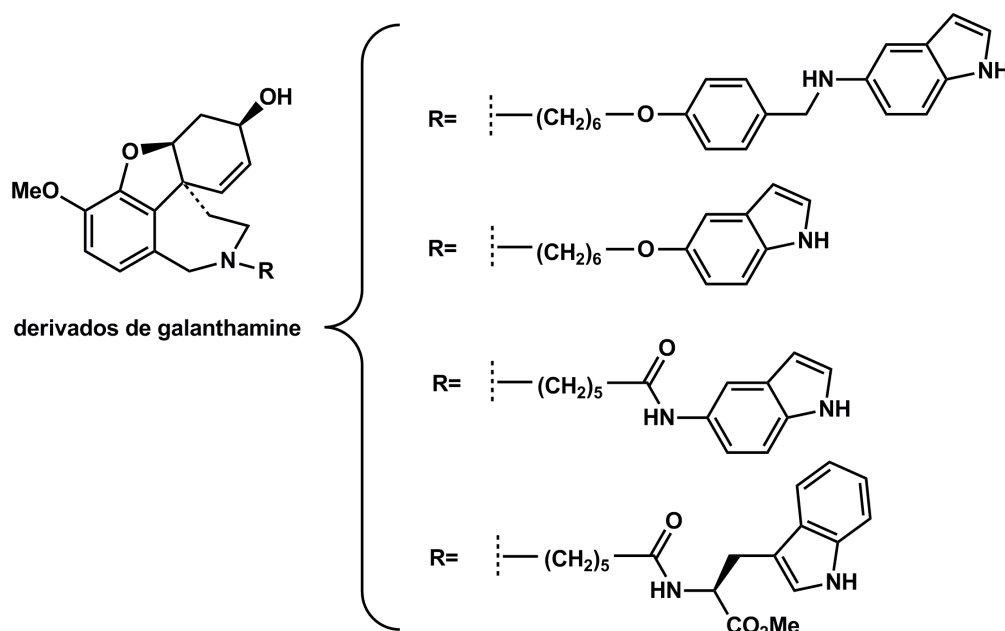


Figura 14. Derivados de galanthamine *versus* docking molecular.

¹² “Human” ACE

¹³ “Human” BuCE

¹⁴ “Torpedo californica” ACE

El extracto de la especie *Zephyranthes carinata*, recolectada en Colombia, presentó capacidad de inhibir la ACE (*eeACE*¹⁵) y, para entender mejor estos resultados, se identificaron los alcaloides presentes en esta muestra por CG-EM y se realizaron estudios de docking molecular (utilizando el programa AutoDockTools). A partir de los resultados obtenidos *in silico*, se observó que 3-epimacronine y lycoramine (**Figura 15**) teóricamente presentan potencial para inhibir la ACE (*TcACE*, estructura: 1DX6). El complejo 3-epimacronine-*TcACE* realizaría interacciones con los aminoácidos Ser200, His440, Trp84 y Phe330, que serían similares a las observadas en el complejo galanthamine-*TcACE*, y el complejo lycoramine-*TcACE* se diferenciaría del anterior únicamente por la posición del puente de hidrógeno con el residuo de aminoácido Ser200 (Cortés et al., 2015).

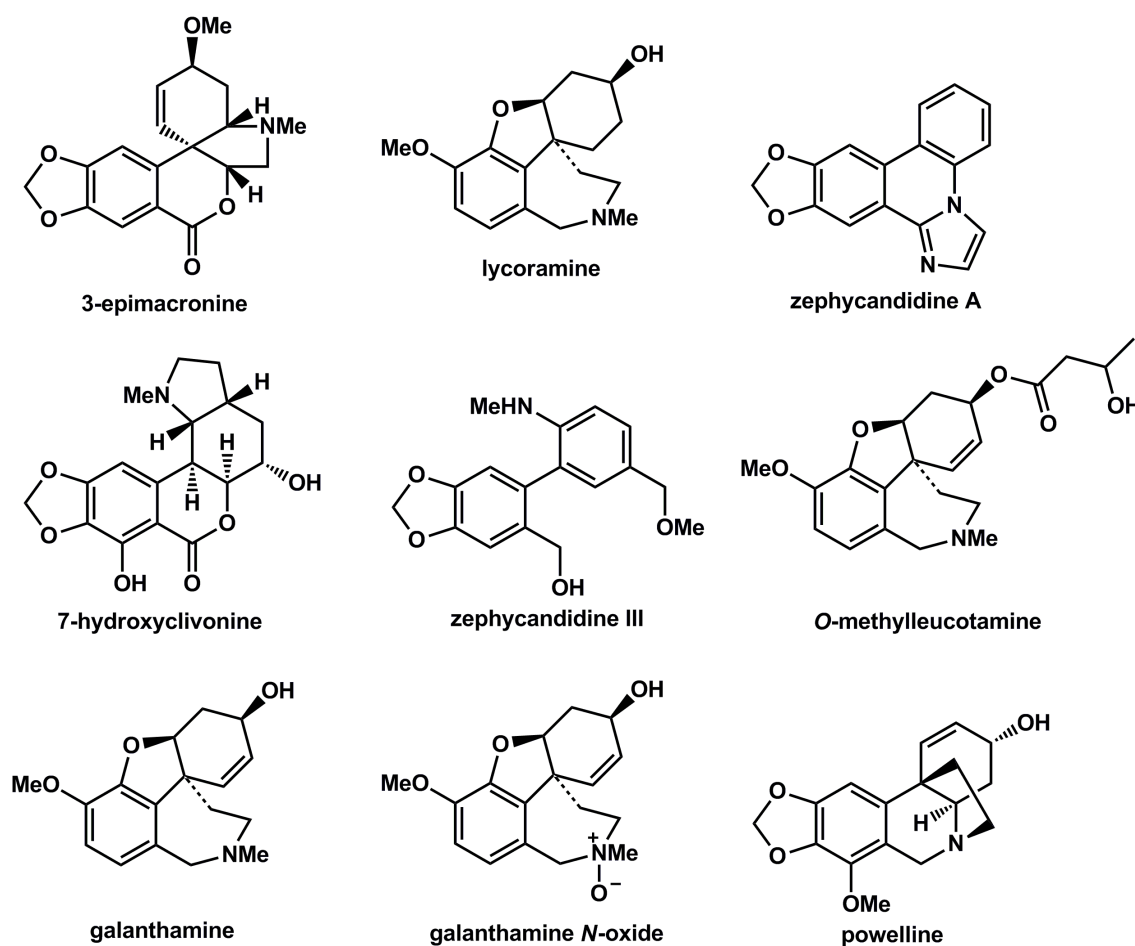


Figura 15. Alcaloides de Amaryllidaceae *versus* docking molecular.

¹⁵ “*Electrophorus electricus*” ACE

Otros estudios revelan que el compuesto zephycandidine A (**Figura 15**), aislado de la especie *Zephyranthes candida*, presenta capacidad de inhibir la enzima ACE (*eeACE*) *in vitro*, con un valor de EC_{50} de 127.99 μ M. Se llevó a cabo el análisis de docking molecular (utilizando el software AutoDock 4.2 en combinación con el método MM/GBSA implementado en AMBER 11) para comprender mejor como el alcaloide zephycandidine A estaría interactuando con cinco estructuras cristalográficas distintas de la enzima *hACE* (estructuras: 4EY5, 4EY6, 4EY7, 4M0E y 4M0F). Se observó que mientras la galanthamine interacciona con el sitio catalítico de la *hACE*, zephycandidine A interacciona con el sitio periférico aniónico de la garganta. Además, se verificó que las interacciones con los aminoácidos Trp286 y Tyr337 son necesarias para que se forme el complejo ligando-*hACE* (Zhang et al., 2016).

El compuesto 7-hydroxyclicivonine (**Figura 15**), aislado de la planta *Hippeastrum argentinum*, presenta actividad inhibitoria sobre la BuCE (*eqBuCE*¹⁶), con una EC_{50} de 67.3 μ M, *in vitro*. Se llevaron a cabo estudios de docking molecular, dinámica molecular y teoría cuántica del átomo (utilizando los softwares AutoDock Vina, Amber y Multiwfn) para entender como 7-hidroxiclicivonine estaría interaccionando con la BuCE (*eqBuCE*, estructura: Q9N1N9). Los resultados indicaron que 7-hydroxyclicivonine y galanthamine estarían interactuando en el mismo sitio activo de la *eqBuCE* e incluso a través de residuos de aminoácidos similares. Sin embargo, los resultados del análisis de teoría cuántica del átomo sugieren que las interacciones que estabilizan el complejo galanthamine-*eqBuCE* son significativamente más fuertes que las que ocurren entre 7-hidroxiclicivonine-*eqBuCE* (Ortiz et al., 2016).

El compuesto zephycandidine III (**Figura 15**), aislado de la especie *Zephyranthes candida*, presentó potencial inhibitorio de la ACE (*eeACE*) *in vitro*, con una EC_{50} de 8.82 μ M. Se han realizado análisis de docking molecular (utilizando el software AutoDock 4.2 en combinación con el método MM/GBSA implementado en AMBER 11) a través de cinco estructuras cristalográficas distintas de la enzima ACE (*hACE*, estructuras: 4EY5, 4EY6, 4EY7, 4M0E y 4M0F). *In silico*, los resultados revelaron que zephycandidine III se une mediante un enlace de hidrógeno fuerte al residuo del aminoácido Gly120 y dos débiles con Gly122 y Tyr337, respectivamente, mientras que la galanthamine se estabiliza gracias a tres enlaces de hidrógeno con Glu202 Tyr337 y Gly122, respectivamente. Los autores también reafirmaron que las

¹⁶ “Equine serum” BuCE

interacciones con los aminoácidos Trp286 e Tyr337 son importantes para que se forme el complejo ligando-ACE (Zhan et al., 2017).

Recientemente, distintas muestras de *Galanthus elwesii*, recolectadas en Turquía, presentaron potencial inhibitorio relevante de la ACE (*ee*ACE), con valores de EC_{50} entre 0.72 y 20.57 $\mu\text{g ml}^{-1}$, y de la BuCE (*eq*BuCE), con valores de EC_{50} entre 6.56 y 15.85 $\mu\text{g ml}^{-1}$. Para entender mejor la actividad de inhibición de la ACE, cada uno de los 15 alcaloides identificados en estas muestras por CG-EM fue evaluado por docking molecular (programa GOLD 5.2.1) utilizando las enzimas *h*ACE (1EVE) y *h*BuCE (4TPK). El alcaloide *O*-methylleucotamine (**Figura 15**) de esta especie presentó *in silico* los mejores resultados de inhibición de ambas enzimas (Bozkurt et al., 2017).

De las especies *Zephyranthes carinata*, *Phaedranassa lehmanni*, *Eucharis bonplandii*, *E. caucana*, *Crinum jagus*, *Hippeastrum elegans* y *Clivia miniata*, recolectadas en Colombia, se obtuvo su perfil alcaloídico por CG-EM y se evaluó el potencial de inhibición de las colinesterasas de cada muestra *in vitro*. El extracto de hojas de *Z. carinata* mostró la inhibición frente a la *ee*ACE (EC_{50} 1.16 $\mu\text{g ml}^{-1}$), el extracto de bulbos de *E. bonplandii* frente a la *h*ACE (EC_{50} 0.72 $\mu\text{g ml}^{-1}$), el extracto de bulbos de *C. jagus* frente a la *h*BuCE (EC_{50} 8.51 $\mu\text{g ml}^{-1}$) y el extracto de hojas de *C. jagus* frente a la *eq*BuCE (EC_{50} 11.04 $\mu\text{g ml}^{-1}$). Estudios de docking molecular (programa AutoDock v.4.2) de los alcaloides descritos en estas muestras concluyen que galanthamine, galanthamine *N*-oxide y powelline (**Figura 15**) son las estructuras con mejor potencial de inhibición de las enzimas *Tc*ACE (1DX6), *h*ACE (4EY7) y *h*BuCE (4BDS) (Cortés et al., 2018).

1.4 Enfermedades tropicales

Las Enfermedades Tropicales Desatendidas (ETD) son un grupo diverso de enfermedades transmisibles que ocurren en áreas tropicales y subtropicales del planeta y afectan a millones de personas, convirtiéndose en un desafío para la salud pública mundial (WHO, 2017). La Organización Mundial de la Salud (WHO) enumera 17 ETD, incluyendo la enfermedad de Chagas, la tripanosomiasis africana humana (enfermedad del sueño) y la leishmaniosis. Junto con la malaria, estas enfermedades son muy temidas en las poblaciones afectadas, donde la pobreza limita el acceso a los métodos de prevención y tratamiento (WHO, 2017). Una de las razones principales por las que estas enfermedades son desatendidas es que hay pocos incentivos para que la industria

farmacéutica invierta en el desarrollo de medicamentos para una población de pacientes que no puede pagarlos. Por lo tanto, la mayor parte de la investigación sobre el tratamiento efectivo de estas enfermedades se lleva a cabo en laboratorios académicos con limitados recursos financieros, de personal y técnicos (Klug et al., 2016).

1.4.1 Alcaloides de las Amaryllidaceae con actividad antiprotozoaria

Los estudios realizados revelan que, entre los alcaloides aislados de la especie *Crinum amabile*, augustine (**Figura 16**) es el más activo frente a cepas fármaco-sensible (cepa D-6) y fármaco-resistente (cepa W-2) del agente causante de la malaria, *Plasmodium falciparum*, con valores de EC_{50} de 140 y 180 ng ml⁻¹, respectivamente (Likhitwitayawuid et al., 1993). Los compuestos 1-*O*-acetylnorpluviine, anhydrolycorin-6-one, sternbergine, crinamine y hamayne (**Figura 16**), aislados de la planta *Brunsvigia radulosa*, demostraron una actividad antiplasmodial moderada frente a las cepas fármaco-sensible (D10) y fármaco-resistente (FAC8) de *P. falciparum*, con valores de EC_{50} entre 2.8 y 28.3 µg ml⁻¹ y entre 3.4 y 34.2 µg ml⁻¹, respectivamente (Campbell et al., 2000).

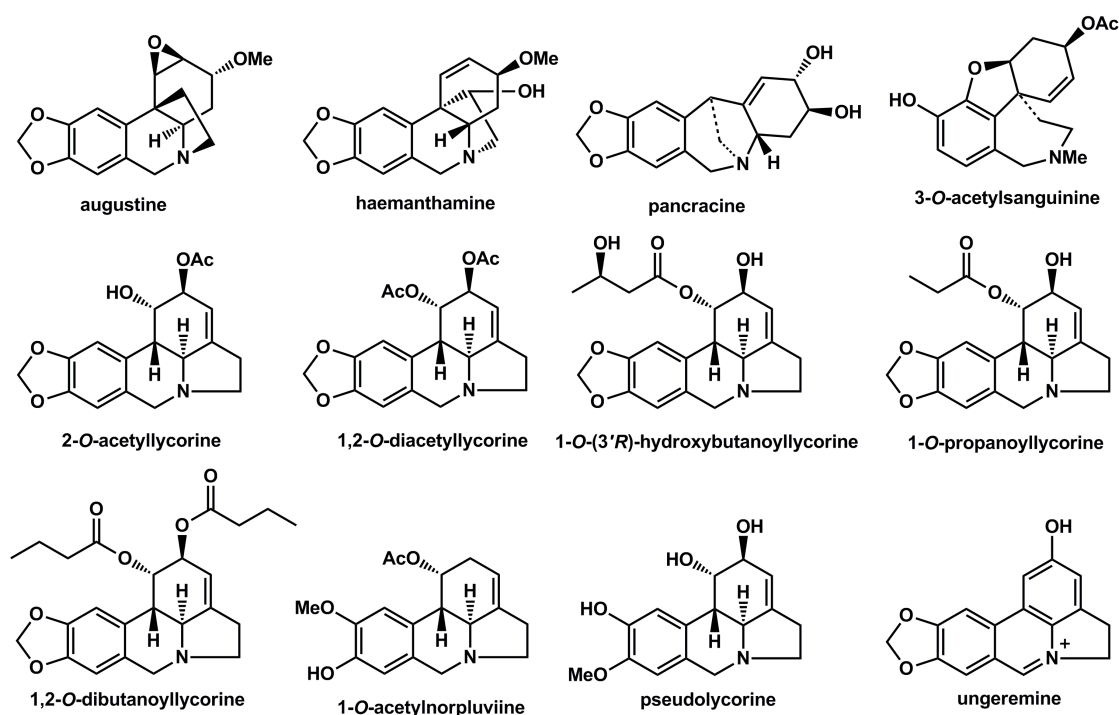


Figura 16. Alcaloides de Amaryllidaceae *versus* potencial antiparasitario.

El compuesto pancracine (**Figura 16**), aislado de la planta *Narcissus angustifolius*, se mostró activo frente al agente causante de la enfermedad del sueño,

Trypanosoma brucei rhodesiense (STIB 900), con una EC_{50} de $0.7 \mu\text{g ml}^{-1}$, y frente al agente causante de la enfermedad de Chagas, *Trypanosoma cruzi* (Tulahuen C4), con una EC_{50} de $7.1 \mu\text{g ml}^{-1}$. Este mismo alcaloide presentó actividad frente a las cepas fármaco-sensible (K1) y fármaco-resistente (NF54) de *P. falciparum*, con valores de EC_{50} de 0.75 y $0.70 \mu\text{g ml}^{-1}$, respectivamente (Labraña et al., 2002).

Los alcaloides 1,2-*O*-diacetyllycorine y 3-*O*-acetylsanguinine (**Figura 16**), aislados de la especie *Crinum kirkii*, son activos frente a *T. b. rhodesiense* (STIB 900), con valores de EC_{50} de 1.0 y $1.1 \mu\text{g ml}^{-1}$, respectivamente. El alcaloide 3-*O*-acetylsanguinine también presentó actividad frente a *T. cruzi* (Tulahuen C4), con un valor de EC_{50} de $2.3 \mu\text{g ml}^{-1}$ (Machocho et al., 2004).

Asimismo, se evaluó *in vitro* el potencial antiparasitario de algunos productos de semisíntesis derivados de lycorine. El producto 2-*O*-acetyllycorine (**Figura 16**) presentó una significativa actividad frente a *T. brucei* (cepa GUTat 3.1) con una EC_{50} de $0.15 \mu\text{g ml}^{-1}$, mientras que fármacos de referencia, como pentamidine, suramine y eflornitine mostraron valores de EC_{50} de 0.00158 , 1.58 y $2.27 \mu\text{g ml}^{-1}$, respectivamente. Los compuestos 1,2-*O*-dibutanoyllycorine, 1-*O*-propanoyllycorine y 1-*O*-(3'*R*)-hydroxybutanoyllycorine (**Figura 16**) mostraron una alta actividad antiplasmodial frente a la cepa fármaco-resistente (K1) de *P. falciparum*, con valores de EC_{50} de 0.67 , 0.37 y $0.62 \mu\text{g ml}^{-1}$, respectivamente, y frente a la cepa fármaco-sensible (FCR3), con valores de EC_{50} de 0.53 , 0.30 y $0.49 \mu\text{g ml}^{-1}$, respectivamente (Toriizuka et al., 2008).

En otro estudio se evaluó el potencial de veintisiete derivados de lycorine frente a la cepa fármaco-sensible (F-32 Tanzania) de *P. falciparum*. Los mejores resultados fueron obtenidos para los derivados de lycorine que presentan grupos hidroxilo en los carbonos C-1 y C-2, así como para los que presentan un grupo acetato o isobutirato (**Figura 16**) (Cedrón et al., 2010).

Los alcaloides haemanthamine, pseudolycorine y ungeremine (**Figura 16**), aislados de la planta *Phaedranassa dubia*, presentaron buenos resultados de actividad antiprotozoaria *in vitro*. Haemanthamine se mostró activa frente a *T. b. rhodesiense* (cepa STIB 900) y *P. falciparum* (cepa K1), con valores de EC_{50} de 1.62 y $2.29 \mu\text{M}$, respectivamente. Pseudolycorine mostró actividad frente a *P. falciparum* (cepa K1), con EC_{50} de $0.83 \mu\text{M}$, mientras que ungeremine resultó activa frente al *T. b. rhodesiense* (cepa STIB 900), *T. cruzi* (cepa Tulahuen C4) y *P. falciparum* (cepa K1), con valores de EC_{50} de 3.66 , 3.20 y $0.33 \mu\text{M}$, respectivamente (Osorio et al., 2010).

También se ha evaluado *in vitro* el potencial antiparasitario de diversos alcaloides aislados de la especie *Galanthus trojanus*. Dihydrolycorine, haemanthamine, arolycoricidine y protopine (**Figura 17**) presentaron actividad inhibitoria del *T. b. rhodesiense* (cepa STIB 900) con valores de EC_{50} entre 3.10 y 8.71 $\mu\text{g ml}^{-1}$. Haemanthamine fue el alcaloide más potente frente a *T. cruzi* (cepa Tulahuen C2C4), con una EC_{50} de 1.80 $\mu\text{g ml}^{-1}$. Los compuestos stylopine, protopine, haemanthamine y arolycoricidine (**Figura 17**) presentaron una actividad significativa frente a una cepa fármaco-resistente de *P. falciparum* (cepa K1), con valores de EC_{50} de 0.23, 0.50, 2.75 y 4.44 $\mu\text{g ml}^{-1}$, respectivamente (Kaya et al., 2011).

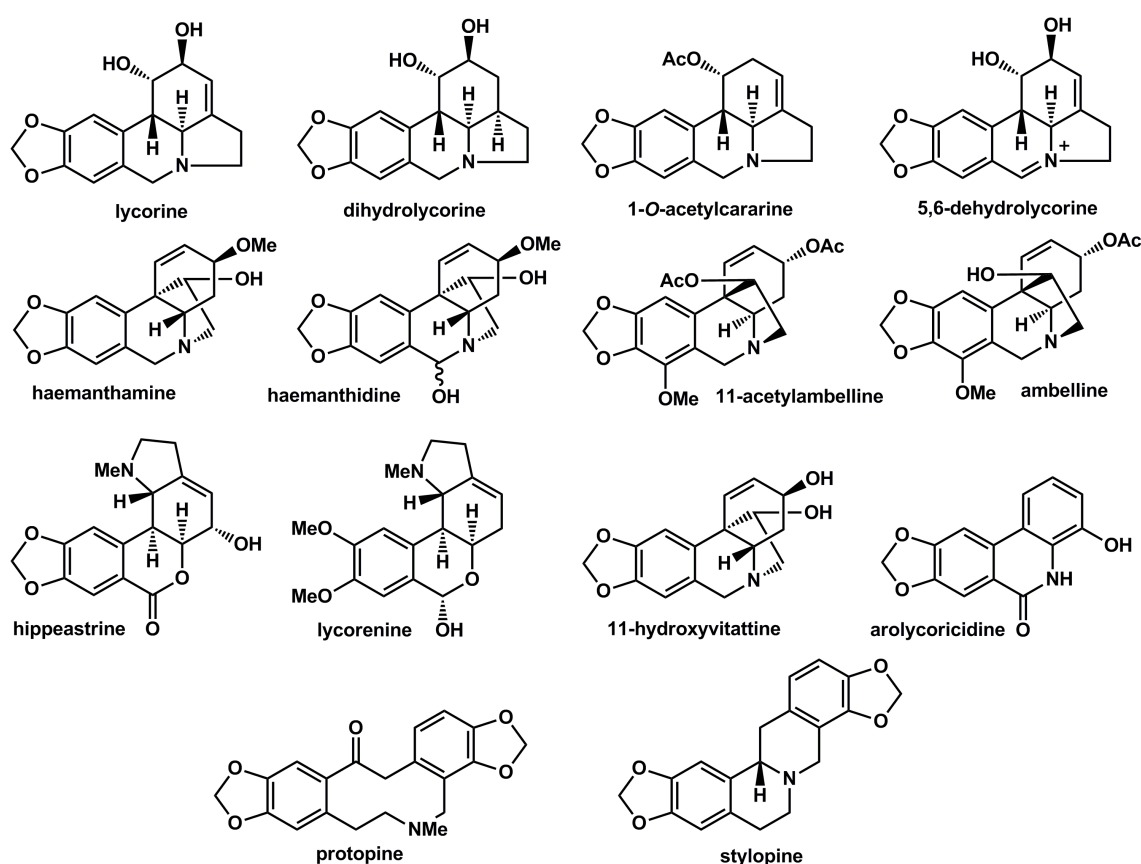


Figura 17. Alcaloides de Amaryllidaceae *versus* potencial antiparasitario.

En otro estudio, se sintetizaron y evaluaron treinta y un derivados de los alcaloides haemanthamine, haemanthidine y 11-hydroxyvittatine (**Figura 17**) frente a una cepa fármaco-sensible de *P. falciparum* (F32). El producto con un grupo nicotinato en C-3 y en C-11 fue el más activo frente a esta cepa, presentando IC_{50} de 0.80 μM (Cedrón et al., 2012).

Por otro lado, se prepararon veintiún derivados de lycorenine a partir del alcaloide hippeastrine y evaluados contra una cepa fármaco-sensible de *P. falciparum* (F32) (**Figura 17**). Las modificaciones realizadas en hippeastrine indican que la eliminación del grupo metilendioxo produce un ligero aumento de la actividad. Sin embargo, los dímeros de hippeastrine (**Figura 6**) fueron los compuestos más activos frente al *P. falciparum*, con valores de EC₅₀ entre 1.3 y 4.0 µM (Cedron et al., 2013).

El compuesto 5,6-dehydrolycorine (**Figura 17**), aislado de la planta *Lycoris radiata*, presentó actividad frente a la cepa fármaco-resistente de *P. falciparum* (D-6), con una EC₅₀ de 2.3 µM, y a la cepa fármaco-sensible (W-2), con una EC₅₀ de 2.3 µM (Hao et al., 2013). Los compuestos 1-*O*-acetylcaranine, ambelline y 11-*O*-acetylambelline (**Figura 17**), aislados de la especie *Amaryllis belladonna*, también presentaron actividad antiplasmodial. 1-*O*-Acetylcaranine se mostró activa frente a la cepa fármaco-resistente de *P. falciparum* (Dd2), con una EC₅₀ de 3.5 µM, mientras que ambelline y 1-*O*-acetylambelline exhibieron una actividad moderada, con una EC₅₀ de 7.3 y 35.0 µM, respectivamente (Cho et al., 2018).

1.5 CG-EM y RMN en el estudio de los alcaloides de Amaryllidaceae

Las técnicas de cromatografía de gases acoplada a espectrometría de masas (CG-EM) y la resonancia magnética nuclear (RMN) son de fundamental importancia para el estudio químico de los alcaloides de Amaryllidaceae. Juntas, estas herramientas analíticas pueden proporcionar una rápida identificación de compuestos ya conocidos y la elucidación estructural detallada en el caso de los productos aislados por primera vez (Bastida et al., 2006).

1.5.1 Cromatografía de Gases – Espectrometría de Masas (CG-EM)

La cromatografía de gases es una herramienta analítica utilizada para separar mezclas de compuestos volátiles a través de una columna como fase estacionaria y un gas inerte como fase móvil, siendo la temperatura una importante variable a considerar. Únicamente las moléculas capaces de ser vaporizadas sin experimentar descomposición son adecuadas para este tipo de análisis. Un espectrómetro de masas es básicamente un instrumento que mide la relación masa-carga (m/z) de iones en fase gaseosa, proporcionando información sobre la abundancia de cada especie iónica, y que ofrece la

posibilidad de ser acoplado como detector a la separación de los compuestos obtenidos por cromatografía de gases (Kitson et al., 1996).

Estudios de CG-EM de extractos de especies de la familia Amaryllidaceae revelan que estas pueden contener un número bastante variado de alcaloides. Cada planta metaboliza una mezcla de alcaloides formada por un pequeño número de alcaloides mayoritarios y un gran número de alcaloides minoritarios. Esto normalmente resulta de la diferencia en la especificidad del sustrato y del nivel de la expresión génica de las enzimas involucradas en los procesos de biosíntesis (Berkov et al., 2011; Berkov et al., 2013; Takos y Rook, 2013).

Generalmente, los compuestos orgánicos presentan patrones de fragmentación característicos al ser ionizados, lo que permite su identificación mediante comparación con datos obtenidos previamente. De esta manera, la CG-EM es una poderosa herramienta analítica para la obtención del perfil químico de compuestos naturales volátiles. Mediante numerosos estudios de espectrometría de masas de impacto electrónico (EIMS) de los alcaloides de Amaryllidaceae, ha sido posible establecer los patrones de fragmentación característicos para los diferentes tipos de esqueletos (Bastida et al., 2006).

1.5.1.1 Patrón de fragmentación de los alcaloides de las Amaryllidaceae por CG-EM

Las estructuras del tipo lycorine con un doble enlace entre los carbonos C-3 y C-4 normalmente sufren la pérdida de los carbonos C-1 y C-2, junto con los respectivos sustituyentes, por medio de una fragmentación del tipo retro-Diels-Alders, llevando a la formación del pico base representado por el fragmento m/z 226 (**Figura 18**). En estructuras con dos grupos metoxilo en el anillo A, en lugar del grupo metilendioxo de lycorine, el pico base del espectro es m/z 242. Curiosamente, los derivados acetilados en C-2 no siguen este patrón de fragmentación (Bastida et al., 2006).

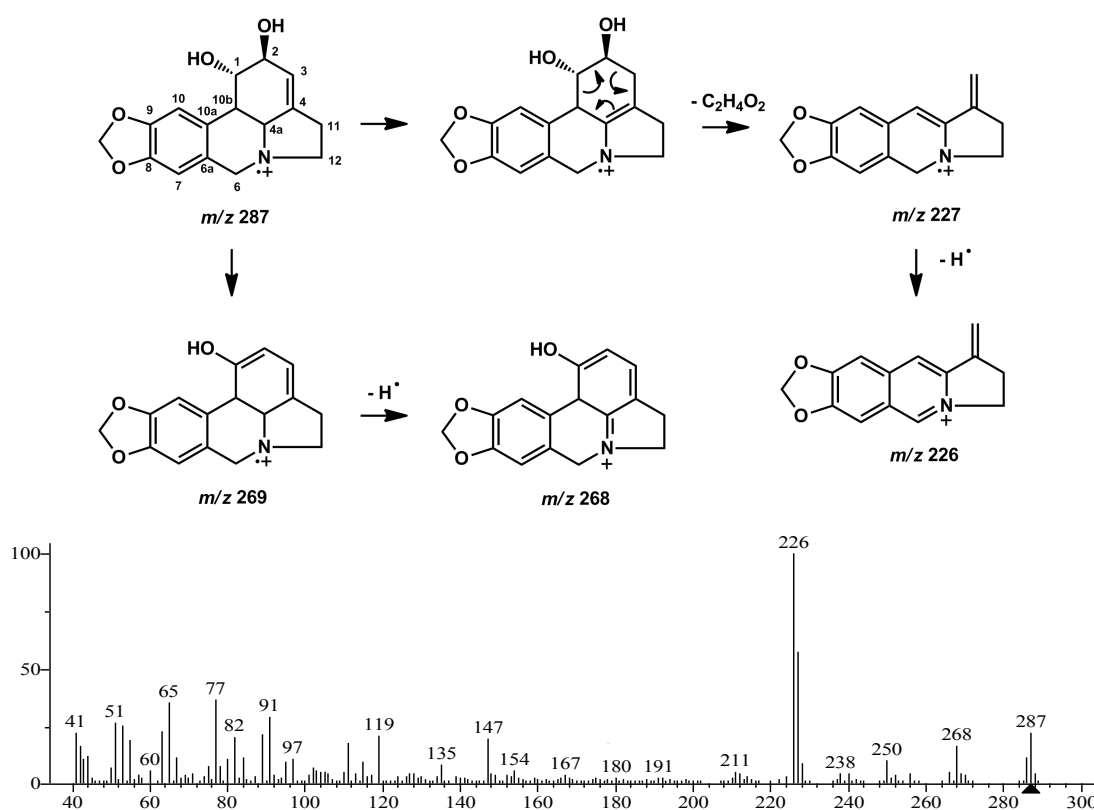


Figura 18. Patrón de fragmentación de lycorine.

El fragmento más abundante del espectro de masas de los compuestos de tipo homolycorine surge de la rotura de los enlaces lábiles del anillo C por una reacción retro-Diels-Alder que genera dos fragmentos, siendo el más característico y abundante el correspondiente al anillo de pirrolidina, unido a los sustituyentes de la posición 2 (Bastida et al., 2006). Por lo tanto, en el caso de homolycorine (con dos hidrógenos en C-2) el pico base se observa a m/z 109, mientras que la hippeastrine (con un grupo hidroxilo en C-2) lo presenta a m/z 125 y la ungerine (con un grupo metoxilo en C-2) a m/z 139 (Figura 19) (Bastida et al., 2006).

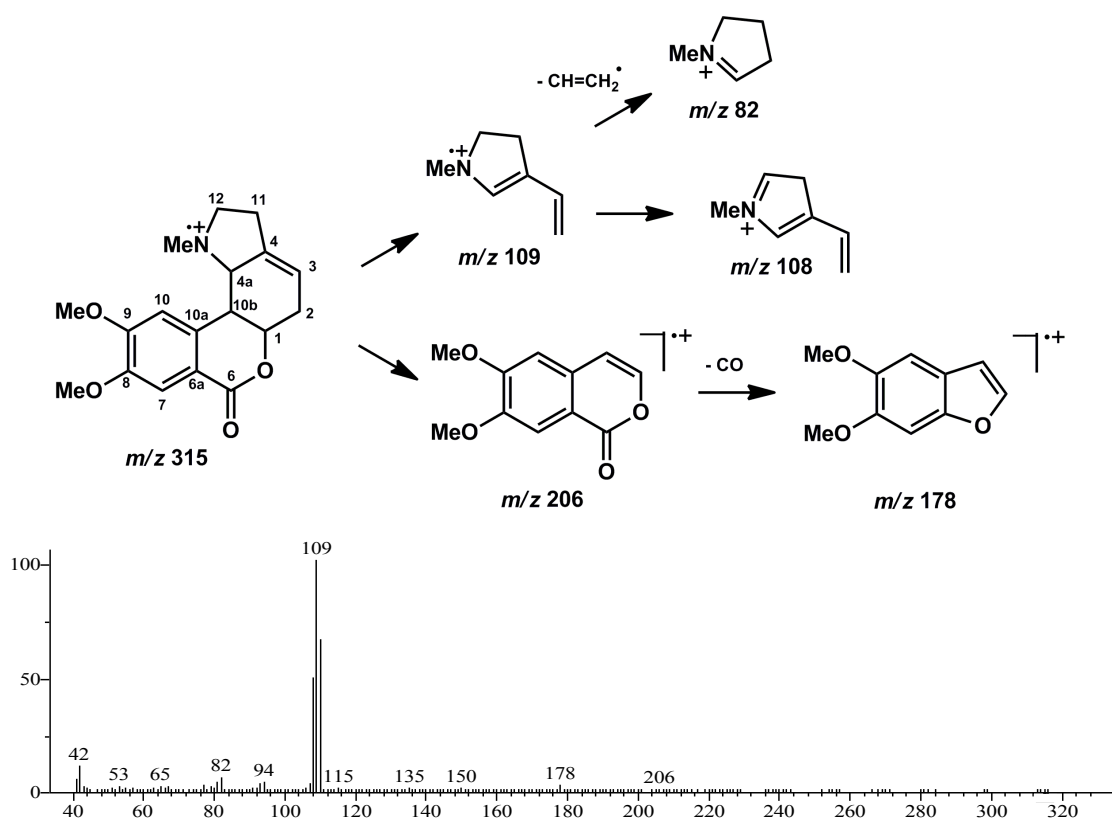


Figura 19. Patrón de fragmentación de homolycorine.

En estructuras del tipo crinine y haemanthamine el anillo aromático usualmente tiene un papel fundamental en la estabilización de los fragmentos, el átomo de nitrógeno suele perderse, y el paso inicial en los mecanismos de fragmentación implica la apertura del puente de dos carbonos, C11-C-12, por lo que en la mayoría de los casos el ion molecular corresponde al pico base. Los patrones de fragmentación de estas estructuras cambian de acuerdo con la presencia del sustituyente en diversas posiciones, la saturación del anillo C y la influencia de la estereoquímica (**Figura 20**) (Bastida et al., 2006; Longevialle et al., 1973).

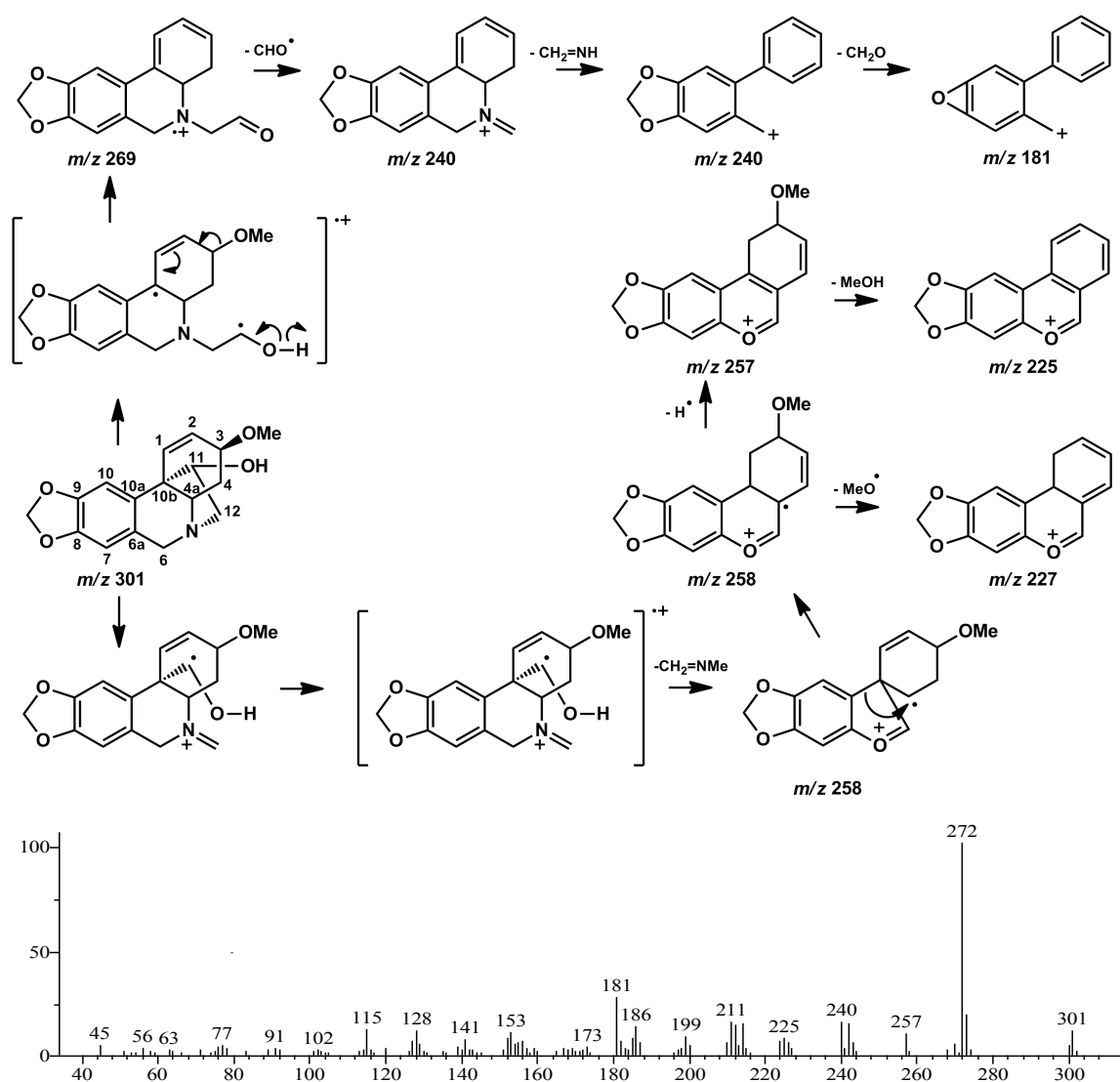


Figura 20. Patrón de fragmentación de haemanthamine.

Sutiles cambios en la estereoquímica son suficientes para causar diferencias apreciables en el perfil de fragmentación en las estructuras del tipo tazettine. En el espectro de masas de tazettine, con un grupo metoxilo β -orientado en C-3, el ion base ocurre a m/z 247, seguido de una fragmentación de tipo retro-Diels-Alder del anillo C (**Figura 21**). Por otro lado, su epímero criwellina contiene un fragmento de baja abundancia a m/z 247 (**Figura 21**). Ambos estereoisómeros experimentan la pérdida sucesiva de un radical metilo y agua, resultando en la formación de los iones a m/z 316 y m/z 298, así como posteriores fragmentaciones (Duffield et al., 1965).

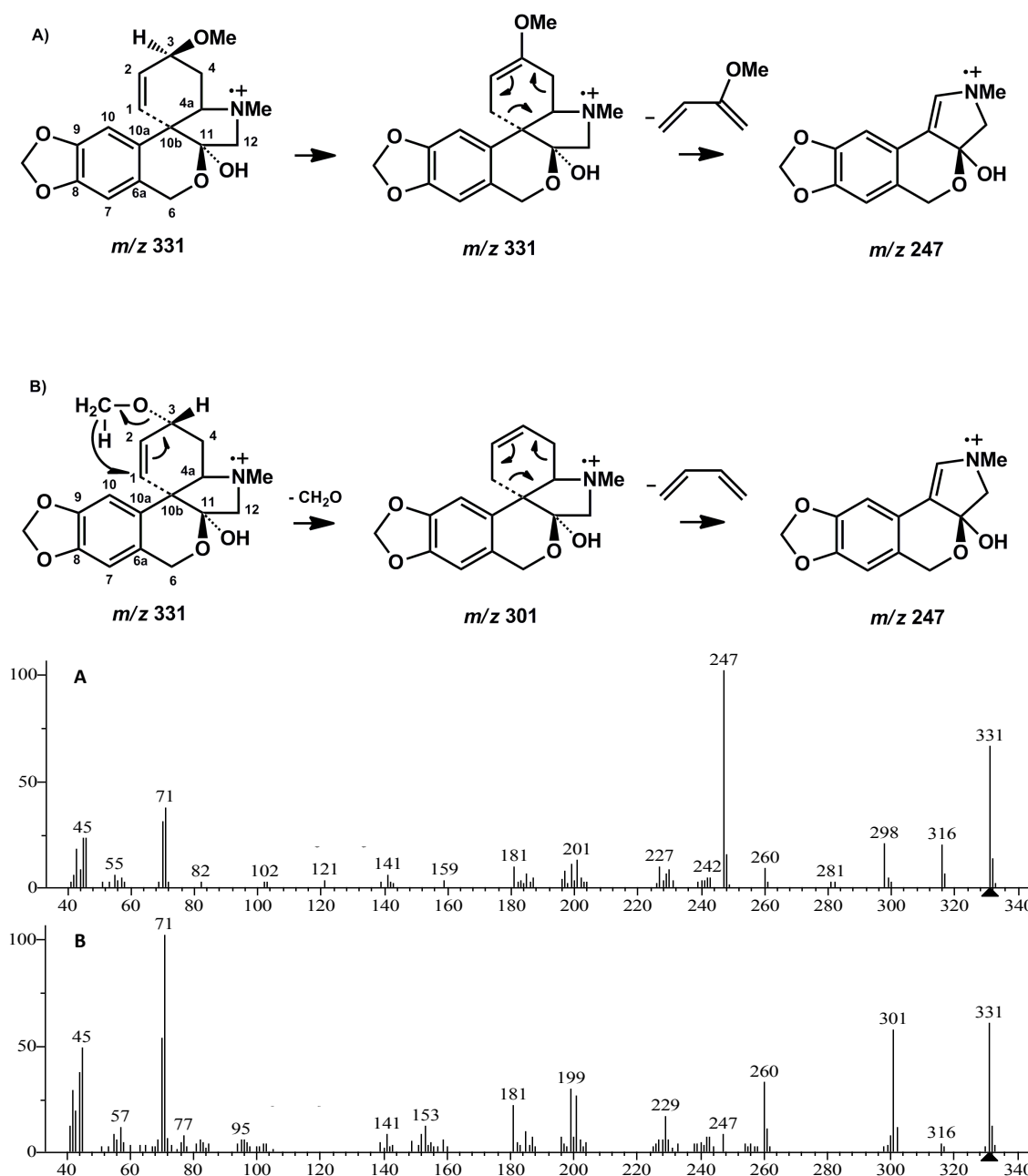


Figura 21. Patrón de fragmentación de tazettine (A) y criwelline (B).

El patrón de fragmentación de los alcaloides con el núcleo 5,11-methanomorphanthridine, que se refiere a las estructuras del tipo montanine, depende en gran parte de la naturaleza y estereoquímica de los sustituyentes en C-2 y C-3. La presencia de un grupo metoxilo en C-2 da lugar a un fragmento de 31 unidades menos que el ion molecular (**Figura 22**). La estereoquímica del sustituyente en C-2 presenta considerable influencia en como transcurre la fragmentación de tipo retro-Diels-Alder, la cual se ve aumentada cuando el sustituyente se encuentra α -orientado (Bastida et al., 2006).

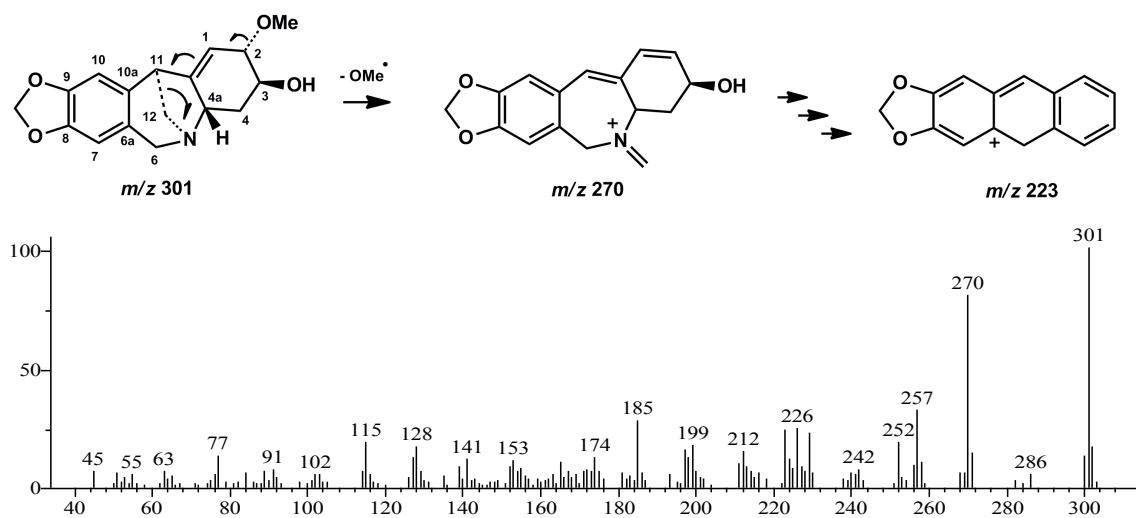


Figura 22. Patrón de fragmentación de montanine.

La intensidad del ion molecular, la ruptura del anillo C y la eliminación de elementos del anillo B, incluido del nitrógeno, son características de las estructuras del tipo galanthamine (**Figura 23**). El compuesto galanthamine y su epímero en la posición 3 presentan diferentes tiempos de retención bajo las mismas condiciones cromatográficas, junto a una sutil pero significativa diferencia en sus espectros: la abundancia del fragmento a m/z 216 (Razakov et al., 1969).

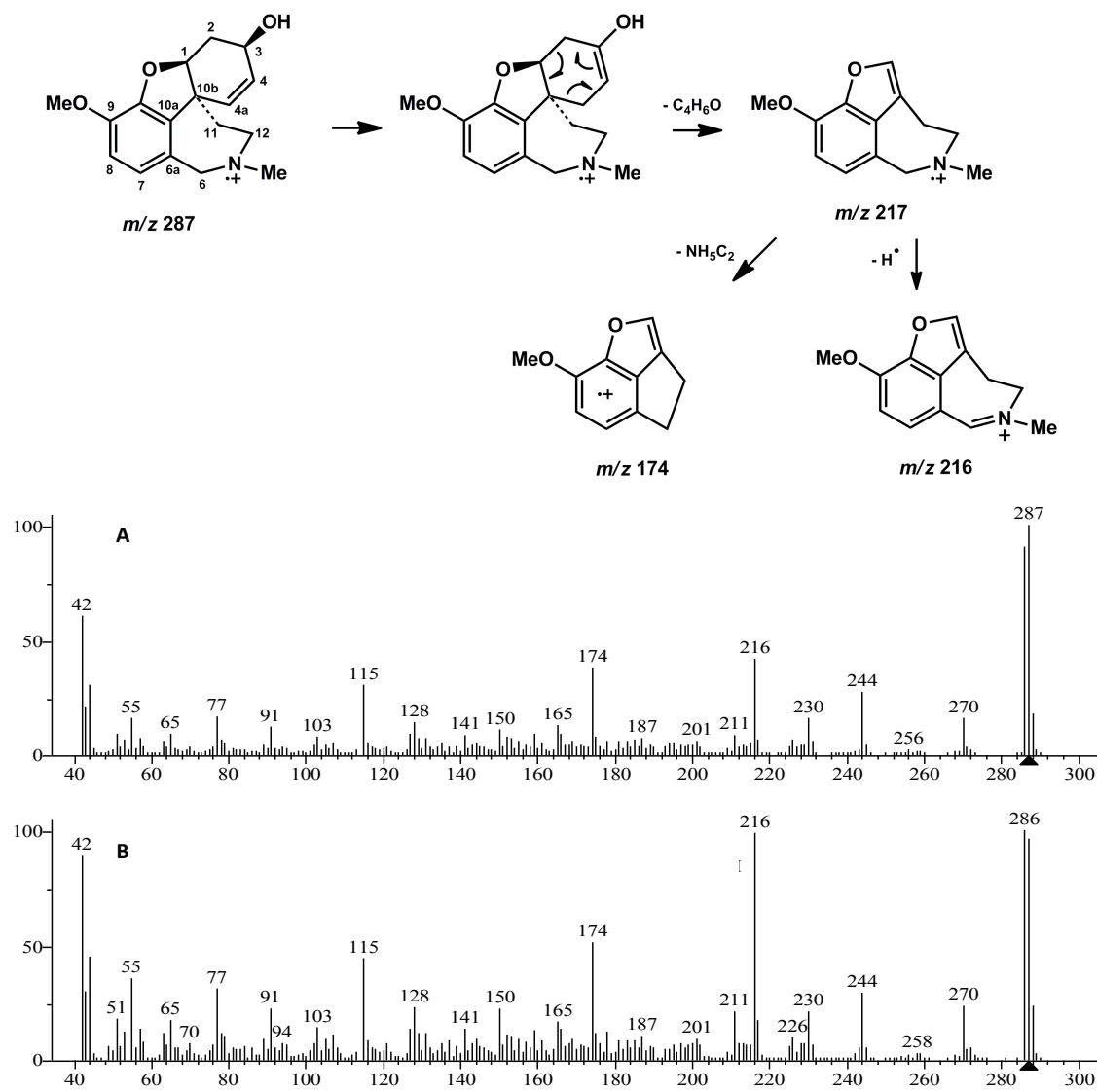


Figura 23. Patrón de fragmentación de galanthamine (A) y 3-epigalanthamine (B).

1.5.2 Resonancia Magnética Nuclear (RMN)

La espectroscopía de Resonancia Magnética Nuclear (RMN) es una herramienta analítica que proporciona información estructural y sobre la estereoquímica de elevado interés en un espacio de tiempo reducido. Se trata de una técnica que estudia el comportamiento de ciertos núcleos atómicos en presencia de un campo magnético externo. El campo magnético aplicado produce un desdoblamiento de los niveles de energía del *spin* nuclear (en este caso ^1H y ^{13}C), de modo que pueden inducirse transiciones entre ellos como consecuencia de la absorción de una radiación electromagnética adecuada. En este momento, los espines, comportándose como pequeños imanes polarizados, comienzan a presionar con su frecuencia característica en torno a un campo magnético externo, induciendo una pequeña corriente oscilante de radiofrecuencia en una bobina receptora situada en las inmediaciones de la muestra. A medida que los núcleos van regresando poco a poco a la situación inicial de equilibrio, alineados con el campo magnético principal, la señal detectada va disminuyendo de intensidad hasta hacerse cero. Esta caída de la señal se conoce como caída libre de la inducción (FID, del inglés “free induction decay”) y da lugar al espectro de RMN (Heinrich et al., 2004).

1.5.2.1 Características de los espectros de ^1H -RMN de los alcaloides de Amaryllidaceae

Las principales características del espectro de ^1H -RMN de los alcaloides del tipo lycorine son la presencia de dos singuletes de los protones aromáticos en *para*, juntamente con un protón olefínico. Para este tipo de estructura, normalmente se puede observar dos dobletes correspondientes a los protones del sistema AB de la posición bencílica 6. Normalmente los protones 6 y 12 β -orientados se encuentran en posiciones más desapantalladas en el espectro que sus homólogos en α lo que se debe al efecto del par de electrones libres del átomo de nitrógeno en *cis*. Generalmente la unión de los anillos B/C presentan una configuración *trans* y, consecuentemente, una constante de acoplamiento grande (>10 Hz) entre los protones 4a-10b elevada (Bastida et al, 2006).

En el espectro de ^1H -RMN de estructuras del tipo homolycorine se suele observar dos singuletes correspondientes a los protones aromáticos en *para*, siendo la señal de H-7 normalmente la más desapantallada debido a la presencia del carbonilo de la posición 6 en *peri*. Por lo general, ocurre una unión *cis* entre los anillos B/C de esta clase de compuestos, lo que es congruente con la pequeña constante de acoplamiento entre los protones 1-10b. En la mayoría de estos compuestos, el anillo C presenta un

protón vinílico. Además, el grupo *N*-metilo normalmente se encuentra en el intervalo de δ 2.0 – 2.2 ppm (Bastida et al., 2006).

La configuración absoluta de los esqueletos del tipo crinine y haemanthamine se determina por dicroísmo circular (DC). Con relación al espectro de ^1H -RMN de las estructuras de este tipo es posible observar que, utilizando CDCl_3 como solvente, la magnitud de las constantes de acoplamiento entre H-3 y cada uno de los protones olefinicos (H-1, H-2), ofrece información sobre la estereoquímica del sustituyente en C-3 en relación al puente etano. Así, en aquellos alcaloides en los que el puente y el sustituyente en C-3 se encuentran en *cis*, H-1 presenta un acoplamiento alílico con H-3 ($J_{2,3} \sim 1\text{-}2$ Hz) y H-2 muestra una constante más pequeña con H-3 ($J_{2,3} \sim 0\text{-}1.5$ Hz), tal como ocurre en crinamine. Por el contrario, en la serie epimérica en 3, como el alcaloide haemanthamine, se observa una constante de acoplamiento mayor entre H-2 y H-3 ($J_{2,3} = 5$ Hz), mientras que el acoplamiento entre H-1 y H-3 no es detectable. Esta regla se aplica tanto para los alcaloides del tipo crinine, como haemanthamine siempre y cuando el solvente utilizado sea CDCl_3 . Las estructuras con un sustituyente en C-6, como papyramine/6-epipapyramine o haemanthidine/6-epihaemanthidine, aparecen como una mezcla de epímeros de difícil separación por métodos convencionales de cromatografía (Bastida et al., 2006).

En el espectro de ^1H -RMN de estructuras del tipo tazettine, la presencia de un grupo *N*-metilo, normalmente en el intervalo de δ 2.4-2.5 ppm, hace posible la distinción de este tipo de alcaloides de aquellos de los tipos haemanthamine o crinine. Además, el espectro de ^1H -RMN siempre muestra la señal correspondiente al grupo metilendioxo (Bastida et al., 2006).

La configuración absoluta de los alcaloides del tipo montanine se determina mediante dicroísmo circular (DC). El espectro de ^1H -RMN de este tipo de estructuras se parece mucho a los del tipo lycorine, sin embargo, se pueden diferenciarse a través de la técnica de COSY. En los alcaloides del tipo montanine, las señales atribuidas a los protones H-4 son las más apantalladas, mientras que en el tipo lycorine las señales más apantalladas corresponden a los protones de la posición 11 y al protón 12α (Bastida et al., 2006).

En el espectro ^1H -RMN de estructuras del tipo galanthamine es posible observar la presencia de dos dobletes correspondientes a los protones aromáticos del anillo A en *orto*, con una constante de acoplamiento de alrededor de 8 Hz. Además, la asignación

de la estereoquímica del sustituyente en C-3 se realiza en base a las constantes de acoplamiento con el protón olefínico H-4. Cuando la constante $J_{3,4}$ tiene un valor de alrededor de 5 Hz, el sustituyente es *pseudo*-axial, mientras que si el valor es próximo a 0 Hz indica que el sustituyente en C-3 es *pseudo*-ecuatorial (Bastida et al., 2006).

1.5.2.2 Características de los espectros de ^{13}C -RMN de los alcaloides de Amaryllidaceae

En general, el espectro de ^{13}C -RMN de los alcaloides de Amaryllidaceae se encuentra dividido en dos regiones principales: a campos más bajos (>90 ppm) se observan las señales correspondientes a los grupos carbonilo, carbonos olefínicos y aromáticos, así como las señales del grupo metilendioxo, mientras que las señales de los carbonos alifáticos se encuentran a campos más altos, siendo la señal del grupo *N*-metilo la única fácilmente reconocible, entre 40-46 ppm. El efecto del sustituyente (OH, Me y OAc) son de gran importancia para la localización de la posición de los grupos funcionales en el espectro de carbono (Bastida et al., 2006; Bastida et al., 2011).

1.5.2.3 Espectros de 2D-RMN en la elucidación estructural

Generalmente, la elucidación estructural de un compuesto desconocido se logra mediante la combinación de diversas técnicas de RMN complementarias. La espectroscopía de ^1H -RMN revela informaciones fundamentales sobre los distintos tipos estructurales, mientras que su combinación con la espectroscopía de ^{13}C -RMN y las técnicas de RMN bidimensional (2D-RMN), suele permitir el establecimiento de su estereoquímica y la identificación inequívoca de la molécula en estudio (Bastida et al., 2006; Bastida et al., 2011).

Los experimentos bidimensionales son de importancia significativa para realizar una correcta asignación de las señales de ^1H -RMN y ^{13}C -RMN, especialmente en el caso de estructuras nuevas. Entre las técnicas de 2D-RMN que se utilizan más ampliamente, pueden citarse las siguientes:

- ^1H - ^1H COSY (COrelated SpectroscopY): las correlaciones observadas corresponden a acoplamientos directos entre los protones involucrados, siendo de gran importancia para la asignación de acoplamientos geminales y vecinales;
- ^1H - ^1H NOESY (Nuclear Overhauser Effect SpectroscopY): técnica de gran utilidad para obtener información en relación a la proximidad espacial entre protones y, por lo tanto, sobre su estereoquímica;

- ^1H - ^{13}C HSQC (**H**eteronuclear **S**ingle **Q**uantum **C**orrelation): muestra las correlaciones entre ^1H - ^{13}C directamente enlazados, permitiendo la adecuada asignación de todos los carbonos, a excepción de los cuaternarios;
- ^1H - ^{13}C HMBC (**H**eteronuclear **M**ultiple **B**ond **C**orrelation): de gran importancia en la determinación de correlaciones a larga distancia entre ^1H - ^{13}C , permitiendo la asignación de los carbonos cuaternarios a través de la observación de su correlación con protones situados a tres enlaces de distancia (Bastida et al., 2006; Bastida et al., 2011).

2. OBJETIVOS

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2.1 Objetivo general

Estudiar el perfil de alcaloides de distintas especies de Amaryllidaceae recolectadas en Sudamérica y promover el desarrollo de nuevas moléculas farmacológicamente activas a partir de productos naturales.

2.1.1 Objetivos específicos

- Estudiar el contenido en alcaloides de los extractos de distintas especies de las Amaryllidaceae recolectadas en Sudamérica por cromatografía de gases acoplada a espectrometría de masas (CG-EM) y determinar su composición.
- Aislar nuevos alcaloides de las especies de Amaryllidaceae y realizar la identificación y elucidación estructural de estas estructuras utilizando distintas técnicas espectroscópicas, principalmente resonancia magnética nuclear (RMN).
- Determinar la actividad biológica de los extractos y de los alcaloides aislados, considerando los ensayos de medición de su actividad inhibitoria de las colinesterasas (ACE y BuCE) y su actividad antiparasitaria.
- Realizar estudios de docking molecular para entender mejor las interacciones que ocurren entre el alcaloide y la enzima en los ensayos de inhibición de las colinesterasas.
- Identificar las especies que presenten un mayor interés farmacéutico para el desarrollo de nuevos fármacos.

3. RESULTADOS

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Los resultados de la presente tesis están reflejados en los artículos científicos que se detallan a continuación y que brevemente se resumen en las páginas posteriores.

Artículo 1. Tallini, L.R.; Andrade, J.P. de; Kaiser, M.; Viladomat, F.; Nair, J.J.; Zuanazzi, J.A.S.; Bastida, J. Alkaloid constituents of the Amaryllidaceae plant *Amaryllis belladonna* L. *Molecules*. 2017, 22:9, 1437-1448.

Artículo 2. Tallini, L.R.; Osorio, E.H.; Santos, V.D. dos; Borges, W. de S.; Kaiser, M.; Viladomat, F.; Zuanazzi, J.A.S.; Bastida, J. *Hippeastrum reticulatum* (Amaryllidaceae): alkaloid profiling, biological activities and molecular docking. *Molecules*. 2017, 22:12, 2191-2204.

Artículo 3. Tallini, L.R.; Torras-Claveria, L.; Borges, W.; Kaiser, M.; Viladomat, F.; Zuanazzi, J.A.S.; Bastida, J. N-oxide alkaloids from *Crinum amabile* (Amaryllidaceae). *Molecules*. 2018, 23:6, 1277-1287.

Artículo 4. Tallini, L.R.; Bastida, J.; Cortés, N.; Osorio, E.H.; Theoduloz, C.; Schmeda-Hirschman, G. Cholinesterase inhibition activity, alkaloid profiling and molecular docking of Chilean *Rhodophiala* (Amaryllidaceae). 2018. *Molecules*. 2018, 23:7, 1532-1558.

3.1 Artículo 1

Alkaloid constituents of the Amaryllidaceae plant *Amaryllis belladonna* L.

Molecules, 22:9, 1437-1448 (2017)

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Article

Alkaloid Constituents of the Amaryllidaceae Plant *Amaryllis belladonna* L.

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Abstract: The plant family Amaryllidaceae is well-known for its unique alkaloid constituents, which exhibit a wide range of biological activities. Its representative, *Amaryllis belladonna*, has a geographical distribution covering mainly southern Africa, where it has significant usage in the traditional medicine of the native people. In this study, *A. belladonna* samples collected in Brazil were examined for alkaloid content. Alkaloid profiles of *A. belladonna* bulbs were generated by a combination of chromatographic, spectroscopic and spectrometric methods, including GC–MS and 2D NMR. In vitro screening against four different parasitic protozoa (*Trypanosoma cruzi*, *T. brucei rhodesiense*, *Leishmania donovani* and *Plasmodium falciparum*) was carried out using the *A. belladonna* crude methanol extract, as well as three of its alkaloid isolates. Twenty-six different Amaryllidaceae alkaloids were identified in the *A. belladonna* bulb samples, and three of them were isolated. Evidence for their respective biosynthetic pathways was afforded via their mass-spectral fragmentation data. Improved data for 1-O-acetylcaranine was provided by 2D NMR experiments, together with new ¹H-NMR data for buphanamine. The crude extract and 3-O-acetylhamayne exhibited good antiprotozoal activity in vitro, although both with a high cytotoxic index.

Keywords: *Amaryllis belladonna*; Amaryllidaceae; alkaloids; biosynthetic pathways; NMR; MS

1. Introduction

The plant family Amaryllidaceae has attracted considerable attention in view of the fascinating structural features and varied biological activities manifested by its alkaloid principles. Anticancer, antimicrobial and anticholinesterase activities represent some of these biological properties [1]. The genus *Amaryllis* L. comprises two species, *A. belladonna* and *A. acuminata*, both native to southern Africa [2]. Also referred to by the folk names “belladonna-lily” and “naked-lady”, *A. belladonna* has been used for several centuries in the medicinal traditions of the Sotho, Xhosa and Zulu peoples of South Africa, and in Java for the treatment of “swelling” (a presumed synonym for cancer) [2,3].

The characteristic isoquinoline alkaloids produced by members of the Amaryllidaceae are derived from the aromatic amino acids phenylalanine and tyrosine, which combine to give the common precursor O-methylnorbelladine [4]. Consequently, alternative ways of oxidative phenolic

coupling of this key intermediate metabolite lead to the three main discernible skeleton types that form the basis for further structural diversity in the Amaryllidaceae. The *ortho-para'* coupling of O-methylnorbelladine results in the formation of the lycorine-type skeleton, which in turn leads to homolycorine-type compounds. The galanthamine-type skeleton originates from *para-ortho'* phenolic oxidative coupling, whilst *para-para'* coupling gives the crinine-, haemanthamine-, tazettine-, narciclasine- and montanine-type structures [5].

Gas chromatography-mass spectrometry (GC-MS) studies of Amaryllidaceae plants show that they contain a quite variable number of alkaloids [6,7]. Each species produces a mixture of alkaloids, often with a few dominant compounds and a larger number of compounds at lower concentrations, which is likely to result from differences in substrate specificity and expression level of the various biosynthetic enzymes [8]. The evolution of the biosynthetic pathways in the production of Amaryllidaceae alkaloids remains unclear [9]. Most research on Amaryllidaceae alkaloid biosynthesis was carried out during the 1960s and early 1970s. More recently, there has been notable interest in the biosynthesis of galanthamine and its congeners, given their success as commercial drug targets [10]. In this regard, in 2001, galanthamine was approved by the FDA (Food and Drug Administration) for the clinical management of mild to moderate Alzheimer's disease [11]. Subsequently, there has been widespread interest in the Amaryllidaceae as a source of novel chemotherapeutics [12].

Thirty-one alkaloids have been previously identified in *A. belladonna*, including 1-O-acetylcaranine, 6-hydroxybuphanisine, 6-hydroxycrinine, 8-demethylmaritidine, 11-hydroxyvittatine, amarbellisine, amaryllidine, amaryllisine, ambelline, anhydrolycorinone, anhydrolycorinium, belladine, bellamarine, buphanamine, caranine, crinine, galanthamine, galanthine, hippadine, hippeastrine, ismine, lycorenine, lycorine, pancracine, parkacine, parkamine, powelline, pratorimine, pratosine, undulatine and vittatine [2,13–21]. Anti-bacterial, anti-fungal and anti-neoplastic effects are associated with some of the alkaloids isolated from *A. belladonna* [2,20].

Protozoal diseases such as leishmaniasis, trypanosomiasis and malaria are still the most prevalent parasitic diseases worldwide, with a significant social and economic impact [22]. In many cases, contemporary chemotherapeutic regimens are not satisfactory due to a lack of effectiveness as well as the toxicity associated with long-term treatment. Furthermore, the emergence of global drug resistance has necessitated the development of new anti-parasitological drugs [23,24].

The aim of this work was to perform a detailed study of the alkaloid constituents of *A. belladonna* utilizing spectroscopic and chromatographic methods, including GC-MS and NMR. In this way, twenty-six alkaloids were identified and evidence presented for their respective biosynthetic pathways. Furthermore, a comprehensive NMR study of 1-O-acetylcaranine is presented, together with new ¹H-NMR data of buphanamine. Finally, anti-parasitological activities of the crude extract and three isolated alkaloids from *A. belladonna* are reported for the first time.

2. Results and Discussion

2.1. Alkaloids Identified in *A. belladonna* by GC-MS

The structural types of the alkaloids from *A. belladonna* were identified by comparing their GC-MS spectra and Kovats retention index (RI) values with those of authentic Amaryllidaceae alkaloids previously isolated and identified by spectrometric methods as nuclear magnetic resonance (NMR), ultraviolet (UV), circular dichroism (CD) and mass spectrometry (MS) in our laboratory. The NIST 05 Database and literature data were also used (Table 1). The MS spectra were deconvoluted by AMDIS 2.64 software (NIST).

Table 1. Alkaloids identified in *A. belladonna* by GC–MS.

Alkaloid	RI	M ⁺	MS
ismine (1)	2288.1	257 (28)	239 (20), 238 (100), 225 (7), 211 (7), 196 (9), 180 (8), 139 (8)
trisphaeridine (2)	2305.0	223 (100)	224 (15), 222 (38), 193 (2), 164 (15), 138 (26), 111 (14)
buphanisine (3)	2447.5	285 (100)	270 (34), 254 (34), 242 (12), 227 (24), 215 (85), 201 (23), 185 (22), 172 (22), 157 (33), 128 (33)
anhydrolycorine (4)	2470.2	251 (42)	250 (100), 220 (3), 192 (18), 163 (3), 125 (2)
caranine (5)	2560.9	271 (59)	270 (37), 252 (53), 240 (10), 227 (44), 226 (100), 212 (6), 181 (1)
1-O-acetylcaranine (6)	2569.6	313 (72)	270 (3), 252 (100), 240 (9), 227 (41), 226 (97)181 (2)
8-O-demethylmaritidine (7)	2575.5	273 (100)	256 (13), 244 (12), 230 (26), 216 (14), 201 (90), 189 (61), 175 (28), , 157 (19), 141 (11), 128 (24)
vittatine (8)	2578.0	271 (100)	254 (11), 242 (11), 228 (22), 214 (13), 199 (58), 187 (63), 173 (24), 157 (18), 141(13), 128 (29)
O-methylnorbelladine (9)	2578.5	273 (-)	166 (36), 137 (100), 122 (8), 94 (6)
11,12-dehydroanhydrolycorine (10)	2638.1	249 (59)	248 (100), 218 (1),190 (26), 163 (8), 137 (1), 123 (5), 95 (13)
6-methoxybuphanidrine (11)	2656.8	345 (58)	330 (33), 298 (14), 287 (32), 259 (68), 227 (30), 181 (10), 145 (13)
3-O-acetylvittatine (12)	2690.6	313 (100)	298 (1), 284 (6), 270 (27), 254 (75), 252 (61), 224 (38), 216 (60), 198 (36), 187 (36), 139 (15), 128 (33)
powelline (13)	2702.7	301 (100)	284 (10), 272 (14), 258 (39), 244 (24), 229 (8), 217 (7), 202 (22), 187 (23), 159 (13), 143 (11), 127 (19)
buphanidrine (14)	2705.2	315 (100)	300 (31), 284 (35), 272 (9), 260 (39), 245 (69), 228 (24), 215 (21), 202 (20), 187 (18), 130 (18)
lycorine (15)	2722.0	287 (18)	286 (10), 268 (19), 250 (16), 238 (7), 227 (78), 226 (100), 211 (6), 147 (5), 119 (3)
buphanamine (16)	2726.5	301 (100)	282 (21), 272 (13), 256 (22), 244 (11), 190 (7), 165 (9)
1-O-acetyllycorine (17)	2754.9	329 (31)	268 (31), 250 (20), 227 (64), 226 (100), 211 (6), 192 (3), 167 (3), 147 (6)
3-O-acetylpowelline (18)	2768.7	343 (100)	314 (6), 300 (25), 284 (78), 283 (24), 268 (9), 254 (21), 246 (81), 228 (39), 217 (18), 202 (6), 183 (9)
hippadine (19)	2775.0	263 (100)	233 (2), 205 (7), 177 (24), 150 (13), 131 (9), 111 (2), 75 (7)
ambelline (20)	2814.6	331 (86)	316 (8), 299 (44), 287 (100), 270 (33), 260 (76), 257 (58), 239 (52), 228 (20), 211 (62), 190 (39)
6-hydroxybuphanidrine (21)	2828.4	331 (44)	314 (5), 300 (10), 282 (7), 276 (100), 261 (33), 258 (13), 243 (12), 228 (8), 217 (21), 216 (22)
11-O-acetylbambelline (22)	2850.2	373 (100)	314 (58), 313 (50), 282 (46), 258 (48), 255 (36), 254 (63), 241 (38), 240 (35), 218 (44)
undulatine (23)	2855.2	331 (100)	316 (9), 300 (9), 286 (15), 258 (33), 244 (14), 232 (17), 217 (28), 205 (60), 189 (32), 173 (30)
3-O-acetylhamayne (24)	2907.7	329 (3)	300 (100), 269 (6), 240 (16), 224 (8), 212 (34), 211 (14), 199 (6), 181 (54), 167 (3), 153 (18), 128 (10)
crinamidine (25)	2954.3	317 (59)	300 (2), 288 (100), 258 (21), 244 (27), 230 (16), 217 (35), 203 (34), 189 (20), 173 (40), 145 (17)
distichamine (26)	2984.3	329 (100)	328 (25), 314 (15), 300 (9), 286 (9), 269 (5), 256 (3), 231 (20), 204 (13), 190 (5), 130 (2)

Fourteen of the identified alkaloids were detected for the first time in this species: trisphaeridine [2], buphanisine [3], anhydrolycorine [4], 11,12-dehydroanhydrolycorine [10], 6-methoxybuphanidrine [11], 3-O-acetylvittatine [12], buphanidrine [14], 1-O-acetyllycorine [17], 3-O-acetylpowelline [18], 6-hydroxybuphanidrine [21], 11-O-acetylbelline [22], 3-O-acetylhamayne [24], crinamidine [25] and distichamine [26]. All the alkaloids found in *A. belladonna* belong to the typical Amaryllidaceae alkaloid groups, including the lycorine, crinine and haemanthamine groups. 1-O-Acetylcaranine [6] was the most abundant alkaloid and the crinine group was the most represented, whilst no galanthamine-type alkaloids were detected in this plant.

Based on their structural identities, it has been possible to show how the alkaloids identified in *A. belladonna* could be linked biosynthetically (Figure 1). We propose that the chemical similarity of the alkaloid types from this species may be due to different combinations of common biosynthetic reactions, such as hydroxylation, hydrogenation, reduction, epoxidation, methylation, methoxylation, acetylation and allylic rearrangements, starting from a common precursor. Precedence for these reactions has been studied in other alkaloid groups; for example, indole alkaloids in *Catharanthus roseus* (Apocynaceae) [26,27].

To date, galanthamine metabolization represents the most studied Amaryllidaceae alkaloid biosynthetic pathway. Experiments involving the application of ^{13}C -labelled *O*-methylnorbelladine to field-grown *Leucojum aestivum* plants indicate that galanthamine biosynthesis involves phenolic oxidative coupling of *O*-methylnorbelladine to a dienone, the adduct of which undergoes spontaneous conversion to *N*-demethylnarwedine. The latter is converted via stereoselective reduction to *N*-demethylgalanthamine, which is then *N*-methylated to galanthamine [10].

Benzylamine *O*-methylnorbelladine (9) was also shown to be a precursor of norpluviine (27), which is known to be integral to lycorine-type alkaloid metabolization [28]. Apart from this, the transformation of caranine (5) into lycorine (15) has been observed in both *Zephyranthes candida* and *Clivia miniata* [29,30]. In *A. belladonna*, it is conceivable that 1-*O*-acetylcaranine (6) and 1-*O*-acetyllycorine (17) are derived via acetylation of the C-1 hydroxy group in caranine (5) and lycorine (15), respectively. Furthermore, we suggest that lycorine (15) could be metabolized to anhydrolycorine (4) by double dehydration of the C-1 and C-2. Also, we suggest that anhydrolycorine (4) may be a precursor of 11,12-dehydroanhydrolycorine (10) by reduction of the D-ring double bond, which could then serve as a precursor to hippadine (19) via hydroxylation at C-6 and subsequent oxidation to the amide functionality.

Crinine (31) can be considered the precursor of all crinine-type alkaloids in possession of the methylenedioxy group, whilst buphanisine (3) and powelline (13) may be easily derived from crinine (31) by methylation and methoxylation reactions at C-3 and C-7, respectively. In addition, C-7 methoxylation of buphanisine (3) and/or C-3 methylation of powelline (13) are possible alternative steps in the buphanidrine (14) biosynthetic pathway. The presence of ambelline (20) and 11-acetylbelline (22) suggests a C-11 hydroxylation of buphanidrine (14) followed by an acetylation reaction. Buphanidrine (14) is a likely precursor of 6-hydroxybuphanidrine (21), which could then lead to 6-methoxybuphanidrine (11) via a methylation reaction. Also in the crinine group, it is conceivable that metabolic acetylation of powelline (13) would afford 3-*O*-acetylpowelline (18). Powelline (13) could also facilitate crinamidine (25) metabolization via C-2, C-3 epoxidation. This would subsequently lead to undulatine (23) by C-3 methylation, which in turn is distinguished as the precursor to distichamine (26) following allylic rearrangement. Further to this, 8-*O*-demethylmaritidine (7) is a plausible precursor of vittatine (8) via methylenedioxy group transformation, from which 3-*O*-acetylvittatine (12) may be readily accessible. The presence of 3-*O*-acetylhamayne (24) suggests that the structurally related alkaloids siculine (28), epivittatine (29) and hamayne (30) may also be present, but in hitherto undetectable quantities in *A. belladonna*.

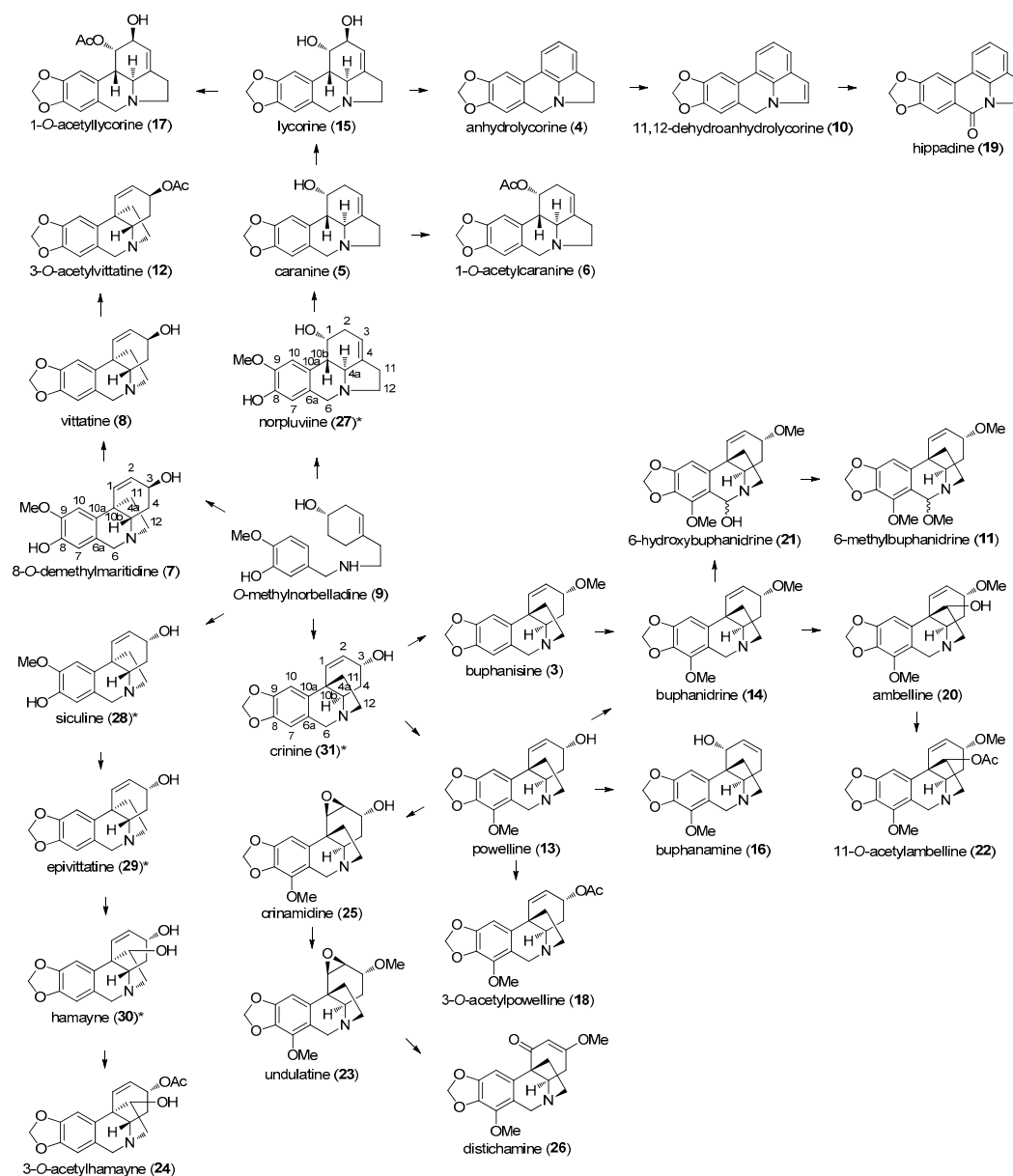


Figure 1. Tentative biosynthetic routes to the alkaloids of *A. belladonna*. * Alkaloids not identified in this plant but which could be involved in the metabolic route include norpluviine (27), siculine (28), epivittatine (29), hamayne (30) and crinine (31).

2.2. 1-O-Acetylcaranine

The base ion signal that occurs at m/z 252 in 1-O-acetylcaranine (6) is characteristic of deacetylation at C-1 and dehydration at C-1 and C-2 (Figure 2). Herein we present the complete NMR data for 1-O-acetylcaranine (6) by both 1D and 2D NMR spectroscopic analysis (see 1-O-acetylcaranine NMR spectra in Supplementary Materials). The ^1H -NMR spectrum (Table 2) was similar to that of 1-O-acetylcaranine and caranine [13,31,32]. The shift of the H-1 proton signal to a lower magnetic field than that observed for caranine suggested a substitution of the hydroxyl group at C-1, which was further substantiated by the presence of a singlet at δ 1.93, indicative of an acetyl group. The ^{13}C -NMR spectrum showed a singlet resonance signal at δ 171.0, which confirmed the presence of one carbonyl group. The COSY spectrum showed an allylic coupling between H-3 and H-4a and between H-3 and H-11, which allowed us to determine the H-10b proton location in the ^1H -NMR spectrum. In addition, the small magnitude of the coupling constants between H-1 and H-10b allowed us to assign the α -orientation to

the acetyl group. The two C-6 protons were differentiated as an AB system with a geminal coupling of around 14 Hz. H-4a showed NOESY correlations with both H-2 α and H-6 α , which turned out to be key correlations in the assignment of their orientation. Furthermore, H-12 α was ascribable to a higher field as a consequence of NOESY contour correlation with H-6 α . The HMBC spectrum allowed us to assign the quaternary carbons C-6a (δ 129.5) and C-10a (δ 127.8) via three-bond correlation with H-10 and H-7, respectively. The aromatic protons were ascribable to H-7 and H-10 due to three-bond HMBC correlations with C-10a and C-6a, respectively, in addition to NOESY correlations observed for the H-6/H-7 and H-1/H-10 proton pairs. The C-8 quaternary carbon (δ 146.2) was located via four-bond HMBC connectivity with both H-6, as well as via three-bond connectivity with H-10. The quaternary carbon C-9 (δ 146.5) was determined via three-bond HMBC connectivity with H-7. Finally, the singlet resonance signal at δ 139.26 was assigned to C-4, taking into account three bond connectivities to H-12 β . All these data are in agreement with the structure of 1-*O*-acetylcaranine (6) (see Figure 4).

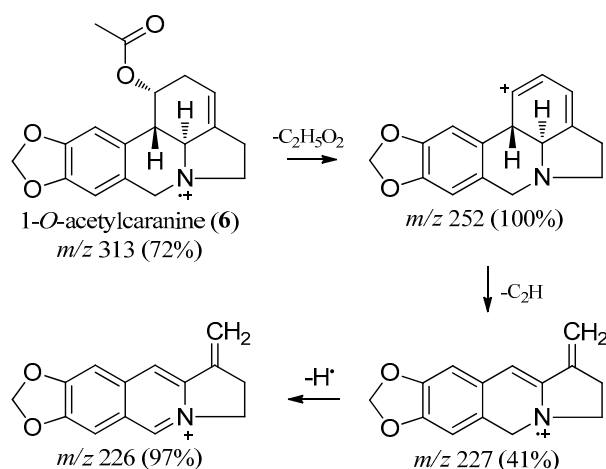


Figure 2. Mass fragmentation pattern of 1-*O*-acetylcaranine (6).

Table 2. ^{13}C -NMR, ^1H -NMR and HMBC data for 1-*O*-acetylcaranine (6) (500 MHz, CDCl_3).

Position	δ_{C} , Type	δ_{H} (J in Hz)	HMBC
1	66.60 d	5.84 br dd (4.5, 2.0)	C-3, C-4a, C-10b, OCOCH_3
2 α	33.55 t	2.39 m	
2 β	33.55 t	2.64 m	
3	114.43 d	5.39 dd (4.9, 2.4)	C-4a, C-1
4	139.56 s		
4a	61.51 d	2.82 d (10.8)	
6 α	57.09 t	3.54 d (14.3)	C-10a
6 β	57.09 t	4.13 d (14.1)	C-4a, C-7, C-10a
6a	129.54 s		
7	107.34 d	6.57 br s	C-6, C-9, C10a
8	146.24 s		
9	146.49 s		
10	105.25 d	6.72 d (1.0)	C-6a, C-8, C-10b
10a	127.79 s		
10b	43.56 d	2.67 m	
11 α	28.70 t	2.61 m	
11 β	28.70 t	2.61 m	
12 α	53.83 t	2.39 m	C-6
12 β	53.83 t	3.33 ddd (9.4, 4.9, 4.4)	C-4, C-4a, C-6
OCH_2O	101.16 t	5.91 d (1.5)–5.92 d (1.5)	
OCOCH_3	171.03 q	1.93 s	
OCOCH_3	21.53 q	1.93 s	

2.3. Buphanamine

For most crinine-type alkaloids, the molecular ion is prominent as the base peak, and the fragmentation mechanism is initiated by opening of the C-11/C-12 ethano-bridge, indicating bond cleavage at the position β to the nitrogen atom (Figure 3) [33].

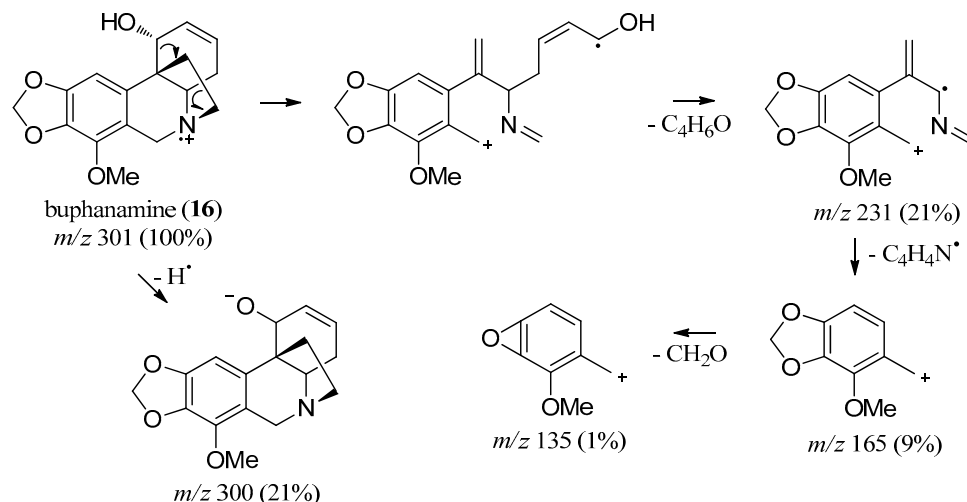


Figure 3. Mass fragmentation pattern for buphanamine (**16**) (adapted from [33]).

The ^1H -NMR data we obtained for buphanamine (**16**) (Table 3) differed slightly from the data available in the literature [34]. The discrepancies we noted relate to the H-4 α , H-4 β , H-4a, H-6 α , H-6 β , H-11endo, H-11exo, H-12endo and H-12exo proton resonances (Δ +0.26, +0.22, +0.36, +0.26, +0.27, +0.14, +0.20, +0.24, +0.51, respectively). These are quite downfield-shifted compared to the corresponding data we obtained in our NMR analysis (see buphanamine NMR spectra in Supplementary Materials). Quaternization of the nitrogen atom via *N*-oxide or salt formation is known to influence the chemical shifts of protons in its vicinity [1].

Table 3. ^{13}C -NMR, ^1H -NMR and HMBC data of buphanamine (**16**) (500 MHz, CDCl_3).

Position	δ_{C} , Type	δ_{H} (J in Hz)	HMBC
1	64.5 d	4.74 d (5.5)	C-3, C-4a, C-10a, C-11
2	125.5 d	6.00 dddd (10.0, 5.5, 2.8, 1.8)	C-10b
3	128.9 d	5.86 ddd (10.0, 4.5, 2.8)	C-4a
4 α	28.2 t	2.57 dddd (19.7, 8.1, 4.5, 1.9)	C-2, C-10b
4 β	28.2 t	1.99 dddd (19.8, 8.6, 1.1, 0.3)	C-2, C-10b
4a	59.2 d	3.43 t (8.3)	C-10a
6a	117.9 s		
6 α	56.9 t	4.17 d (17.2)	C-4a, C-7, C-10a
6 β	56.9 t	3.81 d (17.2)	C-4a, C-7, C-10a
7	140.8 s		
8	133.6 s		
9	148.6 s		
10	98.0 d	6.59 s	C-6a, C-8, C-10b
10a	137.0 s		
10b	48.3 s		
11endo	38.7 t	1.85 dddd (11.9, 8.8, 3.1, 1.3)	C-4a, C-10a
11exo	38.7 t	1.92 ddd (12.1, 10.0, 7.3)	C-4a, C-10a
12endo	51.4 t	2.75 ddd (13.0, 8.8, 7.2)	C-4a, C-6
12exo	51.4 t	3.40 ddd (13.0, 10.0, 3.0)	C-6, C-10b
OCH ₂ O	100.7 t	5.88 d (1.5)–5.89 d (1.5)	C-8, C-9
OCH ₃	59.1 q	3.98 s	C-7

In the ^1H -NMR spectrum, the coupling constants between H-1 and H-2 ($J = 5.5$ Hz), H-2 and H-3 ($J = 10.0$ Hz), H-3 and H-4 α ($J = 4.5$ Hz), together with the geminal coupling of around 19.7 Hz between H-4 α and H-4 β , allowed us to place the hydroxyl group at C-1 and the double bond between C-2 and C-3. The NOE contour between H-1 and 2H-11, and the homoallylic coupling between H-1 and H-4 β , confirmed the α -orientation for the hydroxyl group. Interestingly, the COSY correlation observed between H-2 and 2H-4 also confirmed the allylic coupling between these protons. The two H-6 protons were clearly differentiated as part of an AB system, each with a geminal coupling value of 17.2 Hz. The singlet aromatic proton resonance was ascribable to H-10 due to three-bond HMBC correlations with C-6a and C-10b, in addition to a NOESY correlation with H-1, allowing the aromatic methoxyl group to be placed at C-7. H-6 α was assigned to a lower field via a NOESY contour correlation with H-4a (Figure 4). The H-11 exo and H-12 exo protons were assignable based on the large values of their respective coupling constants. The quaternary carbons C-6a and C-10a were ascribed by means of their three-bond HMBC correlations with H-10 and H-1, respectively. Finally, the singlet resonance signal at $\delta = 48.3$ was assigned to C-10b, taking into account three-bond connectivities to H-10, H-4 α and H-4 β . The absolute configuration of this alkaloid was determined from the CD spectrum, wherein the curve was qualitatively similar to that of buphanamine (16) [35].

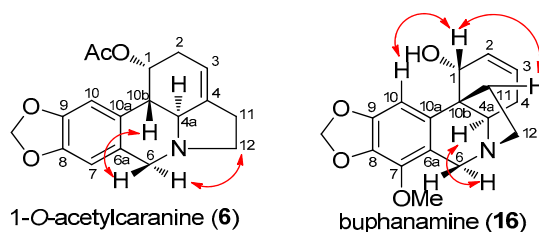


Figure 4. Key NOESY correlations observed for 1-*O*-acetylcaranine (6) and buphanamine (16).

2.4. Biological Activity

The biological activity tests against the parasitic protozoa and for cytotoxicity were performed as described earlier [36]. 3-*O*-Acetylhamayne showed higher activity than the other alkaloids against all protozoan parasites tested. It was active against *Trypanosoma brucei rhodesiense* ($\text{IC}_{50} = 1.51 \mu\text{g mL}^{-1}$), *T. cruzi* ($\text{IC}_{50} = 8.25 \mu\text{g mL}^{-1}$), *Leishmania donovani* ($\text{IC}_{50} = 17.91 \mu\text{g mL}^{-1}$) and *Plasmodium falciparum* ($\text{IC}_{50} = 1.14 \mu\text{g mL}^{-1}$). However, the cytotoxicity against L6 cells (rat skeletal myoblasts, $\text{IC}_{50} = 1.72 \mu\text{g mL}^{-1}$) highlights that 3-*O*-acetylhamayne is not a selective antiparasitic agent. The crude extract exhibited activity against *T. brucei rhodesiense* ($\text{IC}_{50} = 4.67 \mu\text{g mL}^{-1}$), *T. cruzi* ($\text{IC}_{50} = 34.86 \mu\text{g mL}^{-1}$) and *P. falciparum* ($\text{IC}_{50} = 1.17 \mu\text{g mL}^{-1}$), but it was less cytotoxic than 3-*O*-acetylhamayne. There is very little information about the structure–antiprotozoal activity relationship of the Amaryllidaceae alkaloids, but some results suggest that the methylenedioxy group can contribute to increase the antiprotozoal activity in these alkaloids [24]. The crude extract results suggest that *A. belladonna* could be an interesting source of alkaloids with antiparasitic activity. The antiprotozoal activity of *A. belladonna* alkaloids is summarized in Table 4.

Table 4. In vitro antiprotozoal and cytotoxic activities of *A. belladonna*.

Parasite	<i>T. b. rhodesiense</i>	<i>T. cruzi</i>	<i>L. donovani</i>	<i>P. falciparum</i>	Cytotoxicity
Stage	Trypomastigotes	Amastigotes	Amastigotes	IEF (Intraerythrocytic)	
Strain	STIB 900 (IC ₅₀ ^a)	Tulahuen C4 LacZ (IC ₅₀ ^a)	MHOM-ET-67/L82 (IC ₅₀ ^a)	NF54 (IC ₅₀ ^a)	L6 (IC ₅₀ ^a)
melarsoprol	0.0035 ^b and 0.0010 ^c	0.660 ^b and 1.080 ^c	0.085 ^b and 0.091 ^c	0.002 ^{b,c}	0.005 ^b and 0.007 ^c
benznidazole					
miltefosine					
chloroquine					
podophyllotoxin					
1-O-acetylcaranine (6)	1.97	35.9	>100	3.21	14.2
3-O-acetylhamayne (24)	1.51	8.3	17.9	1.14	1.72
buphanamine (16)	28.2	62.9	>100	25.9	>100
crude extract	4.67	34.9	>100	1.17	34.3

^a all values expressed as $\mu\text{g mL}^{-1}$; ^b reference value to 1-O-acetylcaranine; ^c reference value to buphanamine, 3-O-acetylhamayne and crude extract.

3. Materials and Methods

3.1. Plant Material

Bulbs of *Amaryllis belladonna* L. were collected in Canela (Rio Grande do Sul, Brazil) in August 2014. The first sample was authenticated by Dr. Julie H. A. Dutilh, University of Campinas (Unicamp, Campinas, Brazil). A specimen voucher (179860) has been deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (UFRGS, Porto Alegre, Brazil).

3.2. Equipment

About 2 mg of each alkaloid extract was dissolved in 1 mL of MeOH and/or CHCl₃ and injected directly into the GC–MS apparatus (Agilent Technologies 6890N coupled with MSD5975 inert XL) operating in the electron ionization (EI) mode at 70 eV. A Sapiens-X5 MS column (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used. The temperature gradient performed was as follows: 12 min at 100 °C, 100–180 °C at 15 °C/min, 180–300 °C at 5 °C/min and 10 min hold at 300 °C. The injector and detector temperatures were 250 and 280 °C, respectively, and the flow-rate of carrier gas (He) was 1 mL/min. A split ratio of 1:5 was applied and the injection volume was 1 μL .

¹H- and ¹³C-NMR, COSY, NOESY, HMQC and HMBC spectra were recorded on a Varian VNMRS 500 MHz using CDCl₃ as the solvent and TMS as the internal standard. Chemical shifts were reported in units of δ (ppm) and coupling constants (*J*) expressed in Hz. CD, UV and IR spectra were recorded on Jasco-J-810, Dinko UV2310 and Thermo Scientific Nicolet iN10 MX spectrophotometers, respectively. High-resolution electrospray ionization mass spectrometry (HR–ESI–MS) spectra were obtained on an LC/MSD-TOF (2006) mass spectrometer (Agilent Technologies). Silica gel SDS chromagel 60 A CC (6–35 μm) was used for vacuum liquid chromatography (VLC), and silica gel 60 F254 Macherey-Nagel for analytics and prep. Spots on chromatograms were detected under UV light (254 nm) and by Dragendorff's reagent stain.

3.3. Extraction

Fresh bulbs (2.5 kg) of *A. belladonna* were dried at 40 °C for seven days. After this, the dried material (950 g) was macerated with MeOH (4 × 2 L) at room temperature for 48 h, the combined macerate was filtered and the solvent evaporated to dryness under reduced pressure. The bulb crude extract (105 g) was then acidified to pH 3 with diluted H₂SO₄ (5% *v/v*) and the neutral material removed with Et₂O (10 × 200 mL). The aqueous solution was basified up to pH 9–10 with NH₄OH (25% *v/v*) and extracted with *n*-Hexane (12 × 200 mL) to give the *n*-hexane extract (0.47 g), which was followed by extraction with EtOAc (15 × 200 mL) to provide the EtOAc extract (4.07 g).

The extracts were subjected to a combination of chromatographic techniques, including vacuum liquid chromatography (VLC) [25] and semi-preparative thin-layer chromatography (TLC). The general VLC procedure consisted of the use of a silica gel 60 A (6–35 μm) column with a height of 4 cm and a variable diameter according to the amount of sample (2.5 cm for 400–1000 mg; 1.5 cm for 150–400 mg). Alkaloids were eluted with *n*-hexane containing increasing EtOAc concentrations, followed by neat EtOAc, which was gradually enriched with MeOH (reaching a maximum concentration of 20% *v/v*). Fractions of 10–15 mL were collected, monitored by TLC (UV 254 nm, Dragendorff's reagent) and combined according to their profiles. For semi-preparative TLC, silica gel 60F₂₅₄ was used (20 cm $\times$ 20 cm $\times$ 0.25 mm) together with different solvent mixtures depending on each particular sample (EtOAc:MeOH, 9:1 *v/v*, or EtOAc:MeOH, 8:2 *v/v*), always in an environment saturated with ammonia. The alkaloids were each identified by GC–MS and the three known alkaloids were isolated and structurally elucidated by NMR as 1-*O*-acetylcaranine (362.9 mg), 3-*O*-acetylhamayne (2.2 mg) and buphanamine (17.2 mg).

3.4. Characterization of Compounds

1-*O*-Acetylcaranine (6). $[\alpha]_{\text{D}}^{22}$ –161.84 (*c* 0.0009, CHCl_3); UV (MeOH) λ_{max} (log ϵ): 290.5 (3.77), 235.5 (3.76) nm; IR (CHCl_3) ν_{max} 2888, 1726, 1505, 1487, 1487, 1450, 1376, 1238, 1033, 934, 847 cm^{-1} ; ^1H -NMR (CDCl_3 , 500 MHz) and ^{13}C -NMR (CDCl_3 , 125 MHz) see Table 2; ESI–MS data shown in Table 1; HR–ESI–MS of $[\text{M} + \text{H}]^+$ *m/z* 314.1394 (calcd. for $\text{C}_{18}\text{H}_{20}\text{NO}_4$, 314.1387).

Buphanamine (16). $[\alpha]_{\text{D}}^{22}$ –169.03 (*c* 0.0009, CHCl_3); UV (MeOH) λ_{max} (log ϵ): 285.0 (3.17), 207.0 (4.14) nm; CD (MeOH, 20 $^\circ\text{C}$) $\Delta\epsilon_{250}$ +1792, $\Delta\epsilon_{268}$ –247, $\Delta\epsilon_{288}$ +293; IR ν_{max} 3334, 2923, 1619, 1478, 1240, 1080, 1044, 753 cm^{-1} ; ^1H -NMR (CDCl_3 , 500 MHz) and ^{13}C -NMR, 125 MHz) see Table 3; ESI–MS data shown in Table 1; HR–ESI–MS of $[\text{M} + \text{H}]^+$ *m/z* 302.1385 (calcd. for $\text{C}_{17}\text{H}_{20}\text{NO}_4$, 302.1387).

Supplementary Materials: Supplementary Materials are available online.

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Author Contributions: Jaume Bastida designed the experiments, analyzed the data and wrote the paper. Luciana R. Tallini performed the experiments, analyzed the data and wrote the paper. Jean Paulo de Andrade and Marcel Kaiser performed the experiments and wrote the paper. Francesc Viladomat, Jerald J. Nair and José Angelo S. Zuanazzi wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are not available.



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3.2 Artículo 2

***Hippeastrum reticulatum* (Amaryllidaceae): alkaloid profiling, biological activities and molecular docking**

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Article

Hippeastrum reticulatum (Amaryllidaceae): Alkaloid Profiling, Biological Activities and Molecular Docking

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Abstract: The Amaryllidaceae family has proven to be a rich source of active compounds, which are characterized by unique skeleton arrangements and a broad spectrum of biological activities. The aim of this work was to perform the first detailed study of the alkaloid constituents of *Hippeastrum reticulatum* (Amaryllidaceae) and to determine the anti-parasitological and cholinesterase (AChE and BuChE) inhibitory activities of the epimers (6 α -hydroxymaritidine and 6 β -hydroxymaritidine). Twelve alkaloids were identified in *H. reticulatum*: eight known alkaloids by GC-MS and four unknown (6 α -hydroxymaritidine, 6 β -hydroxymaritidine, reticulinine and isoreticulinine) by NMR. The epimer mixture (6 α -hydroxymaritidine and 6 β -hydroxymaritidine) showed low activity against all protozoan parasites tested and weak AChE-inhibitory activity. Finally, a molecular docking analysis of AChE and BuChE proteins showed that isoreticulinine may be classified as a potential inhibitory molecule since it can be stabilized in the active site through hydrogen bonds, π - π stacking and hydrophobic interactions.

Keywords: 6 α -hydroxymaritidine; 6 β -hydroxymaritidine; reticulinine; isoreticulinine; *Hippeastrum reticulatum*

1. Introduction

Natural products offer a wealth of bio-structural information that can be used to guide drug discovery and molecular design projects [1]. Plants have played a huge role in traditional medicine, and plant screening has become an important tool in drug discovery for pharmaceutical companies and university institutes [2]. Alkaloids are plant secondary metabolites that are usually low in nitrogen but high in oxygen [3]. Their three-dimensional complexity and interesting ring systems are of great interest for drug development [3–5].

The Amaryllidaceae, a plant family in the monocot order Asparagales, is composed of three subfamilies: Amaryllidoideae, Agapanthoideae and Allioideae [6,7]. The Amaryllidoideae include about 80 genera [8] that are mainly distributed in tropical and subtropical regions, but also in temperate

zones [9]. A particular feature of plants of the Amaryllidoideae subfamily is their content of an exclusive, numerous and still expanding group of alkaloids, known as Amaryllidaceae alkaloids. These compounds are characterized by unique skeleton arrangements and a broad spectrum of biological activities [10]. Galanthamine is the most important Amaryllidaceae alkaloid, having received FDA approval in 2001 for the clinical management of mild to moderate Alzheimer's disease (AD) [11].

Over 47 million people worldwide were living with dementia in 2016, a figure expected to increase to 131 million by 2050 [12]. The most common symptom of AD is a deterioration of memory and other cognitive domains, leading to death within 3 to 9 years after diagnosis [13]. Multiple factors have been implicated in AD pathology, including a reduced cholinergic neurotransmission level, oxidative stress and aggregated amyloid- β -peptide (A β) [14].

Another global health challenge is tackling the neglected tropical diseases (NTDs), a diverse group of communicable diseases that prevail in tropical and subtropical conditions and affect more than a billion people [15]. The World Health Organization (WHO) lists 17 NTDs, including Chagas disease, human African trypanosomiasis (sleeping sickness) and leishmaniasis. Alongside malaria, these diseases are highly feared in affected populations, where poverty limits access to prevention and treatment interventions [15]. One of the main reasons why these diseases are “neglected” is that there is little incentive for the pharmaceutical industry to invest in developing drugs for a patient population that cannot afford them [16]. Consequently, much of the research on effective treatment of these diseases is carried out in academic laboratories with limited financial, personnel, and technical resources [16].

The *Hippeastrum* Herb. is a genus (Amaryllidaceae) native to South America and comprises approximately 60 species [17], about 30 of which are found in Brazil; the majority are endemic and poorly studied [18]. This genus has been traditionally used to cure piles, tumors and various inflammatory disorders such as asthma [19]. Physiological activities reported for plants of this genus include psychopharmacological [20], against *Trichomonas vaginalis* [21] and cytotoxicity [22,23]. The species *H. reticulatum* is widely used as an ornamental plant due to its colorful flowers ranging from light pink to dark purple [24]. In recent years, some studies have been published about its primary metabolites, pigment constituents (anthocyanins), botanical characterization, karyotypes and phylogenesis [9,24–26].

The aim of this work was to perform the first study of the alkaloid profile and biological activities of *Hippeastrum reticulatum*. Four new alkaloids were isolated and chemically characterized by spectroscopic methods and eight known alkaloids were identified by gas chromatography-mass spectrometry (GC-MS). Anti-parasitological and cholinesterase—acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)—inhibitory activities of the epimers (compounds 1 and 2, Figure 1) are described. Molecular docking studies were also carried out to investigate the affinity of the four new compounds for the active sites of AChE and BuChE based on intermolecular features such as hydrogen bonds, π - π stacking and hydrophobic interactions. Unfortunately, it was not possible to determine the biological activities of the isomers (compounds 3 and 4, Figure 1) due to a lack of samples.

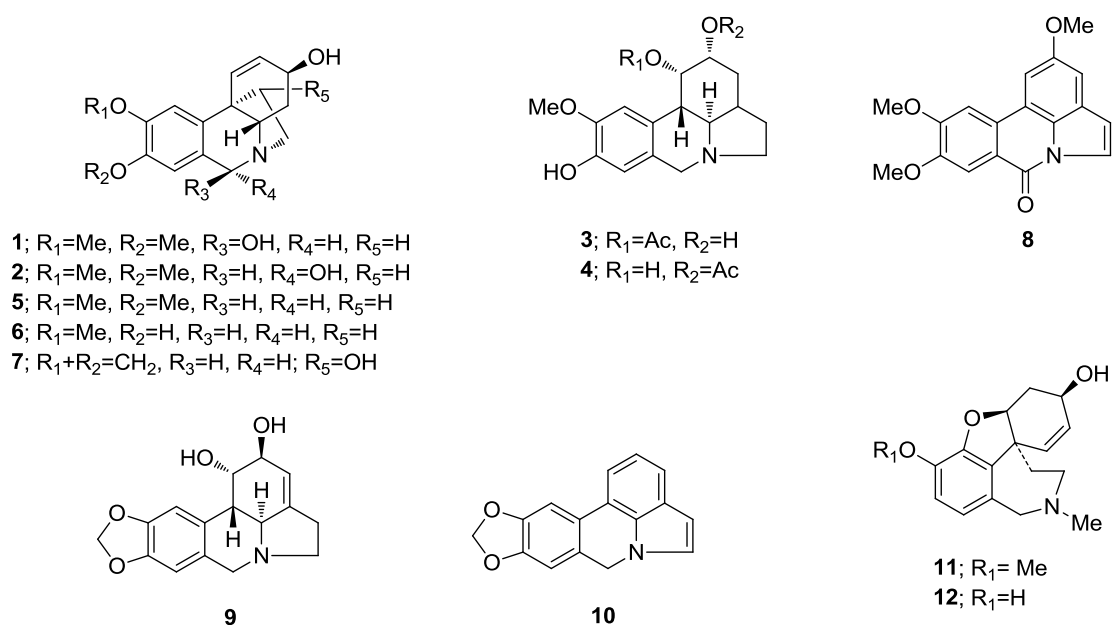
2. Results and Discussion

2.1. Alkaloid Identification by GC-MS

The MeOH extracts of *H. reticulatum* plants were fractioned following the sequence described in the Experimental section. The fractions were analyzed and the structural types of the alkaloids from this species were identified by comparing their GC-MS spectra and Kovats Retention Index (RI) values with those of authentic Amaryllidaceae alkaloids previously isolated and identified by spectrometric methods (NMR, UV, CD, IR, MS) in our laboratory, by the NIST 05 Database or by literature data (Table 1). Eight known alkaloids were identified by GC-MS using our Amaryllidaceae alkaloid library (Figure 1) and four new alkaloids (1–4) were isolated and chemically characterized by spectroscopic methods.

Table 1. Alkaloids identified in *H. reticulatum* by GC-MS.

alkaloid	RI	M ⁺	MS
galanthamine (11)	2410.8	287 (84)	288 (20), 286 (100), 270 (15), 244 (27), 230 (15), 216 (37), 174 (32), 159 (11), 115 (16)
sanguinine (12)	2430.5	273 (100)	274 (16), 272 (77), 256 (23), 202 (31), 197 (15), 165 (14) 160 (45), 152 (15), 115 (19)
6β- and 6α-hydroxymaritidine (1) and (2)	2495.5	303 (20)	286 (9), 274 (12), 260 (21), 259 (100), 258 (12), 256 (20), 241 (30) 128 (16), 115 (20)
8-O-demethylmaritidine (6)	2522.4	273 (100)	274 (18), 230 (24), 203 (19), 202 (25), 201(89), 189 (55), 175 (23), 115 (19), 56 (20)
maritidine (5)	2528.5	287 (100)	288 (20), 244 (35), 217 (20) 216 (25), 215 (95), 203 (55), 189 (18), 128 (17), 115 (20)
11,12-dehydroanhydrolycorine (10)	2629.3	249 (59)	248 (100), 218 (1),190 (26), 163 (8), 137 (1), 123 (5), 95 (13)
<i>m/z</i> 264	2693.6	265 (76)	264 (100), 248 (18), 220 (12), 191 (14),178 (18)
11-hydroxyvittatine (7)	2732.5	287 (5)	259 (16), 258 (100), 242 (9), 214 (9), 212 (8), 211 (13), 186 (13), 181 (14), 128 (11)
lycorine (9)	2771.8	287 (18)	286 (10), 268 (19), 250 (16), 238 (7), 227 (78), 226 (100), 211 (6), 147 (5), 119 (3)
isoreticulinine (4)	2829.1	333 (<1)	291 (37), 290 (100), 274 (11), 272 (6), 256 (5), 228 (5), 147 (13)
reticulinine (3)	2133.4	333 (42)	332 (100), 316 (5) 290 (6), 272 (56), 256 (31), 244 (14), 216 (14), 147 (26)
<i>m/z</i> 294	2950.3	295 (87)	294 (100), 278 (10), 264 (3), 250 (7), 235 (3), 221 (5), 207 (3), 194 (5)
<i>m/z</i> 280	2978.6	281 (75)	280 (100), 264 (12), 250 (3), 236 (11), 219 (4), 207 (4), 194 (8), 178 (4), 167 (3)
2-methoxypratosine (8)	3071.1	309 (100)	310 (20), 294 (18), 284 (7), 266 (24), 251 (14), 236 (6), 164 (4), 152 (6)

**Figure 1.** Alkaloids identified in *H. reticulatum*.

2.2. Structural Elucidation by NMR

2.2.1. 6 β -Hydroxymaritidine (1) and 6 α -hydroxymaritidine (2)

Compounds **1** and **2** were obtained together from an acetate leaf fraction and isolated as a mixture, which was not separated by conventional chromatographic methods. The HRMS data of compounds **1** and **2** suggested the molecular formula $C_{17}H_{22}NO_4$ for the parent ion $[M + H]^+$ at m/z 304.1544 (calcd 304.1543). The absolute configuration of these epimers was determined by circular dichroism (CD). The curve and shape were qualitatively similar to those of known haemanthamine-type alkaloids, with the 5,10b-ethano bridge in an α -orientation, having a minimum of 250 nm and a maximum of around 285 nm. The 1H -NMR data of compounds **1** and **2** (Table 2) were similar to the data published for papyramine and 6-*epi*-papyramine [27] (Figure 2). The spectrum showed two singlets at δ 5.35 and 6.12 due to the benzylic H-6 proton for epimers **1** and **2**, respectively. Two double doublets of the olefinic protons at δ 5.98 and 6.02 were assignable to the H-2 protons of both epimers (compounds **1** and **2**, respectively) due to their coupling with the H-1 doublets at 6.59 and 6.51 ppm, respectively. The value of the H-2/H-3 coupling constant (J = 5.3 and 5.1 Hz, respectively) was in agreement with a β -orientation of the hydroxyl group at C-3. The assignment of the aromatic protons was made on the basis of the relative intensities of the signals of both epimers and the benzylic coupling between H-6 and H-7, observed in the 2D COSY experiment. Two signals for the methoxy groups at δ 3.90 and 3.87 were observed, both of them integrating 4.3 protons (since epimers **1** and **2** were in a ratio of 3:1). The protons H-4a and H-4ax showed a large coupling (J = 13.6 and 13.8 Hz, compound **1** and **2**, respectively), due to their trans diaxial configuration. The NOESY contour between H-6 α and H-12endo allowed us to assign the β -orientation of the hydroxyl group in compound **1** (Figure 3). The coupling between H-12exo and H-4 α allowed us to establish the conformational orientation of these protons in compound **2** (Figure 3). All the signals were confirmed by 2D-NMR.

Table 2. NMR data for compounds **1** and **2** (500 MHz for 1H and 125 Hz for ^{13}C , $CDCl_3$).

No.	1		2	
	δ_C , type	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)
1	130.7, <i>d</i>	6.59, <i>d</i> (10.0)	129.2, <i>d</i>	6.51, <i>d</i> (10.1)
2	127.9, <i>d</i>	5.98, <i>dd</i> (5.3 and 10.4)	128.4, <i>d</i>	6.02, <i>dd</i> (5.1 and 10.0)
3	63.2, <i>d</i>	4.32, <i>m</i>	62.1, <i>d</i>	4.37, <i>m</i>
4 α	31.0, <i>t</i>	1.72, <i>dt</i> (4.0 and 13.7)	30.5, <i>t</i>	1.85, <i>dt</i> (4.0 and 13.4)
4 β	31.0, <i>t</i>	2.14, <i>dd</i> (4.1 and 13.3)	30.5, <i>t</i>	2.29, <i>dd</i> (4.2 and 13.8)
4a	56.4, <i>d</i>	4.15, <i>dd</i> (4.1 and 13.6)	61.7, <i>d</i>	3.89, <i>m</i>
6a	125.2, <i>s</i>	-	123.4, <i>s</i>	-
6	88.8, <i>d</i>	5.35, <i>s</i>	86.6, <i>d</i>	6.12, <i>s</i>
7	111.8, <i>d</i>	6.89, <i>s</i>	110.7, <i>d</i>	7.03, <i>s</i>
8	148.0, <i>s</i>	-	148.4, <i>s</i>	-
9	148.9, <i>s</i>	-	149.2, <i>s</i>	-
10	105.3, <i>d</i>	6.83, <i>s</i>	105.1, <i>d</i>	6.78, <i>s</i>
10a	136.0, <i>s</i>	-	133.7, <i>s</i>	-
10b	44.2, <i>s</i>	-	44.8, <i>s</i>	-
11exo	39.9, <i>t</i>	2.03, <i>m</i>	39.8, <i>t</i>	2.03
11endo	39.9, <i>t</i>	2.03, <i>m</i>	39.8, <i>t</i>	2.03
12exo	47.4, <i>t</i>	3.37, <i>ddd</i> (3.8; 10.0 and 13.6)	41.2, <i>t</i>	3.19, <i>ddd</i> (3.5; 10.4 and 13.6)
12endo	47.4, <i>t</i>	2.87, <i>ddd</i> (6.5; 9.0 and 13.1)	41.2, <i>t</i>	3.94, <i>m</i>
OMe	56.1, <i>q</i>	3.90, <i>s</i>	56.1, <i>q</i>	3.90, <i>s</i>
OMe	55.9, <i>q</i>	3.87, <i>s</i>	55.9, <i>q</i>	3.87, <i>s</i>

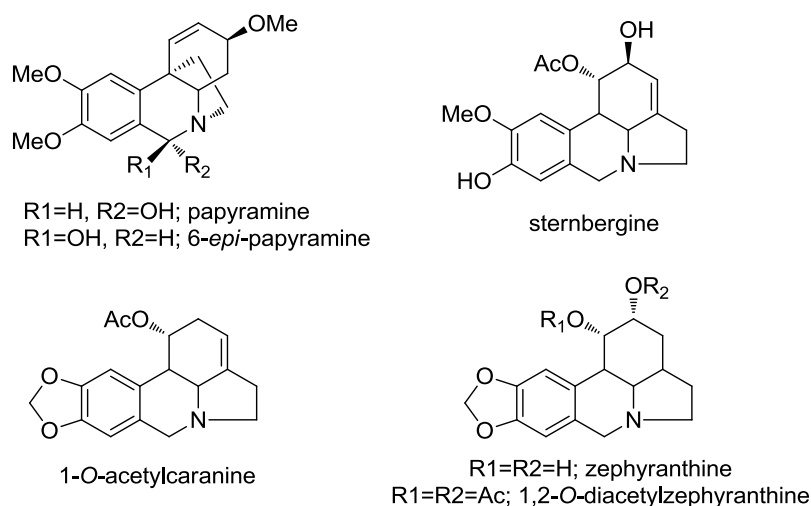


Figure 2. Alkaloids allowing the structural elucidation of compounds 1–4.

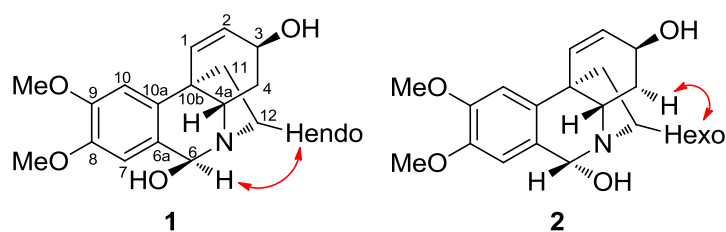


Figure 3. Key NOESY correlations of compounds 1 and 2.

2.2.2. Reticulinine (3) and Isoreticulinine (4)

Compounds **3** and **4** were obtained from an acetate bulb fraction and isolated as a mixture due to limited sample availability. The HRMS data of compounds **3** and **4** suggested the molecular formula $C_{17}H_{22}NO_4$ for the parent ion $[M + H]^+$ at m/z 334.1651 (calcd 334.1649). The 1H -NMR data summarized in Table 3 were similar to those reported for zephyranthine, 1,2-*O*-diacetylzephyranthine [28] and sternbergine [29] and 1-*O*-acetylcaranine [30] (Figure 2). A correlation between the protons of the methoxy group and H-10 in the 2D NOESY spectrum allowed us to assign this group at C-9 in both compounds (Figure 4). The two double doublets at δ 3.11 ($J = 5.9$ and 10.4 Hz) and 3.22 ($J = 5.9$ and 10.7 Hz) were attributed to the H-4a position in compounds **3** and **4**, respectively. The large coupling constant supported a *trans*-fusion of the B/C rings in both structures. The location and axial configuration of the radical group at C-1 in compounds **3** and **4**, respectively, were deduced from COSY and NOESY experiments (Figure 4). In compound **3**, the low magnetic field location of the H-1 and H-2 protons (δ 6.01 and 4.15 ppm, respectively) in the 1H spectrum allowed us to determine the presence of an acetyl group at C-1 and a hydroxyl group at C-2, respectively. Conversely, in compound **4**, the low magnetic field of the H-1 and H-2 protons (δ 4.77 and 5.12 ppm, respectively) indicated a hydroxyl group at C-1 and an acetyl group at C-2, respectively. In these compounds, the equatorial H-1 resonated as a triplet at δ 6.01 ($J = 3.9$ Hz) and 4.77 ($J = 1.5$ Hz), respectively, and was coupled to H-10b and H-2 in the COSY spectrum, and to H-10 in the NOESY experiment (Figure 4). These data and the large constant coupling between H-2 and H-3 α in compounds **3** and **4** ($J = 9.8$ and 11.6 Hz, respectively) allowed us to assign the equatorial configuration of the substituent at C-2. From the HMBC spectrum, three bond correlations were observed for H-7 to C-9, H-10 to C-8, H-7 to C-10a and H-10 to C-6a, enabling us to identify the resonances of the quaternary carbons C-8, C-9, C-6a and C-10a. In addition, a three-bond coupling between the methoxy protons and C-9 confirmed the NOESY result (Figure 4).

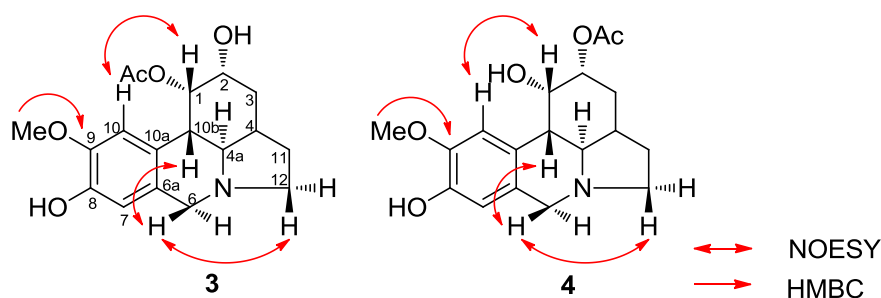


Figure 4. Key NOESY correlations of compounds 3 and 4.

Table 3. NMR data for compounds 3 and 4 (400 MHz for ^1H and 100 Hz for ^{13}C , CDCl_3).

No.	3		4	
	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)
1	70.6 <i>d</i>	6.01, <i>t</i> (3.9)	66.9 <i>d</i>	4.77, <i>t</i> (1.5)
2	68.4 <i>d</i>	4.15, <i>ddd</i> (2.8; 7.1 and 9.8)	72.1 <i>d</i>	5.12, <i>ddd</i> (2.5; 5.4 and 11.6)
3 α	25.8 <i>t</i>	2.02, <i>m</i>	26.8 <i>t</i>	2.23, <i>ddd</i> (6.2; 11.8 and 13.4)
3 β	25.8 <i>t</i>	2.02, <i>m</i>	26.8 <i>t</i>	1.95, <i>m</i>
4	36.7 <i>d</i>	2.68, <i>m</i>	36.4 <i>d</i>	2.68, <i>m</i>
4a	58.0 <i>d</i>	3.11, <i>dd</i> (5.9 and 10.4)	57.4 <i>d</i>	3.22, <i>dd</i> (5.9 and 10.7)
6 α	51.4 <i>t</i>	4.28, <i>d</i> (16.7)	51.6 <i>t</i>	4.29, <i>d</i> (16.4)
6 β	51.4 <i>t</i>	3.73, <i>d</i> (16.7)	51.6 <i>t</i>	3.74, <i>d</i> (16.9)
6a	127.7 <i>s</i>	-	127.7 <i>s</i>	-
7	112.7 <i>d</i>	6.67, <i>s</i>	112.9 <i>d</i>	6.68, <i>s</i>
8	144.0 <i>s</i>	-	144.0 <i>s</i>	-
9	145.4 <i>s</i>	-	145.4 <i>s</i>	-
10	106.4 <i>d</i>	6.73, <i>s</i>	106.4 <i>d</i>	6.82, <i>s</i>
10a	126.7 <i>s</i>	-	126.7 <i>s</i>	-
10b	33.1 <i>d</i>	2.68, <i>m</i>	34.3 <i>d</i>	2.59, <i>d</i> (10.5)
11 α	29.7 <i>t</i>	1.85, <i>m</i>	29.9 <i>t</i>	2.04, <i>m</i>
11 β	29.7 <i>t</i>	1.77, <i>m</i>	29.9 <i>t</i>	1.85, <i>m</i>
12 α	52.7 <i>t</i>	3.39, <i>m</i>	52.9 <i>t</i>	3.39, <i>m</i>
12 β	52.7 <i>t</i>	2.81, <i>m</i>	52.9 <i>t</i>	2.81, <i>m</i>
OMe	56.0 <i>q</i>	3.82, <i>s</i>	56.1 <i>q</i>	3.89, <i>s</i>
MeCOO	172.2 <i>q</i>	2.06, <i>s</i>	170.4 <i>q</i>	2.16, <i>s</i>
MeCOO	21.1 <i>q</i>	2.06, <i>s</i>	21.3 <i>q</i>	2.16, <i>s</i>

2.3. Biological Activity

2.3.1. Antiprotozoal Activity

6 β -Hydroxymaritidine (1) and 6 α -hydroxymaritidine (2) were isolated as an epimer mixture, which showed low activity against all protozoan parasites tested (Table 4). However, this sample also presented low cytotoxicity ($>100 \mu\text{g mL}^{-1}$), so it could be interesting to isolate the epimers and analyze their individual antiprotozoal activity. The quantity of reticuline (3) and isoreticuline (4) was insufficient for an antiprotozoal study.

2.3.2. Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities

6 β -Hydroxymaritidine (1) and 6 α -hydroxymaritidine (2), isolated as an epimer mixture, were tested for *in vitro* AChE- and BuChE-inhibitory activities. With galanthamine as a positive control, the epimers exhibited weak AChE inhibition ($\text{IC}_{50} 90.43 \pm 4.26 \mu\text{M}$) and no BuChE inhibition ($\text{IC}_{50} > 600 \mu\text{M}$), while galanthamine presented AChE and BuChE inhibition ($\text{IC}_{50} 1.56 \pm 0.14$ and $12.96 \pm 0.65 \mu\text{M}$, respectively). As described below in the molecular docking results, it would be of

interest to check the cholinesterase inhibitory activities of reticulinine (3) and isoreticulinine (4), but their low amounts precluded these assays.

Table 4. In vitro antiprotozoal and cytotoxic activities of 1 and 2. Values expressed in $\mu\text{g mL}^{-1}$.

Parasite	<i>T. b. rhodesiense</i>	<i>T. cruzi</i>	<i>L. donovani</i>	<i>P. falciparum</i>	Cytotoxicity
Stage	Trypomastigotes	Amastigotes	Amastigotes	IEF (intraerythrocytic)	
Strain	STIB 900 (IC ₅₀)	Tulahuen C4 (IC ₅₀)	MHOM-ET-67/L82 (IC ₅₀)	NF54 (IC ₅₀)	L6 (IC ₅₀)
melarsoprol	0.0010				
benznidazole		1.080			
miltefosine			0.091		
chloroquine				0.002	
podophyllotoxin					0.007
compounds 1 and 2	30.68	66.11	>100	32.86	>100

2.4. Molecular Docking

All the new structures obtained from *H. reticulatum* had their theoretical acetyl- and butyrylcholinesterase inhibitory potential evaluated by molecular docking. The results of their binding interactions and orientation patterns with the active site gorge of AChE and BuChE are represented in Table 5.

Table 5. Estimated energies of reaction and inhibition constants for the new compounds in the active sites of AChE and BuChE.

Alkaloids	1DX6 ^a (kcal mol ^{−1})	4EY7 ^b (kcal mol ^{−1})	4BDS ^c (kcal mol ^{−1})
6β-hydroxymaritidine (1)	−8.49	−9.44	−8.95
6α-hydroxymaritidine (2)	−8.25	−8.75	−8.72
reticulinine (3)	−8.88	−8.87	−8.10
isoreticulinine (4)	−9.02	−9.80	−8.12
galanthamine (11)	−9.36	−10.10	−8.74

^a 1DX6: *Torpedo californica* Acetylcholinesterase; ^b 4EY7: Human Acetylcholinesterase; ^c 4BDS: Human Butyrylcholinesterase.

The simulated molecular docking on the 1DX6 and 4EY7 structures showed that the alkaloid isoreticulinine (4) theoretically has a higher inhibitory effect against AChE than reticulinine (3), 6β-hydroxymaritidine (1) and 6α-hydroxymaritidine (2), but lower than galanthamine by 0.34 and 0.30 kcal mol^{−1}, respectively. The docking results obtained for isoreticulinine suggest that the presence of a hydroxyl and an acetyl group at the C-1 and C-2 positions, respectively, theoretically improves the AChE inhibition on the 1DX6 and 4EY7 structures in comparison with reticulinine. Structural representations of the best conformation of the complexed active site of the *TcAChE* with galanthamine and isoreticulinine (4) are depicted in Figure 5.

The molecular docking results reported in Table 5 are analyzed here in terms of different interactions between the alkaloids and active site of the *TcAChE* protein: hydrogen bonds, π - π stacking and anionic interactions. Regarding hydrogen bonding, galanthamine showed the highest stability due to two strong interactions with the Glu199 and Ser200 residues, while the isoreticulinine complex presented only one interaction with the Ser200 residue. Both these alkaloids have a similar localization according to π - π stacking, interactions with Trp84, Phe330 and Asp72 residues, and anionic stabilization. The hydrogen bond interaction of reticulinine (3) is as depicted in Figure 5 for isoreticulinine (4), with a distance close to 1.9 Å. The hydrogen bond interactions for 6β-hydroxymaritidine (1) and 6α-hydroxymaritidine (2) are similar, although anionic and π - π stacking interactions suggest a different, more distant localization, in accordance with the binding free energies reported in Table 5.

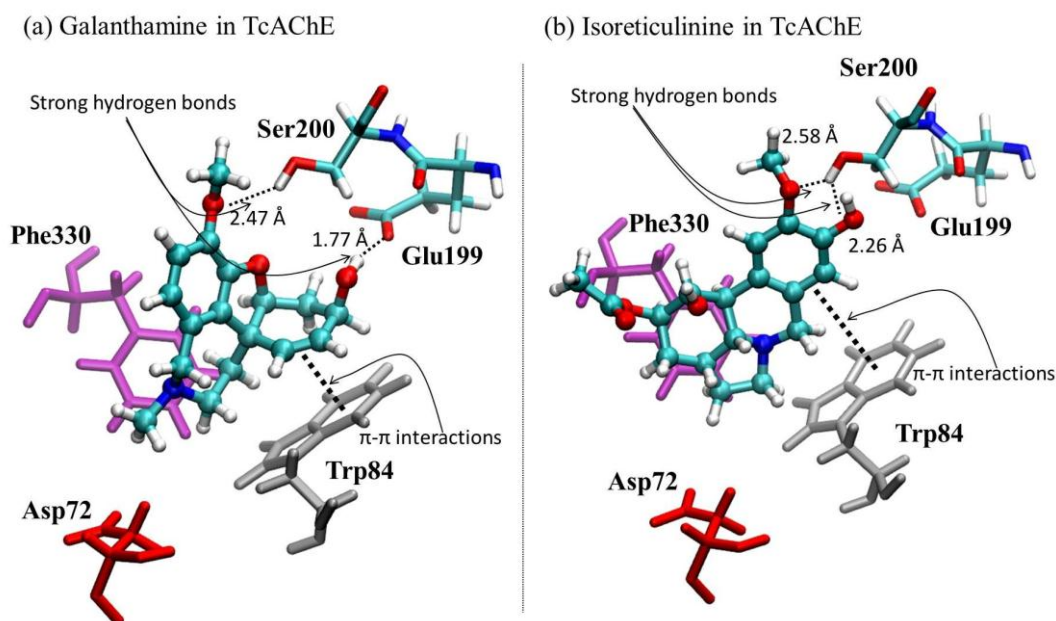


Figure 5. Graphical representations of (a) galanthamine-*TcAChE* and (b) isoreticulnine-*TcAChE* complexes.

The molecular simulation of 6 β -hydroxymaritidine (**1**) on the 4BDS structure theoretically showed more potent enzymatic inhibition against BuChE than 6 α -hydroxymaritidine (**2**), isoreticulnine (**4**) and reticulnine (**3**), and interestingly, it was higher than galanthamine by 0.21 kcal mol^{−1}. These results also indicate that 6 α -hydroxymaritidine (**2**) theoretically has virtually the same inhibitory activity as galanthamine toward BuChE, differing by only 0.02 kcal mol^{−1}. Furthermore, the docking results propose that the β -orientation of the hydroxyl group at the C-6 position in 6 β -hydroxymaritidine (**1**) could improve the butyrylcholinesterase inhibition on the 4BDS structure by 0.23 kcal mol^{−1} compared to the α -orientation of the hydroxyl group at the C-6 position in 6 α -hydroxymaritidine (**2**). Structural representations of the best conformation of the complexed active site of human BuChE with 6 β -hydroxymaritidine (**1**) are presented in Figure 6.

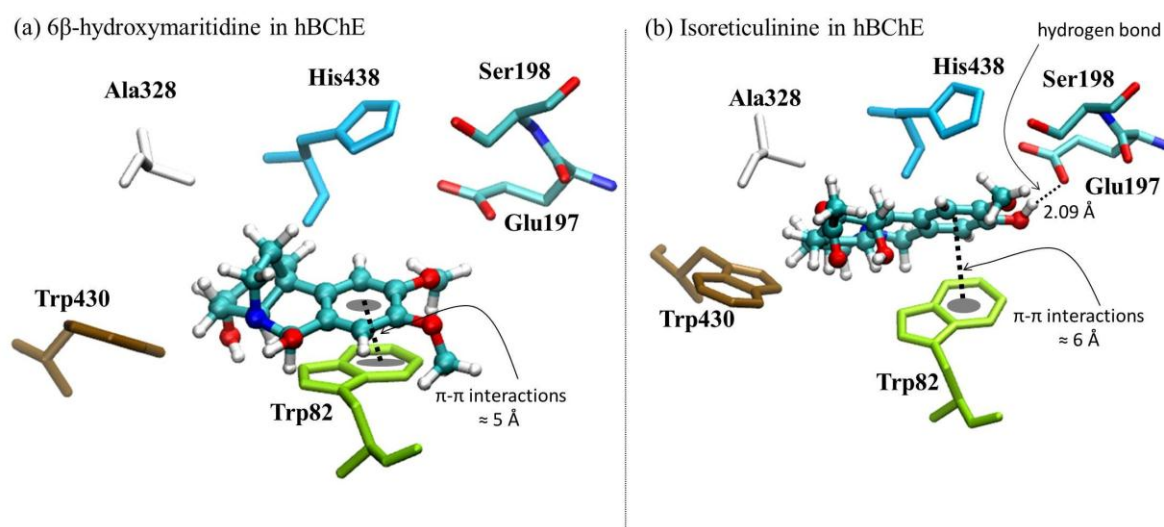


Figure 6. Graphical representations of (a) 6 β -hydroxymaritidine-*hBChE* and (b) isoreticulnine-*hBChE* complexes.

In the case of BuChE, 4BDS in the PDB code, the crystallographic data show that tacrine, a molecule for anti-Alzheimer's drugs targeting acetyl- and butyryl-cholinesterase, is stabilized by π - π stacking interactions between the aromatic rings and the Trp82 residue; additionally, this ligand is stabilized by the presence of hydrophobic interactions with Trp430, Ala328 and His438. On the other hand, similar to TcAChE, the active site shows the presence of two available residues available for hydrogen bond interactions, Glu197 and Ser198. In terms of π - π stacking interactions, Figure 6 shows that 6 β -hydroxymaritidine (1) is more stable than isorecticulnine (4). However, the localization of 6 β -hydroxymaritidine in the active site is not appropriate for forming hydrogen bonds with the Glu197 and Ser198 residues. The results suggest that in an ideal BuChE inhibitor π - π stacking and hydrogen bond interactions should be in equilibrium, and among the tested molecules isorecticulnine was closest to this definition. In terms of hydrophobic interactions, all molecular docking analyses reported similar interactions between the alkaloids and Trp430, Ala328 and His438 residues.

The theoretical results obtained by molecular docking for 6 β -hydroxymaritidine (1) and 6 α -hydroxymaritidine (2) BuChE inhibition are not in agreement with the experimental assays. The difference between the BuChE structure used for the molecular docking (human) and experiments (equine serum), as well as the inability of these compounds to arrive to the active site gorge of BuChE may help understand the difference between the theoretical and practical results. Moreover, the use of an epimer mixture in the assays could also have contributed to the discrepancy between the theoretical and practical results.

According to the molecular interactions shown in the docking analysis, the isorecticulnine alkaloid (4) may be catalogued as a potential inhibitory molecule based on its very good interaction with the active site through strong hydrogen bonds in both of the proteins evaluated, AChE and BuChE. Additionally, the position of isorecticulnine alkaloid (4) on the gorge of the active sites showed that it can be stabilized by π - π stacking and hydrophobic interactions.

AChE is highly selective for ACh hydrolysis, while BuChE is able to metabolize different substrates [31]. AChE activity in the brain of Alzheimer's disease patients tends to decrease, while that of BuChE increases [32]. Consequently, the search for a structure able to simultaneously inhibit both enzymes may provide a better therapeutic response for Alzheimer's disease than AChE-selective agents [33]. This information adds to the importance of the results obtained herein for isorecticulnine (4) by molecular docking.

3. Materials and Methods

3.1. General Experimental Procedures

About 2 mg of each alkaloid extract were dissolved in 1000 μ L of MeOH and/or CHCl₃ and injected directly into the GC-MS apparatus (Agilent Technologies 6890N coupled with MSD5975 inert XL, Santa Clara, CA, USA) operating in the EI mode at 70 eV. A Sapiens-X5 MS column (30 m $\times$ 0.25 mm i.d., film thickness 0.25 μ m, Teknokroma, Barcelona, Spain) was used. The temperature gradient performed was the following: 2 min at 100 $^{\circ}$ C, 100–180 $^{\circ}$ C at 15 $^{\circ}$ C min^{−1}, 180–300 $^{\circ}$ C at 5 $^{\circ}$ C min^{−1} and 10 min hold at 300 $^{\circ}$ C. The injector and detector temperatures were 250 $^{\circ}$ C and 280 $^{\circ}$ C, respectively, and the flow-rate of carrier gas (He) was 1 mL min^{−1}. A split ratio of 1:10 was applied and the injection volume was 1 μ L.

¹H, ¹³C-NMR, COSY, NOESY, HSQC, and HMBC spectra were recorded on a Varian VNMRs 500 MHz (Palo Alto, CA, USA) and a Bruker 400 MHz Avance III (Billerica, MA, USA) equipped with CryoProbe Prodigy, using CDCl₃ as the solvent and TMS as the internal standard. Chemical shifts are reported in units of δ (ppm) and coupling constants (*J*) expressed in Hz. CD, UV and IR spectra were recorded on Jasco-J-810 (Easton, MD, USA), Dinko UV2310 (Barcelona, Spain) and Thermo Scientific Nicolet iN10 MX spectrophotometers (Waltham, MA, USA), respectively. HR-ESI-MS spectra were obtained on an LC/MSD-TOF (2006) mass spectrometer (Agilent technologies, Santa Clara, CA, USA). Silica gel SDS chromagel 60 A CC (6–35 μ m) (Carlo Erba Reagents, Val de Reuil, France) was used for VLC, and silica gel 60 F254 (Merck, Darmstadt, Germany) for analytics and preparative TLC. Spots

on chromatograms were detected under UV light (254 nm) (Spectroline, Westbury, NY, USA) and by Dragendorff's reagent stain.

3.2. Plant Material

Bulbs and leaves of *Hippeastrum reticulatum* Herb. were collected in Floresta Nacional de Goytacazes (Espírito Santo, Brazil) in May 2015. The samples were authenticated by Dr Julie H. A. Dutilh, Universidade de Campinas (UNICAMP, Campinas, Brazil). A specimen voucher (VIES 38724) has been deposited in the Herbarium VIES from Universidade Federal do Espírito Santo (UFES, Vitória, Brazil).

3.3. Extraction and Isolation

Fresh bulbs (1.5 kg) and leaves (400 g) of *H. reticulatum* were collected and macerated with MeOH (2×1.0 L) at room temperature for 4 days, the combined macerate filtered and the solvent evaporated to dryness under reduced pressure. The bulb and leaf crude extracts (117.3 and 53.5 g, respectively) were then acidified to pH 3 with diluted H_2SO_4 (2%, v/v). The neutral material was removed with Et_2O (10×150 mL) and then extracted with EtOAc (3×150 mL) to provide the acid EtOAc extracts (0.60 and 0.44 g, respectively). The aqueous solutions were basified up to pH 9–10 with NH_4OH (25%, v/v) and extracted with *n*-Hex (16×150 mL) to give the *n*-Hex extracts (0.14 and 0.02 g, respectively), which were followed by extraction with EtOAc (15×150 mL) to provide the EtOAc extracts (1.6 and 0.3 g, respectively) and finally extracted with EtOAc:MeOH (3:1, v/v) (4×150 mL) to provide the EtOAc:MeOH extracts (2.10 and 1.56 g, respectively).

The extracts were subjected to a combination of chromatographic techniques, including vacuum liquid chromatography (VLC) [34] and semi-preparative TLC. The general VLC procedure consisted of the use of a silica gel 60 A (6–35 μm) column with a height of 4 cm and a variable diameter according to the amount of sample (2.5 cm for 400–1000 mg; 1.5 cm for 150–400 mg). Alkaloids were eluted with *n*-Hex containing increasing EtOAc concentrations, followed by neat EtOAc, which was gradually enriched with MeOH (reaching a maximum concentration of 20%, v/v). Fractions of 10–15 mL were collected, monitored by TLC (UV 254 nm, Dragendorff's reagent [35,36]), and combined according to their profiles. For semi-preparative TLC, silica gel 60F₂₅₄ was used (20 cm $\times$ 20 cm $\times$ 0.25 mm) together with different solvent mixtures depending on each particular sample (EtOAc:MeOH, 9:1, v/v or EtOAc:MeOH, 8:2, v/v), always in an environment saturated with ammonia. Each known alkaloid was identified by GC-MS and the four new alkaloids were structurally elucidated by NMR.

The EtOAc leaf extract (0.3 g) was subjected to a VLC column (2.5 cm $\times$ 4.0 cm), starting the elution with 100% *n*-Hex, gradually increasing the polarity with EtOAc up to 100% EtOAc, and finally increasing the MeOH percentage in the mixture up to a ratio of EtOAc:MeOH (80:20, v/v), similar to the methodology used in *Hippeastrum aulicum* by Bessa and co-workers [37]. 580 fractions (10 mL each) were collected, analyzed by TLC and grouped in nine fractions. Fraction 5 (72.8 mg), eluted with EtOAc:MeOH (eluted with 95:05 until 90:10, v/v), was subjected to a semi-preparative TLC using 2 plates and a mobile phase consisting of EtOAc:MeOH (80:20, v/v) in an environment saturated with ammonia, and 15.60 mg of the epimer mixture **1** and **2** was isolated.

Exclusion chromatography was carried out using a Sephadex LH-20 column (2.5 cm $\times$ 40 cm) to clean and separate the alkaloids present in the EtOAc bulb extract (1.6 g). It was eluted with 100% MeOH, producing 147 fractions, each one containing about 2 mL, which were monitored by TLC and grouped in four fractions. Fraction 2 (801 mg) was chromatographed again in a Sephadex LH-20 in the same conditions, producing 120 sub-fractions, which were grouped in seven sub-fractions. Sub-fraction 2 (10.2 mg) was subjected to a TLC plate, applying 3 runs over a mobile phase consisting of EtOAc:MeOH (80:20, v/v) in an environment saturated with ammonia to obtain 1.09 mg of the isomers **3** and **4**.

3.4. Characterization of Compounds

6 β -Hydroxymaritidine (1) and 6 α -hydroxymaritidine (2): Amorphous solid; $[\alpha]_D^{22}$ -11.22 (c 0.98, CHCl₃); UV (MeOH) $\lambda_{\max}$ (log ϵ): 282.5 (3.64), 234.0 (3.92) nm; CD (MeOH, 20 °C) $\Delta\epsilon_{250}$ -340 , $\Delta\epsilon_{284}$ $+5938$; IR $\nu_{\max}$ cm⁻¹ 3341, 2958, 1609, 1512, 1464, 1406, 1268, 1246, 1222, 1192, 1141, 1113, 1043, 1017, 941, 866, 779, 746; ¹H-NMR (CDCl₃, 500 MHz) and ¹³C-NMR (CDCl₃, 125 MHz) see Table 2; EIMS data shown in Table 1; HREIMS of [M + H]⁺ at m/z 304.1544 (calcd 304.1543).

Reticulinine (3) and isoreticulinine (4): Amorphous solid; ¹H-NMR (CDCl₃, 400 MHz) and ¹³C-NMR (CDCl₃, 100 MHz) see Table 3; IR $\nu_{\max}$ cm⁻¹ 2852, 1732, 1571, 1513, 1452, 1375, 1240, 1114, 1047; EIMS data shown in Table 1; [M + H]⁺ at m/z 334.1651 (calcd 334.1649).

3.5. Biological Activity

3.5.1. Antiprotozoal Activity

In vitro tests for the biological activity of the epimers 6 β -hydroxymaritidine (1) and 6 α -hydroxymaritidine (2) against *Trypanosoma brucei rhodesiense* (trypomastigotes forms, STIB 900 strain), *Trypanosoma cruzi* (amastigotes forms, Tulahuen C4 strain), *Leishmania donovani* (amastigotes forms, MHOM-ET-67/L82 strain), and *Plasmodium falciparum* (intraerythrocytic forms, IEF, NF54 strain) and a cytotoxicity test against the mammalian L6 cell line from rat skeletal myoblasts were carried out at the Swiss Tropical and Public Health Institute (Swiss TPH, Basel, Switzerland) according to established protocols as described by Orhan and co-workers [38]. The reference drugs used in these assays were melarsoprol, benznidazole, miltefosine, chloroquine and podophyllotoxin, respectively.

3.5.2. Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities

Cholinesterase inhibitory activities were analyzed as by Ellman and co-workers [39] with some modifications as by López and co-workers [40]. Fifty microliters of AChE or BuChE in phosphate buffer (8 mM K₂HPO₄, 2.3 mM NaH₂PO₄, 0.15 NaCl, pH 7.5) and 50 μ L of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 minutes at room temperature before 100 μ L of the substrate solution (0.1 M Na₂HPO₄, 0.5 M DTNB, and 0.6 mM ATCI or 0.24 mM BTCi in Millipore water, pH 7.5) was added. The absorbance was read in a Labsystem microplate reader (Thermo Electron Corporation, Vantaa, Finland) at 405 nm after 10 minutes. Enzyme activity was calculated as a percentage compared to an assay using a buffer without any inhibitor. The cholinesterase inhibitory data were analyzed with the software Microsoft Office Excel 2010. The epimer concentrations used to calculate IC₅₀ values were 10, 20, 40, 60, 80, 100 and 200 μ g mL⁻¹ in both AChE and BuChE assays.

3.6. Molecular Docking

The molecular docking simulations for 6 β -hydroxymaritidine (1), 6 α -hydroxymaritidine (2), reticulinine (3) and isoreticulinine (4) were performed to investigate the binding mode in the active site of three different enzymes, *Torpedo californica* AChE (TcAChE), hBChE, and hAChE, proteins with PDB codes 1DX6 [41], 4BDS [42], and 4EY7 [43], respectively. The 3D structures of the alkaloids were drawn with the Chemcraft program [44] and then submitted to a geometrical optimization procedure using PBE0 [45] /6-311+g* [46] level of theory with the Gaussian 09 program [47]. All optimized alkaloids were confirmed as a minimum on the potential energy surface. The docking simulations for the set of optimized ligands were performed using the AutoDock v.4.2 program [48].

AutoDock combines a rapid energy evaluation through precalculated grids of affinity potentials with a variety of search algorithms to find suitable binding positions for a ligand on a given macromolecule. To compare the results from the docking simulations, the water molecules, cofactors, and ions were excluded from each X-ray crystallographic structure. Likewise, the polar hydrogen atoms of the enzymes were added and the non-polar hydrogen atoms were merged. Finally, the

enzyme was treated as a rigid body. The grid maps of interaction energy for various atom types with each macromolecule were calculated by the auxiliary program AutoGrid choosing a grid box with dimensions of $60 \times 60 \times 60$ Å around the active site, which was sufficiently large to include the most important residues of each enzyme. The docking searches for the best orientations of the ligands binding to the active site of each protein were performed using the Lamarckian Genetic Algorithm, (LGA) [49]. The LGA protocol applied a population size of 2000 individuals, while 2,500,000 energy evaluations were used for the 50 LGA runs. The best conformations were chosen from the lowest docked energy solutions in the cluster populated by the highest number of conformations. The best docking complex solutions (poses) were analyzed according to the potential intermolecular interactions (ligand/enzyme), such as hydrogen bonding and the cation- π , π - π stacking.

4. Conclusions

This is the first report about the alkaloid profile and biological activities of *H. reticulatum*. Twelve alkaloids were identified in this species, eight known and four new, 6 α -hydroxymaritidine, 6 β -hydroxymaritidine, reticulinine and isoreticulinine. The low cytotoxicity of the epimers 6 β -hydroxymaritidine (1) and 6 α -hydroxymaritidine (2) and the very good interactions of isoreticulinine (4) with the active sites of the enzymes AChE and BuChE by molecular docking suggests that *H. reticulatum* has high potential as a source of alkaloids with pharmacological activities. Finally, as the molecular docking results indicate that isoreticulinine alkaloid (4) is a promising molecule in the treatment of Alzheimer's disease, the synthesis of this compound and its analogues will be undertaken in future work in our laboratories.

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Author Contributions: Jaume Bastida designed the experiments, analyzed the data and wrote the paper. Luciana R. Tallini performed the experiments, analyzed the data and wrote the paper. Edison H. Osorio performed the molecular docking experiment and wrote the paper. Vanessa Dias dos Santos and Warley de Souza Borges collected the plant, performed the extraction experiments and wrote the paper. Marcel Kaiser performed the antiprotozoal experiments and wrote the paper. Francesc Viladomat and José Angelo S. Zuanazzi wrote the paper.

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3.3 Artículo 3

***N*-oxide alkaloids from *Crinum amabile* (Amaryllidaceae)**

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Article

N-oxide alkaloids from *Crinum amabile* (Amaryllidaceae)

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Abstract: Natural products play an important role in the development of new drugs. In this context, the Amaryllidaceae alkaloids have attracted considerable attention in view of their unique structural features and various biological activities. In this study, twenty-three alkaloids were identified from *Crinum amabile* by GC-MS and two new structures (augustine N-oxide and buphanisine N-oxide) were structurally elucidated by NMR. Anti-parasitic and cholinesterase (AChE and BuChE) inhibitory activities of six alkaloids isolated from this species, including the two new compounds, are described herein. None of the alkaloids isolated from *C. amabile* gave better results than the reference drugs, so it was possible to conclude that the N-oxide group does not increase their therapeutic potential.

Keywords: *Crinum amabile*; augustine N-oxide; buphanisine N-oxide; biological activities

1. Introduction

Natural products play an important role in the development of new drugs [1]. For example, between 1940 and 2014, 49% of the small molecules approved for the treatment of cancer were developed or directly derived from natural products [1]. The isoquinoline-type alkaloids found in the Amaryllidaceae plant family represent an interesting source of new drugs due to their diverse biological activities [2]. The most important Amaryllidaceae alkaloid is galanthamine, which was approved by the Food and Drug Administration (FDA) for the clinical treatment of mild to moderate Alzheimer's disease (AD) in 2001, due to its potential acetylcholinesterase inhibitory activity [3]. According to the most recent botanical classification, the Amaryllidaceae are now a subfamily known as the Amaryllidoideae, which together with the Agapanthoideae and Allioideae belong to the Amaryllidaceae family [4]. Amaryllidoideae includes 59 genera and about 850 species, with centers of diversity in South Africa, South America, particularly in the Andean region, and in the Mediterranean [5].

Within the Amaryllidoideae, the pantropical *Crinum* genus is of commercial, economical and medicinal importance [6,7]. This genus contains approximately 65 species, which are widely distributed in diverse habitats, including coastal areas, pans (seasonally flooded depressions), sandy and aquatic

areas, and swamps [8]. *Crinum* seeds are highly buoyant, with corky, water-repellent surfaces, allowing them to be dispersed by water [8,9]. Extracts from *Crinum* species have been used in folk medicine to treat fever, pain, swelling, sores, wounds, cancer and malaria [10]. The biological activities of *Crinum* species, including antitumor, immunostimulating, analgesic, antiviral, antibacterial, and antifungal, are attributed to their alkaloid content [7,9].

Known as a decorative plant, *Crinum amabile* has also long been used in Vietnamese folk medicine as an emetic and a remedy for rheumatism and earache [11]. Fifteen alkaloids have been previously identified in *C. amabile*: amabiline, ambelline, augustine, buphanisine, crinamabine, crinamine, crinidine, 4a-dehydrocrinamabine, flexinine, galanthamine, galanthine, hippeastrine, lycorine, narvedine and tazettine [11–13]. Among these, amabiline, augustine, buphanisine, crinamine and lycorine have been isolated from this species and assessed for their antimalarial and cytotoxic potential, with augustine being the most active [13].

Tropical diseases such as malaria, leishmaniasis, Chagas disease and African trypanosomiasis affect more than one billion people and cost developing economies billions of dollars every year [14]. As these diseases prevail in areas where poverty limits access to prevention and treatment interventions, the pharmaceutical industry has little interest in investing in tackling them by drug development [15]. On the other hand, dementia affects around 50 million citizens worldwide, 60–70% of whom suffer from Alzheimer's disease, for which the current clinical treatment offers only palliative effects [16,17]. Thus, all these diseases require more research on effective treatment, in which the Amaryllidaceae alkaloids may potentially play an important role.

The aim of this work was to perform a detailed study of the alkaloid constituents of *C. amabile*, utilizing spectroscopic and chromatographic methods, including GC-MS and NMR. Two new alkaloids were isolated and chemically characterized by spectroscopic methods and twenty-three known alkaloids were identified by GC-MS. Due to the potential of Amaryllidaceae alkaloids in the clinical treatment of Alzheimer's disease [3], as well as the activity of augustine against malaria [13], we decided to check the cholinesterase-acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)-inhibitory activities and the antiprotozoal capacity of six alkaloids isolated from *C. amabile*, including the two new alkaloids. The role of *N*-oxide compounds in these biological activities was explored.

2. Results and Discussion

2.1. Alkaloids Identified by GC-MS

Twenty-three known alkaloids from *Crinum amabile* were identified by GC-MS (Table 1 and Figure 1) by comparison of the *R*_t, fragmentation patterns and spectral data using our home database. This database was built from single alkaloids isolated and identified by spectroscopic and spectrometric methods (NMR, UV, CD, IR, MS) in the Natural Products Laboratory, University of Barcelona, Spain. Also used were the NIST 05 Database and literature data [18–22].

2.2. Structural Elucidation

Two new alkaloids were identified in *C. amabile*: augustine *N*-oxide (1) and buphanisine *N*-oxide (2), both *N*-oxides of the structures augustine (13) and buphanisine (8), respectively. *N*-oxides occur as natural products and are not artefacts formed during the isolation procedures [23,24]. Ungiminatorine *N*-oxide, homolycorine *N*-oxide, *O*-methyllycorenine *N*-oxide, galanthamine *N*-oxide, sanguinine *N*-oxide, lycoramine *N*-oxide and undulatine *N*-oxide are examples of Amaryllidaceae alkaloid *N*-oxides also reported as natural products [25–28].

Table 1. Alkaloids identified in *Crinum amabile* by GC-MS.

Alkaloid	RI	M ⁺	MS
Lycorene (3)	2102.2	255 (52)	254 (100), 227 (17), 226(20), 211 (15), 183(14), 181(10)
Ismine (4)	2124.3	257 (28)	239(16), 238 (100), 196 (10), 168 (10)
Demethylismine (5)	2128.8	243 (22)	225(21), 224 (100), 167 (10), 166 (15), 154 (11), 77 (12)
Demethylmesembrenol (6)	2177.0	275 (5)	206 (9), 205 (76), 115 (6), 70 (100)
Galanthamine (7)	2262.8	287 (85)	286 (100), 244(29), 216 (45), 174(39), 165(16), 141 (14), 128 (21), 115 (31)
Buphanisine (8)	2283.7	285 (95)	273 (54), 272 (43), 254 (40), 215 (100), 157 (39), 129 (35), 128 (55), 115 (64)
Sanguinine (9)	2288.3	273 (100)	272 (85), 202 (40), 165(20), 160 (50), 131 (20), 128 (19), 115 (28), 77(20),
Crinine (10)	2326.7	271 (100)	228 (24), 200 (30), 199(81), 187 (76), 173 (28), 129 (34), 128(44), 115 (47), 56 (32)
8-O-Demethylmaritidine (11)	2373.8	273 (100)	230 (25), 202 (29), 201 (80), 189 (65), 175 (29), 129 (24), 128 (30), 115 (32), 56 (30)
3-O-Acetylsanguinine (12)	2387.1	315 (37)	256 (100), 255 (42), 254 (37), 212(26), 165 (33), 152 (23), 115 (30), 96 (67)
Augustine (13)	2411.6	301 (93)	228 (36), 187 (30), 175 (300), 173 (24), 159 (38), 143 (57), 128 (259), 115 (75)
Buphanisine N-oxide (2)	2429.8	301 (nv)	285 (100), 270 (33), 254 (35), 216 (21), 215 (82), 201 (24), 157 (20), 128 (22)
Haemanthamine (14)	2436.9	301 (55)	257 (54), 227 (80), 225 (98), 224(80), 181 (100), 153 (46), 152 (46), 115 (64)
11,12-Dehydroanhydrolycorine (15)	2448.5	249 (55)	248 (100), 191 (13), 190 (31), 189 (11), 95 (14)
Crinamine (16)	2497.6	273 (17)	272 (100), 242 (12), 214 (11), 186 (12), 128 (15)
Hamayne (17)	2551.7	259 (14)	258 (100), 242 (11), 214 (10), 211 (12), 181 (14), 128 (19)
1-O-Acetyllycorine (18)	2563.1	329 (20)	299(15), 268 (28), 250 (17), 244 (20), 227 (56), 226 (100), 240 (11)
Augustine N-oxide (1)	2571.8	317 (nv)	301 (100), 228 (34), 187 (22), 175 (77), 173 (17), 159 (27), 143 (37), 115 (37)
Lycorine (19)	2592.2	287 (19)	286 (13), 268 (18), 250 (10), 227 (60), 226 (100), 147 (11)
Undulatine (20)	2594.4	331 (100)	258 (37), 219 (22), 217 (36), 205 (71), 203 (37), 189 (43), 173 (39), 115 (35)
Ambelline (21)	2621.1	331 (69)	287 (100), 260 (81), 257 (62), 255 (74), 254 (52), 241 (51), 239 (61), 211 (69)
Augustamine (22)	2628.7	301 (76)	300 (100), 245(16), 244(84), 215(33), 201 (32), 188 (14), 115 (22), 70 (21)
6-Hydroxybuphanidrine (23)	2631.3	331 (35)	277 (16), 276 (100), 261 (30), 218 (17), 217 (23), 216 (24), 115 (18), 56 (25)
N-Formylinorgalanthamine (24)	2649.1	301 (100)	225 (26), 211 (29), 181 (19), 165 (14), 129 (18), 128 (22), 115 (30), 77 (15)
2-O-Acetyllycorine (25)	2676.2	329 (21)	328 (17), 270 (40), 269 (72), 268 (100), 252 (43), 250 (73), 227 (27), 226 (67)

* not visible.

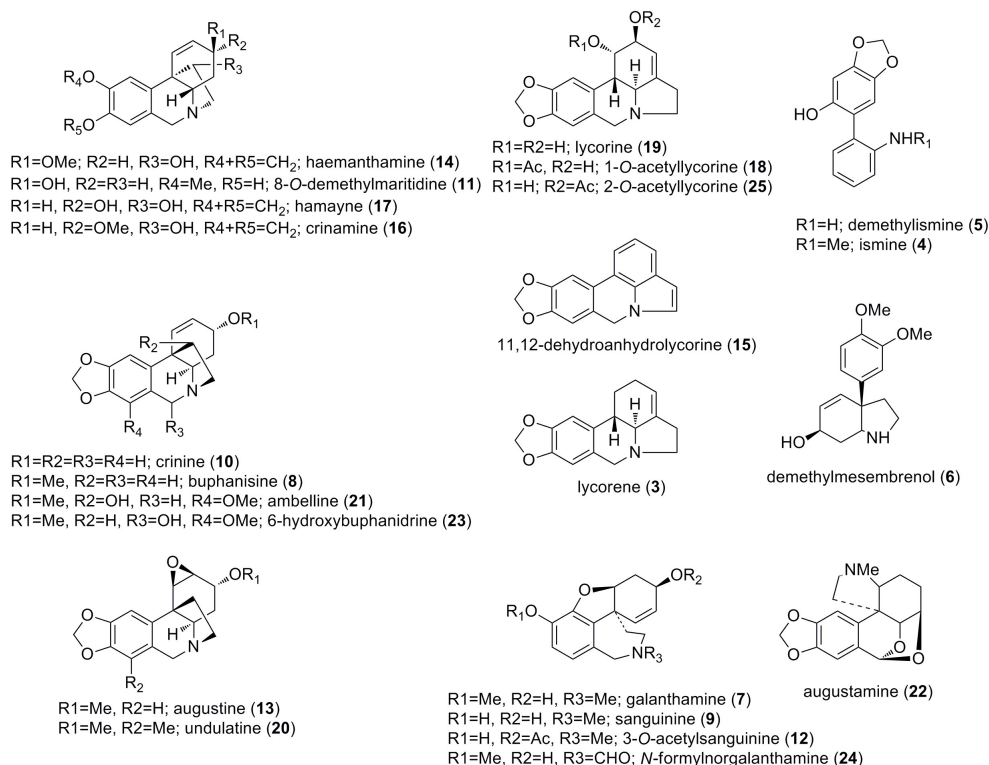


Figure 1. Alkaloids identified in *C. amabile* by GC-MS.

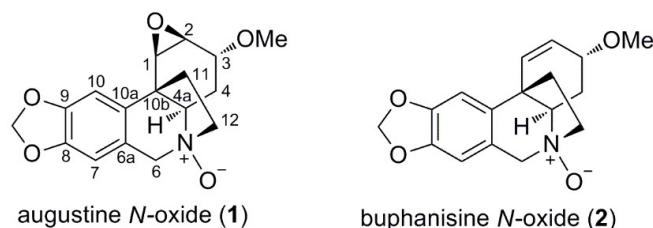
2.2.1. Augustine N-oxide (**1**)

The 1H -NMR signals of compound **1** (Table 2) were consistent with the structure of augustine [29]. However, H-4 α and H-4a and H-6 α , H-6 β , and H-12 $endo$ and H-12 exo were deshielded between 1.21 and 0.36 ppm. These deshielding effects are congruent with the salt or N-oxide form of augustine. HR-ESI-MS analysis was carried out to confirm an additional oxygen atom in the structure. Compound **1** exhibited a parent $[M + H]^+$ ion at m/z 318.1335 in its HR-ESI-MS spectrum, suggesting the molecular formula $C_{17}H_{20}NO_5$ (calcd. 318.1336) and confirming compound **1** as augustine N-oxide.

The absolute configuration of this structure was determined by CD. The curve and shape were qualitatively similar to those of known crinine-type alkaloids, with the 5,10b-ethano bridge in a β -orientation, having a maximum of around 245 nm and a minimum of 295 nm. The COSY spectrum showed a benzylic coupling between H-7 and H-6, which allowed us to determine the H-7 proton location in the 1H -NMR spectrum. The two C-6 protons were differentiated as an AB system with a geminal coupling of around 15.7 Hz. H-4a showed a NOESY correlation with H-6 α , which turned out to be crucial for the assignment of its orientation. We were able to determine the H-4 β orientation from the large coupling constant between H-4a and H-4 β (around 13.8 Hz), and the α -orientation of the methoxy group at C-3 from the small constant between H-4 β and H-3 (around 2.7 Hz). The β -orientation of the epoxy group was assignable based on the low values of the H-1 and H-2 constants (3.5 and 3.2 Hz, respectively). The NOESY contour plot between H-4 β and H-11 exo and H-12 exo allowed us to determine the H-11 exo and H-12 exo locations in the 1H -NMR spectrum. The quaternary carbons C-6a, C-10a, C-8 and C-9 were ascribed by means of their respective three-bond HMBC correlations with H-10, H-7, H-10 and H-7. Finally, the singlet resonance signal at $\delta = 43.95$ ppm in the ^{13}C spectrum was assigned to C-10b, taking into account the three-bond connectivities to H-10, H-4 α and H-4 β (Figure 2) in the HMBC experiment.

Table 2. NMR data for compounds **1** and **2** (400 MHz for ^1H and 100 Hz for ^{13}C , CDCl_3).

No.	1		2	
	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)
1	52.06	3.68, d (3.5)	129.59	6.39, d (10.0)
2	55.06	3.42, ddd (3.2, 2.4, 0.7)	127.18	6.08, ddd (10.0, 5.3, 1.0)
3	73.43	4.12, dd (2.7, 2.5)	71.38	3.95, ddd (5.6, 3.6, 2.0)
4 α	19.72	2.91, dt (14.1, 3.1)	23.46	3.13 ddt (13.6, 4.2, 2.4)
4 β	19.72	1.54, ddd (13.8, 13.8, 2.9)	23.46	1.72 ddd (13.7, 13.7, 4.0)
4a	72.59	3.51, dd (13.4, 3.6)	74.14	3.74, dd (13.2, 4.3)
6 α	67.56	4.83, dd (15.7, 1.8)	76.55	4.84, d (15.6)
6 β	67.56	4.68, d (15.7)	76.55	4.72, d (15.6)
6a	122.33	-	121.71	-
7	106.40	6.57, s	106.44	6.54, s
8	147.13	-	147.20	-
9	147.79	-	147.79	-
10	102.49	6.90, s	102.98	6.81, s
10a	133.92	-	134.75	-
10b	43.95	-	46.60	-
11endo	35.64	1.99, ddd (12.4, 9.4, 5.1)	40.02	2.11, ddd (12.5, 8.0, 6.0)
11exo	35.64	2.79, ddd (12.4, 12.4, 6.9)	40.02	2.26, ddd (12.2, 10.8, 8.6)
12endo	67.56	3.81, ddd (12.8, 9.4, 7.0)	68.97	3.88, m
12exo	67.56	3.73, dddd (12.5, 12.5, 5.0, 2.2)	68.97	3.85, m
OCH ₂ O	101.58	5.99, d (1.3), 5.98 d (1.3)	101.51	5.95, d (1.3), 5.93 d (1.3)
OMe	57.92	3.47, s	57.23	3.39, s

**Figure 2.** Structures of the two new alkaloids elucidated from *C. amabile*.

2.2.2. Buphanisine N-oxide (2)

The ^1H -NMR spectrum of compound **2** (Table 2) was similar to that of buphanisine [30]. However, the H-4 α , H-4a, H-6 α , H-6 β , H-12endo and H-12exo protons were assigned as 0.80, 0.24, 0.32, 0.82, 0.86 and 0.29 ppm more deshielded, respectively, than their homologs in buphanisine. HR-ESI-MS analysis allowed us to confirm the presence of N-oxide in this structure. Compound **2** exhibited a parent $[\text{M} + \text{H}]^+$ ion at m/z 302.1385 in its HR-ESI-MS spectrum, suggesting the molecular formula $\text{C}_{17}\text{H}_{20}\text{NO}_4$ (calcd. 302.1387) and confirming compound **2** as buphanisine N-oxide.

The absolute configuration of this structure was determined by CD. The spectrum curve had a maximum of around 245 nm and a minimum of around 292 nm, confirming a crinine-type alkaloid with the 5,10b-ethano bridge in a β -orientation. The assignment of the aromatic protons was based on the benzylic coupling between H-6 and H-7 observed by a 2D COSY experiment. The ^1H -NMR spectrum showed two doublets at δ 4.84 and 4.72 ppm with a coupling constant of around 15.6 Hz. The first one was assigned as H-6 α due to the NOESY contour plot of the proton H-4a. The large coupling constant between H-4a and H-4 β (around 13.7 Hz) allowed the H-4 β proton location to be determined in the ^1H -NMR spectrum. The NOESY contour plot between H-4 β and H-11exo and H-12exo enabled us to determine their location in the ^1H -NMR spectrum. In the HMBC spectrum, the three-bond correlations observed for H-7 to C-9, H-10 to C-8, H-7 to C-10a and H-10 to C-6a allowed us to identify the location of the quaternary carbons C-8, C-9, C-6a and C-10a in the ^{13}C spectrum.

The three-bond coupling between C-10b and H-2, H-10 and H-4 permitted its location to be assigned at $\delta = 46.60$ ppm in the ^{13}C spectrum (Figure 2).

2.3. Biological Activities

The alkaloid augustine presents significant activity against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* ($\text{IC}_{50} = 0.46$ and $0.60 \mu\text{M}$, respectively) [13]. We consequently decided to verify *in vitro* the activity of six alkaloids isolated from *C. amabile*, augustamine (22), augustine (13), augustine *N*-oxide (1), buphanisine (8), buphanisine *N*-oxide (2) and crinine (10), against four different protozoa, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*, which are related to sleeping sickness, Chagas disease, visceral leishmaniasis and malaria, respectively. These alkaloids are structurally very similar, with the exception of augustamine, which is a unique kind of Amaryllidaceae alkaloid previously isolated from other *Crinum* species, including *C. augustum*, *C. kirkii* and *C. latifolium* [28,31,32], and completely elucidated in 2000 [33]. The rareness of this structure motivated us to isolate it and check its biological activity. In addition, due to the potential effectiveness of Amaryllidaceae alkaloids in the clinical treatment of Alzheimer's disease, the alkaloids were also tested *in vitro* against acetyl- and butyrylcholinesterase.

2.3.1. AChE and BuChE Inhibitory Activities

All the results for cholinesterase inhibitory activities are shown in Table 3. No tested alkaloid presented BuChE inhibitory activity. AChE inhibitory activity was moderate in augustine (13), and low in buphanisine (8). The structures of these two alkaloids are very similar: between C-1 and C-2 augustine (13) presents an epoxy group and buphanisine (8) an olefin group (Figure 1). Interestingly, the augustine (13) epoxy group seems to increase the AChE inhibitory activity, more than the olefin in buphanisine (8). The AChE inhibitory activity is also slightly improved by the presence of a hydroxyl group at C-3, as occurs in the crinine (10) alkaloid, but not by the methoxy group in the same substituent, as occurs in buphanisine (8). Unfortunately, the *N*-oxide group did not increase the AChE inhibitory activities of augustine *N*-oxide (1) and buphanisine *N*-oxide (2). Furthermore, augustamine (22) did not show any cholinesterase inhibitory activities.

Table 3. Results of AChE and BuChE inhibitory activities of the alkaloids isolated from *C. amabile*.

Alkaloid	AChE *	BuChE *
Augustine <i>N</i> -oxide (1)	79.64 ± 5.26	>200
Buphanisine <i>N</i> -oxide (2)	>200	>200
Augustamine (22)	>200	>200
Augustine (13)	45.26 ± 2.11	>200
Buphanisine (8)	183.31 ± 36.64	>200
Crinine (10)	163.89 ± 15.69	>200
Galanthamine (7)	0.45 ± 0.03	3.88 ± 0.19

* all results are in $\mu\text{g mL}^{-1}$.

2.3.2. Antiprotozoal Activity

All the alkaloids isolated from *C. amabile* showed low activity against all the protozoa tested (Table 4). Buphanisine (8) showed significant inhibitory activity against the NF54 strain of *P. falciparum* (with a 50% inhibitory concentration (IC_{50}) of $4.28 \pm 0.18 \mu\text{g mL}^{-1}$). The presence of an *N*-oxide group in augustine *N*-oxide (1) and buphanisine *N*-oxide (2) appears to decrease their activity against *T. brucei* and *P. falciparum* compared to augustine (13) and buphanisine (8), respectively. In this experiment, the epoxy group at C-1 and C-2 probably decreases the activity of augustine (13) against *P. falciparum* compared to buphanisine (8), which has a double bond between C-1 and C-2. Furthermore,

the presence of a methoxy group at C-3 seems to increase the activity of buphanisine (**8**) against *P. falciparum* compared to crinine (**10**), which has a hydroxyl group in the same position.

Table 4. *In vitro* antiprotozoal and cytotoxic activities of **1** and **2**. Values expressed in IC₅₀ (μg mL^{−1}).

Parasite	<i>T. brucei</i> <i>rhodesiense</i>	<i>T. cruzi</i>	<i>L. donovani</i>	<i>P. falciparum</i>	Cytotoxicity
Reference drug	0.003 ± 0.001 ^a	0.865 ± 0.08 ^b	0.515 ± 0.06 ^c	0.004 ± 0.0007 ^d	0.004 ± 0.0007 ^e
Augustine (13)	15.05 ± 1.06	56.00 ± 0.71	>100	14.20 ± 0.14	>100
Augustine N-oxide (1)	58.85 ± 11.53	66.25 ± 11.81	>100	36.65 ± 4.74	>100
Buphanisine (8)	16.5 ± 0.57	55.55 ± 4.60	>100	4.28 ± 0.18	72.85 ± 5.02
Buphanisine N-oxide (2)	55.25 ± 4.31	64.05 ± 1.34	>100	32.55 ± 0.07	>100
Crinine (10)	18.95 ± 0.78	57.45 ± 6.86	>100	30.95 ± 2.19	>100
Augustamine (22)	19.20 ± 2.97	54.00 ± 4.53	>100	20.35 ± 0.21	81.55 ± 0.64

^a melarsoprol; ^b benznidazole; ^c miltefosine; ^d chloroquine; ^e podophyllotoxin.

3. Materials and Methods

3.1. Plant Material

Bulbs of *Crinum amabile* Donn. were collected in Vitoria (Espírito Santo, Brazil) in September 2016. The sample was authenticated by Dr. Alan Meerow at the Subtropical Horticulture Research Station (Miami, FL, USA). A specimen voucher (VIES 39506) has been deposited in the Herbarium of the Universidade Federal do Espírito Santo (UFES; Vitoria, Brazil).

3.2. Equipment

About 2 mg of each alkaloid extract was dissolved in 1000 μL of methanol (MeOH) and/or chloroform (CHCl₃) and injected directly into the GC-MS apparatus (Agilent Technologies, Santa Clara CA, USA) operating in the EI mode at 70 eV. A Sapiens-X5 MS column (30 m × 0.25 mm i.d., film thickness 0.25 μm) was used. The temperature gradient performed was the following: 2 min at 100 °C, 100–180 °C at 15 °C min^{−1}, 180–300 °C at 5 °C min^{−1} and 10 min hold at 300 °C. The injector and detector temperatures were 250 °C and 280 °C, respectively, and the flow-rate of carrier gas (He) was 1 mL min^{−1}. A split ratio of 1:10 was applied and the injection volume was 1 μL. The alkaloids were identified by GC-MS and the mass spectra were deconvoluted using the software AMDIS 2.64. Kovats retention indices (RI) were recorded with a standard calibration *n*-hydrocarbon mixture (C9–C36) using AMDIS 2.64 software.

¹H-NMR, ¹³C-NMR, COSY, NOESY, HSQC, and HMBC spectra were recorded on a Bruker 400 MHz Avance III equipped with CryoProbe Prodigy (Bruker, Billerica, MA, USA), using CDCl₃ as the solvent and tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in units of δ (ppm) and coupling constants (J) are expressed in Hz. CD, UV and IR spectra were recorded on Jasco-J-810 (Jasco, Easton, MD, USA), Dinko UV2310 (Dinko Instruments, Barcelona, USA) and Thermo Scientific Nicolet iN10 MX spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA), respectively. HR-ESI-MS spectra were obtained on an LC/MSD-TOF (2006) mass spectrometer (Agilent Technologies) operating in the positive mode, applying 4 kV in the capillary, 175 V in the fragmentor, a gas temperature of 325 °C, and N₂ as the nebulizing gas (15 psi) and drying gas (flow = 7.0 L min^{−1}). Silica gel SDS chromagel 60 A CC (6–35 μm) was used for VLC, and silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany) for analytics and prep. Spots on chromatograms were detected under UV light (254 nm) and by Dragendorff's reagent stain.

3.3. Extraction

Fresh bulbs (2.2 kg) and leaves (1.3 kg) of *C. amabile* were collected and macerated with MeOH (3 × 1.0 L) at room temperature for 4 days. The combined macerate was filtered and the solvent

evaporated to dryness under reduced pressure. The bulb and leaf crude extracts (485 and 390 g, respectively) were then acidified to pH 3 with diluted sulfuric acid, H_2SO_4 (2%, *v/v*). The neutral material was removed with Et_2O (3×200 mL) and extracted with ethyl acetate (EtOAc) (3×200 mL) to obtain the acid EtOAc extracts (4.58 and 2.8 g, respectively). The aqueous solutions were basified up to pH 9–10 with ammonium hydroxide, NH_4OH (25%, *v/v*) and extracted with *n*-hexane, *n*-Hex (3×150 mL) to give the *n*-Hex extracts (1.16 and 0.40 g, respectively, and finally extracted with EtOAc (2×200 mL) to obtain the EtOAc extracts (5.11 and 1.15 g, respectively).

The extracts were subjected to a combination of chromatographic techniques, including vacuum liquid chromatography (VLC) [34], Sephadex, thin layer chromatography (TLC) and semi-preparative TLC. The VLC is an effective methodology to rapidly and inexpensively separate large or small quantities of compounds from extracts [35]. A silica gel 60 A (6–35 μm) column was used with a height of 4 cm and a variable diameter according to the amount of sample (2.5 cm for 400–1000 mg; 1.5 cm for 150–400 mg). Alkaloids were eluted with *n*-Hex containing increasing EtOAc concentrations, followed by neat EtOAc, which was gradually enriched with MeOH (reaching a maximum concentration of 20%, *v/v*). Fractions of 10–15 mL were collected, monitored by TLC (UV 254 nm, Dragendorff's reagent), and combined according to their profiles. For semi-preparative TLC, silica gel 60F²⁵⁴ was used (20 cm $\times$ 20 cm $\times$ 0.25 mm) together with different solvent mixtures depending on each particular sample (EtOAc:MeOH, 9:1, *v/v*; EtOAc:MeOH, 8:2, *v/v*; or EtOAc: CHCl_3 :MeOH, 6:4:2, *v/v/v*), always in an environment saturated with ammonia. The alkaloids were each identified by GC-MS and the two new alkaloids had their structure elucidated by NMR.

Exclusion chromatography was carried out using a Sephadex LH-20 column (2.5 cm $\times$ 40 cm) to clean and separate the alkaloids in the *n*-Hex bulb extract (1.16 g). It was eluted with 100% MeOH, producing 52 fractions, each one containing about 2 mL, which were monitored by TLC and grouped in four fractions. Fraction 3 (1.00 g) was subjected to a VLC column (2.5 cm $\times$ 4.0 cm), starting the elution with 100% *n*-Hex, and gradually increasing the polarity by adding concentrations of EtOAc up to 100%. The MeOH percentage in the mixture was then increased up to a ratio of EtOAc:MeOH (80:20, *v/v*) and finally, keeping the MeOH percentage stable, the EtOAc percentage was decreased and the CHCl_3 percentage increased to a ratio of EtOAc:MeOH: CHCl_3 (60:20:40, *v/v/v*). 48 fractions (100 mL each) were collected, analyzed by TLC and grouped in twelve fractions.

Fraction B (28.5 mg) - eluted with *n*-Hex:EtOAc (eluted with 70:30 until 60:40, *v/v*), fraction D (26.1 mg)-eluted with *n*-Hex: EtOAc (40:60, *v/v*), fraction F (40.0 mg) - eluted with EtOAc:MeOH (eluted with 96:4 until 92:8, *v/v*), fraction H (10.5 mg) - eluted with EtOAc:MeOH (eluted with 88:12 until 83:17, *v/v*) and fraction J (25.0 mg) - eluted with EtOAc:MeOH: CHCl_3 (eluted with 80:20:0 until 71:20:9, *v/v/v*) were subject to different semi-preparative TLC using a mobile phase consisting of EtOAc:MeOH: CHCl_3 (60:20:40, *v/v/v*) in an environment saturated with ammonia. 12.0 mg of augustamine (22) was isolated from fraction B, 9.1 mg of augustine (13) from fraction D, 6.0 mg of buphanisine (8) from fraction F, 2.9 mg of crinine (10) from fraction H, and 4.0 mg of augustine *N*-oxide (1) and 12.0 mg of buphanisine *N*-oxide (2) from fraction J.

3.4. Characterization of Compounds

Augustine N-oxide (1): Amorphous solid; $[\alpha]_D^{22} -24.0$ (*c* 0.001, CHCl_3); UV (MeOH) λ_{max} (log ϵ): 292.0 (3.69), 240.5 (5.57) nm; CD (MeOH, 20 °C) $\Delta\epsilon_{245} + 5739$, $\Delta\epsilon_{295} - 5169$; IR ν_{max} 3363, 2986, 2851, 1740, 1504, 1489, 1464, 1391, 1374, 1241, 1147, 1079, 1034, 926, 846 and 814 cm^{-1} ; ^1H -NMR (CDCl_3 , 400 MHz) and ^{13}C -NMR (CDCl_3 , 100 MHz) see Table 2; ESI-MS data shown in Table 1; HR-ESI-MS of $[\text{M} + \text{H}]^+$ *m/z* 318.1335 (calcd. for $\text{C}_{17}\text{H}_{20}\text{NO}_5$, 318.1336).

Buphanisine N-oxide (2): Amorphous solid; $[\alpha]_D^{22} -1.0$ (*c* 0.001, CHCl_3); UV (MeOH) λ_{max} (log ϵ): 292.0 (3.57), 240.5 (3.45) nm; CD (MeOH, 20 °C) $\Delta\epsilon_{245} + 7271$, $\Delta\epsilon_{292} - 7223$; IR ν_{max} 3350, 3037, 2977, 2824, 1652, 1488, 1402, 1377, 1255, 1241, 1097, 1071, 1034, 966, 931 and 854 cm^{-1} ; ^1H -NMR (CDCl_3 , 400 MHz) and ^{13}C -NMR (CDCl_3 , 100 MHz) see Table 2; ESI-MS data shown in Table 1; HR-ESI-MS of $[\text{M} + \text{H}]^+$ *m/z* 302.1385 (calcd. for $\text{C}_{17}\text{H}_{20}\text{NO}_4$, 302.1387).

3.5. Biological Activities

3.5.1. Antiprotozoal Activities

In vitro tests for the biological activity of the alkaloids isolated from *C. amabile* against *Trypanosoma brucei rhodesiense* (trypomastigotes forms, STIB 900 strain), *Trypanosoma cruzi* (axenic grown amastigotes forms, Tulahuen C4 strain), *Leishmania donovani* (amastigotes forms, MHOM-ET-67/L82 strain), and *Plasmodium falciparum* (intraerythrocytic forms, IEF, NF54 strain) and a cytotoxicity test against the mammalian L6 cell line from rat skeletal myoblasts were carried out at the Swiss Tropical and Public Health Institute (Swiss TPH, Basel, Switzerland) according to established protocols as described by Orhan and co-workers [36]. The reference drugs used in these assays were melarsoprol, benznidazole, miltefosine, chloroquine and podophyllotoxin, respectively.

3.5.2. Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities

Cholinesterase inhibitory activities were analyzed as by Ellman and co-workers [37] with some modifications as by López and co-workers [38]. Fifty microliters of AChE or BuChE phosphate buffer (8m M K_2HPO_4 , 2.3 mM NaH_2PO_4 , 0.15 M NaCl, pH 7.5) and 50 μ L of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 min at room temperature before 100 μ L of the substrate solution (0.1 M Na_2HPO_4 , 0.5 M DTNB, and 0.6 mM acetylthiocholine iodide, ATCI, or 0.24 mM butyrylthiocholine iodide, BTCl, in Millipore water, pH 7.5) was added. The absorbance was read in a Labsystem microplate reader (Helsinki, Finland) at 405 nm after 10 min. Galanthamine served as positive control. In a first step, samples were assessed at 10, 100 and 200 μ g mL^{-1} towards both enzymes. Samples with an $IC_{50} > 200 \mu g mL^{-1}$ were considered inactive. Samples with an $IC_{50} < 200 \mu g mL^{-1}$ were further analyzed to determine the IC_{50} values. Enzyme activity was calculated as a percentage compared to an assay using a buffer without any inhibitor. The cholinesterase inhibitory data were analyzed with the software Microsoft Office Excel 2010.

4. Conclusions

Twenty-five alkaloids were identified in *C. amabile*, including two new alkaloids, augustine N-oxide and buphanisine N-oxide. This is the first time that augustamine and N-oxide structures have been described in this species. These alkaloids, together with augustine, buphanisine and crinine, were isolated, but none showed remarkable biological activity.

Author Contributions: Jaume Bastida designed the experiments, analyzed the data and wrote the paper. Luciana R. Tallini and Laura Torras-Claveria performed the experiments, analyzed the data and wrote the paper. Warley de Souza Borges collected the plant, performed the extraction and wrote the paper. Marcel Kaiser performed the antiprotozoal experiments and wrote the paper. Francesc Viladomat and José Angelo S. Zuanazzi wrote the paper.

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3.4 Artículo 4

Cholinesterase inhibition activity, alkaloid profiling and molecular docking of Chilean *Rhodophiala* (Amaryllidaceae)

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Article

Cholinesterase Inhibition Activity, Alkaloid Profiling and Molecular Docking of Chilean *Rhodophiala* (Amaryllidaceae)

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Abstract: Amaryllidaceae plants are the commercial source of galanthamine, an alkaloid approved for the clinical treatment of Alzheimer's disease. The chemistry and bioactivity of Chilean representatives of *Rhodophiala* genus from the family of Amaryllidaceae have not been widely studied so far. Ten collections of five different Chilean *Rhodophiala* were analyzed in vitro for activity against enzymes such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) as well as for their alkaloid composition by GC-MS. To obtain an insight into the potential AChE and BuChE inhibitory activity of the alkaloids identified in the most active samples, docking experiments were carried out. Although galanthamine was found neither in aerial parts nor in bulbs of *R. splendens*, these plant materials were the most active inhibitors of AChE (IC₅₀: 5.78 and 3.62 µg/mL, respectively) and BuChE (IC₅₀: 16.26 and 14.37 µg/mL, respectively). Some 37 known alkaloids and 40 still unidentified compounds were detected in the samples, suggesting high potential in the Chilean Amaryllidaceae plants as sources of both novel bioactive agents and new alkaloids.

Keywords: *Rhodophiala*; alkaloids; molecular docking; AChE; BuChE; GC-MS

1. Introduction

The vast structural and chemical diversity of natural products gives them a significant role in drug discovery [1]. Alkaloids are of particular interest in biomedicine and drug discovery research [2] due to their structural diversity and specific biological potential [3]. The Amaryllidaceae is a plant family that contains an exclusive, large and still expanding alkaloid group known as the Amaryllidaceae alkaloids, which are characterized by unique skeleton arrangements and a broad spectrum of biological activities [4,5]. Amaryllidaceae plants have been used in folk medicine for their therapeutic and

toxic properties [6]. Hippocrates of Kos (460–370 BP), considered the father of modern medicine, recommended the oil of *Narcissus* (Amaryllidaceae) species for the treatment of symptoms that today would be recognized as cancer [7].

The Amaryllidaceae alkaloids are classified mainly into nine skeleton types: norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, tazettine, montanine and galanthamine (Figure 1) [4]. The most important Amaryllidaceae alkaloid is galanthamine, which was isolated for the first time from *Galanthus woronowii* in the 1950s and was approved for the clinical treatment of mild to moderate Alzheimer's disease (AD) by the Food and Drug Administration (FDA) at the beginning of this century [8,9]. Alzheimer's disease (AD), characterized by severe and progressive memory loss, is the most common form of dementia and is becoming increasingly prevalent in people older than 65 years [10,11]. It is estimated that 47 million people live with dementia in the world today, with an economic impact estimated at 818 billion dollars [12]. Known factors involved in the development of AD include a reduced cholinergic neurotransmission level, oxidative stress, amyloid- β -peptide ($A\beta$) and tau protein aggregation [13].

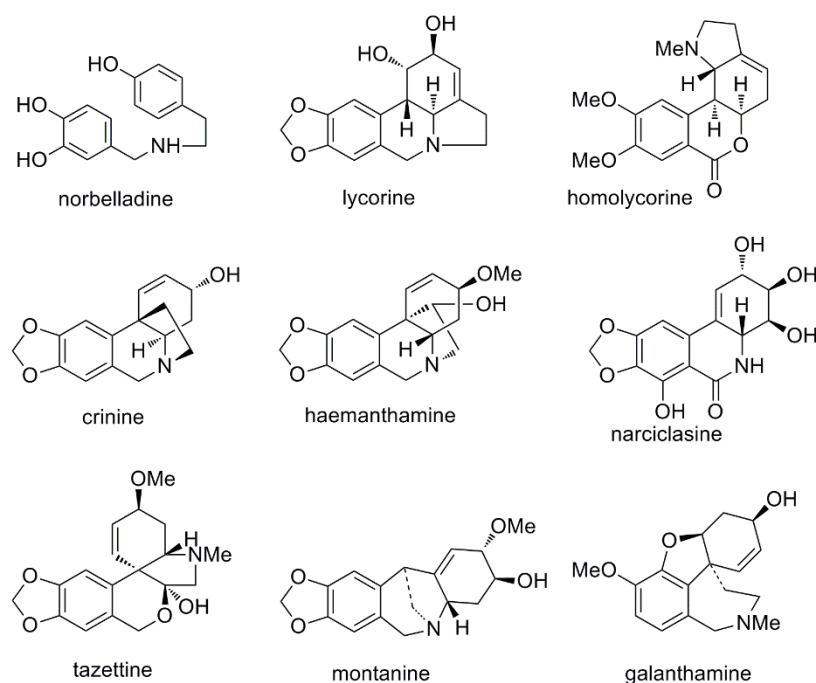


Figure 1. Amaryllidaceae alkaloid types.

Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) [14]. Acetylcholinesterase is highly selective for ACh hydrolysis, and BuChE can metabolize different substrates [15]. In the brain of AD patients, AChE activity tends to decrease while that of BuChE increases [16]. Consequently, cholinesterase inhibitors that suppress both AChE and BuChE may provide a better therapeutic response rather than AChE-selective agents [17].

Little is known on the chemistry and bioactivity of the South American endemic Amaryllidaceae genus *Rhodophiala*. The *Rhodophiala* species have high ornamental potential due to its attractive red, yellow, white or orange flowers [18–22]. The approximately 40 species described at present occur in Argentina, Bolivia, southern Brazil, Chile and Uruguay [22]. *Rhodophiala* plants have a tunicate bulb of 4–6 cm diameter, which is set 20–30 cm underground, a single umbel holding up to six flowers, each flower being 4–6 cm wide and a flower stem 35 to 50 cm long [18].

Rhodophiala species are well known ornamental plants. Propagation of the Chilean species was reported [19] as well as phylogenetic [20] and morphological studies [21,22]. The renewed interest on

galanthamine sources including the search for additional alkaloids with inhibitory activity towards the enzymes AChE and BuChE has prompted research work on the South American species of this family. Brazilian [23] and Argentinean wild Amaryllidaceae [24] showed high structural diversity in the alkaloid patterns and encouraged work on the Chilean species of this family. The knowledge of the chemical composition and anti-cholinesterase activity of the Chilean species belonging to genus *Rhodophiala* is limited. The comparative studies of their chemical composition are needed to identify the best potential sources of bioactive alkaloids in the South American species.

The aim of this work was to disclose the potential of Chilean *Rhodophiala* species as inhibitors of the enzymes AChE and BuChE as well as to analyze the alkaloid content and composition of native species by gas chromatography coupled to mass spectrometry (GC-MS) looking for galanthamine sources and searching for other compounds with effect of AChE and BuChE. Molecular docking studies were also carried out to investigate the affinity of the alkaloids identified in the most promising sample at the active sites of AChE and BuChE.

2. Results and Discussion

Twenty alkaloid extracts from five different Chilean *Rhodophiala* species (Figures 2 and 3) were assessed for inhibitory activity towards the enzymes AChE and BuChE. The alkaloid composition of the extracts was analyzed by GC-MS and the single alkaloid content was quantified and reported as mg GAL/g alkaloid extract (AE). The extracts have been obtained as described in Section 2.2. Briefly, the (fresh) plant material was lyophilized to obtain the dry tissues content (values included in the Table) of the different plant parts investigated. The extraction yields are calculated and reported from the lyophilized plant material.

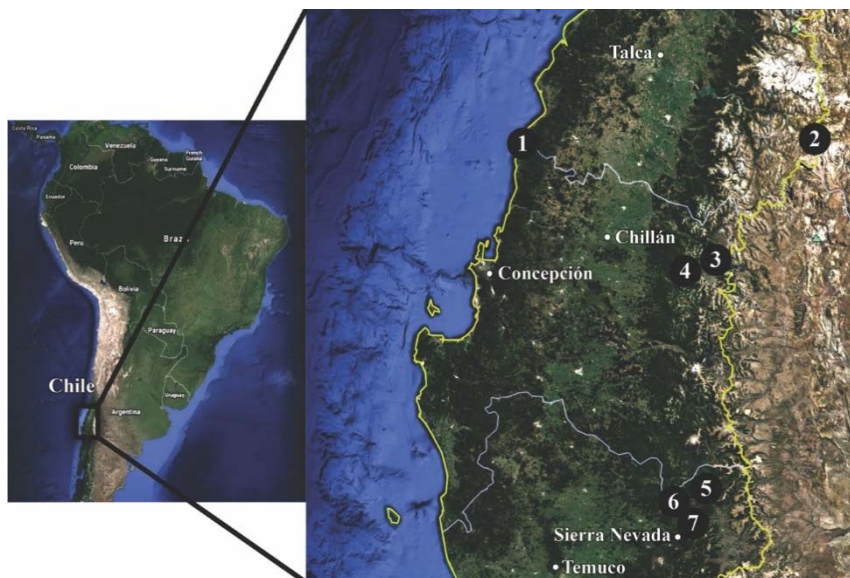


Figure 2. Map of Chile showing the collection sites of the *Rhodophiala* species. 1: Arcos de Calán; 2: Laguna del Maule; 3: Nevado de Chillán; 4: Las Trancas; 5: Volcán Lonquimay; 6: Malalcahuello; 7: Sierra Nevada. Map source: Google Earth.

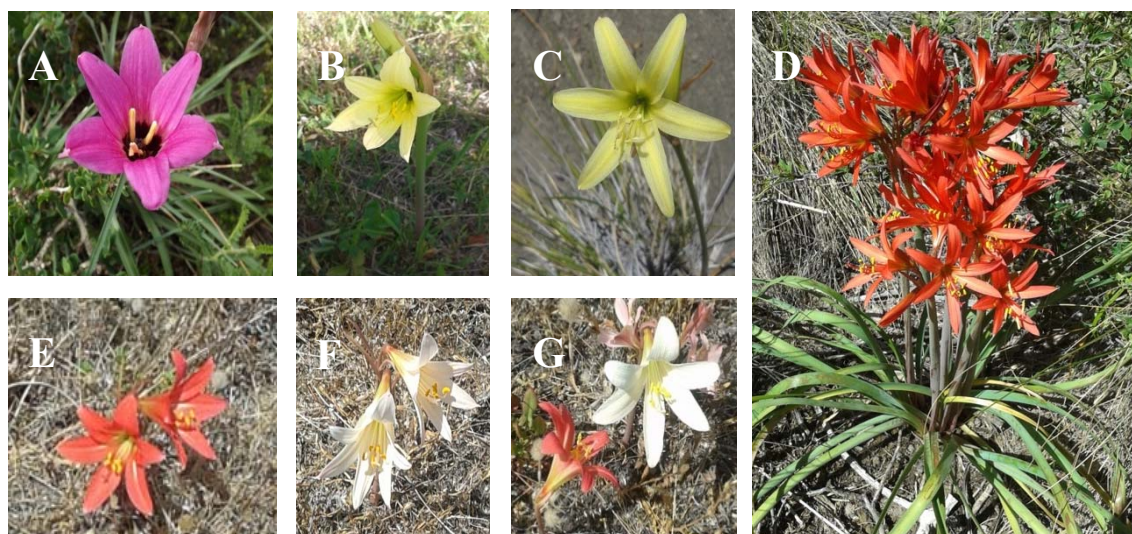


Figure 3. Flowering *Rhodophiala* species from central-southern Chile. (A) *R. andicola* (Poepp.) Traub; (B) *R. araucana* (Phil.) Traub; (C) *R. montana* (Phil.) Traub; (D) *R. splendens* (Renjifo) Traub; (E) *R. pratensis* (Poepp.) Traub; (F) *R. pratensis* white flowers and (G) *R. pratensis* plants with red and white flowers.

2.1. AChE and BuChE Inhibitory Activities

The crude alkaloid samples from the different Chilean *Rhodophiala* were tested in vitro for AChE and BuChE-inhibitory activity. The percent dry weight of the samples, *w/w* extraction yields, and cholinesterase inhibition are summarized in Table 1. Galanthamine was used as a control and presented AChE and BuChE inhibition with IC_{50} values of 0.48 ± 0.07 and 3.70 ± 0.24 $\mu\text{g/mL}$, respectively. All the alkaloid extracts tested were active against AChE. The highest AChE inhibitory potential was found in bulbs of *R. pratensis* (sample Q) followed by *R. splendens* (sample S) with IC_{50} values of 3.32 ± 0.26 and 3.62 ± 0.02 $\mu\text{g/mL}$, respectively. Lowest activity was measured for the aerial part of *R. pratensis* (sample N) (IC_{50} value: 102.27 ± 6.61 $\mu\text{g/mL}$). Nearly 50% of the samples showed some activity against BuChE, with better effect for the bulbs of *R. splendens* (sample S) (IC_{50} 14.37 ± 1.94 $\mu\text{g/mL}$).

The bulb (I) and leaf (J) extracts of *R. montana* presented moderate activity against both enzymes with better effect of (I) against AChE and (J) towards BuChE. The differences in the chemical profiles of I and J could explain these results. However, the high number of unknown alkaloids in the extracts precludes further discussion. The bulb (Q) and leaf (R) extracts of white flowering *R. pratensis* were active towards AChE, with IC_{50} of 3.32 and 8.39 $\mu\text{g/mL}$, respectively but with mild to low effect against BuChE (Table 1), reducing the pharmacological interest of this species. The high AChE and BuChE inhibitory activity of *R. splendens* bulb (S) and aerial parts/leaves (T) renders this plant as the most promising species in the search for active molecules for AD therapy (Table 1).

Table 1. Percent dry weight, *w/w* extraction yields from dry starting material, percent alkaloid extract (from the crude extract), acetyl-(AChE) and butyryl- (BuChE) cholinesterase inhibitory activity of alkaloid-enriched extracts from Chilean *Rhodophiala*.

Scientific Names, Abbreviated Collection Place and Reference Letter	% Dry Weight ^a	<i>w/w</i> Extraction Yield ^b	% Alkaloid Extract ^c	AChE IC ₅₀ (μg/mL) ^d	BuChE IC ₅₀ (μg/mL) ^d
Aerial parts					
<i>R. andicola</i> , SN (B)	15.46	30.63	3.16	18.16 ± 2.94	138.27 ± 6.83
<i>R. andicola</i> , NC (D)	18.70	22.10	2.86	12.30 ± 0.74	43.41 ± 2.64
<i>R. andicola</i> , VL (F)	20.00	17.50	2.07	74.44 ± 5.53	>200
<i>R. araucana</i> , M, (H)	17.70	25.30	3.78	^e	^e
<i>R. montana</i> , LM (J)	21.40	9.40	1.14	33.57 ± 2.16	16.38 ± 0.78
<i>R. pratensis</i> , AC, RF, nl, sand dunes (L)	14.84	27.08	1.93	72.59 ± 4.26	>200
<i>R. pratensis</i> , AC, RF, L (N)	14.53	10.10	3.72	102.27 ± 6.61	>200
<i>R. pratensis</i> , AC, RF, nl (P)	11.68	32.39	2.87	31.97 ± 3.24	>200
<i>R. pratensis</i> , AC, WF (R)	10.67	16.47	1.15	8.39 ± 0.27	>200
<i>R. splendens</i> , LT, (T)	14.81	44.26	1.53	5.78 ± 0.93	16.26 ± 3.34
Bulbs					
<i>R. andicola</i> , SN (A)	25.41	7.53	2.15	13.29 ± 1.01	45.76 ± 9.72
<i>R. andicola</i> , NC (C)	28.90	6.00	2.23	7.26 ± 0.16	47.38 ± 4.08
<i>R. andicola</i> , VL (E)	23.50	7.40	2.37	22.77 ± 1.57	113.24 ± 2.77
<i>R. araucana</i> , M, (G)	19.80	7.90	2.73	6.23 ± 0.24	45.71 ± 3.51
<i>R. montana</i> , LM, (I)	28.00	3.26	1.50	18.13 ± 0.51	40.05 ± 9.03
<i>R. pratensis</i> , AC, RF, nl, sand dunes (K)	18.87	19.73	1.50	11.81 ± 0.17	>200
<i>R. pratensis</i> , AC, RF, L (M)	20.98	11.73	2.84	44.23 ± 4.08	>200
<i>R. pratensis</i> , AC, RF, nl (O)	18.76	7.36	2.33	47.66 ± 1.78	>200
<i>R. pratensis</i> , AC, WF (Q)	19.64	4.84	2.29	3.32 ± 0.26	52.16 ± 0.57
<i>R. splendens</i> , LT, (S)	21.35	35.05	1.60	3.62 ± 0.02	14.37 ± 1.94

^a after lyophilization; ^b from lyophilized material; ^c from the crude extract; ^d all IC₅₀ were calculated using R² ≥ 0.99; ^e insufficient sample; WF: white flowers; RF: red flowers; L: with leaves; nl: no leaves; Collection place: AC: Arcos de Calán, Región del Maule; LM: Laguna del Maule; LT: Las Trancas; M: Malalcahuello, Región de la Araucanía; NC: Nevado de Chillán; SN: Sierra Nevada; VL: volcan Lonquimay.

2.2. Alkaloid Identification by GC-MS

The activity of the Chilean *Rhodophiala* towards acetylcholinesterase is a consequence of the chemical composition of the extracts. Therefore, the alkaloid composition is a key point to understand the chemical diversity of these plants as a source of potential therapies for AD. The alkaloids occurring in the different extracts were identified by comparing their GC-MS spectra and Kovats Retention Index (RI) values with those of authentic samples. Thirty-seven known alkaloids were identified in these samples (Figure 4). About 50% of them belong to three different alkaloid types, namely: lycorine, haemanthamine and crinine. The others belong to six different alkaloid types: tazettine, homolycorine, galanthamine, montanine, mesembrenone and narciclasine. Two unusual alkaloids known as ismine and galanthindole were also found. The occurrence and quantification of the alkaloids in the samples is summarized in Table 2: (A) (aerial parts) and (B) (bulbs). The number of alkaloids detected varied among extracts, ranging from 8 in the aerial parts of *R. andicola* collected in Sierra Nevada (B) to 23 in the bulb of *R. pratensis* (K). Forty structures found in these samples could not be identified, suggesting high potential of Chilean *Rhodophiala* species in the search for new alkaloids. The number of unidentified compounds ranged from 3 in aerial parts of *R. andicola* (A, B and D), *R. pratensis* (P) and *R. splendens* (T) to 12 in aerial parts of *R. montana* (J). Representative chromatograms of the samples are shown in Figures 5–9.

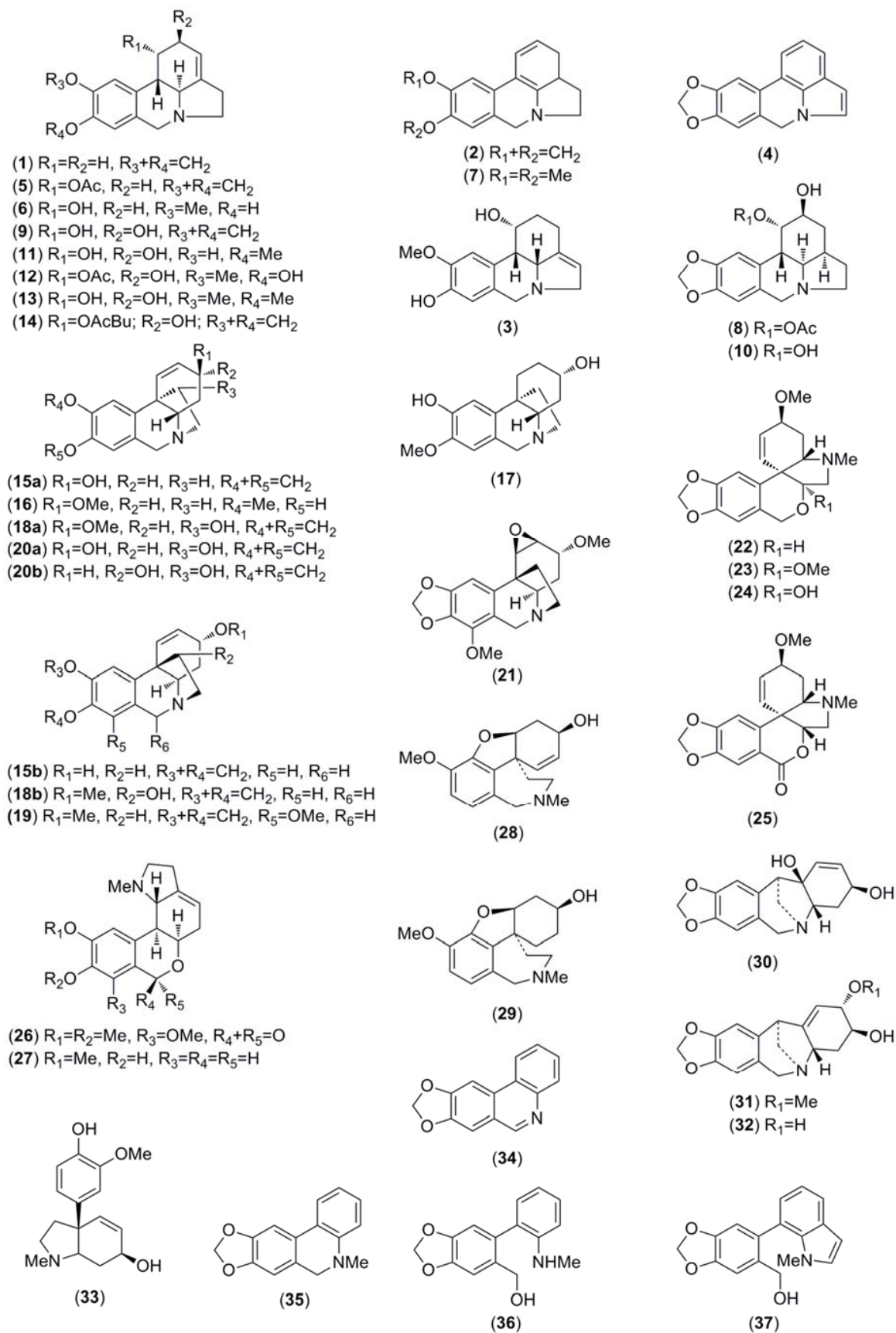
Figure 4. Alkaloids identified in Chilean *Rhodophiala* species by GC-MS.

Table 2. (A) Identification of alkaloids occurring in the aerial parts of Chilean *Rhodophiala* species by GC-MS. Values are expressed as mg GAL/g AE; (B) Identification of alkaloids occurring in the bulbs of Chilean *Rhodophiala* species by GC-MS. Values are expressed as mg GAL/g AE.

(A)													
Alkaloid	M ⁺	BP	RI	B	D	F	H	J	L	N	P	R	T
Lycorine-type				8.6	7.5	25.1	46.0	46.9	35.5	37.3	35.4	60.8	13.1
lycorene (1)	255	254	2346.8	-	-	-	-	9.4	5.1	7.2	T	-	-
anhydrolycorine (2)	251	250	2543.1	-	T	5.9	11.2	9.6	9.9	8.3	11.8	13.2	-
kirkine (3)	253	252	2588.2	-	-	-	-	7.2	5.3	-	6.0	7.2	-
11,12-dehydroanhydrolycorine (4)	249	248	2646.4	8.6	7.5	13.5	8.4	15.6	8.1	21.8	7.5	9.4	7.8
1-O-acetylcaranine (5)	313	226	2653.1	-	-	-	-	-	-	-	-	-	-
norpluviine (6)	273	228	2683.6	-	-	-	-	-	-	-	-	6.2	-
assoanine (7)	267	266	2708.9	-	T	-	-	-	-	-	-	-	-
3,4-dihydro 1-acetyllycorine (8)	331	330	2723.3	-	-	-	-	-	-	-	-	-	-
lycorine (9)	287	226	2791.7	-	T	5.7	14.7	5.1	7.1	-	10.1	24.8	5.3
dihydrolycorine (10)	289	288	2833.9	-	-	-	-	-	-	-	T	T	-
pseudolycorine (11)	289	228	2856.4	-	-	-	-	-	-	-	-	-	-
sternbergine (12)	331	228	2838.8	-	-	-	6.4	-	-	-	T	-	-
methylpseudolycorine (13)	303	242	2911.2	-	-	-	-	-	-	-	-	-	-
1-O-(3'-acetoxybutanoyl)lycorine (14)	415	226	3248.8	-	-	-	5.3	-	-	-	-	-	-
Haemanthamine/crinine type				-	11.6	53.1	34.4	14.8	17.2	21.4	38.7	35.7	48.0
vittatine (15a)/crinine (15b)	271	271	2512.4	-	T	7.7	-	-	-	-	6.2	5.6	-
8-O-demethylmaritidine (16)	273	273	2540.0	-	-	-	-	-	-	-	T	-	6.4
deacetylcantabricine (17)	275	275	2573.1	-	-	-	-	-	-	-	-	-	T
haemanthamine (18a)/crinamine (18b)	301	272	2673.4	-	11.6	45.4	34.4	7.7	17.2	21.4	32.5	30.1	25.0
buphanidrine (19)	315	315	2748.3	-	-	-	-	-	-	-	-	-	-
11-hydroxyvittatine (20a)/hamayne (20b)	287	258	2750.5	-	-	-	-	-	-	-	-	-	16.6
undulatine (21)	331	331	2892.5	-	-	-	-	7.1	-	-	-	-	-
Tazettine-type				60.7	59.1	95.8	22.3	-	11.2	16.3	16.0	29.9	55.6
deoxytazettine (22)	315	231	2575.6	6.1	5.5	6.7	5.0	-	T	5.2	-	5.2	-
O-methyltazettine (23)	345	261	2641.1	54.6	36.0	66.2	10.3	-	11.2	11.1	10.1	14.9	30.7
tazettine (24)	331	247	2685.1	-	12.5	17.0	7.0	-	-	-	5.9	9.8	24.9
epimacronine (25)	329	245	2848.0	-	5.1	5.9	T	-	T	-	-	T	T

Table 2. Cont.

(A)													
Alkaloid	M ⁺	BP	RI	B	D	F	H	J	L	N	P	R	T
Homolycorine-type				-	-	9.1	-	-	8.6	22.4	6.5	19.5	-
nerinine (26)	347	109	2513.5	-	-	-	-	-	8.6	22.4	6.5	19.5	-
8-O-demethylhomolycorine (27)	301	109	2856.4	-	-	9.1	-	-	-	-	-	-	-
Galanthamine-type				-	5.1	14.1	7.1	5.8	-	-	-	-	-
galanthamine (28)	287	286	2519.9	-	-	8.6	7.1	5.8	-	-	-	-	-
lycoramine (29)	289	288	2544.6	-	5.1	5.5	-	-	-	-	-	-	-
Montanine-type				-	-	-	-	8.1	28.3	19.7	15.2	26.7	-
pancratinine C (30)	287	176	2623.5	-	-	-	-	-	-	-	-	-	-
montanine (31)	301	301	2663.1	-	-	-	-	8.1	28.3	19.7	15.2	26.7	-
pancracine (32)	287	287	2737.4	-	-	-	-	-	-	-	-	-	-
Mesembrenone-type				-	-	-	-	-	-	-	-	-	-
demethylmesembrenol (33)	275	205	2343.2	-	-	-	-	-	T	-	T	T	-
Narciclasine-type				12.7	11.2	13.5	5.8	6.2	5.5	44.5	-	10.4	32.2
trisphaeridine (34)	223	223	2322.9	12.7	5.1	6.0	5.8	6.2	5.5	39.4	T	5.2	22.7
dihydrobicolorine (35)	239	238	2366.1	T	6.1	7.5	T	-	T	5.1	T	5.2	9.5
Other-type				5.2	32.9	62.1	8.5	-	5.9	-	5.7	17.7	34.5
ismine (36)	257	238	2304.6	-	7.6	17.0	T	-	T	-	T	6.0	14.3
galanthindole (37)	281	281	2534.8	5.2	25.3	45.1	8.5	-	5.9	-	5.7	11.7	20.2
Not identified				46.0	26.8	46.1	42.0	126.9	35.9	78.5	20.6	73.4	21.8
unknown (ismine-derivate) *	227	226	2232.2	-	-	-	-	-	-	8.8	-	-	-
unknown (ismine-derivate) *	227	225	2232.2	-	5.6	6.8	5.6	-	-	8.9	-	-	6.2
unknown	269	238	2258.9	-	5.1	6.9	-	-	-	-	-	-	6.4
unknown (mesembrenone-type) *	245	175	2280.8	-	-	-	-	-	6.0	-	-	10.5	-
unknown	269	268	2285.5	-	-	-	-	-	-	9.7	-	-	-
unknown	253	252	2313.1	5.9	T	-	-	5.9	-	13.7	T	-	-
unknown	251	251	2335.2	-	-	-	-	6.2	-	9.9	-	-	-

(A)

[illegible]

[illegible]

Table 2. Cont.

(B)													
Alkaloid	M ⁺	BP	RI	A	C	E	G	I	K	M	O	Q	S
haemanthamine (18a)/crinamine (18b)	301	272	2673.4	60.0	31.7	31.7	36.6	5.8	23.0	27.6	38.2	31.0	16.4
buphanidrine (19)	315	315	2748.3	-	-	-	-	5.9	-	-	-	-	-
11-hydroxyvittatine (20a)/ hamayne (20b)	287	258	2750.5	-	7.00	-	T	-	-	-	-	6.6	8.2
undulatine (21)	331	331	2892.5	-	-	-	-	5.7	-	-	-	-	-
Tazettine-type				74.8	56.1	85.9	33.1	-	21.8	18.0	8.9	17.9	21.9
deoxytazettine (22)	315	231	2575.6	8.3	10.7	13.1	9.6	-	-	-	-	-	T
O-methyltazettine (23)	345	261	2641.1	58.7	23.0	61.7	14.8	-	10.9	12.7	8.9	9.9	14.3
tazettine (24)	331	247	2685.1	7.8	22.4	11.1	8.7	-	10.9	5.3	T	8.0	7.6
epimacronine (25)	329	245	2848.0	-	T	T	T	-	-	-	-	-	-
Homolycorine-type				9.4	-	10.6	-	-	13.9	14.1	10.1	28.5	9.8
nerinine (26)	347	109	2513.5	-	-	-	-	-	13.9	14.1	10.1	21.1	-
8-O-demethylhomolycorine (27)	301	109	2856.4	9.4	-	10.6	-	-	-	-	-	7.4	9.8
Galanthamine-type				-	19.0	11.9	12.3	-	-	-	-	-	-
galanthamine (28)	287	286	2519.9	-	5.1	6.8	5.4	T	-	-	-	-	-
lycoramine (29)	289	288	2544.6	-	13.9	5.1	6.9	-	-	-	-	-	-
Montanine-type				-	-	-	5.2	7.8	41.7	23.1	12.5	24.7	6.4
pancratinine C (30)	287	176	2623.5	-	-	-	-	T	5.3	T	-	-	-
montanine (31)	301	301	2663.1	-	-	-	5.2	7.8	29.8	23.1	12.5	24.7	-
pancracine (32)	287	287	2737.4	-	-	-	-	-	6.6	-	-	-	6.4
Mesembrenone-type				-	-	-	-	-	7.2	5.2	-	5.1	-
demethylmesembrenol (33)	275	205	2343.2	-	-	-	-	-	7.2	5.2	T	5.1	-
Narciclasine-type				12.9	12.7	13.3	5.8	5.7	-	-	-	-	5.1
trisphaeridine (34)	223	223	2322.9	5.5	6.3	5.3	T	5.7	T	T	T	T	5.1
dihydrobicolorine (35)	239	238	2366.1	7.4	6.4	8.0	5.8	T	T	T	T	T	T
Other-type				19.2	35.6	28.2	21.8	-	7.9	7.8	5.3	8.0	7.3
ismine (36)	257	238	2304.6	6.6	9.9	9.5	6.6	-	T	T	T	T	T

(B)

[illegible]

Table 2. Cont.

(B)													
Alkaloid	M ⁺	BP	RI	A	C	E	G	I	K	M	O	Q	S
unknown (crinine/haemanthamine-type) *	347	331	2795.8	-	-	-	-	6.9	-	-	-	-	-
unknown (lycorine-type) *	251	250	2846.6	-	-	6.8	9.9	-	-	-	-	-	-
unknown	335	335	2860.6	-	7.6	-	-	-	-	-	-	-	-
unknown (lycorine-type) *	345	242	2868.8	-	-	-	-	-	-	-	-	-	-
unknown	373	372	2881.9	-	-	-	-	-	-	-	-	-	7.8
unknown (lycorine-type) *	357	356	2942.1	-	-	-	-	-	-	-	-	-	7.0
unknown (lycorine-type) *	279	278	3016.0	-	-	-	-	10.2	-	-	-	-	-
unknown (galanthamine-type) *	375	330	3027.5	-	7.0	-	-	-	-	-	-	-	-
unknown (lycorine-type) *	267	266	3032.9	-	9.2	-	7.3	-	-	-	-	-	-
unknown (lycorine-type) *	375	374	3049.2	-	-	-	-	-	-	-	-	-	8.4
unknown (lycorine-type) *	355	226	3066.6	-	-	-	5.8	-	-	-	-	-	-
unknown (lycorine-type) *	373	226	3161.0	-	-	-	21.5	-	-	-	-	-	-
Total				250.3	272.5	265.9	280.4	186.0	256.3	178.6	148.1	246.3	161.9

BP: Base Peak; T: Traces; **B**: *R. andicola*, SN; **D**: *R. andicola*, NC; **F**: *R. andicola*, VL; **H**: *R. araucana*; **J**: *R. montana*; **L**: *R. pratensis*, RF, nl, dunes; **N**: *R. pratensis*, AP, RF, L; **P**: *R. pratensis*, RF, nl; **R**: *R. pratensis*, WF; **T**: *R. splendens*; **A**: *R. andicola*, SN; **C**: *R. andicola*, NC; **E**: *R. andicola*, VL; **G**: *R. araucana*; **I**: *R. montana*; **K**: *R. pratensis*, RF, nl, dunes; **M**: *R. pratensis*, RF, L; **O**: *R. pratensis*, RF, nl; **Q**: *R. pratensis*, WF; **S**: *R. splendens*; - : not detected; * proposed structure-type according to the fragmentation pattern.

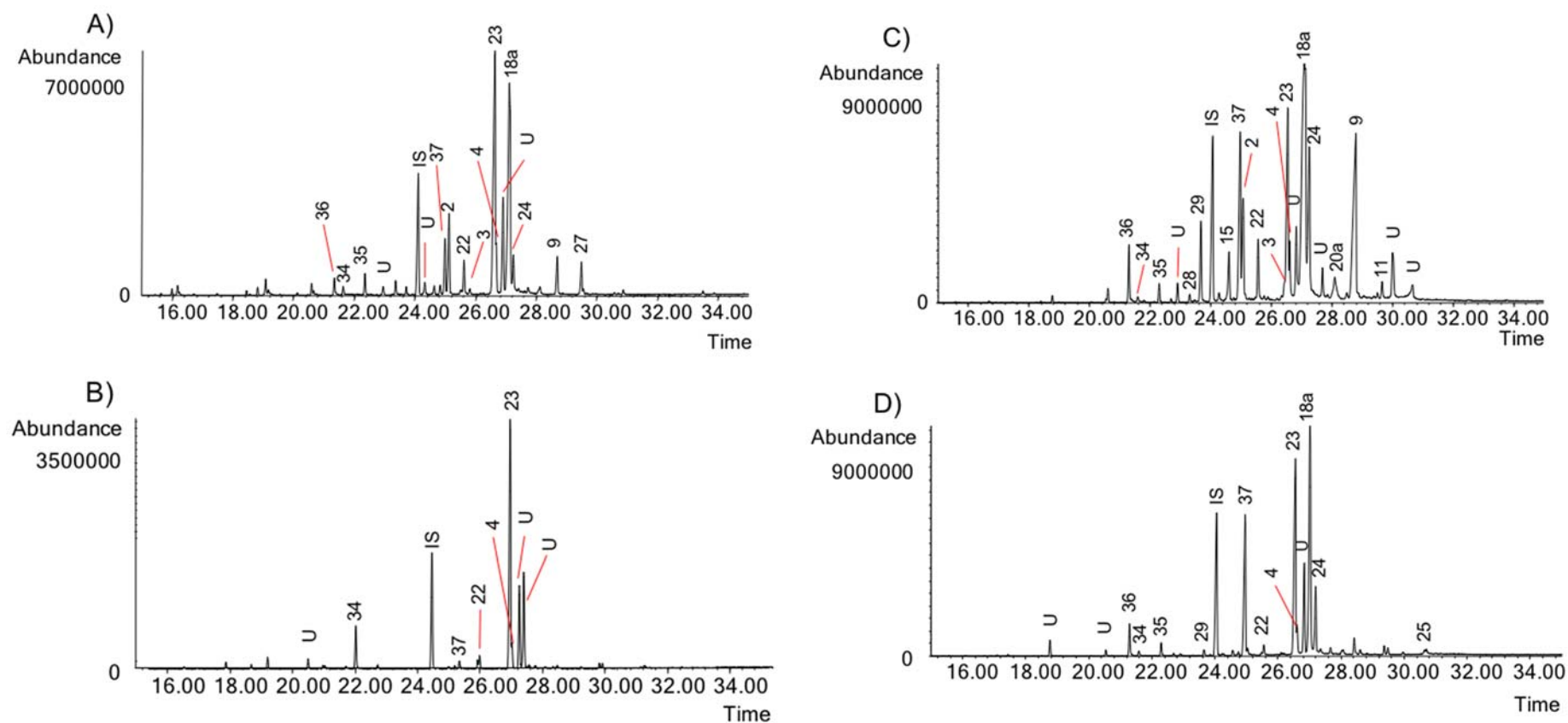


Figure 5. GC chromatograms of the alkaloids from Chilean *Rhodophiala* species. Numbers refer to Table 2. (A) Bulbs of *R. andicola* (Sierra Nevada); (B) Aerial parts of *R. andicola* (Sierra Nevada); (C) Bulbs of *R. andicola* (Nevado de Chillán); (D) Aerial parts of *R. andicola* (Nevado de Chillán). IS: internal standard; U: unknown.

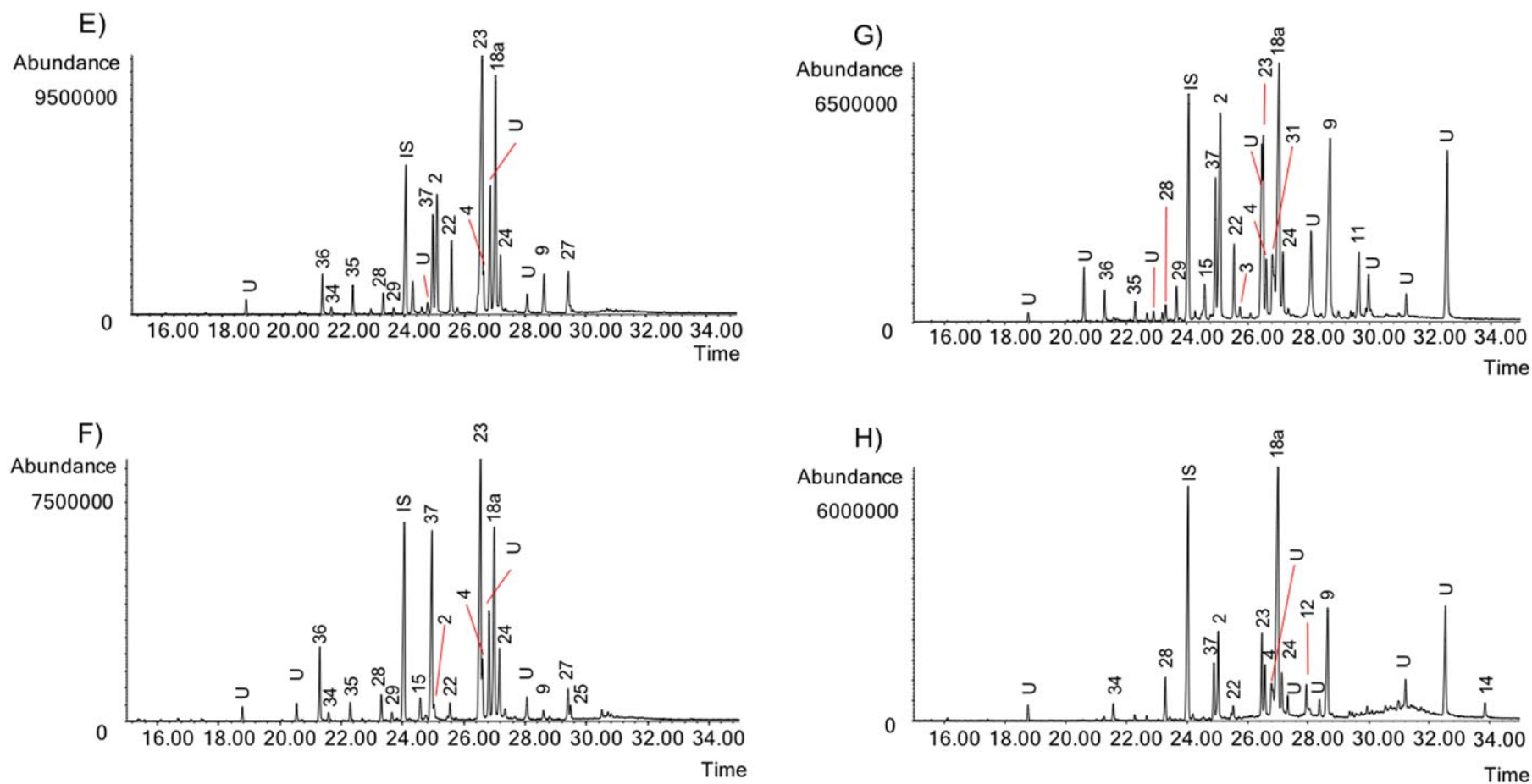


Figure 6. GC chromatograms of the alkaloids from Chilean *Rhodophiala* species. Numbers refer to Table 2. (E) Bulbs of *R. andicola* (Volcán Lonquimay); (F) Aerial parts of *R. andicola* (Volcán Lonquimay); (G) Bulbs of *R. araucana*; (H) Aerial parts of *R. araucana*. IS: internal standard; U: unknown.

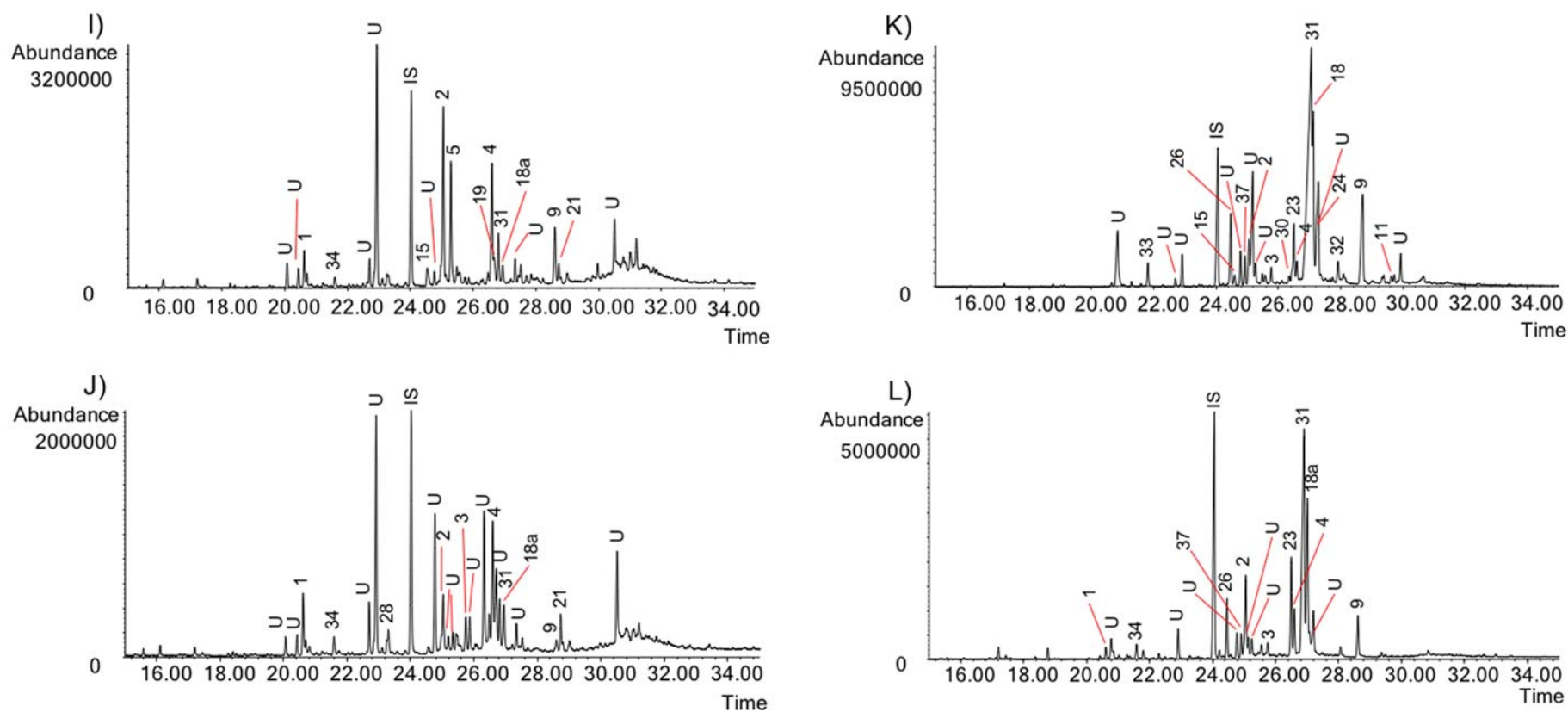


Figure 7. GC chromatograms of the alkaloids from Chilean *Rhodophiala* species. Numbers refer to Table 2. (I) Bulbs of *R. montana*; (J) Aerial parts of *R. montana*; (K) Bulbs of *R. pratensis* (red flowers, without leaves, growing on sand dunes); (L) Aerial parts of *R. pratensis* (red flowers, without leaves, growing on sand dunes). IS: internal standard; U: unknown.

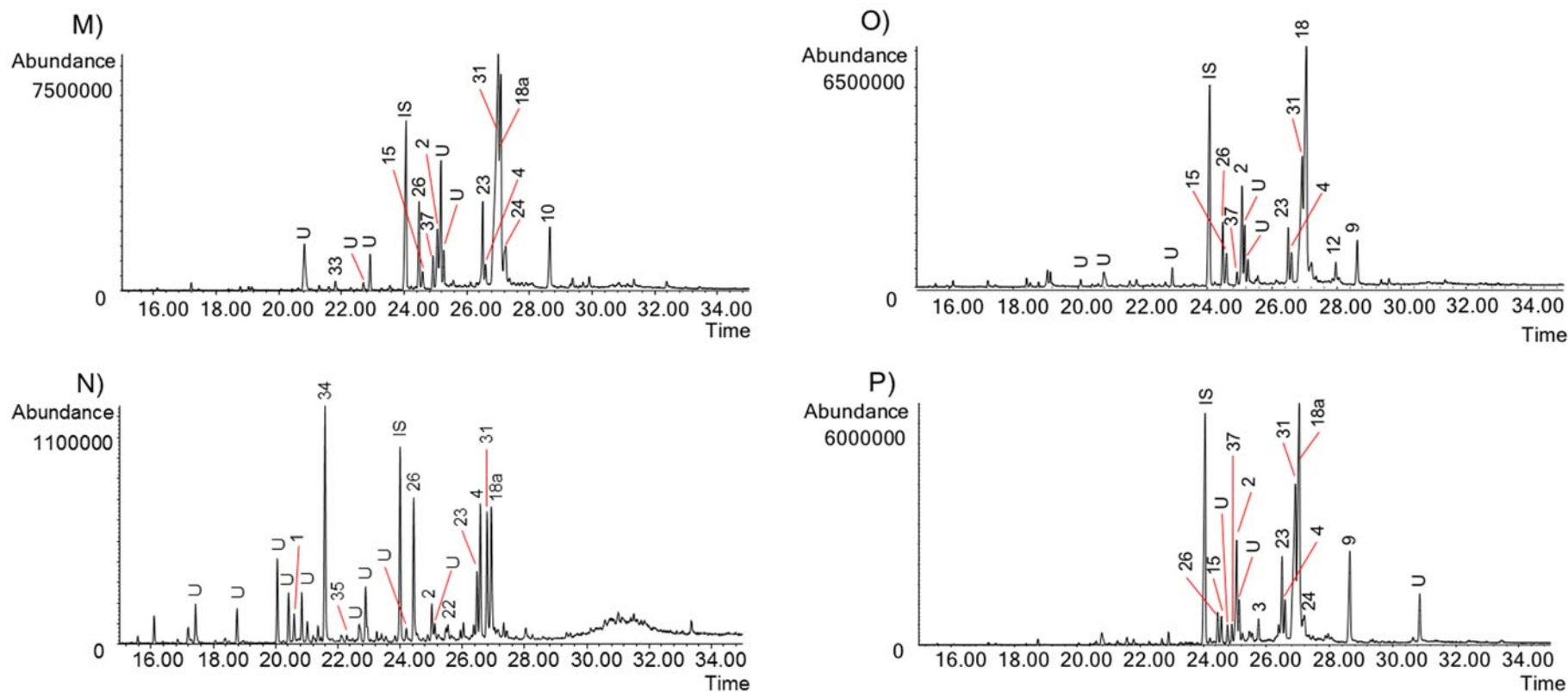


Figure 8. GC chromatograms of the alkaloids from Chilean *Rhodophiala* species. Numbers refer to Table 2. (M) Bulbs of *R. pratensis* (red flowers, with leaves); (N) Aerial parts of *R. pratensis* (red flowers, with leaves); (O) Bulbs of *R. pratensis* (red flowers, without leaves); (P) Aerial parts of *R. pratensis* (red flowers, without leaves). IS: internal standard; U: unknown.

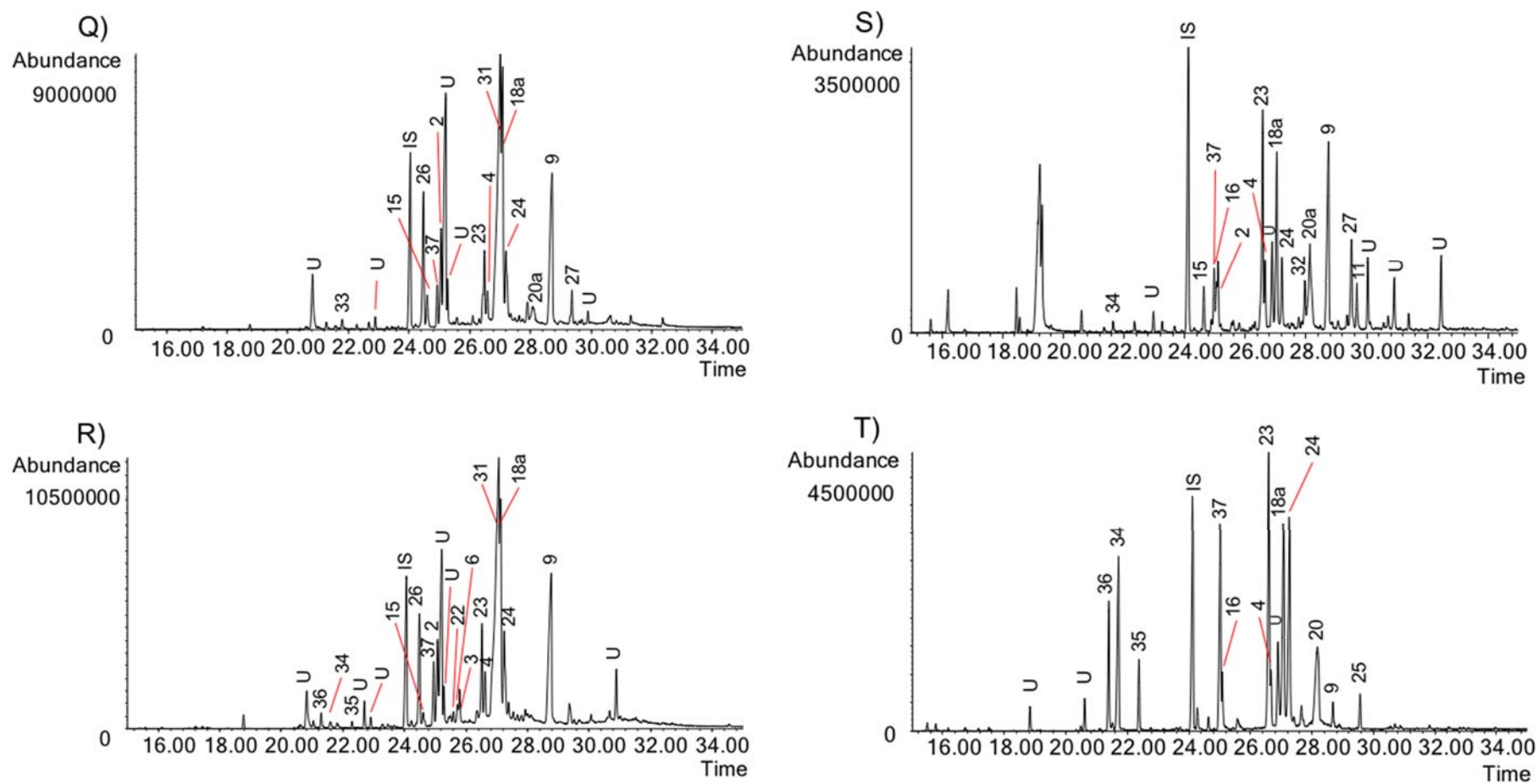


Figure 9. GC chromatograms of the alkaloids from Chilean *Rhodophiala* species. Numbers refer to Table 2. (Q) Bulbs of *R. pratensis* (white flowers); (R) Aerial parts of *R. pratensis* (white flowers); (S) Bulbs of *R. splendens*; (T) Aerial parts of *R. splendens*. IS: internal standard; U: unknown.

The highest alkaloid concentration was detected in the aerial parts of *R. andicola* (F) and in the aerial parts of *R. pratensis* (R) (311.1 and 274.1 mg GAL/g AE, respectively). Lowest content was found in the aerial parts of *R. andicola* (B) and in the aerial parts of *R. pratensis* (P) (133.2 and 138.1 mg GAL/g AE, respectively). In 70% of the samples, lycorine-, haemanthamine/crinine- and tazettine-type alkaloids were predominant. Lycorine-type alkaloids were present in all species with higher content in *R. araucana* (G) and *R. montana* bulbs (I) (79.0 and 78.6 mg GAL/g AE, respectively) and lowest values in the aerial parts of *R. andicola* (B) and (D) (8.6 and 7.5 mg GAL/g AE, respectively).

Haemanthamine/crinine-alkaloids occur in all samples except the aerial parts of *R. andicola* (B). However, the higher content was found in the bulbs of *R. andicola* from the same collection place (A) and in the aerial parts of the plant collected at Volcan Lonquimay (F). Compounds from the tazettine-type were not detected in the aerial parts and bulbs of *R. montana* (I and J). From the different Amaryllidaceae alkaloids groups, tazettine-type alkaloids were the main compounds in several samples, occurring in highest concentration in *R. andicola* (E, F) with values of 85.9 and 95.8 mg GAL/g AE of tazettine-type alkaloids in bulbs and aerial parts, respectively.

Galanthamine-type alkaloids were detected in low quantities in three species, namely *R. andicola*, *R. araucana* and *R. montana* (samples C, D, E, F, G, H and J) ranging between 5.1 to 19.0 mg GAL/g AE. Montanine-type alkaloids were present in all species, except *R. andicola*. The highest level of montanine-type compounds was detected in *R. pratensis* (K) (41.7 mg GAL/g AE), which presented three different alkaloids: pancratine C, montanine and pancracine (5.3, 29.8 and 6.6 mg GAL/g AE, respectively). Mesembrenone-type was the least representative alkaloid-type. It was represented by demethylmesembrenol, detected in low quantities in three different samples of *R. pratensis* (K, M and Q) (7.2, 5.2 and 5.1 mg GAL/g AE, respectively). Narciclasine-type occurs in most samples in a range between 5.1 mg GAL/g AE in bulbs of *R. splendens* (S) to 44.5 and 32.2 mg GAL/g AE in aerial parts of *R. pratensis* with red flowers and leaves (N) and aerial parts of *R. splendens* (T), respectively. All species investigated presented ismine and/or galanthindole alkaloids, except *R. montana*. Forty structures occurring in the extracts could not be identified using the available databases. Three of the unidentified compounds were highly representative among the samples.

The compound with m/z 252 [$M^+ = 253$] (RI 2405.0) occurs in 60% of the samples. The m/z 109 with [$M^+ = 331$] (RI 2557.5), which probably belongs to the homolycorine-type alkaloids, was detected in 40% of the samples and in high amounts in bulbs of *R. pratensis* with white flowers (46.7 mg GAL/g AE). Finally, m/z 261 with [$M^+ = 345$] (RI 2662.6) was detected in 45% of the samples and in high quantity in aerial parts of *R. andicola* collected at Volcan Lonquimay (24.6 mg GAL/g AE).

The highest content of non-identified alkaloids was detected in the aerial parts of *R. montana* (J) and in the bulbs of *R. pratensis* (red flowers and without leaves) collected in the sand dunes at the sea shore (K) (126.9 and 92.6 mg GAL/g AE, respectively). The lowest content was detected in aerial parts of *R. pratensis* with red flowers and without leaves (P) and in aerial parts of *R. splendens* (T) (20.6 and 21.8 mg GAL/g AE, respectively).

2.3. Molecular Docking

In this study, *R. splendens* was the most active inhibitor of AChE and BuChE. Alkaloid analysis by GC-MS allowed the identification of 17 compounds in the leaf extract of *R. splendens* (T) including two unidentified constituents (Table 2). The 15 alkaloids identified in the extract were evaluated for their theoretical AChE and BuChE inhibitory potential by molecular docking (Tables 3 and 4). As expected, no alkaloid identified in sample T presented better theoretical AChE inhibitory activity than galanthamine. Molecular simulation of six alkaloids identified in sample T on the 4BDS structure theoretically showed higher enzymatic inhibition against BuChE than galanthamine by 0.80 kcal/mol.

Table 3. AChE and BuChE inhibitory activities of some alkaloids identified in the aerial parts of *R. splendens* (T) and the reference compound galanthamine. Values are expressed as IC₅₀ (μg/mL).

Alkaloid	AChE	BuChE
11-hydroxyvittatine (20a)	122.17 ± 22.03	>200
lycorine (9)	101.70 ± 23.79	>200
8-O-demethylmaritidine (16)	113.21 ± 8.21	127.87 ± 2.45
hamayne (20b)	135.09 ± 15.33	48.40 ± 1.13
deacetylcantabricine (17)	>200	>200
haemanthamine (18a)	184.68 ± 11.58	>200
galanthamine (28)	0.48 ± 0.07	3.70 ± 0.24

Table 4. Estimated free energy binding of molecular docking between alkaloids identified in aerial parts of *R. splendens* and cholinesterases (AChE and BuChE). Values are expressed in kcal/mol.

Alkaloid	AChE ^{*a}	BuChE ^{*b}
11-hydroxyvittatine (20a)	−8.43	−9.03
lycorine (9)	−8.82 ^{*c}	−8.94
8-O-demethylmaritidine (16)	−8.74 ^{*d}	−8.93
hamayne (20b)	−8.28	−8.54
deacetylcantabricine (17)	−7.90	−8.43
haemanthamine (18a)	−8.80	−8.34
galanthamine (28)	−9.55 ^{*c}	−8.23 ^{*c}
epimacronine (25)	−9.36 ^{*c}	−7.63
tazettine (24)	−8.66 ^{*c}	−7.87
O-methyltazettine (23)	−8.54	−7.87
11,12-dehydroanhydrolycorine (4)	−8.41	−7.44
galanthindole (37)	−7.81	−7.41
trisphaeridine (34)	−7.38	−7.27
dihydrobicolorine (35)	−7.33	−7.38
ismine (36)	−6.78	−7.08

^{*a} PBD code: 1DX6; ^{*b} PBD code: 4BDS; ^{*c} Cortes et al., 2015; ^{*d} Cortes et al., 2017.

To gain further insight into the molecular docking results, an experiment was carried out to check the AChE and BuChE inhibitory activities of 11-hydroxyvittatine (**20**), lycorine (**9**), 8-O-demethylmaritidine (**16**), hamayne (**20b**), deacetylcantabricine (**17**) and haemanthamine (**18a**) (Table 3). The best AChE and BuChE inhibitory activities were obtained for lycorine (**9**) (IC₅₀ 101.70 ± 23.79 μg/mL) and hamayne (**20b**) (IC₅₀ 48.40 ± 1.13 μg/mL), respectively. However, their theoretical BuChE inhibition was not supported by the experimental assays. The difference in origin of the BuChE structure used in the molecular docking (human) and experimental assays (equine serum), as well as the inability of these compounds to arrive at the BuChE active site of the enzyme could help to explain the difference between theoretical and practical results.

Two important regions in the active sites of the hBuChE enzymes have been located: the first corresponding to the catalytic triad composed by the residues His438, Ser198, and Glu325 [25], while the second corresponds to a choline binding site (α-anionic site), composed principally by the residues Trp82 and Phe329 [25]. A graphical representation of molecular binding of 11-hydroxyvittatine (**20a**) and hamayne (**20b**) alkaloids with the hBuChE protein is presented in Figure 10. The alkaloid 11-hydroxyvittatine (**20a**) shows two strong interactions, hydrogen bonds, with the residues Trp82 and Trp430; however, this molecule does not present any interactions close to the catalytic triad His438, Ser198, and Glu325. On the other hand, hamayne (**20b**) shows one hydrogen bond interaction with the residue Gly115, an amino acid located close to the catalytic triad His438, Ser198, and Glu325. In the case of the interactions at the choline binding site (α-anionic site), both alkaloids show the same π–π stacking interaction with the residue Trp82. These molecular interactions suggest that the β-orientation of the hydroxyl group at C-3 in 11-hydroxyvittatine (**20a**) could theoretically increase the BuChE

inhibition on the 4BDS structure by 0.49 kcal/mol, compared to the α -orientation of the hydroxyl group at the C-3 position in hamayne (20b). However, in the experimental assays, hamayne (20b) showed BuChE inhibitory activity ($48.40 \pm 1.13 \mu\text{g/mL}$). It can be hypothesized that the β -orientation of the hydroxyl group at C-3 in 11-hydroxyvittatine (20a) probably makes it difficult for the compound to arrive at the catalytic triad in the active site of the BuChE.

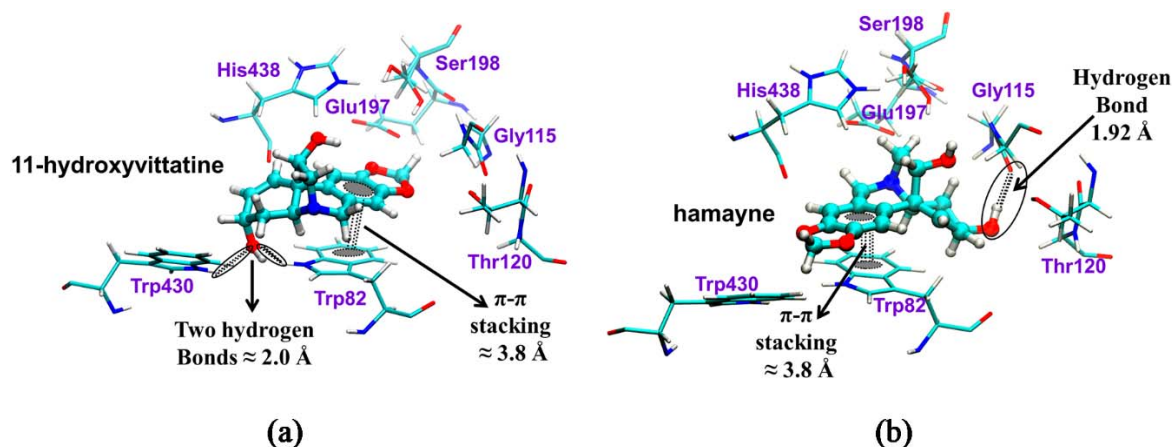


Figure 10. Graphical representations of the binding of (a) 11-hydroxyvittatine (20a) and (b) hamayne (20b) in the gorge of the active site of *hBuChE*.

Studies on alkaloid composition associated with cholinesterase inhibition and binding-mode prediction have been reported [26,27]. A work on Argentinean Amaryllidaceae [24] reported the composition and acetylcholinesterase inhibition of four wild growing species, including *Rhodophiala mendocina*. Two *R. mendocina* samples collected in different locations presented similar activity towards AChE with IC_{50} values of $2.0 \mu\text{g/mL}$ but with relevant differences in the qualitative and quantitative alkaloid composition. The sample from the Provincia de San Juan showed high content of haemanthamine/crinamine (31.2%), tazettine (32.9%) and lycorine (13.3%) while the plant collected in the Provincia de Neuquen presented 6.8% haemanthamine/crinamine and 20.4% of lycorine, respectively. Galanthamine was found in both samples with 0.6 and 0.8% for the San Juan and Neuquen plants, respectively. In a report from acetylcholinesterase inhibitory alkaloids from Brazilian Amaryllidaceae [23] the bulbs of *Rhodophiala bifida* (Herb.) Traub were investigated. The activity on AChE was moderate with an IC_{50} value of $8.45 \mu\text{g/mL}$, being lower than that from *R. mendocina* [24]. The alkaloid extract of *R. bifida* bulbs contained high amounts of montanine (91.94%). The alkaloid composition of the Chilean *Rhodophiala ananuca* (formerly: *Hippeastrum ananuca*) was described [28,29]. The bulbs contained phenantridine alkaloids, including hippeastidine and epi-homolycorine.

The alkaloid montanine isolated from *R. bifida* showed activity towards a panel of eight human cancer cell lines. According to [30], montanine at $2.5 \mu\text{g/mL}$ was more active than doxorubicine on the multi-drug resistant breast cell line NCLADR. Montanine also showed antimicrobial effect with MIC of $5 \mu\text{g/mL}$ against *S. aureus* ATCC 6538 and *E. coli* ATCC 24922 and $20 \mu\text{g/mL}$ against *P. aeruginosa* ATCC 27853, respectively [31]. In a screening towards *Trichomonas vaginalis*, dichloromethane and n-butanol extracts from Brazilian *Hippeastrum* species and *Rhodophiala bifida* showed activity against the protozoa [32]. The most active fractions contained the alkaloids lycorine and lycosinine.

In a study on the alkaloids of *Zephyranthes robusta* (Amaryllidaceae), the compounds isolated were evaluated as inhibitors of human cholinesterases [33]. The authors used human erythrocyte AChE and serum BuChE. The compounds were tested in a range of $0.5\text{--}500 \mu\text{g/mL}$ and the inhibition was reported as IC_{50} values in μMolar concentration. While the activity of the reference compound galanthamine was similar in both studies, 11-hydroxyvittatine, lycorine and haemanthamine were not active on the human AChE and BuChE. 8-O-demethylmaritidine and hamayne were inactive

on human BuChE but presented activity on erythrocyte AChE. The differences in the results can be explained by the biological source of the enzymes (human cholinesterases for [33] and electric eel AChE and horse (equine serum) BuChE in this work. For a better comparison of results, the use of enzymes from the same biological source should be recommended.

In summary, the AChE and BuChE inhibitory activity of the Chilean *Rhodophiala* species investigated led to an interesting source of inhibitors that do not contain the alkaloid galanthamine. Our results suggest that Chilean *Rhodophiala* could be a promising source of new alkaloids with effect towards cholinesterases. The difficulty in finding a species with high activity against AChE and BuChE, the similarity of the AChE and BuChE inhibitory values, the low complexity of the alkaloid profile of aerial parts of *R. splendens*, together with the absence of galanthamine-type alkaloids in this sample, prompted us to further explore the results.

3. Materials and Methods

3.1. Plant Material

The samples were collected in central-southern Chile and were identified following the reference [19,34]. *Rhodophiala andicola* (Poepp.) Traub, was collected at Sierra Nevada (Región de la Araucanía, 27 January 2016), the slopes of Volcán Lonquimay (Región de la Araucanía, Provincia del Malleco, 19 December 2016) and the slopes of Nevado de Chillán (Región del Bio-Bio, 30 December 2016). *Rhodophiala araucana* (Phil.) Traub was collected at Malalcahuello, Región de la Araucanía (19 December 2016), and *Rhodophiala montana* (Phil.) Traub at the roadside to Laguna del Maule, Región del Maule (2 January 2017). Samples from *Rhodophiala pratensis* (Poepp.) Traub were collected at Arcos de Calán, Región del Maule (12 December 2016) including plants growing on sand dunes and grasslands close to the sea. Plants with red and white flowers were collected separately. According to [34], the plant with red flowers fits the description of *R. pratensis*. *Rhodophiala splendens* (Renjifo) Traub was collected at Las Trancas, Región del Bio-Bio (2 January 2016). The plants were identified by Dr. Patricio Peñailillo, Herbario de la Universidad de Talca. Voucher herbarium specimens have been deposited at the Universidad de Talca as follows: *R. andicola* (N° 4081); *R. araucana* (N° 4083); *R. montana* (N° 4080); *R. pratensis* (N° 4084); *R. pratensis* (white flower) (N° 8085); *R. splendens* (N° 4082). A map with the collection places is shown in Figure 2. Pictures of the species investigated are illustrated in Figure 3.

3.2. Extraction

The freshly collected plant material was cleaned and separated into bulbs and aerial parts, frozen and lyophilized before extraction. The dry weight percentage was determined. The lyophilized plant material was extracted with MeOH under sonication for 10 min (3×) changing the solvent each time. The plant to solvent ratio ranged from 1:10 to 1:60 and was selected according to the volume of plant material for extraction. The combined MeOH solubles were taken to dryness under reduced pressure to afford the crude extracts. The crude extracts were then acidified to pH 3 with diluted H₂SO₄ (2%, v/v) and the neutral material was removed with Et₂O. The aqueous solutions were basified up to pH 9–10 with NH₄OH (25%, v/v) and extracted with EtOAc to provide the alkaloid extracts which were used for all experiments (enzyme inhibition assays and chemical analysis by GC-MS).

3.3. Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) Inhibitory Activity

Cholinesterase inhibitory activities were determined according to [35] with some modifications [36]. Stock solutions with 518U of AChE from *Electrophorus electricus* (Merck, Darmstadt, Germany) and BuChE from equine serum (Merck, Darmstadt, Germany), respectively, were prepared and kept at −20 °C. Acetylthiocholine iodide (ATCI), S-butyrylthiocholine iodide (BTCl) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Merck (Darmstadt, Germany). Fifty microliters of AChE or BuChE (both enzymes used at 6.24 U) in phosphate buffer

(8 mM K_2HPO_4 , 2.3 mM NaH_2PO_4 , 0.15 NaCl, pH 7.5) and 50 μ L of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 min at room temperature. Then, 100 μ L of the substrate solution (0.1 M Na_2HPO_4 , 0.5 M DTNB, and 0.6 mM ATCI or 0.24 mM BTCl in Millipore water, pH 7.5) was added. After 10 min, the absorbance was read at 405 nm in a Labsystem microplate reader (Thermo Fischer, Waltham, MA, USA). Enzyme activity was calculated as percent compared to a control using buffer without any inhibitor. Galanthamine served as positive control. In a first step, samples were assessed at 10, 100 and 200 μ g/mL towards both enzymes. Samples with an $IC_{50} > 200$ μ g/mL were considered inactive. Samples with an $IC_{50} < 200$ μ g/mL were further analyzed to determine the IC_{50} values. The cholinesterase inhibitory data were analyzed with the software Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA).

3.4. Alkaloids Identification and Quantification

3.4.1. Equipment

The equipment used for the identification and quantification of the alkaloids was a GC-MS 6890N apparatus (Agilent Technologies, Santa Clara, CA, USA) coupled with MSD5975 inert XL operating in the electron ionization (EI) mode at 70 eV. A Sapiens-X5 MS column (30 m $\times$ 0.25 mm i.d., film thickness 0.25 μ m) was used. The temperature gradient was as follows: 12 min at 100 $^{\circ}$ C, 100–180 $^{\circ}$ C at 15 $^{\circ}$ C/min, 180–300 $^{\circ}$ C at 5 $^{\circ}$ C/min and 10 min hold at 300 $^{\circ}$ C. The injector and detector temperatures were 250 and 280 $^{\circ}$ C, respectively, and the flow-rate of carrier gas (He) was 1 mL/min. Two mg of each alkaloid extract was dissolved in 1 mL of MeOH:CHCl₃ (1:1, v/v) and 1 μ L was injected using the splitless mode. Codeine (0.05 mg/mL) was used as an internal standard in all the samples.

3.4.2. Alkaloids Identification

Amaryllidaceae alkaloids occurring in the samples were identified by comparison of the R_t , fragmentation patterns and data interpretation of the spectra. The database used was built using single alkaloids previously isolated and identified by spectroscopic and spectrometric methods (NMR, UV, CD, IR, MS) in the Natural Products Laboratory, Universidad de Barcelona, the NIST 05 Database and literature data [37–42].

3.4.3. Alkaloid Quantification

To quantify the single constituents, a calibration curve of galanthamine (10, 20, 40, 60, 80 and 100 μ g/mL) was used. The same amount of codeine (0.05 mg/mL) was added to each solution sample as an internal standard. The peak areas were manually obtained considering selected ions for each compound (usually the base peak of their MS, i.e., m/z at 286 for galanthamine, at 299 for codeine). The ratio between the values obtained for galanthamine and codeine in each solution was plotted against the corresponding concentration of galanthamine to obtain the calibration curve and its equation ($y = 0.0224x - 0.2037$; $R^2 = 0.9977$). All data were standardized to the area of the internal standard (codeine) and the equation obtained for the calibration curve of galanthamine was used to calculate the amount of each alkaloid. Results are expressed as mg GAL, which was finally related to the alkaloid extract weight. As the peak area does not only depend on the corresponding alkaloid concentration but also on the intensity of the mass spectra fragmentation, the quantification is not absolute. However, the methodology is considered suitable to compare the specific alkaloid amount between samples [39,42].

3.5. Molecular Docking

The molecular docking simulations for the alkaloids identified in *Rhodophiala splendens*, the most promising species found in this study, were performed to investigate the binding mode into the active site of two different enzymes, namely *Torpedo californica* AChE (TcAChE) and hBuChE: proteins with PDB codes 1DX6 [43] and 4BDS [44], respectively. The 3D-structures of the alkaloids were drawn

using the Chemcraft program [45], and then submitted to a geometrical optimization procedure at PBE0 [46]/6-311+g* [47] level of theory using the Gaussian 09 program [48]. All optimized alkaloids were confirmed as a minimum on the potential energy surface. The docking simulations for the set of optimized ligands were performed using the AutoDock v.4.2 program [49]. AutoDock combines a rapid energy evaluation through pre-calculated grids of affinity potentials with a variety of search algorithms to find suitable binding positions for a ligand on a given macromolecule. To compare the results from the docking simulations, the water molecules, cofactors, and ions were excluded from each X-ray crystallographic structure. Likewise, the polar hydrogen atoms of the enzymes were added, and the non-polar hydrogen atoms were merged. Finally, the enzyme was treated as a rigid body. The grid maps of interaction energy for various atom types with each macromolecule were calculated by the auxiliary program AutoGrid, choosing a grid box with dimensions of $70 \times 70 \times 70$ Å around the active site, which was sufficiently large to include the most important residues of each enzyme. The docking searches for the best orientations of the ligands binding to the active site of each protein were performed using the Lamarckian Genetic Algorithm (LGA) [50]. The LGA protocol applied a population size of 2000 individuals, while 2,500,000 energy evaluations were used for the 50 LGA runs. The best conformations were chosen from the lowest docked energy solutions in the cluster populated by the highest number of conformations. The best docking complex solutions (poses) were analyzed according to the potential intermolecular interactions (ligand/enzyme), such as hydrogen bonding and the cation- π , π - π stacking.

Author Contributions: G.S.-H. and J.B. conceived and designed the experiments; L.R.T., N.C., E.H.O. and C.T. performed the experiments; L.R.T., J.B. and G.S.-H. analyzed the data; C.T. and G.S.-H. collected and processed the samples; G.S.-H., J.B., L.R.T. and E.H.O. wrote the paper.

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Sample Availability: Samples of the compounds are not available from the authors.



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4. DISCUSIÓN

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En el presente trabajo se ha estudiado el perfil alcaloídico de distintas especies de Amaryllidaceae recolectadas en América Latina, realizando el aislamiento de alcaloides y la elucidación estructural de nuevas estructuras presentes en las especies *Amaryllis belladonna*, *Hippeastrum reticulatum* y *Crinum amabile*. Además, se ha verificado el potencial de diferentes especies de Amaryllidaceae como fuente de nuevos productos bioactivos frente a diversas enfermedades tropicales y la enfermedad de Alzheimer. En los siguientes apartados se realiza una breve discusión de los resultados obtenidos.

4.1 *Amaryllis belladonna*

En este estudio se ha obtenido el perfil de alcaloides de la especie *Amaryllis belladonna* por CG-EM y RMN, así como se ha evaluado el potencial anti-protozoario del extracto crudo y de tres alcaloides aislados a partir de esta planta. Veintiséis alcaloides han sido identificados en esta especie y, basándonos en la similitud de las estructuras detectadas, se ha establecido una posible correlación biosintética entre estos alcaloides (**Figura 24**). Además, se han completado los datos espectrales de los compuestos 1-*O*-acetylcaranine y buphanamine.

4.1.1 Alcaloides identificados por CG-EM

Basándonos en las estructuras de los alcaloides identificados en *A. belladonna* y en informaciones ya existentes acerca de la biosíntesis de los alcaloides de las Amaryllidaceae, ha sido posible proponer una ruta de metabolización de los alcaloides identificados en esta planta (**Figura 24**), según la cual 1-*O*-acetylcaranine y 1-*O*-acetyllycorine serían derivados de los alcaloides caranine y lycorine, respectivamente, por medio de una reacción de acetilación del grupo hidroxilo en C-1 de ambas estructuras. A su vez, sugerimos que lycorine podría metabolizarse a anhydrolycorine por deshidratación de las posiciones C-1 y C-2, y que anhydrolycorine podría ser el precursor del alcaloide 11,12-dehydroanhydrolycorine a través de una reacción de reducción en el anillo-D. Este último compuesto serviría como precursor del compuesto hippadine por hidroxilación y oxidación de la posición C-6.

El compuesto crinine sería el precursor de todos los alcaloides del tipo al que da nombre que presentan el grupo metilendioxo. Buphanisine y powelline podrían derivar del alcaloide crinine mediante reacciones de metilación y metoxilación de las posiciones C-3 y C-7, respectivamente, las cuales también podrían ser consideradas etapas alternativas para la biosíntesis del compuesto buphanidine. La presencia de los alcaloides ambelline y 11-acetylambelline sugieren una hidroxilación en C-11 del compuesto buphanidine, seguida de una reacción de acetilación. Buphanidine sería el

precursor del compuesto 6-hydroxybuphanidrine, el cual podría originar 6-methoxybuphanidrine a través de una reacción de metilación. El alcaloide powelline metabolizaría las estructuras 3-*O*-acetylpowelline, por medio de una reacción de acetilación del sustituyente en C-3, y crinamidine, a través de la formación de un grupo epóxido en las posiciones C-2 y C-3. Crinamidine podría ser el precursor del alcaloide undulatine vía metilación del sustituyente en C-3, el cual a su vez, podría servir como vía para la síntesis de distichamine por medio de un rearrreglo de tipo alílico.

Por otro lado, 8-*O*-demethylmaritidine sería el precursor del alcaloide vittatine a través de la formación del grupo metilendioxo. A su vez, la acetilación del compuesto vittatine originaría el compuesto 3-*O*-acetyl vittatine. La presencia del alcaloide 3-*O*-acetylhamayne sugiere que los compuestos siculine, epivittatine y hamayne también deberían estar presentes en el extracto de *A. belladonna*, pero en bajas concentraciones o trazas.

4.1.2 1-*O*-Acetylcaranine

En este trabajo se presenta la completa elucidación estructural del compuesto 1-*O*-acetylcaranine a partir de espectroscopía de RMN mono y bidimensional. El espectro de ¹H-RMN de este compuesto se ha mostrado similar al del compuesto caranine (Mason et al., 1955; Miyakado et al., 1975; Lamoral-Theys et al., 2009). Sin embargo, el protón H-1 del compuesto 1-*O*-acetylcaranine se ha encontrado a campos magnéticos más bajos que su homólogo del compuesto caranine, sugiriendo la acetilación del grupo hidroxilo de la caranine que concuerda con la señal a δ 1.93 que integra para 3 protones en el espectro de ¹H-RMN. La presencia de una señal en δ 171.0 en el espectro de ¹³C-RMN ha servido para confirmar la existencia de un grupo carbonilo en la estructura. La baja magnitud de la constante de acoplamiento entre H-1 y H-10b ha permitido determinar la α -orientación del grupo acetoxilo en C-1. El protón H-4a ha mostrado proximidad especial con H-2 α y H-6 α a través del experimento de NOESY. La presencia de una correlación NOE entre H-6 α y H-12 α ha servido para confirmar la asignación del H-12 α en el espectro de ¹H-RMN.

4.1.3 Buphanamine

En el espectro de ¹H-RMN ha sido posible observar que los desplazamientos de buena parte de los protones de la buphanamine no se han mostrado de acuerdo con los

valores encontrados en la literatura (Sandager et al., 2005). Los hidrógenos H-4 α , H-4 β , H-4a, H-6 α , H-6 β , H-11endo, H-11exo, H-12endo y H-12exo publicados para la buphanamine han estado más desapantallados Δ +0.26, +0.22, +0.36, +0.26, +0.27, +0.14, +0.20, +0.24 y +0.51 ppm, respectivamente, sugiriendo que los datos publicados por Sandager y colaboradores (2005) corresponderían a buphanamine en forma de sal o a su *N*-óxido (Bastida et al., 2006). La constante de acoplamiento entre H-1 y H-2 (J = 5.5 Hz), H-2 y H-3 (J = 10.0 Hz), H-3 y H-4 α (J = 4.5 Hz), junto a una constante de cerca de 19.7 Hz entre H-4 α , y H-4 β , ha hecho posible establecer la presencia del grupo hidroxilo en C-1 y del doble enlace entre C-2 y C-3. La información obtenida mediante el estudio NOESY ha sido de gran importancia para establecer la orientación en α del grupo hidroxilo y la configuración absoluta de este compuesto ha sido determinada por dicroísmo circular (DC).

4.1.4 Actividad biológica

En la **Tabla 1** están descritos los resultados del potencial antiparasitario del extracto crudo de *A. belladonna* y de los tres alcaloides aislados a partir de esta planta. El compuesto 3-*O*-acetylhamayne ha mostrado actividad frente al *Trypanosoma brucei rhodesiense* (IC₅₀ = 1.51 μ g ml⁻¹), *Trypanosoma cruzi* (IC₅₀ = 8.25 μ g ml⁻¹), *Leishmania donovani* (IC₅₀ = 17.91 μ g ml⁻¹) y *Plasmodium falciparum* (IC₅₀ = 1.14 μ g ml⁻¹), sin embargo, este alcaloide ha presentado un bajo grado de selectividad. Por otro lado, el extracto crudo de *A. belladonna* ha mostrado actividad frente a *T. brucei rhodesiense* (IC₅₀ = 4.67 μ g ml⁻¹), *T. cruzi* (IC₅₀ = 34.86 μ g ml⁻¹) y *P. falciparum* (IC₅₀ = 1.17 μ g ml⁻¹), así como un mayor nivel de selectividad que el compuesto 3-*O*-acetylhamayne.

Tabla 1. Resultados *in vitro* de la actividad antiparasitaria y citotóxica de los compuestos aislados y del extracto crudo de *A. belladonna*.

Parasito	<i>T. b. rhodesiense</i>	<i>T. cruzi</i>	<i>L. donovani</i>	<i>P. falciparum</i> IEF (Intraerythrocytic)	Citotoxicidad
Agente	Trypomastigotes	Amastigotes	Amastigotes		
Cepa	STIB 900 (IC ₅₀ ^a)	Tulahuen C4 LacZ (IC ₅₀ ^a)	MHOM-ET- 67/L82 (IC ₅₀ ^a)	NF54 (IC ₅₀ ^a)	L6 (IC ₅₀ ^a)
melarsoprol	0.0035 ^b y 0.0010 ^c				
benznidazole		0.660 ^b y 1.080 ^c			
miltefosine			0.08 ^b y 0.091 ^c		
chloroquine				0.002 ^{b,c}	
podophyllotoxin					0.005 ^b y 0.007 ^c
1- <i>O</i> -acetylcaranine	1.97	35.9	>100	3.21	14.2
3- <i>O</i> -acetylhamayne	1.51	8.3	17.9	1.14	1.72
buphanamine	28.2	62.9	>100	25.9	>100
extracto crudo	4.67	34.9	>100	1.17	34.3

^aTodos los valores se cuantificaron como μ g ml⁻¹; ^bvalores de referencia para 1-*O*-acetylcaranine; ^cvalores de referencia para 3-*O*-acetylhamayne, buphanamine y extracto crudo.

4.2 *Hippeastrum reticulatum*

En esta tesis, se ha realizado el primer estudio acerca del perfil de alcaloides de la especie *H. reticulatum*. Se han identificado doce alcaloides en esta especie por CG-EM, cuatro de los cuales no habían sido reportados previamente en la literatura (**Figura 25**). Además, también se ha evaluado *in vitro* e *in silico* el potencial biológico de esta especie.

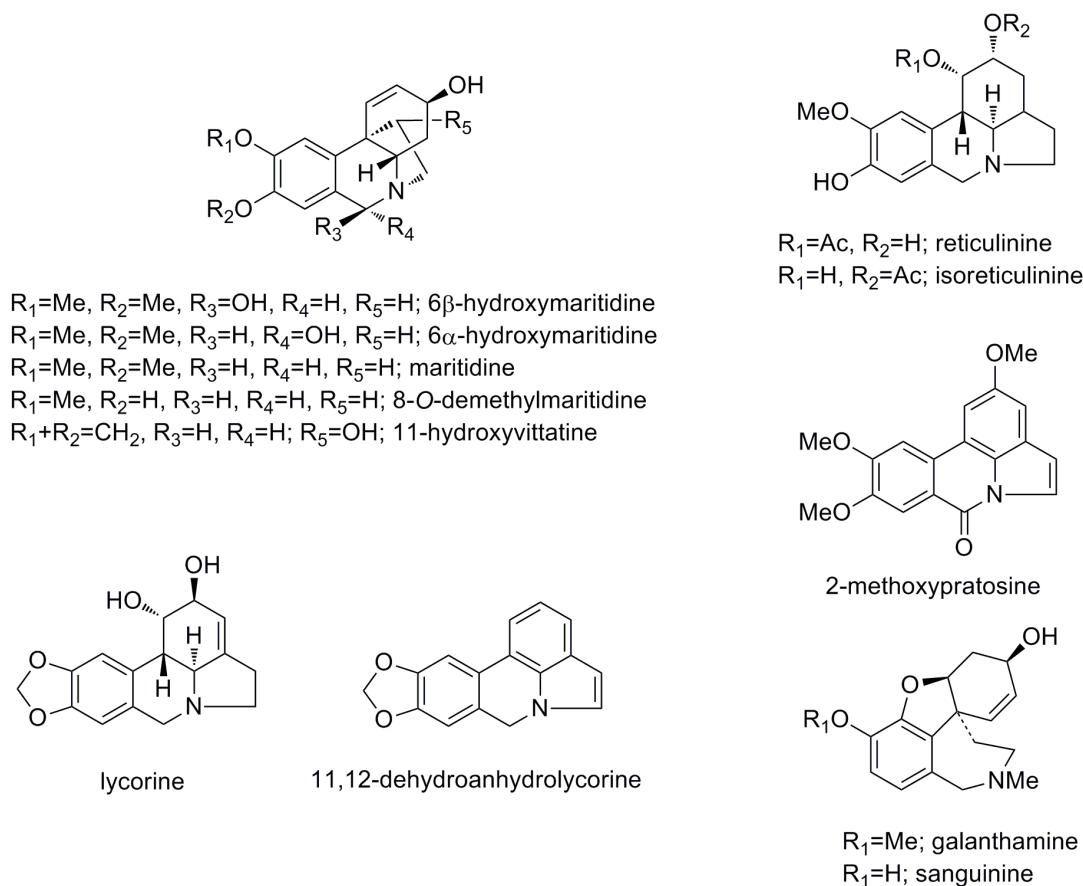


Figura 25. Alcaloides identificados en *H. reticulatum*.

4.2.1 6 β -Hydroxymaritidine y 6 α -hydroxymaritidine

Los compuestos 6 β -hydroxymaritidine y 6 α -hydroxymaritidine se han aislado como una mezcla de epímeros a partir de hojas de la especie *H. reticulatum*, no siendo posible separarlos por técnicas convencionales de cromatografía. El espectro de 1H -RMN ha mostrado similitud al obtenido por Bastida y col. (1990) para los compuestos papyramine y 6-epi-papyramine. La presencia de dos singuletes, a δ 5.35 y 6.12 ppm, se ha asignado a los protones H-6 de los epímeros 6 β - y 6 α -hydroxymaritidine, respectivamente. La existencia de dos metoxilos aromáticos en el espectro, los cuales

integran para 4.3 protones, ha hecho posible determinar la porción de 3:1 entre los epímeros 6 β - y 6 α -hydroxymaritidine, respectivamente. El estudio de NOESY entre los protones H-6 α y H-12endo ha servido para determinar la orientación en β del grupo hidroxilo en C-6 del compuesto 6 β -hydroxymaritidine. La configuración absoluta de estos epímeros ha sido determinada por dicroísmo circular (DC) y se ha confirmado su fórmula molecular (C₁₇H₂₂NO₄) por HRMS.

4.2.2 Reticulinine e isoreticulinine

Los compuestos reticulinine e isoreticulinine son isómeros y han sido aislados como una mezcla la cual no ha sido posible separar debido a la baja cantidad de muestra disponible. Los resultados obtenidos para estos isómeros por ¹H-RMN muestran una notable similitud a los reportados para los compuestos zephyranthine, 1,2-*O*-diacetylzephyranthine, sternbergine y 1-*O*-acetylcaranine (Herrera et al., 2001; Evidente et al., 1984; Tallini et al., 2017). En base a la información obtenida mediante el experimento bidimensional de NOESY, ha sido posible asignar el grupo metoxilo a la posición C-9 de ambas moléculas. La localización de los protones H-1 y H-2 a campos magnéticos bajos (δ 6.01 y 4.15 ppm, respectivamente) ha servido para determinar la presencia de los grupos acetoxilo e hidroxilo en las posiciones C-1 y C-2, respectivamente, del compuesto reticulinine. En el compuesto isoreticulinine, la localización de los protones H-1 y H-2 a bajos campos magnéticos (δ 4.77 y 5.12 ppm, respectivamente) ha indicado la presencia de un grupo hidroxilo en C-1 y acetoxilo en C-2, respectivamente. Entre los hidrógenos H-2 y H-3 α ha sido posible observar una constante de acoplamiento de 9.8 y 11.6 Hz para los compuestos reticulinine e isoreticulinine, respectivamente, compatible con una relación transdiaxial, lo que ha servido como base para determinar la configuración en ecuatorial del sustituyente en C-2 de ambas moléculas.

4.2.3 Actividad biológica

In vitro, se ha evaluado la actividad antiparasitaria e inhibidora de las colinesterasas de la mezcla de epimeros: 6 β - y 6 α -hydroxymaritidine. No se han realizado dichos estudios para los compuestos reticulinine e isoreticulinine debido a la baja cantidad de muestra obtenida de estos dos isómeros.

4.2.3.1 Actividad antiprotozoaria

Los compuestos 6 β - y 6 α -hydroxymaritidine han presentado baja actividad antiparasitaria frente a todos los protozoarios evaluados (**Tabla 2**).

Tabla 2. Resultados *in vitro* de actividad antiparasitaria y citotóxica de *H. reticulatum*. Resultados expresados en IC₅₀ ($\mu\text{g ml}^{-1}$).

Parasito	<i>T. b.</i> <i>rhodesiense</i> ^{*a}	<i>T.</i> <i>cruzi</i> ^{*b}	<i>L.</i> <i>donovani</i> ^{*c}	<i>P.</i> <i>falciparum</i> ^{*d}	Cytotoxicity ^{*e}
melarsoprol	0.0010				
benznidazole		1.080			
miltefosine			0.091		
chloroquine				0.002	
podophyllotoxin					0.007
6 β / 6 α -hydroxymaritidine	30.68	66.11	>100	32.86	>100

^{*a}Trypomastigotes (cepa STIB900); ^{*b}Amastigotes (cepa Tulahuen C4); ^{*c}Amastigotes (cepa MHOM-ET-67/L82); ^{*d}IEF (intraeritrocítica, cepa NF54; ^{*e}(cepa L6).

4.2.3.2 Actividad inhibidora de la ACE y BuCE

Se ha utilizado el alcaloide galanthamine como control para el ensayo de inhibición de la ACE y de la BuCE (IC₅₀ 1.56 ± 0.14 y 12.96 ± 0.65 μM , respectivamente). Los compuestos 6 β - y 6 α -hydroxymaritidine presentaron una baja inhibición de la ACE (IC₅₀ 90.43 ± 4.26 μM) y tampoco han resultado activos frente a la BuCE (IC₅₀ > 600 μM).

4.2.4 Docking molecular

Se han evaluado mediante docking molecular las nuevas estructuras identificadas en la planta *H. reticulatum* para determinar su potencial de inhibición de la ACE y BuCE (**Tabla 3**). *In silico*, isoreticulinine ha presentado una mejor inhibición de las estructuras 1DX6 y 4EY7 de la ACE en relación a los demás compuestos. En la **Figura 26** se encuentra la representación estructural de la mejor conformación del complejo activo de la TcACE con galanthamine (sustancia de referencia) y isoreticulinine. Galanthamine ha presentado una fuerte interacción con los residuos Glu199 y Ser200, mientras que isoreticulinine la ha tenido únicamente con el residuo Ser200.

Tabla 3. Energías estimadas para la inhibición de las enzimas ACE y BuCE por docking molecular. Valores expresados en kcal mol⁻¹.

Alcaloides	1DX6 ^a	4EY7 ^b	4BDS ^c
6 β -hydroxymaritidine	-8.49	-9.44	-8.95
6 α -hydroxymaritidine	-8.25	-8.75	-8.72
reticulinine	-8.88	-8.87	-8.10
isoreticulinine	-9.02	-9.80	-8.12
galanthamine	-9.36	-10.10	-8.74

^a1DX6: ACE (*Torpedo californica*); ^b4EY7: ACE (humana); ^c4BDS: BuCE (humana).

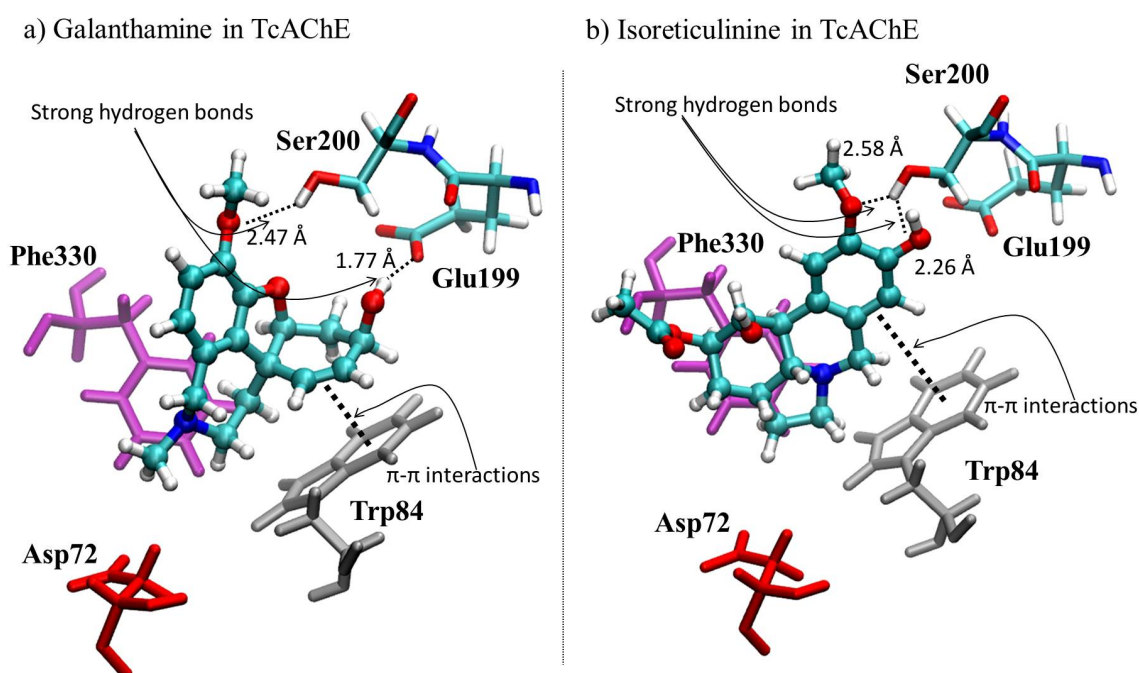


Figura 26. Representación de las interacciones entre los complejos galanthamine-*TcACE* (a) e isoreticulinine-*TcACE* (b).

El compuesto 6 β -hydroxymaritidine ha mostrado mejores resultados de inhibición de la *hBuCE* (estructura 4BDS) que galanthamine. En el sitio activo de esta enzima, es posible observar la presencia de dos residuos de aminoácidos importantes para que ocurra el efecto de inhibición, Glu197 y Ser198. Sin embargo, la interacción del alcaloide 6 β -hydroxymaritidine con el centro activo no favorece la formación de enlaces de hidrógeno con estos dos residuos de aminoácidos. Los resultados sugieren que las interacciones del tipo π - π y los enlaces de hidrógeno deberían estar en equilibrio para que ocurriera una inhibición ideal de la BuCE, siendo así, entre las moléculas testadas, isoreticulinine parece estar más cerca de esta definición. De esta forma, la molécula isoreticulinine podría ser catalogada como promisorio para la inhibición de la

ACE y BuCE debido a las fuertes interacciones mediante puentes de hidrógeno que presenta con los centros activos de estas dos enzimas (**Figura 27**).

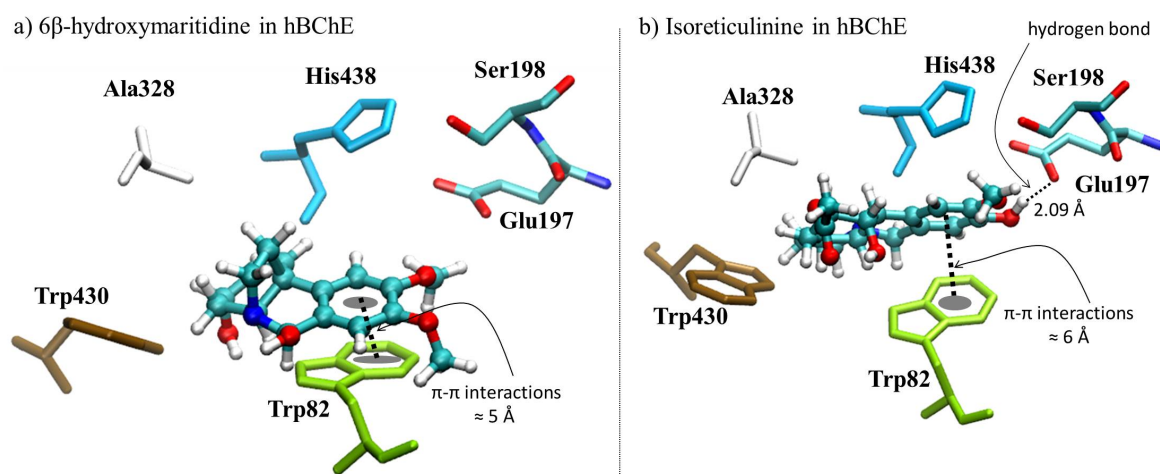


Figura 27. Representación de las interacciones entre los complejos 6 β -hydroxymaritidine-*h*BuCE (**a**) e isoreticulinine-*h*BuCE (**b**).

4.3 *Crinum amabile*

Se han identificado veintitrés alcaloides en la especie *C. amabile* por CG-EM y dos nuevas estructuras han sido caracterizadas químicamente por técnicas espectroscópicas (**Figura 28**). Además, se han aislado seis alcaloides de esta planta, incluyendo los compuestos nuevos.

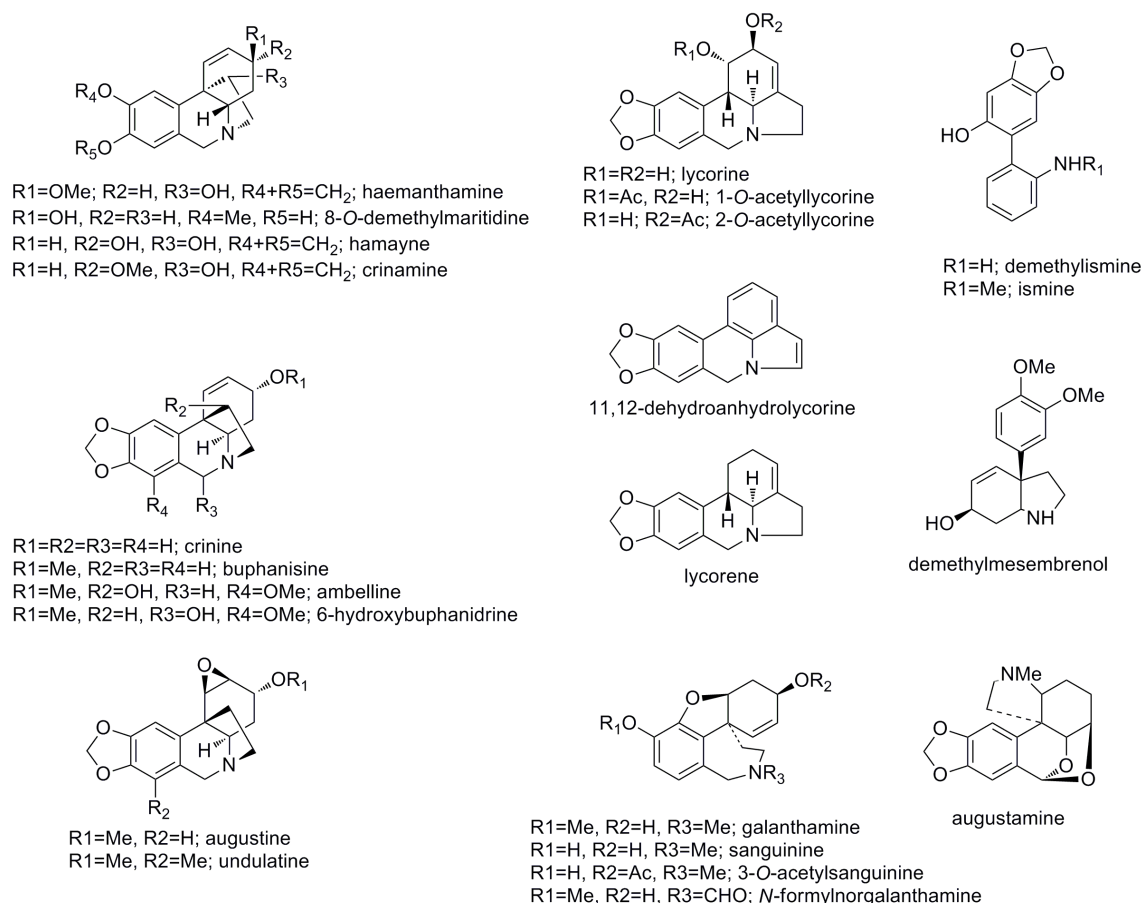


Figura 28. Alcaloides identificados en *C. amabile* por CG-EM.

4.3.1 Augustine *N*-oxide

La mayoría de las señales obtenidas en el espectro de ¹H-RMN para el compuesto augustine *N*-oxide están de acuerdo con los ya descritos en la literatura para augustine (Frahm et al., 1981), con excepción de las posiciones H-4a, H-4, H-6 y H-12. En augustine *N*-oxide, las posiciones H-4a, H-4α, H-6α, H-6β, H-12endo y H-12exo se encuentran 1.21, 0.36, 1.13, 0.26, 1.01 y 0.53 ppm, respectivamente, más desapantalladas que en augustine, lo que podría ser resultado de una estructura en la forma de sal o de la presencia de un grupo *N*-óxido. El análisis de HRMS ha revelado la presencia de un oxígeno adicional en relación a la augustine, lo que confirmaría la existencia de un *N*-óxido en esta estructura. La configuración absoluta de este

compuesto ha sido establecida por DC, siendo la curva obtenida cualitativamente similar a la de los alcaloides del tipo crinine.

4.3.2 Buphanisine *N*-oxide

Del mismo modo que para augustine *N*-oxide, la mayoría de las señales obtenidas en el espectro de ^1H -RMN para el compuesto buphanisine *N*-oxide han mostrado similitud con buphanisine (Viladomat et al., 1995). Sin embargo, las posiciones H-4 α , H-4a, H-6 α , H-6 β , H-12endo y H-12exo, se han encontrado más desapantalladas con variaciones de 0.80, 0.24, 0.32, 0.82, 0.86 y 0.29 ppm, respectivamente. La presencia de un grupo *N*-óxido en este compuesto ha sido confirmada por HRMS y su configuración absoluta ha sido establecida por DC.

4.3.3 Actividades biológicas

Se ha evaluado el potencial antiparasitario e inhibidor de las colinesterasas de seis alcaloides aislados de la especie *C. amabile*: augustamine, augustine, augustine *N*-oxide, buphanisine, buphanisine *N*-oxide y crinine (**Figura 29**).

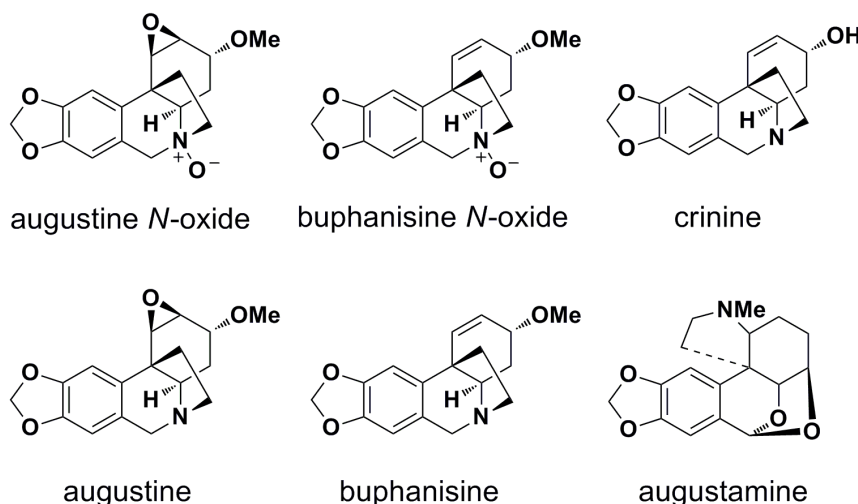


Figura 29. Alcaloides aislados de la especie *C. amabile*

4.3.3.1 Actividad antiprotozoaria

Los alcaloides aislados a partir de la planta *Crinum amabile* han presentado una baja actividad frente a todos los protozoos testados (**Tabla 4**). Los *N*-óxidos, augustine *N*-oxide y buphanisine *N*-oxide, dan lugar a una menor actividad del compuesto frente al *T. brucei* y al *P. falciparum* en relación al compuesto base. La existencia de un grupo

epóxido entre C-1 y C-2, parece disminuir la actividad del compuesto augustine frente al *P. falciparum* comparada con la presencia de un doble enlace en la misma posición como lo que ocurre en el alcaloide buphanisine. Además, la presencia de un grupo metoxilo en la posición C-3, como por ejemplo en buphanisine, parece aumentar la actividad frente al *P. falciparum* comparada con la existencia de un hidroxilo en la misma posición, como ocurre en crinine.

Tabla 4. Actividad antiparasitaria y citotóxica de los alcaloides aislados de *Crinum amabile*. Valores expresados en IC₅₀ (µg ml⁻¹).

Parasito Agente Cepa	<i>T. b. rhodesiense</i> Trypomastigotes STIB 900	<i>T. cruzi</i> Amastigotes Tulahuen C4	<i>L. donovani</i> Amastigotes MHOM-ET-67/L82	<i>P. falciparum</i> IEF (intraerythrocytic) NF54	Cytotoxicity L6
Fármaco de referencia	0.003 ± 0.001 ^a	0.865 ± 0.08 ^b	0.515 ± 0.06 ^c	0.004 ± 0.0007 ^d	0.004 ± 0.0007 ^e
Augustine	15.05 ± 1.06	56.00 ± 0.71	>100	14.20 ± 0.14	>100
Augustine <i>N</i> -oxide	58.85 ± 11.53	66.25 ± 11.81	>100	36.65 ± 4.74	>100
Buphanisine	16.5 ± 0.57	55.55 ± 4.60	>100	4.28 ± 0.18	72.85 ± 5.02
Buphanisine <i>N</i> -oxide	55.25 ± 4.31	64.05 ± 1.34	>100	32.55 ± 0.07	>100
Crinine	18.95 ± 0.78	57.45 ± 6.86	>100	30.95 ± 2.19	>100
Augustamine	19.20 ± 2.97	54.00 ± 4.53	>100	20.35 ± 0.21	81.55 ± 0.64

^amelarsoprol; ^bbenznidazole; ^cmiltefosine; ^dchloroquine; ^epodophyllotoxin

4.3.3.2 Actividad inhibitoria de la ACE y BuCE

Los resultados de actividad inhibitoria de las colinesterasas se encuentran resumidos en la **Tabla 5**. Ninguno de los alcaloides ha presentado actividad inhibitoria de la BuCE, sin embargo, augustine y buphanisine han presentado moderada y baja actividad, respectivamente, frente a la ACE. De esta manera, el grupo epóxido presente en augustine parece tener mejor efecto inhibidor de la ACE que el doble enlace presente en buphanisine. Por otro lado, la presencia del grupo *N*-óxido en los compuestos augustine *N*-oxide y buphanisine *N*-oxide no ha incrementado la actividad inhibitoria de la ACE en comparación con los valores obtenidos para augustine y buphanisine, respectivamente. Entre los resultados obtenidos para crinine y buphanisine, la presencia de un hidroxilo en C-3 da lugar a una mayor actividad comparativamente con el grupo metoxilo.

Tabla 5. Resultados de inhibición de las colinesterasas a partir de los alcaloides aislados de la especie *Crinum amabile*. Valores expresados en IC₅₀ (µg ml⁻¹).

alcaloide	ACE*	BuCE*
augustine <i>N</i> -oxide	79.64 ± 5.26	>200
buphanisine <i>N</i> -oxide	>200	>200
agustamine	>200	>200
augustine	45.26 ± 2.11	>200
buphanisine	183.31 ± 36.64	>200
crinine	163.89 ± 15.69	>200
galanthamine	0.45 ± 0.03	3.88 ± 0.19

4.4 Diferentes especies de *Rhodophiala* recolectadas en Chile como fuente de alcaloides bioactivos para el tratamiento de la enfermedad de Alzheimer

En esta tesis se ha obtenido el perfil de alcaloides de veinte extractos de cinco especies diferentes de *Rhodophiala* recolectadas en Chile mediante CG-EM. Cada compuesto ha sido cuantificado como equivalentes de galanthamine por gramo de extracto rico en alcaloide (GAL g⁻¹ ERA). *In vitro*, se ha evaluado el potencial inhibidor de la ACE y BuCE de los diferentes extractos obtenidos. Además, tanto *in vitro* como *in silico*, se ha verificado el potencial inhibidor de las colinesterasas de algunos alcaloides identificados en estas muestras.

4.4.1 Actividad inhibitoria de la ACE y BuCE

Los mejores resultados de inhibición de la enzima ACE han sido obtenidos para los bulbos de *R. pratensis* con flores blancas (muestra Q) y de *R. splendens* (muestra S), con valores de IC₅₀ de 3.32 ± 0.26 y 3.62 ± 0.02 µg ml⁻¹, respectivamente (**Tabla 6**). Aproximadamente 50% de los extractos han mostrado actividad inhibitoria de la BuCE, obteniéndose los mejores resultados para bulbos y partes aéreas de *R. splendens* (muestras S y T, respectivamente), con valores de IC₅₀ de 14.37 ± 1.94 y 16.26 ± 3.34 µg ml⁻¹. A partir de estos resultados, sugerimos que la especie *R. splendens* sería la más promisorio para el desarrollo de nuevas moléculas para el tratamiento de la enfermedad de Alzheimer.

Tabla 6. Resultados de actividad inhibitoria de las colinesterasas *in vitro* de extractos de varias especies del género *Rhodophiala*. Resultados expresados en IC₅₀ (µg ml⁻¹).

código	Nombre científico	Localidad de recolecta	ACE	BuCE
(A)	<i>R. andicola</i> ^{*a}	Sierra Nevada	13.29 ± 1.01	45.76 ± 9.72
(B)	<i>R. andicola</i> ^{*b}	Sierra Nevada	18.16 ± 2.94	138.27 ± 6.83
(C)	<i>R. andicola</i> ^{*a}	Nevado de Chillán	7.26 ± 0.16	47.38 ± 4.08
(D)	<i>R. andicola</i> ^{*b}	Nevado de Chillán	12.30 ± 0.74	43.41 ± 2.64
(E)	<i>R. andicola</i> ^{*a}	Volcan Lonquimay	22.77 ± 1.57	113.24 ± 2.77
(F)	<i>R. andicola</i> ^{*b}	Volcan Lonquimay	74.44 ± 5.53	>200
(G)	<i>R. araucana</i> ^{*a}	Malalcahuello	6.23 ± 0.24	45.71 ± 3.51
(H)	<i>R. araucana</i> ^{*b}	Malalcahuello	IS*	IS*
(I)	<i>R. montana</i> ^{*a}	Laguna del Maule	18.13 ± 0.51	40.05 ± 9.03
(J)	<i>R. montana</i> ^{*b}	Laguna del Maule	33.57 ± 2.16	16.38 ± 0.78
(K)	<i>R. pratensis</i> ^{*a,c,d}	Dunas	11.81 ± 0.17	>200
(L)	<i>R. pratensis</i> ^{*b,c,d}	Dunas	72.59 ± 4.26	>200
(M)	<i>R. pratensis</i> ^{*a,c,e}	Arcos de Calán	44.23 ± 4.08	>200
(N)	<i>R. pratensis</i> ^{*b,c,e}	Arcos de Calán	102.27 ± 6.61	>200
(O)	<i>R. pratensis</i> ^{*a,c,d}	Arcos de Calán	47.66 ± 1.78	>200
(P)	<i>R. pratensis</i> ^{*b,c,d}	Arcos de Calán	31.97 ± 3.24	>200
(Q)	<i>R. pratensis</i> ^{*a,f}	Arcos de Calán	3.32 ± 0.26	52.16 ± 0.57
(R)	<i>R. pratensis</i> ^{*b,f}	Arcos de Calán	8.39 ± 0.27	>200
(S)	<i>R. splendens</i> ^{*a}	Las Trancas	3.62 ± 0.02	14.37 ± 1.94
(T)	<i>R. splendens</i> ^{*b}	Las Trancas	5.78 ± 0.93	16.26 ± 3.34

*IS insuficiente cantidad de muestra para realizar ensayos biológicos; ^{*a}bulbos; ^{*b}parte aérea; ^{*c}flores rojas; ^{*d}nl: sin hojas; ^{*e}L: con hojas; ^{*f}WF: flores blancas.

4.4.2 Alcaloides identificados por CG-EM

Mediante CG-EM, se han identificado treinta y siete alcaloides conocidos en las distintas muestras de *Rhodophiala* (**Figura 30**). El perfil alcaloídico de cada extracto y la cantidad de cada compuesto están representados en la **Tabla 12** de la sección Anexos. El número de alcaloides detectados en cada extracto ha variado entre 8 (muestra **B**) y 23 (muestra **K**). Asimismo, aparte de los alcaloides conocidos, no se ha podido identificar un buen número de estructuras, sugiriéndose que estas muestras podrían tener un elevado potencial en la búsqueda de nuevos alcaloides de las Amaryllidaceae.

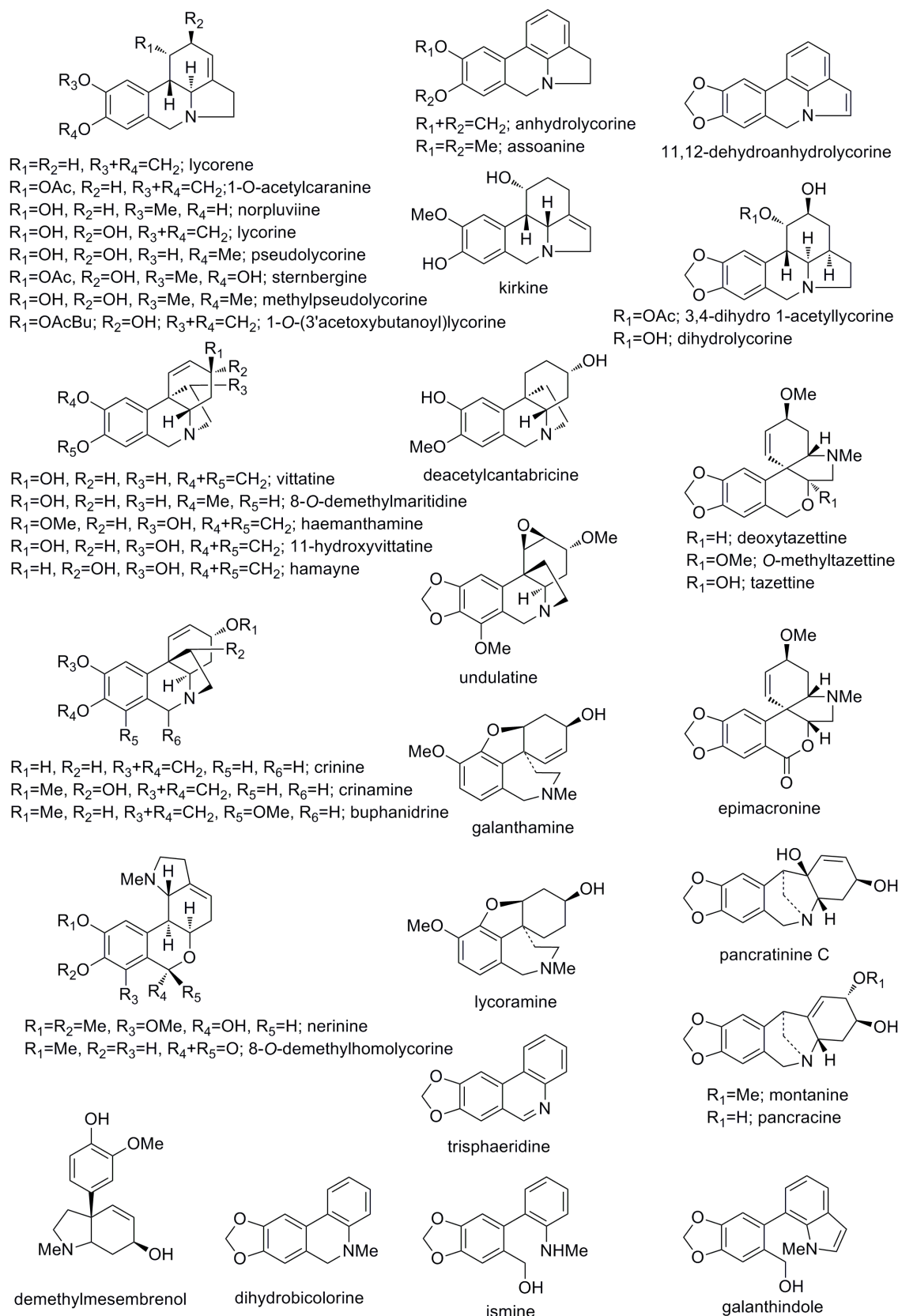


Figura 30. Alcaloides identificados en distintos extractos de diferentes especies del género *Rhodophiala*.

Las concentraciones más elevadas de alcaloides se detectan en las partes aéreas de *R. andicola* recolectada en el Volcan Lonquimay (muestra **F**) y en las partes aéreas de *R. pratensis* con flores color crema (muestra **R**), 311.1 y 274.1 mg GAL g⁻¹ ERA, respectivamente. Por otro lado, las más bajas se han cuantificado en las partes aéreas de *R. andicola* recolectada en Sierra Nevada (muestra **B**) y en las partes aéreas de *R. pratensis* con flores rojas y sin hojas (muestra **P**), 133.2 y 138.1 mg GAL g⁻¹ ERA, respectivamente.

Los alcaloides del tipo lycorine, haemanthamine/crinine y tazettine fueron los predominantes en el 70% de las muestras analizadas. Las estructuras del tipo galathamine han sido detectadas en las especies *R. andicola*, *R. araucana* y *R. montana*, mientras que las del tipo montanine han sido identificadas en todas las especies a excepción de *R. andicola*. En algunas muestras de *R. pratensis* ha sido posible detectar pequeñas concentraciones de demethylmesembrenol, alcaloide del tipo mesembrenone característico del género *Sceletium*.

4.4.3 Docking molecular

En los estudios realizados *in vitro*, *R. splendens* ha sido la especie con un mejor grado de inhibición de ambas colinesterasas. Por CG-EM, ha sido posible detectar 17 compuestos en la parte aérea de esta planta, siendo 15 de ellos fácilmente identificados. En la **Tabla 7**, los estudios de docking molecular de cada uno de estos 15 alcaloides identificados en *R. splendens* (muestra **T**) revelan con valores numéricos cuales de ellos inhibirían en mejor proporción las enzimas ACE y BuCE.

Tabla 7. Resultados de docking molecular para los alcaloides identificados en la parte aérea de *R. splendens* (ACE y BuCE). Valores expresados en kcal mol⁻¹.

Alcaloide	ACE ^{*a}	BuCE ^{*b}
11-hydroxyvittatine	-8.43	-9.03
lycorine	-8.82 ^{*c}	-8.94
8- <i>O</i> -demethylmaritidine	-8.74 ^{*d}	-8.93
hamayne	-8.28	-8.54
deacetylcantabricine	-7.90	-8.43
haemanthamine	-8.80	-8.34
galanthamine	-9.55 ^{*c}	-8.23 ^{*c}
epimacronine	-9.36 ^{*c}	-7.63
tazettine	-8.66 ^{*c}	-7.87
<i>O</i> -methyltazettine	-8.54	-7.87
11,12-dehydroanhydrolycorine	-8.41	-7.44
galanthindole	-7.81	-7.41
trisphaeridine	-7.38	-7.27
dihydrobicolorine	-7.33	-7.38
ismine	-6.78	-7.08

^{*a}PBD código: 1DX6; ^{*b}PBD código: 4BDS; ^{*c}Cortés et al., 2015; ^{*d}Cortés et al., 2018.

Ningún alcaloide identificado en la muestra **T** ha presentado un mejor potencial inhibidor de la ACE que la galanthamine. Sin embargo, seis alcaloides identificados en dicha muestra han exhibido, *in silico*, mejor actividad de inhibición de la *h*BuCE (estructura 4BDS) que galanthamine. Para cada uno de estos seis alcaloides se han llevado a cabo ensayos de inhibición de las colinesterasas *in vitro* para comprobar los resultados obtenidos *in silico* (**Tabla 8**).

Tabla 8. Resultados de actividad inhibitoria de la ACE y BuCE *in vitro* para los alcaloides identificados en la parte aérea de *R. splendens* que han presentado mejor actividad *in silico* en el centro activo de estas dos enzimas. Valores expresados en IC₅₀ (μg ml⁻¹).

Alcaloide	ACE	BChE
11-hydroxyvittatine	122.17 ± 22.03	>200
lycorine	101.70 ± 23.79	>200
8- <i>O</i> -demethylmaritidine	113.21 ± 8.21	127.87 ± 2.45
hamayne	135.09 ± 15.33	48.40 ± 1.13
deacetylcantabricine	>200	>200
haemanthamine	184.68 ± 11.58	>200
galanthamine ^a	0.48 ± 0.07	3.70 ± 0.24

^aSubstancia de referencia

Existen dos lugares activos muy importantes en la enzima *hBChE*: el primero corresponde a la tríada catalítica, compuesta por los residuos de His438, Ser198 y Glu325, y el segundo corresponde al lugar de unión de la colina (sitio α -aniónico) compuesto por los residuos Trp82 y Phe329 (Nicolet et al., 2003). El alcaloide 11-hydroxyvittatine presenta dos interacciones mediante puentes de hidrógeno, con los residuos Trp82 y Trp430; sin embargo, esta molécula no presenta ninguna interacción cerca de la tríada catalítica, mientras que hamayne presenta una interacción por puente de hidrógeno con el residuo Gly115, un aminoácido localizado cerca de la tríada catalítica His438, Ser198 y Glu325. En relación a las interacciones en el lugar de unión de la colina (sitio α -aniónico), ambos alcaloides presentan la misma interacción π - π con Trp82. De esta manera, los resultados *in silico* sugieren que la presencia de un grupo hidroxilo β -orientado en C-3 en el compuesto 11-hydroxyvittatine podría aumentar el potencial teórico de inhibición de la *hBuCE* (estructura 4BDS) en $0.49 \text{ kcal.mol}^{-1}$, al compararlo con hamayne. Sin embargo, los estudios *in vitro* del alcaloide hamayne han presentado mejores resultados de inhibición de la BuCE que 11-hydroxyvittatine (**Tabla 8**). Para explicar estos resultados, se podría sugerir que *in vitro* la β orientación del hidroxilo en C-3 probablemente estaría dificultando el alcance del alcaloide 11-hydroxyvittatine a la tríada catalítica presente en el sitio centro de la BuCE (**Figura 31**).

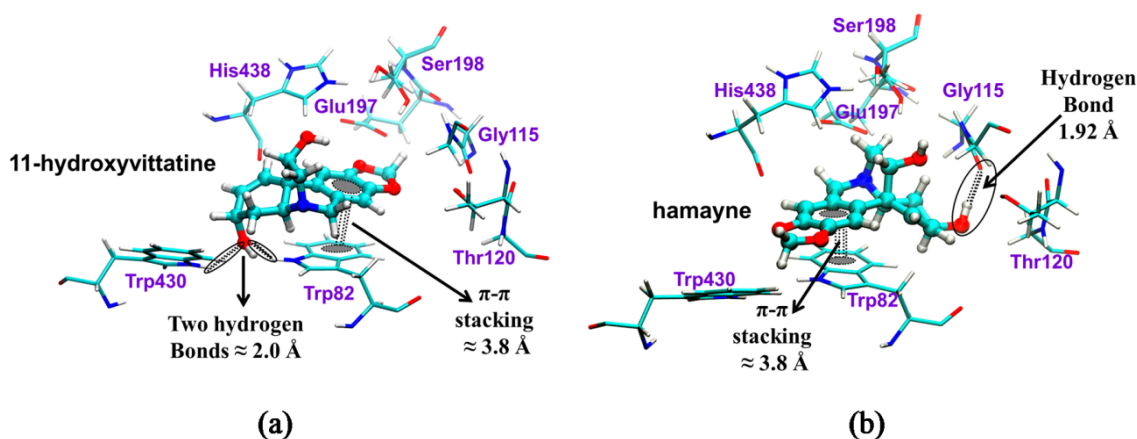


Figura 31. Representación gráfica de las interacciones de 11-hydroxivittatine y hamayne en el centro activo de la enzima *hBuCE*.

5. CONCLUSIONES

5. CONCLUSIONES

1. El estudio de la especie *Amaryllis belladonna* L. ha conducido a la identificación de veintiséis alcaloides y, en base a sus estructuras hemos establecido su relación biosintética. Tanto el compuesto 3-*O*-acetylhamayne como el extracto crudo han presentado una notable actividad antiparasitaria, aunque su índice de selectividad es bajo. Simultáneamente, los estudios de RMN 2D han permitido completar la caracterización estructural de los alcaloides 1-*O*-acetylcaranine y buphanamine.
2. Se ha realizado el primer estudio del contenido en alcaloides de la especie *Hippeastrum reticulatum* Herb., identificándose doce compuestos, cuatro de los cuales no se habían reportado con anterioridad: 6 β -hydroxymaritidine, 6 α -hydroxymaritidine, reticulinine e isoreticulinine. Asimismo, se han llevado a cabo los estudios *in vitro* e *in silico* para evaluar el potencial biológico de los nuevos compuestos. Los resultados *in silico* sugieren que la molécula isoreticulinine podría considerarse como promisorio para la inhibición de la ACE y la BuCE debido a las interacciones observadas entre ligando y proteína.
3. De la especie *Crinum amabile* Donn. se han identificado veinticinco alcaloides y, de ellos, augustine *N*-oxide y buphanisine *N*-oxide se han aislado y caracterizado por primera vez de una fuente natural, lo que confirma el potencial que las especies de la familia Amaryllidaceae mantienen como fuente de nuevos productos bioactivos.
4. Finalmente, hemos obtenido el perfil alcaloídico de distintas especies del género *Rhodophiala* C. Presl. recolectadas en Chile, verificando su potencial como inhibidores de las colinesterasas. Se han podido identificar 37 alcaloides de las distintas muestras y, a pesar de que el alcaloide galanthamine no se encontraba presente en las especies estudiadas, el extracto rico en alcaloides de la parte aérea de *Rhodophiala splendens* ha presentado una notable capacidad inhibidora de la ACE y BuCE. En relación a ello, se ha llevado a cabo estudios *in vitro* e *in silico* con los alcaloides identificados en esta muestra, obteniendo los mejores resultados de inhibición de la BuCE *in vitro* para el compuesto hamayne,

mientras que para el estudio *in silico* lo ha sido la 11-hydroxyvittatine. El hidroxilo en β de la posición 3 de la 11-hydroxyvittatine dificultaría el acceso a la tríada catalítica presente en el centro activo de la BuCE.

6. BIBLIOGRAFÍA

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7. ANEXOS

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7.1 Anexo I: Capítulo de Revisión

Research in natural products: Amaryllidaceae ornamental plants as sources of bioactive compounds

Laura Torras-Claveria, Luciana Tallini, Francesc Viladomat y Jaume Bastida

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5. Research in natural products: Amaryllidaceae ornamental plants as sources of bioactive compounds

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Abstract. Amaryllidaceae plants are known for their ornamental flowers all over the world, but they also have a medicinal value owing to their exclusive group of alkaloids. The Amaryllidaceae alkaloids have a wide range of important biological activities, notably anti-tumoral, anti-parasitic, and acetylcholinesterase inhibition. This review focuses on the chemical characteristics of Amaryllidaceae plants and alkaloids, as well as the different methodologies applied in their study, including promising new docking studies.

Introduction

Amaryllidaceae plants are known for their outstanding attractive flowers (Figure 1), and are widely used and cultivated for ornamental purposes. For example, *Narcissus* species are extensively cultivated and exported for

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ornamental use in the UK and the Netherlands, as are *Lycoris* species in China and Japan, while *Crinum* species are appreciated for their lily-like flowers [1].



Figure 1. *Hymenocallis* spp. (Amaryllidaceae) from Venezuela

Amaryllidaceae plants also have a long history of use for medicinal purposes. The specific type of alkaloids they contain, named Amaryllidaceae alkaloids, display a wide range of biological activities such as antiviral, antimalarial, anticancer and anticholinesterasic [2].

1. Amaryllidaceae family characteristics

The Amaryllidaceae family, of the order Asparagales, consists of bulbous flowering plants, and is divided in three subfamilies (Agapanthoideae, Allioideae and Amaryllidoideae) previously considered as three separate families. The term “Amaryllidaceae”, whether referring to plants or alkaloids, is ubiquitous in the phytochemical and pharmaceutical literature on the subfamily Amaryllidoideae [1, 3]. The monocotyledonous Amaryllidoideae subfamily comprises around 1000 species in 60 genera with a pantropical distribution, above all in three geographic locations: Andean South America, southern Africa and the Mediterranean coast. Amaryllidoideae plants have a high capacity for adaptation. Those of the genera *Leptochiton* and *Paramongaia* survive in areas with a very arid

climate, since bulbs can remain latent for long periods, while *Hippeastrum* and *Hymenocallis* thrive in humid woods [4].

The medicinal use of Amaryllidaceae plants goes back to the Classical period, when Hippocrates and Dioscorides were already using *Narcissus* oil to treat illnesses thought to be linked to uterine tumours. References to *Narcissus* usage against cancer are also found in Pliny the Elder and the Bible. Today Amaryllidaceae plants are still extensively used in traditional medicine in several countries. For example, *Ammocharis* is cooked and used as an enema for blood cleansing or to cure open wounds; bulbs of *Brunsvigia* are applied as antiseptic dressing on fresh wounds and their decoctions treat coughs, colds, and liver diseases; *Clivia* species are used to treat snakebites and wounds, and to facilitate birth; *Crinum* species are applied as a powerful emetic, to treat tumours (Asia and America) and colds, wash wounds and haemorrhoids, as a gynecological remedy (South Africa) and a rubefacient in the treatment of rheumatism (India) [5].

2. Amaryllidaceae alkaloids

Virtually exclusive to the Amaryllidoideae subfamily, the Amaryllidaceae alkaloids have anti-viral, anti-parasitic, anti-cancer and anti-

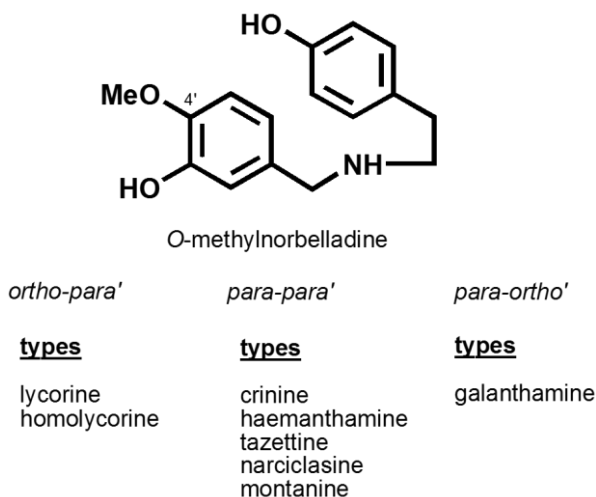


Figure 2. Three options of cyclization of the precursor *O*-methylnorbelladine and the resulting alkaloid types.

cholinesterasic activities, which are probably the origin of many of the traditional medicinal uses of Amaryllidaceae plants. Although structurally diverse, Amaryllidaceae alkaloids are biogenetically related and can be classified into nine basic skeleton groups: norbelladine-, lycorine-, homolycorine-, crinine-, haemanthamine-, narciclasine-, tazettine-, montanine-, and galanthamine-types. However, there are exceptions like graciline or plicamine, which cannot be classified in any of these classical groups, and a new plicamine-type alkaloid was recently identified in *Narcissus broussonetii* [6].

All Amaryllidaceae alkaloids derive from the amino acids L-phenylalanine and L-tyrosine, from which the common precursor *O*-methylnorbellamine is synthesized. A final cyclisation step with different phenol oxidative couplings leads to a diversification of structures (Figure 2) [7].

3. Study of the genus *Narcissus*

The genus *Narcissus* comprises more than one hundred wild species and is mainly distributed around the Mediterranean Sea, including south-western Europe, North Africa, Italy and the Balkans. This genus has an ornamental value, and its easy hybridization has allowed numerous cultivars to be developed.

The traditional medicinal uses of this genus are well documented. The Bible mentions the treatment of symptoms that could now be defined as cancer with *Narcissus poeticus*. Chinese, North African and Arabian medicine continues using this treatment. Today, it is known that *N. poeticus* contains 0.012% of the antineoplastic agent narciclasine [8].

The first isolated Amaryllidaceae alkaloid was lycorine, from *Narcissus pseudonarcissus* by Gerrard in 1877 [9]. Since then, numerous Amaryllidaceae alkaloids have been isolated in more than 40 wild species and over 100 cultivars. The most common alkaloids in *Narcissus* species are lycorine- and homolycorine-types; notably, they do not contain crinine-type alkaloids, as they synthesise alkaloids with the 5,10b-ethano bridge in an α , not β position [8].

Amaryllidaceae alkaloids isolated in the genus *Narcissus* were reviewed by Bastida et al. (2006) [8], and others have been identified by the same group since then [6, 7, 10, 11, 12, 13, 14].

4. Amaryllidaceae plants in traditional medicine in South Africa

Folk medicine still plays a crucial role in the healthcare of a great part of the population in South Africa. It has been estimated that around 27 million South Africans consult traditional healers. Contrary to popular belief, this practice is not restricted to poor and uneducated people, whose access to western medicine is limited by cost or distance, but extends to all sectors of the society [15, 16].

The use of Amaryllidaceae plants in traditional South African medicine by indigenous people has a long history, and some applications were also adopted by early European colonists. Today several concoctions, decoctions, extracts and herbal preparations of Amaryllidaceae species can be found in local traditional medicinal markets. For example, *Apodolirion buchananii* is taken for stomach disorders and *Brunsvigia* species are used against infertility in [16]. Since the application of Amaryllidaceae plants to wounds is widespread, it was suspected that they had antibacterial properties. Indeed, several Amaryllidaceae alkaloids have been isolated and identified in these plants and their antibacterial [17], antifungal [18] antiviral [19], anti-inflammatory and antiparasitic [18] activities have been determined.

5. Amaryllidaceae alkaloids from Amaryllidaceae plants in Ibero-America

Ibero-America, together with South Africa, is a centre of diversification of the Amarylloideae subfamily. While many species remain unexplored, knowledge of Ibero-American Amaryllidaceae plants has advanced considerably in recent years. For example, the novel alkaloid phaedranamine was identified together with 7 known alkaloids in *Phaedranassa dubia* (Colombia) [20]. *Zephyranthes concolor* (Mexico) was studied for the first time in 2011 and 6 Amaryllidaceae alkaloids were identified [21]. Wild Amaryllidaceae species (*Habranthus jamesonii*, *Phycella herbertiana*, *Rhodophiala mendocina*, and *Zephyranthes filifolia*) from the Argentinian Andes were analysed for their alkaloid composition for the first time in 2011, revealing the presence of galanthamine and good acetylcholinesterase activity [22]. The alkaloid composition of the Colombian endemic species *Caliphruria subedentata*, one of only four species of the infrequent genus *Caliphruria*, was analysed by GC-MS and 18 alkaloids were identified, in addition to six others isolated with classical phytochemical methods [23]. The new alkaloid 1-epidemethylbowdensine

was reported for the first time in *Crinum erubescens* collected in Costa Rica [24].

Hippeastrum is a well-known ornamental genus from South America with around 70 species, 34 of them found in Brazil, which according to nrDNA ITS sequences is the area of origin of the genus [25]. In a review focusing on the chemistry and biological activity of Amaryllidaceae alkaloids in the genus *Hippeastrum*, de Andrade et al. (2012) [25] include several new Amaryllidaceae alkaloids they identified for the first time, notably 2 α ,7-dimethoxyhomolycorine and candimine in *Hippeastrum morelianum* [26], and 11 β -hydroxygalanthamine in *Hippeastrum papilio* [27]. Other new alkaloids have been identified for the first time in the Brazilian species *Hippeastrum aulicum* and *H. calyptratum* (aulicine, 3-*O*-methylepipimacowine, 11-oxohaemanthamine and 7-methoxy-*O*-methyllycorenine) [28], and *H. breviflorum* (9-*O*-demethyllycosinine B) [29]. Recently, more new Amaryllidaceae alkaloids have been identified in *H. papilio* (hippapiline, papiline, 3-*O*-demethyl-3-*O*-(3-hydroxybutanoyl)-haemanthamine) [30].

In Argentina, the genus *Hippeastrum* comprises nine widely distributed and poorly studied species, some of them used in traditional medicine by the Toba indigenous community. *H. argentinum* has recently been studied in terms of alkaloid composition and biological activity and two new alkaloids were identified (4-*O*-methylnangustine and 7-hydroxycyclivonine). Furthermore, promising docking studies activities are being carried out [31].

6. Study of galanthamine-producing species

Galanthamine is an Amaryllidaceae alkaloid sold as a drug under the commercial name of Reminyl® in Europe and Razadine® in the USA for the palliative treatment of mild to moderate stages of Alzheimer's disease. Galanthamine can be obtained by chemical synthesis, but the yield is too low to be economically feasible. Instead, it is obtained from natural sources such as *Galanthus nivalis*, *Leucojum aestivum*, *Lycoris radiata* and different species of *Narcissus*. However, several problems, including unsuccessful cultivation or slow regeneration, make it difficult to meet the increasing pharmaceutical demands for this drug [14]. Another issue is the great chemodiversity in one of the main industrial sources of galanthamine, *Leucojum aestivum*. Balearic populations of this species have an alkaloid profile dominated by crinine-type

compounds, while in those close to the Danube River homolycorine-type alkaloids are dominant. In populations from east Bulgaria the main alkaloid is lycorine, and only populations in south Bulgaria were found to predominantly contain galanthamine-type compounds. Thus, the content of galanthamine in *Leucojum aestivum* can range from 0.2 to 95% of total alkaloids [32].

More than 27,000 names of *Narcissus* ornamental cultivars have been registered in the International Daffodil Register and some intersectional cultivars have also been reported as potential sources of galanthamine. These cultivars present some advantages for alkaloid production, as they are less affected by planting depth and density, and large-scale cultivation has already been established for the ornamental plant industry [14]. In this context, 105 ornamental varieties of *Narcissus* were analysed by GC-MS for

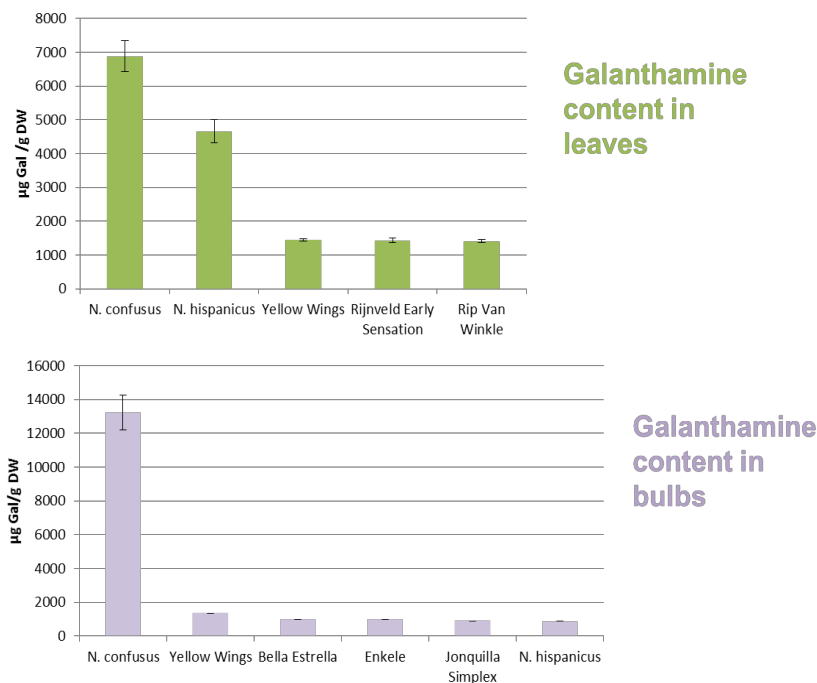


Figure 3. *Narcissus* varieties with highest galanthamine content

their galanthamine content and acetylcholinesterase inhibitory activity, distinguishing between bulbs and leaf tissues. The highest content of galanthamine was found in leaves from *N. hispanicus*, followed by the cultivars ‘Rijnveld Early Sensation’ and ‘Rip van Winkle’, and the bulbs of the cultivars ‘Yellow Wings’ and ‘Bella Estrella’, which could constitute promising new sources of this valuable metabolite (Figure 3) [14].

The total alkaloid composition of these varieties was also determined by GC-MS, comparing alkaloid mass spectra and the Kovats Retention Index with those of authentic standards previously isolated and identified by spectroscopic methods by our group in other Amaryllidaceae plants. Also, a k-means cluster analysis was performed with ornamental varieties according to the alkaloid composition of leaves and bulbs separately, obtaining 5 clusters of cultivars in each tissue. A correspondence analysis was performed to detect a possible relationship between those clusters according to the alkaloid composition and horticultural divisions of *Narcissus* ornamental varieties [7].

7. Pharmacological activity

7.1. Antitumoral

Antitumoral activity was one of the first biological effects attributed to Amaryllidaceae plants. In the early 1980s, several reports on cytotoxic and antineoplastic activities of certain Amaryllidaceae alkaloids appeared, but it was in 1995 when a significant number of Amaryllidaceae alkaloids with different skeleton types were evaluated for their cytotoxicity against a panel of human and murine cell lines. While almost all Amaryllidaceae alkaloids tested were active against fibroblastic LMTK murine cells, pretazettine was the most active against human tumoral Molt4 lymphoid cells, but with no activity against human tumoral hepatoma cells, HepG2. Conversely, lycorenine was the most active against HepG2 cells, and almost inactive against Molt4 cells [33]. Since then, other Amaryllidaceae alkaloids have proved active against other cell lines [34, 35, 36]. Selective apoptosis-inducing effects have been observed in Amaryllidaceae alkaloids of different structural types, leading to structure-activity studies (SAR). For example, an α -ethanobridge and a free hydroxyl at the C-11 position are required for the potent apoptosis induced by crinane- and lycorine-type Amaryllidaceae alkaloids in tumor cells [37, 38, 39]. The

phenanthridinones, exemplified by narciclasine and pancratistatine, have recently attracted considerable interest owing to their potent cell line-specific anticancer activities and minimal effect on normal cells, and a clinical candidate is earmarked for commercialisation in the next decade. Their mechanism is thought to be based on the initiation of cell death via the apoptotic pathway [40].

7.2. Antiparasitic

Antiparasitic properties have been found in some Amaryllidaceae alkaloids. For example, lycorine, augustine and crinamine are the principal antimalarial constituents of *Crinum amabile*, especially augustine, which inhibited both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. Haemanthamine, haemanthidine, 3-epihydroxybulbispermine, galanthine and pancracine are also particularly active against these parasites, some even more so than the standards currently used for treatment [41].

In relation to Chagas disease and Sleeping Sickness, haemanthidine, pancracine, 3-*O*-acetylsanguinine and 1,2-*O*-diacetyllycorine showed biological activity against *Trypanosoma cruzi* and *Trypanosoma brucei-rhodesiense* [20]. The activity of alkaloids isolated in *Phaedranassa dubia* was evaluated against a range of parasitic protozoa, and ungeremine showed the best activity against *T. brucei-rhodesiense*, *T. cruzi* and *P. falciparum* [20]. Compounds from *Galanthus trojanus* also demonstrated antiparasitic properties, protopine being especially promising for its high selectivity and potency against *T. brucei-rhodesiense* and *P. falciparum* K1, which is a drug-resistant strain [42]. Also, the ethanolic extract of *Narcissus broussonetii* demonstrated significant activity against *T. cruzi* [6].

7.3. Acetylcholinesterase inhibition

Inhibition of the acetylcholinesterase enzyme is one of the most important biological activities of certain Amaryllidaceae alkaloids. Galanthamine is currently the only Amaryllidaceae alkaloid commercially sold for the treatment of mild-to-moderate stages of Alzheimer's disease, owing to its capacity to inhibit the enzyme acetylcholinesterase in the brain and, at same time, interact with nicotinic receptors. Thus, the levels of acetylcholine in the brain, which decline in people with Alzheimer's disease, can be maintained [1, 43]. Inhibition of acetylcholinesterase activity has

been reported in other Amaryllidaceae alkaloids, including sanguinine, montanine, 11-hydroxygalanthamine, epinorgalanthamine, and assoanine [44, 45, 46], and some of them, such as sanguinine, are even more potent than galanthamine. However, the extra hydroxyl group of sanguinine, available for the interaction with acetylcholinesterase, makes the molecule more hydrophilic, thus reducing its ability to cross the blood-brain barrier [47]. Also, natural *N*-alkylated galanthamine derivatives such as *N*-allylnorgalanthamine and *N*-(14-methylallyl)norgalanthamine demonstrate a higher inhibition of acetylcholinesterase than galanthamine [48].

7.4. Docking studies

Docking is the use of computational methods to predict the preferred conformation of one molecule to another when they form a stable complex [49]. Docking studies performed with several Amaryllidaceae alkaloids and acetylcholinesterase and butyrylcholinesterase enzymes have revealed, for example, that not only galanthamine-type alkaloids could be useful for the treatment of Alzheimer's disease. Tazettine-type alkaloids should also be considered owing to the high selectivity of 3-epimacronine derivatives for binding the enzyme locus [50]. Docking and molecular dynamics simulation studies have recently shown butyrylcholinesterase inhibitory

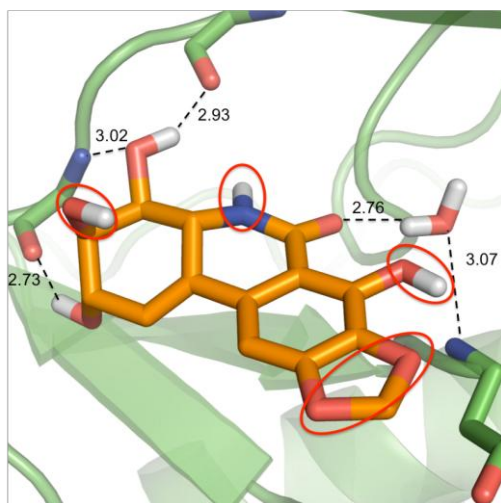


Figure 4. Putative binding mode of narciclasine in GSK-3 β enzyme.

activity in 7- hydroxycyclivonine, which interacts at the same binding site of the enzyme as galanthamine with very similar amino acids (Ortiz et al., 2016). Perspectives for the future include research into glycogen synthase kinase 3 β (GSK3 β) as a new therapeutic target for Amaryllidaceae alkaloids. This enzyme is involved in several cellular processes, including some proteins involved in Alzheimer's disease. Furthermore, there is a growing interest in GSK3 β from unicellular parasites, due to their prominent role in the regulation of circadian rhythms of parasites, which are responsible, for example, for the circadian fever caused by *P. falciparum*, (Figure 4) [51].

8. Conclusion

Amaryllidaceae plants are outstanding, economically important plants, not only for their ornamental value, but also as medicinal plants and sources of drugs and new therapeutic targets. Due to their considerable biodiversification, they are found in almost all the continents: Europe, Asia, Africa and America. Their use in traditional medicine remains important in certain areas, and is supported by numerous scientific studies that have demonstrated the bioactivity of these plants, caused by their content of a particular and exclusive type of alkaloids, named Amaryllidaceae alkaloids.

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7.2 Anexo II: Artículo como co-autora

New Alkaloids from *Hippeastrum papilio* (Ravenna) Van Scheepen

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FULL PAPER

New Alkaloids from *Hippeastrum papilio* (RAVENNA) VAN SCHEEPEN

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A new phytochemical study of the indigenous Brazilian species *Hippeastrum papilio* is reported herein. Three novel Amaryllidaceae alkaloids were isolated, including hippapiline (**1**), papiline (**2**), and 3-*O*-demethyl-3-*O*-(3-hydroxybutanoyl)-haemanthamine (**3**). Their structures were determined by physical and spectroscopic methods. In addition, the known alkaloids, haemanthamine (**4**), galanthamine (**5**), narwedine (**6**), 11 β -hydroxygalanthamine (**7**), apogalanthamine (**8**), and 9-*O*-demethyllycosinine B (**9**) were identified. The unusual *cis*-*B/C*-ring fusion for the new homolycorine representative hippapiline was ratified by NMR and CD spectroscopy.

Introduction. – The plant family Amaryllidaceae comprises ca. 1600 species in 73 genera which are distributed through the tropics and warm, temperate regions of the globe [1][2]. These perennial, bulbous geophytes belong to one of the 20 most significant alkaloid-producing plant families [1][2]. A striking feature of the Amaryllidaceae is the presence of an exclusive group of isoquinoline alkaloids, which are responsible for a wide-range of biological activities [3].

Structurally, these alkaloids have been grouped into nine skeletal types formed *via* specific oxidative phenolic couplings from the common amino acid-derived biogenetic precursor norbelladine [3]. The homolycorine-type skeleton is characterized by a *cis*-*B/C*-ring fusion, in which H–C(1) and H–C(10b) are both α -oriented. The correct configurational characterization of Amaryllidaceae alkaloids thus allows for a better understanding of the biosynthetic pathway diagnostic for a particular skeleton.

Over the past two decades, gas chromatography/mass spectrometry (GC/MS) has been applied successfully in the analysis of Amaryllidaceae alkaloids [4]. A preliminary study of *H. papilio* *via* GC/MS indicated the presence of several unknown structures with MS fragmentation patterns diagnostic of Amaryllidaceae alkaloids [5]. A larger collection of *H. papilio* bulbs was here subjected to a comprehensive phytochemical investigation leading to the identification of hippapiline (**1**), papiline (**2**), and 3-*O*-demethyl-3-*O*-(3-hydroxybutanoyl)haemanthamine (**3**) as the novel constituents, in addition to six other known Amaryllidaceae alkaloids. The β -orientation for both H–C(1) and H–C(10b) in **1**, uncovered by rigorous spectroscopic analysis, is indicative of an unusual *cis*-*B/C*-ring fusion previously not seen for homolycorine-type alkaloids.

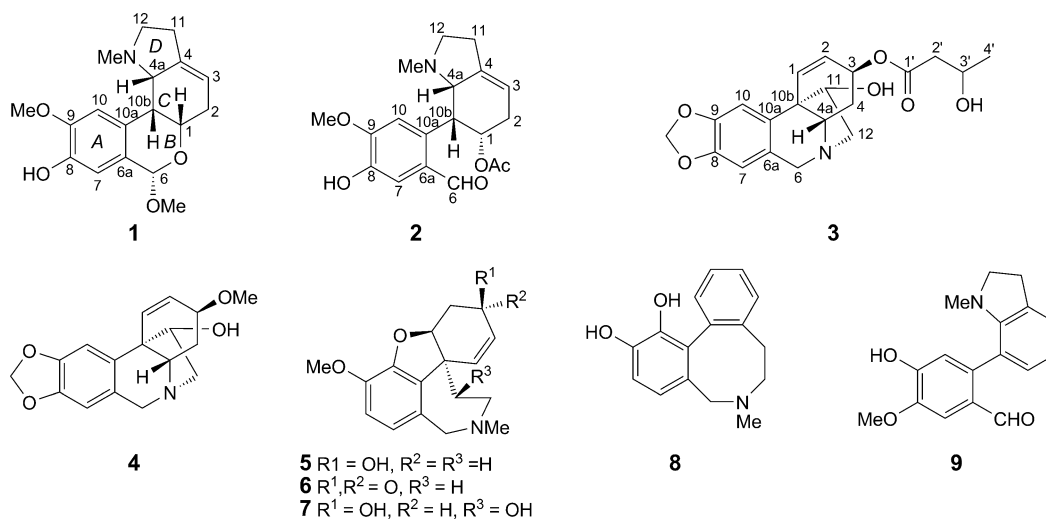
Results and Discussion. – GC/MS Analysis (*Table 1*) revealed that galanthamine (**5**) was the main constituent in the hexane extract (86.3%), also featuring as one of the major components in the AcOEt extract (39.0%) together with haemanthamine (**4**; 26.9%). These results are in agreement with a previous study of *H. papilio*, in which these alkaloids were isolated and identified by NMR and CD spectroscopic techniques [5]. However, apogalanthamine (**8**) and 9-*O*-demethyllycosinine B (**9**) are now reported in this species for the first time (*Fig.*), whilst narwedine (**6**) as in the previous instance [5] was here also detectable only in minimal quantity.

Compound **1** had a HR-ESI-MS signal at m/z 318.1706 ($[M+H]^+$, $C_{18}H_{24}NO_4^+$; calc. 318.1700) and a base peak at m/z 109 ($C_7H_{11}N^+$) in its GC/MS spectrum, arising from a *retro-Diels–Alder* reaction, which is characteristic for a hexahydroindole ring in the homolycorine series lacking substitution at C(2) [4]. Although the basic structure of a homolycorine-type alkaloid for compound **1** was readily established by NMR evidence, the unusual chemical shift and splitting pattern were ratified *via* comparisons with the data for 8-*O*-demethyl-6-*O*-methyllycorenine found in the literature [6]. The 1H -NMR data of **1** was atypical in the following three ways: *i*) two *para*-oriented aromatic H-atoms attributed to H–C(7) and H–C(10), the latter of which was assignable to the highly deshielded resonance *singlet* at $\delta(H)$ 8.41 (confirmed by NOESY correlation with the MeN group); *ii*) an uncommon coupling constant ($J = 4.5$ Hz) observed between H–C(1) and H–C(10b) and the absence of the distinctive *trans*-diaxial coupling ($J \sim 10$ Hz) between H–C(10b) and H–C(4a); *iii*) a NOESY correlation between H–C(4a), H–C(10b), and H–C(1). This data was thus pivotal in assigning both H–C(1) and H–C(10b)

Table 1. GC/MS Analysis of the Alkaloid Content of *Hippeastrum papilio*

Alkaloid	RI	% A ^a)	% B ^b)	M ⁺	MS
Apogalanthamine (8)	2253	–	3.12	269 (88)	268 (32), 254 (26), 226 (61), 211 (54), 194 (32), 193 (50), 166 (29), 165 (100), 152 (30)
Hippapiline (1)	2301	–	1.68	317 (–)	110 (8), 109 (100), 108 (18), 107 (2), 94 (24), 81 (2), 77 (2), 42 (2)
Galanthamine (5)	2335	86.26	39.01	287 (82)	288 (14), 286 (100), 270 (13), 244 (26), 230 (13), 216 (36), 174 (30), 115 (13)
Narwedine (6)	2402	1.31	0.52	285 (85)	286 (15), 284 (100), 242 (22), 216 (23), 214 (10), 199 (26), 185 (13), 181 (12), 178 (15), 174 (43), 161 (11), 153 (13), 141 (10), 128 (22), 115 (22), 77 (13), 42 (22)
9- <i>O</i> -Demethyllycosinine B (9)	2499	0.24	5.72	283 (100)	284 (19), 256 (11), 255 (70), 254 (72), 240 (30), 239 (13), 223 (11), 222 (33), 210 (11), 194 (17), 167 (10), 44 (16)
11 β -Hydroxygalanthamine (7)	2510	traces	7.02	303 (21)	302 (12), 231 (21), 230 (100), 213 (28), 181 (13), 174 (13), 115 (13), 44 (13)
Haemanthamine (4)	2556	1.92	26.87	301 (12)	273 (18), 272 (100), 242 (15), 240 (16), 214 (13), 212 (14), 211 (15), 181 (26), 153 (10), 128 (12), 115 (11)
Papiline (2)	2565	1.08	10.98	345 (–)	286 (3), 177 (3), 165 (1), 122 (1), 110 (6), 109 (100), 108 (14), 96 (1), 82 (3), 81 (2), 44 (1), 43 (3), 42 (2)
3- <i>O</i> -Demethyl-3- <i>O</i> -(3-Hydroxybutanoyl)-haemanthamine (3)	3030	–	1.00	373 (5)	345 (21), 344 (95), 270 (24), 269 (37), 268 (25), 240 (55), 226 (20), 225 (30), 224 (25), 212 (53), 211 (27), 210 (16), 182 (20), 181 (100), 153 (33), 128 (16), 115 (17), 45 (21)

^a) % A: Alkaloid percentage in the total mixture of alkaloids extracted with hexane. ^b) % B: Alkaloid percentage in the total mixture of alkaloids extracted with AcOEt. All values are expressed as a relative percentage of TIC.

Figure. Alkaloids identified in *Hippeastrum papilio*

to the β -face. CD Analysis showed that there were positive, negative, and positive Cotton effects at around 225, 250, and 290 nm, respectively, which are antipodal to those observed for typically *cis*-B/C-ring-fused homolycorine-type alkaloids [7]. Taken together, the CD and NMR data (Table 2) are in agreement with this novel stereochemical arrangement involving *cis*-B/C-ring fusion in **1**, for which the name hippapiline is proposed.

The HR-ESI-MS of **2** suggested a molecular formula C₁₉H₂₄NO₅⁺ for [M+H]⁺ with the parent ion at *m/z* 346.1643 (calc. 346.1649). The EI-MS showed a signal ion at *m/z* 286 ([M–59]⁺) diagnostic for the loss of an AcO group. Characteristic NMR signals included: *i*) a singlet H-atom resonance at δ (H) 9.91, indicative of an aldehyde functionality which appeared in nonfused dihydroindole

lycosinine derivatives [8][9], with the corresponding signal at δ (C) 191.7 (*d*) in the ¹³C-NMR; *ii*) two *para*-orientated aromatic H-atoms at δ (H) 7.32 and 7.29, the more deshielded of which was assigned to H–C(7) due to its NOESY correlation with H–C(6); *iii*) the AcO substituent was assigned to C(1) due to the strong deshielding of H–C(1) (δ (H) 5.45), confirmed by HMBC; *iv*) the magnitude of the coupling constant (*J*(4a,10b) = *J*(1,10b) = 4.5 Hz) together with the observed NOESY correlations were congruent with the *syn*-disposition for H–C(1), H–C(10b), and H–C(4a). The IR spectrum of **2** displayed strong absorbances at 1738 and 1674 cm^{–1} for two C=O groups ascribed to the AcO and conjugated CHO moieties, respectively. All spectroscopic data together were in agreement with a new compound bearing a nonfused

Table 2. NMR Data (500 MHz for ^1H - and 125 MHz for ^{13}C -NMR, CDCl_3) of **1**. δ in ppm, J in Hz.

Position	$\delta(\text{H})$	COSY	NOESY	$\delta(\text{C})$	HMBC
1	4.29 (<i>ddd</i> , $J = 9.8, 6.8, 4.5$)	H-2 α , H-2 β , H-10b	H-2 α , H-2 β , H-4 α , H-10b	70.5 (<i>d</i>)	
2 α	2.41–2.47 (<i>m</i>)	H-1, H-2 β , H-3, H-4 α , H-11	H-1, H-2 β , H-3, 6-MeO	28.9 (<i>t</i>)	
2 β	2.25–2.29 (<i>m</i>)	H-1, H-2 α , H-3, H-4 α , H-11	H-1, H-2 α , H-3		
3	5.07 (<i>br. s</i>)	H-2 α , H-2 β , H-4 α , H-11	H-2 α , H-2 β , H-11	115.2 (<i>d</i>)	
4				138.4 (<i>s</i>)	
4 α	2.94 (<i>br. s</i>)	H-2 α , H-2 β , H-3, H-10b, H-11	H-1, H-10b, H-12 β , MeN	70.6 (<i>d</i>)	
6 β	5.44 (<i>s</i>)	H-7	H-7, H-10b, 6-MeO	97.1 (<i>d</i>)	C-1, C-10a, 6-MeO
6 α				127.8 (<i>s</i>)	
7	6.88 (<i>s</i>)	H-6 β	H-6 β	112.4 (<i>d</i>)	C-6, C-8, C-9, C-10a
8				143.7 (<i>s</i>)	
9				145.9 (<i>s</i>)	
10	8.41 (<i>s</i>)	H-10b	9-MeO, MeN	110.6 (<i>d</i>)	C-6a, C-8
10a				126.7 (<i>s</i>)	
10b	3.32 (<i>t</i> , $J = 4.5$)	H-1, H-4 α , H-10	H-1, H-4 α , H-6 β , MeN	35.4 (<i>d</i>)	C-1, C-4, C-4a, C-6a
11 (2H)	2.28–2.36 (<i>m</i>)	H-2 α , H-2 β , H-3, H-4 α , H-12 α , H-12 β	H-3, H-12 α , H-12 β	28.6 (<i>t</i>)	
12 α	3.21 (<i>ddd</i> , $J = 8.7, 6.7, 2.0$)	H-11, H-12 β	H-11, H-12 β , MeN	56.1 (<i>t</i>)	C-4, C-4a
12 β	2.18 (<i>dt</i> , $J = 9.8, 8.6$)	H-11, H-12 α	H-4 α , H-11, H-12 α , MeN		
6-MeO	3.57 (<i>s</i>)		H-2 α , H-6 β	55.3 (<i>q</i>)	C-6
9-MeO	3.80 (<i>s</i>)		H-10	55.7 (<i>q</i>)	C-9
MeN	2.44 (<i>s</i>)		H-4 α , H-10, H-10b, H-12 α , H-12 β	40.0 (<i>q</i>)	C-4a, C-12

hexahydroindole nucleus, for which the name papiline has been proposed. The complete NMR data for compound **2** are listed in Table 3.

The new alkaloid **3** exhibited a parent ion $[M + \text{H}]^+$ at m/z 374.1603 in its HR-ESI-MS spectrum, suggesting the molecular formula $\text{C}_{20}\text{H}_{24}\text{NO}_6^+$ (calc. 374.1598). The EI-MS fragmentation showed the loss of 105 mass units characteristic of a 3-hydroxybutanoyl substituent [10], which was confirmed by corresponding resonances in the ^1H - and ^{13}C -NMR spectra (Table 4). The remaining NMR data

were very similar to those of haemanthamine [3]. Furthermore, the loss of 29 mass units from the molecular ion observed by GC/MS (m/z 344, $[M - 29]^+$) is typical of haemanthamine derivatives bearing a OH group at C(11) [4]. The magnitude of the coupling constant between H–C(2) and H–C(3) ($J(2,3) = 5.1$ Hz) suggested a *trans* relationship between the substituent at C(3) and the 5,10b-ethano bridge [3]. The CD spectrum confirmed **3** as a haemanthamine-type derivative, showing positive and negative Cotton effects at 275 and 249 nm, respectively. A

Table 3. NMR Data (500 MHz for ^1H - and 125 MHz for ^{13}C -NMR, CDCl_3) of **2**. δ in ppm, J in Hz.

Position	$\delta(\text{H})$	COSY	NOESY	$\delta(\text{C})$	HMBC
1	5.45 (<i>ddd</i> , $J = 10.0, 5.0, 5.0$)	H-2 α , H-2 β , H-10b	H-2 β , H-4 α , H-10b	70.9 (<i>d</i>)	C-10a, MeCO
2 α	2.00–2.06 (<i>m</i>)	H-1, H-2 β , H-3, H-4 α , H-11	H-2 β , H-3, H-10	27.9 (<i>t</i>)	MeCO
2 β	2.44 (<i>dddd</i> , $J = 16.5, 5.5, 4.5, 2.5$)	H-1, H-2 α , H-3, H-4 α , H-11	H-1, H-2 α , H-3		
3	5.54 (<i>m</i>)	H-2 α , H-2 β , H-4 α , H-11	H-2 α , H-2 β , H-11	116.1 (<i>d</i>)	
4				131.1 (<i>s</i>)	
4 α	3.10 (<i>s</i>)	H-2 α , H-2 β , H-3, H-10b, H-11	H-1, H-10b, H-12 β , MeN	69.5 (<i>d</i>)	
6	9.91 (<i>s</i>)		H-7, H-10b	191.7 (<i>d</i>)	
6 α				127.2 (<i>s</i>)	
7	7.32 (<i>s</i>)		H-6	116.2 (<i>d</i>)	C-8, C-9, C-10a
8				144.6 (<i>s</i>)	
9				150.1 (<i>s</i>)	
10	7.29 (<i>s</i>)	9-MeO	H-2 α , H-11, 9-MeO	112.5 (<i>d</i>)	C-6a, C-8, C-9, C-10b
10a				129.0 (<i>s</i>)	
10b	4.74 (<i>dd</i> , $J = 4.5, 4.5$)	H-1, H-4 α	H-1, H-4 α , H-6, MeN	35.3 (<i>d</i>)	C-1, C-4a, C-10
11 (2H)	2.55 (<i>m</i>)	H-2 α , H-2 β , H-3, H-4 α , H-12 α , H-12 β	H-3, H-10, H-12 α , H-12 β	28.0 (<i>t</i>)	
12 α	3.26 (<i>br. s</i>)	H-11, H-12 β	H-11, H-12 β , MeN	56.3 (<i>t</i>)	
12 β	2.33 (<i>q</i> , $J = 9.0$)	H-11, H-12 α	H-4 α , H-11, H-12 α , MeN		MeN
9-MeO	3.84 (<i>s</i>)	H-10	H-10	55.8 (<i>q</i>)	C-9
MeCO	1.90 (<i>s</i>)			21.2 (<i>q</i>)	MeCO
MeCO				170.6 (<i>s</i>)	
MeN	2.20 (<i>s</i>)		H-4 α , H-10b, H-12 α , H-12 β	40.9 (<i>q</i>)	C-4a, C-12

Table 4. NMR Data (500 MHz for ^1H - and 125 MHz for ^{13}C -NMR, CDCl_3) of **3**. δ in ppm, J in Hz.

Position	$\delta(\text{H})$	COSY	NOESY	$\delta(\text{C})$	HMBC
1	6.52 (<i>d</i> , $J = 10.1$)	H-2	H-2, H-10	129.3 (<i>d</i>)	C-3, C-4a, C-10a, C-10b
2	6.31 (<i>ddd</i> , $J = 10.1, 5.1, 0.7$)	H-1, H-3	H-1, H-3	130.2 (<i>d</i>)	C-3, C-4, C-10b
3	5.44 (<i>td</i> , $J = 4.8, 1.7$)	H-2, H-4 α , H-4 β	H-2, H-4 α , H-4 β	67.2 (<i>d</i>)	C-1, C-2, C-4a, C-1'
4 α	2.37 (<i>td</i> , $J = 14.0, 4.6$)	H-3, H-4 β , H-4a	H-3, H-4 β , H-4a, H _{exo} -12	29.5 (<i>t</i>)	C-4a
4 β	1.91 (<i>dd</i> , $J = 14.0, 4.6$)	H-3, H-4 α , H-4a	H-3, H-4 α , H-4a		C-2, C-3, C-4a, C-10b
4a	3.36 (<i>dd</i> , $J = 13.5, 4.5$)	H-4 α , H-4 β	H-4 α , H-4 β , H-6 β	63.0 (<i>d</i>)	C-6, C-12, C-11
6 α	3.72 (<i>d</i> , $J = 17.0$)	H-6 β , H-7	H-6 β , H _{endo} -12, H-7	61.5 (<i>t</i>)	C-4a, C-6a, C-7, C-10a
6 β	4.35 (<i>d</i> , $J = 17.0$)	H-6 α , H-7	H-4a, H-6 α , H-7		C-6a, C-7, C-8, C-10a, C-11, C-12
6a				126.9 (<i>s</i>)	
7	6.51 (<i>s</i>)	H-6 α , H-6 β	H-6 α , H-6 β	107.1 (<i>d</i>)	C-6, C-9, C-10a
8				146.6 (<i>s</i>)	
9				146.8 (<i>s</i>)	
10	6.84 (<i>s</i>)		H-1	103.4 (<i>d</i>)	C-6a, C-8, C-10b
10a				134.8 (<i>s</i>)	
10b				50.2 (<i>s</i>)	
11	4.03 (<i>ddd</i> , $J = 6.5, 3.5, 1.5$)	H _{exo} -12, H _{endo} -12	H _{endo} -12	80.3 (<i>d</i>)	C-4a
12 _{exo}	3.27 (<i>dd</i> , $J = 14.0, 3.0$)	H-11, H _{endo} -12	H-4a, H _{endo} -12	63.6 (<i>t</i>)	C-4a, C-6, C-11
12 _{endo}	3.41 (<i>dd</i> , $J = 14.0, 6.5$)	H-11, H _{exo} -12	H-6 α , H-11, H _{exo} -12		C-6, C-4a, C-10b, C-11
OCH ₂ O	5.91 (<i>d</i> , $J = 7.5$)			101.1 (<i>t</i>)	C-8, C-9
1'				172.4 (<i>s</i>)	C-2'
2'a	2.45 (<i>dd</i> , $J = 16.5, 3.0$)	H-2'b, H-3'	H-2'b	43.0 (<i>t</i>)	C-1', C-3', C-4'
2'b	2.36 (<i>dd</i> , $J = 16.5, 9.0$)	H-2'a, H-3'	H-2'a		C-1', C-3', C-4'
3'	4.17 (<i>ddq</i> , $J = 9.5, 6.3, 3.3$)	H-2'a, H-2'b, H-4'	H-4'	64.4 (<i>d</i>)	
4'	1.19 (<i>d</i> , $J = 6.3$)	H-3'	H-3'	22.6 (<i>q</i>)	C-2', C-3'

full set of NMR data for 3-*O*-demethyl-3-*O*-(3-hydroxybutanoyl)haemanthamine (**3**) is shown in Table 4.

In summary, a new phytochemical investigation of *H. papilio* led to the identification of nine alkaloids, among which hippapiline, papiline, and 3-*O*-demethyl-3-*O*-(3-hydroxybutanoyl)haemanthamine are reported for the first time. The unusual β -orientation for both H–C(1) and H–C(10b) in the homolycorine-type skeleton of hippapiline was confirmed by NMR and CD spectroscopy. This finding provides new insight on the biosynthesis of homolycorine compounds in particular and Amaryllidaceae alkaloids in general.

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Experimental Part

General. Thin layer chromatography (TLC): silica gel F_{254} as the stationary phase with plate dimensions of 20 cm $\times$ 20 cm $\times$ 0.20 mm for anal. TLC, and 20 cm $\times$ 20 cm $\times$ 0.25 mm for semi-prep. TLC (SPTLC; Val-de-Reuil, France). Vacuum liquid chromatography (VLC): silica gel (Kieselgel – mesh 0.15/0.30, Val-de-Reuil, France). Optical rotations: PerkinElmer 241 polarimeter (Waltham, MA, USA) in CHCl_3 at 22°. CD Spectra: Jasco-J-810 spectrophotometer (Easton, MD, USA) in MeOH. UV Spectra: Dinko UV2310 instrument (Barcelona, Spain); λ_{max} (log ϵ) in nm. IR Spectra: Nicolet Avatar 329 FT-IR spectrophotometer (Waltham, MA, USA); $\tilde{\nu}_{\text{max}}$ in cm^{-1} . NMR Spectra: Varian VNMRs 500 MHz (Palo Alto, CA, USA), using CDCl_3 as solvent; δ in ppm rel. to Me_4Si as internal standard, J in Hz.

EI-MS: Agilent 6890 + MSD 5975 GC/MS spectrometer (Agilent Technologies, Santa Clara, CA, USA) operating in EI mode at 70 eV. An HP-5 MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) was used. The temp. program was as follows: 100–180° at 15° min^{-1} , 1 min hold at 180°, 180–300° at 5° min^{-1} , and 1 min hold at 300°. The injector temp. was 280°. The flow rate of carrier gas (He) was 0.8 ml min^{-1} . The 1:20, 1:10, and 1:5 split ratios were applied, depending on sample concentration. GC/MS Results were analyzed using AMDIS 2.64 software (NIST). HR-ESI-MS: LC/MSD-TOF spectrometer (Agilent Technologies, Santa Clara, CA, USA) through direct injection of pure compounds dissolved in $\text{H}_2\text{O}/\text{MeCN}$ (1:1).

Plant Material. *Hippeastrum papilio* was collected during the flowering period (May, 2012) in the south of Brazil (Caxias do Sul - RS). A voucher specimen (ICN-149428) was authenticated by Dr. Julie Dutilh (University of Campinas) and deposited with the Institute of Botany, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre.

Identification of Alkaloids by GC/MS. The alkaloids were identified by comparing their GC/MS spectra and Kovats retention index (*RI*) with those of authentic Amaryllidaceae alkaloids previously isolated and identified by spectrometric methods (NMR, UV, CD, MS) [5][8][11], the NIST 05 Database or literature data. *RI* Values of compounds were measured with a standard *n*-hydrocarbon mixture (C_9 – C_{36}) using AMDIS 2.64 software. The proportion of each individual compound in the alkaloid fractions analyzed by GC/MS (Table 1) is expressed as a percentage of the total alkaloids (TIC – total ion current). The area of the GC/MS peaks depends on the concentration of the corresponding compound and the intensity of their mass spectral fragmentation. Although data given in Table 1 do not express a real quantification, they can be used for a relative comparison of the amount of the respective alkaloids present in *Hippeastrum papilio*.

Extraction and Isolation of Alkaloids. Dried bulbs (220 g) of *H. papilio* were crushed and extracted with MeOH at r.t. as following: twice using 900 ml for 48 h and twice with 400 ml for 72 h. The extract was evaporated under reduced pressure to yield 55 g. This crude extract was acidified to pH 2 with H_2SO_4 (2% v/v), and extracted with Et_2O (170 ml $\times$ 6) to remove neutral material. The aq. soln. was basified with

25% $\text{NH}_3 \cdot \text{H}_2\text{O}$ up to pH 10, and extracted with hexane (170 ml $\times$ 13) to give extract *A* (1.3 g). Another extraction using AcOEt (170 ml $\times$ 20) gave extract *B* (1.5 g). Extract *A* yielded galanthamine (**5**; 130.71 mg) and haemanthamine (**4**; 49.5 mg) by SPTLC (CH_2Cl_2 /acetone 10:7; in NH_3 atmosphere). Extract *B* was subjected to vacuum liquid chromatography (VLC) using a silica gel (7 g) column with a diameter of 2.5 cm and a height of 4.5 cm, eluting with hexane gradually enriched with AcOEt (0 $\rightarrow$ 100%), and then with AcOEt gradually enriched with MeOH (0 $\rightarrow$ 20%). Fractions of 40 ml were collected (340 in total) monitored by TLC (*Dragendorff's* reagent, UV 254 nm) and combined according to their profiles. From fractions 11–20, hippapiline (**1**; 22.2 mg) was directly obtained as a crystal. Fractions 90–160 were subjected to SPTLC (hexane/AcOEt 1:2; and a second time hexane/AcOEt/MeOH 5:10:7; in NH_3 atmosphere) to give papiline (**2**; 26.7 mg). 9-*O*-Demethylcosinine B (**9**; 7.4 mg) was isolated from fractions 61–85 by SPTLC (hexane/AcOEt 4:1; in NH_3 atmosphere), while 11 β -hydroxygalanthamine (**7**; 1.2 mg) was obtained *via* SPTLC (hexane/AcOEt/MeOH 10:3:3; in NH_3 atmosphere) from fractions 206–210. A third VLC column (1.5 $\times$ 4.5 cm) was performed with fractions 161–205 yielding 176 fractions (10 ml each), of which fractions 69–92 were further purified by SPTLC (hexane/AcOEt/MeOH 2:4:1; in NH_3 atmosphere) to furnish 3-*O*-demethyl-3-*O*-(3-hydroxybutanoyl)haemanthamine (**3**; 12.5 mg). In addition, a small amount of narwedine (**6**) and apogalanthamine (**8**) was identified by comparing their GC/EI-MS spectra and *Kovats* retention indices (*RI*) with our own library database. All known alkaloids isolated were identified by comparing their physical and spectroscopic data with those of alkaloids previously isolated and characterized by our group [5][8][11].

Hippapiline (= (7 α ,13 β ,16 β)-7,10-Dimethoxy-1-methyllycorenan-9-ol; **1**). White crystals. $[\alpha]_{\text{D}}^{25} = +33$ ($c = 0.15$, CHCl_3). UV (MeOH): 316 (2.90), 282 (3.30), 228 (3.64). CD (MeOH, 20°): $\Delta\epsilon_{227} + 1330$, $\Delta\epsilon_{247.5} - 455$, $\Delta\epsilon_{281.5} + 89$, $\Delta\epsilon_{305.5} - 176$. IR (CHCl_3): 3380, 2924, 1733, 1509, 1449, 1275, 1130, 1058, 1043, 960, 913, 880, 802, 758. $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): Table 2. EI-MS: Table 1. HR-EI-MS: 318.1706 ($[M + \text{H}]^+$, $\text{C}_{18}\text{H}_{24}\text{NO}_4^+$; calc. 318.1700).

Papiline (= (6*S*,7*R*,7*aS*)-7-(2-Formyl-4-hydroxy-5-methoxyphenyl)-2,3,5,6,7,7*a*-hexahydro-1-methyl-1*H*-indol-6-yl Acetate; **2**). Amorphous solid. $[\alpha]_{\text{D}}^{25} = -29$ ($c = 0.26$, CHCl_3). UV (MeOH): 318 (3.27), 280 (3.48), 236 (3.76). IR (CHCl_3): 2925, 2854, 1738, 1674, 1595, 1557, 1514, 1455, 1378, 1260, 1148, 1099, 1020, 800. $^1\text{H-NMR}$ (CDCl_3 ,

500 MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): Table 3. EI-MS: Table 1. HR-EI-MS: 346.1643 ($[M + \text{H}]^+$, $\text{C}_{19}\text{H}_{24}\text{NO}_5^+$; calc. 346.1649).

3-*O*-Demethyl-3-*O*-(3-Hydroxybutanoyl)haemanthamine (= (3 β ,13 β)-1,2-Didehydro-11-hydroxycrinan-3-yl 3-Hydroxybutanoate; **3**). Amorphous solid. $[\alpha]_{\text{D}}^{25} = -13$ ($c = 0.49$, CHCl_3). UV (MeOH): 292 (3.46), 238 (3.36). CD (MeOH, 20°): $\Delta\epsilon_{249} - 2375$, $\Delta\epsilon_{275} + 1569$. IR (CHCl_3): 3377, 2925, 1727, 1504, 1484, 1376, 1295, 1239, 1173, 1064, 1037, 988, 936, 853, 758. $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): Table 4. EI-MS: Table 1. HR-EI-MS: 374.1603 ($[M + \text{H}]^+$, $\text{C}_{20}\text{H}_{24}\text{NO}_6^+$; calc. 374.1598).

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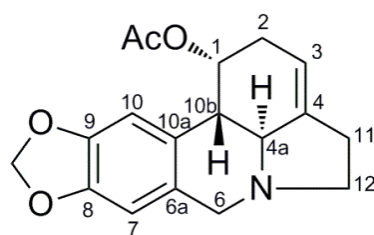
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7.3 Anexo III

A continuación se adjuntan las tablas con datos de CG-EM y RMN de los compuestos identificados en el presente trabajo.

7.3.1 *Amaryllis belladonna***Tabla 1.** Alcaloides identificados en *A. belladonna* por CG-EM.

Alcaloide	IR	M ⁺	EM
ismine	2288.1	257 (28)	239 (20), 238 (100), 225 (7), 211 (7), 196 (9), 180 (8), 139 (8)
trisphaeridine	2305.0	223 (100)	224 (15), 222 (38), 193 (2), 164 (15), 138 (26), 111 (14)
buphanisine	2447.5	285 (100)	270 (34), 254 (34), 242 (12), 227 (24), 215 (85), 201 (23), 185 (22), 157 (33), 128 (33)
anhydrolycorine	2470.2	251 (42)	250 (100), 220 (3), 192 (18), 163 (3), 125 (2)
caranine	2560.9	271 (59)	270 (37), 252 (53), 240 (10), 227 (44), 226 (100), 212 (6), 181 (1)
1- <i>O</i> -acetylcaranine	2569.6	313 (72)	270 (3), 252 (100), 240 (9), 227 (41), 226 (97)181 (2)
8- <i>O</i> -demethylmaritidine	2575.5	273 (100)	256 (13), 244 (12), 230 (26), 216 (14), 201 (90), 189 (61), 175 (28), , 157 (19), 128 (24)
vittatine	2578.0	271 (100)	254 (11), 228 (22), 214 (13), 199 (58), 187 (63), 173 (24), 157 (18), 141(13), 128 (29)
<i>O</i> -methylnorbelladine	2578.5	273 (-)	166 (36), 137 (100), 122 (8), 94 (6)
11,12-dehydroanhydrolycorine	2638.1	249 (59)	248 (100), 218 (1),190 (26), 163 (8), 137 (1), 123 (5), 95 (13)
6-methoxylbuphanidrine	2656.8	345 (58)	330 (33), 298 (14), 287 (32), 259 (68), 227 (30), 181 (10), 145 (13)
3- <i>O</i> -acetylvittatine	2690.6	313 (100)	270 (27), 254 (75), 252 (61), 224 (38), 216 (60), 198 (36), 187 (36), 139 (15), 128 (33)
powelline	2702.7	301 (100)	284 (10), 272 (14), 258 (39), 244 (24), 229 (8), 202 (22), 187 (23), 159 (13), 143 (11), 127 (19)
buphanidrine	2705.2	315 (100)	300 (31), 284 (35), 272 (9), 260 (39), 245 (69), 228 (24), 215 (21), 202 (20), 187 (18), 130 (18)
lycorine	2722.0	287 (18)	286 (10), 268 (19), 250 (16), 238 (7), 227 (78), 226 (100), 211 (6), 147 (5), 119 (3)
buphanamine	2726.5	301 (100)	282 (21), 272 (13), 256 (22), 244 (11), 190 (7), 165 (9)
1- <i>O</i> -acetyllycorine	2754.9	329 (31)	268 (31), 250 (20), 227 (64), 226 (100), 211 (6), 192 (3), 167 (3), 147 (6)
3- <i>O</i> -acetylpowelline	2768.7	343 (100)	300 (25), 284 (78), 283 (24), 268 (9), 254 (21), 246 (81), 228 (39), 217 (18), 183 (9)
hippadine	2775.0	263 (100)	205 (7), 177 (24), 150 (13), 131 (9), 111 (2), 75 (7)
ambelline	2814.6	331 (86)	299 (44), 287 (100), 270 (33), 260 (76), 257 (58), 239 (52), 228 (20), 211 (62), 190 (39)
6-hydroxybuphanidrine	2828.4	331 (44)	300 (10), 282 (7), 276 (100), 261 (33), 258 (13), 243 (12), 228 (8), 217 (21), 216 (22)
11- <i>O</i> -acetylabelline	2850.2	373 (100)	314 (58), 313 (50), 282 (46), 258 (48), 255 (36), 254 (63), 241 (38), 240 (35), 218 (44)
undulatine	2855.2	331 (100)	316 (9), 300 (9), 286 (15), 258 (33), 244 (14), 232 (17), 217 (28), 205 (60), 189 (32), 173 (30)
3- <i>O</i> -acetylhamayne	2907.7	329 (3)	300 (100), 240 (16), 224 (8), 212 (34), 211 (14), 181 (54), 153 (18), 128 (10)
crinamidine	2954.3	317 (59)	288 (100), 258 (21), 244 (27), 230 (16), 217 (35), 203 (34), 189 (20), 173 (40), 145 (17)
distichamine	2984.3	329 (100)	328 (25), 314 (15), 300 (9), 286 (9), 269 (5), 256 (3), 231 (20), 204 (13), 190 (5)



1-O-acetylcaranine

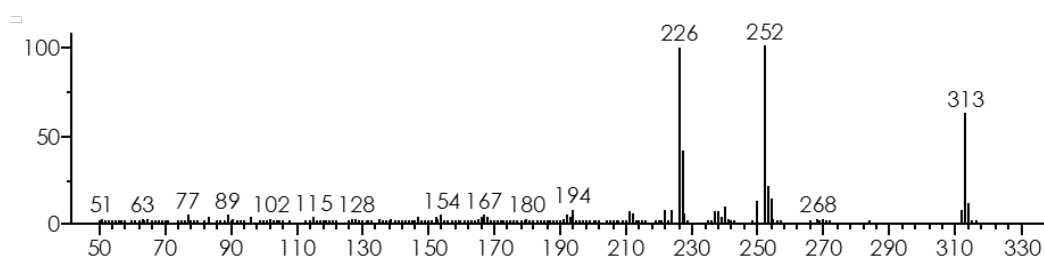


Figura 1. Patrón de fragmentación de masas del alcaloide 1-O-acetylcaranine por EM.

Tabla 2. Resultados de RMN obtenidos para el compuesto 1-*O*-acetylcaranine (CDCl₃, 500MHz).

Posición	δ_H (J en Hz)	δ_C , tipo	COSY	NOESY	HMBC
1	5.84 <i>br dd</i> (4.5, 2.0)	66.60 <i>d</i>	H-2 α , H-2 β , H-10b	H-2 α , H-2 β , H-10, H-10b	C-3, C-4a, C-10b, OCOCH ₃
2 α	2.39 <i>m</i>	33.55 <i>t</i>	H-1, H-2 β , H-3, H-4a	H-1, H-2 β , H-3	-
2 β	2.64 <i>m</i>	33.55 <i>t</i>	H-1, H-2 α , H-3	H-1, H-2 α , H-3	-
3	5.39 <i>dd</i> (4.9, 2.4)	114.43 <i>d</i>	H-2 α , H-2 β , H-4a, H-11 α , H-11 β	H-2 α , H-2 β	C-4a, C-1
4		139.56 <i>s</i>	-	-	-
4a	2.82 <i>d</i> (10.8)	61.51 <i>d</i>	H-2 α , H-3	H-6 α	-
6 α	3.54 <i>d</i> (14.3)	57.09 <i>t</i>	H-6 β , H-7	H-4a, H-6 β , H-7, H-12 α	C-10a
6 β	4.13 <i>d</i> (14.1)	57.09 <i>t</i>	H-6 α , H-7	H-6 α , H-7, H-10b, H-12 β	C-4a, C-7, C-10a
6a		129.54 <i>s</i>	-	-	-
7	6.57 <i>br s</i>	107.34 <i>d</i>	H-6 α , H-6 β	H-6 α , H-6 β	C-6, C-9, C10a
8		146.24 <i>s</i>	-	-	-
9		146.49 <i>s</i>	-	-	-
10	6.72 <i>d</i> (1.0)	105.25 <i>d</i>	H-10b	H-1, H-10b	C-6a, C-8, C-10b
10a		127.79 <i>s</i>	-	-	-
10b	2.67 <i>m</i>	43.56 <i>d</i>	H-1, H-10	H-1, H-6 β , H-10	-
11 α	2.61 <i>m</i>	28.70 <i>t</i>	H-3, H-11 β , H-12 α , H-12 β	H-11 β , H-12 α , H-12 β	-
11 β	2.61 <i>m</i>	28.70 <i>t</i>	H-3, H-11 α , H-12 α , H-12 β	H-11 α , H-12 α , H-12 β	-
12 α	2.39 <i>m</i>	53.83 <i>t</i>	H-11 α , H-11 β , H-12 β	H-6 α , H-11 α , H-11 β , H-12 β	C-6
12 β	3.33 <i>ddd</i> (9.4, 4.9, 4.4)	53.83 <i>t</i>	H-11 α , H-11 β , H-12 α	H-6 β , H-11 α , H-11 β , H-12 α	C-4, C-4a, C-6
OCH ₂ O	5.91 <i>d</i> (1.5) - 5.92 <i>d</i> (1.5)	101.16 <i>t</i>	-	-	-
OCOCH ₃	1.93 <i>s</i>	171.03 <i>q</i>	-	-	-
OCOCH ₃	1.93 <i>s</i>	21.53 <i>q</i>	-	-	-

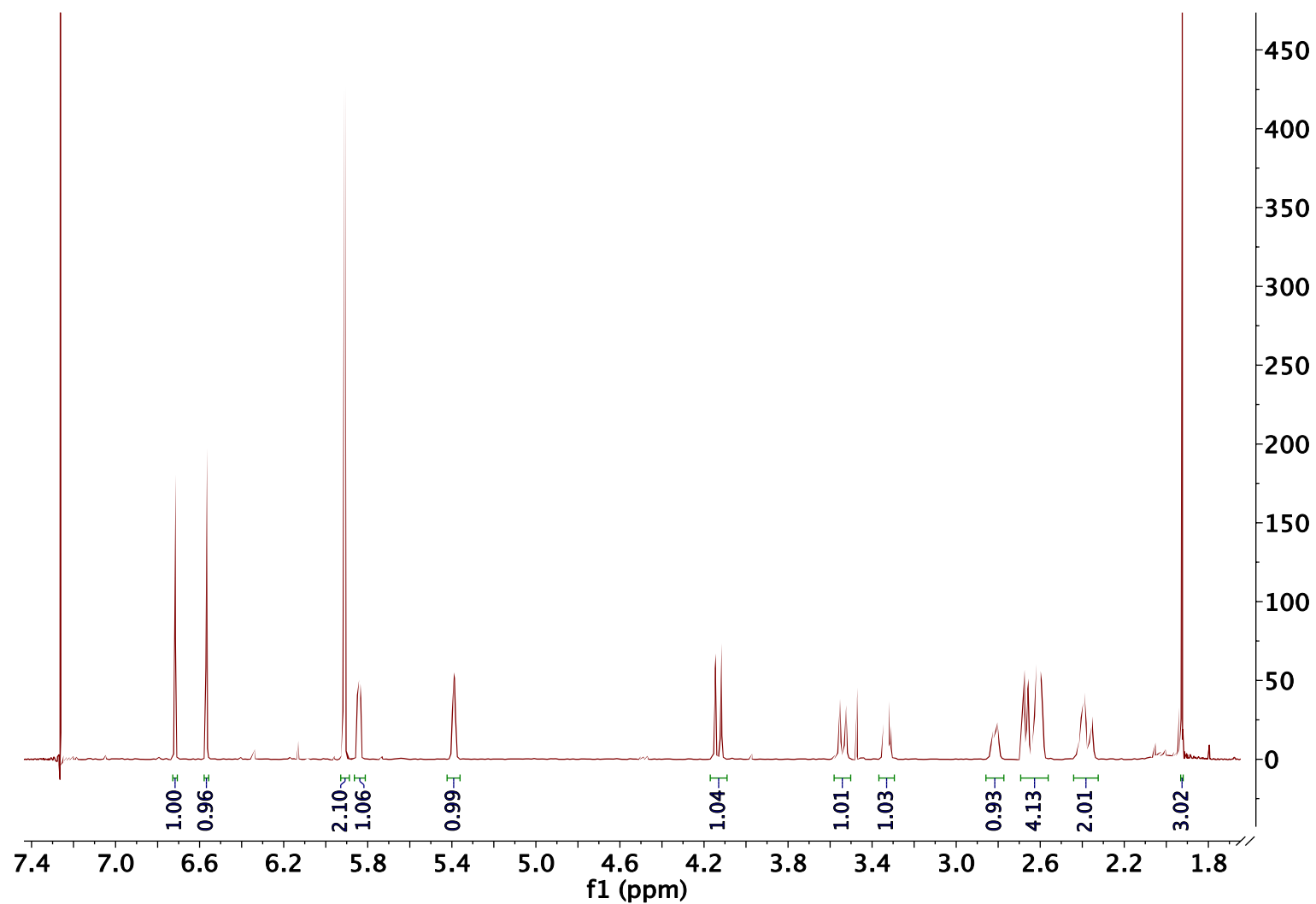


Figura 2. Espectro de ^1H -RMN del compuesto 1-O-acetylcaranine (CDCl_3 , 500MHz).

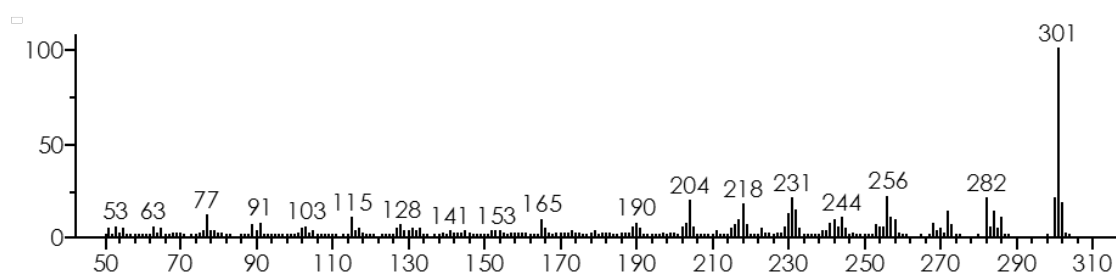
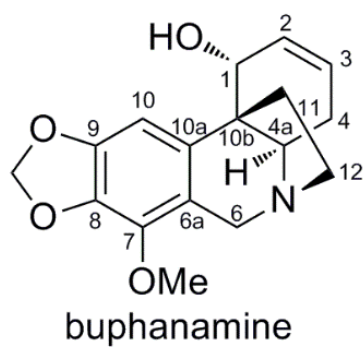


Figura 3. Patrón de fragmentación de masas del alcaloide buphanamine por EM.

Tabla 3. Resultados de RMN obtenidos para el compuesto buphanamine (CDCl₃, 500MHz).

Posición	δ_H (J en Hz)	δ_C , tipo	COSY	NOESY	HMBC
1	4.74 <i>d</i> (5.5)	64.5 <i>d</i>	H-2, H-4a, H-10	H-2, H-10, H-11endo, H-11exo	C-3, C-4a, C-10a, C-11
2	6.00 <i>dddd</i> (10.0, 5.5, 2.8, 1.8)	125.5 <i>d</i>	H-1, H-3, H-4 α , H-4 β	H-1, H-3, H-4 β	C-10b
3	5.86 <i>ddd</i> (10.0, 4.5, 2.8)	128.9 <i>d</i>	H-2, H-4 α , H-4 β	H-2, H-4 α , H-4 β	C-4a
4 α	2.57 <i>dddd</i> (19.7, 8.1, 4.5, 1.9)	28.2 <i>t</i>	H-2, H-3, H-4 β , H-4a	H-3, H-4 β , H-4a	C-2, C-10b
4 β	1.99 <i>dddd</i> (19.8, 8.6, 1.1, 0.3)	28.2 <i>t</i>	H-2, H-3, H-4 α , H-4a	H-2, H-3, H-4 α , H-4a	C-2, C-10b
4a	3.43 <i>t</i> (8.3)	59.2 <i>d</i>	H-1, H-4 α , H-4 β	H-4 α , H-4 β , H-6 α	C-10a
6a		117.9 <i>s</i>	-	-	-
6 α	4.17 <i>d</i> (17.2)	56.9 <i>t</i>	H-6 β	H-4a, H-6 β	C-4a, C-7, C-10a
6 β	3.81 <i>d</i> (17.2)	56.9 <i>t</i>	H-6 α	H-6 α , H-12endo	C-4a, C-7, C-10a
7		140.8 <i>s</i>	-	-	-
8		133.6 <i>s</i>	-	-	-
9		148.6 <i>s</i>	-	-	-
10	6.59 <i>s</i>	98.0 <i>d</i>	H-1	H-1	C-6a, C-8, C-10b
10a		137.0 <i>s</i>	-	-	-
10b		48.3 <i>s</i>	-	-	-
11 endo	1.85 <i>dddd</i> (11.9, 8.8, 3.1, 1.3)	38.7 <i>t</i>	H-11exo, H-12endo, H-12exo	H-1, H-11exo, H-12endo, H12exo	C-4a, C-10a
11 exo	1.92 <i>ddd</i> (12.1, 10.0, 7.3)	38.7 <i>t</i>	H-11endo, H-12endo, H-12exo	H-1, H-11endo, H-12endo, H-12exo	C-4a, C-10a
12 endo	2.75 <i>ddd</i> (13.0, 8.8, 7.2)	51.4 <i>t</i>	H-11endo, H-11exo, H-12exo	H-6 β , H-11endo, H-11exo H-12exo	C-4a, C-6
12 exo	3.40 <i>ddd</i> (13.0, 10.0, 3.0)	51.4 <i>t</i>	H-11endo, H-11exo, H-12endo	H-11endo, H-11exo, H-12exo	C-6, C-10b
OCH ₂ O	5.88 <i>d</i> (1.5) - 5.89 <i>d</i> (1.5)	100.7 <i>t</i>	-	-	C-8, C-9
OCH ₃	3.98 <i>s</i>	59.1 <i>q</i>	-	-	C-7

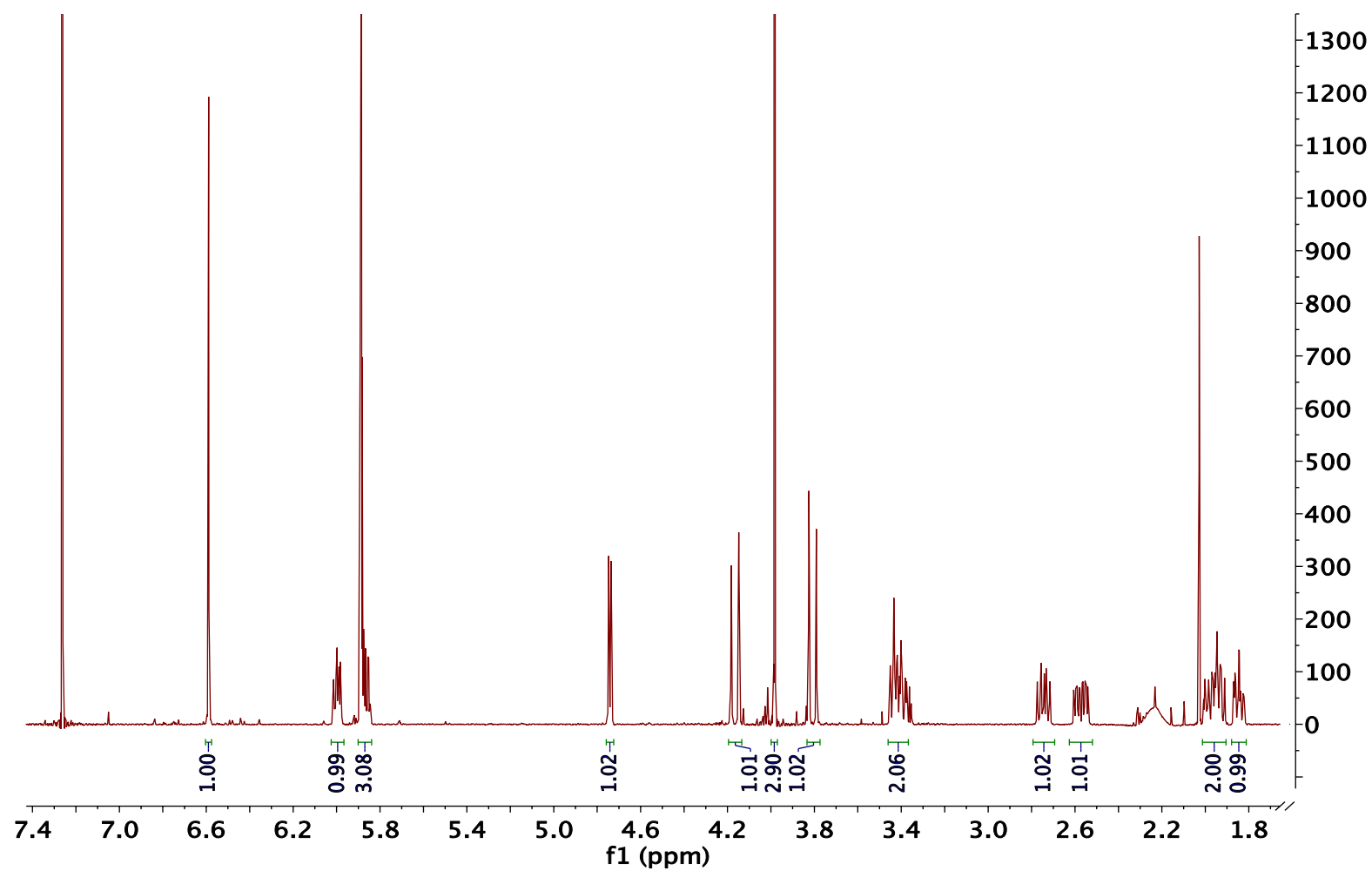


Figura 4. Espectro de ^1H -RMN del compuesto buphanamine (CDCl_3 , 500MHz).

7.3.2 *Hippeastrum reticulatum***Table 4.** Alcaloides identificados en *H. reticulatum* por CG-EM.

Alcaloides	RI	M ⁺	MS
galanthamine	2410.8	287 (84)	288 (20), 286 (100), 270 (15), 244 (27), 230 (15), 216 (37), 174 (32), 159 (11), 115 (16)
sanguinine	2430.5	273 (100)	274 (16), 272 (77), 256 (23), 202 (31), 197 (15), 165 (14), 160 (45), 152 (15), 115 (19)
6β- y 6α-hydroxymaritidine	2495.5	303 (20)	286 (9), 274 (12), 260 (21), 259 (100), 258 (12), 256 (20), 241 (30), 128 (16), 115 (20)
8- <i>O</i> -demethylmaritidine	2522.4	273 (100)	274 (18), 230 (24), 203 (19), 202 (25), 201(89), 189 (55), 175 (23), 115 (19), 56 (20)
maritidine	2528.5	287 (100)	288 (20), 244 (35), 217 (20), 216 (25), 215 (95), 203 (55), 189 (18), 128 (17), 115 (20)
11,12-dehydroanhydrolycorine	2629.3	249 (59)	248 (100), 218 (1), 190 (26), 163 (8), 137 (1), 123 (5), 95 (13)
<i>m/z</i> 264	2693.6	265 (76)	264 (100), 248 (18), 220 (12), 191 (14), 178 (18)
11-hydroxyvittatine	2732.5	287 (5)	259 (16), 258 (100), 242 (9), 214 (9), 212 (8), 211 (13), 186 (13), 181 (14), 128 (11)
lycorine	2771.8	287 (18)	286 (10), 268 (19), 250 (16), 238 (7), 227 (78), 226 (100), 211 (6), 147 (5), 119 (3)
isoreticulinine	2829.1	333 (<1)	291 (37), 290 (100), 274 (11), 272 (6), 256 (5), 228 (5), 147 (13)
reticulinine	2133.4	333 (42)	332 (100), 316 (5), 290 (6), 272 (56), 256 (31), 244 (14), 216 (14), 147 (26)
<i>m/z</i> 294	2950.3	295 (87)	294 (100), 278 (10), 264 (3), 250 (7), 235 (3), 221 (5), 207 (3), 194 (5)
<i>m/z</i> 280	2978.6	281 (75)	280 (100), 264 (12), 250 (3), 236 (11), 219 (4), 207 (4), 194 (8), 178 (4), 167 (3)
2-methoxypratosine	3071.1	309 (100)	310 (20), 294 (18), 284 (7), 266 (24), 251 (14), 236 (6), 164 (4), 152 (6)

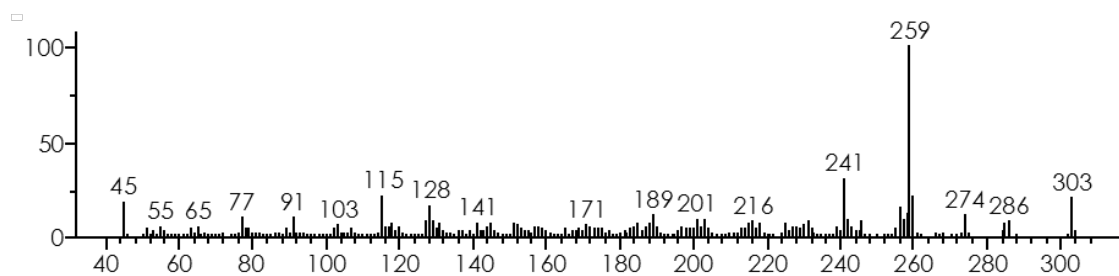
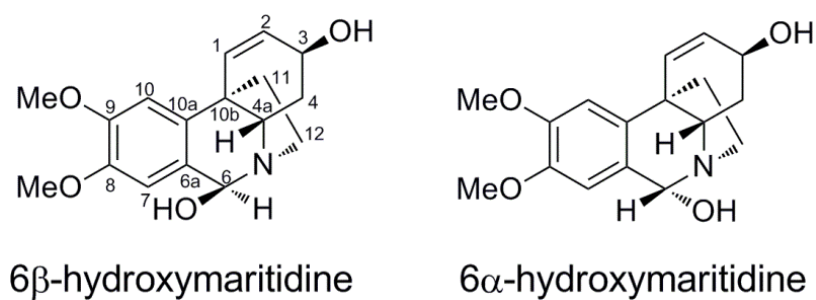


Figure 5. Patrón de fragmentación de masas de los alcaloides 6 β - y 6 α -hydroxymaritidine por EM.

Tabla 5. Resultados de RMN obtenidos para el compuesto 6 β -hydroxymaritidine (CDCl₃, 500MHz).

Posición	(J en Hz)	δ_C , tipo	COSY	NOESY	HMBC
1	6.59, <i>d</i> (10.0)	130.7, <i>d</i>	H-2	H-2	C-4a, C-10a
2	5.98, <i>dd</i> (5.3 and 10.4)	127.9, <i>d</i>	H-1, H-3	H-1, H-3	C-4, C-10b
3	4.32, <i>m</i>	63.2, <i>d</i>	H-2, H-4 α , H-4 β	H-2, H-4 α , H-4 β	C-1, C-4a
4 α	1.72, <i>dt</i> (4.0 and 13.7)	31.0, <i>t</i>	H-3, H-4 β , H-4a	H-3, H-4 β , H-4a	C-10b
4 β	2.14, <i>dd</i> (4.1 and 13.3)	31.0, <i>t</i>	H-3, H-4 α , H-4a	H-3, H-4 α , H-4a	C-10b
4a	4.15, <i>dd</i> (4.1 and 13.6)	56.4, <i>d</i>	H-4 α , H-4 β	H-4 α , H-4 β	C-11
6a	-	125.2, <i>s</i>	-	-	-
6	5.35, <i>s</i>	88.8, <i>d</i>	H-7	H-7, H-12endo	C-7, C-10a, C-12
7	6.89, <i>s</i>	111.8, <i>d</i>	H-6	H-6	C-6, C-9, C-10a
8	-	148.0, <i>s</i>	-	-	-
9	-	148.9, <i>s</i>	-	-	-
10	6.83, <i>s</i>	105.3, <i>d</i>	-	-	C-6a, C-8, C-10b
10a	-	136.0, <i>s</i>	-	-	-
10b	-	44.2, <i>s</i>	-	-	-
11exo	2.03, <i>m</i>	39.9, <i>t</i>	H-11endo, H-12endo, H-12exo	H-11endo, H-12endo, H-12exo	C-10a
11endo	2.03, <i>m</i>	39.9, <i>t</i>	H-11exo, H-12endo, H-12exo	H-11exo, H-12endo, H-12exo	C-10a
12exo	3.37, <i>ddd</i> (3.8; 10.0 and 13.6)	47.4, <i>t</i>	H-12endo, H-11endo, H-11exo	H-12endo, H-11endo, H-11exo	C-6
12endo	2.87, <i>ddd</i> (6.5; 9.0 and 13.1)	47.4, <i>t</i>	H-12exo, H-11endo, H-11exo	H-6, H-12exo, H-11endo, H-11exo	C-6
OMe	3.90, <i>s</i>	56.1, <i>q</i>	-	-	-
OMe	3.87, <i>s</i>	55.9, <i>q</i>	-	-	-

Tabla 6. Resultados de RMN obtenidos para el compuesto 6 α -hydroxymaritidine (CDCl₃, 500MHz).

Posición	δ_H (J en Hz)	δ_C , tipo	COSY	NOESY	HMBC
1	6.51, <i>d</i> (10.0)	129.2, <i>d</i>	H-2	H-2	C-4a, C-10a
2	6.02, <i>dd</i> (5.3 and 10.4)	128.4, <i>d</i>	H-1, H-3	H-1, H-3	C-4, C-10b
3	4.37, <i>m</i>	62.1, <i>d</i>	H-2, H-4 α , H-4 β	H-2, H-4 α , H-4 β	C-1, C-4a
4 α	1.85, <i>dt</i> (4.0 and 13.7)	30.5, <i>t</i>	H-3, H-4 β , H-4a	H-3, H-4 β , H-4a	C-10b
4 β	2.29, <i>dd</i> (4.1 and 13.3)	30.5, <i>t</i>	H-3, H-4 α , H-4a	H-3, H-4 α , H-4a	C-10b
4a	3.89, <i>dd</i> (4.1 and 13.6)	61.7, <i>d</i>	H-4 α , H-4 β	H-4 α , H-4 β , H-6	C-11
6a	-	123.4, <i>s</i>	-	-	-
6	6.12, <i>s</i>	86.6, <i>d</i>	H-7	H-4a, H-7	C-7, C-10a, C-12
7	7.03, <i>s</i>	110.7, <i>d</i>	H-6	H-6	C-6, C-9, C-10a
8	-	148.4, <i>s</i>	-	-	-
9	-	149.2, <i>s</i>	-	-	-
10	6.78, <i>s</i>	105.1, <i>d</i>	-	-	C-6a, C-8, C-10b
10a	-	133.7, <i>s</i>	-	-	-
10b	-	44.8, <i>s</i>	-	-	-
11exo	2.03, <i>m</i>	39.8, <i>t</i>	H-11endo, H-12endo, H-12exo	H-11endo, H-12endo, H-12exo	C-10a
11endo	2.03, <i>m</i>	39.8, <i>t</i>	H-11exo, H-12endo, H-12exo	H-11exo, H-12endo, H-12exo	C-10a
12exo	3.19, <i>ddd</i> (3.8; 10.0 and 13.6)	41.2, <i>t</i>	H-12endo, H-11endo, H-11exo	H-12endo, H-11endo, H-11exo	C-6
12endo	3.94, <i>ddd</i> (6.5; 9.0 and 13.1)	41.2, <i>t</i>	H-12exo, H-11endo, H-11exo	H-12exo, H-11endo, H-11exo	C-6
OMe	3.90, <i>s</i>	56.1, <i>q</i>	-	-	-
OMe	3.87, <i>s</i>	55.9, <i>q</i>	-	-	-

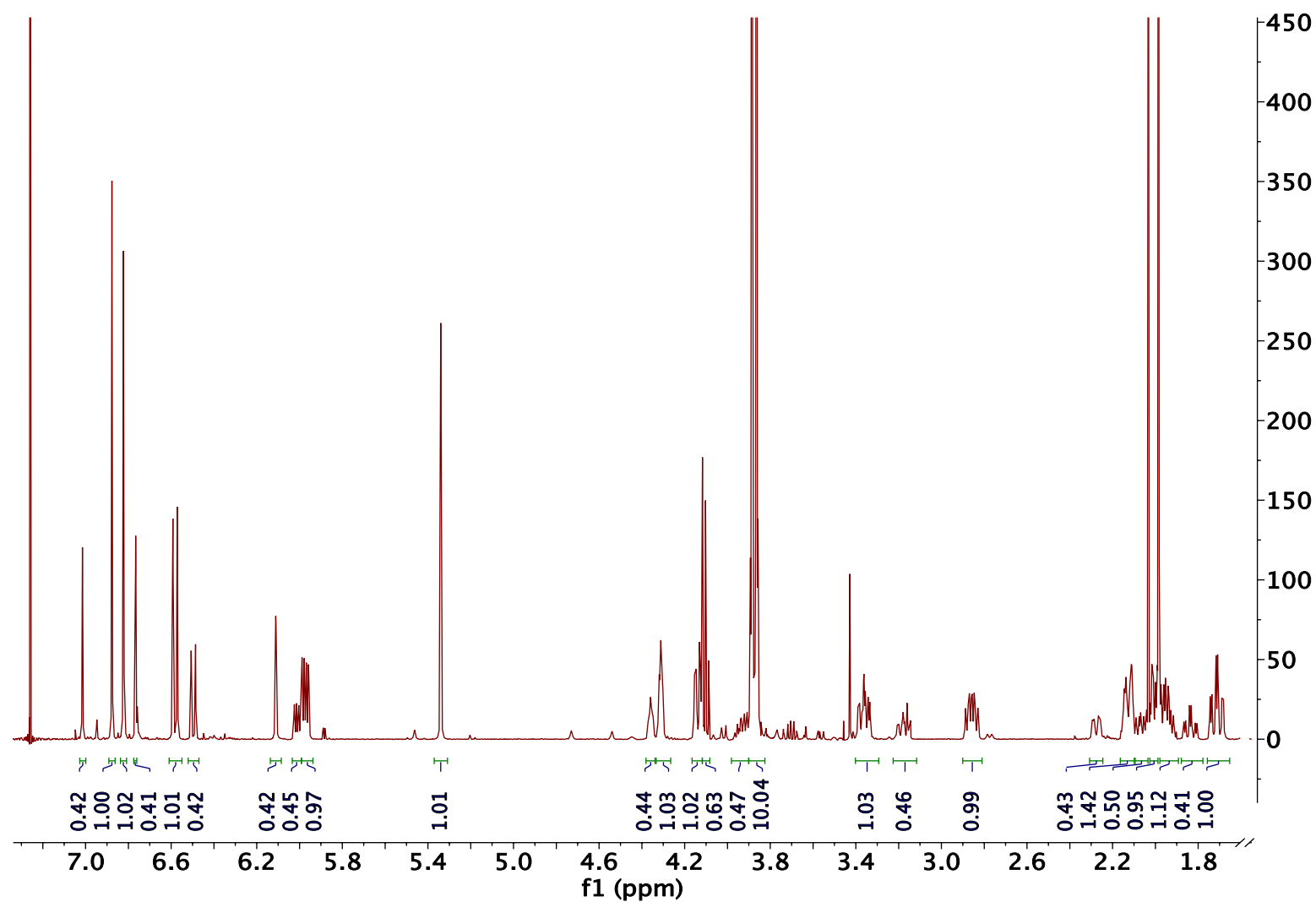


Figura 6. Espectro de ^1H -RMN de los compuestos 6β -hydroxymaritidine y 6α -hydroxymaritidine (CDCl_3 , 500MHz).

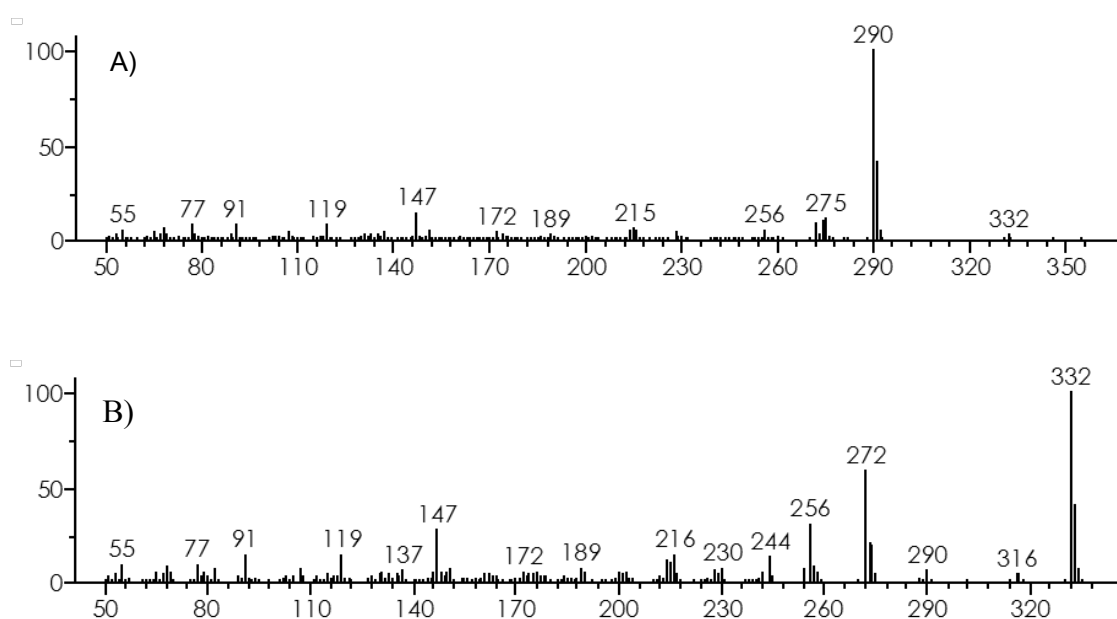
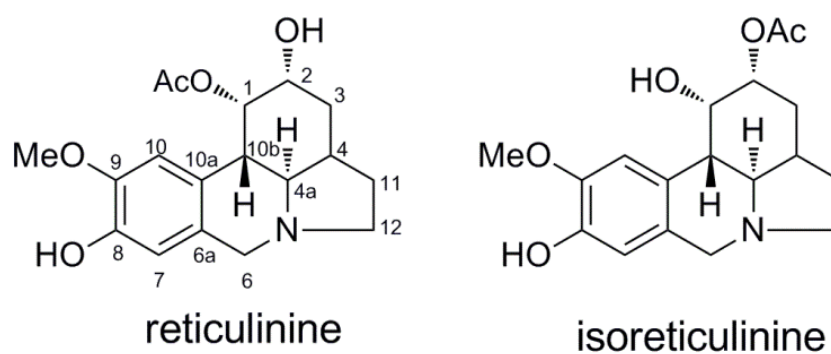


Figura 7. Patrón de fragmentación de los alcaloides reticuline e isoreticuline por EM.

Tabla 7. Resultados de RMN obtenidos para el compuesto reticuline (CDCl₃, 400MHz).

Posición	δ_H (J en Hz)	δ_C , tipo	COSY	NOESY	HMBC
1	6.01, <i>t</i> (3.9)	70.6 <i>d</i>	H-2; H-10; H-10b	H-2; H-10; H-10b	C-4a
2	4.15, <i>ddd</i> (2.8; 7.1 and 9.8)	68.4 <i>d</i>	H-1, H-3 α ; H-3 β	H-1, H-3 α ; H-3 β ; H-10b	-
3 α	2.02, <i>m</i>	25.8 <i>t</i>	H-2; H-3 β , H-4	H-2; H-3 β ; H-4	-
3 β	2.02, <i>m</i>	25.8 <i>t</i>	H-2; H-3 α ; H-4	H-2; H-3 α ; H-4	-
4	2.68, <i>m</i>	36.7 <i>d</i>	H-3 α ; H-3 β ; H-4a	H-3 α ; H-3 β ; H-4a	-
4a	3.11, <i>dd</i> (5.9 and 10.4)	58.0 <i>d</i>	H-4; H-10b	H-4, H-6 α ; H-10b	C-3, C-12
6 α	4.28, <i>d</i> (16.7)	51.4 <i>t</i>	H-6 β , H-7	H-4a; H-6 β ; H-7	C-4a; C-6a; C-7; C-10a; C-12
6 β	3.73, <i>d</i> (16.7)	51.4 <i>t</i>	H-6 α ; H-7	H-6 α ; H-7; H-10b; H-12 β	C-4a; C-6a; C-7; C-10a; C-12
6a	-	127.7 <i>s</i>	-	-	-
7	6.67, <i>s</i>	112.7 <i>d</i>	H-6 α ; H-6 β	H-6 α ; H-6 β	C-6; C-9; C-10a
8	-	144.0 <i>s</i>	-	-	-
9	-	145.4 <i>s</i>	-	-	-
10	6.73, <i>s</i>	106.4 <i>d</i>	H-1, H-10b	H-1; H-10b; OCH ₃	C6a; C-8; C-10b
10a	-	126.7 <i>s</i>	-	-	-
10b	2.68, <i>m</i>	33.1 <i>d</i>	H-1; H-4a; H-10	H-1; H-2; H-4a; H-6 β , H10	C-6a
11 α	1.85, <i>m</i>	29.7 <i>t</i>	H-11 β ; H-12 α ; H-12 β	H-11 β ; H-12 α ; H-12 β	C-4a
11 β	1.77, <i>m</i>	29.7 <i>t</i>	H-11 α ; H-12 α ; H-12 β	H-11 α ; H-12 α ; H-12 β	C-4a
12 α	3.39, <i>m</i>	52.7 <i>t</i>	H-12 β ; H-11 α ; H-11 β	H-12 β ; H-11 α ; H-11 β	C-4a
12 β	2.81, <i>m</i>	52.7 <i>t</i>	H-12 α ; H-11 α ; H-11 β	H-6 β ; H-12 α ; H-11 α ; H-11 β	C-6
OCH ₃	3.82, <i>s</i>	56.0 <i>q</i>	-	H-10	C-9
OCOCH ₃	2.06, <i>s</i>	21.1 <i>q</i>	-	-	OCOCH ₃
OCOCH ₃	-	172.2 <i>q</i>	-	-	-

Tabla 8. Resultados de RMN obtenidos para el compuesto isoreticulinine (CDCl₃, 400MHz).

Posición	δ_H (J en Hz)	δ_C , tipo	COSY	NOESY	HMBC
1	4.77, <i>t</i> (1.5)	66.9 <i>d</i>	H-2; H-10; H-10b	H-2; H-10; H-10b	C-4a
2	5.12, <i>ddd</i> (2.5; 5.4 and 11.6)	72.1 <i>d</i>	H-1, H-3 α ; H-3 β	H-1, H-3 α ; H-3 β ; H-10b	-
3 α	2.23, <i>ddd</i> (6.2; 11.8 and 13.4)	26.8 <i>t</i>	H-2; H-3 β , H-4	H-2; H-3 β ; H-4	-
3 β	1.95, <i>m</i>	26.8 <i>t</i>	H-2; H-3 α ; H-4	H-2; H-3 α ; H-4	-
4	2.68, <i>m</i>	36.4 <i>d</i>	H-3 α ; H-3 β ; H-4a	H-3 α ; H-3 β ; H-4a	-
4a	3.22, <i>dd</i> (5.9 and 10.7)	57.4 <i>d</i>	H-4; H-10b	H-4, H-6 α ; H-10b	C-3, C-12
6 α	4.29, <i>d</i> (16.4)	51.6 <i>t</i>	H-6 β , H-7	H-4a; H-6 β ; H-7	C-4a; C-6a; C-7; C-10a; C-12
6 β	3.74, <i>d</i> (16.9)	51.6 <i>t</i>	H-6 α ; H-7	H-6 α ; H-7; H-10b; H-12 β	C-4a; C-6a; C-7; C-10a; C-12
6a	-	127.7 <i>s</i>	-	-	-
7	6.68, <i>s</i>	112.9 <i>d</i>	H-6 α ; H-6 β	H-6 α ; H-6 β	C-6; C-9; C-10a
8	-	144.0 <i>s</i>	-	-	-
9	-	145.4 <i>s</i>	-	-	-
10	6.82, <i>s</i>	106.4 <i>d</i>	H-1, H-10b	H-1; H-10b; OCH ₃	C6a; C-8; C-10b
10a	-	126.7 <i>s</i>	-	-	-
10b	2.59, <i>d</i> (10.5)	34.3 <i>d</i>	H-1; H-4a; H-10	H-1; H-2; H-4a; H-6 β , H-10	C-6a
11 α	2.04, <i>m</i>	29.9 <i>t</i>	H-11 β ; H-12 α ; H-12 β	H-11 β ; H-12 α ; H-12 β	C-4a
11 β	1.85, <i>m</i>	29.9 <i>t</i>	H-11 α ; H-12 α ; H-12 β	H-11 α ; H-12 α ; H-12 β	C-4a
12 α	3.39, <i>m</i>	52.9 <i>t</i>	H-12 β ; H-11 α ; H-11 β	H-12 β ; H-11 α ; H-11 β	C-4a
12 β	2.81, <i>m</i>	52.9 <i>t</i>	H-12 α ; H-11 α ; H-11 β	H-6 β ; H-12 α ; H-11 α ; H-11 β	C-6
OCH ₃	3.89, <i>s</i>	56.1 <i>q</i>	-	H-10	C-9
OCOCH ₃	2.16, <i>s</i>	21.3 <i>q</i>	-	-	OCOCH ₃
OCOCH ₃	2.16, <i>s</i>	170.4 <i>q</i>	-	-	-

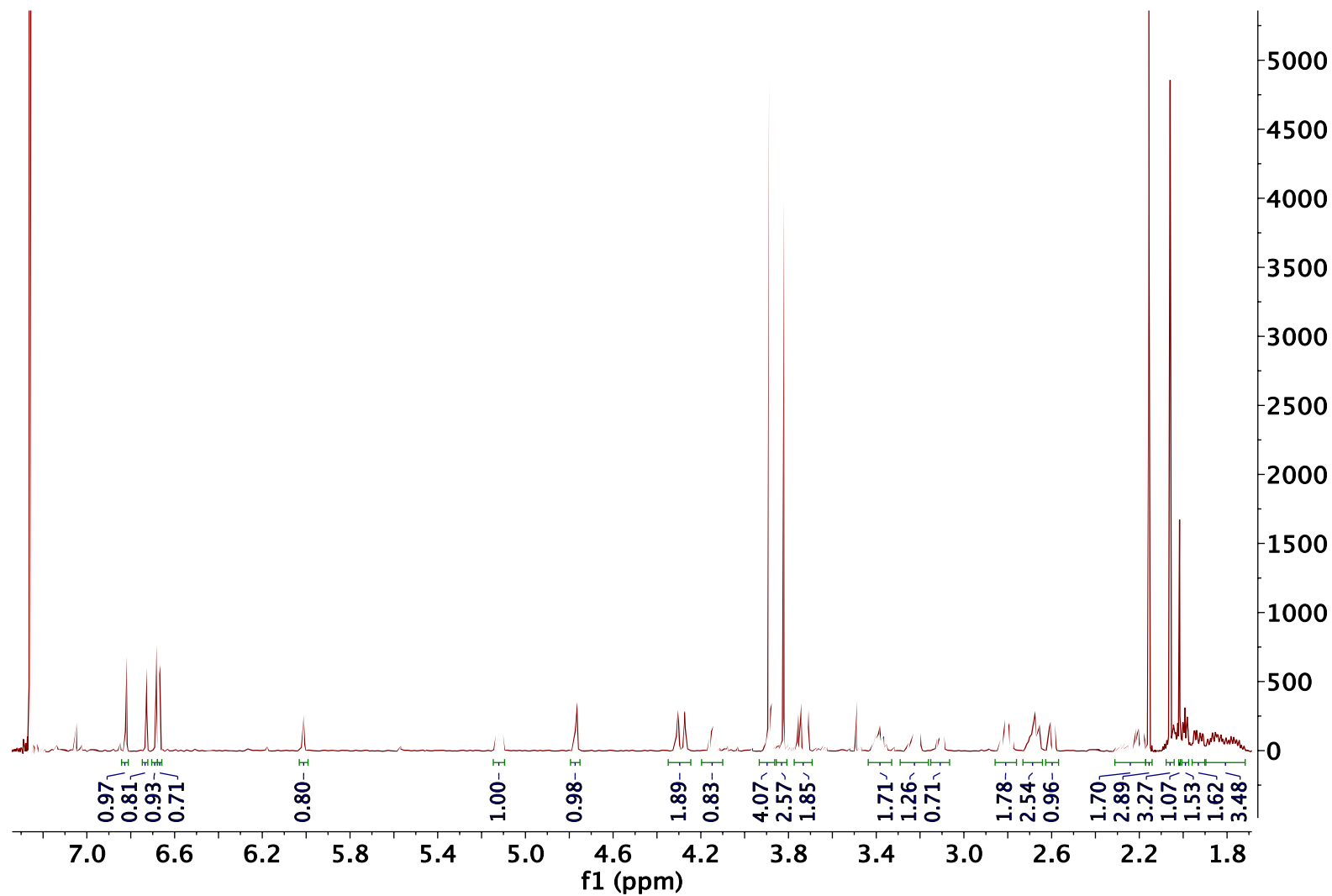


Figura 8. Espectro de ^1H -RMN de los compuestos reticuline e isoreticuline (CDCl_3 , 400MHz).

7.3.3 *Crinum amabile***Tabla 9.** Alcaloides identificados en *Crinum amabile* por CG-EM.

Alcaloide	RI	M+	MS
lycorene	2102.2	255 (52)	254 (100), 227 (17), 226(20), 211 (15), 183(14), 181(10)
ismine	2124.3	257 (28)	239(16), 238 (100), 196 (10), 168 (10)
demethylismine	2128.8	243 (22)	225(21), 224 (100), 167 (10), 166 (15), 154 (11), 77 (12)
demethylmesembrenol	2177.0	275 (5)	206 (9), 205 (76), 115 (6), 70 (100)
galanthamine	2262.8	287 (85)	286 (100), 244(29), 216 (45), 174(39), 165(16), 141 (14), 128 (21), 115 (31)
buphanisine	2283.7	285 (95)	273 (54), 272 (43), 254 (40), 215 (100), 157 (39), 129 (35), 128 (55), 115 (64)
sanguinine	2288.3	273 (100)	272 (85), 202 (40), 165(20), 160 (50), 131 (20), 128 (19), 115 (28), 77(20),
crinine	2326.7	271 (100)	228 (24), 200 (30), 199(81), 187 (76), 173 (28), 129 (34), 128(44), 115 (47), 56 (32)
8- <i>O</i> -demethylmaritidine	2373.8	273 (100)	230 (25), 202 (29), 201 (80), 189 (65), 175 (29), 129 (24), 128 (30), 115 (32), 56 (30)
3- <i>O</i> -acetylsanguinine	2387.1	315 (37)	256 (100), 255 (42), 254 (37), 212(26), 165 (33), 152 (23), 115 (30), 96 (67)
augustine	2411.6	301 (93)	228 (36), 187 (30), 175 (300), 173 (24), 159 (38), 143 (57), 128 (25), 115 (75)
buphanisine <i>N</i> -oxide	2429.8	301 (nv)	285 (100), 270 (33), 254 (35), 216 (21), 215 (82), 201 (24), 157 (20), 128 (22)
haemanthamine	2436.9	301 (55)	257 (54), 227 (80), 225 (98), 224(80), 181 (100), 153 (46), 152 (46), 115 (64)
11,12-dehydroanhydrolycorine	2448.5	249 (55)	248 (100), 191 (13), 190 (31), 189 (11), 95 (14)
crinamine	2497.6	273 (17)	272 (100), 242 (12), 214 (11), 186 (12), 128 (15)
hamayne	2551.7	259 (14)	258 (100), 242 (11), 214 (10), 211 (12), 181 (14), 128 (19),
1- <i>O</i> -acetyllycorine	2563.1	329 (20)	299(15), 268 (28), 250 (17), 244 (20), 227 (56), 226 (100), 240 (11)
augustine <i>N</i> -oxide	2571.8	317 (nv)	301 (100), 228 (34), 187 (22), 175 (77), 173 (17), 159 (27), 143 (37), 115 (37)
lycorine	2592.2	287 (19)	286 (13), 268 (18), 250 (10), 227 (60), 226 (100), 147 (11)
undulatine	2594.4	331 (100)	258 (37), 219 (22), 217 (36), 205 (71), 203 (37), 189 (43), 173 (39), 115 (35)
ambelline	2621.1	331 (69)	287 (100), 260 (81), 257 (62), 255 (74), 254 (52), 241 (51), 239 (61), 211 (69)
augustamine	2628.7	301 (76)	300 (100), 245(16), 244(84), 215(33), 201 (32), 188 (14), 115 (22), 70 (21)
6-hydroxybuphanidrine	2631.3	331 (35)	277 (16), 276 (100), 261 (30), 218 (17), 217 (23), 216 (24), 115 (18), 56 (25)
<i>N</i> -formylnorgalanthamine	2649.1	301 (100)	225 (26), 211 (29), 181 (19), 165 (14), 129 (18), 128 (22), 115 (30), 77 (15)

nv = no visible

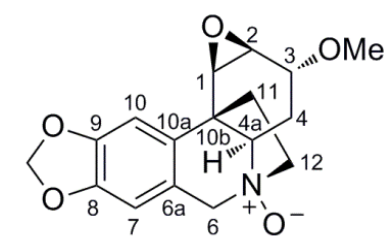
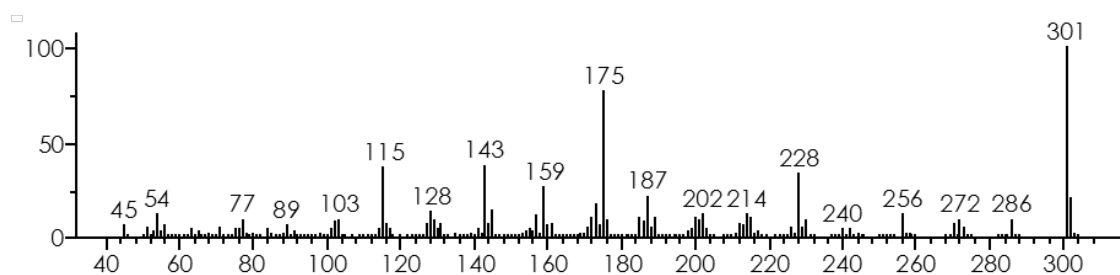
augustine *N*-oxide**Figura 9.** Patrón de fragmentación del alcaloide augustine *N*-oxide por EM.

Tabla 10. Resultados de RMN obtenidos para el compuesto augustine *N*-oxide (CDCl₃, 400MHz).

Posición	δ_H (J en Hz)	δ_C , tipo	COSY	NOESY	HMBC
1	3.68, <i>d</i> (3.5)	52.06	H-2	H-2, H-10	C-3, C-10b
2	3.42, <i>ddd</i> (3.2, 2.4, 0.7)	55.06	H-1, H-3	H-1, H-3	C-4
3	4.12, <i>dt</i> (2.7, 2.5)	73.43	H-2, H-4 α , H-4 β	H-2, H-4 α , H-4 β	C-1, OMe
4 α	2.91, <i>dt</i> (14.1, 3.1)	19.72	H-3, H-4 β , H-4a	H-3, H-4 β , H-4a	C-2
4 β	1.54, <i>ddd</i> (13.8, 13.8, 2.9)	19.72	H-3, H-4 α , H-4a	H-3, H-4 α , H-4a, H-11 _{exo}	-
4a	3.51, <i>dd</i> (13.4, 3.6)	72.59	H-4 α , H-4 β	H-4 α , H-4 β , H-6 α	C-10a
6 α	4.83, <i>dd</i> (15.7, 1.8)	67.56	H-7	H-4a, H-6 β , H-7	C-7, C-10a, C-12
6 β	4.68, <i>d</i> (15.7)	67.56	H-7	H-6 α , H-7, H-12 _{endo}	C-7, C-10a, C-12
6a	-	122.33	-	-	-
7	6.57, <i>s</i>	106.40	H-6 α , H-6 β	H-6 α , H-6 β	C-6, C-9, C-10a
8	-	147.13	-	-	-
9	-	147.79	-	-	-
10	6.90, <i>s</i>	102.49	-	H-1	C-6a, C-8, C-10b
10a	-	133.92	-	-	-
10b	-	43.95	-	-	-
11 _{endo}	1.99, <i>ddd</i> (12.4, 9.4, 5.1)	35.64	H-11 _{exo} , H-12 _{endo} , H-12 _{exo}	H-11 _{exo} , H-12 _{endo} , H-12 _{exo}	C-10a
11 _{exo}	2.79, <i>ddd</i> (12.4, 12.4, 6.9)	35.64	H-11 _{endo} , H-12 _{endo} , H-12 _{exo}	H-4 β , H-11 _{endo} , H-12 _{endo} , H-12 _{exo}	C-10a
12 _{endo}	3.81, <i>ddd</i> (12.8, 9.4, 7.0)	67.56	H-11 _{endo} , H-11 _{exo} , H-12 _{exo}	H-6 β , H-11 _{endo} , H-11 _{exo} , H-12 _{exo}	C-6
12 _{exo}	3.73, <i>dddd</i> (12.5, 12.5, 5.0, 2.2)	67.56	H-11 _{endo} , H-11 _{exo} , H-12 _{endo}	H-11 _{endo} , H-11 _{exo} , H-12 _{endo}	C-6
OCH ₂ O	5.99, <i>d</i> (1.3), 5.98 <i>d</i> (1.3)	101.58	-	-	C-8, C-9
OMe	3.47, <i>s</i>	57.92	-	-	C-3

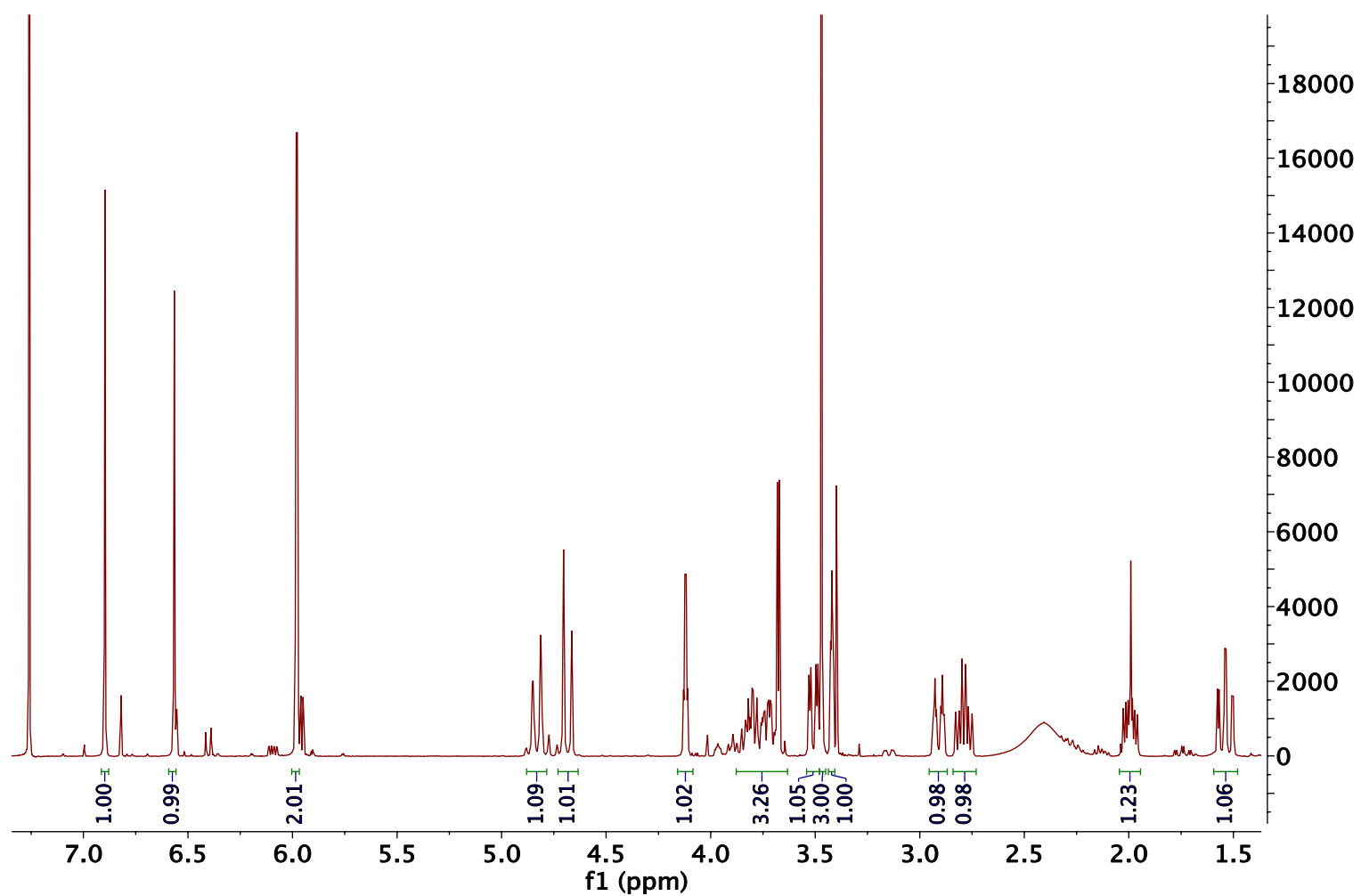
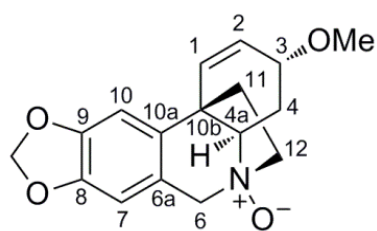


Figura 10. Espectro de ^1H -RMN del compuesto augustine *N*-oxide (CDCl_3 , 400MHz).



buphanisine *N*-oxide

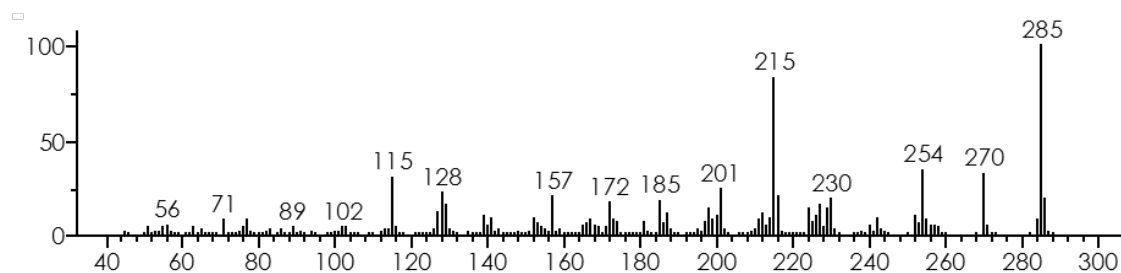


Figura 11. Patrón de fragmentación del alcaloide buphanisine *N*-oxide por EM.

Tabla 11. Resultados de RMN obtenidos para el compuesto buphanisine *N*-oxide (CDCl₃, 400MHz).

Posición	δ_H (J en Hz)	δ_C , tipo	COSY	NOESY	HMBC
1	6.39, <i>d</i> (10.0)	129.59	H-2	H-2, H-10	C-3, C-4a, C-10a, C-11
2	6.08, <i>ddd</i> (10.0, 5.3, 1.0)	127.18	H-1, H-3	H-1, H-3	C-10b
3	3.95, <i>ddd</i> (5.6, 3.6, 2.0)	71.38	H-2, H-4 β	H-2, H-4 β , OMe	C-1, C-4a, OMe
4 α	3.13 <i>ddt</i> (13.6, 4.2, 2.4)	23.46	H-4 β , H-4a	H-4 β , H-4a	C-2, C-10b
4 β	1.72 <i>ddd</i> (13.7, 13.7, 4.0)	23.46	H-3, H-4 α , H-4a	H-3, H-4 α , H-4a, H-11exo	C-10b
4a	3.74, <i>dd</i> (13.2, 4.3)	74.14	H-4 α , H-4 β	H-4 α , H-4 β , H-6 α	C-3, C-6, C-11, C-12
6 α	4.84, <i>d</i> (15.6)	76.55	H-6 β , H-7	H-4a, H-6 β , H-7	C-4a, C-7, C-10a, C-12
6 β	4.72, <i>d</i> (15.6)	76.55	H-6 α , H-7	H-6 α , H-7, H-12endo	C-4a, C-7, C-10a, C-12
6a	-	121.71	-	-	-
7	6.54, <i>s</i>	106.44	H-6 α , H-6 β	H-6 α , H-6 β	C-6, C-9, C-10a
8	-	147.20	-	-	-
9	-	147.79	-	-	-
10	6.81, <i>s</i>	102.98	-	H-1	C-10b, C-6a, C-8
10a	-	134.75	-	-	-
10b	-	46.60	-	-	-
11endo	2.11, <i>ddd</i> (12.5, 8.0, 6.0)	40.02	H-11exo, H-12endo, H-12exo	H-11exo, H-12endo, H-12exo	C-10a
11exo	2.26, <i>ddd</i> (12.2, 10.8, 8.6)	40.02	H-11endo, H-12endo, H-12exo	H-4 β , H-11endo, H-12endo, H-12exo	C-1, C-10a
12endo	3.88, <i>m</i>	68.97	H-11endo, H-11exo, H-12exo	H-6 β , H-11endo, H-11exo, H-12exo	C-4a, C-6, C-10b
12exo	3.85, <i>m</i>	68.97	H-11endo, H-11exo, H-12endo	H-11endo, H-11exo, H-12endo	C-4a, C-6, C-10b
OCH ₂ O	5.95, <i>d</i> (1.3), 5.93 <i>d</i> (1.3)	101.51	-	-	C-8, C9
OMe	3.39, <i>s</i>	57.23	-	-	C-3

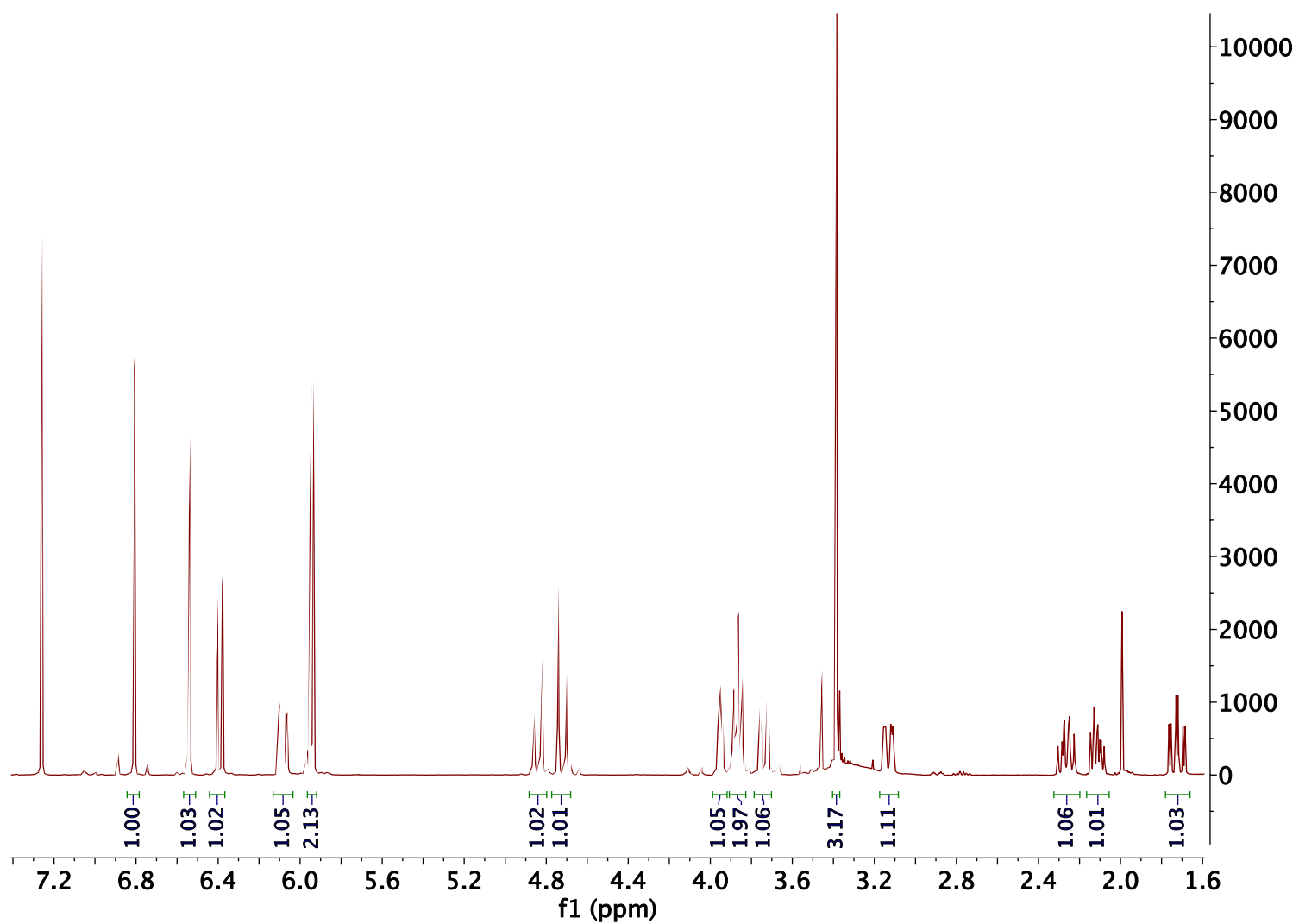


Figura 12. Espectro de ^1H -RMN del compuesto buphanisine *N*-oxide (CDCl_3 , 400MHz).

7.3.4 Rhodophiala

Tabla 12. Alcaloides detectados en distintas especies de *Rhodophiala* por CG-EM. Valores expresados en mg GAL g⁻¹ ERA.

Alcaloide	M ⁺	Ion Base	IR	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Tipo lycorine				44.1	8.6	58.2	7.5	43.2	25.1	79.0	46.0	78.6	46.9	42.6	35.5	26.1	37.3	33.8	35.4	41.2	60.8	38.5	13.1
lycorene (1)	255	254	2346.8	-	-	-	-	-	-	-	-	7.6	9.4	-	5.1	trazas	7.2	-	trazas	-	-	-	-
anhydrolycorine (2)	251	250	2543.1	18.1	-	15.4	trazas	25.0	5.9	27.3	11.2	24.6	9.6	9.4	9.9	10.1	8.3	13.3	11.8	14.9	13.2	7.7	-
kirkine (3)	253	252	2588.2	5.0	-	6.0	-	-	-	5.1	-	trazas	7.2	6.1	5.3	-	-	-	6.0	-	7.2	trazas	-
11,12-dehydroanhydrolycorine (4)	249	248	2646.4	11.0	8.6	8.6	7.5	8.1	13.5	8.5	8.4	19.0	15.6	6.6	8.1	6.4	21.8	7.3	7.5	6.6	9.4	8.5	7.8
1- <i>O</i> -acetylcaranine (5)	313	226	2653.1	-	-	-	-	-	-	-	-	17.4	-	-	-	-	-	-	-	-	-	-	-
norpluviine (6)	273	228	2683.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.2	-	-
assoanine (7)	267	266	2708.9	-	-	-	trazas	-	-	trazas	-	-	-	-	-	-	-	-	-	-	-	-	-
3,4-dihydro 1-acetyllycorine (8)	331	330	2723.3	-	-	-	-	-	-	-	-	-	-	-	-	trazas	-	-	-	-	-	trazas	-
lycorine (9)	287	226	2791.7	10.0	-	21.8	trazas	10.1	5.7	27.5	14.7	10.0	5.1	15.4	7.1	trazas	-	7.4	10.1	19.7	24.8	16.3	5.3
dihydrolycorine (10)	289	288	2833.9	-	-	-	-	-	-	-	-	-	-	trazas	-	9.6	-	trazas	trazas	trazas	trazas	trazas	-
pseudolycorine (11)	289	228	2856.4	-	-	6.4	-	-	-	10.6	-	-	-	5.1	-	-	-	-	-	-	-	6.0	-
sternbergine (12)	331	228	2838.8	-	-	-	-	-	-	-	6.4	-	-	-	-	-	-	5.8	trazas	-	-	-	-
methylpseudolycorine (13)	303	242	2911.2	-	-	-	-	-	-	trazas	-	-	-	-	-	-	-	-	-	-	-	-	-
1- <i>O</i> -(3'-acetoxybutanoyl)lycorine (14)	415	226	3248.8	-	-	-	-	-	-	-	5.3	-	-	-	-	-	-	-	-	-	-	-	-
Tipo haemanthamine/crinine				60.0	-	49.5	11.6	31.7	53.1	43.7	34.4	17.4	14.8	28.6	17.2	33.8	21.4	45.5	38.7	44.9	35.7	36.9	48.0
vittatine (15a)/crinine (15b)	271	271	2512.4	-	-	10.8	trazas	-	7.7	7.1	-	trazas	-	5.6	-	6.2	-	7.3	6.2	7.3	5.6	6.3	-
8- <i>O</i> -demethylmaritidine (16)	273	273	2540.0	-	-	trazas	-	-	-	-	-	trazas	-	-	-	-	-	trazas	-	-	-	6.0	6.4
deacetylcantabricine (17)	275	275	2573.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	trazas
haemanthamine (18a)/crinamine (18b)	301	272	2673.4	60.0	-	31.7	11.6	31.7	45.4	36.6	34.4	5.8	7.7	23.0	17.2	27.6	21.4	38.2	32.5	31.0	30.1	16.4	25.0
buphanidrine (19)	315	315	2748.3	-	-	-	-	-	-	-	-	5.9	-	-	-	-	-	-	-	-	-	-	-
11-hydroxyvittatine (20a)/ hamayne (20b)	287	258	2750.5	-	-	7.00	-	-	-	trazas	-	-	-	-	-	-	-	-	-	6.6	-	8.2	16.6
undulatine (21)	331	331	2892.5	-	-	-	-	-	-	-	-	5.7	7.1	-	-	-	-	-	-	-	-	-	-
Tipo tazettine				74.8	60.7	56.1	59.1	85.9	95.8	33.1	22.3	-	-	21.8	11.2	18.0	16.3	8.9	16.0	17.9	29.9	21.9	55.6
deoxytazettine (22)	315	231	2575.6	8.3	6.1	10.7	5.5	13.1	6.7	9.6	5.0	-	-	-	trazas	-	5.2	-	-	-	5.2	trazas	-
<i>O</i> -methyltazettine (23)	345	261	2641.1	58.7	54.6	23.0	36.0	61.7	66.2	14.8	10.3	-	-	10.9	11.2	12.7	11.1	8.9	10.1	9.9	14.9	14.3	30.7
tazettine (24)	331	247	2685.1	7.8	-	22.4	12.5	11.1	17.0	8.7	7.0	-	-	10.9	-	5.3	-	trazas	5.9	8.0	9.8	7.6	24.9
epimacronine (25)	329	245	2848.0	-	-	trazas	5.1	trazas	5.9	trazas	trazas	-	-	-	trazas	-	-	-	-	-	trazas	-	trazas
Tipo homolycorine				9.4	-	-	-	10.6	9.1	-	-	-	-	13.9	8.6	14.1	22.4	10.1	6.5	28.5	19.5	9.8	-
nerinine (26)	347	109	2513.5	-	-	-	-	-	-	-	-	-	-	13.9	8.6	14.1	22.4	10.1	6.5	21.1	19.5	-	-
8- <i>O</i> -demethylhomolycorine (27)	301	109	2856.4	9.4	-	-	-	10.6	9.1	-	-	-	-	-	-	-	-	-	-	7.4	-	9.8	-
Tipo galanthamine				-	-	19.0	5.1	11.9	14.1	12.3	7.1	-	5.8	-	-	-	-	-	-	-	-	-	-
galanthamine (28)	287	286	2519.9	-	-	5.1	-	6.8	8.6	5.4	7.1	trazas	5.8	-	-	-	-	-	-	-	-	-	-
lycoramine (29)	289	288	2544.6	-	-	13.9	5.1	5.1	5.5	6.9	-	-	-	-	-	-	-	-	-	-	-	-	-
Tipo montanine				-	-	-	-	-	-	5.2	-	7.8	8.1	41.7	28.3	23.1	19.7	12.5	15.2	24.7	26.7	6.4	-
pancratinine C (30)	287	176	2623.5	-	-	-	-	-	-	-	-	trazas	-	5.3	-	trazas	-	-	-	-	-	-	-
montanine (31)	301	301	2663.1	-	-	-	-	-	-	5.2	-	7.8	8.1	29.8	28.3	23.1	19.7	12.5	15.2	24.7	26.7	-	-
pancracine (32)	287	287	2737.4	-	-	-	-	-	-	-	-	-	-	6.6	-	-	-	-	-	-	-	6.4	-
Tipo mesembrenone				-	-	-	-	-	-	-	-	-	-	7.2	-	5.2	-	-	-	5.1	-	-	-
demethylmesembrenol (33)	275	205	2343.2	-	-	-	-	-	-	-	-	-	-	7.2	trazas	5.2	-	trazas	trazas	5.1	trazas	-	-
Tipo narciclasine				12.9	12.7	12.7	11.2	13.3	13.5	5.8	5.8	5.7	6.2	-	5.5	-	44.5	-	-	-	10.4	5.1	32.2
trisphearidine (34)	223	223	2322.9	5.5	12.7	6.3	5.1	5.3	6.0	trazas	5.8	5.7	6.2	trazas	5.5	trazas	39.4	trazas	trazas	trazas	5.2	5.1	22.7
dihydrobicolorine (35)	239	238	2366.1	7.4	trazas	6.4	6.1	8.0	7.5	5.8	trazas	trazas	-	trazas	trazas	trazas	5.1	trazas	trazas	trazas	5.2	trazas	9.5
Otro tipo				19.2	5.2	35.6	32.9	28.2	62.1	21.8	8.5	-	-	7.9	5.9	7.8	-	5.3	5.7	8.0	17.7	7.3	34.5
ismine (36)	257	238	2304.6	6.6	-	9.9	7.6	9.5	17.0	6.6	trazas	-	-	trazas	trazas	trazas	-	trazas	trazas	trazas	6.0	trazas	14.3
galanthindole (37)	281	281	2534.8	12.6	5.2	25.7	25.3	18.7	45.1	15.2	8.5	-	-	7.9	5.9	7.8	-	5.3	5.7	8.0	11.7	7.3	20.2
No identificado				29.9	46.0	41.4	26.8	41.2	46.1	79.8	42.0	76.8	126.9	92.6	35.9	50.5	78.5	32.0	20.6	76.0	73.4	36.0	21.8

unknown	227	226	2232.2	-	-	-	-	-	-	-	-	-	-	-	-	-	8.8	-	-	-	-	-	-
unknown	227	225	2232.2	-	-	trazas	5.6	6.2	6.8	5.1	5.6	-	-	-	-	-	8.9	trazas	-	-	-	-	6.2
unknown	269	238	2258.9	-	-	trazas	5.1	-	6.9	-	-	-	-	-	-	-	-	-	-	-	-	6.4	
unknown	245	175	2280.8	-	-	-	-	-	-	-	-	-	-	15.3	6.0	11.8	-	6.1	-	11.4	10.5	-	-
unknown	269	268	2285.5	-	-	-	-	-	-	-	-	-	-	-	-	-	9.7	-	-	-	-	-	-
unknown	253	252	2313.1	5.4	5.9	-	trazas	-	-	-	-	6.7	5.9	-	-	-	13.7	5.1	trazas	-	-	-	-
unknown	251	251	2335.2	-	-	-	-	-	-	-	-	6.2	6.2	-	-	-	9.9	-	-	-	-	-	-
unknown	257	256	2379.0	-	-	-	-	-	-	8.0	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	253	252	2405.0	-	-	6.4	-	-	-	5.1	-	6.9	8.9	8.3	6.2	8.0	10.3	6.0	-	5.4	5.5	5.5	-
unknown	269	211	2480.5	-	-	-	-	-	-	trazas	trazas	34.5	28.1	5.3	-	5.1	6.1	-	trazas	-	7.1	-	-
unknown	271	238	2492.7	5.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	331	109	2557.5	-	-	-	-	-	-	-	-	-	-	19.7	5.3	18.5	5.7	8.7	7.0	46.7	33.0	-	-
unknown	329	329	2564.1	-	-	-	-	-	-	-	-	-	-	6.2	5.3	7.1	-	6.1	trazas	7.2	7.3	-	-
unknown	257	225	2579.2	-	-	-	-	-	-	-	trazas	-	-	-	-	-	5.4	-	-	-	-	-	-
unknown	299	238	2584.0	-	-	-	-	trazas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	315	254	2616.4	-	-	-	-	-	-	-	-	5.4	17.5	8.6	6.0	-	-	-	5.7	-	-	-	-
unknown	315	231	2616.9	-	-	-	-	5.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	329	268	2646.1	-	-	-	-	-	-	-	-	-	5.1	7.9	-	-	-	-	-	5.3	-	-	-
unknown	297	297	2655.7	-	-	-	-	-	-	-	-	-	5.4	-	-	-	-	-	-	-	-	-	-
unknown	345	261	2662.6	18.8	17.9	11.2	16.1	23.0	24.6	-	5.6	-	-	-	-	-	-	-	-	-	-	7.3	9.2
unknown	345	272	2671.8	-	22.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	283	283	2692.5	-	-	-	-	-	-	-	-	-	6.9	-	-	-	-	-	-	-	-	-	-
unknown	303	302	2703.2	-	-	-	-	-	-	-	-	-	-	21.3	7.1	-	-	-	-	-	-	-	-
unknown	315	315	2724.2	-	-	-	-	-	-	-	-	-	16.8	-	-	-	-	-	-	-	-	-	-
unknown	253	252	2735.3	-	-	-	-	-	-	17.1	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	345	109	2735.6	-	-	-	-	-	-	-	-	-	5.9	-	-	-	-	-	-	-	-	-	-
unknown	347	331	2795.5	-	-	-	-	-	-	-	-	-	6.5	-	-	-	-	-	-	-	-	-	-
unknown	345	331	2795.7	-	-	-	-	-	-	-	5.6	-	-	-	-	-	-	-	-	-	-	-	-
unknown	331	331	2795.8	-	-	-	-	-	-	-	-	6.9	-	-	-	-	-	-	-	-	-	-	-
unknown	251	250	2846.6	-	-	-	-	6.8	7.8	9.9	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	335	335	2860.6	-	-	7.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	345	242	2868.8	-	-	-	-	-	-	-	5.3	-	-	-	-	-	-	-	-	-	-	-	-
unknown	373	372	2881.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.8	-
unknown	357	356	2942.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.9	-	10.0	7.0	-	-
unknown	279	278	3016.0	-	-	-	-	-	-	-	-	10.2	13.7	-	-	-	-	-	-	-	-	-	-
unknown	375	330	3027.5	-	-	7.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	267	266	3032.9	-	-	9.2	-	-	-	7.3	-	-	-	-	-	-	-	-	-	-	-	-	-
unknown	375	374	3049.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8.4	-
unknown	355	226	3066.6	-	-	-	-	-	-	5.8	6.1	-	-	-	-	-	-	-	-	-	-	-	-
unknown	373	226	3161.0	-	-	-	-	-	-	21.5	13.8	-	-	-	-	-	-	-	-	-	-	-	-
Total				250.3	133.2	272.5	154.2	265.9	311.1	280.4	166.1	186.0	208.7	256.3	148.1	178.6	240.1	148.1	138.1	246.3	274.1	161.9	205.3

A: *R. andicola*, B, SN; **B:** *R. andicola*, AP, SN; **C:** *R. andicola*, B, NC; **D:** *R. andicola*, AP, NC; **E:** *R. andicola*, B, VL; **F:** *R. andicola*, AP, VL; **G:** *R. araucana*, B; **H:** *R. araucana*, AP; **I:** *R. montana*, B; **J:** *R. montana*, AP; **K:** *R. pratensis*, B, RF, nl, dunes; **L:** *R. pratensis*, AP, RF, nl, dunes; **M:** *R. pratensis*, B, RF, L; **N:** *R. pratensis*, AP, RF, L; **O:** *R. pratensis*, B, RF, nl; **P:** *R. pratensis*, AP, RF, nl; **Q:** *R. pratensis*, B, WF; **R:** *R. pratensis*, AP, WF; **S:** *R. splendens*, B; **T:** *R. splendens*, AP; - : no detectado.

7.4 Anexo IV: Especies estudiadas



Figura 13. *Amaryllis belladonna*.



Figura 14. *Hippeastrum reticulatum*.



Figura 15. *Crinum amabile*.



Figura 16. *Rhodophiala splendens*.

