Biodiversidad y Conservación de Recursos Fitogenéticos. Las Amarillidáceas como Fuente de Productos Bioactivos

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Memòria presentada per Natalia Belén Pigni per optar al títol de doctor per la Universitat de Barcelona

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1. INTRODUCCIÓN

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1.1. Productos Naturales y Desarrollo de Fármacos

A lo largo de la historia, el hombre ha tenido que valerse de una gran variedad de recursos naturales para sobrevivir. En el campo de la medicina tradicional, por ejemplo, el uso de hierbas medicinales para el tratamiento de diversas afecciones de la salud se remonta a miles de años atrás, con registros que datan de la antigua Babilonia, Egipto, India y China. En la industria farmacéutica moderna, a pesar de la gran variedad de moléculas derivada de los progresos en el ámbito de la química combinatoria, los productos naturales continúan desempeñando un papel fundamental en el desarrollo de fármacos (Ngo *et al.*, 2013). Tal como señalan Newman y Cragg (2012) en su última revisión sobre las fuentes de nuevos fármacos aprobados durante el período de 30 años comprendido entre 1981 y 2010, la contribución de esta área de investigación ha sido muy significativa. Por mencionar un ejemplo, de todas las pequeñas moléculas aprobadas como fármacos, cerca del 35% corresponde a compuestos de origen natural y derivados semisintéticos, mientras que el 30% son moléculas sintéticas inspiradas en productos naturales o con un farmacóforo desarrollado a partir de un compuesto natural.

Entre los recursos naturales disponibles, los vegetales representan una fuente importante de moléculas estereoespecíficas con estructuras complejas. Esta capacidad para producir metabolitos secundarios con diversas actividades biológicas suele explicarse como parte de una estrategia de supervivencia contra el ataque de herbívoros, o bien para facilitar una adecuada dispersión. La gran influencia de las plantas en el desarrollo de fármacos se ejemplifica adecuadamente con el conocido alcaloide morfina, aislado por primera vez a principios del siglo XIX de *Papaver somniferum* y todavía ampliamente aplicado en la medicina actual para el tratamiento del dolor (Houghton, 2001).

A pesar del enorme desarrollo en el campo de la síntesis orgánica, aún hoy existen casos de fármacos que se obtienen directamente a partir del material vegetal, lo que resulta en una necesidad creciente de generar fuentes renovables y promover la protección de la biodiversidad vegetal (Lubbe y Verpoorte, 2011). El uso racional de los recursos naturales es esencial para evitar un posible deterioro irreversible.

1.2. Plantas de la Familia Amaryllidaceae y sus Alcaloides

La familia botánica Amaryllidaceae, un grupo de monocotiledóneas pertenecientes al orden Asparagales, ha sido objeto de debate taxonómico durante mucho tiempo. Según la última clasificación actualizada del APG (*Angiosperm Phylogeny Group*), sustentada por varios análisis moleculares y morfológicos (Meerow *et al.*, 1999; Meerow y Snijman, 2006), la familia Amaryllidaceae J.St.-Hil. comprende tres subfamilias: Agapanthoideae, Allioideae y Amaryllidoideae las cuales, a su vez, habían sido previamente consideradas como Agapanthaceae (comúnmente conocidos como agapantos), Alliaceae (incluyendo al ajo y las especies afines) y Amaryllidaceae (Chase *et al.*, 2009; APG III, 2009). Cabe señalar que, a pesar de que la estrecha relación entre estos tres grupos está generalmente aceptada, la modificación de la nomenclatura puede dar lugar a malentendidos, ya que el concepto más ampliamente utilizado relacionado con el término "Amaryllidaceae" implica sólo una subfamilia del taxón actual.

Las plantas de la subfamilia Amaryllidoideae, objeto de este estudio, son hierbas perennes y bulbosas que suelen presentar flores llamativas, lo que les proporciona valor ornamental. Se han reconocido 59 géneros, tales como *Crinum*, *Hippeastrum*, *Zephyranthes*, *Narcissus*, *Galanthus*, entre otros, y alrededor de 800 especies (Stevens, 2012). Su distribución geográfica es cosmopolita, incluyendo principalmente regiones tropicales y subtropicales, pero también es común encontrar algunos géneros en el área del Mediterráneo y en zonas templadas de Asia (Figura 1.1). Estudios filogenéticos apuntan a Sudáfrica y América del Sur como centros de diversificación primaria y secundaria, respectivamente (Ito *et al.*, 1999). Dado que numerosas especies de este grupo son endémicas y muy vulnerables, existe la necesidad de promover su conservación y mejorar el conocimiento existente sobre las mismas.

Una de las características más interesantes de las plantas de Amaryllidoideae es la presencia de un grupo de alcaloides exclusivo, los cuales han sido objeto de investigación durante más de 150 años (Bastida *et al.*, 2006). Teniendo en cuenta los recientes cambios en su clasificación taxonómica ya mencionados, es importante hacer hincapié en que estos compuestos no son típicos de las subfamilias Allioideae y Agapanthoideae, aunque continúan siendo ampliamente conocidos como alcaloides de

"Amaryllidaceae". Estos alcaloides se han aislado a partir de todos los géneros de la subfamilia Amaryllidoideae, y pueden ser considerados como marcadores químicos.

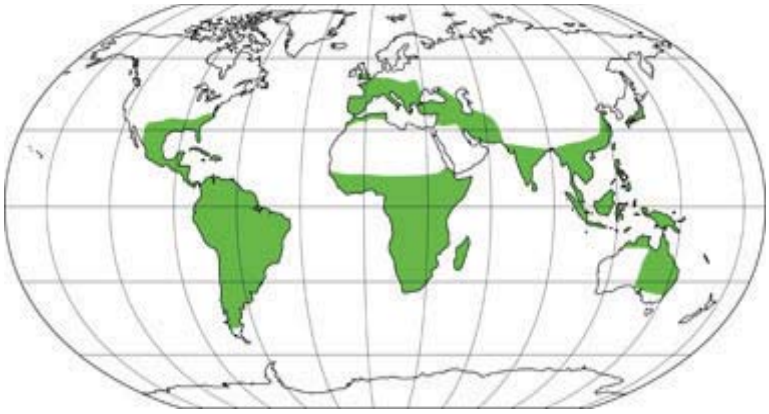

Figura 1.1: Distribución mundial de la familia Amaryllidaceae (subfam. Amaryllidoideae). Imagen tomada de *www.thecompositaehut.com*.

El gran interés en el estudio de los alcaloides de Amaryllidaceae, especialmente en el ámbito de la farmacología, se debe a su amplio rango de actividades biológicas, que incluyen, entre otras, propiedades antivirales, antitumorales y antiparasitarias (Bastida *et al.*, 2011). De hecho, estas plantas han sido utilizadas como hierbas medicinales durante miles de años. En el siglo IV a. C., el aceite de *Narcissus poeticus* L. ya era conocido por Hipócrates de Cos por ser adecuado para el tratamiento de tumores, mientras que en el siglo I d. C., se había establecido para este fin en Oriente Medio y en el Imperio Romano (Pettit *et al.*, 1986). En la actualidad, uno de los compuestos más interesantes del grupo es la galantamina, un inhibidor potente, reversible y competitivo de la enzima acetilcolinesterasa (AChE), aprobado y comercializado como estrategia para el tratamiento paliativo de la enfermedad de Alzheimer (Reminyl®, Razadyne®).

1.2.1. Química y Biosíntesis

Desde el aislamiento de licorina a partir de *Narcissus pseudonarcissus* en 1877 hasta el presente, se han caracterizado más de 400 alcaloides de las Amaryllidoideae (Jin, 2009), y todos ellos están relacionados a nivel biosintético. En general, se clasifican en nueve grupos diferentes representados por: norbelladina, licorina,

homolicorina, crinina, hemantamina, narciclasina, tazetina, montanina y galantamina (Figura 1.2) (Bastida *et al.*, 2011). Sus particularidades químicas más notables se resumen a continuación (Bastida y Viladomat, 2002):

1. Una estructura base C_6-C_1-N-C_2-C_6, en la que la porción C_6-C_1 deriva del aminoácido L-fenilalanina y el fragmento N-C_2-C_6 proviene de L-tirosina.
2. Son bases moderadamente débiles con pKa entre 6 y 9.
3. La mayoría contiene un solo átomo de nitrógeno, el cual puede ser secundario, terciario o incluso cuaternario. Típicamente, el número de carbonos varía entre 16 y 20, según los sustituyentes del sistema policíclico.

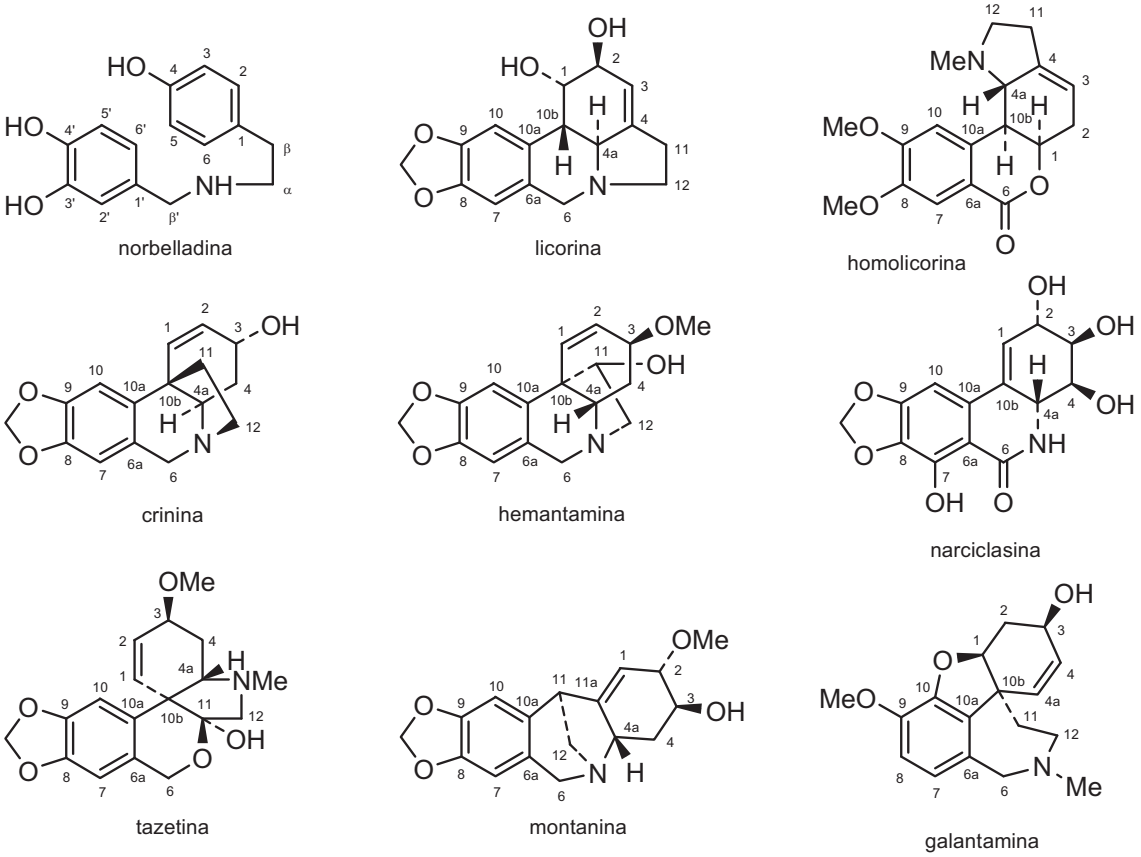

Figura 1.2: Alcaloides de Amaryllidaceae representativos de los 9 grupos. La numeración corresponde a la propuesta por Ghosal *et al.* (1985).

La ruta biosintética que origina esta variedad de estructuras sigue un esquema general de cuatro etapas que comienza con la preparación enzimática de los precursores a partir de los aminoácidos L-fenilalanina (L-phe) y L-tirosina (L-tyr). En los alcaloides de amarillidáceas, L-phe sirve como precursor primario del fragmento C_6-C_1, que corresponde al anillo A y la posición bencílica (C-6), mientras que L-tyr es precursor del anillo C, la cadena lateral de dos carbonos (C-11 y C-12) y el nitrógeno, C_6-C_2-N. Para formar la porción C_6-C_1, L-phe es convertida en aldehído protocatéquico a través de la vía de ácidos cinámicos, involucrando la participación de la enzima fenilalanina amonio liasa (PAL). Por otro lado, L-tyr es mínimamente modificada mediante un paso de descarboxilación antes de ser incorporada (Figura 1.3) (Bastida *et al.*, 2011).

La segunda etapa de la biosíntesis implica la fusión entre tiramina y el aldehído protocatéquico, que da lugar a norbelladina por medio de la formación de una base de Schiff. Esta reacción clave representa la entrada de los metabolitos primarios a una vía metabólica secundaria. La posterior metilación de norbelladina en la posición *orto* (4´) del anillo A se considera el tercer paso.

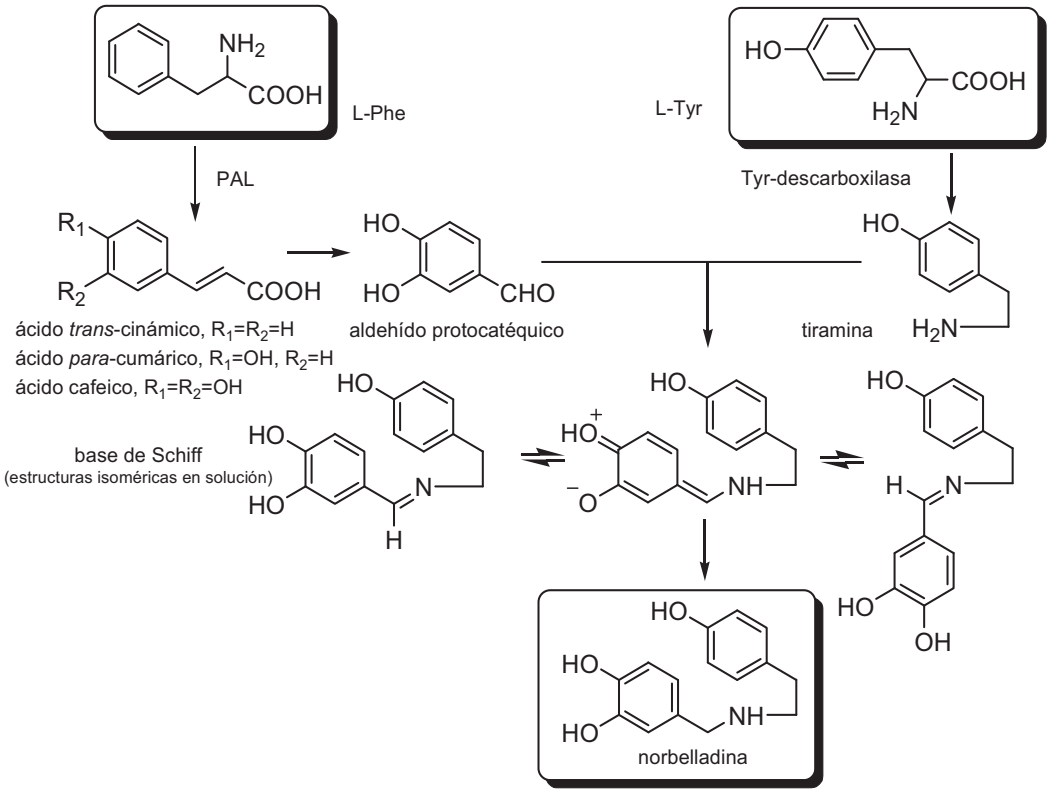

Figura 1.3: Ruta biosintética hasta norbelladina.

Finalmente, la última etapa incluye una serie de reacciones secuenciales que resultan en la diversificación hacia los ocho esqueletos restantes mostrados en la Figura 1.2. El paso inicial consiste en una ciclación secundaria producida por el acoplamiento oxidativo de *O*-metilnorbelladina, el cual puede transcurrir a través de tres vías diferentes para dar lugar a las distintas estructuras. El acoplamiento fenol oxidativo *orto-para´* resulta en la formación del esqueleto tipo licorina, a partir del cual se originan los compuestos de tipo homolicorina. Por otro lado, la ciclación secundaria *para-para´* produce los alcaloides con estructura base 5,10b-etanofenantridina (tipos crinina y hemantamina), los cuales pueden sufrir ciertas modificaciones para generar las estructuras de tipo tazetina, narciclasina y montanina. De manera similar, los alcaloides de tipo galantamina, con su núcleo dibenzofurano característico, derivan de un acoplamiento fenol oxidativo *para-orto´* (Figura 1.4).

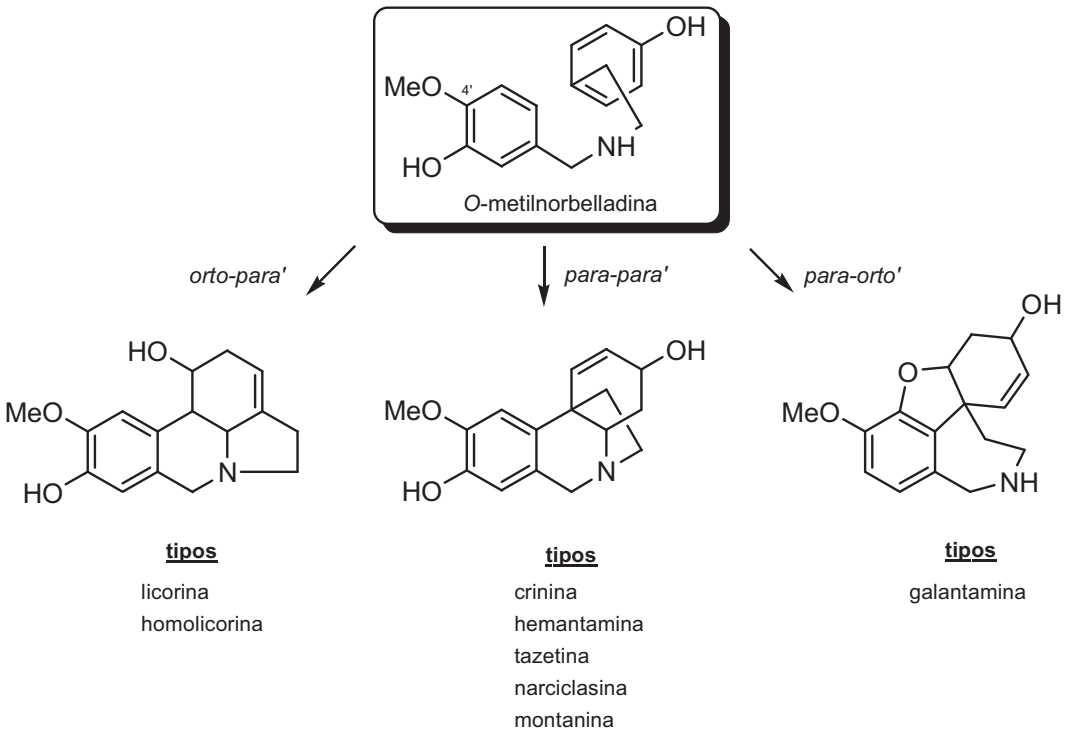

Figura 1.4: Vías alternativas de acoplamiento fenol oxidativo.

Recientemente, a partir del aislamiento de nuevos alcaloides de especies de los géneros *Cyrtanthus*, *Narcissus* y *Galanthus* (Brine *et al.*, 2002; de Andrade *et al.*, 2012a; Ünver *et al.*, 1999), se han propuesto algunos subgrupos adicionales de estructuras, tales como las gracilinas, que incorporan un esqueleto 10b,4a-

etanoiminodibenzo[*b,d*]pirano; las plicaminas, compuestos dinitrogenados en los que el oxígeno del anillo B de tazetina está reemplazado por un nitrógeno que, a su vez, presenta un sustituyente de tipo hidroxifenetil; y el galantindol (Figura 1.5) (Ünver, 2007). Con respecto a su biosíntesis, las gracilinas posiblemente se originan a partir de alcaloides de tipo hemantamina, mientras que las plicaminas proceden muy probablemente del tipo tazetina (de Andrade *et al.*, 2012b).

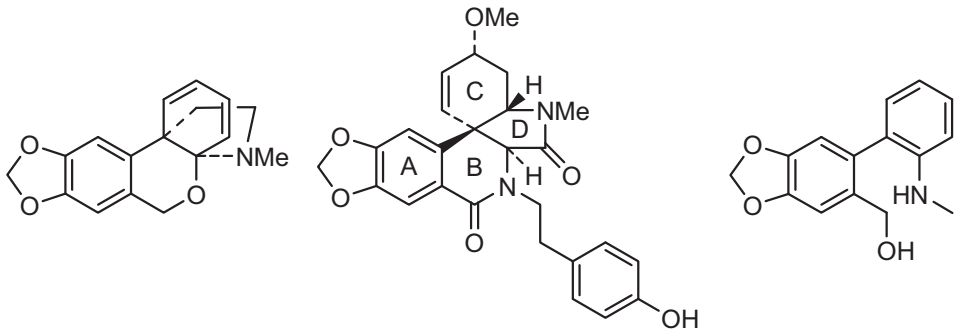

Figura 1.5: Estructuras representativas de los subgrupos adicionales propuestos.

Además, se han encontrado otras estructuras inusuales en plantas de la subfamilia Amaryllidoideae, tales como (-)-capnoidina y (+)-bulbocapnina, ambos considerados alcaloides isoquinolínicos típicos, que han sido identificados en la especie *Galanthus nivalis* subsp. *cilicicus* de Turquía (Kaya *et al.*, 2004). Sin embargo, hasta la fecha, los compuestos inusuales siempre se han hallado en compañía de alcaloides típicos de Amaryllidaceae.

Otro caso curioso corresponde a los alcaloides de tipo mesembrano, característicos del género *Sceletium* (Aizoaceae) de Sudáfrica (Figura 1.6) (Smith *et al.*, 1996). Estos compuestos han sido encontrados en algunas especies de Amaryllidoideae, incluyendo *Narcissus pallidulus*, *Crinum oliganthum,* y *Narcissus triandrus* (Bastida *et al.*, 1989; Döpke y Sewerin, 1981; Seijas *et al.*, 2004). En un principio, su similitud estructural con los alcaloides de tipo crinano sugirió la posibilidad de una ruta biosintética en común con los alcaloides de Amaryllidaceae (Jeffs *et al.*, 1971a), pero estudios posteriores revelaron procesos fundamentalmente diferentes, aunque con la participación de los mismos aminoácidos precursores (Gaffney, 2008).

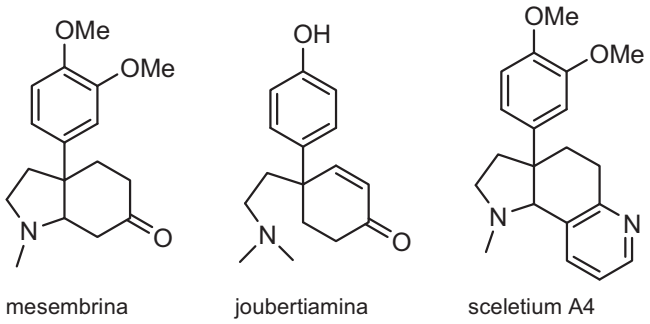

Figura 1.6: Estructuras representativas de los alcaloides de tipo mesembrano.

1.2.2. Actividad Biológica

Tal como ocurre con muchos grupos de plantas, el estudio detallado de los componentes activos de las especies de Amaryllidoideae ha sido ocasionado gracias a la observación de su uso tradicional en la medicina popular, y el género *Narcissus* es un buen ejemplo. Como ya se ha mencionado, algunas especies se han utilizado para el tratamiento de tumores desde hace más de dos mil años, pero también se ha descrito, entre otros, su uso en aplicaciones locales para heridas, tratamiento de trastornos respiratorios y como antieméticos (Bastida *et al.*, 2006). Si bien un gran número de extractos vegetales han sido ensayados demostrando un amplio rango de actividades biológicas, el aislamiento de sus alcaloides puros, junto con su síntesis en algunos casos, así como los resultados obtenidos de estudios de relación estructura-actividad (SAR), han permitido establecer ciertas actividades para los diversos tipos de estructuras.

El alcaloide hallado con más frecuencia dentro de este grupo es licorina, que fue el primero en ser aislado. Se ha reportado su actividad como potente inhibidor de la biosíntesis de ácido ascórbico, del crecimiento y la división celular, y de la organogénesis en plantas superiores, algas y levaduras. En animales, ha demostrado una importante actividad antitumoral, siendo considerado un agente quimioterapéutico prometedor debido a su efecto antiproliferativo selectivo, más marcado en células cancerosas que en células normales (Lamoral-Theys *et al.*, 2009; McNulty *et al.*, 2009). Otra actividad destacable de los alcaloides de tipo licorina es su potente efecto inhibidor sobre parásitos, habiéndose reportado que su actividad frente a *Trichomonas vaginalis* transcurre a través de la muerte celular mediada por un

mecanismo conocido como parapoptosis (Giordani *et al.*, 2011), además de numerosos ensayos que señalan la inhibición de *Plasmodium falciparum* y *Trypanosoma brucei* (Toriizuka *et al.*, 2008). Licorina y pseudolicorina también han demostrado, entre otras propiedades, efectos antivirales, así como la capacidad de interaccionar con el ADN. Al igual que otros tipos de alcaloides del grupo, licorina y sus derivados presentan efectos analgésicos e hipotensores (Bastida *et al.*, 2011).

Algunos compuestos de tipo homolicorina muestran actividad citotóxica e hipotensora, mientras que la propia homolicorina también ha demostrado actividad antirretroviral. La hipeastrina, alcaloide activo frente a *Herpes simplex* tipo I, muestra propiedades antifúngicas frente a *Candida albicans* y posee una débil acción disuasoria de alimentación en insectos (Bastida *et al.*, 2011). Por otra parte, candimina presenta actividad frente a *T. vaginalis*. Sin embargo, la bioactividad de este tipo de alcaloides se desconoce en gran medida (de Andrade *et al.*, 2012b).

Los compuestos de la serie hemantamina han demostrado efectos inhibidores significativos del crecimiento de una gran variedad de células tumorales, siendo potentes inductores de apoptosis a concentraciones micromolares (McNulty *et al.*, 2007). Entre otras actividades destacadas descritas para este tipo de estructuras, los efectos antimaláricos son particularmente notables, y se ha propuesto que la presencia del grupo metilendioxi, junto con el nitrógeno terciario no metilado, contribuyen a un aumento de la actividad (Osorio *et al.*, 2008). Por otro lado, vitatina ha demostrado actividad antibacteriana frente a organismos Gram-positivos, *Staphylococcus aureus*, y Gram-negativos, *E. coli* (Evidente *et al.*, 2004). Con respecto a los alcaloides tipo crinina, con su característica configuración β del puente 5,10b-etano, también han mostrado efectos antiproliferativos sobre células tumorales humanas (Berkov *et al.*, 2011c), pero se requieren estudios adicionales para corroborar las propiedades de este esqueleto tipo.

Tanto narciclasina como pancratistatina han sido objeto de numerosos estudios debido a su prometedora actividad antitumoral y otros efectos biológicos (Bastida *et al.*, 2011). McNulty *et al.* (2010) reportan la inhibición potente y selectiva del citocromo humano P450 3A4 por análogos sintéticos de pancratistatina. Asimismo, a pesar de que algunos ensayos preclínicos con narciclasina resultaron desalentadores debido a su toxicidad, recientemente se ha propuesto la aplicación potencial de este

alcaloide y estructuras relacionadas para el tratamiento de tumores cerebrales (Van Goietsenoven *et al.*, 2013).

El alcaloide tazetina ha demostrado ser un artefacto del aislamiento originado a partir de pretazetina, un compuesto químicamente lábil (de Andrade *et al.*, 2012a). Aunque ambos han mostrado propiedades citotóxicas, pretazetina resulta mucho más interesante dada su actividad como antiviral y antitumoral (Bastida *et al.*, 2011).

Uno de los esqueletos más relevantes debido a su amplio espectro de acción es la montanina. Su actividad antioxidante se ha evaluado mediante ensayos con el radical DPPH (2,2-difenil-1-picril-hidracilo), reportándose su actividad inhibidora del crecimiento de algunos microorganismos, como *Staphylococcus aureus*, *Pseudomonas aeruginosa* y *E. coli* (Castilhos *et al.*, 2007). Otros estudios también han indicado efectos de tipo ansiolítico, antidepresivo y anticonvulsivante en ratones, así como citotoxicidad e inhibición de la AChE (de Andrade *et al.*, 2012b). Además, pancracina ha demostrado una amplia gama de actividades antibacterianas.

Desde el punto de vista farmacológico, el alcaloide galantamina merece un capítulo exclusivo, dado que es el único de este grupo que ha sido aprobado y que actualmente se comercializa para el tratamiento sintomático de la enfermedad de Alzheimer, un tipo de demencia con un enorme y creciente impacto sobre la población mundial. Este interesante compuesto se descubrió durante la década de 1950 en la especie *Galanthus woronowii*, y rápidamente atrajo la atención de la industria farmacéutica (Berkov *et al.*, 2009a). En la actualidad, se comercializa como sal de hidrobromuro con la denominación de Razadyne® (o Reminyl®). Aunque su actividad inhibidora de la enzima AChE es ampliamente conocida, se ha propuesto un mecanismo de acción dual que implica su participación en la modulación alostérica de los receptores nicotínicos (Farlow, 2003). Es interesante citar que se ha evaluado la actividad inhibidora de AChE de varios compuestos estructuralmente relacionados con el alcaloide galantamina, como algunos derivados *N*-alquilados y sanguinina (con un hidroxilo en posición 9), y resultaron ser unas diez veces más activos que dicho compuesto (Berkov *et al.*, 2008a).

A pesar de que la síntesis química de galantamina se ha logrado de manera exitosa, las plantas continúan siendo la fuente principal de este producto. Mientras que en Europa Central y Occidental se obtiene mayoritariamente a partir de cultivares

de *Narcissus*, en Europa del Este es obtenida a partir de *Leucojum aestivum*, y en China se extrae de *Lycoris radiata*. Además, se ha descrito su presencia en plantas de diversos géneros, incluyendo *Hippeastrum*, *Hymenocallis*, *Zephyranthes*, *Ungernia* y *Haemanthus* (Berkov *et al.*, 2009a). Debido a la continua demanda de este alcaloide, existe un gran interés en la búsqueda de nuevas fuentes que sean altamente productoras de galantamina.

Entre las estructuras inusuales encontradas en las plantas de la subfamilia Amaryllidoideae, los alcaloides de tipo mesembrano han demostrado propiedades biológicas destacables. Estos compuestos son característicos de las plantas del género *Sceletium* N.E.Br. (anteriormente *Mesembryanthemun* L.), de Sudáfrica, y su descubrimiento fue impulsado gracias al interés en un preparado comúnmente utilizado por grupos étnicos de la región, conocido como "Kanna", o también llamado "Channa" o "Kougoed" (Popelak y Lettenbauer, 1967; Smith *et al.*, 1996). Se han llevado a cabo numerosos estudios sobre la química y las aplicaciones de los alcaloides de tipo mesembrano, mostrando una marcada actividad como inhibidores de la recaptación de serotonina y, consecuentemente, con un considerable potencial para ser usados como antidepresivos (Gericke y Viljoen, 2008; Harvey *et al.*, 2011). En efecto, existe una patente desarrollada en Estados Unidos para el uso de preparados farmacéuticos que contienen mesembrina y compuestos relacionados en el tratamiento de estados depresivos y otros trastornos tales como ansiedad o drogodependencia (Gericke y Van Wyk, 2001).

Tal como ya se ha mencionado, los alcaloides de amarillidáceas son marcadores químicos característicos sintetizados exclusivamente por plantas de la subfamilia Amaryllidoideae. Esta cualidad, unida a sus actividades biológicas distintivas, ha conducido a un interesante estudio que reveló una correlación significativa entre filogenia, variabilidad de alcaloides y ensayos de actividad biológica relacionados con el Sistema Nervioso Central (SNC), para este grupo de plantas (Rønsted *et al.*, 2012). En dicho trabajo se utilizaron más de un centenar de especies de Amaryllidaceae, combinando el análisis de secuencias de ADN nuclear, plastídico y mitocondrial, con ensayos *in vitro* de inhibición de AChE y de unión al transportador de recaptación de serotonina, así como con un análisis del contenido de alcaloides. A pesar de que estos resultados no son extrapolables a otros sistemas, poseen una importante aplicación

potencial, por ejemplo, en la selección de taxones candidatos para el desarrollo de fármacos.

1.3. GC-MS y RMN: Técnicas Clave en el Estudio de Alcaloides de Amaryllidaceae

El análisis de la composición de alcaloides de extractos vegetales, así como la identificación de nuevos compuestos de especies de Amaryllidoideae, ha sido posible en gran medida gracias al desarrollo de dos técnicas de estudio fundamentales que han pasado a formar parte de los procedimientos de aplicación rutinaria: la cromatografía de gases acoplada a espectrometría de masas (GC-MS) y la resonancia magnética nuclear (RMN). La extensa investigación llevada a cabo durante los últimos 50 años en el ámbito de alcaloides de amarillidáceas ha dado lugar a la caracterización de ciertos patrones para los diversos tipos estructurales, posibilitando la rápida identificación de los compuestos ya conocidos y la elucidación estructural detallada en el caso de los productos aislados de *novo*.

1.3.1. Cromatografía de Gases - Espectrometría de Masas (GC-MS)

La cromatografía de gases (GC), introducida en los años 50, es una conocida técnica con la capacidad de separar componentes de una mezcla, que implica la volatilización de la muestra mediante su calentamiento. El equipo incluye una columna con la fase estacionaria, un gas portador inerte, y un detector. Sólo las moléculas que pueden ser vaporizadas sin experimentar descomposición son adecuadas para este análisis. Por otra parte, 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 (Kitson *et al.*, 1996). Generalmente, los compuestos orgánicos presentan patrones de fragmentación característicos después de ser ionizados, lo que permite su identificación mediante comparación con los datos obtenidos previamente. La combinación de ambas técnicas es una poderosa herramienta comúnmente conocida como GC-MS, la cual tiene un costo relativamente bajo, unido a una alta resolución y eficiencia.

Los extractos de plantas Amaryllidoideae suelen ser mezclas complejas con un número elevado de compuestos. La técnica de GC-MS, ya sea en modo de impacto electrónico (EI) o de ionización química (CI), ha demostrado ser muy útil para la rápida separación y detección de sus componentes. Los alcaloides de amarillidáceas pueden ser analizados sin necesidad de una derivatización previa ya que retienen sus patrones de fragmentación particulares bajo las condiciones de GC, permitiendo la identificación de compuestos ya caracterizados, o la obtención de información estructural valiosa cuando se trata de nuevas moléculas (Berkov *et al.*, 2005). Especialmente significativa resulta la observación de que pequeños cambios en la estereoquímica de estos alcaloides suelen ser suficientes para causar apreciables diferencias en el espectro de masas de los estereoisómeros (Berkov *et al.*, 2012; Duffield *et al.*, 1965).

Al abordar el estudio del contenido de alcaloides de una especie vegetal o variedad particular, es de gran utilidad obtener un perfil general mediante GC-MS, tanto del extracto crudo como de las fracciones derivadas del mismo. Además de proporcionar información sobre la presencia de alcaloides conocidos y posibles nuevas estructuras, permite el análisis de rendimientos y potenciales pérdidas que pueden surgir durante la aplicación de las diferentes técnicas de aislamiento. Recientemente, se ha reportado la validación de GC-MS como método de elección para el control de calidad de materias primas vegetales usadas en la producción de galantamina (Berkov *et al.*, 2011b), lo que demuestra las ventajas de su utilización en el análisis cualitativo y cuantitativo de estas plantas, incluso en comparación con otras metodologías (Gotti *et al.*, 2006).

Durante los años 60 y 70 se llevaron a cabo numerosos estudios de espectrometría de masas de impacto electrónico (EIMS) de los alcaloides de Amaryllidaceae, permitiendo establecer patrones de fragmentación característicos para varios esqueletos tipo (Bastida *et al.*, 2006). Además, el posterior desarrollo de nuevas metodologías, así como la caracterización de otras estructuras, ha dado lugar a la generación de información bien documentada con valor diagnóstico considerable para la identificación de este grupo de alcaloides. Por ello, vale la pena realizar algunos comentarios sobre los casos más representativos. Los ejemplos que se describirán a continuación demuestran el gran valor de la metodología de GC-MS en la identificación de alcaloides de amarillidáceas, aunque cabe destacar que no todas las estructuras

pertenecientes a este grupo pueden ser asignadas inequívocamente, como ocurre en el caso de los derivados *N*-óxidos.

1.3.1.1. Tipo licorina

El patrón de fragmentación de este esqueleto tipo se mantiene en condiciones de GC. El pico molecular aparece con una intensidad apreciable y suele experimentar la pérdida de agua, así como de C-1 y C-2, junto con los respectivos sustituyentes, por medio de una fragmentación de tipo retro-Diels-Alder (Figura 1.7). Curiosamente, la pérdida de agua a partir del ión molecular depende de la estereoquímica del grupo hidroxilo en C-2, y no ocurre en derivados acetilados (Bastida *et al.*, 2006). En estructuras con dos grupos metoxilo en el anillo A en lugar del grupo metilendioxi de licorina (como es el caso de galantina) el pico base del espectro es 16 unidades mayor que *m/z* 226, apareciendo a *m/z* 242.

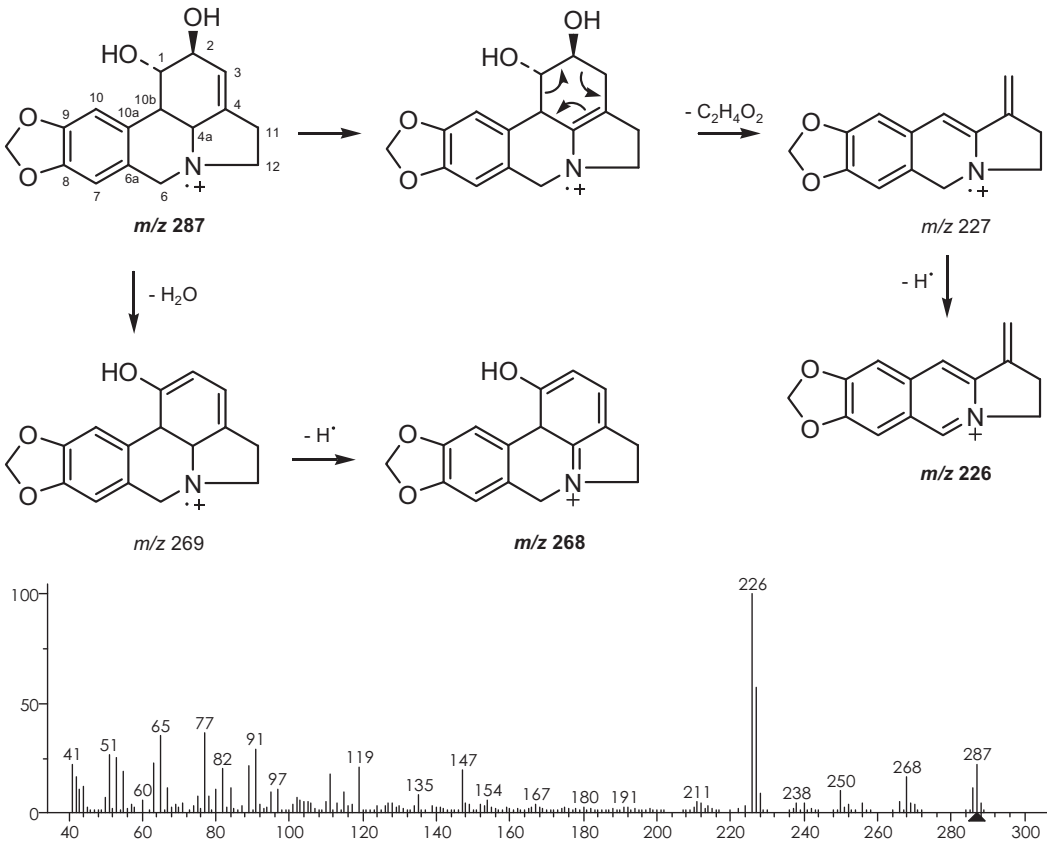

Figura 1.7: Patrón de fragmentación de licorina.

1.3.1.2. Tipo homolicorina

El fragmento dominante en el espectro de masas de los compuestos de tipo homolicorina surge de la rotura de los enlaces lábiles del anillo C por una reacción retro-Diels-Alder que genera dos fragmentos (Figura 1.8), 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 homolicorina el pico base se observa a *m/z* 109, mientras que hipeastrina (con un grupo hidroxilo en C-2) lo presenta a *m/z* 125. Los alcaloides de esta serie suelen ser difíciles de diferenciar debido a la reducida abundancia del ión molecular y los demás fragmentos.

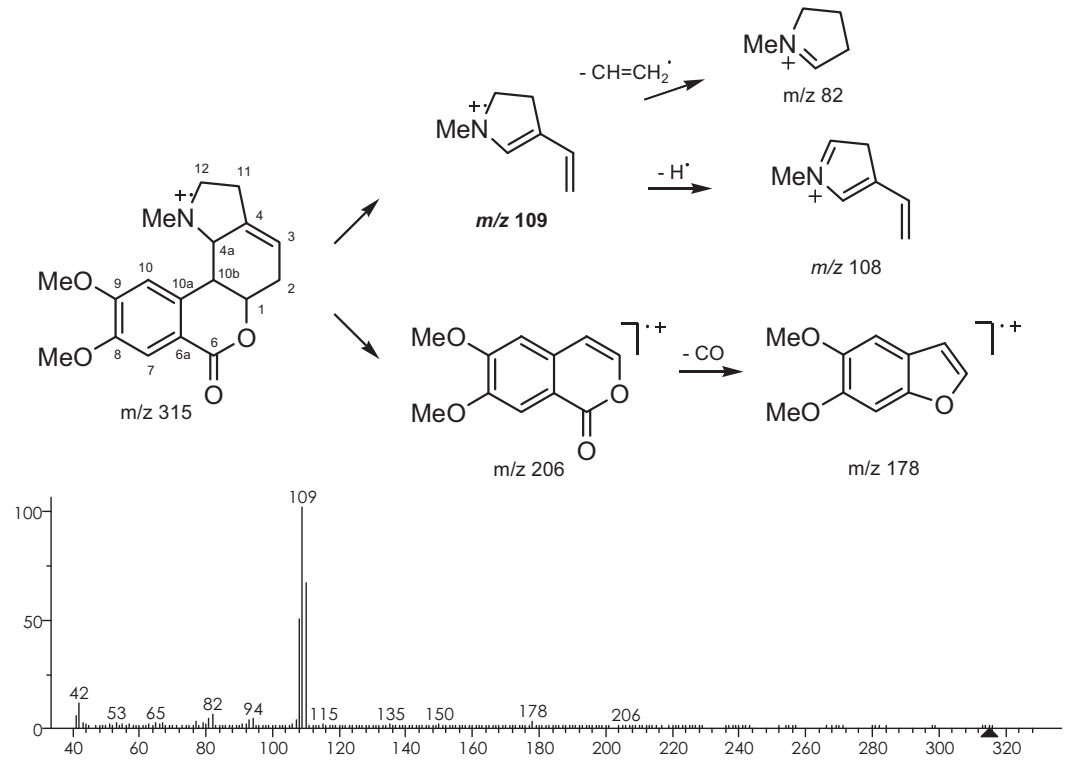

Figura 1.8: Patrón de fragmentación de homolicorina.

1.3.1.3. Tipos crinina y hemantamina

La fragmentación de este tipo de alcaloides ha sido estudiada en detalle para varias estructuras. En la mayoría de los casos, el ión molecular corresponde al pico base, el anillo aromático tiene un papel fundamental en la estabilización de los fragmentos, mientras que el átomo de nitrógeno suele perderse, y el paso inicial en los mecanismos de fragmentación implica la apertura del puente de dos carbonos 11-12. Se han descrito varios patrones característicos teniendo en cuenta la presencia de sustituyentes en diversas posiciones, la saturación del anillo C, y la influencia de la estereoquímica (Bastida *et al.*, 2006; Longevialle *et al.*, 1973). Sin embargo, con respecto a la aplicación de la metodología de GC-MS, es interesante destacar que hemantamina es susceptible a la descomposición térmica, lo que modifica el espectro observado en condiciones de GC, en comparación con el obtenido mediante inyección directa en un espectrómetro de masas (Figura 1.9). Kreh *et al.* (1995) han propuesto un mecanismo para explicar los iones producidos por el efecto de una alta temperatura, involucrando la ruptura del puente 5,10b-etano, seguida de una rotura en α.

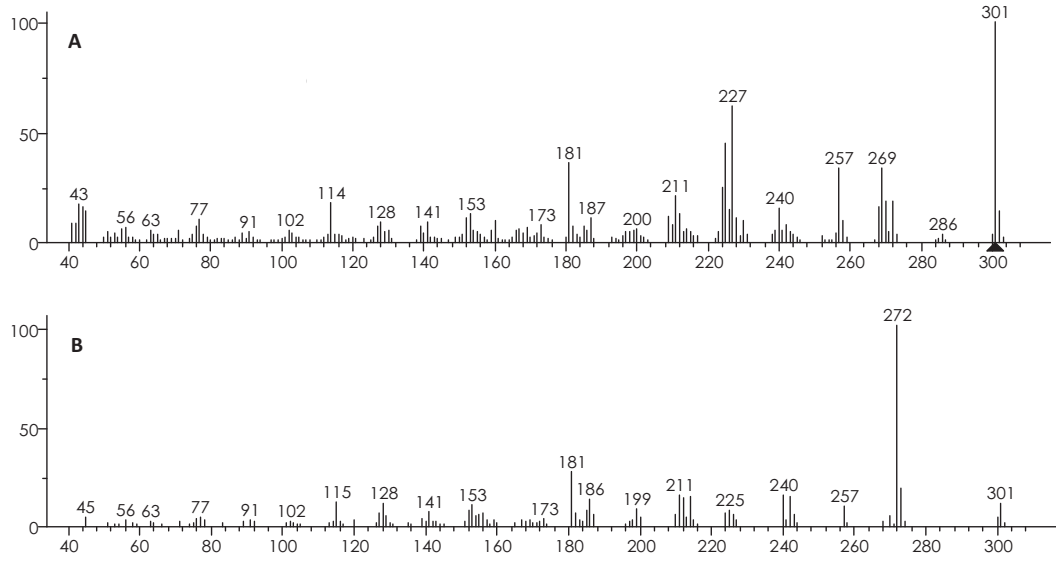

Figura 1.9: MS de hemantamina, mediante inyección directa (**A**) y en condiciones de GC (**B**).

1.3.1.4. Tipo tazetina

El esqueleto de tipo tazetina es un buen ejemplo para ilustrar cómo pequeños cambios en la estereoquímica pueden verse reflejados en los patrones de fragmentación. Tazetina y criwellina difieren sólo en la configuración del grupo metoxilo en C-3, pero ello es suficiente para producir variaciones notables en sus espectros de masas. La reacción principal involucra una fragmentación de tipo retro-Diels-Alder, la cual en criwellina está precedida por la pérdida del grupo metoxilo, dado que su configuración la favorece, mientras que en tazetina ocurre tras una simple reorganización de protones (Figura 1.10). Además, ambas estructuras 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).

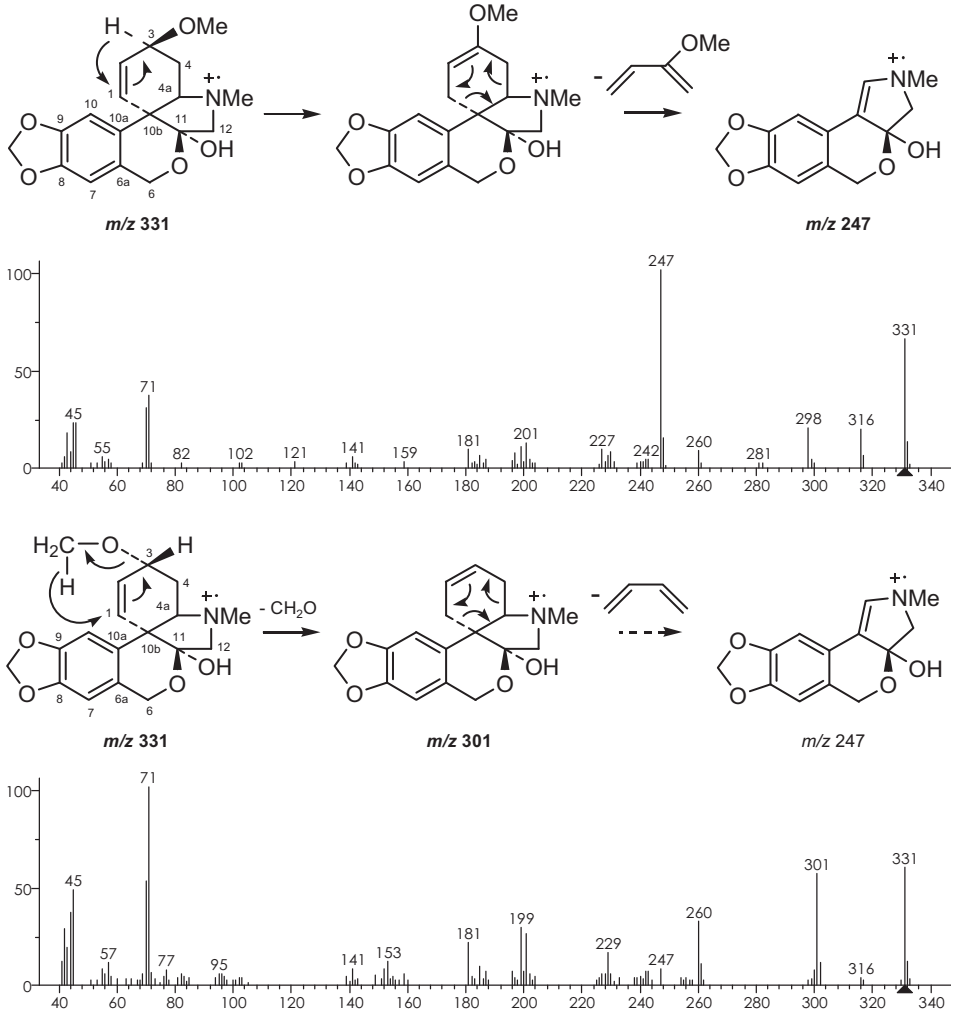

Figura 1.10: Patrones de fragmentación de tazetina (**A**) y criwellina (**B**).

1.3.1.5. Tipo montanina

El patrón de fragmentación de alcaloides con el núcleo 5,11-metanomorfantridina depende en gran medida de la naturaleza y configuración de los sustituyentes en C-2 y C-3. Las estructuras que contienen un grupo metoxilo dan lugar a un fragmento 31 unidades menor que el ión molecular (Figura 1.11). Por otra parte, la configuración del sustituyente en C-2 tiene una considerable influencia en la medida en como transcurre la fragmentación de tipo retro-Diels-Alder, que se ve aumentada cuando el sustituyente se encuentra en posición α (Bastida *et al.*, 2006).

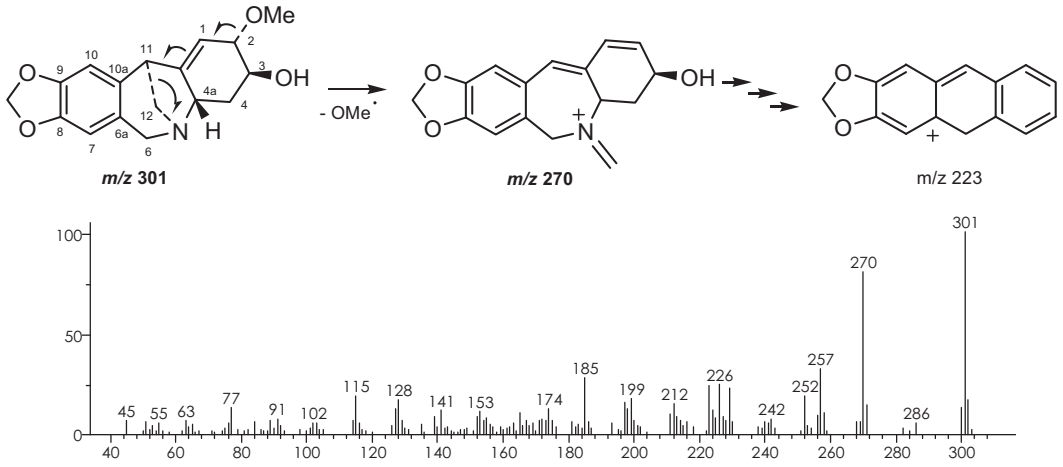

Figura 1.11: Patrón de fragmentación de montanina.

1.3.1.6. Tipo galantamina

Las estructuras de esta serie son probablemente las más estudiadas entre los alcaloides de Amaryllidaceae. Durante la década de 1970, se propuso que la fragmentación de algunos compuestos de este grupo incluía tres pasos principales que comprenden la eliminación del sustituyente en C-3, del anillo C, y del átomo de nitrógeno (Figura 1.12). Más recientemente, se ha utilizado la metodología de GC-MS para el análisis detallado del comportamiento de varios esqueletos de tipo galantamina, demostrando que sus patrones de fragmentación se mantienen en dichas condiciones. Esto ha permitido su establecimiento como una técnica de rutina para el estudio de extractos vegetales que contienen este tipo de alcaloides (Berkov *et al.*, 2012).

En la citada referencia se reportaron además numerosos factores que influyen en la fragmentación, como por ejemplo la posición de diversos sustituyentes. Una vez más, es interesante mencionar cómo una modificación estereoquímica puede afectar el espectro de masas de un compuesto, tal es el caso de galantamina y su epímero en posición 3. Ambos 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, la cual se explica por la presencia de un puente de hidrógeno intramolecular que estabiliza el ión molecular de galantamina (Figura 1.13).

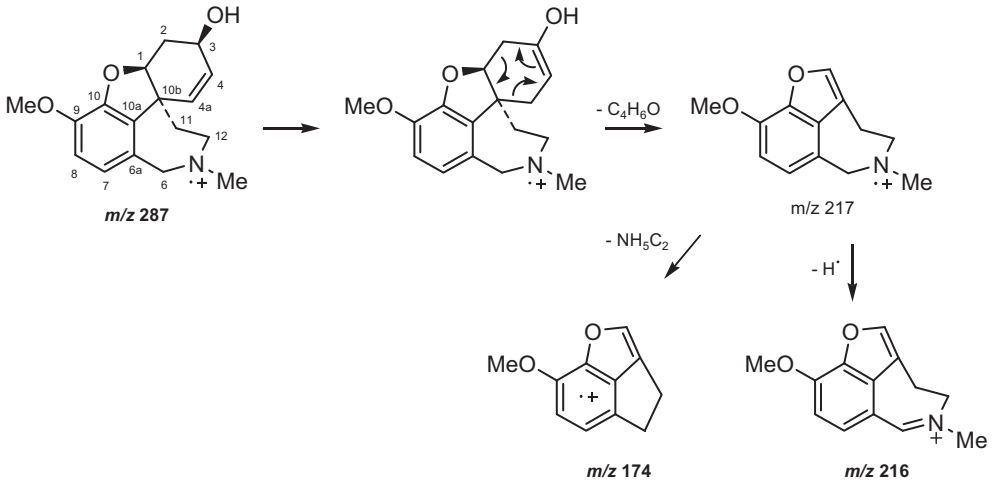

Figura 1.12: Fragmentación de galantamina.

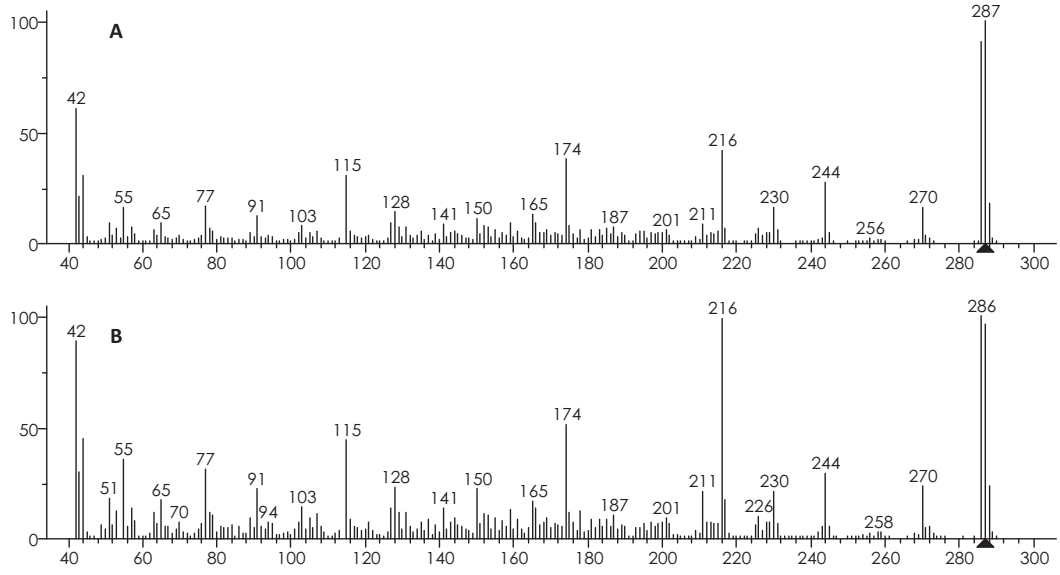

Figura 1.13: Espectro de masas de galantamina (**A**) y 3-epigalantamina (**B**).

1.3.2. Resonancia Magnética Nuclear (RMN)

La resonancia magnética nuclear (RMN) es un tipo de espectroscopia de absorción, tal como la de infrarrojo (IR) o ultravioleta (UV). Básicamente, bajo la influencia de un campo magnético y en las condiciones apropiadas, una muestra es capaz de absorber radiación electromagnética en la región de las radiofrecuencias dependiendo de sus características particulares. La absorción es una función que depende de ciertos núcleos presentes en la molécula (Silverstein y Webster, 1998). La técnica de RMN se aplica principalmente en la identificación de compuestos orgánicos puros, pero durante los últimos años también se ha extendido al análisis de mezclas complejas en el ámbito de la metabolómica, siendo utilizada en numerosos estudios de extractos vegetales, incluyendo el análisis de especies y variedades de amarillidáceas (Kim *et al.*, 2010; Lubbe *et al.*, 2010).

Además de los datos obtenidos a partir de IR, UV, dicroísmo circular (CD) y espectrometría de masas de alta resolución (HRMS), entre otras metodologías, la elucidación estructural de un compuesto desconocido se logra generalmente mediante la combinación de diversas técnicas de RMN complementarias. Con respecto a los alcaloides de Amaryllidaceae, la espectroscopia de ^{1}H-RMN otorga información fundamental sobre los distintos tipos estructurales, mientras que su combinación con la espectroscopia de ^{13}C-RMN y las técnicas de RMN bidimensional (2D-RMN), suele permitir la identificación inequívoca de la molécula en estudio, así como el establecimiento de su estereoquímica en muchos casos.

Las características más significativas de los espectros de ^{1}H-RMN de los alcaloides de amarillidáceas han sido esbozadas, detallando las claves para su identificación (Bastida *et al.*, 2011). En general, la región aromática (δ 6.5-8.5 ppm) contribuye a definir el tipo de esqueleto, mientras que la sustitución del anillo aromático suele ser evidente por la observación de las señales correspondientes a uno o más grupos metoxilo alrededor de δ 3.6-4.0 ppm, o la presencia de la señal típica de un grupo metilendioxi sobre δ 6.0 ppm. En muchas estructuras en las que la posición bencílica en C-6 está saturada, como licorina, hemantamina y galantamina, la presencia de un sistema AB, es característica de dichos protones. Curiosamente, su desplazamiento químico se ve influenciado por la orientación del par de electrones libre del átomo de

nitrógeno. Además de estos rasgos comunes, vale la pena mencionar algunas particularidades para cada tipo de alcaloide.

1.3.2.1. Tipo licorina

Entre las características principales del espectro de ^{1}H-RMN de licorina y sus derivados, se encuentran los dos singuletes de los protones aromáticos en orientación *para*, junto con un único protón olefínico, y los dobletes correspondientes a la posición bencílica 6. El desapantallamiento observado en las señales de los protones β de las posiciones 6 y 12, en relación a sus homólogos en α, se debe al efecto del par de electrones libre en *cis* del átomo de nitrógeno.

Generalmente, los alcaloides aislados del género *Narcissus* muestran una configuración *trans* en la unión de los anillos B/C, con una constante de acoplamiento entre los protones 4a-10b de unos 11 Hz. Sólo kirkina presenta una configuración *cis*, con una constante de acoplamiento menor (8 Hz).

1.3.2.2. Tipo homolicorina

Estos alcaloides incluyen un grupo característico que puede ser una lactona, un hemiacetal o un éter cíclico. En su espectro de ^{1}H-RMN se suelen observar dos singuletes correspondientes a los protones aromáticos en *para*, siendo la señal de H-7 la que usualmente se encuentra más desapantallada debido al grupo carbonilo en *peri*.

La mayoría de estos compuestos pertenecen a una única serie enantiomérica con la unión *cis* entre los anillos B/C, lo cual es congruente con el reducido valor de la constante de acoplamiento entre los protones 1-10b. En el género *Narcissus* no se ha encontrado ninguna excepción a esta regla. Por otra parte, el elevado valor de la constante entre H-4a y H-10b (J ~10 Hz) sólo es compatible con una relación *trans*-diaxial.

Por lo general, el anillo C presenta un protón vinílico. Si la posición 2 se encuentra sustituida por un grupo hidroxilo, metoxilo o acetilo, siempre muestra una disposición α. El grupo *N*-metilo suele hallarse en el intervalo de δ 2.0-2.2 ppm, pero en el caso de alcaloides con el anillo C saturado, se han descrito

algunas correlaciones empíricas para la estereoquímica de las uniones entre los anillos B/C y C/D, en las que se reportan señales más desapantalladas (Jeffs *et al.*, 1988).

1.3.2.3. Tipos crinina y hemantamina

La configuración absoluta de estos alcaloides se determina mediante dicroísmo circular (CD). Los alcaloides del género *Narcissus* son exclusivamente del tipo hemantamina, mientras que en los géneros como *Brunsvigia* y *Boophane*, entre otros, los alcaloides de tipo crinina son predominantes. Además, es importante mencionar que los alcaloides aislados del género *Narcissus* no muestran sustituciones adicionales en el anillo aromático, aparte de las de C-8 y C-9, mientras que en los géneros dominados por los esqueletos del tipo crinina es bastante común la presencia de compuestos con un sustituyente metoxilo en C-7.

Utilizando $CDCl_3$ como solvente, la magnitud de las constantes de acoplamiento entre H-3 y cada uno de los protones olefínicos (H-1, H-2) ofrece información sobre la configuración del sustituyente en C-3. En aquellos alcaloides en los que el puente de dos carbonos (C-11 y C-12) se halla en configuración *cis* respecto al sustituyente en C-3, H-1 presenta un acoplamiento alílico con H-3 ($J_{1,3}$ ~1-2 Hz) y H-2 muestra una constante más pequeña con H-3 ($J_{2,3}$ ~0-1.5 Hz), tal como ocurre en crinamina. Por el contrario, en la serie epimérica de la hemantamina, 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 también se aplica a los alcaloides tipo crinina.

Los compuestos con un sustituyente hidroxilo en C-6, como papiramina/6-epipapiramina o hemantidina/6-epihemantidina, aparecen como una mezcla de epímeros que no pueden ser separados ni siquiera mediante HPLC.

1.3.2.4. Tipo tazetina

La presencia de un grupo *N*-metilo (δ 2.4-2.5 ppm) distingue a este tipo de alcaloides de los tipos hemantamina o crinina, a partir de los que proceden biosintéticamente. Por lo demás, el espectro de ^{1}H-RMN siempre muestra la señal correspondiente al grupo metilendioxi.

1.3.2.5. Tipo montanina

La configuración absoluta de los alcaloides de tipo montanina debe determinarse mediante CD. Su espectro de ^{1}H-RMN es muy similar a los alcaloides con esqueleto de tipo licorina, aunque las estructuras de tipo montanina pueden ser distinguidas a través del análisis del espectro COSY. Las señales atribuidas a los protones H-4 (las más apantalladas) muestran correlación con las correspondientes a H-3 y H-4a, mientras que en el espectro de un esqueleto de tipo licorina, las señales más apantalladas corresponden a los dos protones de la posición 11 y al protón 12α.

1.3.2.6. Tipo galantamina

Entre los alcaloides de Amaryllidaceae, sólo los de tipo galantamina muestran una constante de acoplamiento en *orto* (~8 Hz) entre los protones aromáticos del anillo A.

La asignación de la estereoquímica del sustituyente en C-3 se realiza en base a las constantes de acoplamiento del 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.

Este tipo de alcaloides suele mostrar la presencia de un grupo *N*-metilo aunque, ocasionalmente, también se ha reportado la existencia de grupos *N*-formilo. La presencia del anillo furano provoca un efecto de desapantallamiento sobre H-1.

Con respecto a la espectroscopia de ^{13}C-RMN, la misma se ha utilizado ampliamente en la determinación del esqueleto carbonado de estos alcaloides. En líneas generales, el espectro de ^{13}C-RMN de los alcaloides de amarillidáceas puede dividirse en dos regiones principales: a campos más bajos (>90 ppm) se observan las señales correspondientes a grupos carbonilo, carbonos olefínicos y aromáticos, así como la señales del grupo metilendioxi; 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, OMe, OAc) en las resonancias de carbono es de gran importancia para la localización de la posición de los grupos funcionales.

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

- **^{1}H-^{1}H COSY** (**CO**rrelated **S**pectroscop**Y**), en la cual las correlaciones observadas corresponden a acoplamientos directos entre los protones involucrados, siendo de gran utilidad en la asignación de acoplamientos geminales y vecinales.

- **^{1}H-^{1}H NOESY** (**N**uclear **O**verhauser **E**ffect **S**pectroscop**Y**), de gran valor para obtener información sobre la proximidad espacial entre protones y, por lo tanto, sobre la estereoquímica.

- **^{1}H-^{13}C HSQC** (**H**eteronuclear **S**ingle **Q**uantum **C**orrelation), que muestra las correlaciones entre ^{1}H-^{13}C directamente enlazados, permitiendo la adecuada asignación de todos los carbonos, a excepción de los cuaternarios.

- **^{1}H-^{13}C HMBC** (**H**eteronuclear **M**ultiple **B**ond **C**orrelation), muy útil en la determinación de correlaciones a larga distancia entre ^{1}H-^{13}C. Permite la identificación de los carbonos cuaternarios a través de la observación de su correlación con protones situados a tres enlaces de distancia.

2. OBJETIVOS

2. OBJETIVOS

El objetivo general planteado para este proyecto de tesis ha sido la bioprospección de la diversidad vegetal de especies de la familia Amaryllidaceae (subfam. Amaryllidoideae) del área mediterránea e iberoamericana, analizando sus alcaloides como marcadores químicos, con el fin último de utilizar esta información para el aprovechamiento de estos recursos en la obtención de productos farmacológicamente activos.

Objetivos Específicos:

- Estudio del contenido de alcaloides de especies de la familia Amaryllidaceae, incluyendo la determinación de la composición de extractos mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS), así como el aislamiento de sus componentes en los casos en que se disponga de material suficiente, para la posterior identificación y caracterización estructural aplicando diversas técnicas espectroscópicas, como resonancia magnética nuclear (RMN).

- Determinación de la actividad biológica de los extractos vegetales y de sus alcaloides, considerando ensayos de inhibición de la enzima acetilcolinesterasa (AChE) y de actividad antiparasitaria, entre otros.

- Identificación de especies de potencial interés farmacéutico por su alto contenido en compuestos con destacable actividad biológica.

- Contribución a la revisión taxonómica de las especies estudiadas basándose en la presencia de ciertos alcaloides como marcadores químicos.

2.1. Objectives

The general aim of the present work has been bioprospecting among the diverse plant species of the family Amaryllidaceae (subfam. Amaryllidoideae) found in the Mediterranean and Iberoamerican areas by analyzing alkaloids as chemical markers. The ultimate goal is to make use of this information to favour the rational exploitation of these resources for the production of pharmacologically active compounds.

Specific Objectives:

- Study the alkaloid content of species belonging to the family Amaryllidaceae, including the determination of composition of plant extracts by gas chromatography coupled to mass spectrometry (GC-MS), as well as the isolation of their components, if sufficient material were available, for their subsequent identification and structural elucidation using a combination of spectroscopic methodologies such as nuclear magnetic resonance (NMR).

- Determine the biological activities of plant extracts and their alkaloids, considering assays of acetylcholinesterase (AChE) inhibition and antiparasitic activity, among others.

- Identify species with potential pharmaceutical interest due to a high content of compounds showing remarkable bioactivity.

- Contribute to the taxonomical revision of the species under study, based on the presence of certain types of alkaloids as chemical markers.

3. RESULTADOS

3. RESULTADOS

Los resultados de la presente tesis están reflejados en los siguientes artículos científicos, los cuales se presentan a continuación acompañados por un breve resumen en castellano:

Artículo 1. Pigni, N.B., Berkov, S., Elamrani, A., Benaissa, M., Viladomat, F., Codina, C., Bastida, J. (2010). Two new alkaloids from *Narcissus serotinus* L. *Molecules, 15*, 7083-7089.

Artículo 2. Pigni, N.B., Ríos-Ruiz, S., Martínez-Francés, V., Nair, J.J., Viladomat, F., Codina, C., Bastida, J. (2012). Alkaloids from *Narcissus serotinus*. *Journal of Natural Products, 75*(9), 1643-1647.

Artículo 3. Pigni, N.B., Ríos-Ruiz, S., Luque, F.J., Viladomat, F., Codina, C., Bastida, J. (2013). Wild daffodils of the section Ganymedes from the Iberian Peninsula as a source of mesembrane alkaloids. Enviado para su publicación en *Phytochemistry*.

Artículo 4. Ortiz, J.E., Berkov, S., Pigni, N.B., Theoduloz, C., Roitman, G., Tapia, A., Bastida, J., Feresin, G.E. (2012). Wild Argentinian Amaryllidaceae, a new renewable source of acetylcholinesterase inhibitor galanthamine and other alkaloids. *Molecules, 17*, 13473-13482.

3.1. Artículo 1

Two new alkaloids from *Narcissus serotinus* L.

Molecules, 15, 7083-7089 (2010)

Narcissus serotinus L. es una especie perteneciente a la familia botánica Amaryllidaceae que presenta una distribución geográfica localizada principalmente en zonas costeras del Mediterráneo. En el presente artículo se reporta el aislamiento y elucidación estructural de dos nuevos alcaloides a partir de ejemplares de dicha especie recolectados cerca de Casablanca (Marruecos).

A partir de 350 g de material vegetal fresco consistente en planta entera, se aplicaron técnicas estandarizadas de extracción y separativas, incluyendo cromatografía líquida de vacío (VLC) y cromatografía en capa fina semi-preparativa (pTLC), con la finalidad de obtener fracciones y compuestos purificados adecuados para su análisis mediante GC-MS y RMN.

El análisis del extracto utilizando GC-MS permitió la identificación de cinco alcaloides conocidos por comparación con los patrones de fragmentación reportados en la literatura: licorina, galantina, 1-*O*-(3´-hidroxibutanoil)licorina, asoanina e hipeastrina. Además, se describe el aislamiento y elucidación estructural de dos nuevos alcaloides: 1-*O*-(3´-acetoxibutanoil)licorina y narseronina.

Narseronina, alcaloide mayoritario del extracto, es el primero de la serie homolicorina que presenta un doble enlace en la unión entre los anillos B y C, en posición 1-10b. Curiosamente, corresponde al mismo compuesto previamente reportado por Vrondeli *et al.* (2005) caracterizado como un isómero de 3-epimacronina, una asignación que no concuerda con los resultados aquí expuestos.

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Article

Two New Alkaloids from *Narcissus serotinus* L.

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Abstract: The Amaryllidaceae family is well known for the presence of an exclusive group of alkaloids with a wide range of biological activities. *Narcissus serotinus* L. is a plant belonging to this family and its geographical distribution is mainly located along the Mediterranean coast. In the present work, specimens collected near Casablanca (Morocco) were used to study the alkaloid content of this species. Starting with 350 g of the whole plant we used standard extraction and purification procedures to obtain fractions and compounds for GC-MS and NMR analysis. As well as five known alkaloids, we isolated two new compounds: 1-*O*-(3′-acetoxybutanoyl)lycorine and narseronine. The latter has been previously published, but with an erroneous structure.

Keywords: *Narcissus serotinus*; Amaryllidaceae; alkaloids; narseronine; 1-*O*-(3′-acetoxybutanoyl)lycorine

1. Introduction

Plants belonging to the Amaryllidaceae family are well known for the presence of an exclusive group of alkaloids with a wide range of biological activities [1]. Within this group, the genus *Narcissus* has been extensively used in traditional medicine to treat a variety of health problems.

Antiviral, antifungal and antitumoral activities are just some of the phamacological effects that have been proven for these alkaloids.

Narcissus serotinus L. is an autumn flowering species and the only member of the monotypic section *Serotini*. It grows mostly in calcareous sandy soil or maquis in dry coastal areas, and its geographical distribution extends over the coastal southern Mediterranean region, including southern Portugal, southern and eastern Spain, western and eastern Italy, Croatia, much of Greece and Israel, almost all the Mediterranean islands, north-west Morocco, Algeria, Tunisia and Libya [2,3].

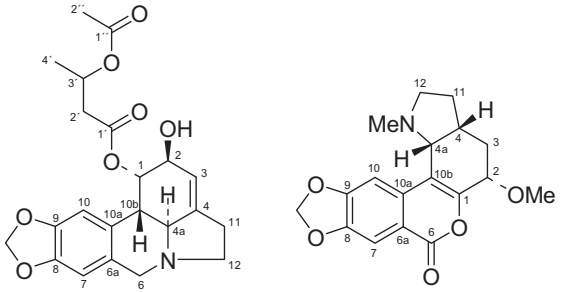
The aim of this work is to investigate the alkaloid content of this species through the analysis of specimens collected in Morocco. In a previously published article, Vrondeli *et al.* [4] described the isolation of a new alkaloid from this species, suggesting a 3-epimacronine isomer. Based on the results reported herein, we propose an alternative structure, which also represents a new compound within the Amaryllidaceae alkaloid family.

2. Results and Discussion

The MeOH extract of the fresh aerial parts and bulbs of *N. serotinus* L. was fractioned according to the methodology described in the experimental section. The GC-MS analysis of fraction B revealed the presence of lycorine. The analysis of fraction A showed a more complex mixture: in addition to lycorine [1,5] we determined the presence of galanthine [1,6], 1-*O*-(3′-hydroxybutanoyl)lycorine [7], assoanine [8] and hippeastrine [9] together with two new alkaloids (Figure 1). Identification of known compounds and structural elucidation of the new ones were achieved through the combined use of GC-MS, HRMS and one and two-dimensional NMR techniques.

The HRMS of **1** suggested a molecular formula $C_{22}H_{26}NO_7$ for $[M+H]^+$ with a parent ion at *m/z* 416.1702 (calc. 416.1704). The EIMS showed a molecular ion $[M]^+$ at *m/z* 415 (18%) with a base peak at *m/z* 226. It is interesting to note that the isomer 2-*O*-(3′-acetoxybutanoyl)lycorine, isolated from *Galanthus nivalis* [10], shows a very similar fragmentation pattern but with a base peak at *m/z* 250. However, the pattern observed for **1** shows the base peak at *m/z* 226 [7]. These empirical cases prove that the GC-MS technique is useful for differentiating between isomers with substituents at position 1 or 2. The ^{1}H-NMR spectral data of compound **1** and the isomer, 2-*O*-(3′-acetoxybutanoyl)lycorine, are very similar too, showing the major difference in proton shielding at positions 1 and 2: in **1** H-1 is more deshielded (δ 5.68) than the same proton in the isomer (δ 4.51) and the inverse situation occurs for H-2, which appears at δ 4.23 in the spectrum of **1** and at δ 5.31 for the isomer. Considering its coupling constant values, we assume that the configuration of **1** is the same as that proposed for 2-*O*-(3′-acetoxybutanoyl)lycorine and 1-*O*-(3′-hydroxybutanoyl)lycorine. The high coupling constant (10.4) observed between H-4a and H-10b suggests a *trans*-diaxial configuration. Protons 6β and 12β are more deshielded than 6α and 12α, respectively, because of the *cis*-lone pair of the nitrogen atom. The combined data suggested for compound **1** the structure of 1-*O*-(3′-acetoxybutanoyl)lycorine. The ^{1}H-NMR, COSY and HSQC data are recorded in Table 1.

Figure 1. New alkaloids isolated from *N. serotinus* L. 1-*O*-(3′-acetoxybutanoyl)lycorine (**1**) and narseronine (**2**).



1 **2**

Table 1. ^{1}H-NMR, COSY and HSQC data of 1-*O*-(3′-acetoxybutanoyl)lycorine (**1**).

Position	^{1}H δ (J in Hz)	COSY	HSQC
1	5.68 *s*	H-2, H-10b	72.5 *d*
2	4.23 *dt* (3.3, 1.7)	H-1, H-3, H-11	69.4 *d*
3	5.56 *m*	H-2, H-11	116.9 *d*
4a	2.76 *d* (10.4)	H-10b	61.9 *d*
6α	3.54 *d* (14.1)	H-6β	56.6 *t*
6β	4.16 *d* (14.1)	H-6α	56.6 *t*
7	6.58 *s*		107.3 *d*
10	6.72 *s*		104.8 *d*
10b	2.91 *d* (10.4)	H-1, H-4a	38.8 *d*
11 (2H)	2.65 *m*	H-2, H-3, H-12α, H-12β	28.4 *t*
12α	2.42 *dd* (9.3, 5.0)	H-11, H-12β	53.4 *t*
12β	3.38 *dt* (9.2, 4.8)	H-11, H-12α	53.4 *t*
OCH_2O	5.92 *s*		100.8 *t*
$2'_A$	2.43 *dd* (15.5, 5.4)	$H-2'_B$, H-3′	40.5 *t*
$2'_B$	2.53 *dd* (15.5, 7.8)	$H-2'_A$, H-3′	40.5 *t*
3′	5.10 *m*	$H-2'_A$, $H-2'_B$, H-4′	66.9 *d*
4′	1.14 *d* (6.3)	H-3′	19.3 *q*
AcO (2′′)	1.95 *s*		20.7 *q*

The HRMS analysis of narseronine (**2**) suggested a molecular formula $C_{18}H_{20}NO_5$ for the parent ion $[M+H]^+$ at *m/z* 330.1340 (calc. 330.1336). This indicates a molecular formula $C_{18}H_{19}NO_5$, in accordance with a molecular weight of 329. The EIMS showed a molecular ion $[M]^+$ at *m/z* 329 (20%). The mass spectral fragmentation pattern is not similar to those commonly shown by the homolycorine type compounds, because of the absence of a double bond between C-3 and C-4. The unusual occurence of a double bond at position C-1/C-10b probably drastically changes this pattern. Its ^{1}H NMR spectrum exhibited two singlets at δ 7.66 and 7.29 for the *para*-oriented aromatic prontons H-7 and H-10, respectively, with H-7 more deshielded due to the *peri*-carbonyl group [1]. Also, the NOESY experiment showed the spatial proximity between H-10 and the *N*-methyl group. Two doublets appeared at δ 6.10 and δ 6.12 for the protons of the methylendioxy group. A triplet at δ 4.22 was assigned to H-2, coupled to H-3 with a J = 6.1 Hz, suggesting an equatorial orientation with a similar dihedral angle between H-2 and the two H-3 protons; this is consistent with the α position of the methoxy group at C-2 and with the NOESY correlation of this substituent with H-11. A doublet at

δ 3.94, was undoubtedly assigned to H-4a for a 3JC-H HMBC correlation with the *N*-methyl group; COSY experiment showed its only correlation with H-4, with a J = 6.4 Hz suggesting a *cis*- C/D ring fusion [11,12]. The spectrum also showed, between the most significant signals, a singlet integrating for 3 protons at δ 3.57 assigned to the methoxy group at C-2, a doublet of triplets at δ 3.05, assigned to H-12α, more deshielded than H-12β because of the *cis*-lone pair of the nitrogen atom [1], a singlet corresponding to the *N*-methyl group at δ 2.41, also supporting the *cis*-C/D ring junction if we consider the empirical correlations of *N*-methyl chemical shifts with stereochemical assignments suggested by Jeffs *et al.* [11]; and a doublet of triplets at δ 2.01 assigned to H-3α, showing spatial proximity with the *O*-CH_3 group in the NOESY experiment. The NMR spectral data is shown in Table 2.

Narseronine was previously isolated by Vrondeli *et al.* [4] but published with an erroneous structure. They suggested a 3-epimacronine isomer, a tazettine type alkaloid but their mass spectral fragmentation proposal does not explain the occurrence of the most abundant peaks of the mass spectrum, such as *m/z* 240 or 241. Also, the ^{1}H-NMR assignment is not adequate, including, for instance, the protons in an α-position to the *N*-methyl group (H-6 in their numbering system) at δ 2.30-1.80 ppm, a more shielded displacement than can be expected for a proton in such an electronic environment.

Table 2. ^{1}H-NMR, COSY, NOESY, ^{13}C-NMR (HSQC) and HMBC data of narseronine (**2**).

Position	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ	HMBC
1	-	-	-	152.9 *s*	-
2	4.22 *t* (6.1)	H-3α, H-3β	H-3α, H-3β, OCH_3	74.9 *d*	C-1, C-3, C-4, C-10b, OCH_3
3α	2.01 *dt* (13.5, 5.5)	H-2, H-3β, H-4	H-2, H-3β, H-4, OCH_3	31.4 *t*	C-1, C-2, C-4, C-4a, C-11
3β	2.22 - 2.13 *m* *(overlapped)*	H-2, H-3α, H-4	H-2, H-3α, H-4, OCH_3	31.4 *t*	C-1, C-2, C-4, C-4a, C-11
4	2.64 *m*	H-3α, H-3β, H-4a, H-11α, H-11β	H-3α, H-3β, H-4a, H-11α, H-11β	35.1 *d*	C-12
4a	3.94 *d* (6.4)	H-4	H-4, H-10, NCH_3	61.6 *d*	C-1, C-3, C-4, C-10a, C-10b, C-11, C-12, NCH_3
6	-	-	-	161.5 *s*	-
6a	-	-	-	116.4 *s*	-
7	7.66 *s*	-	-	107.8 *d*	C-6, C-8, C-9, C-10a
8	-	-	-	148.4 *s*	-
9	-	-	-	153.8 *s*	-
10	7.29 *s*	-	H-4a, NCH_3	103.3 *d*	C-6a, C-8, C-9, C-10b
10a	-	-	-	135.1 *s*	-
10b	-	-	-	110.8 *s*	-
11α	2.22 - 2.13 *m* *(overlapped)*	H-4, H-11β, H-12α, H-12β	H-4, H-11β, H-12α, H-12β, OCH_3	29.6 *t*	C-3, C-4a
11β	1.90 *ddd* (12.6, 8.3, 4.2)	H-4, H-11α, H-12α, H-12β	H-4, H-11α, H-12α, H-12β, OCH_3	29.6 *t*	C-3, C-4a
12α	3.05 *dt* (11.0, 7.6)	H-11α, H-11β, H-12β	H-11α, H-11β, H-12β, NCH_3	54.3 *t*	C-4, C-4a, C-11, NCH_3
12β	2.81 *m*	H-11α, H-11β, H-12α	H-11α, H-11β, H-12α, NCH_3	54.3 *t*	C-4, C-4a, C-11, NCH_3
OCH_2O	6.10 *d* (1.2) 6.12 *d* (1.2)	-	-	102.4 *t*	C-8, C-9
OCH_3	3.57 *s*	-	H-2, H-3α, H-3β, H-11α, H-11β	58.3 *q*	C-2
NCH_3	2.41 *s*	-	H-2, H-3α, H-3β, H-11α, H-11β	41.8 *q*	C-4a, C-12

3. Experimental

3.1. General

NMR spectra were recorded in a Mercury 400 MHz or a Varian VXR 500 MHz, instrument using $CDCl_3$ as the solvent and TMS as the internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (*J*) in Hz. EIMS were obtained on a GC-MS Agilent 6890 + MSD 5975 operating in EI mode at 70 eV. A HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100-180 ºC at 15 ºC min^{-1}, 1 min hold at 180 ºC, 180-300 ºC at 5 ºC min^{-1} and 1 min hold at 300 ºC. Injector temperature was 280 ºC. The flow rate of carrier gas (Helium) was 0.8 mL min^{-1}. In most cases the split ratio was 1:20, but with more diluted samples a split ratio of 1:5 was applied. UV spectra were obtained on a DINKO UV2310 instrument and IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer.

3.2. Plant material

Whole plants of *Narcissus serotinus* L. (Amaryllidaceae) were collected in October 2009 during the flowering period in Ben Slimane, near Casablanca (Morocco), and identified by Professor El Ghazi. A voucher sample (MB-026/2009) was deposited at the Herbarium of the Faculty of Sciences Ain Chock, University Hassan II.

3.3. Extraction and isolation of alkaloids

The fresh whole plant (350 g) was crushed and extracted with methanol (1 × 800 mL, 24 h; 1 × 800 mL, 72 h; and 2 × 400 mL, 48 h each). The extract was evaporated under reduced pressure to yield 5.5 g. This crude extract was dissolved in 100 mL of H_2SO_4 1% (v/v) and neutral material was removed with Et_2O (6 × 100 mL). The acidic soln. was basified with 25% ammonia up to pH 9-10 and extracted with EtOAc (3 × 100 mL) to give extract A (149.4 mg). Another extraction with EtOAc (2 × 100 ml) gave extract B (23 mg). Both fractions were dried with anhydrous Na_2SO_4, filtered and completely dried under reduced pressure. Referred to the fresh weight, the sum of these two extracts represents approximately 0.05%. After dissolving A and B in MeOH, lycorine crystallized directly. Extract A was subjected to a vacuum liquid chromatography (VLC) [13] using a silica gel 60 A (6-35 μ) column with a diameter of 1 cm and a height of 4 cm. Alkaloids were eluted using hexane gradually enriched with EtOAc, and then EtOAc gradually enriched with MeOH. Fractions of 10 mL were collected (105 in total) monitored by TLC (Dragendorff´s reagent, UV 254 nm) and combined according to their profiles. Five main fractions were obtained and subjected to preparative TLC (20 cm × 20 cm × 0.25 mm, silica gel 60F254). Narseronine (**2**, 4.5 mg) and 1-*O*-(3´-acetoxybutanoyl)lycorine (**1**, 1.5 mg) were obtained in major quantities from fractions *32-38* (eluted from VLC with hexane-EtOAc, 30:70 to 20:80) through preparative TLC (EtOAc-hexane 4:1 + 25% ammonia).

1-O-(3´-Acetoxybutanoyl)lycorine (**1**). UV (MeOH) λ_{max} nm: 368.0, 260.0. IR ($CHCl_3$) ν_{max} cm^{-1}: 2959, 2924, 2853, 1735, 1461, 1371, 1244, 1170, 1038, 776. ^{1}H-NMR, COSY, HSQC (400 MHz, 500 MHz, $CDCl_3$) see Table 1. EIMS 70eV (rel. int.): 416 (4), 415 (18), 354 (1), 269 (12), 268 (37), 250

(27), 227 (75), 226 (100), 192 (4), 147 (5), 96 (4), 69 (15), 43 (27). HRMS of $[M+H]^+$ *m/z* 416.1702 (Calc. 416.1704 for $C_{22}H_{26}NO_7$).

Narseronine (**2**). UV (MeOH) λ_{max} nm: 322.5, 286.0, 241.5. IR ($CHCl_3$) ν_{max} cm^{-1}: 2928, 1718, 1503, 1482, 1415, 1283, 1256, 1162, 1106, 1035, 935, 754. ^{1}H-NMR, COSY, NOESY, HSQC, HMBC and ^{13}C-NMR (500 MHz, $CDCl_3$) see Table 2. EI-MS 70eV (rel. int.): 329 (20), 328 (21), 314 (2), 299 (28), 272 (38), 271 (18), 256 (46), 255 (16), 254 (30), 242 (34), 241 (98), 240 (100), 228 (13), 213 (18), 212 (13), 59 (42), 57 (60), 44 (37). HRMS of $[M+H]^+$ *m/z* 330.1340 (Calc. 330.1336 for $C_{18}H_{20}NO_5$).

4. Conclusions

These results lead us to conclude that *N. serotinus* L. is an interesting source of alkaloids with potential pharmacological activities. Lycorine type alkaloids have shown notable properties as potent antimalarial and antitrypanosomal agents [7]. Recent investigations, including structure-activity studies, have also demonstrated they are potent inducers of apoptosis with good antitumoral activities [5,14]. In this sense, 1-*O*-(3′-acetoxybutanoyl)lycorine (**1**) is an attractive candidate for research in these areas. The isolation of narseronine (**2**) is also promising; this is the first report of a double bond between C-1 and C-10b in a homolycorine type structure, a feature that confers rigidity to the portion of the molecule formed by A-B rings, and also has a stabilising effect due to the extended conjugated system. This could be an interesting characteristic for potential biological activities related with pharmocophores with such requirements. In other respects, previous reports of antifungal activity of homolycorine-related structures such as hippeastrine [15], suggest narseronine has potential activity as an antifungal agent.

Acknowledgements

The authors are grateful for the collaboration of SCT-UB technicians. N. Pigni thanks the Spanish Ministerio de Educación for a FPU fellowship. This work was performed within the framework of project AECID A/019023/08. The authors also thank Generalitat de Catalunya (2009-SGR1060) for financial support.

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Sample Availability: Not available.

3.2. Artículo 2

Alkaloids from *Narcissus serotinus*

Journal of Natural Products, 75(9), 1643-1647 (2012)

El objetivo de de este trabajo consistió en identificar los alcaloides presentes en ejemplares de la especie *Narcissus serotinus* recolectados cerca de Vinarós (Castellón, España). Tras la extracción y fraccionamiento de 2.43 kg de material vegetal fresco, el análisis de GC-MS permitió la identificación de cinco alcaloides: narseronina, galantina, incartina, masonina e hipeastrina. Conjuntamente, se reporta el aislamiento y elucidación de seis nuevas estructuras dentro del grupo de alcaloides típicos de amarillidáceas, cuya caracterización se ha logrado mediante la combinación de diversas técnicas espectroscópicas, incluyendo HRMS, GC-MS y RMN.

Los principales componentes del extracto corresponden a dos nuevos alcaloides derivados de narcisidina: 3-*O*-metilnarcisidina y 1-*O*-acetil-3-*O*-metilnarcisidina. Además, se aisló un tercer derivado presente en cantidades minoritarias, 1-*O*-acetil-3-*O*-metil-2-oxonarcisidina. La estereoquímica de estos tres compuestos ha sido determinada con la ayuda de las correlaciones observadas en el espectro de RMN bidimensional NOESY.

Del mismo modo, se reporta la caracterización detallada de otros tres alcaloides con estructuras novedosas: 2-metoxipratosina, 11-hidroxigalantina y 2-*O*-metil-clivonina. Todos los componentes identificados en el extracto de *N. serotinus* corresponden a alcaloides de las series licorina y homolicorina.

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Note

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Alkaloids from *Narcissus serotinus*

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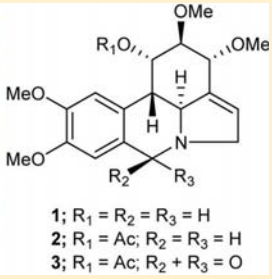
Supporting Information

ABSTRACT: *Narcissus serotinus* belongs to the Amaryllidaceae family, a group well known for an exclusive variety of alkaloids with interesting biological activities. This study was aimed at identifying the alkaloid constituents of *N. serotinus* collected in the Spanish region of Valencia, using a combination of chromatographic, spectroscopic, and spectrometric methods, including GC-MS and 2D NMR techniques. GC-MS analysis allowed for the direct identification of five known compounds. In addition, the isolation and structure elucidation of six new Amaryllidaceae alkaloids are described.



Narcissus serotinus L. is an autumn flowering species belonging to the Amaryllidaceae (subgen. *Hermione*, sect. *Serotini*), whose morphological and genetic variability ($2n$ = 10, $2n$ = 20, and $2n$ = 30) has stirred debate about the precise standing of this taxon as separate from other closely allied taxa such as *N. obsoletus* (Haw.) Steud., *N. deficiens* Herbert,[1,2] and *N. miniatus* Koop., Donnison-Morgan, Zonn.[3,4]

This plant family is well known for the presence of an exclusive array of alkaloids with a wide range of biological activities. Galanthamine is the standout example of these alkaloids, having received FDA approval in 2001 due to its marked ability to selectively and reversibly inhibit the enzyme acetylcholinesterase of significance in the progression of neurodegeneration associated with Alzheimer's disease (AD).[5,6] At present, under the commercial names Razadyne and Reminyl, it is prescribed for the treatment of AD in its mild to moderate stages. Apart from this, alkaloids of the genus *Narcissus* have been shown to possess antifungal, antitumoral, and antiparasitic activities.[7]

We previously described the isolation of two new alkaloids from whole plants of *N. serotinus* L. collected in Morocco.[8] During this investigation,[8] GC-MS analysis indicated the presence of several unknown structures with MS fragmentation patterns characteristic of Amaryllidaceae alkaloids. However, due to sample quantity limitations, we were unable to isolate these compounds. Thus, for the present study, a larger collection was made in the Spanish region of Valencia in order to identify these targets of interest and to compare the chemical constituents of the Moroccan and Spanish populations.

The MeOH extract of whole *N. serotinus* L. plants was fractioned according to the sequence described in the Experimental Section. GC-MS analysis of extracts H1 and A1 revealed the presence of 11 compounds with typical fragmentation patterns of Amaryllidaceae alkaloids of the lycorine and homolycorine series (Table 1). Identification of the known compounds narseronine, galanthine, incartine, masonine, and hippeastrine was made by comparison of their MS data with those gleaned from the literature.[8,9] In addition, six new alkaloids of the Amaryllidaceae group (**1**–**5** and **11**) were identified.

The main components of the extracts correspond to two new structures related to narcissidine: 3-*O*-methylnarcissidine (**1**) and 1-*O*-acetyl-3-*O*-methylnarcissidine (**2**).

The HRMS data of **1** suggested the molecular formula $C_{19}H_{26}NO_5$ for the parent ion $[M + H]^+$ at m/z 348.1808 (calcd 348.1805), while in **2**, a parent ion at m/z 390.1913 indicated the formula $C_{21}H_{28}NO_6$ for its $[M + H]^+$ (calcd 390.1911). The EIMS data of **1**, with a base peak at m/z 284, showed a fragmentation pattern similar to that of narcissidine, with the only difference of the $[M]^+$ peak being 14 mass units higher at m/z 347 (8%), suggesting the presence of an additional methyl group.[10] The MS fragmentation observed for **2**, with a molecular ion $[M]^+$ at m/z 389 (3%), does not coincide with the typical narcissidine pattern, probably due to the presence of an acetyl substituent at C-1.

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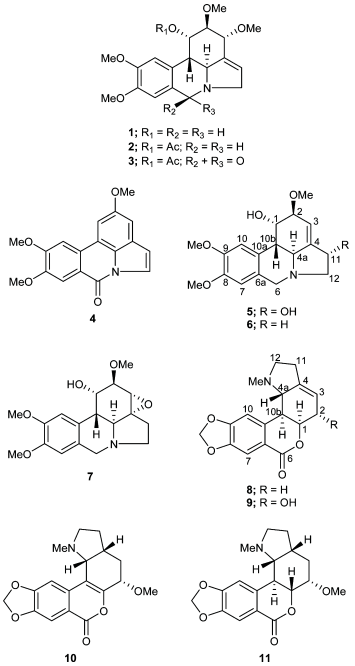
Table 1. GC-MS Analysis of the Alkaloid Content of *N. serotinus* L. Extracts

alkaloid	RI	% H1	% A1	M^+	MS
masonine (**8**)	2670	0.26		299 (−)	190 (1), 162 (2), 134 (2), 109 (100), 108 (25), 94 (3), 82 (3)
galanthine (**6**)	2678	<0.20	8.78	317 (22)	316 (15), 298 (10), 268 (18), 243 (96), 242 (100), 228 (8)
1-*O*-acetyl-3-*O*-methylnarcissidine (**2**)	2718	57.22		389 (3)	388 (5), 357 (50), 326 (98), 314 (3), 298 (35), 294 (20), 284 (9), 272 (19), 266 (100), 258 (31), 228 (40)
incartine (**7**)	2731	<0.20	1.16	333 (42)	332 (100), 315 (25), 259 (73), 258 (97), 244 (17), 214 (9), 172 (5)
3-*O*-methylnarcissidine (**1**)	2800	9.94	31.98	347 (8)	348 (2), 346 (16), 315 (47), 298 (6), 284 (100), 266 (35), 258 (22), 242 (8), 230 (38), 228 (30)
hippeastrine (**9**)	2859		<0.20	315 (−)	162 (4), 134 (3), 125 (100), 96 (36), 82 (3)
11-hydroxygalanthine (**5**)	2870		5.09	333 (16)	332 (15), 316 (11), 314 (11), 302 (5), 284 (13), 266 (8), 259 (100), 258 (89), 240 (13), 228 (4), 162 (11), 141 (12)
2-*O*-methylclivonine (**11**)	2886	0.41		331 (13)	316 (6), 162 (3), 134 (2), 126 (2), 115 (2), 96 (39), 83 (100)
narseronine (**10**)	2909	13.86		329 (23)	328 (24), 314 (2), 299 (31), 272 (42), 256 (47), 241 (100), 240 (97), 228 (12), 213 (17), 59 (50), 57 (70), 44 (45)
2-methoxypratosine (**4**)	3025	0.25	<0.20	309 (100)	310 (20), 294 (16), 278 (2), 266 (22), 251 (12), 236 (7), 222 (5), 208 (7), 193 (4), 164 (4), 125 (2)
1-*O*-acetyl-3-*O*-methyl-6-oxonarcissidine (**3**)	3055	0.40		403 (1)	371 (10), 340 (19), 312 (4), 298 (17), 280 (100), 272 (8), 255 (10)



The ^{1}H NMR spectra of **1** and **2** (Table 2) showed the H-10b double doublet resonance between δ 2.70 and 3.00 ($J \approx$ 11.0, 2.0), characteristic of narcissidine and its derivatives. The spectrum of **1** is in accordance with the data published for narcissidine and 3-*O*-acetylnarcissidine.[11,12] The assignment of the methoxy substituent at C-3 is supported by its spatial correlation with H-11 (NOESY, see Figure 1). Interestingly, the COSY correlation between H-1 and H-3 suggests the existence of "*W*-coupling" between these protons. The ^{1}H NMR data of **2** revealed significant differences compared to **1**, including the presence of a singlet for the acetyl group at δ 1.99, the deshielding of H-1 from δ 4.71 to 5.81, and the shielding of H-10 from δ 6.98 to 6.57.

The designation α/β of H-6 and H-12 relates to the orientation of the electron lone pair on the nitrogen atom, through which the vicinal *cis* protons are markedly deshielded.[7] Although this is not always defined for the narcissidine-type structures, in the majority of cases, the more deshielded proton is assigned to H-β, assuming that the orientation of the lone pair of N is also β. However, for compounds **1** and **2**, the spectroscopic data prompted us to reconsider this assumption, bearing in mind the NOESY correlation between the β-oriented H-10b with the less deshielded H-6. Thus, an α orientation has been assigned to the more deshielded proton and, therefore, to the lone pair of the nitrogen atom.

The HRMS data of compound **3** suggested the molecular formula $C_{21}H_{26}NO_7$ for $[M + H]^+$ with a parent ion at 404.1706 (calcd 404.1704). The NMR data (Table 2) revealed that this structure is consistent with 1-*O*-acetyl-3-*O*-methyl-6-oxonarcissidine. Comparing the ^{1}H NMR spectra of **3** and **2**, the absence of H-6 is evident and H-7 is strongly deshielded due to the effect of the *peri*-carbonyl group. The EIMS data showed a molecular ion $[M]^+$ at m/z 403 (1%) and a base peak at m/z 280, a fragment that could be attributed to an ion stabilized by a conjugated system.

Compound **4** has been identified as the novel 2-methoxypratosine, a derivative of pratosine first isolated from *Crinum latifolium*.[13] The HRMS analysis suggested a molecular formula of $C_{18}H_{16}NO_4$ for $[M + H]^+$ with a parent ion at 310.1074 (calcd 310.1073). The EIMS data exhibited a base peak coincident with the molecular ion $[M]^+$ at m/z 309 as well as a low degree of fragmentation, characteristic of an ion stabilized by an extended conjugated system. The MS pattern is in accordance with the reported data,[13,14] showing similar relative intensities of the fragments, each peak being 30 mass units higher than those of pratosine, which could be explained by the presence of an additional methoxy group. The ^{1}H NMR data (Table 3) reveal the presence of two sets of aromatic protons [δ 8.03 d (3.5) to 6.84 d (3.5) and δ 7.55 d (2.0) to 7.30 d (2.0)] assigned to H-12/H-11 and H-1/H-3, respectively, as well as two aromatic singlets (δ 8.01 for H-7 and δ 7.59 for H-10) and three signals corresponding to methoxy groups attached to aromatic rings. The occurrence of this alkaloid is presumably a byproduct of the other lycorine-type structures found in the extract. However, in a recently published article describing the isolation of two analogous compounds named lycoranines from *Lycoris radiata*, the authors propose a different biosynthetic pathway for Amaryllidaceae alkaloids related with these structures.[15]

The MS fragmentation pattern observed for compound **5** is similar to that of galanthine with a difference of 16 units for the main peaks.[9,16] A molecular ion $[M]^+$ at m/z 333 (16%) (m/z 317 for galanthine) as well as a pair of peaks (with the highest

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Table 2. NMR Data for Compounds 1−3

	1[a]			2[a]			3[b]		
position	δ_H mult (J in Hz)	δ_C		δ_H mult (J in Hz)	δ_C		δ_H mult (J in Hz)	δ_C	
1	4.71, br s	68.1	CH	5.81, br t (2.6)	68.2	CH	5.78, br t (2.7)	66.8	CH
2	3.80, t (2.9)	80.5	CH	3.73, dd (2.8, 2.0)	79.1	CH	3.77, dd (2.9, 2.0)	79.1	CH
3	4.29, br d (2.0)	77.8	CH	4.08, br d (1.7)	76.6	CH	4.06, br d (1.9)	75.8	CH
4		137.2	qC		137.6	qC		136.1	qC
4a	3.87, m (overlapped)	62.6	CH	3.92, m	62.7	CH	4.72, m	60.1	CH
6α	4.17, d (12.8)	54.9	CH_2	4.21, d (13.1)	54.5	CH_2		162.6	qC
6β	3.67, d (12.4)	54.9	CH_2	3.70, d (13.0)	54.5	CH_2			
6a		128.8	qC		128.4	qC		130.8	qC
7	6.75, s	111.0	CH	6.74, s	110.8	CH	7.57, s	111.4	CH
8		147.2	qC		147.4	qC		148.0	qC
9		148.3	qC		148.2	qC		151.9	qC
10	6.98, s	107.9	CH	6.57, s	107.0	CH	6.58, d (0.8)	105.7	CH
10a		130.1	qC		128.5	qC		124.6	qC
10b	2.81, dd (11.2, 1.7)	41.6	CH	2.98, dd (11.0, 2.0)	39.8	CH	3.30, ddd (12.8, 2.5, 0.9)	41.6	CH
11	5.87, q (1.8)	125.9	CH	5.87, q (1.8)	125.9	CH	5.97, q (1.8)	125.4	CH
12α	4.21, br d (14.7)	62.5	CH_2	4.18, m (overlapped)	62.1	CH_2	4.40, ddd (16.0, 3.2, 1.6)	52.6	CH_2
12β	3.65, ddd (14.5, 6.0, 2.0)	62.5	CH_2	3.66, ddd (14.4, 5.7, 2.1)	62.1	CH_2	4.68, ddd (16.1, 5.1, 2.0)	52.6	CH_2
OMe (2)	3.47, s	58.4	CH_3	3.52, s	58.7	CH_3	3.51, s	58.9	CH_3
OMe (3)	3.27, s	56.4	CH_3	3.24, s	56.3	CH_3	3.27, s	56.5	CH_3
OMe (8)	3.86, s	56.3	CH_3	3.86, s	56.1	CH_3	3.93, s	56.2	CH_3
OMe (9)	3.91, s	56.3	CH_3	3.81, s	56.1	CH_3	3.86, s	56.2	CH_3
OCOMe				1.99, s	21.2	CH_3	2.01, s	21.1	CH_3
OCOMe					171.3	qC		171.1	qC

[a]500 MHz for ^{1}H, 125 MHz for ^{13}C. [b]400 MHz for ^{1}H, 100 MHz for ^{13}C; $CDCl_3$.

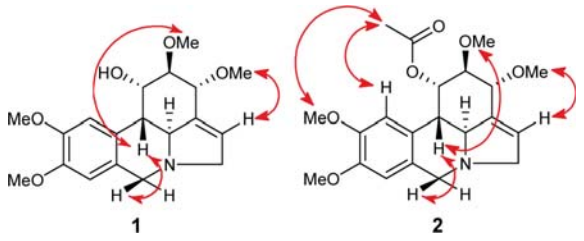

Figure 1. Key NOESY correlations of compounds **1** and **2**.

relative abundance of the spectrum) at m/z 259 and 258 (m/z 243 and 242 for galanthine) suggested the presence of an additional oxygen atom. The HRMS data confirmed the expected molecular formula $C_{18}H_{24}NO_5$ for $[M + H]^+$ with a parent ion at m/z 334.1652 (calcd 334.1649). NMR data analysis (Table 3) allowed the unambiguous identification of this component as 11-hydroxygalanthine. Comparatively, the reported ^{1}H and ^{13}C NMR data of galanthine support the structural assignment.[17]

The α orientation of the hydroxy group is supported by the allylic coupling observed for H-3 and H-11. The J values are correlated to the values of the dihedral angle (φ): angles of 30° usually correspond to small coupling constants (1.0−1.5), whereas larger angles (60−90°) are associated with larger J values (2.2−2.8).[18] A Dreiding model of **5** showed that the angle defined by H-11 and the plane formed by C-3/C-4/C-11 is about 30° if we consider a β orientation for H-11, while it is around 80° in the opposite situation. Thus, a $J_{11,3}$ of 1.5 Hz confirms the β orientation of H-11 and the α position of the substituent.

The EIMS data of compound **11** revealed the characteristic fragmentation of a homolycorine-type structure. A base peak at m/z 83 and a peak at m/z 96 (39%) were similar to the MS fragmentation of clivonine.[19] The molecular ion $[M]^+$ at m/z 331, which is 14 units higher than that observed for clivonine, as well as the result of the HRMS analysis indicated a molecular formula of $C_{18}H_{22}NO_5$ for $[M + H]^+$ with the parent ion at m/z 332.1491 (calcd 332.1492), suggesting the presence of an additional methyl group. A structure similarity search on SciFinder (accessed on July 2011) identified "dihydroungerine", a hydrogenation product of the alkaloid ungerine (isolated from the genus *Ungernia*).[20] The configuration described for ungerine includes a *cis*-B/C ring junction. However, the ^{1}H NMR spectrum of **11** showed the signal of the *N*-methyl group near δ 2.5 ppm, a position that suggests a *trans*-B/C ring fusion if we consider the correlations between stereochemical assignments and *N*-methyl shifts.[21] Additionally, the CD spectrum of **11** had the same shape as that reported for clivonine,[22,23] with observed negative and positive Cotton effects supporting a *trans*-B/C *anti*, *cis*-C/D configuration for the ring junctions. With the differences expected for substitution with a methoxy rather than hydroxy group, the ^{1}H and ^{13}C NMR data recorded in Table 4 is analogous to that reported for synthetic clivonine.[24] As such, our finding is the first for 2-*O*-methylclivonine from a natural source.

In conclusion, *N. serotinus* L. is an interesting source of Amaryllidaceae alkaloids. Six of the components isolated are reported for the first time, five of which (**1**, **2**, **3**, **4**, and **5**) are structurally related to lycorine, while **11** belongs to the homolycorine series. Additionally, five known alkaloids have been identified.

By comparison with the analysis of *N. serotinus* L. plants obtained from Morocco,[8] it is worth mentioning the absence of lycorine and its derivative 1-*O*-(3′-acetoxybutanoyl)lycorine in the Spanish population. The presence of narseronine, an unusual homolycorine-type structure reported for the first time in the Moroccan plants, is also confirmed in the Spanish

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Table 3. NMR Data for Compounds 4 and 5

	4[a]			5[a]		
position	δ_H mult (J in Hz)	δ_C		δ_H mult (J in Hz)	δ_C	
1	7.55, d (2.0)	106.2	CH	4.68, br s	69.0	CH
2		157.7	qC	3.88, ddd (3.0, 3.0, 1.5)	80.9	CH
3	7.30, d (2.0)	106.9	CH	5.94, m	119.4	CH
4		129.1	qC		146.0	qC
4a		126.6	qC	3.03, dd (10.5, 1.4)	59.8	CH
6α		158.3	qC	3.60, br d (14.0)	56.1	CH_2
6β				4.07, d (13.9)	56.1	CH_2
6a		121.2	qC		129.4	qC
7	8.01, s	110.4	CH	6.64, s	111.0	CH
8		149.9	qC		148.0	qC
9		153.7	qC		148.1	qC
10	7.59, s	104.1	CH	6.85, s	107.5	CH
10a		129.3	qC		125.9	qC
10b		117.1	qC	2.65, br d (10.6)	41.7	CH
11	6.84, d (3.5)	110.7	CH	4.89, br ddd (6.5, 1.5)	71.6	CH
12α	8.03, d (3.5)	124.2	CH	2.35, dd (9.2, 6.7)	63.2	CH_2
12β				3.68, dd (9.2, 6.5)	63.2	CH_2
OCH_3 (2)	3.98, s	56.5	CH_3	3.56, s	58.1	CH_3
OCH_3 (8)	4.07, s	56.4	CH_3	3.86, s	56.1	CH_3
OCH_3 (9)	4.12, s	56.4	CH_3	3.90, s	56.3	CH_3

[a]500 MHz for ^1H, 125 MHz for ^{13}C; $CDCl_3$.

Table 4. NMR Data for Compound 11[a]

position	δ_H mult (J in Hz)	δ_C	
1	4.09, dd (12.6, 2.6)	81.4	CH
2	3.78, q (2.9)	76.8	CH
3α	2.37, ddd (15.5, 2.8, 1.6)	26.3	CH_2
3β	1.61, ddd (15.4, 6.5, 3.1)	26.3	CH_2
4	2.62−2.46, m (overlapped)	33.6	CH
4a	2.87, dd (9.8, 6.7)	70.3	CH
6		164.9	qC
6a		118.9	qC
7	7.50, s	109.4	CH
8		146.8	qC
9		152.6	qC
10	7.86, br s	107.4	CH
10a		141.1	qC
10b	3.27, dd (12.8, 9.5)	34.2	CH
11α	2.29−2.15, m	30.5	CH_2
11β	2.15−2.02, m	30.5	CH_2
12α	3.35−3.21, m (overlapped)	53.1	CH_2
12β	2.62−2.46, m (overlapped)	53.1	CH_2
OCH_3 (2)	3.49, s	58.2	CH_3
NCH_3	2.54, s	45.5	CH_3
OCH_2O	6.02, d (1.3) to 6.03, d (1.3)	101.9	CH_2

[a]300 MHz for ^1H, 125 MHz for ^{13}C; $CDCl_3$.

species, being among the three most abundant alkaloids of the extract.

The genetic and morphological variability of *Narcissus serotinus* L. s.l. has been interpreted differently by some authors. Since Fernandes first recognized variations in the genetic endowment ($2n$ = 10, $2n$ = 20, and $2n$ = 30) inside the taxon,[25] subsequent studies considered that the diploid plants ($2n$ = 10), one-flowered with a six-lobed yellow crown, correspond to *N. serotinus* L. s.s.,[1,2] while $2n$ = 30 plants, with (1) 2−3 (4) flowers per scape and an orange three-lobed crown, correspond to *N. deficiens* Herbert. According to these authors, and due to its geographical distribution and morphological characteristics, the material studied here (*N. serotinus* L. s.l.) would correspond to *N. deficiens* Herbert s.s., while the plants from Morocco can be classified as *N. serotinus* L. s.s. This taxonomic division could explain the chemical differences observed within the *N. serotinus* L. s.l. group.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in $CHCl_3$ at 22 °C using a Perkin-Elmer 241 polarimeter. UV spectra were obtained on a Dinko UV2310 instrument. The CD spectrum was recorded at room temperature at a concentration of 1 mg/mL on a JASCO J-810 spectropolarimeter using HPLC grade MeOH as the solvent. IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer. NMR spectra were recorded on a Gemini 300 MHz, a Varian VNMRS 400 MHz, or a Varian VNMRS 500 MHz, using $CDCl_3$ or methanol-d_4 as solvent and TMS as the internal standard. Chemical shifts are reported in δ units (ppm) and coupling constants (J) in Hz. EIMS were obtained on a GC-MS Agilent 6890 + MSD 5975 operating in EI mode at 70 eV. A DB-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was 100−180 at 15 °C min^{-1}, 1 min hold at 180 °C, 180−300 at 5 °C min^{-1}, and 1 min hold at 300 °C. The injector temperature was 280 °C. The flow rate of He carrier gas was 0.8 mL min^{-1}. In most cases the split ratio was 1:20, but with more diluted samples a split ratio of 1:5 was applied. A hydrocarbon mixture (C9−C36, Restek, cat no. 31614) was used for performing the RI calibration. GC-MS results were analyzed using AMDIS 2.64 software (NIST). The proportion of each compound in the alkaloid fractions was expressed as a percentage of the total alkaloids (Table 1). These data do not express a real quantification, although they can be used to compare the relative quantities of each component. HRESIMS data were obtained on an LC/MSD-TOF (Agilent 2006).

Plant Material. Whole plants of *N. serotinus* L. were collected in October 2010 during the flowering period from a population located near Vinarós, Castellón Province (Spain), and identified by S.R.-R. and E.L. This population, growing in an industrial area close to a road, is under threat. It has survived because it receives extra water from nearby crops, which favors an extraordinary growth unusual in the other Valencian populations.

Voucher specimens (BCN-83312) have been deposited in the Herbarium of Barcelona University (CeDocBiV). Some live plants have been included in the Iberian *Narcissus* Collection of the Field Station of Torretes (Ibi, Spain) for conservation and further studies.

Extraction and Isolation. The fresh whole plant (2.43 kg) was crushed and extracted with MeOH (1 × 4.5 L, 36 h; 1 × 2.5 L, 48 h; and 1 × 2.5 L, 96 h). The extract was evaporated under reduced pressure and freeze-dried to yield 63.5 g. This crude extract was dissolved in 400 mL of H_2SO_4 2% (v/v), and neutral material was removed with Et_2O (9 × 400 mL). The acidic solution was basified with 25% ammonia up to pH 9−10 and extracted with *n*-hexane (15 × 400 mL) to give extract H1 (694 mg). Another extraction with EtOAc (12 × 400 mL) gave extract A1 (1.02 g). Both fractions were dried over anhydrous Na_2SO_4, filtered, and completely dried under reduced pressure. Referred to as fraction weight, the sum of these two extracts represents approximately 0.07%.

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The extracts were subjected to a combination of chromatographic techniques, including vacuum liquid chromatography (VLC)[26] and semipreparative TLC. The general VLC procedure consisted in 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 using *n*-hexane gradually enriched with EtOAc and then EtOAc gradually enriched with MeOH (reaching a maximum concentration of 20%). Fractions of 15 mL were collected, monitored by TLC (UV 254 nm, Dragendorff's reagent), and combined according to their profiles. For semipreparative TLC, silica gel 60F254 was used (20 cm × 20 cm × 0.25 mm) together with different solvent mixtures depending on each particular sample, always using an environment saturated with ammonia. The purification of the new reported compounds is described in detail in the Supporting Information.

3-O-Methylnarcissidine (**1**): $[\alpha]^{22}_{D}$ −7 (*c* 0.09, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 279 (3.44), 224 (3.81) nm; IR ($CHCl_3$) ν_{max} 3476, 2924, 2854, 1732, 1670, 1515, 1464, 1374, 1262, 1100, 932 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 2; EIMS data shown in Table 1; HREIMS of $[M + H]^+$ *m*/*z* 348.1808 (calcd for $C_{19}H_{26}NO_5$, 348.1805).

1-O-Acetyl-3-O-methylnarcissidine (**2**): $[\alpha]^{22}_{D}$ −30 (*c* 0.18, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 272 (3.83), 221 (4.05) nm; IR ($CHCl_3$) ν_{max} 2924, 2854, 1732 1610, 1516, 1464, 1373, 1242, 1101, 1047, 941, 757 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 2; EIMS data shown in Table 1; HREIMS of $[M + H]^+$ *m*/*z* 390.1913 (calcd for $C_{21}H_{28}NO_6$, 390.1911).

1-O-Acetyl-3-O-methyl-6-oxonarcissidine (**3**): $[\alpha]^{22}_{D}$ −123 (*c* 0.26, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 298 (3.54), 263 (3.59), 221 (4.25) nm; IR ($CHCl_3$) ν_{max} 3454, 2928, 1735, 1666, 1644, 1604, 1512, 1457, 1432, 1371, 1284, 1241, 1215, 1100, 1053, 1008, 941, 756 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) see Table 2; EIMS data shown in Table 1; HREIMS of $[M + H]^+$ *m*/*z* 404.1706 (calcd for $C_{21}H_{26}NO_7$, 404.1704).

2-Methoxypratosine (**4**): $[\alpha]^{22}_{D}$ −4 (*c* 0.27, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 369 (2.79), 296 (3.36), 253 (3.60), 225 (3.62) nm; IR ($CHCl_3$) ν_{max} 2923, 2854, 1733, 1672, 1604, 1509, 1463, 1376, 1309, 1267, 1145, 1100 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 3; EIMS data shown in Table 1; HREIMS of $[M + H]^+$ *m*/*z* 310.1074 (calcd for $C_{18}H_{16}NO_7$, 310.1073).

11-Hydroxygalanthine (**5**): $[\alpha]^{22}_{D}$ +47 (*c* 0.06, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 282 (3.62), 225 (3.92) nm; IR ($CHCl_3$) ν_{max} 3330, 2926, 1611, 1515, 1465, 1353, 1258, 1214, 1091, 998, 960, 850, 756 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 3; EIMS data shown in Table 1; HREIMS of $[M + H]^+$ *m*/*z* 334.1652 (calcd for $C_{18}H_{24}NO_5$, 334.1649).

2-O-Methylclivonine (**11**): $[\alpha]^{22}_{D}$ +14 (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 297 (3.51), 254 (3.64), 225 (3.99) nm; CD (MeOH, $[\theta]_{\lambda}$) $[\theta]_{302}$ −195, $[\theta]_{291}$ 0, $[\theta]_{273.5}$ +1761, $[\theta]_{261.5}$ 0, $[\theta]_{251}$ −1242, $[\theta]_{239.3}$ 0, $[\theta]_{233.5}$ +1255; IR ($CHCl_3$) ν_{max} 2924, 2854, 1714, 1476, 1274, 1036 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 4; EIMS data shown in Table 1; HREIMS of $[M + H]^+$ *m*/*z* 332.1491 (calcd for $C_{18}H_{22}NO_5$, 332.1492).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A detailed description of the isolation procedure, as well as tables with complete COSY, NOESY, and HMBC data, and ^1H and ^{13}C NMR spectra of alkaloids **1**−**5** and **11** are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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3.3. Artículo 3

Wild daffodils of the section Ganymedes from the Iberian Peninsula as a source of mesembrane alkaloids

Enviado a *Phytochemistry* (2013)

El presente trabajo se realizó con la finalidad de llevar a cabo un estudio detallado de los alcaloides de la especie *Narcissus triandrus* L., así como un análisis del perfil de alcaloides de 18 poblaciones silvestres incluyendo muestras de todos los taxones de la sección Ganymedes. Las especies pertenecientes a esta sección presentan una distribución geográfica que abarca la Península Ibérica y las islas Glenan, en Francia. Estudios previos de *N. triandrus* y *N. pallidulus* han reportado la presencia de alcaloides de tipo mesembrano, típicos del género *Sceletium* (Sudáfrica) e inusuales en plantas de la familia Amaryllidaceae.

Partiendo de 600 g de material vegetal fresco de *N. triandrus*, recolectado cerca de Proaza (Asturias, España), se abordó el fraccionamiento y purificación del extracto con el objeto de estudiar su composición de alcaloides. En el análisis de GC-MS se detectó la presencia de ocho componentes, tres de los cuales se identificaron rápidamente por comparación de sus patrones de fragmentación con los datos de la literarura: 4´-*O*-demetilmesembrenona, mesembrina y mesembrenona, siendo este último el componente mayoritario. Del mismo modo, con la ayuda de los datos de ^{1}H-RMN para la determinación correcta de la estereoquímica, se identificaron 6-epimesembrenol y 6-epimesembranol. Los tres componentes minoritarios restantes detectados mediante GC-MS, permanecieron sin identificar, aunque sus espectros de masas sugieren que se trata de estructuras del mismo tipo.

Por otra parte, se logró el aislamiento de tres alcaloides obtenidos por primera vez de una fuente natural, cuya presencia no se detectó en el análisis de GC-MS. Así, los datos complementarios de HRMS, GC-MS y RMN, permitieron la elucidación estructural de 2-oxomesembrenona, 7,7a-dehidromesembrenona y 2-oxoepi-mesembranol, representando este trabajo una contribución actualizada a la química de estos alcaloides.

El análisis mediante GC-MS de ejemplares de 18 poblaciones silvestres de narcisos de la sección Ganymedes, recolectados en diversas localidades de la Península

Ibérica, ha permitido confirmar la presencia de alcaloides de tipo mesembrano en todos los taxones descritos, sin detectarse trazas de alcaloides habituales de plantas de la familia Amaryllidaceae. En todas las poblaciones analizadas el componente mayoritario se identificó como mesembrenona y, si bien la variabilidad observada no demostró tendencias marcadas para proponer agrupamientos definidos entre las muestras estudiadas, la especie *N. iohannis* destacó por presentar una baja abundancia de alcaloides en comparación con las demás. Asimismo, se observó una reducida proporción de alcaloides en las muestras de *N. pallidulus* (Segovia), diferencia que puede atribuirse a un estado prematuro en el desarrollo, dado que dichas plantas no estaban en floración en el momento de la recolección.

Mediante los resultados de este estudio se confirma que los narcisos de la sección Ganymedes son una fuente alternativa de alcaloides de tipo mesembrano, conocidos inhibidores de la recaptación de serotonina y con potencial aplicación farmacológica en el tratamiento de trastornos psíquicos tales como depresiones, ansiedad o drogodependencia.

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Title: Wild daffodils of the section Ganymedes from the Iberian Peninsula as a source of mesembrane alkaloids

Article Type: Full Length Article

Section/Category: Chemistry

Keywords: Narcissus triandrus; section Ganymedes; Amaryllidaceae; GC-MS; NMR; alkaloid profile; 2-oxomesembrenone; 7,7a-dehydromesembrenone; 2-oxoepimesembranol.

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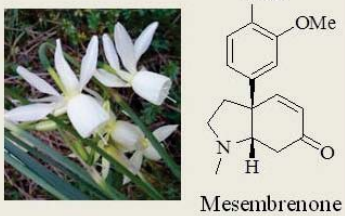
Abstract: The aim of this work was to perform a detailed study of the alkaloid content of Narcissus triandrus, as well as a metabolomic analysis of the alkaloid profile of 18 wild populations, comprising all the taxa of the section Ganymedes. Through the application of a combination of spectroscopic and chromatographic methods, the isolation and structural elucidation of 3 compounds are reported for the first time from a natural source (2-oxomesembrenone, 7,7a-dehydromesembrenone and 2-oxoepimesembranol), together with the identification of 5 major common mesembrane alkaloids. Additionally, the GC-MS analysis of the alkaloid profile demonstrated the regular presence of mesembranes in all the studied plants, showing mesembrenone as the predominant compound without any typical Amaryllidaceae alkaloid being detected.

Graphical abstract

Wild daffodils of the section Ganymedes from the Iberian Peninsula as a source of mesembrane alkaloids

Natalia B. Pigni, Segundo Ríos-Ruiz, F. Javier Luque, Francesc Viladomat, Carles Codina, Jaume Bastida*

Narcissus triandrus L. and other species from the section Ganymedes are atypical members of the Amaryllidaceae family, being characterized by the presence of mesembrane alkaloids.



Highlights

Wild daffodils of the section Ganymedes from the Iberian Peninsula as a source of mesembrane alkaloids

Natalia B. Pigni, Segundo Ríos-Ruiz, F. Javier Luque, Francesc Viladomat, Carles Codina, Jaume Bastida*

- The first detailed reporting of the alkaloid composition of *N. triandrus*.
- Structural elucidation of 3 new mesembrane alkaloids (with GC-MS and NMR data).
- Conformational analysis of 2-oxoepimesembranol.
- GC-MS of the alkaloid profile of 18 wild populations (*Narcissus* sp., Ganymedes).
- Confirmation of the occurrence of mesembrane alkaloids in all samples analyzed.

Graphical abstract

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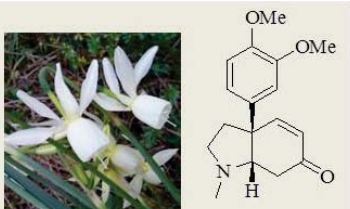

Wild daffodils of the section Ganymedes from the Iberian Peninsula as a source of mesembrane alkaloids

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Abstract

The aim of this work was to perform a detailed study of the alkaloid content of *Narcissus triandrus*, as well as a metabolomic analysis of the alkaloid profile of 18 wild populations, comprising all the taxa of the section Ganymedes. Through the application of a combination of spectroscopic and chromatographic methods, the isolation and structural elucidation of 3 compounds are reported for the first time from a natural source (2-oxomesembrenone, 7,7a-dehydromesembrenone and 2-oxoepimesembranol), together with the identification of 5 major common mesembrane alkaloids. Additionally, the GC-MS analysis of the alkaloid profile demonstrated the regular presence of mesembranes in all the studied plants, showing mesembrenone as the predominant compound without any typical Amaryllidaceae alkaloid being detected.

Keywords

Narcissus triandrus; section Ganymedes; Amaryllidaceae; GC-MS; NMR; alkaloid profile; 2-oxomesembrenone; 7,7a-dehydromesembrenone; 2-oxoepimesembranol.

1. Introduction

Within the Amaryllidaceae family (subfam. Amaryllidoideae), the genus *Narcissus* L. comprises around a hundred wild species with a center of diversity in the Iberian Peninsula and North Africa (Fernandes, 1968; Meerow et al., 1999; Ríos et al., 1999). The great majority of these species are characterized by the presence of a group of alkaloids virtually exclusive to the family, such as lycorine, galanthamine and homolycorine (Bastida et al., 2006). However, other types of alkaloids have also been reported in some Amaryllidaceae species, albeit infrequently, as in the case of *Narcissus pallidulus* and *N. triandrus* (section Ganymedes), from which some mesembrane alkaloids have been identified (Bastida et al., 1989; Berkov et al., in preparation; Seijas et al., 2004).

Many bioactive secondary metabolites have been discovered by observing traditional uses of their sources. The mesembrane alkaloids were found due to the interest on a drug preparation named “Kanna” (also known as “Channa” or “Kougoed”), commonly used by ethnic groups in South Africa and prepared from plants belonging to the genus *Sceletium* N.E.Br. (formerly *Mesembryanthemun* L.) from the Aizoaceae family (Popelak and Lettenbauer, 1967; Smith et al., 1996). Several studies have focused on the chemistry and application of these species, revealing a marked pharmacological activity of their alkaloids, notably as serotonin-uptake inhibitors, which gives them considerable potential as antidepressants (Gericke and Viljoen, 2008; Harvey et al., 2011). In fact, a US Patent has been developed for the use of pharmaceutical preparations containing mesembrine and related compounds for the treatment of depressive states and other disorders like anxiety or drug dependence (Gericke and Van Wyk, 2001).

These alkaloids are usually divided into three different skeleton types represented by

mesembrine, joubertiamine and sceltium A4 (Fig. 1) (Gaffney, 2008). Their structural similarity with crinane-type alkaloids of Amaryllidaceae plants originally led researchers to think of a common biosynthetic route, involving tyrosine and phenylalanine (Jeffs et al., 1971a), but subsequent studies revealed a different pathway, although with the same amino acids as precursors (Gaffney, 2008; Jeffs et al., 1978). The section Ganymedes (*Narcissus* sp.) has been the subject of considerable debate among taxonomists. Whereas some authors consider that it includes only a single species (*N. triandrus* L.) divided into 3 subspecies and some varieties (Barra Lázaro, 2000), studies based on molecular data and genome size support the idea of three different species: *N. triandrus* L., *N. pallidulus* Graells, and *N. lusitanicus* Dorda & Fern. Casas (Santos-Gally et al., 2011; Vives et al., 2010; Zonneveld, 2008); and, more recently, a fourth one has been added, *N. iohannis* Fern. Casas (Fernández Casas, 2011). Geographical distribution of these species is known to comprise the Iberian Peninsula and the Iles Glenans (France). Only a few studies on the alkaloid composition of two of these species have been reported, describing the isolation of mesembrenone and roserine from *N. pallidulus* (Bastida et al., 1989, 1992), a GC-MS analysis of some populations of *N. pallidulus* demonstrating the presence of more than 95% of *Sceletium* alkaloids (Berkov et al., in preparation), as well as a brief summary of the identification of mesembrine, mesembrenol and mesembrenone from *N. triandrus* (Seijas et al., 2004). The application of GC-MS for the detection of these compounds has been previously reported (Shikanga et al., 2012; Smith et al., 1998). In addition, the analysis of metabolic patterns by GC-MS applied to the field of Amaryllidaceae alkaloids with phytochemical differentiation purposes has been described, as in the case of *Galanthus elwesii* and *G. nivalis* (Berkov et al., 2008, 2011). Although the production of specialized metabolites in plants could be influenced by many factors, not only genetic

but also ontogenic and environmental, Rønsted et al. (2012) have demonstrated a significant correlation between phylogenetic and chemical diversity in the Amaryllidoideae subfamily.

The aim of this work was to perform a detailed study on the alkaloid content of *N. triandrus* through the application of a combination of spectroscopic and chromatographic methods, including GC-MS and NMR. The identification of 5 major mesembrane alkaloids has been achieved, together with the isolation and structural elucidation of 3 minor compounds reported for the first time from a natural source: 2-oxomesembrenone (**6**), 7,7a-dehydromesembrenone (**7**) and 2-oxoepimesembranol (**8**) (Fig. 2). Additionally, a metabolomic approach was developed to obtain the GC-MS alkaloid profile of 18 wild populations, comprising all the varieties of the section, with the purpose of studying chemical differences and to confirm the regular presence of mesembrane alkaloids.

2. Results and discussion

2.1. Alkaloids from N. triandrus L.

The alkaloid fractions "H" and "A" from *N. triandrus*, obtained by the extraction procedure described in section 4.3, were analyzed by GC-MS, which allowed the detection of 8 compounds (Table 1). The most abundant alkaloid in both samples was mesembrenone (**5**), which was identified by comparison with its characteristic mass fragmentation pattern, together with 4′-*O*-demethylmesembrenone (**2**) and mesembrine (**4**) (Fig. 3) (Bastida et al., 1989; Jeffs et al., 1974; Martin et al., 1976; Shikanga et al., 2012). On the other hand, although the MS of compounds **1** and **3** were similar to previously reported data for mesembrenol and mesembranol, respectively, their ^{1}H NMR spectra suggested an α orientation for the hydroxyl substituent, typical of the

corresponding epimers. Consequently, compound **1** was identified as 6-epimesembrenol after observing the diagnostic pattern for the two olefinic protons, a *ddd* at δ6.15 ppm (H5; J = 9.8, 5.5, 0.6 Hz) coupled to a *dd* at δ5.76 ppm (H4; J = 9.8, 1.2 Hz), in complete agreement with the data reported by Jeffs et al. (1970). Compound **3** was recognized as 6-epimesembranol by the complete assignment of its ^{1}H NMR spectrum, together with 2D NMR, in which the signal corresponding to H6 appears as a broad singlet at δ 3.95 ppm, suggesting an equatorial orientation. The ^{1}H NMR and NOESY data obtained (Table 2) are consistent with the conformation proposed by Jeffs et al. (1969), involving the six-membered saturated ring in a ground-state chair conformation, with the dimethoxyphenyl substituent occupying an axial position in favour of the formation of an intramolecular hydrogen bond between the proton of the hydroxyl group and the nitrogen atom.

Additionally, three minority compounds were detected in low abundance in fraction A (M1, M2 and M3). The mass spectra observed for M1 and M2, both showing the same peaks but with different abundances, were very similar to the data described for 4′-*O*-demethylmesembranol (Jeffs et al., 1970), suggesting that one of them could correspond to this alkaloid, whereas the other could be an isomer. On the other hand, the mass spectrum of M3 remains unclear: although some of its most notable fragments are described for mesembrane alkaloids, such as *m/z* 244 and *m/z* 256, which are reported for some joubertiamine derivatives (Martin et al., 1976), the base peak at *m/z* 60 is unusual. Thus, with these data it was not possible to propose a structure for M3.

After the application of a combination of chromatographic methods, such as VLC and TLC, three additional compounds not detected by GC-MS were isolated. This led to the elucidation of 2-oxomesembrenone (**6**), which can be considered a new alkaloid, 7,7a-dehydromesembrenone (**7**) and 2-oxoepimesembranol (**8**), both of them reported for the

first time from a natural source.

The HRESIMS of **6** suggested the molecular formula of $C_{17}H_{20}NO_4$ for the parent ion $[M+H]^+$ at *m/z* 302.1382 (calcd. 302.1387). The mass fragmentation pattern showed a base peak coincident with the molecular ion at *m/z* 301, together with less abundant fragments not previously described for mesembrane alkaloids, possibly owing to the uncommon carbonyl moiety at C2 (Fig. 4). The ^{1}H NMR spectrum showed 3 aromatic protons with characteristic *ortho* (8.4 Hz) and *meta* (2.2 Hz) *J* value couplings, two methoxyl groups in an aromatic environment (δ3.88 ppm), a singlet at δ2.81 ppm corresponding to an *N*-methyl group, and a pair of coupled olefinic protons (δ6.71, 6.24 ppm). The α/β orientation of H3 was determined through the observation of NOESY correlations between H3β and the aromatic protons H2′and H6′. The carbonyl groups appear in the ^{13}C NMR spectrum as deshielded signals at δ171.9 and 195.3 ppm. All the data were in accordance with the proposed structure (Table 3).

Compound **7** was first obtained through the oxidation of **5** with diethyl azodicarboxylate as reported by Jeffs et al. (1971b). The reported ^{1}H NMR data were in accordance with our results, as well as its mass fragmentation pattern (Martin et al., 1976). HRESIMS data support the formula $C_{17}H_{20}NO_3$ for the parent ion $[M+H]^+$ at *m/z* 286.1431 (calcd. 286.1438). In order to complete the characterization, the whole structure was confirmed with 2D NMR techniques, and complementary ^{13}C NMR data is reported (Table 4).

The HRESIMS of **8** indicated the molecular formula $C_{17}H_{24}NO_4$ for the parent ion $[M+H]^+$ at *m/z* 306.1691 (calcd. 306.1700). Ishibashi et al. (1991) developed a stereoselective synthesis to obtain mesembranol, which involved a mixture of **8** and its 6-epimer in a ratio of 1 to 3.7, respectively, as intermediary compounds. The ^{1}H NMR data reported for the epimer with the hydroxyl substituent in β orientation shows a signal at δ3.72 as a double triplet (*J* = 11.0, 4.0 Hz) corresponding to H6, where the

high coupling value indicates an axial position for this proton. In contrast, our results revealed smaller *J* values for H6 (δ3.95, *tt*, *J* = 6.1, 3.6 Hz), suggesting a different situation. Moreover, the evidence of spatial correlations in the NOESY experiment pointed to the presence of more than one conformer, such as the correlation observed between H3β and H7a, which supported a chair conformation for the six-membered C-ring with the dimethoxyphenyl group in an axial position similar to that proposed for mesembranol by Jeffs et al. (1969). There was also evidence of spatial proximity among H3α, H5α and H7α, which could only be explained by a chair conformation with the dimethoxyphenyl in an equatorial position (Fig. 5).

In order to confirm our proposal that there are at least two conformers in equilibrium, the conformational preferences of compound **8** in chloroform were determined by means of high-level quantum mechanical calculations. To this end, the population of conformers was estimated by combining the relative stabilities in the gas phase determined at the MP2/aug-cc-pVDZ level, with the solvation free energy in chloroform determined by using both MST and SMD solvation continuum models (see section 4.5). Calculations were done for a model compound in which the methoxy groups in the phenyl ring were replaced by protons. This is justified by the lack of direct contact between the methoxy groups with the rest of the molecule and by the concomitant saving in the cost of computations. Besides the conformational flexibility of the six-membered ring (chair versus boat), the conformational study also explored the optimal orientations of the phenyl ring, which was found to adopt two main orientations, and of the hydroxyl group, which may form an intramolecular hydrogen bond with the lone pair of the amide nitrogen. Finally, additional calculations were done for a compound in which the phenyl ring was replaced by hydrogen with the aim of examining the intrinsic conformational preferences of the bicyclic ring.

The free energy differences between conformers of the two model compounds are reported as Supplementary Information (see Tables S1 and S2). The results confirmed the energetic destabilization of the conformations where the six-membered ring adopts a boat structure (Table 5). Furthermore, the six-membered ring adopts two main chair conformations, where the phenyl ring (or the hydrogen atom) adopts an equatorial or axial position (Fig. 5). The axial arrangement is clearly predominant in the gas phase due to the stabilization afforded by the intramolecular hydrogen bond formed between the hydroxyl group and the lone pair of the amide nitrogen. However, both axial and equatorial arrangements are similarly populated upon solvation in chloroform.

Previous studies have shown that the measured coupling constants can reflect the population-weighted average of the *J* values determined for individual conformers of a given compound (Arnó et al., 2000). Therefore, we have performed similar calculations for the theoretical *J* values obtained for each conformation of the model compound with a phenyl substituent using the graphical tool MestRe-J (Navarro-Vázquez et al., 2004), based on the values of the dihedral angles and the HLA generalization of the Karplus equation (Haasnoot et al., 1980). As noted in Table 6 (see also Fig. 5), these calculations reveal a close correspondence between the calculated and observed *J* values for each vicinal proton of the six-membered C-ring. Overall, the existence of two major chair conformations of compound **8** allows us to explain the observed NOESY correlations, as well as the intermediate values of the coupling constants.

Finally, the fragments observed in the mass spectrum of **8** coincide with those reported for an isomer of this compound, obtained through a synthetic procedure (Keck and Webb, 1982).

Interestingly, the main alkaloids from *N. triandrus* with a hydroxyl substituent at position 6 identified in this work showed the same stereochemistry.

2.2. Section Ganymedes: alkaloid profile analysis

The alkaloid extracts of plants from 18 wild populations were analyzed by GC-MS. The sample distribution comprised a wide geographical range with locations from 3 NW Spanish Communities (Castilla y León, Galicia and Asturias) and N of Portugal. The analysis included plants belonging to all the taxa described in the section Ganymedes (Fernández Casas, 2011). Three repetitions were performed for each population, except for NT-B, NI-B and NL-P6, which were analyzed using 2 samples for each one.

The chromatograms were manually analyzed, recording the peak area. The mass spectra of 8 different compounds were detected (**1-7** and the unidentified M3). The data obtained were normalized to the area of the internal standard (codeine) and to the dry wt (g) of plant material. Finally, the mean and s.d. of the repetitions were calculated for each population. In order to focus on the most significant information, minority components with values lower than 1 (**6**, **7** and M3) were discarded from the graphical representation of the data. The results are summarized in Fig. 6.

All samples revealed the presence of mesembrane alkaloids, and no typical Amaryllidaceae alkaloids were detected. Mesembrenone (**5**) was predominant in all populations, present in notably higher amounts than the second most abundant component: the relation between the mean values calculated for **5** and the second compound oscillated from 3 for NT15 to 89 for NI-B.

Although the variability within populations was considerable, reflected by the high s.d., some grouping trends are noticeable. *N. pallidulus* (NP1, NP2) and *N. iohannis* (NI-B) showed markedly lower alkaloid contents than the other groups. In the case of *N. pallidulus* from Segovia, this could be attributed to the early developmental stage of the plants, which were not flowering at the time of collection; however, other factors such

as location or taxonomic divisions cannot be discarded. On the other hand, *N. iohannis*, which belongs to a taxon previously reported as *N. triandrus* var. alejandrei by Barra Lázaro (2000), but recently classified as a new species with a putative hybrid origin between *N. triandrus* and *N. pallidulus* (Fernández Casas, 2011), showed the lowest quantity of alkaloid content, which together with its distinct habitat and morphology could support its independence as a species.

In the remaining populations, the alkaloid profile and abundance were comparatively similar, with a subtle variation in the populations of *N. triandrus* (Galicia-Asturias and Burgos) and one population from Portugal (NP-P2), which showed a higher contribution of the minority compounds.

3. Concluding remarks

The analysis of the alkaloid content of *N. triandrus* led to the identification of eight mesembrane alkaloids, three of which were isolated from a natural source for the first time. The GC-MS data of all the compounds are reported, as well as the NMR data of the alkaloids 6-epimesembranol (**3**), 2-oxomesembrenone (**6**), 7,7a-dehydromesembrenone (**7**) and 2-oxoepimesembranol (**8**), which represents a detailed and updated contribution to the structural chemistry of these alkaloids.

These results confirm for the first time the presence of mesembrane alkaloids in all the *Narcissus* taxa of the section Ganymedes, without any trace of typical alkaloids of the Amaryllidaceae group. However, the studied populations were not definitely clustered by their alkaloid patterns, neither in accordance with taxonomic groups, nor with geographical locations, except for *N. iohannis*, which is endemic to a very small area and the only one growing on alkaline substrates.

This study, together with a previous analysis of *N. pallidulus* and some hybrids (Berkov

et al., in preparation), reveals an important aspect of the phylogeny of *Narcissus*: Ganymedes is the only section within the Amaryllidaceae with an atypical alkaloid biosynthetic pathway. According to Santos-Gally et al. (2011), this group has a relatively recent onset (about 4 million years ago) and, although it shows a high morphological variability, its alkaloid chemistry seems to be relatively conservative. Finally, it is interesting to note that the presence of mesembrane alkaloids is a chemical feature shared by this group of Mediterranean daffodils with the very distantly related dicotyledonous plants of the genus *Sceletium* from South Africa. Thus, *Narcissus* taxa belonging to the section Ganymedes represent an alternative source of these compounds, whose potential therapeutic applications have already been demonstrated.

4. Experimental and Computational Section

4.1. General experimental procedures

Optical rotations were measured in $CHCl_3$ at 22 °C using a Perkin-Elmer 241 polarimeter. UV spectra were obtained on a Dinko UV2310 instrument. IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer. NMR spectra were recorded on a Varian VNMRS 500 MHz, using $CDCl_3$ as the solvent and TMS as the internal standard. Chemical shifts are reported in δ units (ppm) and coupling constants (*J*) in Hz. EIMS were obtained on a GC-MS Agilent 6890 + MSD 5975 operating in EI mode at 70 eV. A HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was 100−180 at 15 °C min^{-1}, 1 min hold at 180 °C, 180−300 at 5 °C min^{-1}, and 1 min hold at 300 °C. The injector temperature was 280 °C. The flow rate of He carrier gas was 0.8 mL min^{-1}. A hydrocarbon mixture (C9−C36, Restek, cat no. 31614) was used for performing the RI calibration. GC-MS results were analyzed using AMDIS 2.64 software (NIST). The proportion of each compound in the alkaloid

fractions is reported as a percentage of the total alkaloids (Table 1). These data do not express a real quantification, although they can be used to compare the relative quantities of each component. HRESIMS data were obtained on an LC/MSD-TOF (Agilent 2006).

4.2. Plant Material

Plant material was collected in March 2012 by S.R.-R. and N.B.P., according to the flowering period reported for the target populations. Beginning in Segovia, the expedition covered Castilla y León (Spain), the center and North of Portugal, Galicia and Asturias (Spain). As the flowering of wild populations depends on the specific climatic conditions of each year, the search for some reference locations, mainly in the interior, was unsuccessful. Samples from Burgos (Spain) were collected in April 2012 by Rafa Díez, as a kind collaboration to our work. Data regarding location, labeling and additional information are detailed in Table 7.

An abundant and disperse population of *Narcissus triandrus* L. located near Proaza, Asturias (Spain), allowed the collection of an adequate quantity of material for the alkaloid isolation, without threatening its survival. Voucher specimens (BCN-102933) have been deposited in the Herbarium of Barcelona University (CeDocBiV), as well as specimens of the majority of the populations analyzed (BCN-102921-BCN-102932). Live plants have been included in the Iberian Narcissus Collection of the Field Station of Torretes (Ibi, Spain) for conservation and further studies.

4.3. Extraction and isolation of alkaloids from N. triandrus

The fresh whole plant (600 g) was crushed and extracted with MeOH (1 × 1.5 l, 72 h; 1 × 1.5 l, 48 h; and 1 × 1.5 l, 48 h). The extract was evaporated under reduced pressure to

yield 27.5 g. This crude extract was dissolved in 200 ml of H_2SO_4 2% (v/v) and neutral material was removed with Et_2O (5 × 200 ml). The acidic solution was then basified with 25% ammonia up to pH 9−10 and extracted with *n*-hexane (10 × 200 ml) to give extract H (350 mg). Another extraction with EtOAc (7 × 200 ml) gave extract A (118 mg). Both fractions were dried over dry Na_2SO_4, filtered, and completely dried under reduced pressure. Referred to fraction wt, the sum of these two extracts represents approximately 0.08%.

The extracts were subjected to a combination of chromatographic techniques, including vacuum liquid chromatography (VLC) (Coll and Bowden, 1986) and semiprep. 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 using *n*-hexane gradually enriched with EtOAc, and then EtOAc gradually enriched with MeOH (reaching a maximum concentration of 20%). Fractions of 10-15 ml were collected, monitored by TLC (UV 254 nm, Dragendorff's reagent), and combined according to their profiles. For semiprep. TLC, silica gel 60F254 was used (20 cm × 20 cm × 0.25 mm) together with different solvent mixtures depending on each particular sample, always using an environment saturated with ammonia.

*4.3.1. 2-Oxomesembrenone (**6**)*

Amorphous solid; $[\alpha]_D^{22}$ +3.6 (c 0.24, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 279.0 (2.88), 219.0 (3.67), 209.5 (3.78) nm; IR ($CHCl_3$) υ_{max}: 1693, 1519, 1464, 1257, 1148, 1025, 757 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 3; EIMS data shown in Table 1; ESI-TOF-MS *m/z* 302.1382 $[M+H]^+$ (calcd. for $C_{17}H_{20}NO_4$, 302.1387).

4.3.2. 7,7a-Dehydromesembrenone (7)

Amorphous solid; $[\alpha]_D^{22}$ -6.7 (c 0.07, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 342.5 (2.89), 284.5 (2.95), 223.0 (3.77) nm; IR ($CHCl_3$) υ_{max}: 1733, 1637, 1566, 1515, 1464, 1256, 1145, 1025, 761 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 4; EIMS data shown in Table 1; ESI-TOF-MS *m/z* 286.1431 $[M+H]^+$ (calcd. for $C_{17}H_{20}NO_3$, 286.1438).

*4.3.3. 2-Oxoepimesembranol (**8**)*

Amorphous solid; $[\alpha]_D^{22}$ -9.7 (c 0.15, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 278.0 (3.29), 227.5 (3.71) nm; IR ($CHCl_3$) υ_{max}: 3399, 1673, 1520, 1464, 1255, 1149, 1026, 758 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 4; EIMS data shown in Table 1; ESI-TOF-MS *m/z* 306.1691 $[M+H]^+$ (calcd. for $C_{17}H_{24}NO_4$, 306.1700). Supplementary information file contains complete NMR data (Table S4).

4.4. Section Ganymedes: alkaloid profile analysis

Fresh plant material from one or two individual whole plants was macerated with 10 ml MeOH in 15 ml glass vials at the time of collection, with 3 repetitions per population. After 7 days, the solvent was transferred to a new vial and dried on a hot plate at 60 ºC. The crude extracts were dissolved in 4 ml of H_2SO_4 2% (v/v), and 1 ml of codeine in MeOH (0.1 mg/ml) was added as an internal standard. The solution was defatted with Et_2O (3 x 5 ml) and the aq. layer was basified with 450 µl of 32% ammonia to extract the alkaloids with EtOAc (3 x 5 ml). After evaporation of the org. solvent, the dried alkaloid fractions were dissolved in 1 ml of $CHCl_3$ from which 500 µl were transferred to a GC-MS vial. The split ratio was 1:10 and the methodology was the same as

previously described in this section 4.1. The design of the assay included the randomization of the order of samples for both the extraction procedure and the GC-MS analysis.

4.5. Quantum mechanical calculations.

Geometry optimizations were carried out at the MP2 level with the 6-31+G(d) basis (Clark et al., 1983). The minimum energy nature of the stationary points was confirmed by inspection of the vibrational frequencies, which were positive in all cases. The relative stabilities in the gas phase were determined from single-point calculations at the MP2 level with the aug-cc-pVDZ basis (Dunning, 1989), which has been shown to predict well the conformational preferences of flexible compounds (Riley et al., 2007; Forti et al., 2012). Zero-point energy, thermal and entropic effects (at 298 K) were estimated by using the harmonic oscillator-rigid rotor formalism. Finally, the relative stability in chloroform was determined by adding the solvation free energy estimated by using the B3LYP/6-31G(d) parametrized versions of both MST (Curutchet et al., 2001; Soteras et al., 2005) and SMD (Marenich et al., 2009) continuum solvation models. All calculations were performed using Gaussian 03 (Frisch et al., 2009).

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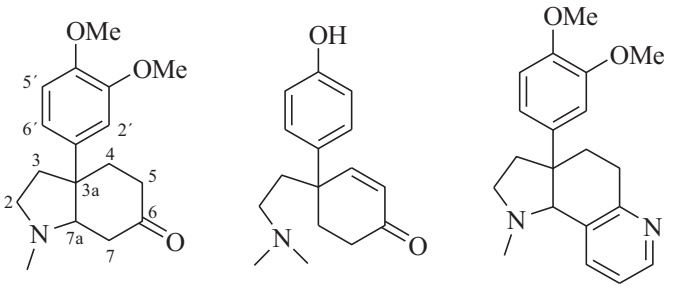
and SAF2011-27642 (DGICYT). Finally, the Centre de Serveis Científics i Acadèmics (CESCA) is acknowledged for computational facilities.

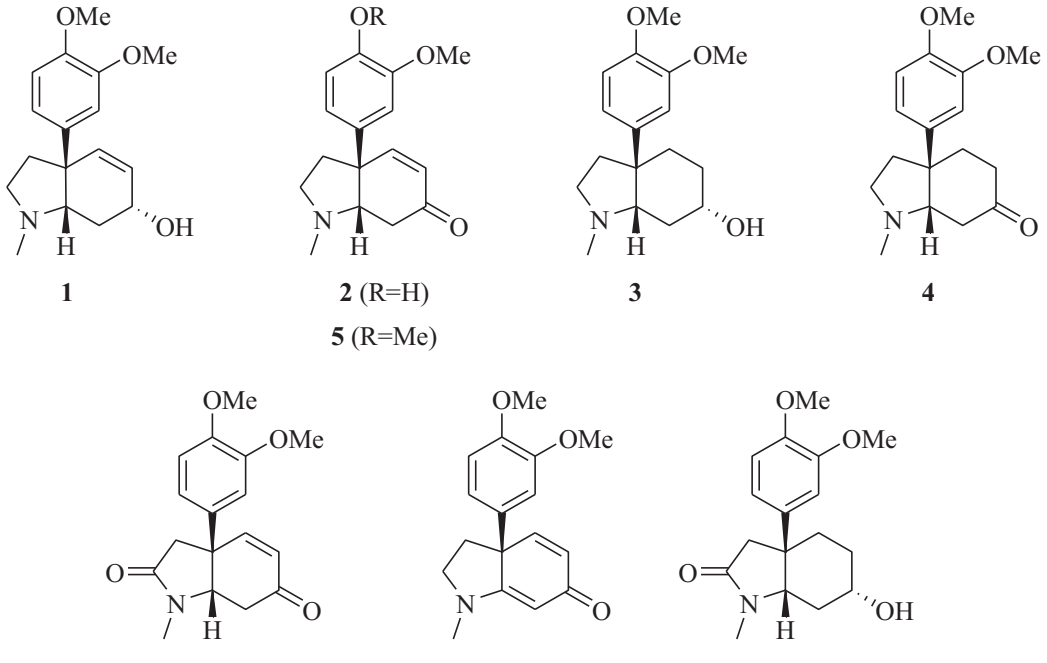
Figures and Legends

Fig. 1. Representative structures of mesembrane alkaloids.



Mesembrine **Joubertiamine** **Sceletium A4**

Figure 2. Alkaloids identified in *N. triandrus* extracts.



1 **2** (R=H) **3** **4**

5 (R=Me)

6 **7** **8**

Figure 3. MS of the alkaloids identified in *N. triandrus* extracts.

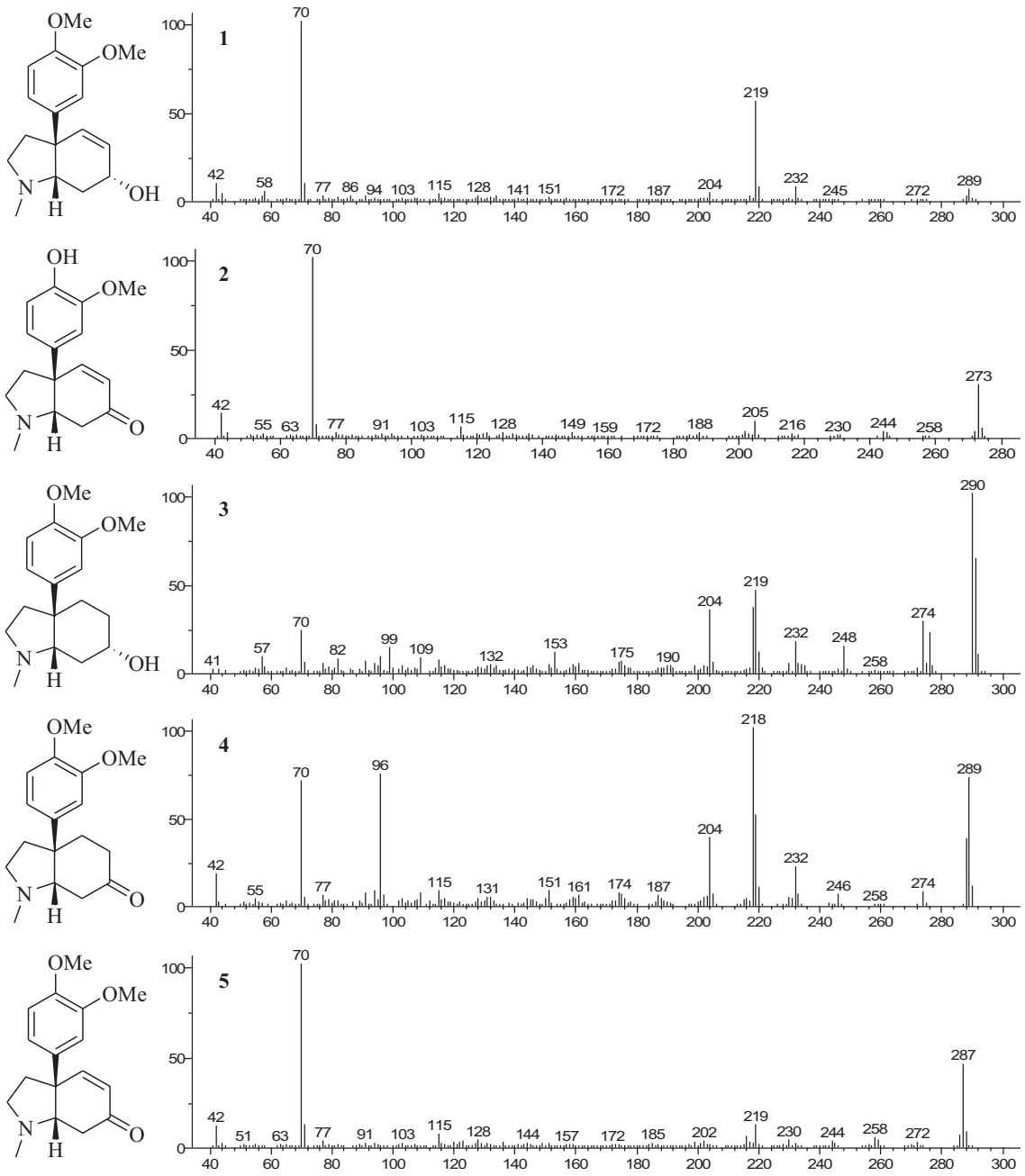


Figure 4. MS of the 3 minority compounds isolated from *N. triandrus*.

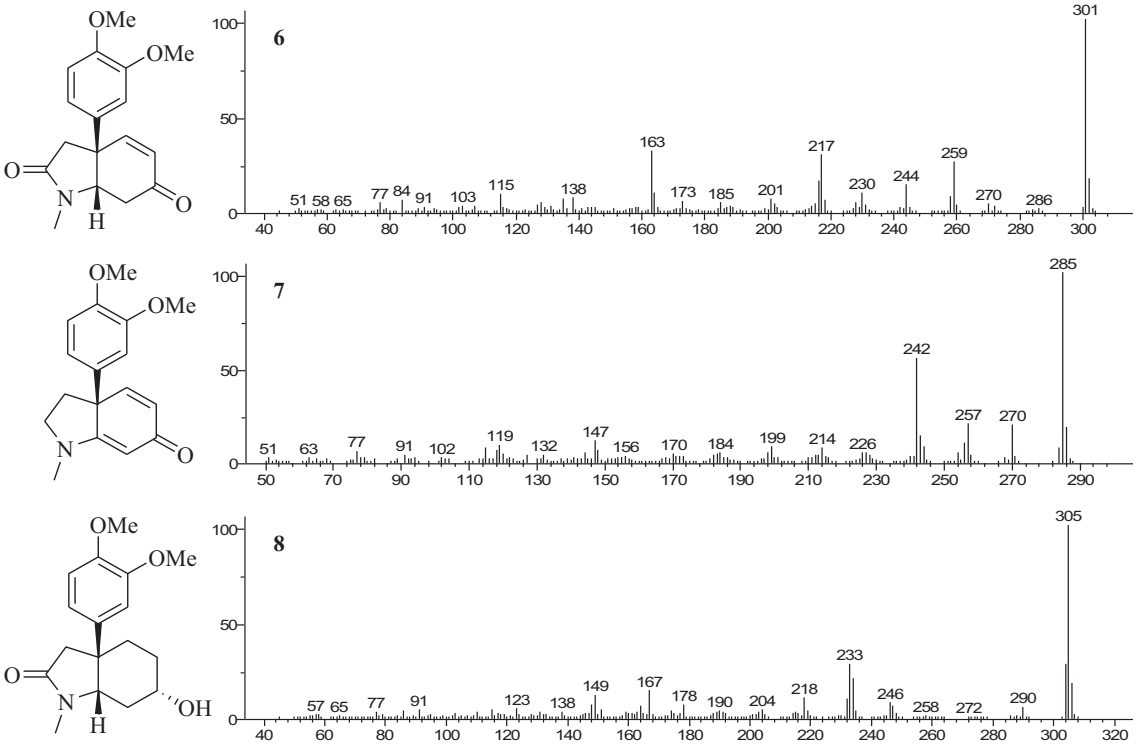


Figure 5. Two representative conformers of compound **8** showing key NOESY correlations (note that the methoxyl groups have been removed for the sake of simplicity).

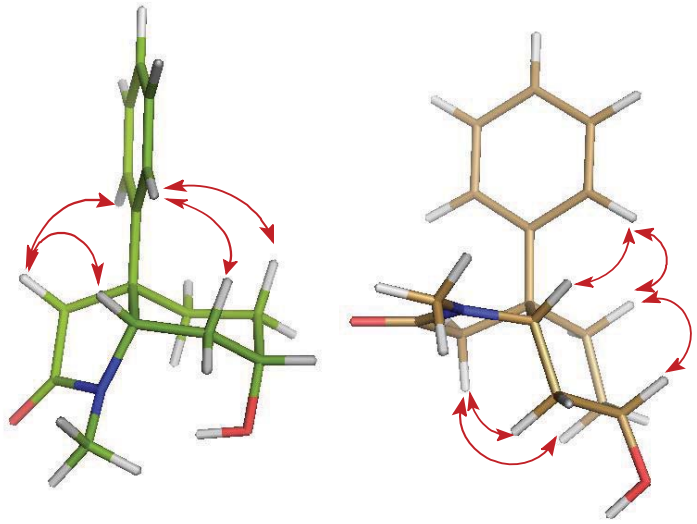
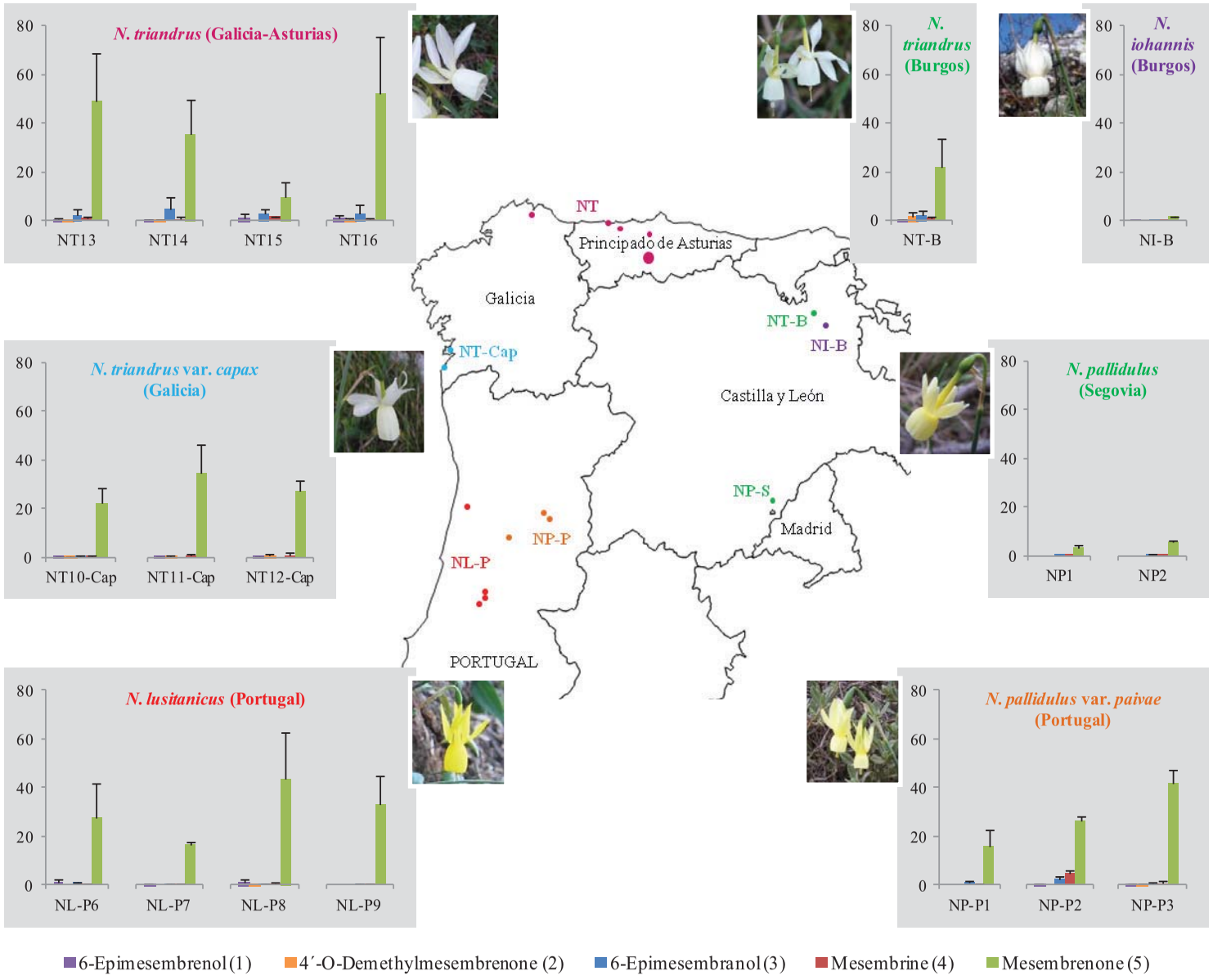


Fig. 6. Alkaloid profile analysis. The approximate locations of populations analyzed are indicated as colored dots. The larger fuchsia point corresponds to *N. triandrus* used for alkaloid isolation. Colored bars represent the mean of the measurements of each population (s.d. in black). Data are the peak area (GC-MS analysis) normalized to the area of the internal standard and to the dry wt (g) of plant material.


N. triandrus (Galicia-Asturias)
NT13
NT14
NT15
NT16
N. triandrus (Burgos)
NT-B
N. iohannis (Burgos)
NI-B
NT
Principado de Asturias
Galicia
NT-B
NI-B
NT-Cap
N. triandrus var. capax (Galicia)
NT10-Cap
NT11-Cap
NT12-Cap
Castilla y León
N. pallidulus (Segovia)
NP1
NP2
NP-S
Madrid
NP-P
NL-P
PORTUGAL
N. lusitanicus (Portugal)
NL-P6
NL-P7
NL-P8
NL-P9
N. pallidulus var. paivae (Portugal)
NP-P1
NP-P2
NP-P3
6-Epimesembrenol (1)
4´-O-Demethylmesembrenone (2)
6-Epimesembranol (3)
Mesembrine (4)
Mesembrenone (5)

Tables

Table 1. Components of the alkaloid extracts of *N. triandrus.*

Compound	RI	% H	% A	M^+	MS
6-Epimesembrenol (**1**)	2317	3.96	ND	289 (7)	232 (8), 219 (55), 204 (5), 115 (4), 70 (100)
M1	2325	ND	< 5	277 (74)	276 (100), 260 (41), 218 (27), 205 (96), 153 (21), 44 (58)
4′-*O*-Demethylmesembrenone (**2**)	2343	ND	9.28	273 (29)	258 (1), 244 (3), 205 (9), 115 (6), 70 (100), 42 (13)
6-Epimesembranol (**3**)	2350	17.74	ND	291 (64)	290 (100), 274 (29), 248 (15), 232 (17), 219 (46), 204 (35), 70 (24)
Mesembrine (**4**)	2354	7.07	ND	289 (72)	274 (8), 232 (22), 218 (100), 204 (38), 96 (74), 70 (70)
Mesembrenone (**5**)	2375	70.49	69.92	287 (45)	258 (5), 219 (12), 115 (7), 70 (100)
M2	2382	ND	< 5	277 (58)	276 (100), 260 (26), 205 (40), 190 (35), 44 (22)
M3	2440	ND	< 5	303 (31)	256 (10), 244 (85), 232 (10), 213 (26), 151 (31), 115(21), 60(100)
2-Oxomesembrenone (**6**)	2675	ND	ND	301 (100)	259 (26), 244 (14), 217 (30), 163 (32), 115 (10)
7,7a-Dehydromesembrenone (**7**)	2697	ND	ND	285 (100)	270 (20), 257 (20), 242 (55), 147 (11), 119 (9)
2-Oxoepimesembranol (**8**)	2755	ND	ND	305 (100)	290 (6), 246 (8), 233 (28), 218 (11), 167 (14), 149 (12)

Table 2. NMR data of **3** (500 MHz, $CDCl_3$)

	3		
Position	**δ_H (mult., *J* in Hz)**	**COSY**	**NOESY**
2α	3.44 (*br s*)	H2α, H3α/β	H2β, H3α/β, H4α, NMe
2β	2.41 (*m*)	H2α, H3α/β	H2α, H3α/β, H7a
3α	1.95 (*m*)	H3β, H2α/β	H2α/β, H3β, H4α, H2′, H6′
3β	1.86 (t*d*, 12.0, 6.7)	H3α, H2α/β	H2α/β, H3α, H7a, H2′, H6′
4α	2.33 (*td*, 14.3, 3.7)	H4β, H5α/β	H2α, H3α, H4β, H5α
4β	1.95 (*m*)	H4α, H5α/β	H4α, H5α/β, H2′, H6′
5α	1.73 (*m*)	H5β, H4α/β, H6	H4α/β, H5β, H6
5β	1.42 (*tdd*, 14.0, 3.7, 2.3)	H5α, H4α/β, H6	H4β, H5α, H6, H7β, H2′, H6′
6	3.95 (*br s*)	H5α/β, H7α/β	H5α/β, H7α/β
7α	2.19 (*br d*, 15.0)	H7β, H6, H7a	H6, H7β, H7a, NMe
7β	1.65 (*dt*, 15.0, 3.0)	H7α, H6, H7a	H5β, H6, H7α, H7a, H2′, H6′
7a	2.94 (*br s*)	H7α/β	H2β, H3β, H7α/β, H2′, H6′, NMe
2′	6.87 (*d*, 2.2)	H6′	H3α/β, H4β, H5β, H7β, H7a
5′	6.81 (*d*, 8.3)	H6′	-
6′	6.88 (*dd*, 8.3, 2.2)	H2′, H5′	H3α/β, H4β, H5β, H7β, H7a
NMe	2.52 (*s*)	-	H2α, H7α, H7a
OMe (3′, 4′)	3.87, 3.89 (*s*)	-	-

Table 3. NMR data of **6** (500 MHz, $CDCl_3$)

	6				
Position	**δ_H (mult., *J* in Hz)**	**δ_C**	**COSY**	**NOESY**	**HMBC**
2	-	171.9	-	-	-
3α	2.66 (*d*, 17.1)	44.2	H3β	H3β, H4	C2, C3a, C4, C7a, C1′
3β	3.18 (*d*, 17.1)	44.2	H3α	H3α, H7a, H2′, H6′	C2, C3a, C4, C1′
3a	-	45.9	-	-	-
4	6.71 (*dd*, 10.2, 1.6)	150.7	H5, H7a	H3α, H5, H2′, H6′	C3, C3a, C6, C7a, C1′
5	6.24 (*d*, 10.2)	129.0	H4	H4	C3a, C7
6	-	195.3	-	-	-
7 (2H)	2.72 (*d*, 3.8)	36.5	H7a	H7a	C3a, C5, C6, C7a
7a	4.06 (*td*, 3.8, 1.6)	65.7	H7, H4	H3β, H7, H2′, H6′, NMe	C3a, C4, C6, C1′
1′	-	131.6	-	-	-
2′	6.85 (*d*, 2.2)	109.9	H6′	H3β, H4, H7a	C3a, C4′, C6′
3′	-	149.7	-	-	-
4′	-	149.1	-	-	-
5′	6.87 (*d*, 8.4)	111.6	H6′	OMe	C1′, C3′
6′	6.92 (*dd*, 8.4, 2.2)	119.4	H2′, H5′	H3β, H4, H7a	C3a, C2′, C4′
NMe	2.81 (*s*)	27.4	-	H7a	C2, C7a
OMe (3′, 4′)	3.88 (*s*)	56.1, 56.2	-	H5′	C3′, C4′

Table 4. NMR data of **7** and **8** (500 MHz, $CDCl_3$)

	7		**8**	
Position	**δ_H (mult., *J* in Hz)**	**δ_C**	**δ_H (mult., *J* in Hz)**	**δ_C**
2α	3.31 (*dd*, 10.3, 8.1)	52.7	-	173.8
2β	3.41 (*ddd*, 10.6, 10.3, 5.0)	52.7		
3α	2.27 (*ddd*, 11.7, 10.8, 8.1)	35.9	2.65 (*d*, 16.4)	45.7
3β	2.56 (*dd*, 11.9, 5.0)	35.9	2.54 (*d*, 16.4)	45.7
3a	-	53.5	-	42.9
4α	6.77 (*d*, 9.6)	142.8	2.14 (*ddd*, 15.0, 10.0, 3.6)	30.0
4β			1.85 (*ddd*, 14.5, 7.4, 3.6)	30.0
5α	6.03 (*dd*, 9.6, 1.4)	128.8	1.60 (*m*)	30.1
5β			1.73 (*ddtd*, 14.0, 10.0, 3.6, 1.0)	30.1
6	-	185.6	3.95 (*tt*, 6.1, 3.6)	66.1
7α	5.46 (br *s*)	93.7	1.96 (*dddd*, 14.6, 6.0, 5.5, 1.0)	32.8
7β			2.14 (*dddd*, 14.7, 5.0, 3.6, 1.0)	32.8
7a	-	171.5	3.91 (*dd*, 5.5, 5.0)	62.3
1′	-	133.2	-	137.4
2′	6.84 (*d*, 2.2)	109.9	6.81 (*d*, 2.4)	110.0
3′	-	149.1	-	149.2
4′	-	148.6	-	148.0
5′	6.78 (*d*, 8.3)	111.3	6.82 (*d*, 8.4)	111.2
6′	6.86 (*dd*, 8.3, 2.3)	118.4	6.86 (*dd*, 8.4, 2.3)	118.2
NMe	2.98 (*s*)	33.1	2.90 (*s*)	28.1
OMe (3′)	3.84 (*s*)	56.2	3.88 (*s*)	56.2
OMe (4′)	3.85 (*s*)	56.1	3.87 (*s*)	56.1

Table 5. Conformer distribution (in percentage) of the model compounds (obtained by replacing the dimethoxyphenyl ring by either hydrogen or phenyl) used to examine the conformational preferences of **8**.

Conformer	Gas	Chloroform		
		MST	SMD	Average
R=H				
boat_axial	0.4	0.8	0.8	0.8
boat-equatorial	0.7	0.7	0.5	0.6
chair_axial	79.1	56.7	43.3	50.0
chair_equatorial	19.8	41.8	55.4	48.6
R=phenyl				
boat_axial	0.5	0.9	1.0	0.9
boat-equatorial	0.5	0.8	0.6	0.7
chair_axial	86.0	67.4	50.3	58.9
chair_equatorial	13.0	31.0	48.1	39.6

Table 6. Coupling constants of protons of the six-membered C-ring of compound **8**.

Vicinal protons	*J* (Hz) calc.[a]	*J* (Hz) obs.	Difference[b]
6 - 5β	2.7	3.6	0.9
6 - 5α	5.9	6.1	0.2
6 - 7β	3.2	3.6	0.4
6 - 7α	5.5	6.1	0.6
7a - 7β	4.7	5.0	0.3
7a - 7α	4.8	5.5	0.7
4α - 5β	9.7	10.0	0.3
4α - 5α	3.2	3.6	0.4
4β - 5β	3.1	3.6	0.5
4β - 5α	6.4	7.4	1.0

[a] For details see Table S3 in Supplementary Information.

[b] Difference = *J* calc. - *J* obs.

Table 7. Plant material information. P: Portugal, S: Spain.

Label	Species	Near Location (Country)	n
NP1	*N. pallidulus* Graells var. *pallidulus*	El Espinar - Segovia (S)	3
NP2	*N. pallidulus* Graells var. *pallidulus*	El Espinar - Segovia (S)	3
NP-P1	*N. pallidulus* Graells var. *paivae* Barra	Prado (P)	3
NP-P2	*N. pallidulus* Graells var. *paivae* Barra	Casas do soeiro (P)	3
NP-P3	*N. pallidulus* Graells var. *paivae* Barra	Fiais da Beira (P)	3
NL-P6	*N. lusitanicus* Dorda & Fern. Casas	Figueiró dos Vinhos (P)	2
NL-P7	*N. lusitanicus* Dorda & Fern. Casas	Ferreira do Zêzere (P)	3
NL-P8	*N. lusitanicus* Dorda & Fern. Casas	Ferreira do Zêzere (P)	3
NL-P9	*N. lusitanicus* Dorda & Fern. Casas	Albergaria-a-Velha (P)	3
NT10-Cap	*N. triandrus* L. var. *capax* (Salisbury) Barra & G. López[a]	Baiona, Galicia (S)	3
NT11-Cap	*N. triandrus* L. var. *capax* (Salisbury) Barra & G. López[a]	Marín, Galicia (S)	3
NT12-Cap	*N. triandrus* L. var. *capax* (Salisbury) Barra & G. López[a]	Marín, Galicia (S)	3
NT13	*N. triandrus* L. var. *triandrus*	Viveiro, Galicia (S)	3
NT14	*N. triandrus* L. var. *triandrus*	Luarca, Asturias (S)	3
NT15	*N. triandrus* L. var. *triandrus*	Trevías, Asturias (S)	3
NT16	*N. triandrus* L. var. *triandrus*	Peñaflor, Asturias (S)	3
NT-B	*N. triandrus* L. var. *triandrus*	Montorio, Burgos (S)	2
NI-B	*N. iohannis* Fern. Casas[b]	Peñahorada, Burgos (S)	2

[a] *N. loiseleuri* Rouy

[b] *N. triandrus* L. subsp. *triandrus* var. *alejandrei* Barra

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Supplementary information

Wild daffodils of the section Ganymedes from the Iberian Peninsula as a source of mesembrane alkaloids

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Table S1. Free energy differences[a] (kcal/mol) between conformers of a model compound in which the dimethoxyphenyl unit present in **8** has been replaced by a hydrogen atom.

Conformer[b]		Gas	Chloroform	
6-membered ring	OH		MST	SMD
boat_ax	-53.0	3.35	2.39	2.32
boat_ax	66.5	3.55	2.62	2.57
boat_ax	175.6	4.20	3.24	2.88
boat_eq	-68.1	3.23	2.62	2.77
boat_eq	41.1	3.89	2.80	3.02
boat_eq	-170.5	3.12	2.95	2.85
chair_ax	-61.7	0.89	0.03	0.20
chair_ax	47.8	1.52	0.53	0.52
chair_ax	170.0	0.00	0.00	0.00
chair_eq	-63.0	1.15	0.16	-0.07
chair_eq	63.0	1.38	0.36	0.12
chair_eq	-179.7	1.47	0.50	0.14

[a] Free energy differences in the gas phase were estimated from single-point energy calculations at the MP2/aug-cc-pVDZ level using the molecular geometries optimized at the MP2/6-31+G(d) level, with inclusion of zero-point energy, thermal and entropy corrections estimated at the MP2/6-31+G(d) level. The solvation free energy in chloroform was determined from continuum solvation calculations by using the MST and SMD models.

[b] Conformers are denoted by the conformation of the six-membered ring (boat; chair), the orientation of the hydrogen atom that replaces the dimethoxyphenyl unit (ax: axial; eq: equatorial), and the orientation of the hydroxyl group (determined by the dihedral angle H–O–C–H).

Table S2. Free energy differences[a] (kcal/mol) between conformers of a model compound in which the dimethoxyphenyl unit present in **8** has been replaced by a phenyl ring.

Conformer[b,c]			Gas	Chloroform	
6-membered ring	Phenyl	OH		MST	SMD
boat_ax	-46.7	-53.1	3.41	2.66	2.55
boat_ax	-46.5	65.7	3.68	2.98	2.84
boat_ax	-46.3	176.2	4.39	3.49	3.24
boat_ax	-14.5	-53.6	3.71	2.79	2.73
boat_ax	-14.5	66.0	4.00	3.11	3.04
boat_ax	-14.8	176.6	4.68	3.72	3.41
boat_eq	-55.2	-67.2	3.63	2.90	2.96
boat_eq	-55.1	41.3	4.25	3.04	3.13
boat_eq	-56.2	172.9	3.59	3.24	3.20
boat_eq	27.0	-68.1	3.77	3.02	3.15
boat_eq	26.4	40.4	4.48	3.21	3.39
boat_eq	28.7	-169.6	3.78	3.60	3.36
chair_ax (1)	-49.1	42.9	2.42	1.39	1.52
chair_ax (2)	-52.6	171.2	0.99	1.10	1.08
chair_ax (3)	-49.3	-60.3	1.76	0.97	1.20
chair_ax (4)	5.9	46.7	1.70	0.62	0.72
chair_ax (5)	5.4	170.7	0.00	0.00	0.00
chair_ax (6)	4.8	-61.4	0.97	0.11	0.35
chair_eq (1)	-0.3	-179.5	2.54	1.39	1.02
chair_eq (2)	-0.6	-62.9	2.20	1.06	0.78
chair_eq (3)	-0.6	62.2	2.42	1.21	0.95
chair_eq (4)	67.0	-63.7	1.53	0.61	0.30
chair_eq (5)	67.2	63.7	1.73	0.79	0.47
chair_eq (6)	67.1	-179.8	1.87	0.96	0.51

[a] Free energy differences in the gas phase were estimated from single-point energy calculations at the MP2/aug-cc-pVDZ level using the molecular geometries optimized at the MP2/6-31+G(d) level, with inclusion of zero-point energy, thermal and entropy corrections estimated at the MP2/6-31+G(d) level. The solvation free energy in chloroform was determined from continuum solvation calculations by using the MST and SMD models.

[b] Conformers are denoted by the conformation of the six-membered ring (boat; chair), the orientation of the phenyl ring that replaces the dimethoxyphenyl unit (ax: axial; eq: equatorial), the orientation of the phenyl ring (determined by the dihedral angle C(N)–C–C_{phenyl}–C'_{phenyl}), and the orientation of the hydroxyl group (determined by the dihedral angle H–O–C–H).

[b] The number in brackets indicates the chair conformations used for computation of *J* coupling constants in Table S3.

Table S3. Coupling constants determined from the molecular geometries of the chair conformations of the model compound in which the dimethoxyphenyl unit of **8** has been replaced by a phenyl ring.

Conformers[a]	chair_eq (1-3)		chair_eq (4-6)		chair_ax (1, 3)		chair_ax (2)		2chair_ax (4, 6)		chair_ax (5)		
Mole fraction[b]	0.1000		0.2094		0.0744		0.0403		0.3043		0.2545		
Vicinal H	φ [c]	J [d]	φ [c]	J [d]	φ [c]	J [d]	φ [c]	J [d]	φ [c]	J [d]	φ [c]	J [d]	J calc.[e]
6 - 5β	60.8	3.40	62.1	3.20	307.2	3.00	305.5	2.71	304.5	2.61	302.4	2.33	2.73
6 - 5α	177.5	11.36	178.6	11.41	63.8	3.13	62.1	3.39	62.1	3.39	59.7	3.76	5.88
6 - 7β	298.8	3.53	298.3	3.45	51.4	2.99	56.2	2.30	47.2	3.65	53.2	2.72	3.19
6 - 7α	182.0	11.40	181.6	11.42	295.5	2.85	299.4	3.43	295.4	2.84	297.3	3.11	5.54
7a - 7β	48.1	5.34	45.2	5.83	311.9	4.34	306.4	3.45	315.3	4.92	309.1	3.88	4.70
7a - 7α	165.0	11.07	162.3	10.78	67.1	2.21	62.3	2.84	70.0	1.87	65.4	2.42	4.83
4α - 5β	60.8	2.70	62.9	2.37	179.6	13.29	174.8	13.23	181.7	13.27	176.5	13.27	9.70
4α - 5α	302.7	3.50	304.8	3.89	60.1	3.01	55.9	3.76	62.5	2.62	58.0	3.38	3.20
4β - 5β	305.2	3.96	307.4	4.38	64.5	2.31	60	3.03	64.2	2.35	60.6	2.93	3.07
4β - 5α	187.1	13.16	189.3	13.04	305.3	3.76	301.2	3.03	305.1	3.73	302.1	3.19	6.40

[a] See Table S2 for numbering of chair conformations.
[b] Mole fraction derived from the relative stabilities reported in Table S2.
[c] Torsion angle between vicinal protons.
[d] J calculated with MestRe-J, based on HLA generalization of the Karplus equation (Haasnoot, et al., 1980).
[e] Calculated according to the mole fraction of the conformers.

Table S4. NMR data **8** (500 MHz, $CDCl_3$)

	8				
Position	**δ_H (mult., *J* in Hz)**	**δ_C**	**COSY**	**NOESY**	**HMBC**
2	-	173.8	-	-	-
3α	2.65 (*d*, 16.4)	45.7	H3β	H3β, H4α/β, H5α , H7α, H2′, H6′	C2, C3a, C4, C7a, C1′
3β	2.54 (*d*, 16.4)	45.7	H3α	H3α, H4α, H7a, H2′, H6′	C2, C3a, C4, C7a, C1′
3a	-	42.9	-	-	-
4α	2.14 (*ddd*, 15.0, 10.0, 3.6)	30.0	H4β, H5α/β	H3α/β, H4β, H5α/β, H2′, H6′	C3, C3a, C5, C6, C7a, C1′
4β	1.85 (*ddd*, 14.5, 7.4, 3.6)	30.0	H4α, H5α/β	H3α, H4α, H5α/β, H6, H2′, H6′	C3, C3a, C5, C6, C7a, C1′
5α	1.60 (*m*)	30.1	H4α/β, H5β, H6	H3α, H4α/β, H5β, H6	C3a, C6, C7
5β	1.73 (*ddtd*, 14.0, 10.0, 3.6, 1.0)	30.1	H4α/β, H5α, H6	H4α/β, H5α, H6, H2′, H6′	C3a, C6, C7
6	3.95 (*tt*, 6.1, 3.6)	66.1	H5α/β, H7α/β	H4β, H5α/β, H7α/β	-
7α	1.96 (*dddd*, 14.6, 6.0, 5.5, 1.0)	32.8	H6, H7β,H7a	H3α, H6, H7β, H7a, NMe	C3a, C4, C5, C6, C7a
7β	2.14 (*dddd*, 14.7, 5.0, 3.6, 1.0)	32.8	H6, H7α,H7a	H6, H7α, H7a, H2′, H6′, NMe	C3a, C4, C5, C6, C7a
7a	3.91 (*dd*, 5.5, 5.0)	62.3	H7α/β	H3β, H7α/β, H2′, H6′, NMe	C2, C3a, C4, C6, C1′
1′	-	137.4	-	-	-
2′	6.81 (*d*, 2.4)	110.0	H6′	H3α/β, H4α/β, H5β, H7β, H7a	C3a, C4′, C6′
3′	-	149.2	-	-	-
4′	-	148.0	-	-	-
5′	6.82 (*d*, 8.4)	111.2	H6′	-	C1′, C3′
6′	6.86 (*dd*, 8.4, 2.3)	118.2	H2′, H5′	H3α/β, H4α/β, H5β, H7β, H7a	C3a, C2′, C4′
NMe	2.90 (*s*)	28.1	-	H7α/β, H7a	C2, C7a
OMe (3′)	3.88 (*s*)	56.2	-	-	C3′
OMe (4′)	3.87 (*s*)	56.1	-	-	C4′

3.4. Artículo 4

Wild Argentinian Amaryllidaceae, a new renewable source of acetylcholinesterase inhibitor galanthamine and other alkaloids

Molecules, 17, 13473-13482 (2012)

La región andina del continente americano es conocida como uno de los principales centros de diversificación de la familia Amaryllidaceae. En Argentina se han descrito 61 especies y, hasta el momento, no se conocían reportes de estudios sobre el contenido de alcaloides de las mismas. En este trabajo se ha abordado por primera vez el análisis de la composición de alcaloides de cuatro especies silvestres: *Habranthus jamesonii*, *Phycella herbertiana*, *Rhodophiala mendocina* y *Zephyranthes filifolia*. Al mismo tiempo, se ha evaluado la capacidad inhibidora de la enzima AChE de dichos extractos.

Los extractos clorofórmicos de bulbo se analizaron mediante GC-MS, con el objetivo de estudiar la composición de alcaloides de cada especie. Los resultados se expresaron en valores porcentuales referidos al área total del cromatograma (% TIC), útiles para la comparación entre diversas muestras. Todos demostraron la presencia de galantamina en cantidades variables, desde 0.6 a 17.8% del total del contenido alcaloídico, siendo *Z. filifolia* la especie más destacada en cuanto a la abundancia de dicho alcaloide. Además, se detectó la presencia de tazetina, licorina, galantina, licoramina y algunos alcaloides de tipo hemantamina, entre los componentes mayoritarios de las diversas especies estudiadas.

Los resultados del ensayo de inhibición de AChE variaron en un rango de valores de IC_{50} entre 1 y 2 μg/mL. Los extractos más prometedores fueron el de *Z. filifolia* (San Juan) y el de *H. jamesonii* (Mendoza), que mostraron valores de IC_{50} (1.0 ± 0.08 y 1.0 ± 0.01 μg/mL, respectivamente) alrededor de 3 veces menos activos en comparación con el control positivo, galantamina (0.29 ± 0.07 μg/mL).

Este estudio demuestra el potencial de especies silvestres argentinas de la familia Amaryllidaceae procedentes de la región andina como fuente renovable de alcaloides bioactivos, como galantamina.

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Article

Wild Argentinian Amaryllidaceae, a New Renewable Source of the Acetylcholinesterase Inhibitor Galanthamine and Other Alkaloids

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Abstract: The Amaryllidaceae family is well known for its pharmacologically active alkaloids. An important approach to treat Alzheimer's disease involves the inhibition of the enzyme acetylcholinesterase (AChE). Galanthamine, an Amaryllidaceae alkaloid, is an effective, selective, reversible, and competitive AChE inhibitor. This work was aimed at studying the alkaloid composition of four wild Argentinian Amarillydaceae species for the first time, as well as analyzing their inhibitory activity on acetylcholinesterase. Alkaloid content was characterized by means of GC-MS analysis. Chloroform basic extracts from *Habranthus jamesonii*, *Phycella herbertiana*, *Rhodophiala mendocina* and *Zephyranthes filifolia* collected in the Argentinian Andean region all contained galanthamine, and showed a strong AChE inhibitory activity (IC_{50} between 1.2 and 2 μg/mL). To our knowledge, no previous reports on alkaloid profiles and AChEIs activity of wild Argentinian Amarillydaceae species have been publisihed. The demand for renewable

sources of industrial products like galanthamine and the need to protect plant biodiversity creates an opportunity for Argentinian farmers to produce such crops.

Keywords: Argentinian Amaryllidaceae wild; alkaloids; galanthamine; lycorine; tazettine; acetylcholinesterase inhibitors

1. Introduction

Many species of medicinal and aromatic plants are cultivated for such industrial uses, but most are still collected in the wild. The demand for renewable sources of industrial products and the need to protect plant biodiversity create an opportunity for farmers to produce such plants as crops. More than 25% of the pharmaceutical drugs used in the World today are derived from plant natural products [1]. In the conventional pharmaceutical industry, pharmaceutical companies produce drugs from compounds extracted from plant material, or use plant derived compounds as starting material to produce drugs semi-synthetically [2]. Examples of the former include the anti-cancer alkaloid paclitaxel from Pacific yew (*Taxus brevifolia*), vinblastine from the Madagascar periwinkle (*Cataranthus roseus*), and digoxin from the foxglove (*Digitalis lanata*) [1].

The alkaloids of the Amaryllidaceae family are extensively studied for their biological activities in several pharmaceutical areas, for example, Alzheimer's disease (AD), a neurodegenerative problem of enormous economic and social impact (15 million people, mainly in developed countries, suffer from the symptoms of this disease). The treatment is based on drugs that increase levels of acetylcholine. Galanthamine is a long-acting, selective, reversible and competitive inhibitor of acetylcholinesterase (AChE) and an allosteric modulator of the neuronal nicotinic receptor for acetylcholine. AChE is responsible for the degradation of acetylcholine at the neuromuscular junction, in peripheral and central cholinergic synapses. Galanthamine has the ability to cross the blood-brain barrier and to act within the central nervous system [3,4]. According to data presented by the Alzheimer's Association in 2007, the prevalence of Alzheimer's disease will quadruple by 2050. Galanthamine hydrobromide has superior pharmacological profiles and higher tolerance as compared to the original AChE inhibitors, physostigmine or tacrine [5]. This alkaloid galanthamine (biosynthesized exclusively by species of Amaryllidaceae family) is the treatment for mild and moderate stages of the AD. Galanthamine, approved in 2001 by FDA (Razadyne®), was originally isolated from *Galanthus woronowii*. While several total syntheses of the alkaloid galanthamine are available [6–10], current marketing is done mainly by the limited extraction of natural populations of *Leucojum aestivum* from Turkey (of varying quality and low content of active principle), or from small plantations of this species in Bulgaria, which are insufficient to meet current pharmaceutical company demand. The worldwide production of galanthamine is about 250 kg per year. Around 61 species of the Amaryllidaceae family grow in Argentina, covering a wide variety of genera (*Chlidanthus*, *Crinum*, *Habranthus*, *Haylockia*, *Hieronymiella*, *Hippeastrum*, *Phycella*, *Rhodophiala*, *Stenomesson* and *Zephyranthes*) [11]. To our knowledge, there are no reports on the chemistry and biological activity of Argentinian species belonging to the Amaryllidaceae group.

Our search for plant raw materials for medicinal products is now aimed at investigating the acetylcholinesterase inhibitory activity (AChE) of basic chloroform extracts (BCE) obtained from *Habranthus jamesonii*, *Phycella herbertiana*, *Rhodophiala mendocina*, and *Zephyranthes filifolia* (Amaryllidaceae species that grow in Argentine) to find new sources of production of galanthamine, and other potential alkaloids for treating AD. AChE inhibitory activity was determined by the spectrophotometric method by Ellman *et al.* [12]. Alkaloid profiles were analyzed by gas chromatography-mass spectrometry (GC–MS).

2. Results and Discussion

The AChE inhibitory activity of the BCE from *Habranthus jamesonii*, *Phycella herbertiana*, *Rodophiala mendocina* and *Zephyranthes filifolia* species, collected from the Andean region of San Juan (SJ), Mendoza (MDZ), and Neuquén (NQN) provinces (Argentine), were tested according to the methodology developed by Ellman *et al.* [12] with some modifications [13]. Galanthamine was used as a positive control. The results, expressed as IC_{50} values (μg/mL) are shown in Table 1. BCE showed the highest acetylcholinesterase inhibitory activity, with IC_{50} values ranging from 1 to 2 μg/mL (reference compound: galanthamine 0.29 ± 0.07 μg/mL). The BCE-*Z. filifolia* MZA and BCE-*H. jamesonii* SJ displayed the highest inhibition towards AChE with similar values (IC_{50} 1 ± 0.01 and 1 ± 0.08 μg/mL respectively) only three times higher than that of galanthamine. BCE-*P. herbertiana* SJ was found to have the second highest inhibition on AChE (IC_{50} values 1.2 ± 0.12 μg/mL). Acetylcholinesterase inhibition was similar to that of specie *R. mendocina* regardless of collection site (IC_{50} values 2 ± 0.15, 2 ± 0.20 μg/mL). BCE-*H. jamesonii* SJ showed a similar AChE inhibitory activity (IC_{50} values 2 ± 0.11 μg/mL). The yield percentages of the basic chloroform extract (BCE) (g/100 g dry bulbs) are reported in Table 1. BCE-*Zephyranthes filifolia* SJ had the lowest percentage at 0.21%, whereas BCE-*Rhodophiala mendocina* SJ gave the highest one at 0.38%.

Table 1. Acetylcholinesterase Enzyme Inhibition of Wild Argentinian Amaryllidaceae extracts expressed as IC_{50} [μg/mL].

Samples (voucher number)	BCE [a]	
	Yield [%] [b]	IC_{50} [μg/mL]
Phycella herbertiana SJ (IBT-Arg1)	0.34	1.2 ± 0.12
Habranthus jamesonii SJ (IBT-Arg2)	0.25	2.0 ± 0.11
Rhodophiala mendocina SJ (IBT-Arg3)	0.38	2.0 ± 0.15
Zephyranthes filifolia SJ (IBT-Arg4)	0.21	1.0 ± 0.08
Habranthus jamesonii MZA (IBT-Arg5)	0.27	1.0 ± 0.01
Rhodophiala mendocina NQN (IBT-Arg6)	0.26	2.0 ± 0.20
Galanthamine [c]		0.29 ± 0.07

[a] Basic chloroform extract, [b] Percentage yield BCE [w/w], [c] Reference compound.

The alkaloids detected by GC-MS in the BCE-*H. jamesonii* MZA, BCE-*H. jamesonii* SJ, BCE-*P. herbertiana* SJ, BCE-*R. mendocina* NQN, BCE-*R. mendocina* SJ and BCE-*Z. filifolia* SJ are listed in Table 2.

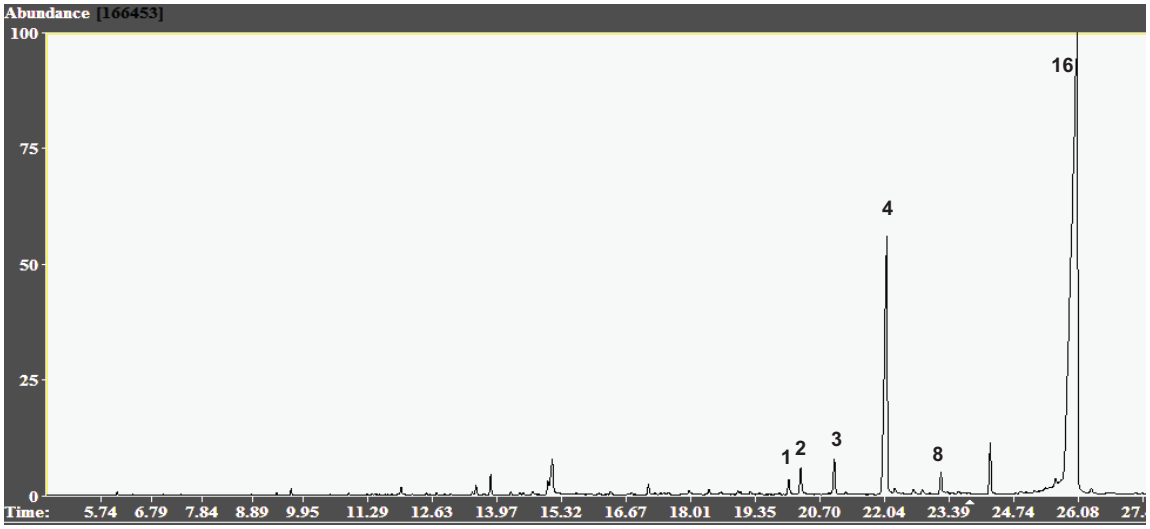
Table 2. Alkaloid composition of four Amaryllidaceae plants.

Compound	*H. jamesonii* [a]		*P. herbertiana* [a]	*R. mendocina* [a]		*Z. filifolia* [a]
	SJ	*MZA*	*SJ*	*SJ*	*NQN*	*SJ*
Trisphaeridine (**1**)	0.7			0.1		1.2
Ismine (**2**)						0.7
5,6-Dihydrobicolorine (**3**)						1.7
Galanthamine (**4**)	1.4	4.3	4.2	0.6	0.8	17.8
Lycoramine (**5**)	1.9		27.4	3.2		
Lycoraminone (**6**)			0.5			
Vittatine (**7**)	13.2		0.1	1.2	0.2	
Narwedine (**8**)		0.8	0.2		0.4	0.9
Anhydrolycorine (**9**)	2.5	1.5	0.4	0.9		
A-289 (**10**)	1.1					
A-315 (**11**)			0.4			
A-249 (**12**)	1.3	0.8				
A-319 (**13**)	1.1					
Montanine (**14**)	5.7		1.8	9.1		
Haemanthamine/Crinamine(**15**) [b]	2.9		2.5	31.2	6.8	
Tazettine (**16**)	28.1		5.4	32.9		69.7
A-301 (**17**)	2.0					
Pancracine (**18**)				0.3		
11-Hydroxyvittatine (**19**)	18.7	3.1	4.6			
Galanthine (**20**)	4.9		17.2			
Lycorine (**21**)	8.2	43.6	33.2	13.3	20.4	
Incartine (**22**)			1.1			
Methylpseudolycorine (**23**)			0.2			
Epimacronine (**24**)				0.6		
8-*O*-Demethylhomolycorine (**25**)			0.6			
Homolycorine type (**26**)				3.9		
2-*O*-Acetyllycorine (**27**)			0.2	2.6		
A-345 (**28**)		2.7				
Tazettamide (**29**)		3.5				
m/z 109 (Homolycorine type) (**30**)		7.7				
Sanguinine (**31**)		0.5				
m/z 83 (285) (**32**)		1.1				
m/z 297(**33**)		11.8				
m/z 281 (**34**)		0.9				
m/z 283 (**35**)		2.3				
N-Demethylgalanthamine (**36**)					0.2	
2-O-Methylpancracine (**37**)					3.8	
N-Formylnorgalanthamine (**38**)					0.1	
Total Alkaloids identified	40.0	84.6	99.1	99.9	32.7	92.0

[a] Values are expressed as GC-MS area %, [b] Cannot be distinguished by GC-MS.

Galanthamine (**4**) was found in all the species and it ranged from 0.6 to 17.8% of total ion current (TIC). *Zephyranthes filifolia* presented the highest galanthamine (**4**) content (17.8% TIC, Figure 1, whereas *R. mendocina* showed the lowest one (0.6% TIC). The highest AChE inhibitory activity of these species (IC_{50} 1.0 ± 0.08 μg/mL) belonged to BCE-*Z. filifolia* SJ a fact that could be related to the high content of galanthamine (**4**).

Figure 1. Representative GC-MS Chromatogram of Wild Argentinian Amaryllidaceae BCE-*Z. filifolia* SJ. Peaks: **1**: Trisphaeridine; **2**: Ismine; **3**: 5,6-Dihydrobicolorine; **4**: Galanthamine **8**: Narwedine; **16**: Tazettine.



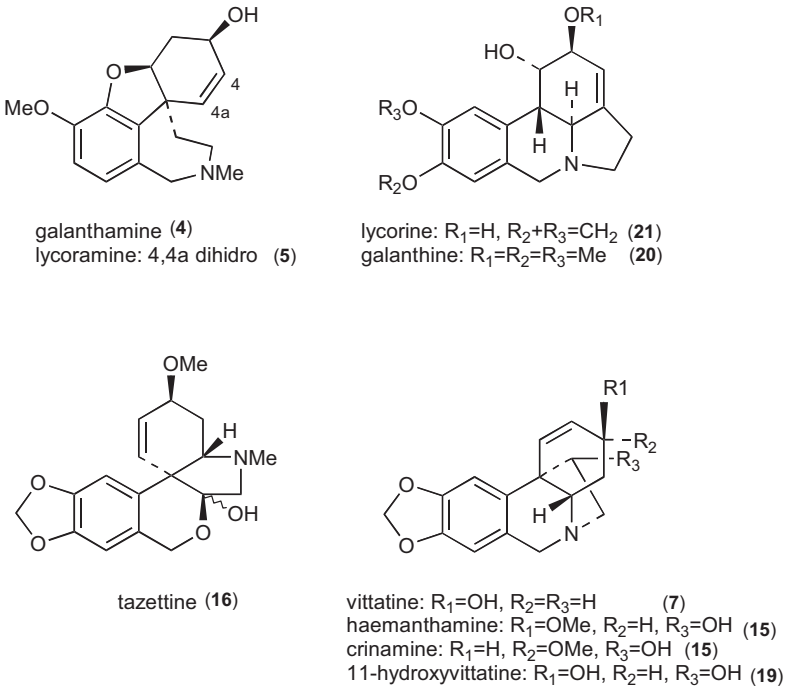
Galanthamine (**4**) content was collection site dependent: the BCE-*H. jamesonii* SJ sample presented 1.4% galanthamine TIC, while the BCE-*H. jamesonii* MZA with 4.3% TIC was four times higher. The differences in alkaloid content, depending on geographical distribution of *H. jamesonii* populations, coincides with a previous report on the European species. Berkov *et al.* [14], reported an intraspecies diversity in alkaloid profiles in *Galanthus elwesii* and *G. nivalis* populations collected in different locations in Bulgaria. They presented galanthamine TIC between 0 and 46%. The main alkaloid types (chemotypes) showed a wide variation in the number of compounds comprising their alkaloid mixture. Genetic and environmental factors and their interaction play a role in determining alkaloid profiles.

Additionally, sanguinine (**31**), identified in BCE-*H. jamesonii* MZA (0.5% TIC), has a hydroxyl group at C9 instead of a methoxyl group, and is around 10 times more active than galanthamine (**4**). Although the differential content could indicate that some environmental parameters might be influencing galanthamine (**4**) production, this specie could be considered for the sustainable production of galanthamine. A similar galanthamine (**4**) content (4.2% TIC) has been found in BCE-*P. herbertiana* SJ. BCE-*R. mendocina* SJ and BCE-*R. mendocina* NQN, showed a similar galanthamine content (<1% TIC). Narwedine (**8**), another AChE inhibitor [15] was found in all the populations studied (with the exception of the *H. jamesonii* and *R. mendocina* collected in San Juan province), but this compound comprised no more than 1% TIC. Other main alkaloids characterized by GC-MS in the BCE-*P. herbertiana* SJ were lycorine (**21**) (33%), lycoramine (**5**) (27%) and tazettine (**16**) (5.4%).

The occurrence of trisphaeridine (**1**), galanthamine (**4**), lycoramine (**5**), vittatine (**7**), anhydrolycorine (**9**), montanine (**14**), haemanthamine/crinamine (**15**), tazettine (**16**), 11-hydroxyvittatine (**19**), galanthine (**20**), lycorine (**21**) and tazettamide (**29**) are reported for the first time in *Habranthus jamesonii* from Argentina. According to the literature, haemanthamine (**15**) and galanthine (**20**), have been reported previously in *Habranthus brachyandrus,* a specie of the genus [16]. At the same time, thirteen alkaloids were characterized in BCE-*R. mendocina* SJ, whereas five of them montanine (**14**), vittatine (**7**), haemanthamine (**15**), tazettine (**16**) and lycorine (**21**) have been previously reported as constituents

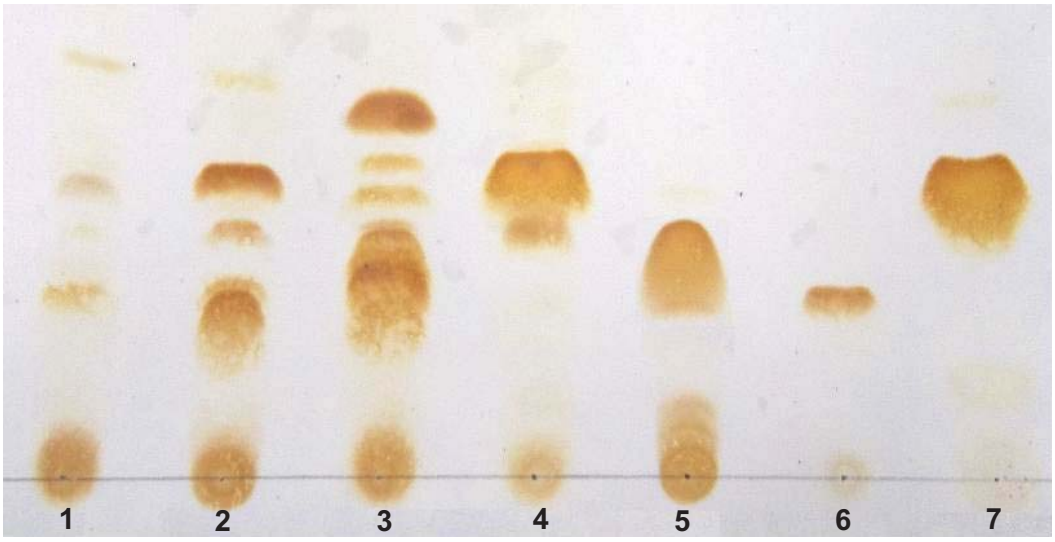
of *Rhodophiala bifida* [17]. The main alkaloids identified in native Amaryllidaceae species from San Juan, Mendoza and Neuquén (Argentina) are shown in Figure 2.

Figure 2. Main alkaloids in Wild Amaryllidaceae Species from Argentina.



The presence of main alkaloids identified by GCMS (lycorine (**21**) and tazettine (**16**) as percentage of the total ion current (TIC) is consistent with that observed in TLC alkaloid profiles of chloroform basic extract (Figure 3).

Figure 3. TLC Analysis of Argentinian Amaryllidaceae (BCE). **1**: *H. jamesonii*, **2**: *P. herbertiana*, **3**: *R. mendocina*, **4**: *Z. filifolia*, **5**: Galanthamine, **6**: Lycorine, **7**: Tazettine.



The AChE inhibitory activity of these species can be explained by the presence of other AChE inhibitors in the alkaloid mixtures. Montanine (**4**) has shown significant AChE inhibitory activity [18],

while a weak activity has been reported for lycorine (**21**) and haemanthamine (**15**) [19]. The other major alkaloids, lycoramine (**5**) and tazettine (**16**) have no AChE inhibitory activity [13] while, to the best of our knowledge, no AChE inhibitory activity assays have been performed for galanthine (**20**), 11-hydroxyvittatine, 2-*O*-acetyllycorine (**27**).

The alkaloids found in the Argentinian species studied possess other interesting biological properties besides their AChE inhibitory activity. Haemanthamine (**15**) is a potent inducer of apoptosis [20], and has antimalarial activity [3]. Vittatine (**7**) has shown cytotoxic activity [3]. Antibacterial activity has been reported for vittatine (**7**) and 11-hydroxyvittatine (**19**) [21]. Lycorine (**21**) exhibits citotoxic, apoptotic, antiviral, antifungal, anti-protozoan [22], and anti-inflammatory activities [23]. It is a good candidate for a therapeutic agent against leukemia [24].Analgesic and hypotensive effects have been reported for galanthine (**20**) [3]. Moderate cytotoxic activity has been reported for tazettine (**16**) [25] which is an isolation artefact of chemically labile pretazettine. This compound, which is indeed present in plants, has shown remarkable cytotoxicity against a number of tumor cell lines [3]. In addition to the alkaloids identified in the species studied, other unknown compounds (**10**, **11**, **12**, **13**, **17**, **26**, **28**, **30**, **32**, **33**, **34**, and **35**) were detected in minor quantities, showing mass spectral patterns that also suggest structures related to the Amaryllidaceae alkaloids. Isolation studies are currently being developed.

3. Experimental

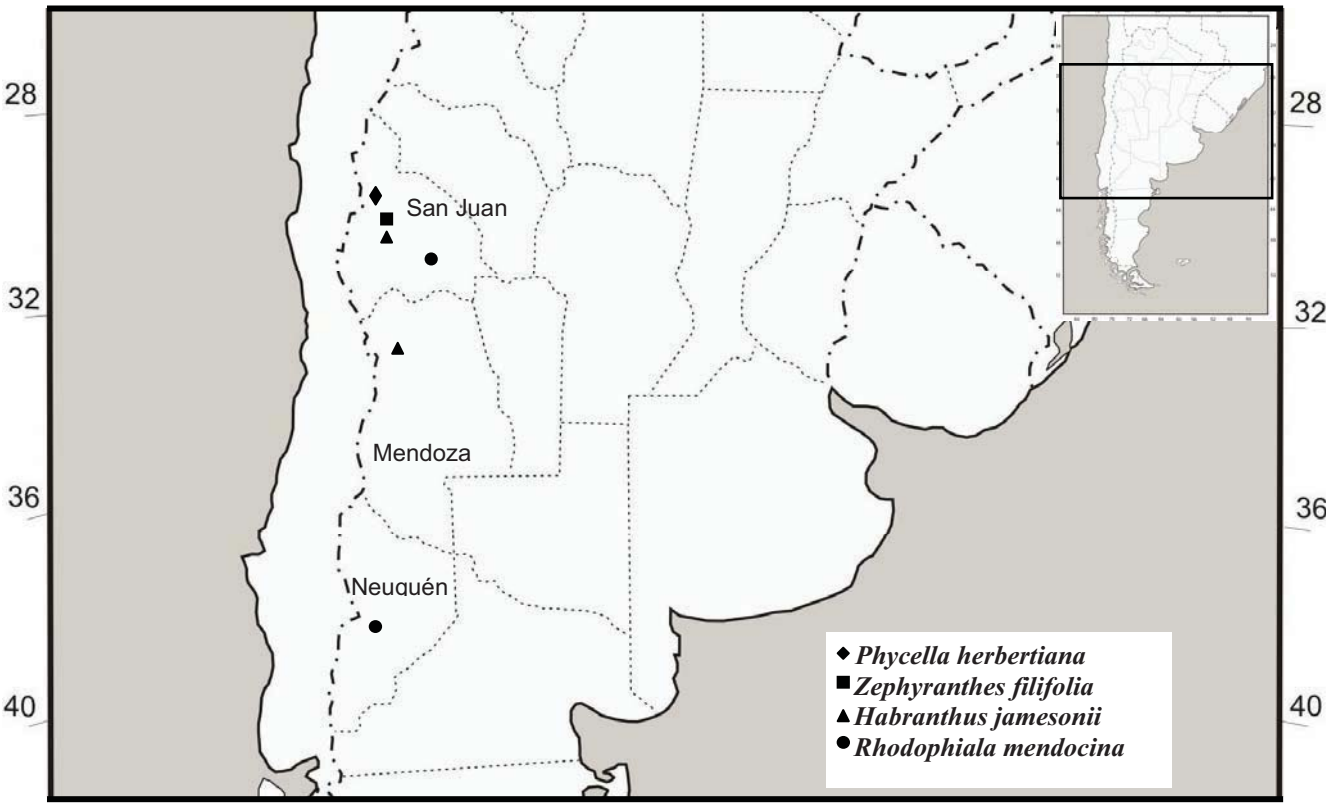
3.1. Plant Material

Wild plants of the species *Habranthus jamesonii* (BAK) Rav, *Phycella herbertiana* LINDL, *Rhodophiala mendocina* (PHIL.) Rav., and *Zephyranthes filifolia* (HERB.) ex Kraenzlin (Amaryllidaceae) were collected in the Andean regions of San Juan (SJ), Mendoza (MZA), and Neuquén (NQN) provinces (Argentina), during the flowering period between October and March 2009-2010 and then transferred to flowerpots and kept under greenhouse conditions. The species collected and identified, and voucher numbers are shown in Table 1. Figure 4 shows a map of the collection area. All plant species were authenticated by MCS German Roitman when they were collected. Voucher specimens were deposited at the Instituto de Biotecnología (UNSJ) with the codes: IBT-UNSJ- Arg1-6.

3.2. Alkaloid Extraction

Dried bulbs (100 g per each plant) were extracted under reflux three times with MeOH (300 mL) for 1 h each. The solvent was evaporated under reduced pressure to give the methanolic crude extracts (MCEs). MCEs were dissolved in H_2SO_4 (2% v/v) and neutral material was removed with $CHCl_3$ (200 mL). Then, the aqueous solutions were basified with 25% NaOH up to pH 10–12 and the alkaloids were extracted with $CHCl_3$ (3 × 500 mL) to obtain the basic chloroform extract (BCE). After evaporation of the organic solvent, the dry alkaloid fractions were dissolved in MeOH for GC/MS analysis. The BCE were named as BCE-*H. jamesonii* MZA, BCE-*H. jamesonii* SJ, BCE-*P. herbertiana* SJ, BCE-*R. mendocina* NQN, BCE-*R. mendocina* SJ and BCE-*Z. filifolia* SJ after their origin.

Figure 4. Collection areas of Argentinian wild Amaryllidaceae.



3.3. Gas Chromatography-Mass Spectroscopy Analyses

GC-MS analyses were performed on a Hewlett Packard 6890/MSD 5975 instrument (Hewlett Packard, Palo Alto, CA, USA) operating in EI mode at 70 eV. A DB-5 MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100–180 °C at 15 °C min^{-1}, 1 min hold at 180 °C, 180–300 °C at 5 °C min^{-1}, and 1 min hold at 300 °C. Injector temperature was 280 °C. The flow rate of carrier gas (He) was 0.8 mL min^{-1}. The split ratio was 1:20. The results obtained were analyzed using AMDIS 2.64 software (NIST). Compounds were identified through the comparison of their mass spectral patterns and retention indexes, with the data recorded in literature.

3.4. Microplate Assay for Acetylcholinesterase Activity

AChE activity was assayed as described by Ellman *et al.* [12] with some modifications [13]. Fifty μL of AChE in buffer phosphate (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) and 50 μL of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 minutes at room temperature before the addition of 100 μL of the substrate solution (0.1 M Na_2HPO_4, 0.5 M DTNB, 0.6 mM ATCI in Millipore water, pH 7.5). The absorbance was read in a Labsystems microplate reader (Helsinki, Finland) at 405 nm after three minutes. Enzyme activity was calculated as a percentage compared to an assay using a buffer without any inhibitor. The AChE inhibitory data were analyzed with the software package Prism (Graph Pad Inc., San Diego, CA, USA). IC_{50} values are means ± SD of three individual determinations each performed in triplicate.

3.5. TLC Analysis of BCE

TLC was carried out on Merck Silica gel 60 F254 plates, using chloroform-methanol-ammonia (99:9:1) mixtures as mobile phase. TLC plates were sprayed with Dragendorff's reagent; main alkaloids gave orange spots.

4. Conclusions

The findings of the present study demonstrate the potential of wild Argentinian Amaryllidaceae species collected in the central Andean region, as a new renewable source of galanthamine. The most promising species seen to be *H. jamesonii* MDZ and *Z. filifolia* SJ. The demand for renewable sources of galanthamine and the need to protect plant biodiversity create an opportunity for Argentinian farmers to produce such crops. Studies of domestication of some of these species are currently in progress in order to determine which crops can be cultivated outdoors in the particular climate and soil, and which can be grown in greenhouses. Production cost and galanthamine levels in traditional cultivars are also being analyzed.

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Sample Availability: Samples of the compounds are available from the authors.

4. DISCUSIÓN

4. DISCUSIÓN

Los resultados expuestos en el presente trabajo comprenden el estudio del contenido de alcaloides de especies silvestres de plantas de la familia Amaryllidaceae (subfam. Amaryllidoideae) del área Mediterránea y de Argentina. En líneas generales, se pueden considerar tres secciones diferentes: por un lado, el aislamiento y caracterización de nuevos alcaloides de la especie *Narcissus serotinus* L.; en segundo lugar, el estudio de los alcaloides inusuales de los narcisos pertenecientes a la sección Ganymedes, como *N. triandrus* L.; y, finalmente, el análisis del contenido de alcaloides de especies silvestres argentinas como fuente de compuestos bioactivos.

4.1. Alcaloides de *Narcissus serotinus* L.

Narcissus serotinus L. es una especie de floración otoñal perteneciente a la sección monotípica Serotini, con una distribución geográfica que se extiende a lo largo del área Mediterránea, incluyendo el sur y este de la Península Ibérica, regiones costeras de Italia, Croacia, Grecia, Israel y el norte de África, así como casi todas las islas del Mediterráneo (Blanchard, 1990; Díaz Lifante *et al.*, 2007). A pesar de que se han llevado a cabo numerosos estudios analizando la variabilidad genética y morfológica de esta especie, sólo existía una publicación disponible en la literatura haciendo referencia a su contenido de alcaloides (Vrondeli *et al.*, 2005), previa al presente trabajo. Curiosamente, en dicho artículo se reporta el aislamiento de un nuevo alcaloide al que se le asigna una estructura del tipo tazetina, una asignación que, en base a nuestros resultados, hemos considerado incorrecta.

En términos generales, en los artículos 1 y 2 incluidos en la sección de Resultados se describe la identificación y elucidación estructural de ocho alcaloides nuevos aislados de *N. serotinus*. Para el primer estudio (Pigni *et al.*, 2010) se utilizaron 350 g de material vegetal fresco recolectado en Marruecos durante Octubre de 2009, lo que permitió la caracterización de narseronina y 1-*O*-(3´-acetoxibutanoil)licorina. De manera adicional, en el análisis de GC-MS se detectó la presencia de licorina, galantina, 1-*O*-(3´-hidroxibutanoil)licorina, asoanina e hipeastrina, así como de algunos compuestos no identificados que presentaron patrones de fragmentación típicos de alcaloides de Amaryllidaceae. Consecuentemente, se planificó una segunda

recolección de material vegetal durante Octubre de 2010 en localidades de la Comunidad Valenciana (España), con el objetivo de identificar dichos componentes y, a la vez, obtener una idea general de las posibles diferencias en el perfil de alcaloides de poblaciones con diferente ubicación geográfica. El hallazgo de una población abundante de *N. serotinus* cerca de Vinarós (Castellón) permitió obtener 2.43 kg de material vegetal, que resultaron en el aislamiento y elucidación estructural de seis nuevos alcaloides: 3-*O*-metilnarcisidina, 1-*O*-acetil-3-*O*-metilnarcisidina, 1-*O*-acetil-3-*O*-metil-6-oxonarcisidina, 2-metoxipratosina, 11-hidroxigalantina, y 2-*O*-metilclivonina, además de la identificación de los ya conocidos narseronina, galantina, incartina, masonina e hipeastrina mediante GC-MS (Pigni *et al.*, 2012). Todos los alcaloides detectados en *N. serotinus* pertenecen a las series licorina y homolicorina (Figura 4.1).

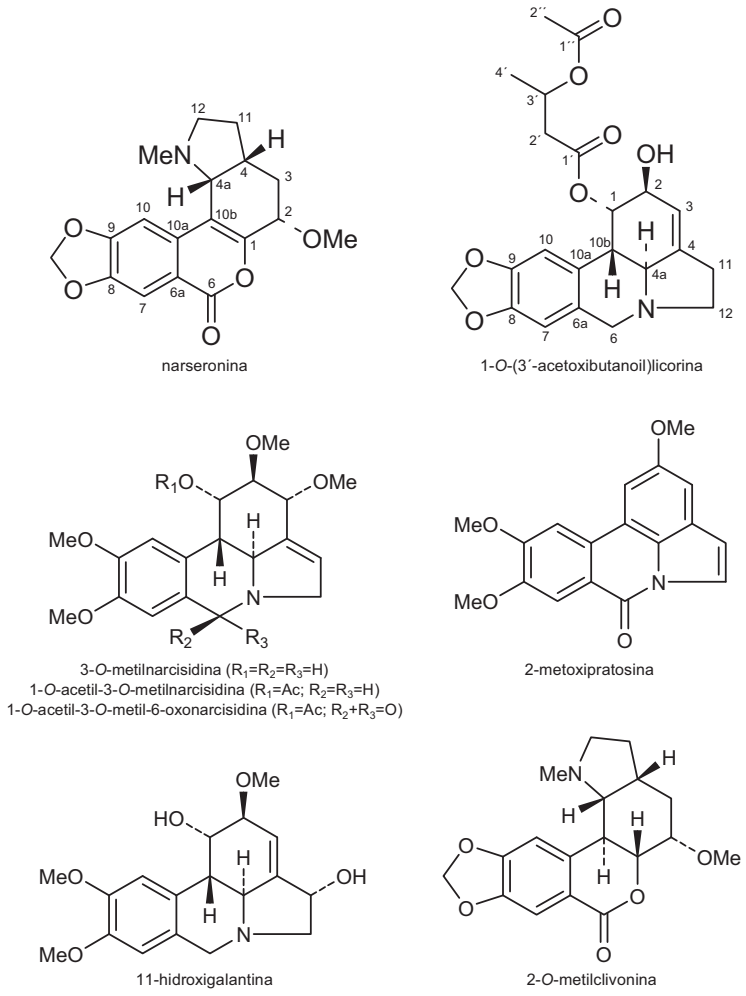

Figura 4.1: Nuevos alcaloides aislados de *N. serotinus.*

4.1.1. Narseronina

Este alcaloide presenta una estructura de tipo homolicorina inusual debido a la presencia de un doble enlace en la posición 1-10b, una característica que no se había reportado anteriormente. Los datos espectrales obtenidos indicaron que se trataba del mismo compuesto aislado de ejemplares griegos de *N. serotinus* por Vrondeli *et al.* (2005), identificado erróneamente como un isómero de 3-epimacronina.

La fórmula molecular $C_{18}H_{19}NO_5$ se confirmó mediante HRMS. Su espectro de masas no mostraba los rasgos típicos de una estructura del tipo homolicorina, probablemente debido que la existencia del doble enlace 1-10b afecta el patrón de fragmentación. Su espectro de ^{1}H-RMN demostró la presencia de dos protones aromáticos en posición *para*, un grupo metilendioxi y un sustituyente metoxilo. La observación de un grupo *N*-metilo relativamente desapantallado (δ 2.41 ppm) sugirió una posible configuración *cis* para la unión de los anillos C/D (Jeffs *et al.*, 1988), también apoyada por el valor de la constante de acoplamiento entre los protones 4-4a de 6.4 Hz, no lo suficientemente elevada como para indicar una relación *trans*-diaxial. El triplete asignado a H-2 (J = 6.1 Hz) apuntó a una orientación *pseudo*-ecuatorial para dicho protón, de acuerdo con la orientación α de grupo metoxilo.

La presencia de un doble enlace en la unión entre los anillos B/C es una característica estructural que confiere rigidez a la porción de la molécula formada por los anillos A-B, con un efecto estabilizante adicional debido a la extensión del sistema conjugado. Esta particularidad podría resultar interesante para farmacóforos con dichos requerimientos. Resultados preliminares de ensayos de actividad biológica no demostraron que este alcaloide tuviese propiedades destacables como inhibidor de la enzima acetilcolinesterasa, ni como antiparasitario. Sin embargo, es importante destacar que después de nuestra publicación, un grupo de investigación australiano desarrolló un proceso de obtención de narseronina mediante síntesis quimio-enzimática, confirmando la estructura con análisis de rayos X, y facilitando que en el futuro puedan ensayarse otro tipo de bioactividades (Schwartz *et al.*, 2011).

4.1.2. 1-O-(3´-acetoxibutanoil)licorina

La observación del espectro de masas de este alcaloide indicó que se trataba de un derivado de licorina, con el pico base característico (*m/z* 226) y un peso molecular de 415 unidades. Su isómero con el sustituyente en posición 2, aislado de *Galanthus nivalis* (Berkov *et al.*, 2007), presenta un patrón de fragmentación muy similar con la diferencia de que el ión más abundante de su MS es *m/z* 250. Esta particularidad también se ha observado en isómeros del mismo tipo con otros grupos funcionales, como acetil o 3´-hidroxibutanoil (Berkov *et al.*, 2009b; Cedrón *et al.*, 2010; de Andrade *et al.*, 2012a; Toriizuka *et al.*, 2008), demostrando que es posible diferenciar varios derivados de licorina con enlaces tipo éster en las posiciones 1 o 2 mediante GC-MS.

El espectro de ^{1}H-RMN es similar al reportado para el isómero en posición 2, excepto por la esperable diferencia en los desplazamientos químicos de H-1 y H-2, ya que el protón de la posición sustituida aparece más desapantallado: δ 5.68 y δ 4.23 ppm, para los protones respectivos del derivado en posición 1; y, δ 4.51 y δ 5.31 ppm, para el isómero en 2. La relación *trans*-diaxial entre los protones 4a y 10b, fue confirmada por el elevado valor de su constante de acoplamiento (10.4 Hz).

4.1.3. Derivados de Narcisidina

Los dos componentes más abundantes del extracto de *N. serotinus* recolectado en la Comunidad Valenciana se identificaron como 3-*O*-metilnarcisidina y 1-*O*-acetil-3-*O*-metilnarcisidina, ambos derivados del alcaloide narcisidina, una estructura de tipo licorina con un doble enlace en posición 4-11 del anillo D (Kihara *et al.*, 1995). Además, se aisló un tercer derivado, 1-*O*-acetil-3-*O*-metil-6-oxonarcisidina, hallado en cantidades muy minoritarias.

El espectro de masas de 3-*O*-metilnarcisidina mostró un patrón muy similar al reportado para narcisidina, aunque con un pico molecular 14 unidades mayor (*m/z* 347) indicando la presencia del grupo metilo adicional. Contrariamente, las fragmentaciones de los otros dos derivados no se correspondían con el patrón característico de narcisidina, probablemente a causa de la sustitución en C-1 y el carbonilo en C-6.

Los espectros de ^{1}H-NMR de 3-*O*-metilnarcisidina y 1-*O*-acetil-3-*O*-metilnarcisidina presentaban el doble doblete característico de este tipo de alcaloides asignado a H-10b entre δ 2.70 y δ 3.00 ppm, con constantes de acoplamiento cercanas a 11.0 y 2.0 Hz. El elevado valor de *J*, asignado al acoplamiento entre H-10b y H-4a, es un indicio de la configuración *trans*-diaxial. En ambos casos, la información obtenida del espectro bidimensional NOESY, ha sido de gran utilidad para resolver la configuración y posición de los grupos funcionales, como lo demuestra el caso del grupo metilo en 3, que evidenció una proximidad espacial con H-11. Los dos derivados con el grupo acetilo en C-1, presentaron el singulete correspondiente en δ 2.00 ppm. Es interesante mencionar también que en el espectro de ^{1}H-RMN de 1-*O*-acetil-3-*O*-metil-6-oxonarcisidina, el protón aromático en posición 7 se encuentra fuertemente desapantallado (δ 7.57 ppm) debido al efecto del grupo carbonilo en *peri*, tal como ocurre en los alcaloides de lactónicos de tipo homolicorina.

A nivel biosintético, ya en los años 70 se propuso que las estructuras de tipo narcisidina derivan de galantina, involucrando la participación del alcaloide incartina como compuesto intermediario epoxidado (Fuganti *et al.*, 1974). Precisamente, ambos alcaloides se han detectado en el extracto de *N. serotinus*.

4.1.4. 2-Metoxipratosina

Los datos espectrales de este alcaloide indicaron la presencia de un sistema estable de dobles enlaces conjugados. Por un lado, su espectro de masas presentaba un pico base coincidente con el pico molecular (*m/z* 309), así como un reducido grado de fragmentación. Por otra parte, en el espectro de ^{1}H-RMN se observaron las 6 señales correspondientes a los protones aromáticos, con sus respectivos acoplamientos, y los 3 grupos metoxilo mostrando el desplazamiento característico de un entorno aromático (δ 3.90-4.20 ppm).

Si bien la presencia de este componente puede atribuirse a un subproducto de licorina y derivados, recientemente se ha propuesto que estructuras de este tipo podrían ser parte de una ruta biosintética alternativa de alcaloides de amarillidáceas, que transcurriría sin la descarboxilaxión previa de L-tirosina (Wang *et al.*, 2009).

4.1.5. 11-Hidroxigalantina

El patrón de fragmentación de este compuesto es muy similar al de galantina (Bastida *et al.*, 1990; Kobayashi *et al.*, 1977), con la notable diferencia de 16 unidades entre los iones mayoritarios de ambos compuestos, sugiriendo la presencia de un átomo de oxígeno adicional. Los datos de RMN concuerdan con los equivalentes reportados para galantina. La orientación α del grupo hidroxilo se asignó teniendo en cuenta el valor de la constante de acoplamiento alílico entre H-3 y H-11, que depende del ángulo diedro definido por H-11 y el plano formado por C-4/C-3/C-11. La constante observada de 1.5 Hz concuerda con la orientación β de H-11.

4.1.6. 2-O-Metilclivonina

El espectro de masas de este alcaloide mostró los dos iones mayoritarios característicos de clivonina (Ali *et al.*, 1983), *m/z* 83 (100%) y *m/z* 96 (39%), con un pico molecular 14 unidades mayor (*m/z* 331) indicando la presencia de un grupo metilo adicional. Con respecto a la estereoquímica, tanto los datos de RMN como los del espectro de dicroísmo circular, sostuvieron una configuración *trans*-B/C *anti*, *cis*-C/D para las uniones de los anillos, en coincidencia con los datos reportados para clivonina (Haning *et al.*, 2011; Wagner *et al.*, 1996).

Un aspecto destacable del espectro de ^{1}H-RMN de este alcaloide es el desplazamiento químico inusualmente desapantallado de los protones aromáticos, especialmente de H-10, cuya señal aparece a campos más bajos que la de H-7, a pesar de la presencia del grupo carbonilo en *peri*. Jeffs *et al.* (1971c) han propuesto que dicho desplazamiento, también observado en otras estructuras similares, se debe a la proximidad espacial entre H-10 y el átomo de nitrógeno del anillo D.

En conjunto, estos resultados permiten concluir que la especie *N. serotinus* es una fuente interesante de alcaloides típicos de Amaryllidaceae, siendo los componentes mayoritarios de los extractos estructuras novedosas. A grandes rasgos, si se compara el análisis de las plantas de Marruecos con las de la Comunidad Valenciana es destacable mencionar la ausencia de licorina y 1-*O*-(3´-acetoxibutanoil)licorina en

estas últimas. No obstante, el alcaloide narseronina se confirmó como uno de los más abundantes en ambos casos. Es importante comentar que, teniendo en cuenta la variabilidad morfológica y genética, así como su distribución geográfica, los ejemplares recolectados en la Comunidad Valenciana (*N. serotinus* L. s.l.) corresponderían a *N. deficiens* Herbert s.s. según algunos autores (Díaz Lifante *et al.*, 2007; Fernández Casas, 2008), mientras que las plantas marroquíes se clasifican como *N. serotinus* L. s.s. Esta separación taxonómica podría explicar la variación del contenido de alcaloides, sin embargo, dado que la producción de metabolitos secundarios en un organismo vegetal suele estar condicionada por un gran número de factores, no es conveniente realizar una generalización de este caso en particular a partir de nuestros datos.

4.2. Alcaloides de Narcisos de la Sección Ganymedes

La clasificación taxonómica de los narcisos pertenecientes a la sección Ganymedes ha sido objeto de debate; así, mientras algunos autores consideran que se trata de una única especie (*N. triandrus* L.) junto con tres subespecies y algunas variedades (Barra Lázaro, 2000), estudios moleculares sostienen la idea de tres especies diferentes: *N. triandrus* L., *N. pallidulus* Graells, y *N. lusitanicus* Dorda & Fern. Casas (Santos-Gally *et al.*, 2011; Vives *et al.*, 2010; Zonneveld, 2008), a las que recientemente se agregó una cuarta, *N. iohannis* Fern. Casas (Fernández Casas, 2011). La distribución geográfica de estas especies abarca la Península Ibérica y las islas Glenan (Francia), siendo su época de floración habitual entre los meses de Marzo y Abril. Estudios previos sobre su contenido de alcaloides reportan la presencia de estructuras de tipo mesembrano, inusuales entre las plantas de la familia Amaryllidaceae, en *N. pallidulus* (Bastida *et al.*, 1989; Berkov *et al.*, en preparación) y *N. triandrus* (Seijas *et al.*, 2004).

En el tercer artículo presentado en la sección de Resultados, se aborda el estudio del contenido de alcaloides de especies de la sección Ganymedes, incluyendo la identificación y aislamiento de alcaloides de la especie *N. triandrus*, así como un análisis por GC-MS del extracto de alcaloides de plantas recolectadas en 18 localidades de la Península Ibérica, que comprenden muestras de todos los taxones descritos para la sección.

4.2.1. Alcaloides de N. triandrus L.

El acceso a una población silvestre abundante de *N. triandrus* cercana a la localidad de Proaza (Asturias, España) permitió la recolección de 600 g de material vegetal fresco que resultaron en la identificación de 8 alcaloides de tipo mesembrano (Figura 4.2).

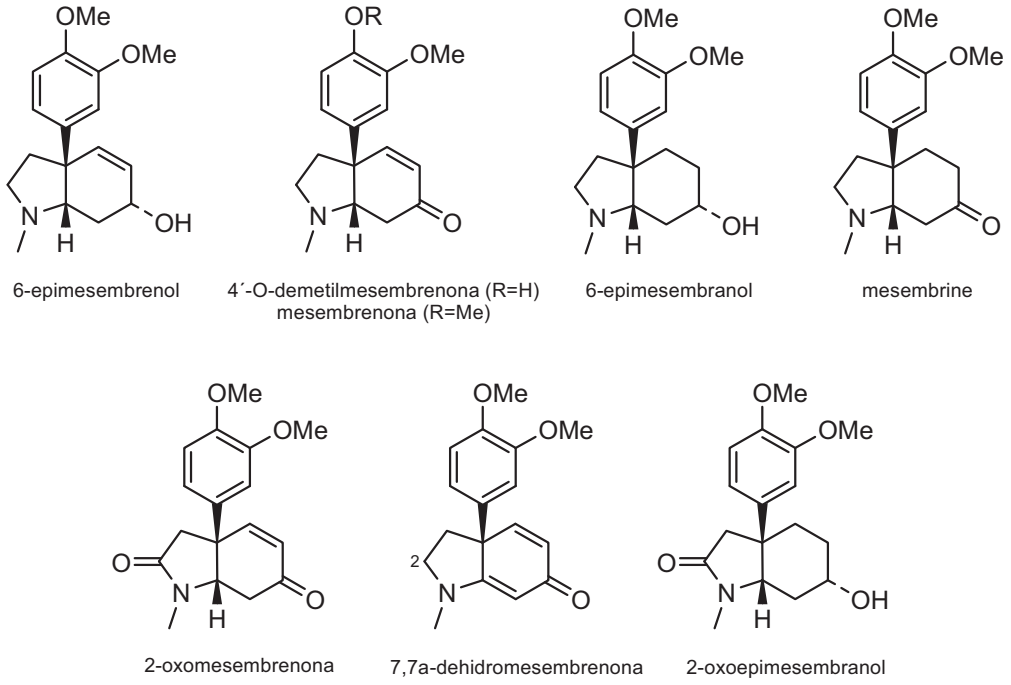

Figura 4.2: Alcaloides identificados en *N. triandrus.*

Los compuestos mesembrenona, 4´-*O*-demetilmesembrenona y mesembrina se identificaron directamente mediante el análisis de GC-MS, por comparación con los patrones de fragmentación previamente reportados (Bastida *et al.*, 1989; Jeffs *et al.*, 1974; Shikanga *et al.*, 2012). Sin embargo, en el caso de los compuestos hidroxilados en la posición 6 (6-epimesembrenol y 6-epimesembranol), el espectro de masas no fue suficiente para definir la estereoquímica, siendo necesaria la información complementaria de ^{1}H-RMN. Las señales correspondientes a los protones olefínicos de 6-epimesembrenol, así como el análisis de las constantes de acoplamiento de H-6 en el caso de 6-epimesembranol, permitieron la asignación del grupo hidroxilo en α, en acuerdo con los datos previos reportados (Jeffs *et al.*, 1969, 1970). Conjuntamente, en el análisis de GC-MS se detectaron tres componentes minoritarios que no fue posible identificar.

Por otra parte, los otros tres alcaloides restantes caracterizados no se detectaron por GC-MS y corresponden a estructuras reportadas por primera vez a partir de una fuente natural. Su aislamiento permitió la identificación y elucidación estructural mediante la combinación de las técnicas espectroscópicas de rutina.

4.2.1.1. 2-Oxomesembrenona

Este alcaloide presenta una estructura novedosa con un carbonilo en la posición 2 y un sistema conjugado de insaturaciones en el anillo de seis miembros. Esta particularidad se ve reflejada en su espectro de masas por la estabilidad del ión molecular (*m/z* 301) y el reducido grado de fragmentación. Al igual que todos los alcaloides identificados en *N. triandrus*, la región aromática de su espectro de ^{1}H-RMN es característica, presentando tres protones aromáticos con acoplamientos típicos de disposiciones *orto* y *meta* (*J* = 8.4, 2.2 Hz, respectivamente). Además, se observan señales correspondientes a dos metoxilos aromáticos, un grupo *N*-metilo (δ 2.81 ppm) y un par de protones olefínicos. El espectro de ^{13}C-RMN presenta las dos señales desapantalladas de los carbonilos (δ 171.9, 195.3 ppm), que muestran las correlaciones respectivas de larga distancia con protones a dos y tres enlaces (HMBC).

4.2.1.2. 7,7a-Dehidromesembrenona

Esta estructura ha sido previamente reportada como producto de la oxidación sintética de mesembrenona con azodicarboxilato de dietilo (DEAD) (Jeffs et al., 1971b). Su espectro ^{1}H-RMN y su fragmentación concuerdan con los datos de la literatura. La asignación ha sido confirmada con técnicas de RMN bidimensionales, además del espectro de ^{13}C-RMN. Es interesante mencionar que el carbono metínico de la posición olefínica 7, se observa inusualmente apantallado (δ 93.7 ppm).

4.2.1.3. 2-Oxoepimesembranol

Este alcaloide se había obtenido previamente por procedimientos sintéticos (Ishibashi *et al.*, 1991), pero los datos espectrales reportados no estaban

completos. Su espectro de masas presenta un abundante pico molecular (*m/z* 305) y un reducido grado de fragmentación.

Al analizar los datos de los espectros de RMN de este compuesto surgieron algunos aspectos controvertidos sobre la elucidación estructural, como la asignación de la disposición del sustituyente hidroxilo, o la observación de correlaciones de proximidad espacial que apuntaban a la presencia de, al menos, dos confórmeros diferentes para el anillo saturado de seis miembros. Además, los valores de las constantes de acoplamiento de H-6 (*tt*, *J* = 6.1, 3.6 Hz) no eran lo suficientemente elevados como para indicar una posición axial, ni se justificaban completamente con una posición ecuatorial.

Consecuentemente, se planteó la realización de un estudio de estabilidad de los posibles confórmeros mediante cálculos de mecánica cuántica, cuyos resultados indicaron la contribución de dos conformaciones de silla principales: una con el grupo dimetoxifenilo en posición axial (en concordancia con estudios conformacionales reportados por Jeffs *et al.*, 1969) y otra con dicho grupo en posición ecuatorial. Asimismo, dado que estudios previos han mostrado que las constantes de acoplamiento observadas pueden reflejar la media ponderada de los valores de *J* determinados para cada confórmero individual de un compuesto en particular (Arnó *et al.*, 2000), se realizaron cálculos similares que revelaron mínimas diferencias entre los valores de *J* calculados y los observados, permitiendo confirmar la presencia de dichas conformaciones en equilibrio.

4.2.2. Análisis del Contenido de Alcaloides de Especies de la Sección Ganymedes

Se recolectaron plantas de 18 poblaciones silvestres de especies de la sección Ganymedes con el objetivo de llevar a cabo un análisis mediante GC-MS para investigar la composición de sus extractos de alcaloides. Las muestras incluyeron ejemplares de todos los taxones descritos para la sección, procedentes de diversas ubicaciones geográficas, comprendiendo tres Comunidades Autónomas de España (Castilla y León, Galicia y Asturias) y la mitad norte de Portugal.

Los cromatogramas obtenidos se analizaron manualmente registrando el área de cada pico. Los datos fueron normalizados respecto al área del estándar interno

agregado (codeína) y al peso seco de material vegetal (g), con el objeto de posibilitar una comparación adecuada. Los resultados se representaron gráficamente tomando la media y desviación estándar de los datos obtenidos de los individuos de cada población.

En todas las muestras analizadas se detectó la presencia de alcaloides de tipo mesembrano, sin observar indicios de estructuras típicas de amarillidáceas. Todas las poblaciones analizadas presentaron el mismo alcaloide mayoritario, mesembrenona, con una gran diferencia respecto de los demás componentes. En líneas generales, los perfiles de alcaloides resultaron similares entre la mayoría de las poblaciones estudiadas.

A pesar de la alta variabilidad, reflejada en los elevados valores de desviación estándar de los datos, se destacaron algunas tendencias sutiles de agrupamiento con respecto a la cantidad de alcaloides. Tanto las muestras de *N. pallidulus* de Segovia, como la de *N. iohannis* de Burgos, mostraron una reducida abundancia de alcaloides en comparación con las demás. En el primer caso, la diferencia puede atribuirse a la variabilidad ontogénica, dado que dichas plantas eran las únicas del muestreo que no estaban en la etapa de floración durante la recolección, aunque tampoco se pueden descartar otros factores de variabilidad. En el caso de *N. iohannis*, la diferencia observada podría estar relacionada con el hecho de que esta especie se considera un caso aislado, por ser endémica de una pequeña región, por sus diferencias morfológicas y por las particularidades ambientales de su hábitat, que llevaron a considerar su clasificación independiente (Fernández Casas, 2011).

Para las demás poblaciones, el perfil y abundancia de alcaloides resultaron comparativamente similares, exceptuando una ligera diferencia en poblaciones de *N. triandrus* (Galicia-Asturias y Burgos) y una población *N. pallidulus* var. *paivae* (NP-P2, Portugal), que presentaron una contribución un poco más elevada de los componentes minoritarios.

Este estudio confirma por primera vez la presencia de alcaloides de tipo mesembrano en todos los taxones de la sección Ganymedes, sin detectar estructuras típicas de Amaryllidaceae. En conjunto con un análisis previo de ejemplares de *N. pallidulus*, que también reveló la presencia de mesembranos (Berkov *et al.*, en preparación), demuestra un proceso interesante en la filogenia del género *Narcissus*,

dado que esta sección es la única que presenta exclusivamente este tipo inusual de alcaloides. Curiosamente, esta es una característica fitoquímica compartida con el distante grupo de dicotiledóneas sudafricanas del género *Sceletium*.

4.3. Amaryllidaceae Argentinas como Fuente de Alcaloides Bioactivos

El cuarto artículo incluido en el presente trabajo forma parte de un conjunto de proyectos dirigidos al estudio de la composición de alcaloides de amarillidáceas latinoamericanas, con el objetivo de investigar fuentes de compuestos bioactivos con potencial aplicación farmacológica. El grupo de investigación del Departamento de Productos Naturales, Biología Vegetal y Edafología de la Facultad de Farmacia (UB), mantiene numerosas colaboraciones con países latinoamericanos como Argentina, Brasil, Colombia, Costa Rica y México, que permiten el desarrollo de investigaciones de especies no estudiadas, favoreciendo el conocimiento de los recursos disponibles y promoviendo su conservación.

En Argentina hay descritas 61 especies de plantas de la familia Amaryllidaceae, incluyendo una gran variedad de géneros. Hasta el momento de la publicación de este artículo (Ortiz *et al.*, 2012), no se conocían reportes de estudios sobre la composición de alcaloides y actividad biológica de dichas especies. Dada la potencialidad ya conocida de estas plantas para la producción de alcaloides bioactivos, junto a la creciente demanda de fuentes renovables y altamente productoras de compuestos medicinales, tales como galantamina, se planteó abordar el estudio del contenido de alcaloides y la capacidad inhibidora de la enzima AChE de cuatro especies silvestres andinas: *Habranthus jamesonii*, *Phycella herbertiana*, *Rhodophiala mendocina* y *Zephyranthes filifolia*.

En el estudio se incluyeron muestras de las cuatro especies recolectadas en la provincia de San Juan (SJ), así como una muestra adicional de *H. jamesonii* de Mendoza (MZA) y una de *R. mendocina* de Neuquén (NQN).

La actividad inhibidora de AChE se evaluó mediante una modificación del método colorimétrico de Ellman *et al.* (1961) (López *et al.*, 2002). Los datos obtenidos se expresaron en valores de concentración inhibitoria del 50% (IC_{50}) y se utilizó galantamina como control positivo. Los resultados de inhibición de los extractos

clorofórmicos mostraron valores de IC_{50} en el rango de 1 a 2 µg/mL. Los extractos más destacables, con valores que demostraron una actividad sólo tres veces menor que galantamina (IC_{50} = 0.29 ± 0.07 µg/mL), fueron los de *Z. filifolia* (IC_{50} = 1.0 ± 0.08 µg/mL) y *H. jamesonii* MZA (IC_{50} = 1.0 ± 0.01 µg/mL).

El contenido de alcaloides de los extractos se analizó mediante GC-MS y los resultados se expresaron en valores porcentuales referidos al área del cromatograma (% TIC, *Total Ion Current*), los cuales no se corresponden exactamente con una cuantificación real pero pueden ser utilizados con fines comparativos (Berkov *et al.*, 2008b). En todas las muestras se detectó el alcaloide galantamina, con una variación entre 0.6 y 17.8 % del total de alcaloides. El extracto de *Z. filifolia* mostró el valor más alto, coincidiendo con una de las mayores actividades inhibidoras de AChE.

En las dos muestras de *H. jamesonii*, tanto el contenido de alcaloides como la actividad biológica ensayada, presentaron diferencias importantes según el lugar de recolección. La mayor capacidad de inhibición de AChE del extracto de *H. jamesonii* MZA, podría explicarse por la presencia de un mayor contenido de galantamina, 4.3% (frente a 1.4% de *H. jamesonii* SJ), sumada a la de otros ya conocidos inhibidores, como sanguinina (10 veces más activo que galantamina), narwedina, y licorina. Las variaciones en el perfil de alcaloides dependientes de la ubicación geográfica de especies silvestres han sido previamente estudiadas, como en el caso de poblaciones búlgaras de *Galanthus nivalis* y *G. elwesii* en las que se detectó una amplia variabilidad intraespecífica en el contenido de alcaloides, ya que la interacción entre factores genéticos y ambientes diversos puede ejercer una gran influencia en la producción de alcaloides de una especie (Berkov *et al.*, 2011a).

La técnica de GC-MS presenta algunas limitaciones para la identificación inequívoca de algunos tipos de alcaloides de amarillidáceas, tal como ocurre en el caso de estructuras de tipo crinano, para la cuales no siempre es posible diferenciar entre compuestos que tienen el puente 5-10b en α (hemantamina) y los de orientación β (crinina). Sin embargo, en muchos casos, los valores de RI de compuestos patrón y la información de investigaciones previas sobre el género bajo estudio, pueden permitir proponer los compuestos más probables.

Teniendo en cuenta lo expuesto, dado que estudios previos de especies de los géneros *Habranthus* y *Rhodophiala* han reportado la presencia de alcaloides de la serie

hemantamina (Jitsuno *et al.*, 2009; Wildman *et al.*, 1967), se ha propuesto que los alcaloides detectados en las muestras estudiadas corresponden a dicha serie. Sin embargo, en el caso de hemantamina y crinamina, epímeros en la posición 3, los datos disponibles no permiten afirmar que se trate de uno u otro. Tanto vitatina como 11-hidroxivitatina, se detectaron en cantidades abundantes en la muestra de *H. jamesonii* SJ (13.2% y 18.7%, respectivamente), mientras que hemantamina/crinamina resultó ser uno de los dos componentes mayoritarios del extracto de *R. mendocina* SJ (31.2%).

Además de galantamina, el alcaloide más abundante en el extracto de *Z. filifolia* corresponde a tazetina (~70%), que también demostró una participación importante en los extractos de *H. jamesonii* SJ (28.1%) y *R. mendocina* SJ (32.9%). Licorina se detectó en abundancia variable (8.2-43.6%) en todas las muestras, excepto *Z. filifolia.* En el caso de *P. herbertiana*, los componentes mayoritarios fueron galantina (17.2%) y licoramina (27.4%), además de licorina (33.2%). En adición a los compuestos identificados, se detectó la presencia de componentes minoritarios con patrones de fragmentación que sugieren estructuras relacionadas con los esqueletos típicos de alcaloides de amarillidáceas.

Este estudio demuestra el potencial de especies silvestres argentinas de la familia Amaryllidaceae procedentes de la región andina, uno de los centros principales de diversificación de este grupo de plantas, como fuente de alcaloides bioactivos. Según el presente análisis, las especies más prometedoras en cuanto a la producción de galantamina, resultaron ser *Z. filifolia* SJ y *H. jamesonii* MZA. La demanda de fuentes renovables y la necesidad de proteger la biodiversidad vegetal de estas especies representan una oportunidad potencialmente productiva para los agricultores argentinos de la región.

5. CONCLUSIONES

5. CONCLUSIONES

1. El estudio de la composición de alcaloides de la especie mediterránea *Narcissus serotinus* L. ha demostrado la presencia de componentes cuyas estructuras pueden clasificarse dentro de las series licorina y homolicorina del grupo de alcaloides característicos de la familia Amaryllidaceae. Se ha reportado el aislamiento y elucidación estructural de 8 compuestos nuevos: narseronina, 1-*O*-(3´-acetoxibutanoil)licorina, 3-*O*-metilnarcisidina, 1-*O*-acetil-3-*O*-metilnarcisidina, 1-*O*-acetil-3-*O*-metil-6-oxonarcisidina, 2-metoxipratosina, 11-hidroxigalantina, y 2-*O*-metilclivonina, indicando que esta especie representa una fuente interesante de alcaloides novedosos para ser ensayados en estudios de actividad biológica. Al mismo tiempo, la caracterización del alcaloide narseronina ha planteado la reconsideración de una estructura previamente publicada de manera errónea como un isómero de 3-epimacronina.

2. El estudio de alcaloides de especies de la sección Ganymedes ha confirmado la presencia de alcaloides de tipo mesembrano, estructuras típicas de las dicotiledóneas sudafricanas del género *Sceletium* e inusuales dentro del grupo de las amarillidáceas, en todos los taxones descritos para la sección. La conocida capacidad de estos alcaloides como inhibidores de la recaptación de serotonina, posibilita su aplicación en el tratamiento farmacológico de estados depresivos y otros trastornos tales como ansiedad y drogodependencia.

3. La caracterización de los compuestos aislados de la especie *Narcissus triandrus* L. representa una contribución detallada y actualizada a la química estructural de estos alcaloides, en la que se aportan datos de GC-MS y RMN. Se destaca el aislamiento de 2-oxomesembrenona, 7,7a-dehidromesembrenona y 2-oxoepimesembranol, reportados por primera vez a partir de una fuente natural.

4. El análisis de especies silvestres de amarillidáceas argentinas de la región andina representa el primer reporte en el que se aborda el estudio del contenido de alcaloides de *Habranthus jamesonii*, *Phycella herbertiana*, *Rhodophiala*

mendocina y *Zephyranthes filifolia*. Los resultados del ensayo de la actividad inhibidora de la enzima AChE de los extractos clorofórmicos de dichas especies, unidos a la caracterización de su composición de alcaloides mediante GC-MS, han demostrado su potencial como fuentes renovables de compuestos activos tales como galantamina. En este sentido, las muestras de *H. jamesonii* y *Z. filifolia* recolectadas en las provincias de Mendoza y San Juan, respectivamente, han resultado especialmente prometedoras.

En líneas generales, este estudio en su conjunto demuestra una vez más el gran potencial de las especies vegetales de la familia Amaryllidaceae (subfam. Amaryllidoideae) como fuente de productos bioactivos y alcaloides con estructuras novedosas, confirmando la importancia de promover la continua caracterización de especies que aún no han sido exploradas.

5.1. Conclusions

1. The study of the alkaloid composition of the Mediterranean species *Narcissus serotinus* L. has shown the occurrence of components whose structures can be classified within the lycorine and homolycorine series, among the typical alkaloids of the Amaryllidaceae plants. The isolation and structural elucidation of 8 new compounds have been reported: narseronine, 1-*O*-(3'-acetoxybutanoyl)lycorine, 3-*O*-methylnarcissidine, 1-*O*-acetyl-3-*O*-methyl-narcissidine, 1-*O*-acetyl-3-*O*-methyl-6-oxonarcissidine, 2-methoxypratosine, 11-hydroxygalanthine, and 2-*O*-methylclivonine, indicating that this species represents an interesting source of novel alkaloids to be assayed in further studies of biological activities. At the same time, the characterization of the alkaloid narseronine has prompted a reconsideration of its previously published structure, which was incorrectly assigned as an isomer of 3-epimacronine.

2. The study of alkaloids from species of the section Ganymedes has confirmed the presence of mesembrane alkaloids, typical structures of the dicotyledonous South African genus *Sceletium* but unusual within the Amaryllidaceae group, in all taxa described for the section. The known activity of these alkaloids as inhibitors of serotonin reuptake indicates their potential application in the pharmacological treatment of depressive states and other disorders, such as anxiety and drug dependence.

3. The characterization of compounds isolated from *Narcissus triandrus* L. represents a detailed and updated contribution to the structural chemistry of these alkaloids, providing complete GC-MS and NMR data. The isolation of 2-oxomesembrenone, 7,7a-dehydromesembrenone and 2-oxoepimesembranol is reported for the first time from a natural source.

4. The analysis of wild Argentinian Amaryllidaceae from the Andean region constitutes the first reported study of alkaloid content of *Habranthus jamesonii*, *Phycella herbertiana*, *Rhodophiala mendocina* and *Zephyranthes filifolia*. The

results of the activity assay of AChE enzyme inhibition of the chloroform extracts from these species, together with the characterization of the alkaloid composition by GC-MS, have demonstrated their potential as renewable sources of active compounds such as galanthamine. In this regard, samples of *H. jamesonii* and *Z. filifolia* collected in the provinces of Mendoza and San Juan, respectively, have been particularly promising.

This study as a whole demonstrates once again the great potential of plant species of the Amaryllidaceae family (subfam. Amaryllidoideae) as a source of bioactive compounds and alkaloids with novel structures, confirming the importance of promoting the continuous characterization of unexplored species.

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7. ANEXOS

7. ANEXOS

7.1. Anexo I: Capítulo de Revisión

Chemical and biological aspects of Amaryllidaceae alkaloids

Jaume Bastida, Strahil Berkov, Laura Torras, Natalia Belén Pigni, Jean Paulo de Andrade, Vanessa Martínez, Carles Codina y Francesc Viladomat

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3. Chemical and biological aspects of Amaryllidaceae alkaloids

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Abstract. The Amaryllidaceae alkaloids represent a large (over 300 alkaloids have been isolated) and still expanding group of biogenetically related isoquinoline alkaloids that are found exclusively in plants belonging to this family. In spite of their great variety of pharmacological and/or biological properties, only galanthamine is used therapeutically. First isolated from *Galanthus* species, this alkaloid is a long-acting, selective, reversible and competitive inhibitor of acetylcholinesterase, and is used for the treatment of Alzheimer's disease. Other Amaryllidaceae alkaloids of pharmacological interest will also be described in this chapter.

Introduction

The Amaryllidaceae are richly represented in the tropics and have pronounced centers of diversity in South-Africa and the Andean region.

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Some genera are also found in the Mediterranean area and temperate regions of Asia.

A particular characteristic of Amaryllidaceae is a consistent presence of an exclusive group of alkaloids, which have been isolated from the plants of all the genera of this family. The Amaryllidaceae alkaloids represent a large and still expanding group of isoquinoline alkaloids, the majority of which are not known to occur in any other family of plants. Since the isolation of the first alkaloid, lycorine, from *Narcissus pseudonarcissus* in 1877, substantial progress has been made in examining the Amaryllidaceae plants, although they still remain a relatively untapped phytochemical source [1]. At present, over 300 alkaloids have been isolated from plants of this family [2] and, although their structures vary considerably, these alkaloids are considered to be biogenetically related.

The large number of structurally diverse Amaryllidaceae alkaloids are classified mainly into nine skeleton types, for which the representative alkaloids are: norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, tazettine, montanine and galanthamine (Fig. 1). With the aim of unifying the numbering system of the different skeleton types, Ghosal's model will be used in this review [3].

As the alkaloids of the Amaryllidaceae family species fall mainly into one of these subgroups, they can serve as a classifying tool for including genera and species in this family. Recently, Unver and Jin have proposed subgroups for some skeleton types, according to the structures of new alkaloids isolated from *Galanthus* species [4,5]. Furthermore, although it is unusual to find other types of alkaloids in this family, if present, they are always accompanied by typical Amaryllidaceae alkaloids. The classical example is the reported presence of the mesembrane (*Sceletium*) alkaloids, generally found in the Aizoaceae family [6,7], in a few species of Amaryllidaceae such as *Hymenocallis arenicola*, *Crinum oliganthum*, *Narcissus pallidulus* and *Narcissus triandrus* [8-10]. In turn, the unexpected isolation of (−)-capnoidine and (+)-bulbocapnine from *Galanthus nivalis* subsp. *cilicicus* is the first report of the occurrence of classical isoquinoline alkaloids in a typical member of the Amaryllidaceae [11].

Plants of the Amaryllidaceae family have been used for thousands of years as herbal remedies. The alkaloids from their extracts have been the object of active chemical investigation for nearly 200 years. Over the past three decades many have been isolated, screened for different biological activities, and synthesized by a number of research groups.

The structural elucidation of the Amaryllidaceae alkaloids and their biological profiles, as well as their synthesis, have been summarized in the last few years [12-14], which, together with the regular publications of the journal

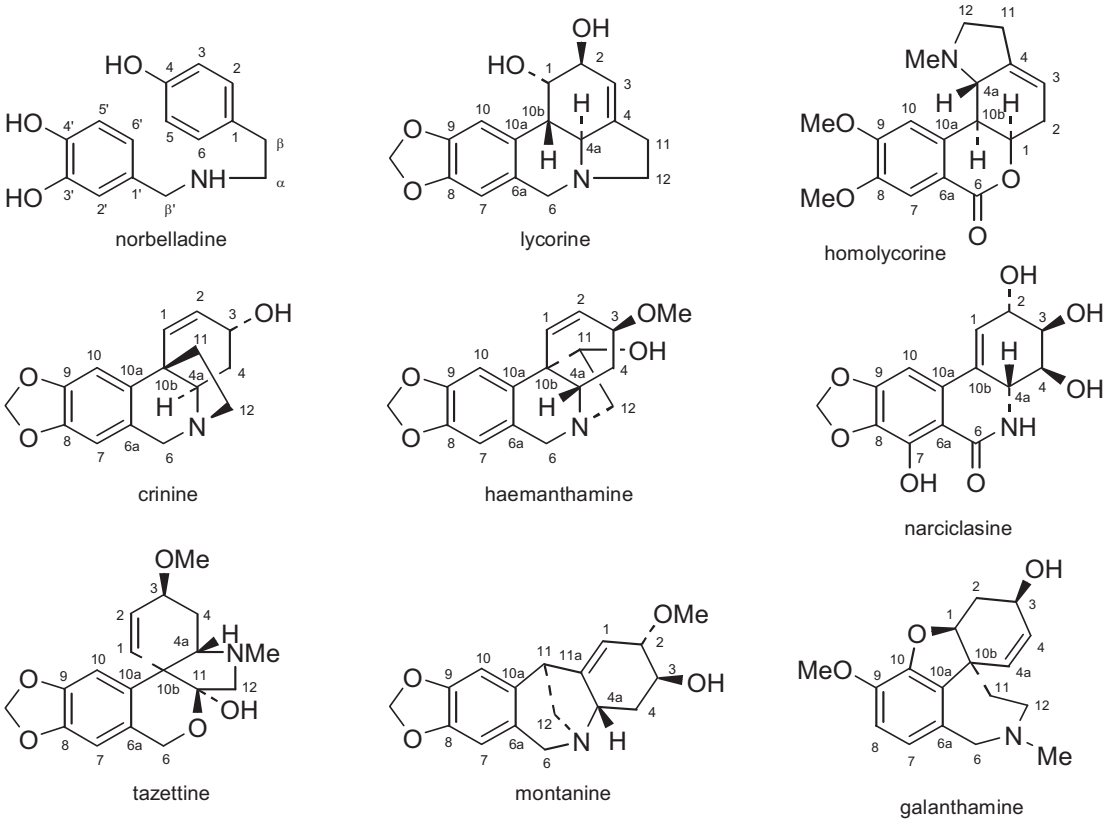

Figure 1. Amaryllidaceae alkaloid types.

Natural Products Reports [5,15-17] over the last decade, represents a valuable source of information.

The present review provides coverage of the biosynthesis, NMR spectroscopy and biological activity of the Amaryllidaceae alkaloids up to the end of 2010.

1. Biosynthetic pathways

Most of the biosynthetic research done on Amaryllidaceae alkaloids was carried out in the sixties and early seventies. Since then, the only noteworthy study has been the biosynthesis of galanthamine and related alkaloids [18]. As in most alkaloid biosyntheses, that of the Amaryllidaceae follows a pattern made up of certain steps.

1.1. Enzymatic preparation of the precursors

Although L-phenylalanine (L-phe) and L-tyrosine (L-tyr) are closely related in chemical structure, they are not interchangeable in plants. In the Amaryllidaceae alkaloids, L-phe serves as a primary precursor of the C_6-C_1

fragment, corresponding to ring A and the benzylic position (C-6), and L-tyr is the precursor of ring C, the two-carbon side chain (C-11 and C-12) and nitrogen, C_6-C_2-N. The conversion of L-phe to the C_6-C_1 unit requires the loss of two carbon atoms from the side chain as well as the introduction of at least two oxygenated substituents into the aromatic ring, which is performed via cinnamic acids. The presence of the enzyme phenylalanine ammonia lyase (PAL) has been demonstrated in Amaryllidaceae plants [19] and the elimination of ammonia mediated by this enzyme is known to occur in an antiperiplanar manner to give *trans*-cinnamic acid, with loss of the β-*pro*-S hydrogen [20]. Thus, it may be expected that L-phe would be incorporated into Amaryllidaceae alkaloids with retention of the β-*pro*-R hydrogen. However, feeding experiments in *Narcissus* 'King Alfred' showed that tritium originally present at C-β of L-phe, whatever the configuration, was lost in the formation of several haemanthamine and homolycorine type alkaloids, which led to the conclusion that fragmentation of the cinnamic acids involves oxidation of C-β to ketone or acid level, the final product being protocatechuic aldehyde or its derivatives (Fig. 2). On the other hand, L-tyr is degraded no further than tyramine before incorporation into the Amaryllidaceae alkaloids.

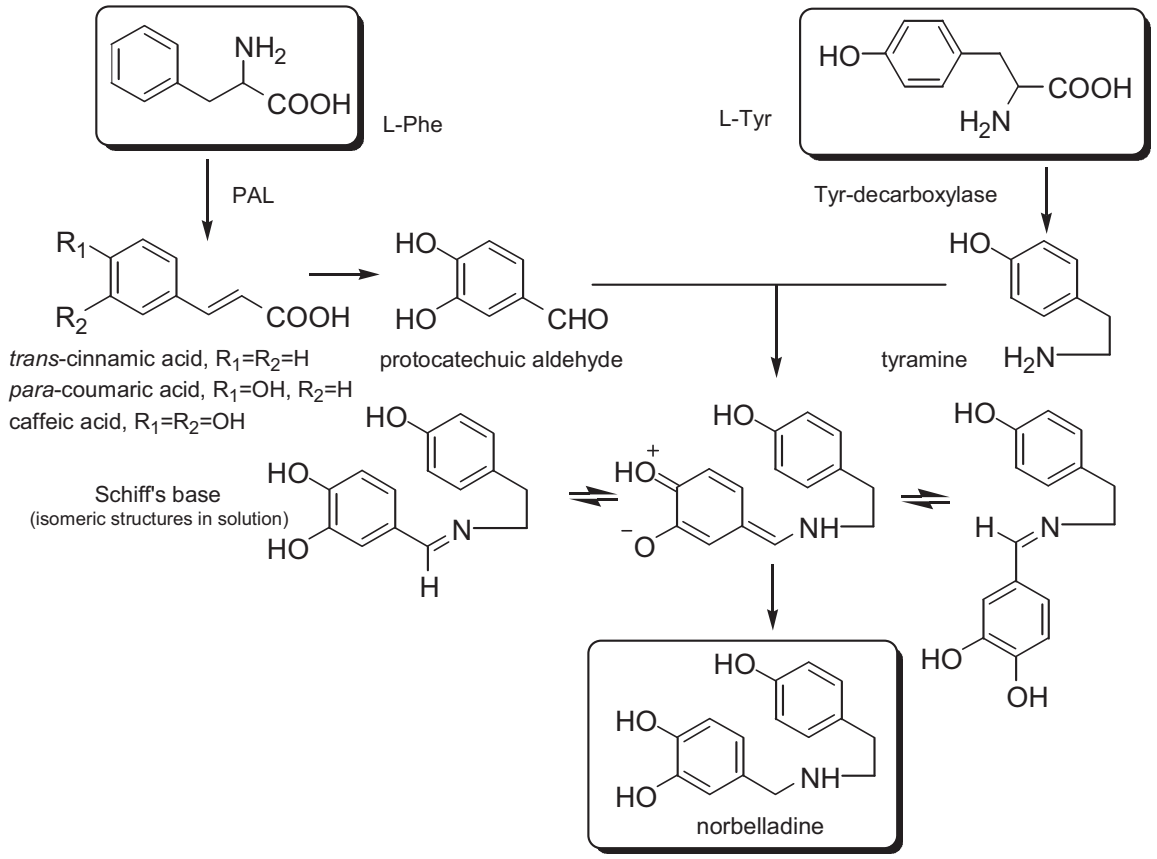

Figure 2. Biosynthetic pathway to norbelladine.

1.2. Primary cyclization mechanisms

Tyramine and protocatechuic aldehyde or its derivatives are logical components for the biosynthesis of the precursor norbelladine. This pivotal reaction represents the entry of primary metabolites into a secondary metabolic pathway. The junction of the amine and the aldehyde results in a Schiff's base, two of which have been isolated up to now from several *Crinum* species: craugsodine [21] and isocraugsodine [22]. The existence of Schiff's bases in nature as well as their easy conversion into the different ring-systems of the Amaryllidaceae alkaloids suggest that the initial hypothesis about this biosynthetic pathway was correct.

1.3. Enzymatic preparation of intermediates

In 1957, Barton and Cohen [23] proposed that norbelladine or related compounds could undergo oxidative coupling in Amaryllidaceae plants, once ring A had been suitably protected by methylation, resulting in the different skeletons of the Amaryllidaceae alkaloids (Fig. 3). The key intermediate in most of cases is *O*-methylnorbelladine.

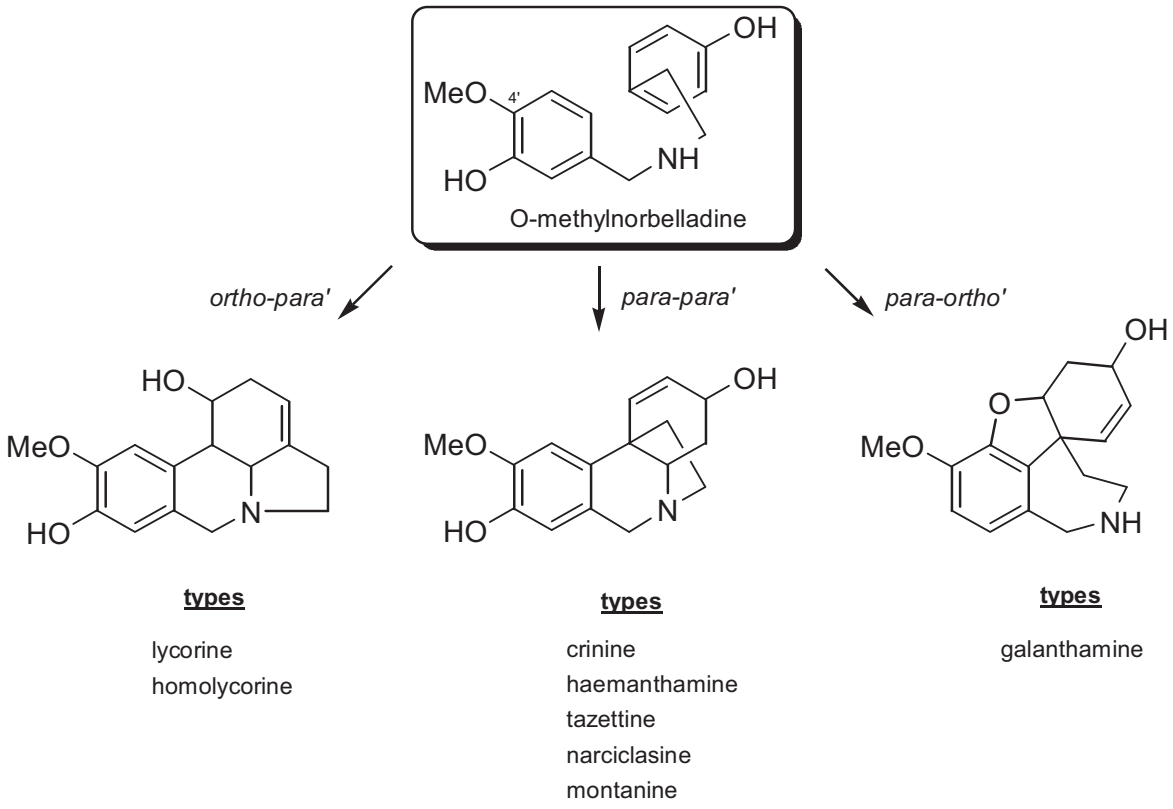

Figure 3. Phenol oxidative coupling in Amaryllidaceae.

1.4. Secondary cyclization, diversification and restructuring

Secondary cyclization is produced by an oxidative coupling of *O*-methylnorbelladine.

1.4.1. Lycorine and homolycorine types

The alkaloids of this group are derivatives of the pyrrolo[*de*]phenanthridine (lycorine type) and the 2-benzopirano-[3,4-*g*]indole (homolycorine type) skeletons, and both types originate from an *ortho-para*' phenol oxidative coupling (Fig. 4).

The biological conversion of cinnamic acid via hydroxylated cinnamic acids into the C_6-C_1 unit of norpluviine has been used in a study of hydroxylation mechanisms in higher plants [24]. When [3-^{3}H, β-^{14}C] cinnamic acid was fed to *Narcissus* 'Texas' a tritium retention in norpluviine of 28% was observed, which is very close to the predicted value resulting from para-hydroxylation with hydrogen migration and retention.

In the conversion of *O*-methylnorbelladine into lycorine, the labelling position [3-^{3}H] on the aromatic ring of L-tyr afterwards appears at C-2 of norpluviine, which is formed as an intermediate, the configuration of the tritium apparently being β [25]. This tritium is retained in subsequently formed

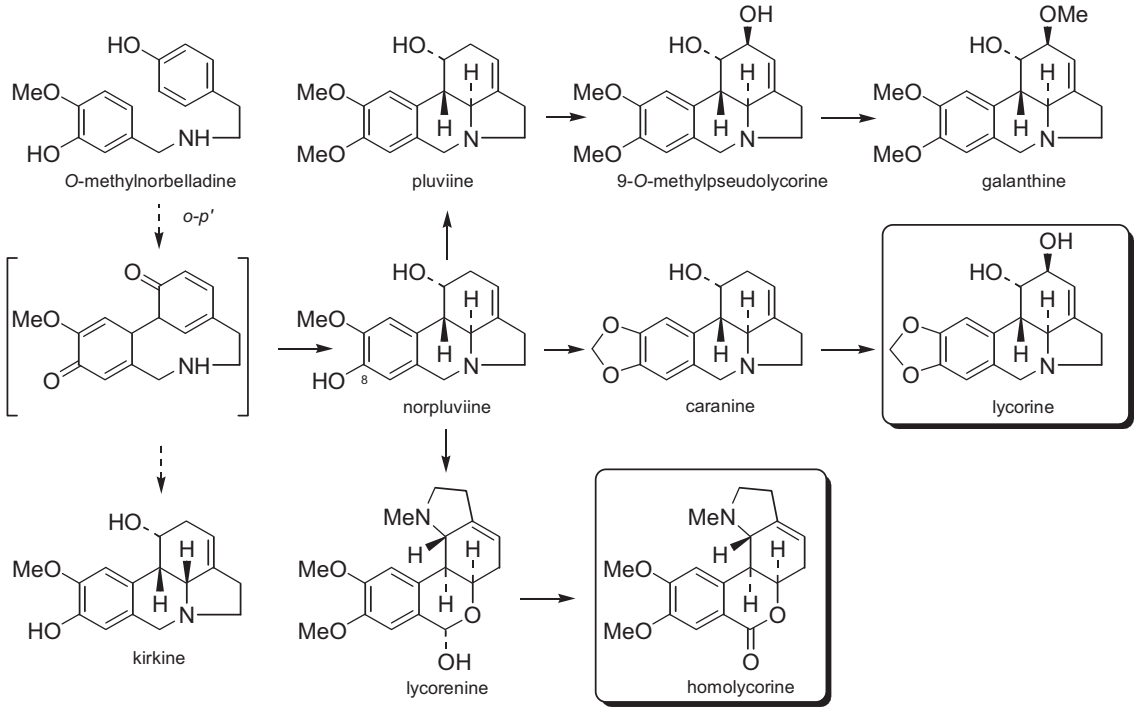

Figure 4. Alkaloids proceeding from an *ortho-para'* coupling.

lycorine, which means that hydroxylation at C-2 proceeds with an inversion of configuration [26] by a mechanism involving an epoxide, with ring opening followed by allylic rearrangement of the resulting alcohol (Fig. 5). Supporting evidence comes from the incorporation of [2β-^{3}H]caranine into lycorine in *Zephyranthes candida* [27]. However, an hydroxylation of caranine in *Clivia miniata* occuring with retention of configuration was also observed [28]. Further, [2α-^{3}H; 11-^{14}C]caranine was incorporated into lycorine with high retention of tritium at C-2, indicating that no 2-oxo-compound can be implicated as an intermediate.

The conversion of the *O*-methoxyphenol to the methylenedioxy group may occur late in the biosynthetic pathway. Tritiated norpluviine is converted to tritiated lycorine by *Narcissus* 'Deanna Durbin', which not only demonstrates the previously mentioned conversion but also indicates that the C-2 hydroxyl group of lycorine is derived by allylic oxidation of either norpluviine or caranine [29].

Regarding the conversion of [2β-^{3}H, 8-OMe-^{14}C]pluviine into galanthine, in *Narcissus* 'King Alfred', the retention of 79% of the tritium label confirms that hydroxylation of C-2 may occur with inversion of configuration [30].

It was considered [31] that another analogous epoxide could give narcissidine in the way shown by loss of the *pro*-S hydrogen from C-11, galanthine being a suitable substrate for epoxidation. Labelled [α-^{14}C, β-^{3}H]-*O*-methylnorbelladine, when fed to *Narcissus* 'Sempre Avanti' afforded galanthine (98% of tritium retention) and narcissidine (46% tritium retention). Loss of hydrogen from C-11 of galanthine was therefore stereospecific. In the nineties, Kihara et al. [32] isolated a new alkaloid, incartine, from flowers of *Lycoris incarnata*, which could be considered as the biosynthetic intermediate of this pathway (Fig. 6).

The biological conversion of protocatechuic aldehyde into lycorenine, which proceeds via *O*-methylnorbelladine and norpluviine, first involves a reduction of the aldehyde carbonyl, and afterwards, in the generation of lycorenine, oxidation of this same carbon atom. The absolute stereochemistry

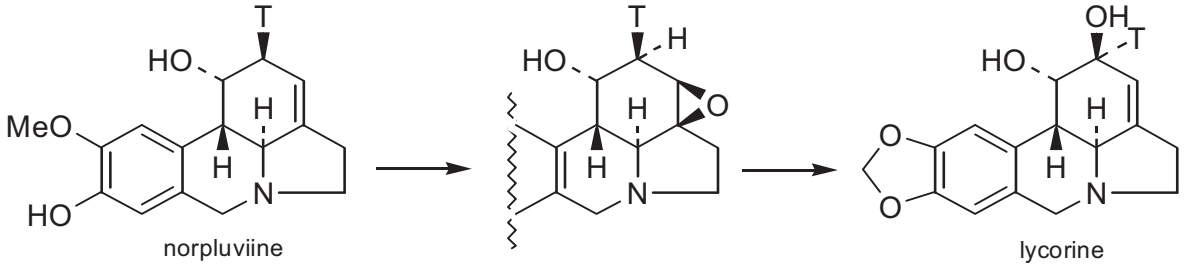

Figure 5. Biosynthesis of lycorine with inversion of the configuration.

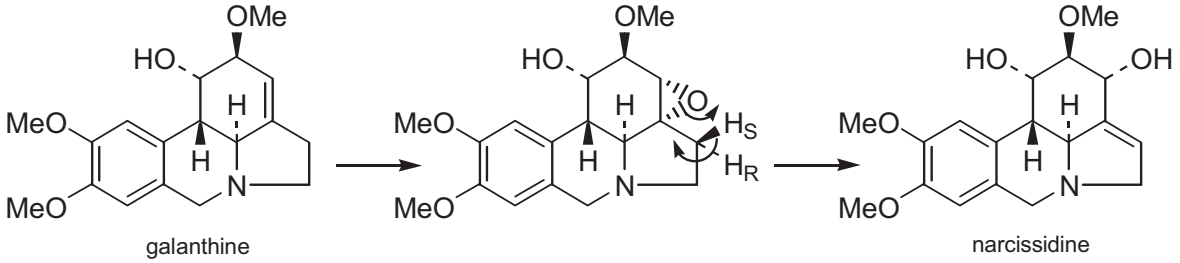

Figure 6. Conversion of galanthine to narcissidine *via* epoxide.

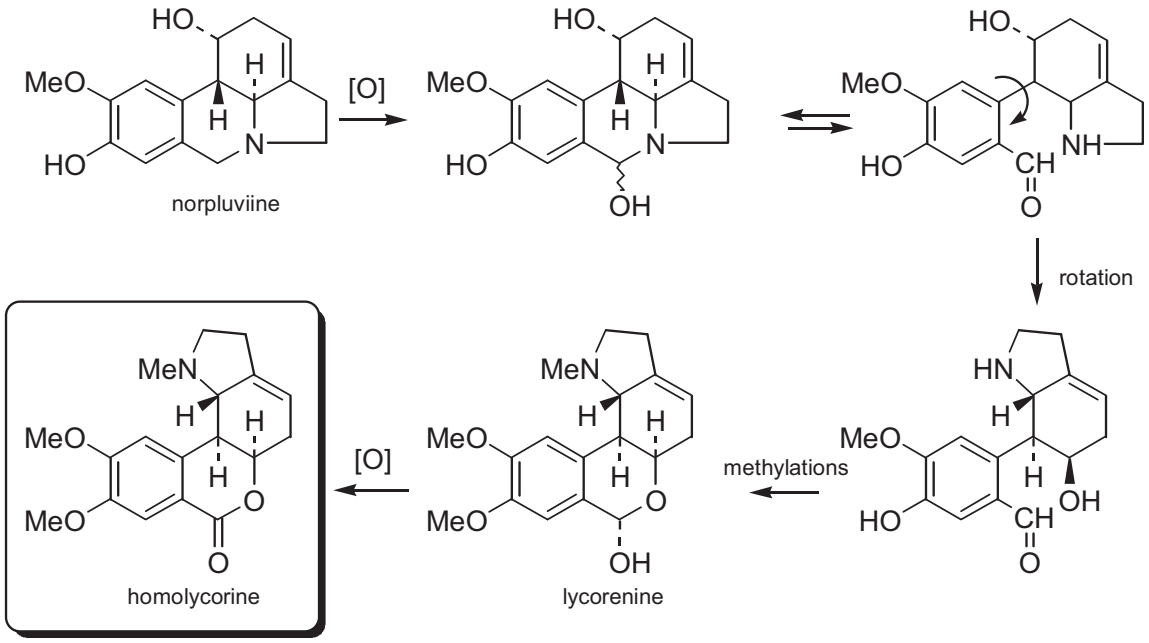

Figure 7. Conversion of norpluviine to homolycorine type alkaloids.

of these processes has been elucidated in subsequent experiments [33], and the results show that hydrogen addition and removal take place on the re-face of the molecules concerned [34], the initially introduced hydrogen being the one later removed [35]. It is noteworthy that norpluviine, unlike pluviine, is converted in *Narcissus* 'King Alfred' primarily to alkaloids of the homolycorine type. Benzylic oxidation at position 6 followed by a ring opening forms an amino aldehyde; the formation of hemiacetal and subsequent methylation provides lycorenine [30], which after oxidation gives homolycorine, as shown in Fig. 7.

1.4.2. Crinine, haemanthamine, tazettine, narciclasine and montanine types

This group includes the alkaloids derived from 5,10b-ethanophenanthridine (crinine and haemanthamine types), 2-benzopyrano[3,4-*c*]indole (tazettine type), phenanthridine (narciclasine type) and 5,11-

methanomorphanthridine (montanine type) skeletons, originating from a *para-para'* phenol oxidative coupling (Fig. 8).

Results of experiments with labelled crinine, and less conclusively with oxovittatine, indicate that the two naturally occurring enantiomeric series, represented in Fig. 8 by crinine and vittatine, are not interchangeable in *Nerine bowdenii* [36].

Incorporation of *O*-methylnorbelladine, labelled in the methoxy carbon and also in positions [3,5-^{3}H], into the alkaloid haemanthamine was without loss of tritium, half of which was at C-2. Consideration of the possible mechanisms involved in relation to tritium retention led to the suggestion that the tritium which is expected at C-4 of haemanthamine might not be stereospecific [37]. The conversion of *O*-methylnorbelladine into haemanthamine involves loss of the *pro-R* hydrogen from the C-β of the tyramine moiety, as well as a further entry of a hydroxyl group at this site [38]. The subsequent benzylic oxidation results in an epimeric mixture that even HPLC cannot separate. The epimeric forms were proposed to be interchangeable. The biosynthetic conversion of the 5,10b-ethanophenanthridine alkaloids to the 2-benzopyrano[3,4-*c*]indole was demonstrated by feeding tritium-labelled alkaloids to *Sprekelia formosissima*. It was shown that this plant converts haemanthamine to haemanthidine/epihaemanthamine and subsequently to pretazettine in an essentially irreversible manner [39]. This transformation was considered to proceed through an intermediate but it has never been detected by spectral methods [40] (Fig. 9).

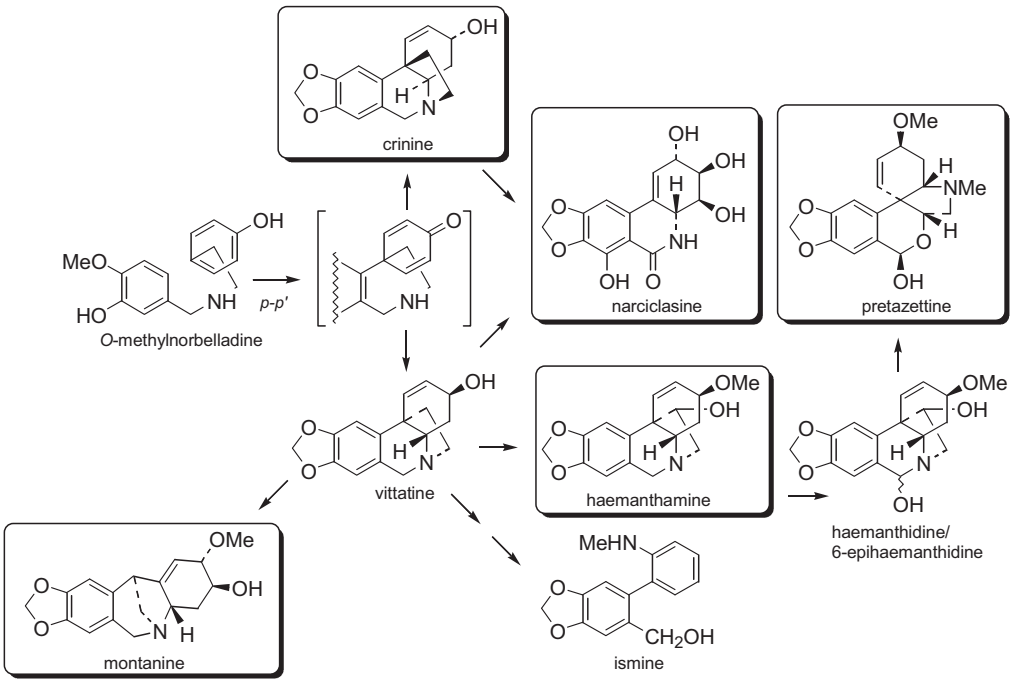

Figure 8. Alkaloids proceeding from a *para-para'* coupling.

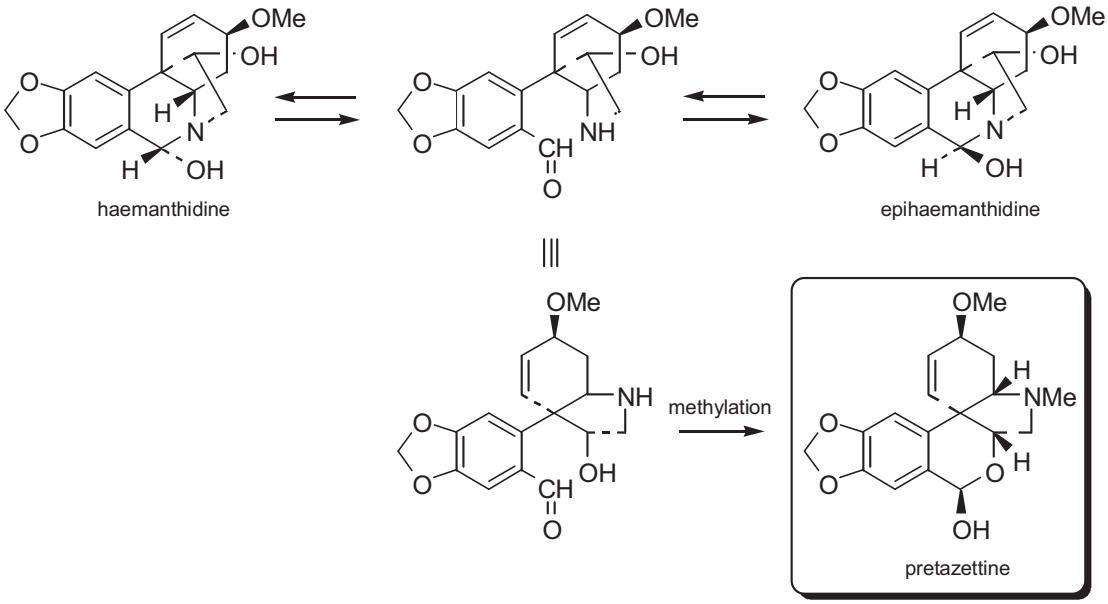

Figure 9. Biosynthesis of pretazettine.

It has also been proved that the alkaloid narciclasine proceeds from the pathway of the biosynthesis of crinine and haemanthamine type alkaloids and not through norpluviine and lycorine derivatives. In fact, in view of its structural affinity to both haemanthamine and lycorine, narciclasine could be derived by either pathway. When *O*-methylnorbelladine labelled in the methoxy carbon and in both protons of position 3 and 5 of the tyramine aromatic ring, was administered to *Narcissus* plants, all four alkaloids incorporated activity. The isotopic ratio [^{3}H:^{14}C] for norpluviine and lycorine was, as expected, 50% that of the precursor, because of its *ortho-para'* coupling. On the contrary, in haemanthamine the ratio was unchanged. These results show clearly that the methoxy group of *O*-methylnorbelladine is completely retained in the alkaloids mentioned, providing a satisfactory internal standard and also, the degree of tritium retention is a reliable guide to the direction of phenol coupling. Narciclasine showed an isotopic ratio (75%) higher than that of lycorine or norpluviine though lower than that of haemanthamine. However, the fact that more than 50% of tritium is retained suggests that *O*-methylnorbelladine is incorporated into narciclasine via *para-para'* phenol oxidative coupling.

O-methylnorbelladine and vittatine are implicated as intermediates in the biosynthesis of narciclasine [41-43], and the loss of the ethane bridge from the latter could occur by a retro-Prins reaction on 11-hydroxyvittatine. Strong support for this pathway was obtained by labelling studies. 11-Hydroxyvittatine has also been proposed as an intermediate in the biosynthesis of haemanthamine and montanine (a 5,11-methanomorphanthridine alkaloid) following the observed specific incorporation of vittatine into the two alkaloids in *Rhodophiala bifida* [36] (Fig. 10).

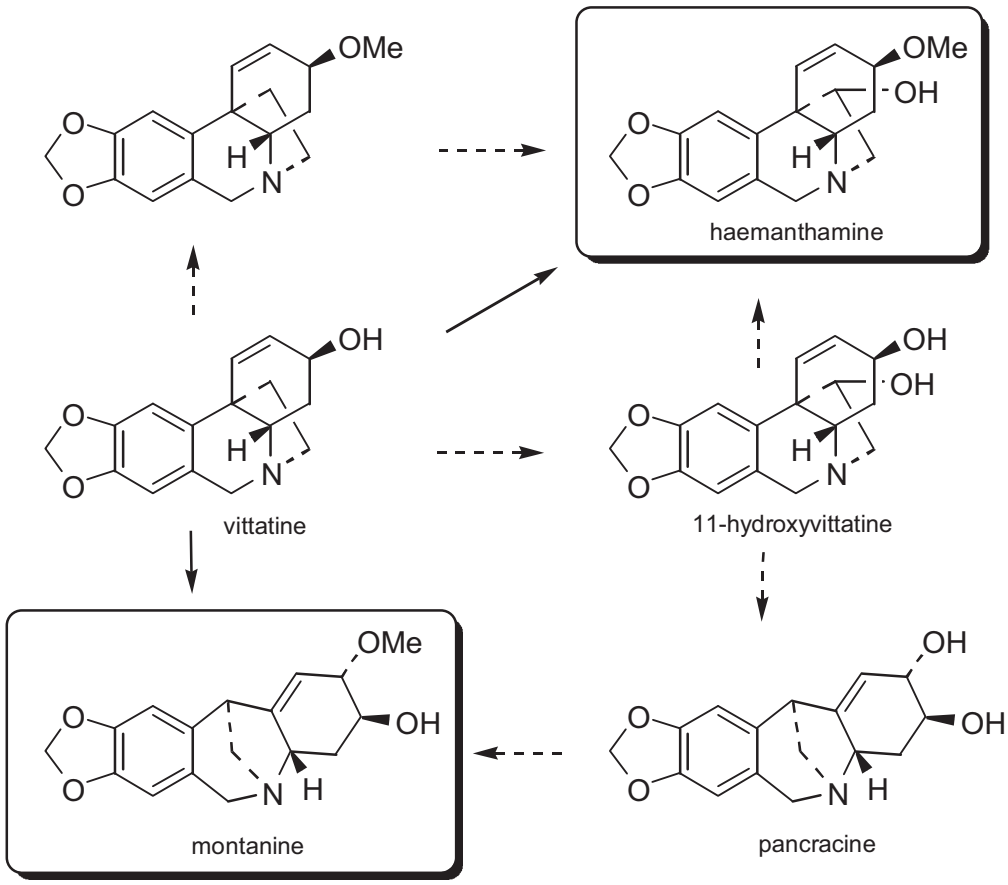

Figure 10. Proposed biosynthetic pathways to haemanthamine and montanine.

Fuganti and Mazza [42,43] concluded that in the late stages of narciclasine biosynthesis, the two-carbon bridge is lost from the oxocrinine skeleton, passing through intermediates bearing a pseudoaxial hydroxy-group at C-3 position and further hydrogen removal from this position does not occur. Noroxomaritidine was also implicated in the biosynthesis of narciclasine and further experiments [44] showed that it is also a precursor for ismine.

The alkaloid ismine has also been shown [45] to be a transformation product of the crinine-haemanthamine series. The precursor, oxocrinine labelled with tritium in the positions 2 and 4, was administered to *Sprekelia formosissima* plants and the radiactive ismine isolated was shown to be specifically labelled at the expected positions.

1.4.3. Galanthamine type

This type of alkaloids have a dibenzofuran nucleus (galanthamine type) and are obtained from a *para-ortho'* phenol oxidative coupling.

The initial studies of this pathway suggested that the *para-ortho'* coupling does not proceed from *O*-methylnorbelladine but from *N,O*-dimethylnorbelladine to finally give galanthamine [46]. *N,O*-dimethylnorbelladine was first isolated from *Pancratium maritimum* [47] a species that also contains galanthamine.

However, the most recent study seems to contradict the evidence set forth here. Experiments carried out with application of ^{13}C-labelled *O*-methylnorbelladine to organs of field grown *Leucojum aestivum* have shown that the biosynthesis of galanthamine involves the phenol oxidative coupling of *O*-methylnorbelladine to a postulated dienone, which undergoes spontaneous closure of the ether bridge to yield *N*-demethylnarwedine, giving norgalanthamine after stereoselective reduction. Furthermore, it was shown that norgalanthamine is *N*-methylated to galanthamine in the final step of biosynthesis [18] (Fig. 11). In contrast with the literature, *N,O*-dimethylnorbelladine was metabolized to a lesser extent in *L. aestivum* and incorporated into galanthamine as well as norgalanthamine at about 1/3 of the rate of *O*-methylnorbelladine.

According to Eichhorn et al. [18], narwedine is not the direct precursor of galanthamine, and could possibly exist in equilibrium with galanthamine, a reaction catalyzed by a hypothetically reversible oxido-reductase.

Chlidanthine, by analogy with the known conversion of codeine to morphine, might be expected to arise from galanthamine by *O*-demethylation. This was shown to be true when both galanthamine and narwedine, with tritium labels, were incorporated into chlidanthine [48].

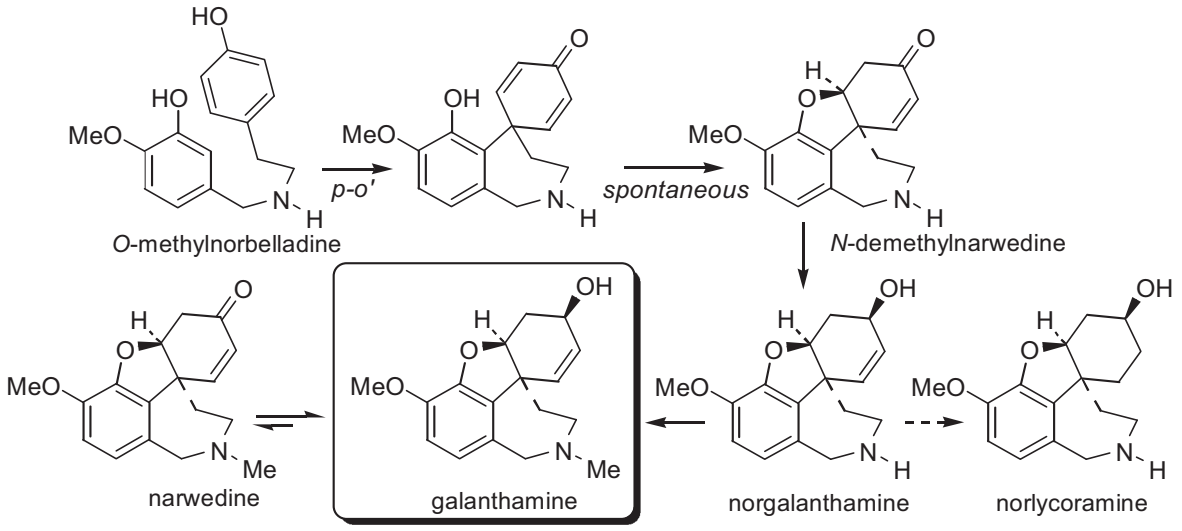

Figure 11. Biosynthesis of galanthamine and derivatives.

2. NMR studies

In a discussion of Proton Nuclear Magnetic Resonance (^{1}H NMR) and Carbon Nuclear Magnetic Resonance (^{13}C NMR), the most significant characteristics of each of Amaryllidaceae alkaloid-type are outlined, indicating the keys for their identification.

2.1. Proton nuclear magnetic resonance

^{1}H NMR spectroscopy gives the most extensive and important information about the different types of Amaryllidaceae alkaloids. In the last 25 years, the routine use of 2D NMR techniques has facilitated the structural assignments and the settling of their stereochemistry.

2.1.1. Lycorine type

This group has been subjected to several ^{1}H NMR studies and lycorine, as well as its main derivatives, has been completely assigned. The general characteristics of the ^{1}H NMR spectra are:

a. Two singlets for the *para*-oriented aromatic protons, together with a unique olefinic proton.
b. Two doublets as an AB system corresponding to the benzylic protons of C- 6. The deshielding observed in the β-protons of positions 6 and 12 in relation to their α-homologues is due to the effect of the *cis*-lone pair of the nitrogen atom.
c. Like almost all other lycorine type examples, the alkaloids isolated from the *Narcissus* genus show a *trans* B/C ring junction, the coupling constant being $J_{4a,10b}$~11 Hz. Only kirkine shows a *cis* B/C ring junction, with a smaller coupling constant $J_{4a,10b}$ 8 Hz.

In the plant, the alkaloid lycorine is particularly vulnerable to oxidation processes, giving several ring-C aromatized products.

2.1.2. Homolycorine type

This group includes lactone, hemiacetal or the more unusual cyclic ether alkaloids. The general traits for this type of compounds could be summarized as follows:

a. Two singlets for the *para*-oriented aromatic protons. In lactone alkaloids, the deshielding of H-7 is caused by the *peri*-carbonyl group.
b. The hemiacetal alkaloids always show the substituent at C-6 in α-disposition.
c. The majority of compounds belong to a single enantiomeric series containing a *cis* B/C ring junction, which is congruent with the small size of the coupling constant $J_{1,10b}$. In the *Narcissus* genus no exception to this rule has been observed.
d. The large coupling constant between H-4a and H-10b ($J_{4a,10b}$~10 Hz) is only consistent with a *trans*-diaxial relationship.
e. In general, ring C presents a vinylic proton. If position 2 is substituted by an OH, OMe or OAc group, it always displays an α-disposition.
f. The singlet corresponding to the *N*-methyl group is in the range of δ 2.0-2.2 ppm, its absence being very unusual.
g. The H-12α is more deshielded than H-12β as a consequence of the *cis*-lone pair of the nitrogen atom.

Homolycorine type alkaloids with a saturated ring C have been studied by Jeff and co-workers [49]. They describe empirical correlations of *N*-methyl chemical shifts with stereochemical assignments of the B/C and C/D ring junction.

2.1.3. Haemanthamine and crinine types

The absolute configuration of these alkaloids is determined through the circular dichroism spectrum. The alkaloids of the *Narcissus* genus are exclusively of the haemanthamine type, while in genera such as *Brunsvigia*, *Boophane* etc., the crinine type alkaloids are predominant. It is also noteworthy that the alkaloids isolated from the Narcissus genus do not show additional substitutions in the aromatic ring apart from those of C-8 and C-9. On the contrary, in the genera where crinine type alkaloids predominate, the presence of compounds with a methoxy substituent at C-7 is quite common. Thus, haemanthamine type alkaloids show the following characteristics:

a. Two singlets for the *para*-oriented aromatic protons, although of course only one for crinane type alkaloids substituted at C-7.
b. Using $CDCl_3$ as the solvent, the magnitude of the coupling constants between each olefinic proton (H-1 and H-2) and H-3 gives information about the configuration of the C-3 substituent. Thus, in those alkaloids in which the two-carbon bridge (C-11 and C-12) is *cis* to the substituent at

C-3, H-1 shows an allylic coupling with H-3 ($J_{1,3}$~1-2 Hz) and H-2 shows a smaller coupling with H-3 ($J_{2,3}$~0-1.5 Hz), as occurs in crinamine. On the contrary, in the corresponding C-3 epimeric series, e.g. haemanthamine, a larger coupling between H-2 and H-3 ($J_{2,3}$ 5 Hz) is shown, the coupling between H-1 and H-3 not being detectable. This rule is also applicable to the crinane type alklaoids.

c. In the haemanthamine series there is frequently an additional W coupling of H-2 with the equatorial H-4β, while the proton H-4α shows a large coupling with H-4a ($J_{4\alpha,4a}$~13 Hz) due to their *trans*-diaxial disposition. The same is applicable in the crinane series.
d. Two doublets for an AB system corresponding to the benzylic protons of position C-6.
e. The pairs of alkaloids with a hydroxy substituent at C-6, like papyramine/ 6-epipapyramine, haemanthidine/6-epihaemanthidine etc, appear as a mixture of epimers not separable even by HPLC.
f. Also in relation with position C-6, it is interesting to note that ismine, a catabolic product from the haemanthamine series, shows a restricted rotation around the biarylic bond, which makes the methylenic protons at the benzylic position magnetically non-equivalent.

2.1.4. Tazettine type

Although tazettine is one of the most widely reported alkaloids in the Amaryllidaceae family, it was found to be an extraction artifact from pretazettine [50].

The presence of an *N*-methyl group (2.4-2.5 ppm) in tazettine type alkaloids immediately distinguishes them from the haemanthamine or crinine types, from which they proceed biosynthetically. Moreover, the ^{1}H NMR spectrum always shows the signal corresponding to the methylenedioxy group.

2.1.5. Montanine type

The absolute configuration of Montanine-type alkaloids is determined through the circular dichroism spectrum. Their ^{1}H NMR data are very similar to those of alkaloids with a lycorine skeleton, but Montanine-type alkaloids can be distinguished by the analysis of a COSY spectrum. The signals attributable to the H-4 hydrogens (the most upfield signals) show correlation with those corresponding to H-3 and H-4a, while in a lycorine skeleton the most upfield signals correspond to the H-11 hydrogens.

2.1.6. Narciclasine type

The narciclasine-type alkaloids present the highest degree of oxidation. The absolute configuration of the most studied alkaloid of this group pancratistatin, was determined by X-ray diffraction [51]. The main ^{1}H NMR characteristics of the narciclasine-type alkaloids are:

a. The only aromatic hydrogen appears as a singlet with a chemical shift higher than 7 ppm.
b. Those alkaloids with a hydrogenated double bond C-1/C-10b possess a *trans* stereochemistry for the B-C ring junction and, consequently, a large coupling constant value for $J_{4a\text{-}10b}$.
c. The hydrogen attached to the nitrogen atom appears as a broad singlet with a chemical shift around 5 ppm, which disappears on the addition of D_2O.

2.1.7. Galanthamine type

Among the Amaryllidaceae alkaloids, only the galanthamine type shows an *ortho*-coupling constant between both aromatic protons of ring A. The general characteristics of their ^{1}H NMR spectra are:

a. Two doublets for the two *ortho*-oriented aromatic protons with a coupling constant of $J_{7,8}$~8 Hz.
b. The assignment of the substituent stereochemistry at C-3 is made in relation with the coupling constants of the olefinic protons H-4 and H-4a. When coupling constant $J_{3,4}$ is about 5 Hz, the substituent is pseudoaxial, while if it is ~0 Hz this indicates that the substituent at C-3 is pseudo-equatorial.
c. Two doublets corresponding to the AB system of the C-6 benzylic protons.
d. The existence of the furan ring results in a deshielding effect in H-1.
e. This type of alkaloids often shows an *N*-methyl group but occasionally an *N*-formyl group has been reported.

2.2. Carbon^{13} nuclear magnetic resonance

^{13}C NMR spectroscopy has been extensively used for determining the carbon framework of Amaryllidaceae alkaloids, and there are several major contributions [52-54]. The assignments are made on the basis of chemical shifts and multiplicities of the signals (by DEPT experiment). The use of 2D

NMR techniques such as HMQC and HMBC allow the assignments to be corroborated.

The ^{13}C NMR spectra of Amaryllidaceae alkaloids can be divided in two regions. The low-field region (>90 ppm) contains signals of the carbonyl group, the olefinic and aromatic carbons as well as that of the methylenedioxy group. The other signals corresponding to the saturated carbon resonances are found in the high-field region, the *N*-methyl being the only characteristic group, easily recognizable by a quartet signal between 40-46 ppm.

The effect of the substituent (OH, OMe, OAc) on the carbon resonances is of considerable importance in localizing the position of the functional groups.

The analysis of the spectra allows conclusions to be drawn about the following aspects:

- The number of methine olefinic carbons.
- The presence and nature of the nitrogen substituent.
- The existence of a lactonic carbonyl group.
- The presence of a quaternary carbon signal assignable to C-10b in the chemical shift range of 42-50 ppm.

3. Biological and pharmacological activities

This section covers the pharmacological and/or biological properties of the most representative Amaryllidaceae alkaloids. Until now only galathamine is being marketed, but the significant activities of other alkaloids in the family demonstrated in recent years could favour their therapeutic use in the near future.

3.1. Lycorine type

The most characteristic and common Amaryllidaceae alkaloid is lycorine, reported to be a powerful inhibitor of ascorbic acid (L-Asc) biosynthesis [55,56], and thus a useful tool in studying Asc-dependent metabolic reactions in L-Asc-synthesising organisms [57,58]. Specifically, lycorine is a powerful inhibitor of the activity of L-galactono-γ-lactone dehydrogenase, the terminal enzyme of L-Asc biosynthesis [59-62], which is thought to be localised in the mitochondrial membrane [63,64]. Galanthine also has a high capacity to inhibit ascorbic acid biosynthesis [56].

Lycorine is a powerful inhibitor of cell growth, cell division and organogenesis in higher plants, algae, and yeasts, inhibiting the cell cycle during interphase, which seems to be related with the L-Asc levels [57,65-69]. In plants, it also inhibits cyanide-insensitive respiration, peroxidase activity

and protein synthesis [70-72]. The effects of lycorine on L-Asc biosynthesis have been reported to occur at concentrations below those at which protein synthesis is affected, but it seems difficult to completely rule out non-specific effects of this alkaloid since it has been reported that, at least in yeasts, lycorine is able to interact directly with mitocondrial DNA. Thus, differing sensitivity to the alkaloid among cells devoid of mitochondrial DNA (*rho*^{0}) and cells with mitochondrial DNA either *rho*$^+$ or *rho*$^-$ has been found in yeasts [59,67,73,74], *rho*^{0} cells being resistant to high concentrations of the drug [69,75-77]. Some strains can even adapt to the presence of lycorine, because they are able to degrade the alkaloid and use its biotransformation products as growth stimulating factors [77]. In contrast, lycorine-1-*O*-β-D-glucoside promotes cell growth, seed germination, and rate of development of root and root hairs in higher plants. The glucosyloxy derivatives of lycorine and pseudolycorine and their aglicones form stable complexes with phytosterols and also with divalent metal ions and are able to translocate them from the rhizosphere to the aerial part [78]. Palmilycorine and some acylglucosyloxy conjugates of lycorine, in turn, are frequently encountered among the phytosterols exhibiting membrane-stabilizing action. Plants also use lycorine-1-*O*-β-D-glucoside and acylglucosyloxy conjugates of lycorine to recognize and reject microorganisms and parasites [79].

The antitumor activity of lycorine in animals [80,81] has been demonstrated by the inhibition of *in vivo* and *in vitro* growth of diverse tumor cells, such as BL6 mouse melanoma, Lewis lung carcinoma, murine ascite or HeLa cells [3,79,82-86]. It induces flat morphology in K-ras-NRK cells (transformed fibroblasts) [87], and reduces the cellular activity in femoral bone marrow tissue that results in granulocytic leucopenia and a decrease in the number of erythrocytes. This alkaloid's mechanism of action is thought to be through inhibition of protein synthesis at the ribosomal level, even though the cytotoxic effects of calprotectin can also be suppressed using lycorine [80,81,88-90]. Lycorine also inhibits murine macrophage production of Tumor Necrosis Factor alpha (TNF-α) [91], and shows inhibitory effects on nitric oxide production and induction of inducible nitric oxide synthase (NOS) in lipopolysaccharide-activated macrophages [92]. The molecular mechanism of lycorine against leukaemia (human cell line HL-60) shows that it can suppress cell growth and reduce cell survival by arresting the cell cycle at the G_2/M phase and inducing apoptosis of tumor cells [93]. Recent studies show that the TNF-α signal transduction pathway and p21-mediated cell-cycle inhibition are involved in the apoptosis of HL-60 cells induced by lycorine [94]. The effects of lycorine on the human multiple myeloma cell line KM3, and the possible mechanisms of these effects have also been studied [95]. The growth rates of the KM3 cells exposed to lycorine clearly slowed down. Cell

fluorescent apoptotic morphological changes, DNA degradation fragments, and a sub-G_1 peak were detected, indicating the occurrence of cell apoptosis after lycorine treatment. Furthermore, the release of mitochondrial cytochrome c, the augmentation of Bas with the attenuation of Bcl-2, and the activation of Caspase-9, -8, and -3 were also observed, suggesting that the mitochondrial pathway and the death acceptor pathway were involved. The results also showed that lycorine was able to block the cell cycle at the G_0/G_1 phase through the downregulation of both cyclin D1 and CDK4. In short, lycorine can suppress the proliferation of KM3 cells and cell survival by arresting cell cycle progression as well as inducing cell apoptosis [96]. A recent paper describes the preparation of a mini-library comprised of synthetic and natural lycorane alkaloids and the investigation of apoptosis-inducing activity in human leukemia (Jurkat) cells. Further insights into the nature of this apoptosis-inducing pharmacophore are described, including the requirement of both free hydroxyl groups in ring-C [97]. Another recent study describes the induction of apoptosis in human leukemia cells by lycorine *via* an intrinsic mitochondria pathway, causing a rapid turnover of protein level of Mcl-1 before Caspases activation. Pronounced apoptosis accompanied by the down-regulation of Mcl-1 was also observed in blasts from patients with acute myeloid leukemia. Lycorine also displays pronounced cell growth inhibitory activities against both parental and multidrug resistant L5178 mouse lymphoma cell lines, but is almost inactive in inhibiting the glycoprotein responsible for the efflux-pump activity of tumor cells. Assays for interactions with tRNA revealed that the antiproliferative effects of lycorine result from their complex formation with tRNA [98]. Interaction of lycorine, pseudolycorine and 2-*O*-acetylpseudo-lycorine with DNA has been observed [99,100]. Most of the alkaloids that showed promising antiproliferative activities have also proved to be efficient apoptosis inducers [14].

Some other alkaloids of this series, such as caranine, galanthine, pseudolycorine and 2-*O*-acetylpseudolycorine, are also active against a variety of tumor cells [84,101,102]. Pseudolycorine inhibits the protein synthesis in tumor cells at the step of peptide bond formation, but it has a different binding site than lycorine [89,103]. Ungeremine, a natural metabolite of lycorine, is responsible, at least partially, for the growth-inhibitory and cytotoxic effects of lycorine, being active against leukemia [104,105]. Lycorine-1-*O*-β-D-glucoside, in turn, has the reverse effect of lycorine, and may produce mitogenic activity in animal cells [106].

A mini-panel of semi-synthetic analogs of lycorine was screened for cytochrome P450 3A4 (CYP3A4) inhibitory activity, the most potent of which (1-*O*-acetyl-2-*O*-*tert*-butyldimethylsilyllycorine) exhibited inhibition at a concentration as low as 0.21 μM. Elements of this unraveled novel

pharmacophore include bulky lipophilic substitution at C-2 in conjunction with a small hydrogen donor/acceptor bond at C-1, or bulky electron-rich substitution at C-1 in conjunction with a vicinal hydrogen donor/acceptor bond [107]. Two semisynthetic silylated lycorane analogs, accesed via a chemoselective silylation strategy from lycorine exhibited low micromolar activities [108].

Lycorine and pseudolycorine exert antiviral effects on several RNA and DNA-containing viruses [109]. Antiviral activity has been observed in tests with flaviviruses, and to a slightly lesser degree, bunyaviruses. Lycorine and pseudolycorine also show inhibitory activity against the Punta Toro and Rift Valley fever viruses, but with low selectivity [110,111]. Lycorine, in turn, acts as an anti-SARS-CoV (Severe Acute Respiratory Syndrome-associated Coronavirus) and shows pronounced activity against poliomyelitis, coxsackie and herpes type 1 [3,112]. It possesses high antiretroviral activity accompanied by low therapeutic indices [113]. The relationship between its structure and the mechanism of activity has been studied in the *Herpes simplex* virus, suggesting that alkaloids that may eventually prove to be antiviral agents have a hexahydroindole ring with two functional hydroxyl groups [114]. The activity was found to be due to the inhibition of multiplication, and not to the direct inactivation of extracellular viruses, and the mechanism of the antiviral effect was partially explained as a blocking of viral DNA polymerase activity [109,115-117].

Lycorine has appreciable inhibitory activity against acetylcholinesterase [118]. Cholinesterase activity appears to be associated with the two free hydroxyl groups present in some of the alkaloids of this structural type [119]. The higher acetylcholinesterase inhibitory activity of assoanine and oxoassoanine with respect to the other lycorine-type alkaloids could be explained by an aromatic ring C, which gives a certain planarity to those molecules [120]. Another alkaloid, galanthine, exhibits powerful cholinergic activity and has therefore attracted much interest in the treatment of myasthenia gravis, myopathy and diseases of the central nervous system [121]. Caranine, pseudolycorine, ungiminorine, and in particular, ungeremine, also show an inhibitory effect on acetylcholinesterase [120,122,123]. Recently, the synthesis of differentially functionalized analogs of lycorine, accessed via a concise chemoselective silylation strategy, has allowed two of the most potent inhibitors of acetylcholinesterase to be described. Important elements of this novel pharmacophore were elucidated through SAR studies [94].

Lycorine is analgesic, more so than aspirin, and hypotensive [124,125], as are caranine and galanthine. The analgesic activity exhibited by the Amaryllidaceae alkaloids is attributed to their similarity with the morphine

and codeine skeletons. Lycorine also has antiarrhythmic action, and lycorine hydrochloride is a strong broncholytic [126]. In fact, lycorine shows a relaxant effect on an isolated epinephrine-precontracted pulmonary artery and increases contractility and the rate of an isolated perfused heart. These effects are mediated by stimulation of β-adrenergic receptors [127].

Lycorine also has a strong inhibitory effect on parasite (*Encephalitozoon intestinalis*) development [128] and antifungal activity against *Candida albicans* [129]. Recently, several lycorine derivatives were examined for their activity against *Trypanosoma brucei* and *Plasmodium falciparum*. Among them, 2-*O*-acetyllycorine showed the most potent activity against parasitic *T. brucei*, while 1-*O*-(3*R*)hydroxybutanoyllycorine, 1,2-di-*O*-butanoyllycorine, and 1-*O*-propanoyllycorine showed significant activity against *P. falciparum* in an *in vitro* experiment [130], although the antimalarial activity of lycorine was already known [131-133]. Galanthine, in turn, shows mild *in vitro* activity against *Tripanosoma brucei rhodesiense* and *Plasmodium falciparum* [134]. Additionally, lycorine has antifeedant [135], emetic [136], anti-inflammatory [137], antiplatelet [138] as well as antifertility [125] activities.

3.2. Homolycorine type

It is reported that some alkaloids of this series, such as homolycorine, 8-*O*-demethylhomolycorine, dubiusine, 9-*O*-demethyl-2α-hydroxyhomoly-corine, hippeastrine, lycorenine or *O*-methyllycorenine present cytotoxic effects against non-tumoral fibroblastic LMTK cells [84], also being moderately active in inhibiting the *in vivo* and *in vitro* growth of a variety of tumor cells, such as Molt 4 lymphoma, HepG2 human hepatoma, LNCaP human prostate cancer or HT [84,125,139]. Dubiusine, lycorenine, 8-*O*-demethylhomolycorine and 9-*O*-demethyl-2α-hydroxyhomolycorine also show DNA binding activity comparable to that of vinblastine [99]. Homolycorine possesses high antiretroviral activity, accompanied by low therapeutic indices [113]. Hippeastrine, in turn, displays antiviral activity against *Herpes simplex* type 1 [114].

Dubiusine, homolycorine, 8-*O*-demethylhomolycorine and lycorenine have a hypotensive effect on the arterial pressure of normatensive rats [140]. Lycorenine also shows a vasodepressor action ascribed to the maintenance of its α-adrenergic blocking action, and produces bradycardia by modifying vagal activity [141]. Another feature of lycorenine is its analgesic activity [3].

Homolycorine and masonine are other inductors of delayed hypersensitivity in animals [142]. Hippeastrine, in turn, shows antifungal activity against *Candida albicans* and it also possesses a weak insect antifeedant activity [129].

3.3. Haemanthamine and crinine types

Haemanthamine, haemanthidine, crinamine, maritidine and papyramine display pronounced cell growth inhibitory activities against a variety of tumor cells, such as Rauscher viral leukaemia, Molt 4 lymphoma, BL6 mouse melanoma, HepG2 human hepatoma, HeLa, LNCaP human prostate cancer or HT [82-84,88,139,143,144]. Some of these alkaloids, namely crinamine, haemanthamine and papyramine, also present a cytotoxic effect against non-tumoral fibroblastic LMTK cells [84]. The mechanism of action of haemanthamine is thought to be through inhibition of protein synthesis, blocking the peptide bond formation step on the peptidyl transferase centre of the 60S ribosomal subunit [89,103]. Haemanthamine and haemanthidine also display the same pronounced cell growth inhibitory activities against both parental and multidrug resistant L5178 mouse lymphoma cell lines as described above for lycorine [98]. Crinamine, in turn, shows inhibitory effects on nitric oxide (NO) production and induction of inducible nitric oxide synthase (NOS) in lipopolysaccharide-activated macrophages [92]. Crinamine and haemanthamine are potent inducers of apoptosis in tumor cells at micromolecular concentrations [145]. The pharmacophoric elements are the alpha-C-2 bridge as well as a small substituent (H, or OH) at C-11. Studies have also shown that α- or β-methoxy or the hydroxyl H-bond acceptor are all tolerated at C-3, and that a C-1/C-2 double bond modulates, but is not a requirement, for apoptosis-inducing activity [146].

The antimalarial activity against strains of chloroquine-sensitive *Plasmodium falciparum* observed in haemanthamine and haemanthidine can be attributed to the methylenedioxybenzene part of the molecule and the terciary nitrogen without methyl [131]. Crinamine also exhibits moderate antimalarial activity [132,147]. Haemanthidine also works *in vitro* against *Trypanosoma brucei rhodesiense* and to a lesser extend against *Trypanosoma cruzi* [134]. Vittatine has antibacterial activity against the Gram-positive *Staphylococcus aureus* and the Gram-negative *Escherichia coli* [129], and the alkaloid crinamine shows strong activity against *Bacillus subtilis* and *Staphylococcus aureus* [148].

Like lycorine, haemanthidine has stronger analgesic and anti-inflammatory activity than aspirin [118,137], and vittatine has been found to potentiate the analgesic effect of morphine [149]. Moreover, some alkaloids of this series, such as haemanthamine or papyramine have a hypotensive effect [140,150], and haemanthamine strong antiretroviral activity [113].

3.4. Tazettine type

Tazettine is mildly active against certain tumor cell lines [88,139,151], with a slight cytotoxicity when tested on fibroblastic LMTK cell lines [84]. Tazettine also displays weak hypotensive and antimalarial activities and interacts with DNA [99,138,140]. Its chemically labile precursor, pretazettine, is far more interesting owing to its antiviral and anticancer activities. In fact, when pretazettine is stereochemically rearranged to tazettine, the biological activity of the precursor is to a large extent inactivated [152,153].

Pretazettine shows cytotoxicity against fibroblastic LMTK cell lines and inhibits HeLa cell growth, being therapeutically effective against advanced Rauscher leukaemia, Ehrlich ascites carcinoma, spontaneous AKR lymphocytic leukaemia and Lewis lung carcinoma [151,154-159]. It is one of the most active of the Amaryllidaceae alkaloids against Molt4 lymphoid cells [84], and is used in combination with DNA-binding and alkylating agents in treating the Rauscher leukaemia virus [151,154]. In fact, pretazettine strongly inhibits the activity of reverse transcriptase from various oncogenic viruses by binding to the enzyme [3]. It inhibits both the growth of the Rauscher virus and cellular protein synthesis in eukaryotic cells by a mechanism that does not affect DNA and RNA synthesis, even though it has a pronounced DNA binding activity [88,89,99,101,111,156,160]. Pretazettine on human MDR1-gene-transfected L5158 mouse lymphoma significantly increased the intracellular concentration of Rh-123 and enhanced the antiproliferative activity of doxorubicin in the L5178 MDR cell line [161]. This alkaloid has also been shown to be active against selected RNA-containing flavoviruses (Japanese encephalitis, yellow fewer and dengue) and bunyaviruses (Punta Toro and Rift Valley fever) in organ culture [111]. It also possesses pronounced activity against *Herpes simplex* type 1 virus [114]. This activity may reflect a general ability to inhibit protein synthesis during viral replication [162].

3.5. Narciclasine type

Narciclasine, an antimitotic and antitumoral alkaloid [163], affects cell division at the metaphase stage and inhibits protein synthesis in eukaryotic ribosomes by directly interacting with the 60s subunit and inhibiting peptide bond formation by preventing binding of the 3' terminal end of the donor substrate to the peptidyl transferase center [89,103,164-166]. It also retards DNA synthesis [167] and inhibits calprotectin-induced cytotoxicity at a more than 10-fold lower concentration than lycorine [90]. The peculiar effects of

narciclasine seem to arise from the functional groups and conformational freedom of its C-ring [168], with the 7-hydroxyl group believed to be important in its biological activity [169]. This alkaloid, related to pancratistatin [167], is one of the most important antineoplastic Amaryllidaceae alkaloids [80] and shows some promise as an anticancer agent. It inhibits HeLa cell growth, has antileukaemic properties and is active against a variety of tumor cells, such as human and murine lymphocytic leukaemia, larynx and cervix carcinomas and Ehrlich tumor cells [115,167,170-172]. One hemisynthetic derivative of narciclasine demonstrated higher *in vivo* antitumor activity in human orthotopic glioma models in mice than narciclasine in nontoxic doses [173], by both the i.v and oral routes. No effect has been observed on solid tumors. Narciclasine-4-*O*-β-D-glucopiranoside shows a very similar cytotoxic and antitumoral activity to narciclasine [174]. The anticancer activity and preclinical studies of narciclasine and its congeners has been gathered by Kornienko and Evidente in a recent review [175]. Melanomas display poor response rates to adjuvant therapies because of their intrinsic resistance to proapoptotic stimuli. Such resistance can be overcome, at least partly, through the targeting of the eEF1A elongation factor with narciclasine [176]. This alkaloid directly binds to human recombinant and yeast-purified eEF1A in a nanomolar range, but not to actin or elongation factor 2. Thus, eEF1A is a potential target to combat melanomas regardless of their apoptosis-sensitivity, which has renewed interest in the pleiotropic cytostatic activity of narciclasine. Apoptosis in Jurkat cells was triggered by narciclasine, narciclasine tetraacetate, *C*-10b-R-hydroxypancratistatin, *cis*-dihydronarciclasine and *trans*-dihydronarciclasine [177].

The effect of pancratistatin treatment on cancerous and normal cells has also been reported [178]. The results indicated that pancratistatin selectively induced apoptosis in cancer cells, and the mitochondria may be the site of action. To further explore the structure-activity relationship of pancratistatin-related compounds, the anticancer efficacy and specificity of two related natural alkaloids were investigated. Both of these compounds lack the polyhydroxylated lycorane element of pancratistatin, instead having a methoxy-substituted crinane skeleton. These results indicated that the phenanthridone skeleton in natural Amaryllidaceae alkaloids may be a significant common element for selectivity against cancer cells. The synergy of pancratistatin and tamoxifen on breast cancer cells in inducing apoptosis by targeting mitochondria has been also reported [179]. The 3,4-*O*-cyclic phosphate salt of pancratistatin is a novel, water soluble synthetic derivative of pancratistatin that *in vivo* caused statistically significant tumor growth delays at its maximum-tolerated dose. Significant vascular shutdown and tumor necrosis were also observed [180],

offering a way forward for improved clinical treatment by greatly enhancing solubility without loss of antitumor activity.

Narciclasine has a prophylactic effect on the adjuvant arthritis model in rats, significantly suppressing the degree of swelling of adjuvant-treated as well as untreated feet [90]. This alkaloid is also active against *Corynebacterium fascians*, inhibits the pathogenic yeast *Cryptococcus neoformans*, and modifications like 2,3,4,7-tetra-*O*-acetylnarciclasine inhibit the growth of the pathogenic bacterium *Neisseria gonorrhoeae* [181]. Antiviral activity has been observed against RNA-containing flaviviruses and bunyaviruses [111].

At the plant level, narciclasine is a potent inhibitor, showing a broad range of effects, including the ability to inhibit seed germination and seedling growth of some plants in a dose-dependent manner, interacting with hormones in some physiological responses [182]. Thus, indole-3-acetic acid cannot overcome the inhibition of elongation of wheat coleoptile sections caused by narciclasine. Additionally, narciclasine suppresses the gibberellin-induced α-amylase production in barley seeds and cytokinin-induced expansion and greening of excised radish cotyledons [183]. Like lycorine, narciclasine also inhibits ascorbic acid biosynthesis [184]. Narciclasine, present in daffodil mucilage, can delay tepal senescence in cut *Iris* flowers by attenuation of protease activity, which, in turn, is apparently related with the inhibition of the protein synthesis involved in senescence [185]. At the organelle level, narciclasine inhibits both isocitrate lyase (ICL) activity in glyoxysomes and hydroxypyruvate reductase (HPR) activity in peroxisomes. It also blocks the formation of chloroplasts, markedly reducing the chlorophyll content of light-grown wheat seedlings, probably due to the inhibition of the formation of 5-aminolevulinic acid, an essential chlorophyll precursor [186]. The formation of light harvesting chlorophyll a/b binding protein (LHCP) is also inhibited by this alkaloid [187].

Some alkaloids of this series, such as trisphaeridine, possess high antiretroviral activities, accompanied by low therapeutic indices [113]. Ismine, in turn, shows a significant hypotensive effect on the arterial pressure of normotensive rats [140] and is cytotoxic against Molt 4 lymphoid and LMTK fibroblastic cell lines [84].

3.6. Montanine type

There is little information about the montanine type alkaloids, only some data about pancracine, which shows antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [129], as well as weak activity against *Trypanosoma brucei rhodesiense, T. cruzi* and *Plasmodium falciparum* [188]. Montanine inhibited, in a dose-dependent manner, more

than 50% of the enzyme acetylcholinesterase at 1 mM concentration. With the concentrations 500 μM and 100 μM, 30-45% of inhibition was detected [189].

3.7. Galanthamine type

Galanthamine, originally isolated from *Galanthus nivalis* L. in the 1940s, is a long-acting, selective, reversible and competitive inhibitor of acetylcholinesterase. This enzyme is responsible for the degradation of acetylcholine at the neuromuscular junction, in peripheral and central cholinergic synapses and in parasympathetic target organs [190-192]. Galanthamine has the ability to cross the blood-brain barrier and act within the central nervous system [193,194]. It binds at the base of the active site gorge of acetylcholinesterase, interacting with both the choline-binding site and the acyl-binding pocket, having a number of moderate-to-weak interactions with the protein [195-197]. In addition, galanthamine stimulates pre- and postsynaptic nicotinic receptors which can, in turn, increase the release of neurotransmitters, thus directly stimulating neuronal function [192,198]. It is also suggested that the stimulation of nicotinic receptors protects against apoptosis induced by β-amyloid toxicity [192,199,200]. Its dual mode of action [195], coupled with the evidence that galanthamine has reduced side effects, make it a promising candidate for the treatment of nervous diseases, paralysis syndrome, schizophrenia and other forms of dementia, as well as Alzheimer's disease [192,195,196].

Other significant pharmacological actions of Galanthamine include an ability to amplify the nerve-muscle transfer [3], affecting membrane ionic processes [201]. It is also known to cause bradycardia or atrioventricular conduction disturbances [150], has long been used as a reversal agent in anaesthetic practice [18], inhibits traumatic shock and has been patented for use in the treatment of nicotine dependence. Besides this, galanthamine acts as a mild analeptic, shows an analgesic power as strong as morphine, compensates for the effects of opiates on respiration, relieves jet lag, fatigue syndrome, male impotence and alcohol dependence, and when applied in eye drops, reduces the intraocular pressure [3,202-204]. It also acts as a hypotensive and has a weak antimalarial activity [138,140].

At present, Alzheimer's disease cannot be prevented or cured, so the symptomatic relief offered by AChEI therapy is the only approved therapeutic option. Due to the relative lack of alternative treatment, galanthamine is a reasonable approximation of the ideal concept of symptomatic Alzheimer's disease therapy [191,205]. Galanthamine hydrobromide (a third-generation cholinesterase inhibitor used against Alzheimer’s disease) offers superior pharmacological profiles and increased tolerance compared to the original

acetylcholinesterase inhibitors, physostigmine or tacrine [193,206-209]. Galanthamine is effective and well tolerated, resulting in short-term improvements in cognition, function and daily life activities in patients with mild to moderate symptoms [198,210,211]. However, there is doubt about its long-term benefits [212] since persistent elevation of acetylcholine beyond 6 months may lead to over-stimulation of both nicotinic and muscarinic acetylcholine receptors, the former causing receptor desensitisation and the latter potentially causing an increased frequency of cholinergic side effects [192,198,213]. The safety profile of galanthamine as well as its clinical effectiveness will only be demonstrated after large-scale clinical trials [213-215].

The development of galanthamine into a widely used Alzheimer's drug can be divided into three main periods: 1- the early development in Eastern Europe for its use in the treatment of poliomyelitis; 2- the pre-clinical development in the 1980s; 3- the clinical development in the 1990s [213]. Galanthamine hydrobromide was first used by Bulgarian and Russian researchers in the 1950s and exploited for a variety of clinical purposes. It has been used clinically for postsurgery reversal of tubocurarine-induced muscle relaxation and for treating post-polio paralysis, *myasthenia gravis* and other neuromuscular diseases, as well as traumatic brain injuries [216,217]. As early as 1972, Soviet researchers demonstrated that galanthamine could reverse scopolamine-induced amnesia in mice, a finding that was demonstrated in man 4 years later. However, this compound was not applied to Alzheimer's disease until 1986, long after the widely accepted cholinergic hypothesis had been first postulated, when researchers in Western Europe switched their attention to galanthamine due to its ability to penetrate the blood-brain barrier and specifically to augment the central cholinergic function [213,218]. This led to clinical trials of galanthamine in the treatment of Alzheimer's disease. In 1996, Sanochemia Pharmazeutika in Austria first launched galanthamine as 'Nivalin®', but its strictly limited availability meant the international pharmaceutical community adopted a cautions approach [18,194], until Sanochemia Pharmazeutika developed a method to synthetically produce the compound in 1997 [219]. Later, galanthamine was co-developed by Shire Pharmaceuticals (Great Britain) and the Janssen Research Foundation (Belgium), who have launched galanthamine as 'Reminyl®' in many countries [192,213]. This renewed interest is reflected in the increasing number of scientific reviews dealing exclusively with galanthamine and its derivatives [220-223].

Sanguinine has a more potent acetylcholinesterase inhibitory activity than galanthamine due to an extra hydroxyl group available for potential interaction with acetylcholinesterase [120]. Sanguinine, in turn, is 10-fold

more selective than galanthamine for acetylcholinesterase (AChE) vs. butyrylcholinesterase (BuChE) [224]. The lack of AChE inhibitory activity of lycoramine and epinorlycoramine could be due to the occurrence of a double bond in ring C, which does not allow these compounds to have the same spatial configuration as the active alkaloids of this series [120].

Narwedine, the biogenic precursor of galanthamine, has been studied as a respiratory stimulator. It increases the amplitude and decreases the frequency of cardiac contractions and would therefore be of value in reducing blood loss during surgery [150]. It also inhibits the action of narcotics and hypnotics, and increases the analgesic effect of morphine [149], as well as the pharmacological effects of caffeine, carbazole, arecoline and nicotine [126].

3.8. Other alkaloids

Cherylline is a 4-arylisoquinoline derivative, a group with several potential medicinal properties [188], including a weak acetylcholinesterase inhibitory activity [118]. Mesembrenone, in turn, is mildly active against Molt 4 lymphoid and non-tumoral fibroblastic LMTK cells [84], has a moderate hypotensive effect on arterial pressure and interacts slightly with DNA [99,140].

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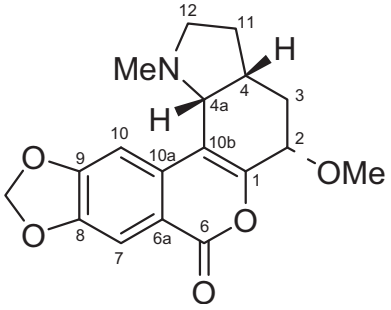
7.2. Anexo II

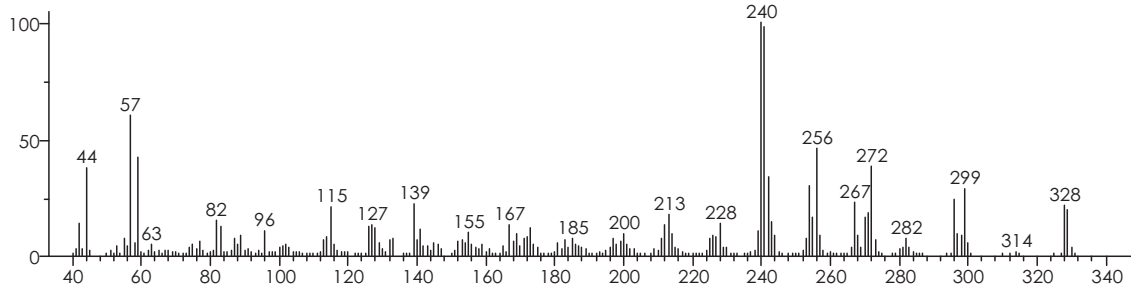
A continuación se adjuntan las tablas con datos de RMN, espectros de masas y espectros de ^{1}H-RMN de los compuestos nuevos caracterizados en el presente trabajo.

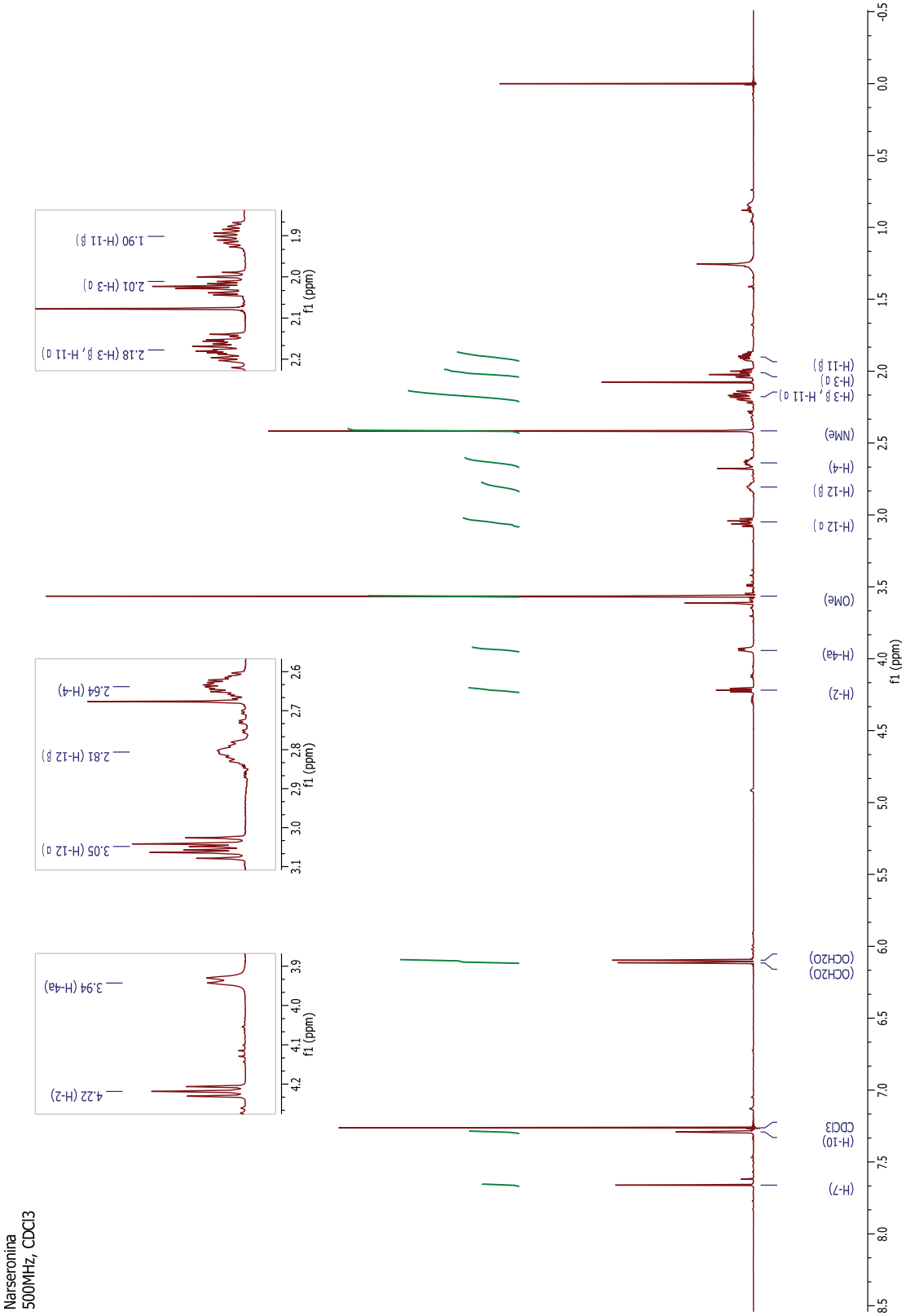
7.2.1. Narseronina

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ	HMBC
1	-	-	-	152.9 *s*	-
2	4.22 *t* (6.1)	H3α/β	H3α/β, OMe	74.9 *d*	C1, C3, C4, C10b, OMe
3α	2.01 *dt* (13.5, 5.5)	H2, H3β, H4	H2, H3β, H4, OMe	31.4 *t*	C1, C2, C4, C4a, C11
3β	2.22 - 2.13 *m (solap.)*	H2, H3α, H4	H2, H3α, H4, OMe	31.4 *t*	C1, C2, C4, C4a, C11
4	2.64 *m*	H3α/β, H4a, H11α/β	H3α/β, H4a, H11α/β	35.1 *d*	C12
4a	3.94 *d* (6.4)	H4	H4, H10, NMe	61.6 *d*	C1, C3, C4, C10a, C10b, C11, C12, NMe
6	-	-	-	161.5 *s*	-
6a	-	-	-	116.4 *s*	-
7	7.66 *s*	-	-	107.8 *d*	C6, C8, C9, C10a
8	-	-	-	148.4 *s*	-
9	-	-	-	153.8 *s*	-
10	7.29 *s*	-	H4a, NMe	103.3 *d*	C6a, C8, C9, C10b
10a	-	-	-	135.1 *s*	-
10b	-	-	-	110.8 *s*	-
11α	2.22 - 2.13 *m (solap.)*	H4, H11β, H12α/β	H4, H11β, H12α/β, OMe	29.6 *t*	C3, C4a
11β	1.90 *ddd* (12.6, 8.3, 4.2)	H4, H11α,H12α/β	H4, H11α, H12α/β, OMe	29.6 *t*	C3, C4a
12α	3.05 *dt* (11.0, 7.6)	H11α/β, H12β	H11α/β, H12β, NMe	54.3 *t*	C4, C4a, C11, NMe
12β	2.81 *m*	H11α/β, H12α	H11α/β, H12α, NMe	54.3 *t*	C4, C4a, C11, NMe
OCH_2O	6.10 *d* (1.2), 6.12 *d* (1.2)	-	-	102.4 *t*	C8, C9
OMe	3.57 *s*	-	H2, H3α/β, H11α/β	58.3 *q*	C2
NMe	2.41 *s*	-	H4a, H10, H12α/β	41.8 *q*	C4a, C12



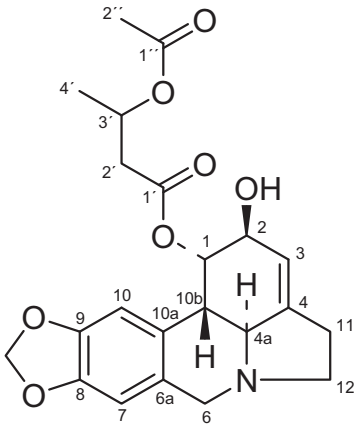
Espectro de Masas



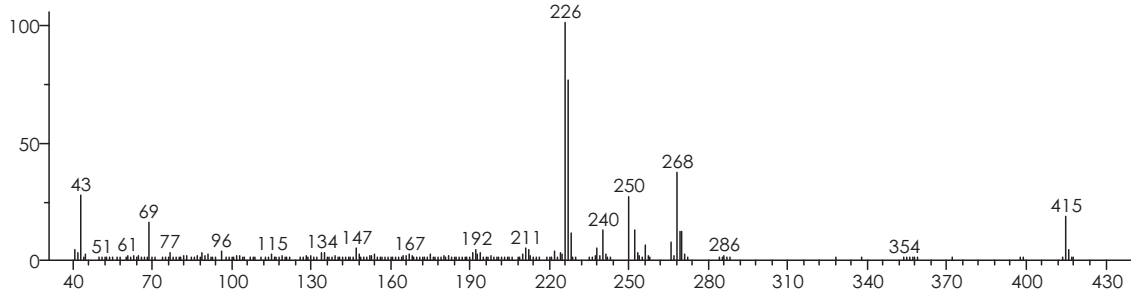

Narseronina
500MHz, CDCl3
4.22 (H-2)
3.94 (H-4a)
3.05 (H-12 α)
2.81 (H-12 β)
2.64 (H-4)
2.18 (H-3 β, H-11 α)
2.01 (H-3 α)
1.90 (H-11 β)
(H-7)
CDCl3
(H-10)
(OCH2O)
(OCH2O)
(H-2)
(H-4a)
(OMe)
(H-12 α)
(H-12 β)
(H-4)
(NMe)
(H-3 β, H-11 α)
(H-3 α)
(H-11 β)
f1 (ppm)

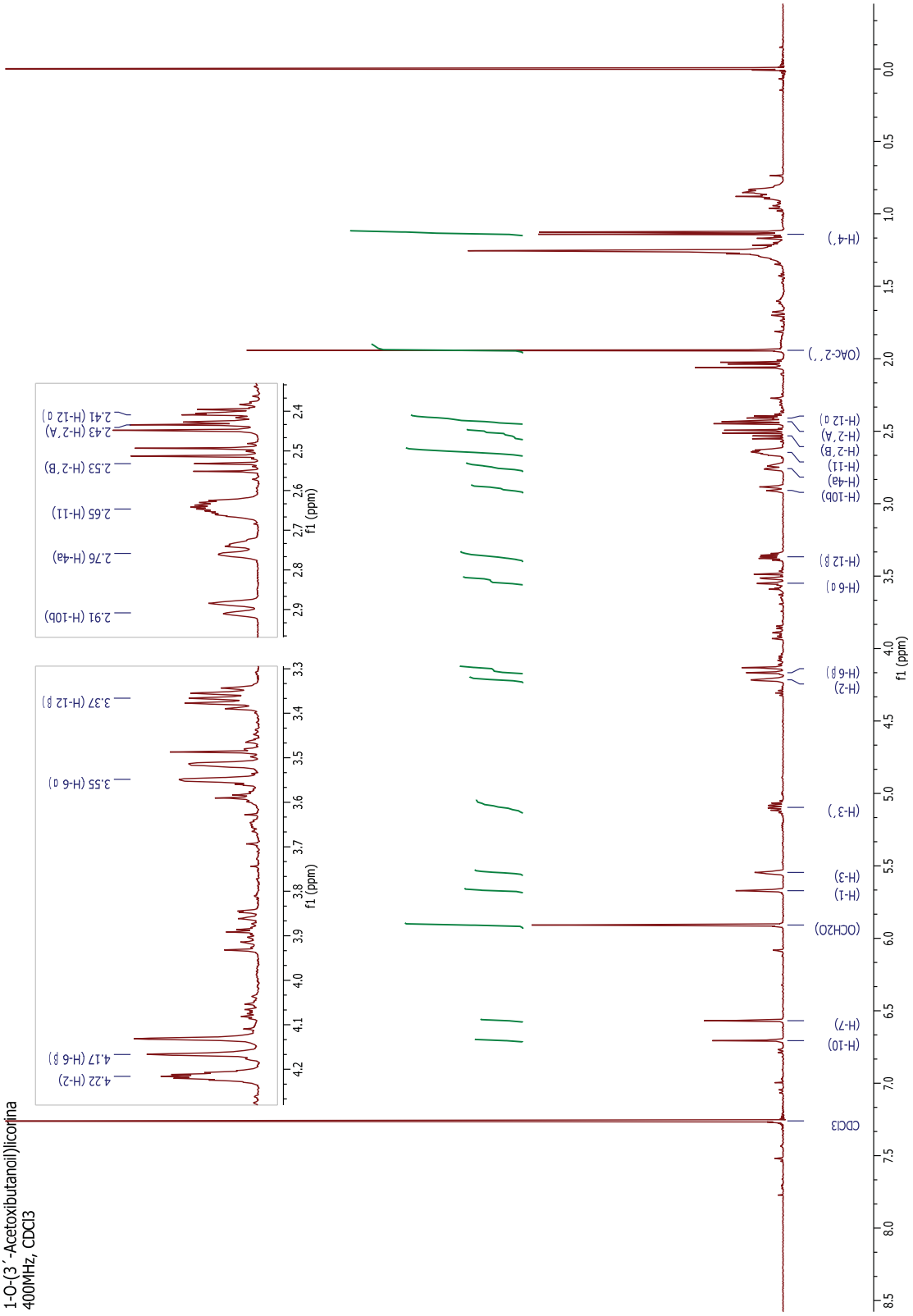
7.2.2. 1-O-(3´-Acetoxibutanoil)licorina

Posición	^{1}H δ (J in Hz)	COSY	^{13}C δ	
1	5.68 *s*	H2, H10b	72.5	*d*
2	4.23 *dt* (3.3, 1.7)	H1, H3, H11	69.4	*d*
3	5.56 *m*	H2, H11	116.9	*d*
4a	2.76 *d* (10.4)	H10b	61.9	*d*
6α	3.54 *d* (14.1)	H6β	56.6	*t*
6β	4.16 *d* (14.1)	H6α	56.6	*t*
7	6.58 *s*	-	107.3	*d*
10	6.72 *s*	-	104.8	*d*
10b	2.91 *d* (10.4)	H1, H4a	38.8	*d*
11 (2H)	2.65 *m*	H2, H3, H12α/β	28.4	*t*
12α	2.42 *dd* (9.3, 5.0)	H11, H12β	53.4	*t*
12β	3.38 *dt* (9.2, 4.8)	H11, H12α	53.4	*t*
OCH_2O	5.92 *s*	-	100.8	*t*
$2'_A$	2.43 *dd* (15.5, 5.4)	$H2'_B$, H3´	40.5	*t*
$2'_B$	2.53 *dd* (15.5, 7.8)	$H2'_A$, H3´	40.5	*t*
3´	5.10 *m*	$H\text{-}2'_A$, $H\text{-}2'_B$, H-4´	66.9	*d*
4´	1.14 *d* (6.3)	H3´	19.3	*q*
OAc (2´´)	1.95 *s*	-	20.7	*q*



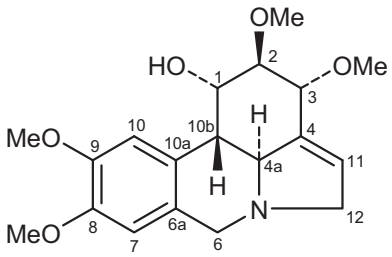
Espectro de Masas



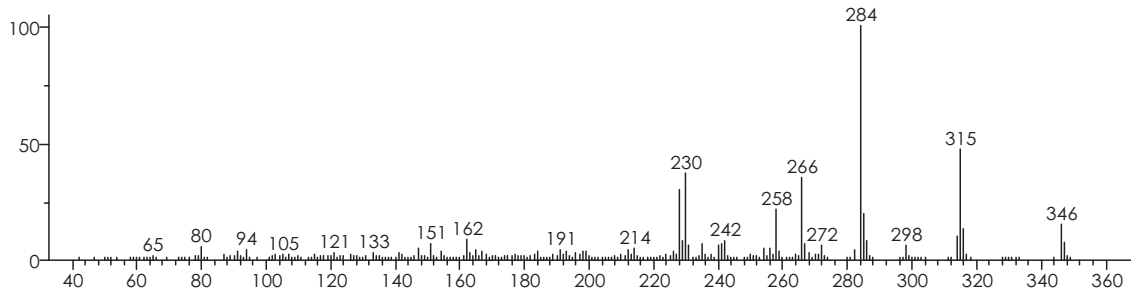

1-O-(3´´-Acetoxibutanoil)licorina
400MHz, CDCl3
CDCl3
(H-10)
(H-7)
(OCH2O)
(H-1)
(H-3)
(H-3´)
(H-2)
(H-6 β)
(H-6 α)
(H-12 β)
(H-10b)
(H-4a)
(H-11)
(H-2´B)
(H-2´A)
(H-12 α)
(OAc-2´´)
(H-4´)
f1 (ppm)
4.22 (H-2)
4.17 (H-6 β)
3.55 (H-6 α)
3.37 (H-12 β)
2.91 (H-10b)
2.76 (H-4a)
2.65 (H-11)
2.53 (H-2´B)
2.43 (H-2´A)
2.41 (H-12 α)

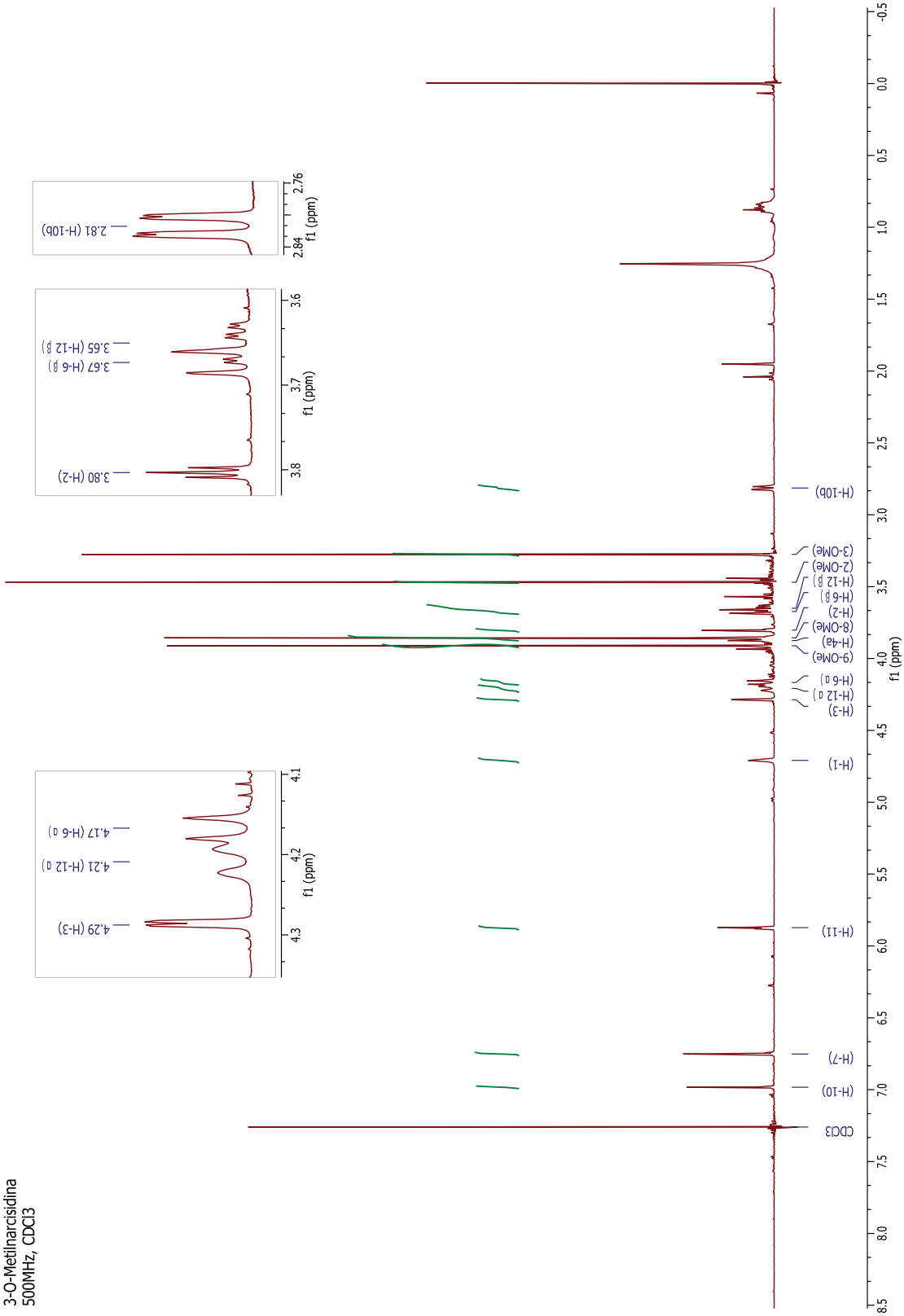
7.2.3. 3-O-Metilnarcisidina

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ	HMBC
1	4.71 *br s*	H2, H3, H10b	H2, H10, H10b, OMe (2)	68.1 *d*	C2, C3, C4a
2	3.80 *t* (2.9)	H1, H3	H1, H3, OMe (2/3)	80.5 *d*	C1, C3, C4, C10b, OMe (2)
3	4.29 *br d* (2.0)	H1, H2	H2, H11, OMe (2/3)	77.8 *d*	C1, C2, C4a, C11, OMe (3)
4	-	-	-	137.2 *s*	-
4a	3.87 *m (solap.)*	H10b, H11, H12β	-	62.6 *d*	C12
6α	4.17 *d* (12.8)	H6β, H7	H6β, H7	54.9 *t*	C4a, C6a, C7, C10a, C12
6β	3.67 *d* (12.4)	H6α, H7	H6α, H7, H10b	54.9 *t*	C4a, C6a, C7, C10a, C12
6a	-	-	-	128.8 *s*	-
7	6.75 *s*	H6α/β, OMe (8)	H6α/β, OMe (8)	111.0 *d*	C6, C9, C10a
8	-	-	-	147.2 *s*	-
9	-	-	-	148.3 *s*	-
10	6.98 *s*	H10b, OMe (9)	H1, H10b, OMe (9)	107.9 *d*	C6a, C8, C10b
10a	-	-	-	130.1 *s*	-
10b	2.81 *dd* (11.2, 1.7)	H1, H4a, H10	H1, H6β, H10, OMe (2)	41.6 *d*	C1, C4a, C10, C10a
11	5.87 *q* (1.8)	H4a, H12α/β	H3, H12α/β, OMe (3)	125.9 *d*	C3, C4, C4a
12α	4.21 *br d* (14.7)	H11, H12β	H11, H12β	62.5 *t*	C4, C4a, C6, C11
12β	3.65 *ddd* (14.5, 6.0, 2.0)	H4a, H11, H12α	H11, H12α	62.5 *t*	C4, C4a, C6, C11
OMe (2)	3.47 *s*	-	H1, H2, H3, H10b	58.4 *q*	C2
OMe (3)	3.27 *s*	-	H2, H3, H11	56.4 *q*	C3
OMe (8)	3.86 *s*	H7	H7	56.3 *q*	C8
OMe (9)	3.91 *s*	H10	H10	56.3 *q*	C9



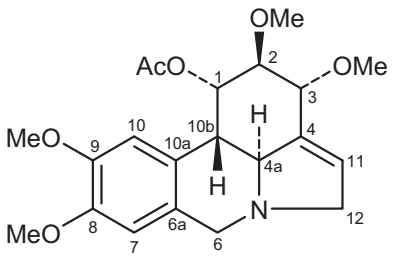
Espectro de Masas



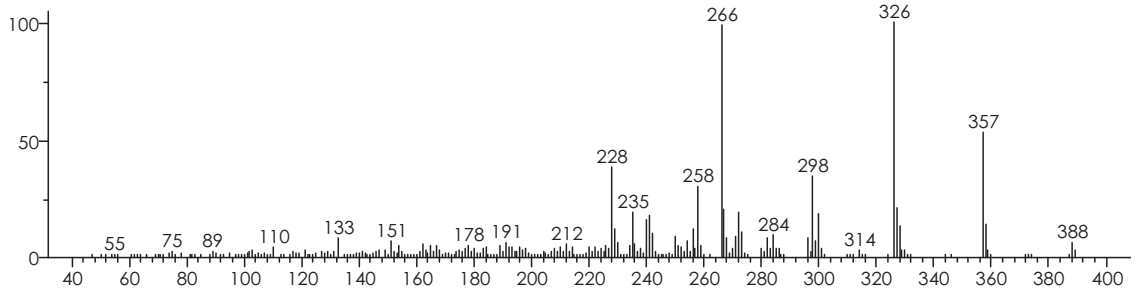

3-O-Metilnarcisidina
500MHz, CDCl3
CDCl3
(H-10)
(H-7)
(H-11)
(H-1)
(H-3)
(H-12 α)
(H-6 α)
(9-OMe)
(H-4a)
(8-OMe)
(H-2)
(H-6 β)
(H-12 β)
(2-OMe)
(3-OMe)
(H-10b)
f1 (ppm)
4.29 (H-3)
4.21 (H-12 α)
4.17 (H-6 α)
3.80 (H-2)
3.67 (H-6 β)
3.65 (H-12 β)
2.81 (H-10b)

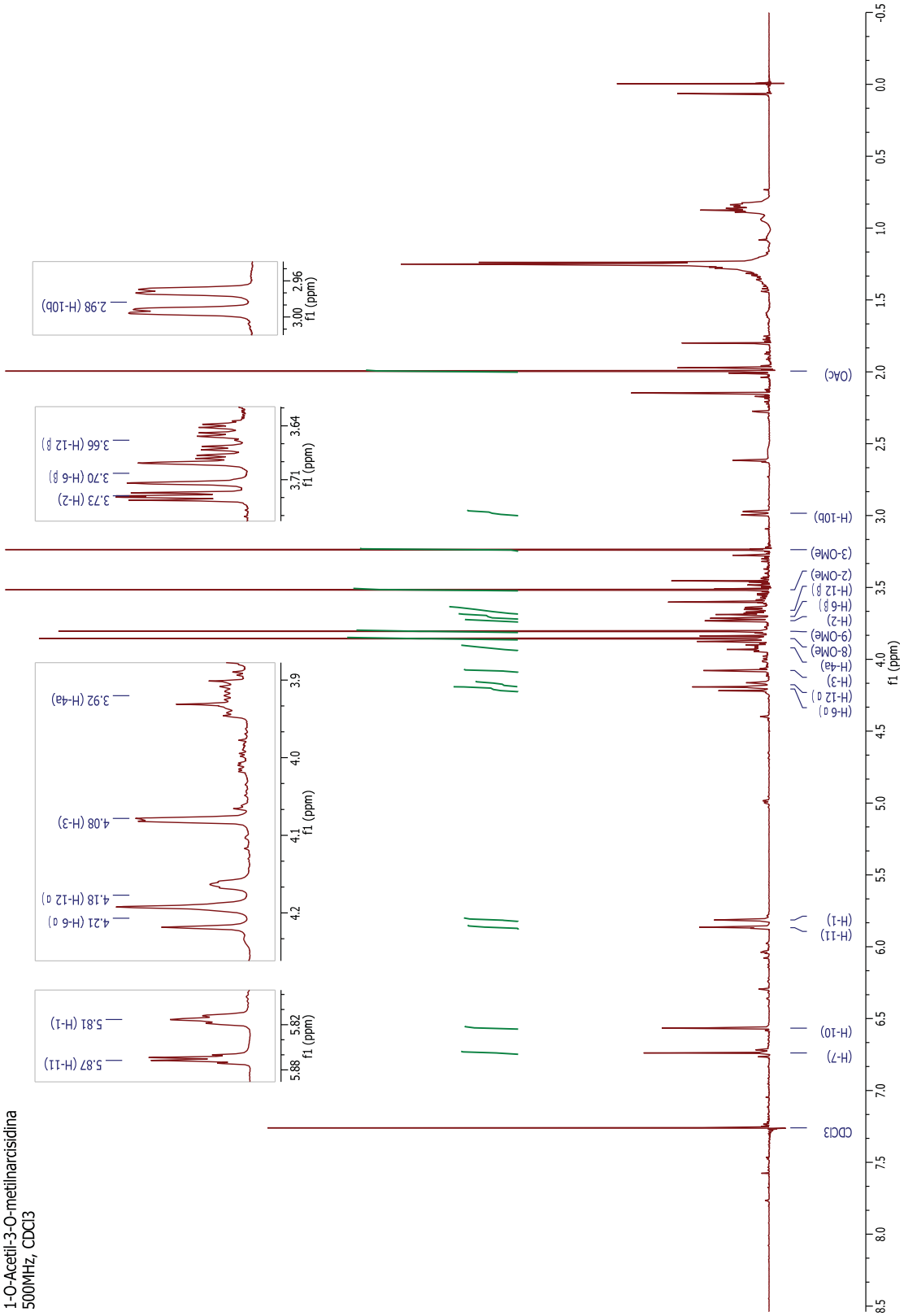
7.2.4. 1-O-Acetil-3-O-metilnarcisidina

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ	HMBC
1	5.81 *br t* (2.6)	H2, H3, H10b	H2, H10, H10b, OMe (2), OCOMe	68.2 *d*	C2, C3, C4a, OCOMe
2	3.73 *dd* (2.8, 2.0)	H1, H3	H1, H3, OMe (2/3), OCOMe	79.1 *d*	C1, C3, C4, C10b, OMe (2)
3	4.08 *br d* (1.7)	H1, H2, H11	H2, H11, OMe (2/3)	76.6 *d*	C1, C2, C4, C4a, C11, OMe (3)
4	-	-	-	137.6 *s*	-
4a	3.92 *m*	H10b, H11, H12β	OMe (3)	62.7 *d*	-
6α	4.21 *d* (13.1)	H6β, H7	H6β, H7	54.5 *t*	C4a, C7, C10a, C12
6β	3.70 *d* (13.0)	H6α, H7	H6α, H7, H10b	54.5 *t*	C4a, C7, C10a, C12
6a	-	-	-	128.4 *s*	-
7	6.74 *s*	H6α/β, OMe (8)	H6α/β, OMe (8)	110.8 *d*	C6, C9, C10, C10a, C10b
8	-	-	-	147.4 *s*	-
9	-	-	-	148.2 *s*	-
10	6.57 *s*	H10b, OMe (9)	H1, H10b, OMe (9), OCOMe	107.0 *d*	C6, C6a, C7, C8, C10b
10a	-	-	-	128.5 *s*	-
10b	2.98 *dd* (11.0, 2.0)	H1, H4a, H10	H1, H6β, H10, OMe (2)	39.8 *d*	C4, C4a, C6a, C8, C10, C10a
11	5.87 *q* (1.8)	H3, H4a, H12α/β	H3, H12α/β, OMe (3)	125.9 *d*	C3, C4, C4a, C12
12α	4.18 *m (solap.)*	H11, H12β	H11, H12β	62.1 *t*	C4, C6, C10b, C11
12β	3.66 *ddd* (14.4, 5.7, 2.1)	H4a, H11, H12α	H11, H12α	62.1 *t*	C4, C6, C10b, C11
OMe (2)	3.52 *s*	-	H1, H2, H3, H10b	58.7 *q*	C2
OMe (3)	3.24 *s*	-	H2, H3, H4a, H11, OCOMe	56.3 *q*	C3
OMe (8)	3.86 *s*	H7	H7	56.1 *q*	C8
OMe (9)	3.81 *s*	H10	H10, OCOMe	56.1 *q*	C9
OCOMe	1.99 *s*	-	H1, H2, H10, OMe (3/9)	21.2 *q*	C1, OCOMe
OCOMe	-	-	-	171.3 *s*	-



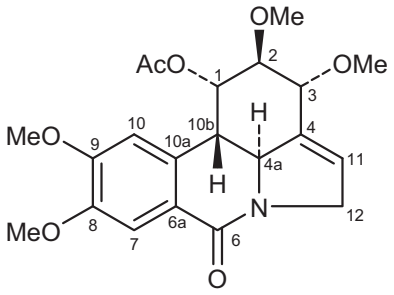
Espectro de Masas



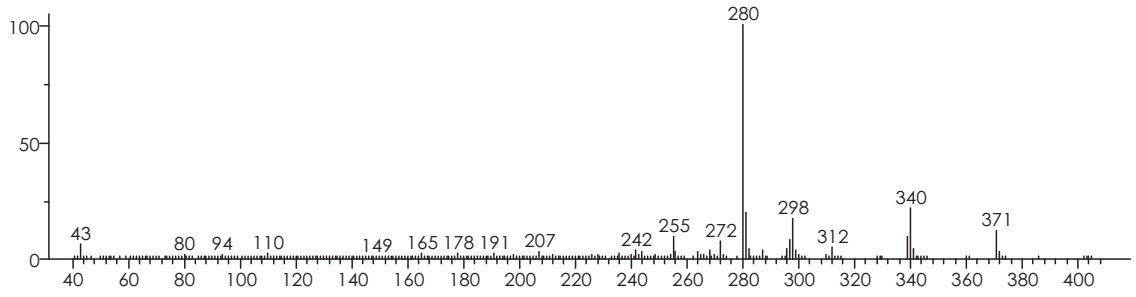

1-O-Acetil-3-O-metilnarcisidina
500MHz, CDCl3
f1 (ppm)
CDCl3
(H-7)
(H-10)
(H-11)
(H-1)
(H-6 α)
(H-12 α)
(H-3)
(H-4a)
(8-OMe)
(9-OMe)
(H-2)
(H-6 β)
(H-12 β)
(2-OMe)
(3-OMe)
(H-10b)
(OAc)
5.87 (H-11)
5.81 (H-1)
4.21 (H-6 α)
4.18 (H-12 α)
4.08 (H-3)
3.92 (H-4a)
3.73 (H-2)
3.70 (H-6 β)
3.66 (H-12 β)
2.98 (H-10b)

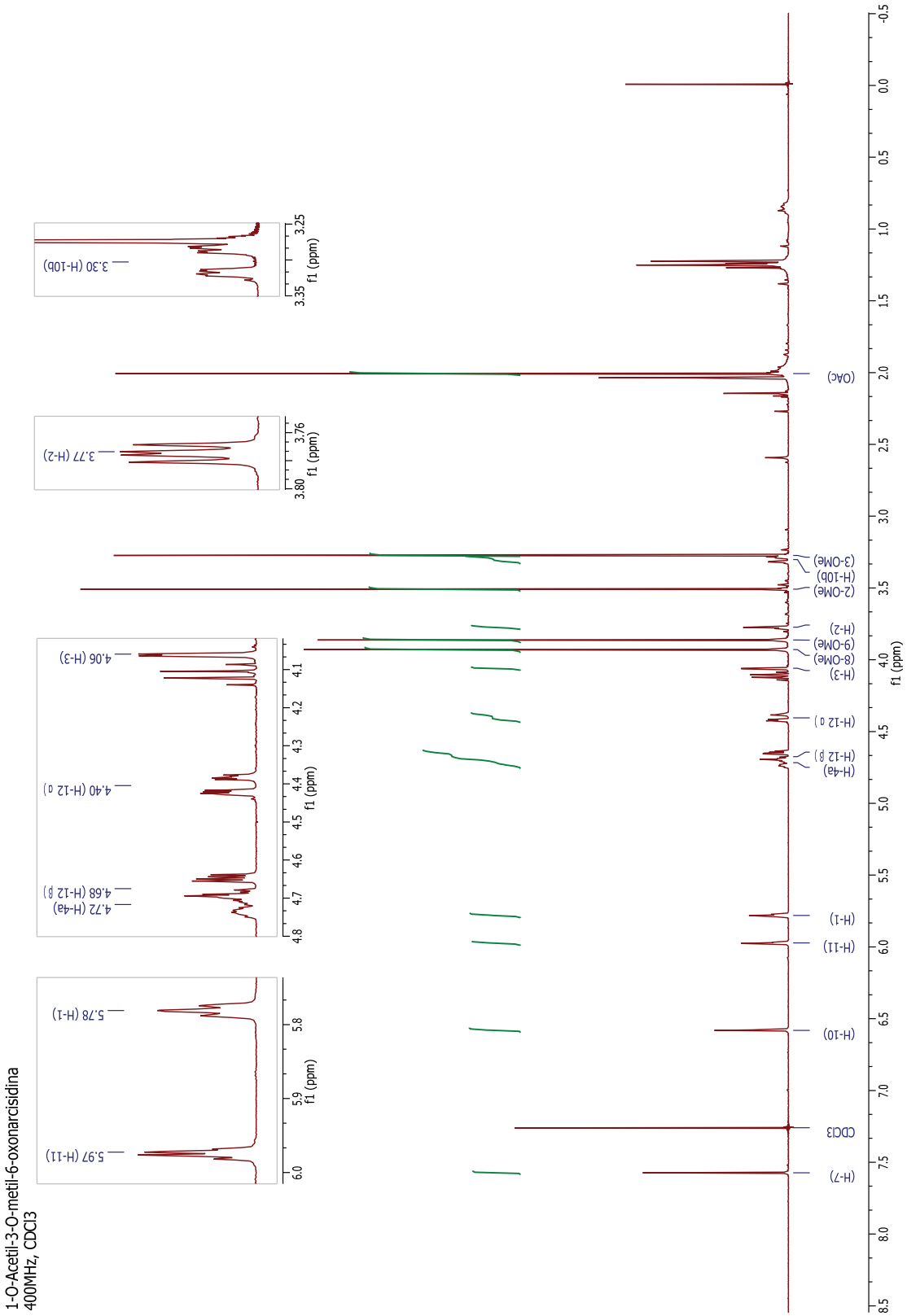
7.2.5. 1-O-Acetil-3-O-metil-6-oxonarcisidina

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ		HMBC
1	5.78 *br t* (2.7)	H2, H3, H10b	H2, H10, H10b, OMe (2), OCOMe	66.8	*d*	C2, C3, C4a, OCOMe
2	3.77 *dd* (2.9, 2.0)	H1, H3	H1, H3, OMe (2/3), OCOMe	79.1	*d*	C1, C3, C4, C10b, OMe (2)
3	4.06 *br d* (1.9)	H1, H2	H2, H11, OMe (2/3)	75.8	*d*	C1, C2, C4a, C11, OMe (3)
4	-	-	-	136.1	*s*	-
4a	4.72 *m*	H10b, H11, H12α/β	H10b, H12α, OMe (3), OCOMe	60.1	*d*	C4, C11
6	-	-	-	162.6	*s*	-
6a	-	-	-	130.8	*s*	-
7	7.57 *s*	OMe (8)	OMe (8)	111.4	*d*	C6, C6a, C8, C9, C10a
8	-	-	-	148.0	*s*	-
9	-	-	-	151.9	*s*	-
10	6.58 *d* (0.8)	H10b, OMe (9)	H1, H10b, OMe (9), OCOMe	105.7	*d*	C6, C8, C9, C10a, C10b
10a	-	-	-	124.6	*s*	-
10b	3.30 *ddd* (12.8, 2.5, 0.9)	H1, H4a, H10	H1, H4a, H10, OMe (2)	41.6	*d*	C4a, C6a
11	5.97 *q* (1.8)	H4a, H12α/β	H3, H12α/β, OMe (3)	125.4	*d*	C3, C4a, C12
12α	4.40 *ddd* (16.0, 3.2, 1.6)	H4a, H11, H12β	H4a, H11, H12β	52.6	*t*	C4, C11
12β	4.68 *ddd* (16.1, 5.1, 2.0)	H4a, H11, H12α	H11, H12α	52.6	*t*	C4, C11
OMe (2)	3.51 *s*	-	H1, H2, H3, H10b, OCOMe	58.9	*q*	C2
OMe (3)	3.27 *s*	-	H2, H3, H4a, H11, OCOMe	56.5	*q*	C3
OMe (8)	3.93 *s*	H7	H7	56.2	*q*	C8
OMe (9)	3.86 *s*	H10	H10, OCOMe	56.2	*q*	C9
OCOMe	2.01 *s*	-	H1, H2, H4a, H10, OMe (2/3/9)	21.1	*q*	OCOMe
OCOMe	-	-	-	171.1	*s*	-



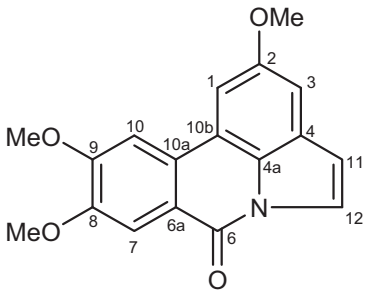
Espectro de Masas



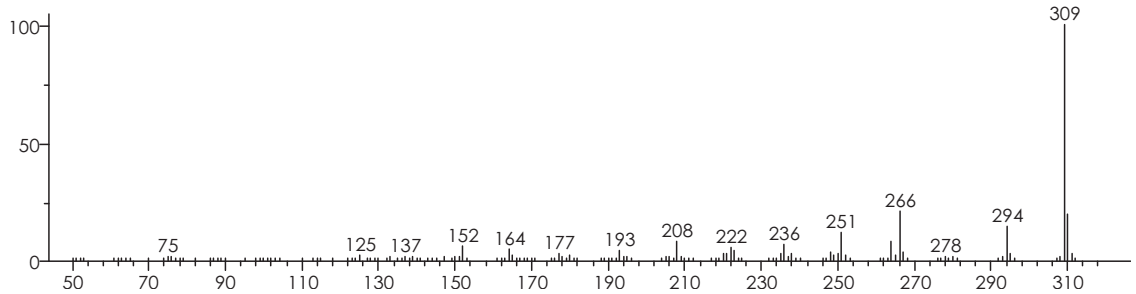

1-O-Acetil-3-O-metil-6-oxonarcisidina
400MHz, CDCl3
5.97 (H-11)
5.78 (H-1)
4.72 (H-4a)
4.68 (H-12 β)
4.40 (H-12 α)
4.06 (H-3)
3.77 (H-2)
3.30 (H-10b)
(OAc)
(3-OMe)
(H-10b)
(2-OMe)
(H-2)
(9-OMe)
(8-OMe)
(H-3)
(H-12 α)
(H-4a)
(H-12 β)
(H-1)
(H-11)
(H-10)
CDCl3
(H-7)
f1 (ppm)

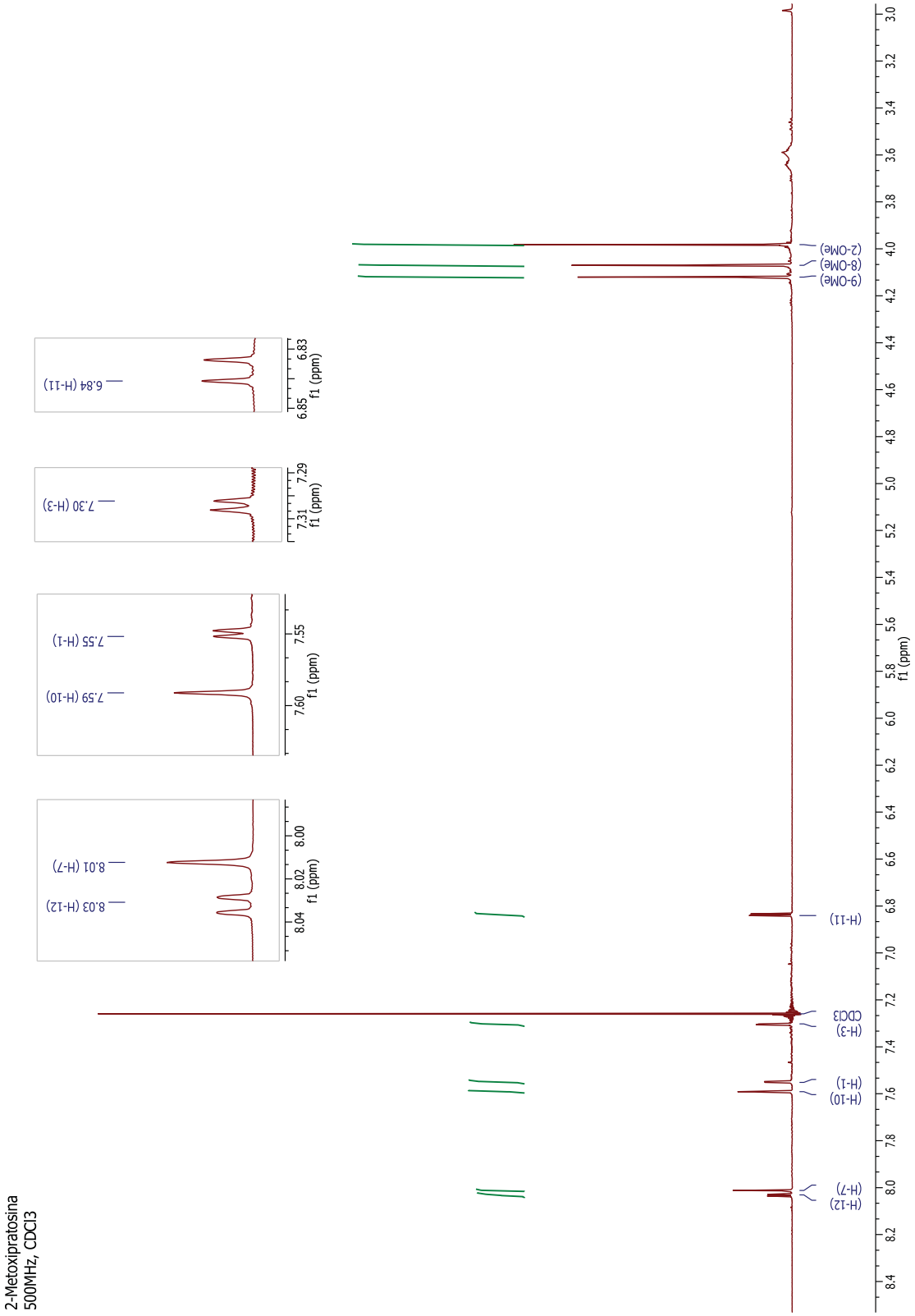
7.2.6. 2-Metoxipratosina

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ		HMBC
1	7.55 *d* (2.0)	H3	OMe (2)	106.2	*d*	C2, C3, C4a, C10a
2	-	-	-	157.7	*s*	-
3	7.30 *d* (2.0)	H1	H11, OMe (2)	106.9	*d*	C1, C2, C4a, C11
4	-	-	-	129.1	*s*	-
4a	-	-	-	126.6	*s*	-
6	-	-	-	158.3	*s*	-
6a	-	-	-	121.2	*s*	-
7	8.01 *s*	OMe (8)	OMe (8)	110.4	*d*	C6, C6a, C8, C9, C10a
8	-	-	-	149.9	*s*	-
9	-	-	-	153.7	*s*	-
10	7.59 *s*	OMe (9)	OMe (9)	104.1	*d*	C6a, C8, C9, C10b
10a	-	-	-	129.3	*s*	-
10b	-	-	-	117.1	*s*	-
11	6.84 *d* (3.5)	H12	H3, H12	110.7	*d*	C4, C4a
12	8.03 *d* (3.5)	H11	H11	124.2	*d*	-
OMe (2)	3.98 *s*	-	H1, H3	56.5	*q*	C2
OMe (8)	4.07 *s*	H7	H7	56.4	*q*	C8
OMe (9)	4.12 *s*	H10	H10	56.4	*q*	C9



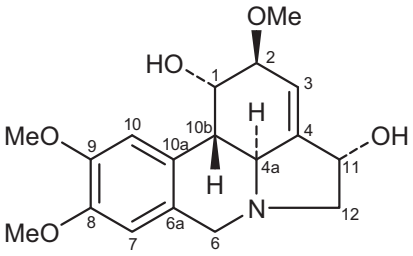
Espectro de Masas



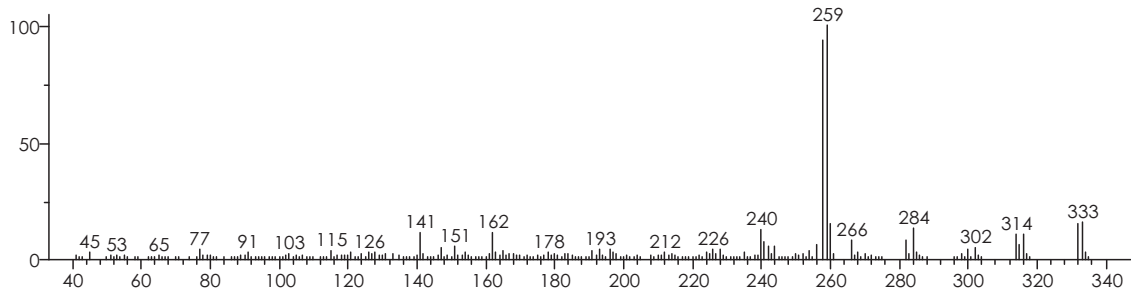

2-Metoxipratosina
500MHz, CDCl3
(9-OMe)
(8-OMe)
(2-OMe)
(H-11)
CDCl3
(H-3)
(H-1)
(H-10)
(H-12)
(H-7)
f1 (ppm)
6.84 (H-11)
7.30 (H-3)
7.55 (H-1)
7.59 (H-10)
8.01 (H-7)
8.03 (H-12)

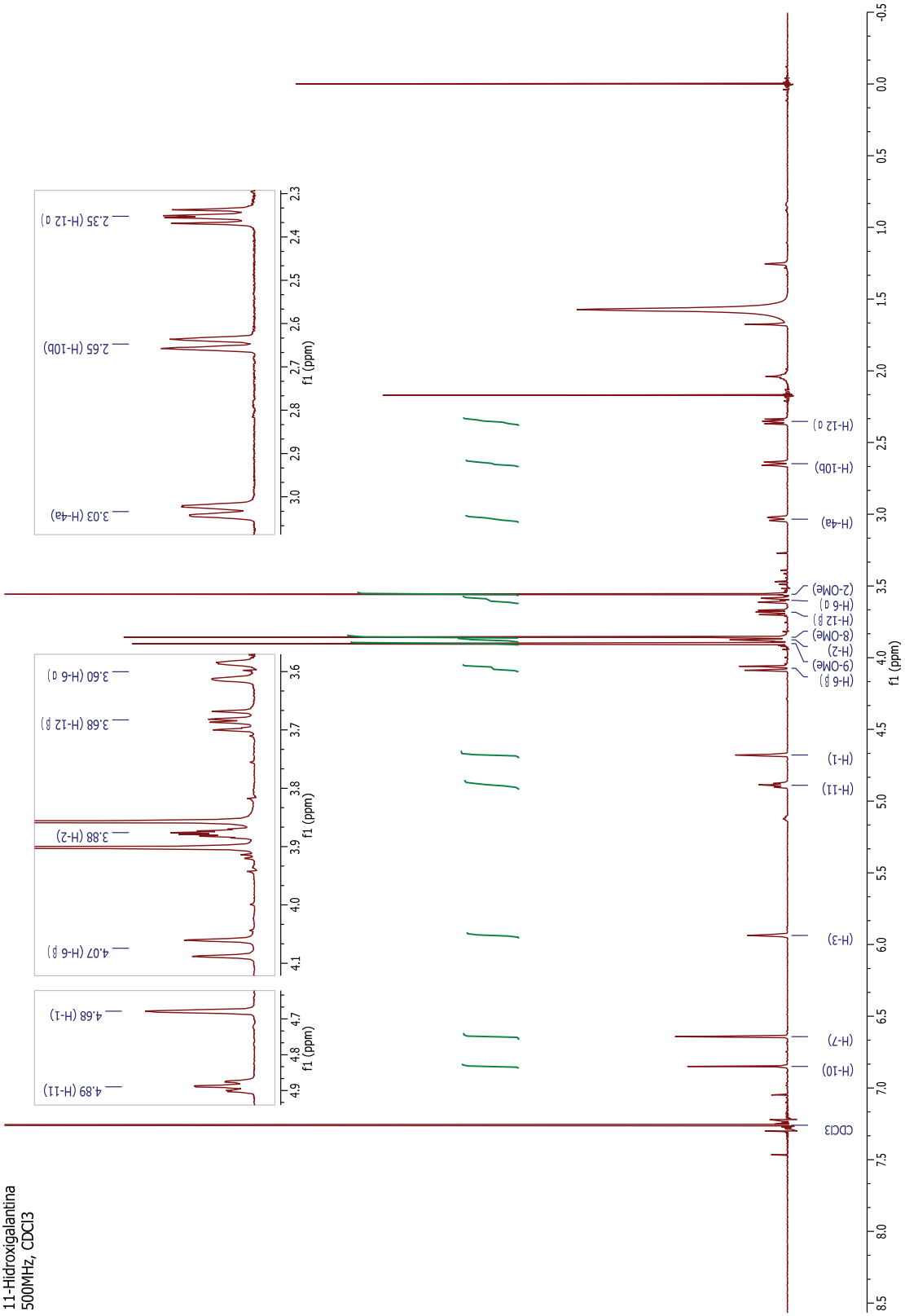
7.2.7. 11-Hidroxigalantina

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ		HMBC
1	4.68 *br s*	H2, H3, H10b	H2, H10, H10b, OMe (2)	69.0	*d*	C2, C3, C4a
2	3.88 *ddd* (3.0, 3.0, 1.5)	H1, H3, H4a, H11	H1, H3, OMe (2)	80.9	*d*	C1, C4, OMe (2)
3	5.94 *m*	H1, H2, H4a, H11	H2, H11, OMe (2)	119.4	*d*	C4a
4	-	-	-	146.0	*s*	-
4a	3.03 *dd* (10.5, 1.4)	H2, H3, H10b, H11	H6α, H12α	59.8	*d*	C4
6α	3.60 *br d* (14.0)	H6β, H7, H10b	H4a, H6β, H7, H12α	56.1	*t*	C4a, C6a, C10a, C12
6β	4.07 *d* (13.9)	H6α, H7, H10b	H6α, H7, H10b, H12β	56.1	*t*	C4a, C6a, C7, C10a
6a	-	-	-	129.4	*s*	-
7	6.64 *s*	H6α/β, OMe (8)	H6α/β, OMe (8)	111.0	*d*	C6, C9, C10a
8	-	-	-	148.0	*s*	-
9	-	-	-	148.1	*s*	-
10	6.85 *s*	H10b, OMe (9)	H1, H10b, OMe (9)	107.5	*d*	C6a, C8, C10b
10a	-	-	-	125.9	*s*	-
10b	2.65 *br d* (10.6)	H1, H4a, H6α/β , H10	H1, H6β, H10	41.7	*d*	C4a, C10a
11	4.89 *br ddd* (6.5, 1.5)	H2, H3, H4a, H12α/β	H3, H12α/β	71.6	*d*	C3, C4
12α	2.35 *dd* (9.2, 6.7)	H11, H12β	H4a, H6α, H11, H12β	63.2	*t*	C6, C11
12β	3.68 *dd* (9.2, 6.5)	H11, H12α	H6β, H11, H12α	63.2	*t*	C4, C4a, C11
OMe (2)	3.56 *s*	-	H1, H2, H3	58.1	*q*	C2
OMe (8)	3.86 *s*	H7	H7	56.1	*q*	C8
OMe (9)	3.90 *s*	H10	H10	56.3	*q*	C9



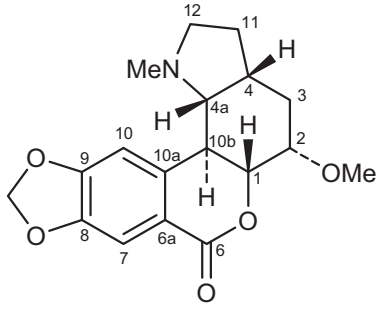
Espectro de Masas



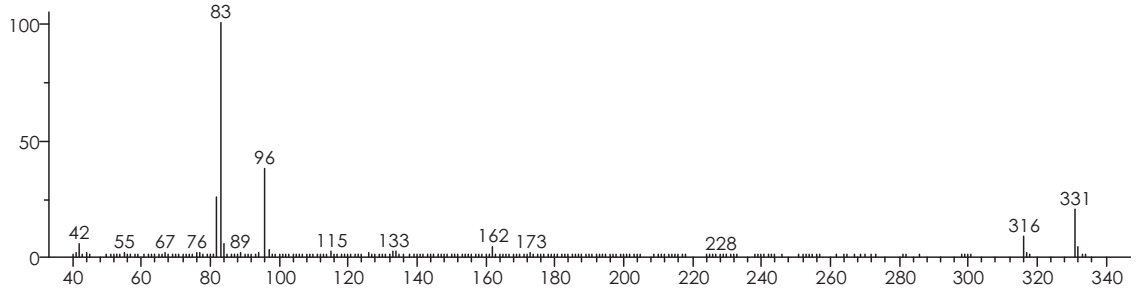

11-Hidroxigalantina
500MHz, CDCl3
CDCl3
(H-7)
(H-10)
(H-3)
(H-1)
(H-11)
(H-6 β)
(9-OMe)
(H-2)
(8-OMe)
(H-12 β)
(H-6 α)
(2-OMe)
(H-4a)
(H-10b)
(H-12 α)
f1 (ppm)
4.89 (H-11)
4.68 (H-1)
4.07 (H-6 β)
3.88 (H-2)
3.68 (H-12 β)
3.60 (H-6 α)
3.03 (H-4a)
2.65 (H-10b)
2.35 (H-12 α)

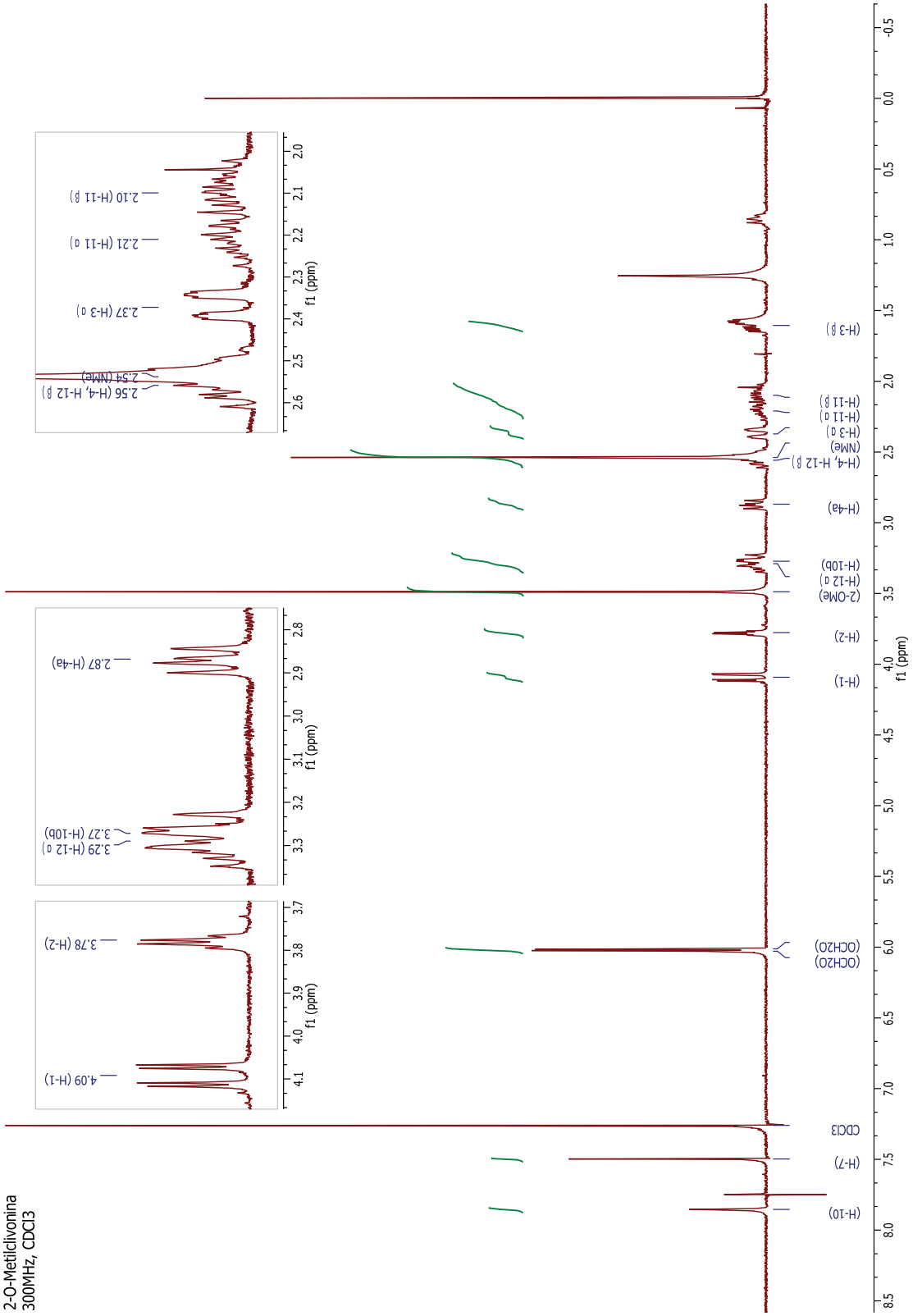
7.2.8. 2-O-Metilclivonina

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ	HMBC
1	4.09 *dd* (12.6, 2.6)	H2, H10b	H2, H3β, H4a	81.4 *d*	-
2	3.78 *q* (2.9)	H1, H3α/β	H1, H3α/β, OMe (2)	76.8 *d*	C1, C4, OMe (2)
3α	2.37 *ddd* (15.5, 2.8, 1.6)	H2, H3β, H4	H2, H3β, OMe (2)	26.3 *t*	C1, C2, C4, C4a, C11
3β	1.61 *ddd* (15.4, 6.5, 3.1)	H2, H3α, H4	H1, H2, H3α, H4	26.3 *t*	C4, C11
4	2.62-2.46 *m (solap.)*	H3α/β	H3β, H-4a, H11β	33.6 *d*	-
4a	2.87 *dd* (9.8, 6.7)	H10b	H1, H4, H10, NMe	70.3 *d*	-
6	-	-	-	164.9 *s*	-
6a	-	-	-	118.9 *s*	-
7	7.50 *s*	-	-	109.4 *d*	C6, C6a, C8, C9, C10a
8	-	-	-	146.8 *s*	-
9	-	-	-	152.6 *s*	-
10	7.86 br *s*	-	H4a, NMe	107.4 *d*	-
10a	-	-	-	141.1 *s*	-
10b	3.27 *dd* (12.8, 9.5)	H1, H4a	H11α, OMe (2)	34.2 *d*	-
11α	2.29-2.15 *m*	H11β, H12α/β	H10b, H11β, H12α, OMe (2)	30.5 *t*	-
11β	2.15-2.02 *m*	H11α, H12α/β	H4, H11α , H12β	30.5 *t*	-
12α	3.35-3.21 *m (solap.)*	H11α/β, H12β	H11α, H12β	53.1 *t*	-
12β	2.62-2.46 *m (solap.)*	H11α/β, H12α	H11β, H12α	53.1 *t*	-
OMe (2)	3.49 *s*	-	H2, H3α, H10b, H11α	58.2 *q*	C2
NMe	2.54 *s*	-	H4a, H10	45.5 *q*	-
OCH_2O	6.02 *d* (1.3) - 6.03 *d* (1.3)	-	-	101.9 *t*	C8, C9



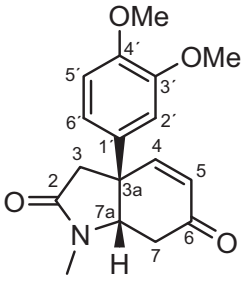
Espectro de Masas



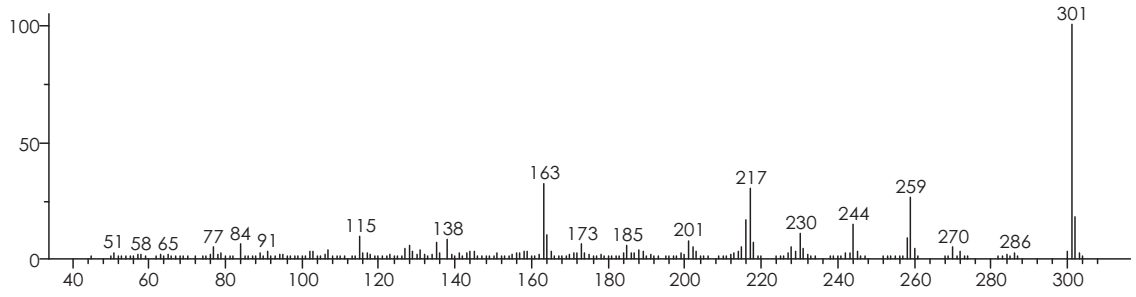

2-O-Metilclivonina
300MHz, CDCl3
(H-10)
(H-7)
CDCl3
(OCH2O)
(H-1)
(H-2)
(2-OMe)
(H-12 α)
(H-10b)
(H-4a)
(H-4, H-12 β)
(NMe)
(H-3 α)
(H-11 α)
(H-11 β)
(H-3 β)
f1 (ppm)
4.09 (H-1)
3.78 (H-2)
3.29 (H-12 α)
3.27 (H-10b)
2.87 (H-4a)
2.56 (H-4, H-12 β)
2.54 (NMe)
2.37 (H-3 α)
2.21 (H-11 α)
2.10 (H-11 β)

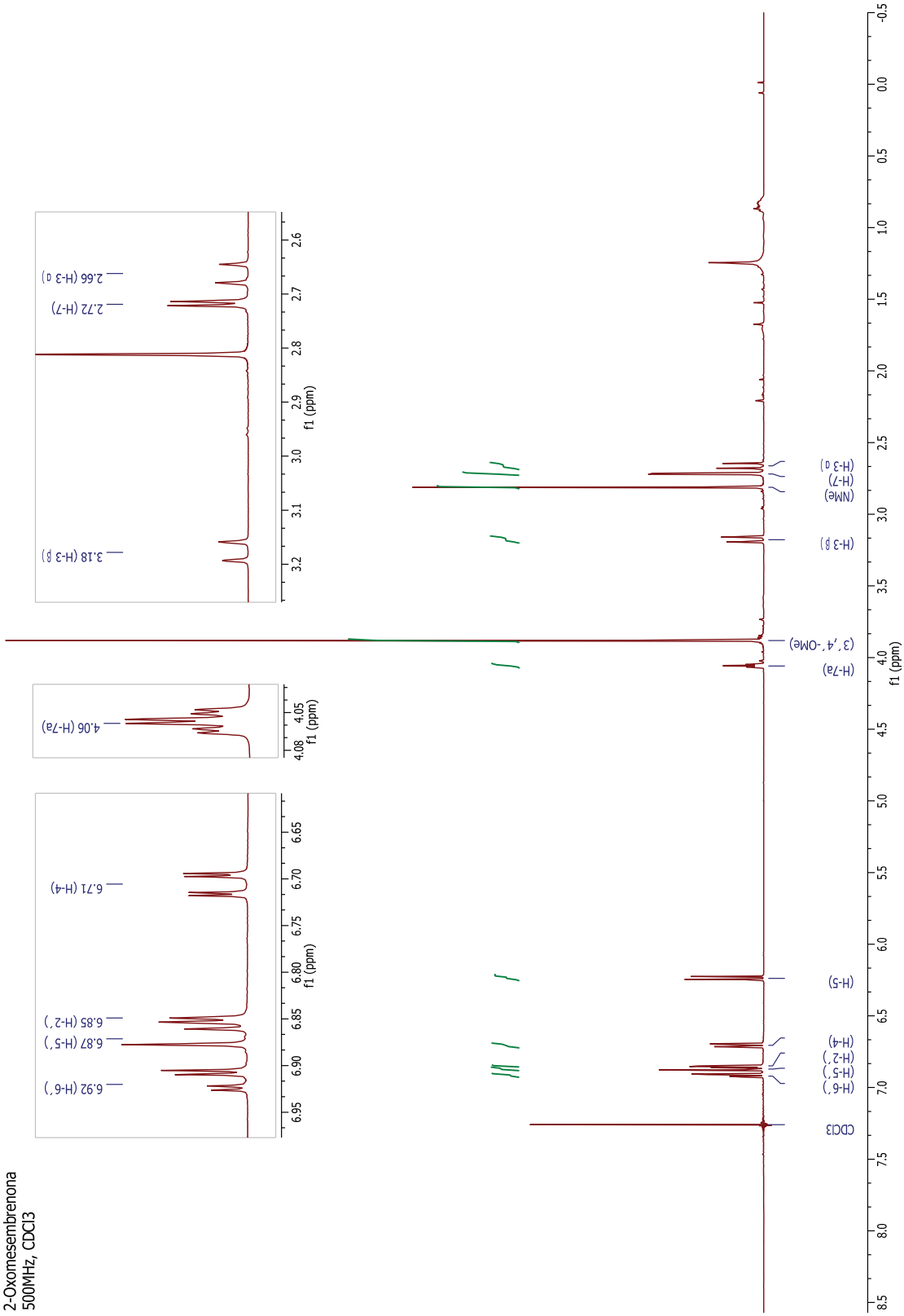
7.2.9. 2-Oxomesembrenona

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ		HMBC
2	-	-	-	171.9	*s*	-
3α	2.66 *d* (17.1)	H3β	H3β, H4	44.2	*t*	C2, C3a, C4, C7a, C1´
3β	3.18 *d* (17.1)	H3α	H3α, H7a, H2´, H6´	44.2	*t*	C2, C3a, C4, C1´
3a	-	-	-	45.9	*s*	-
4	6.71 *dd* (10.2, 1.6)	H5, H7a	H3α, H5, H2´, H6´	150.7	*d*	C3, C3a, C6, C7a, C1´
5	6.24 *d* (10.2)	H4	H4	129.0	*d*	C3a, C7
6	-	-	-	195.3	*s*	-
7 (2H)	2.72 *d* (3.8)	H7a	H7a	36.5	*t*	C3a, C5, C6, C7a
7a	4.06 *td* (3.8, 1.6)	H7, H4	H3β, H7, H2´, H6´, NMe	65.7	*d*	C3a, C4, C6, C1´
1´	-	-	-	131.6	*s*	-
2´	6.85 *d* (2.2)	H6´	H3β, H4, H7a	109.9	*d*	C3a, C4´, C6´
3´	-	-	-	149.7	*s*	-
4´	-	-	-	149.1	*s*	-
5´	6.87 *d* (8.4)	H6´	OMe	111.6	*d*	C1´, C3´
6´	6.92 *dd* (8.4, 2.2)	H2´, H5´	H3β, H4, H7a	119.4	*d*	C3a, C2´, C4´
NMe	2.81 *s*	-	H7a	27.4	*q*	C2, C7a
OMe (3´, 4´)	3.88 *s*	-	H5´	56.1, 56.2	*q*	C3´, C4´



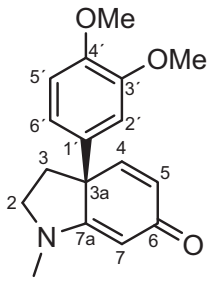
Espectro de Masas



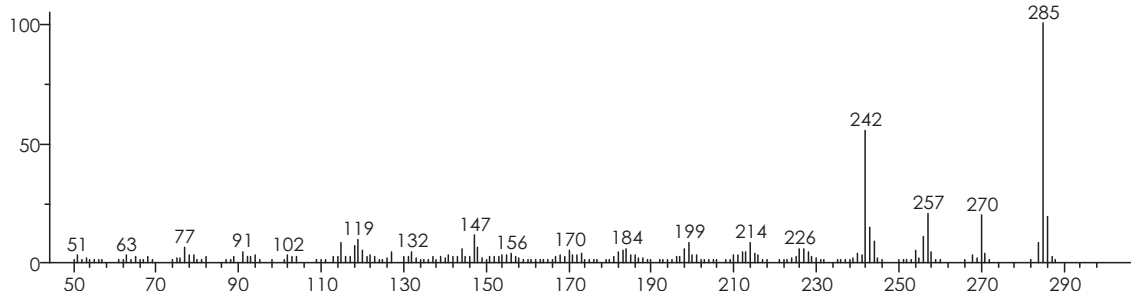

2-Oxomesembrenona
500MHz, CDCl3
f1 (ppm)
CDCl3
(H-6')
(H-5')
(H-2')
(H-4)
(H-5)
(H-7a)
(3',4'-OMe)
(H-3 β)
(NMe)
(H-7)
(H-3 α)
6.92 (H-6')
6.87 (H-5')
6.85 (H-2')
6.71 (H-4)
4.06 (H-7a)
3.18 (H-3 β)
2.72 (H-7)
2.66 (H-3 α)

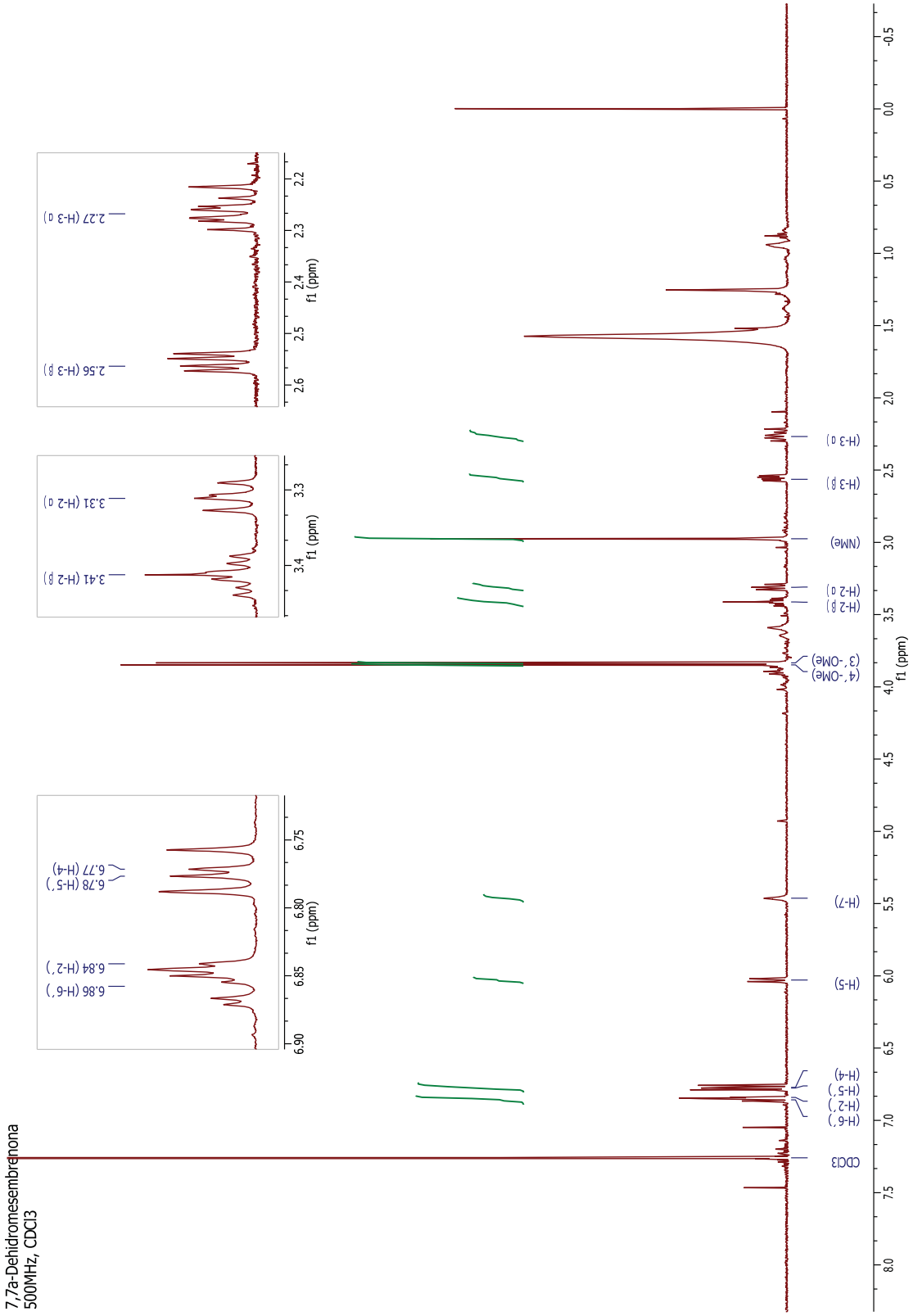
7.2.10. 7,7a-Dehidromesembrenona

Posición	^{1}H δ (J in Hz)	COSY	^{13}C δ	HMBC
2α	3.31 *dd* (10.3, 8.1)	H2β, H3α	52.7 *t*	C3, C3a
2β	3.41 *ddd* (10.6, 10.3, 5.0)	H2α, H3α/β	52.7 *t*	-
3α	2.27 *ddd* (11.7, 10.8, 8.1)	H2α/β, H3β	35.9 *t*	C3a, C1´
3β	2.56 *dd* (11.9, 5.0)	H2β, H3α	35.9 *t*	C3a, C7a, C1´
3a	-	-	53.5 *s*	-
4	6.77 *d* (9.6)	H5	142.8 *d*	C3a, C6, C7a, C1´
5	6.03 *dd* (9.6, 1.4)	H4	128.8 *d*	C3a
6	-	-	185.6 *s*	-
7	5.46 *br s*	-	93.7 *d*	-
7a	-	-	171.5 *s*	-
1´	-	-	133.2 *s*	-
2´	6.84 *d* (2.2)	H6´	109.9 *d*	C3a, C4´, C6´
3´	-	-	149.1 *s*	-
4´	-	-	148.6 *s*	-
5´	6.78 *d* (8.3)	H6´	111.3 *d*	C1´, C3´
6´	6.86 *dd* (8.3, 2.3)	H2´, H5´	118.4 *d*	C3a, C2´, C4´
NMe	2.98 *s*	-	33.1 *q*	C2, C7a
OMe (3´)	3.84 *s*	-	56.2 *q*	C3´
OMe (4´)	3.85 *s*	-	56.1 *q*	C4´



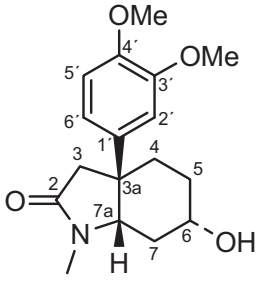
Espectro de Masas



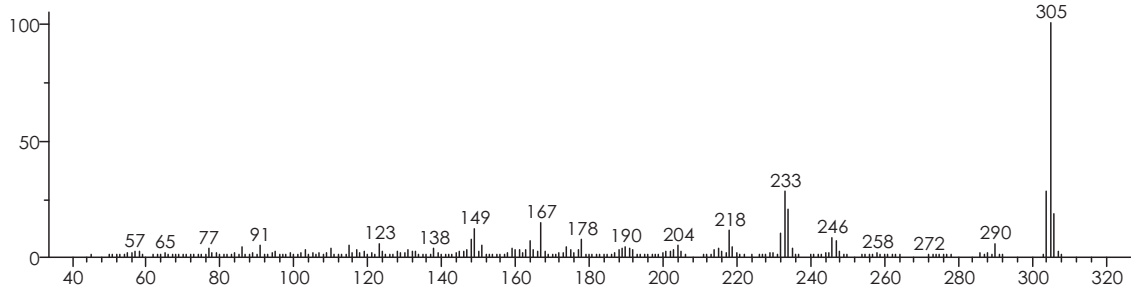

7,7a-Dehidromesembrenona
500MHz, CDCl3
CDCl3
(H-6')
(H-2')
(H-5')
(H-4)
(H-5)
(H-7)
(4'-OMe)
(3'-OMe)
(H-2 β)
(H-2 α)
(NMe)
(H-3 β)
(H-3 α)
6.86 (H-6')
6.84 (H-2')
6.78 (H-5')
6.77 (H-4)
3.41 (H-2 β)
3.31 (H-2 α)
2.56 (H-3 β)
2.27 (H-3 α)
f1 (ppm)

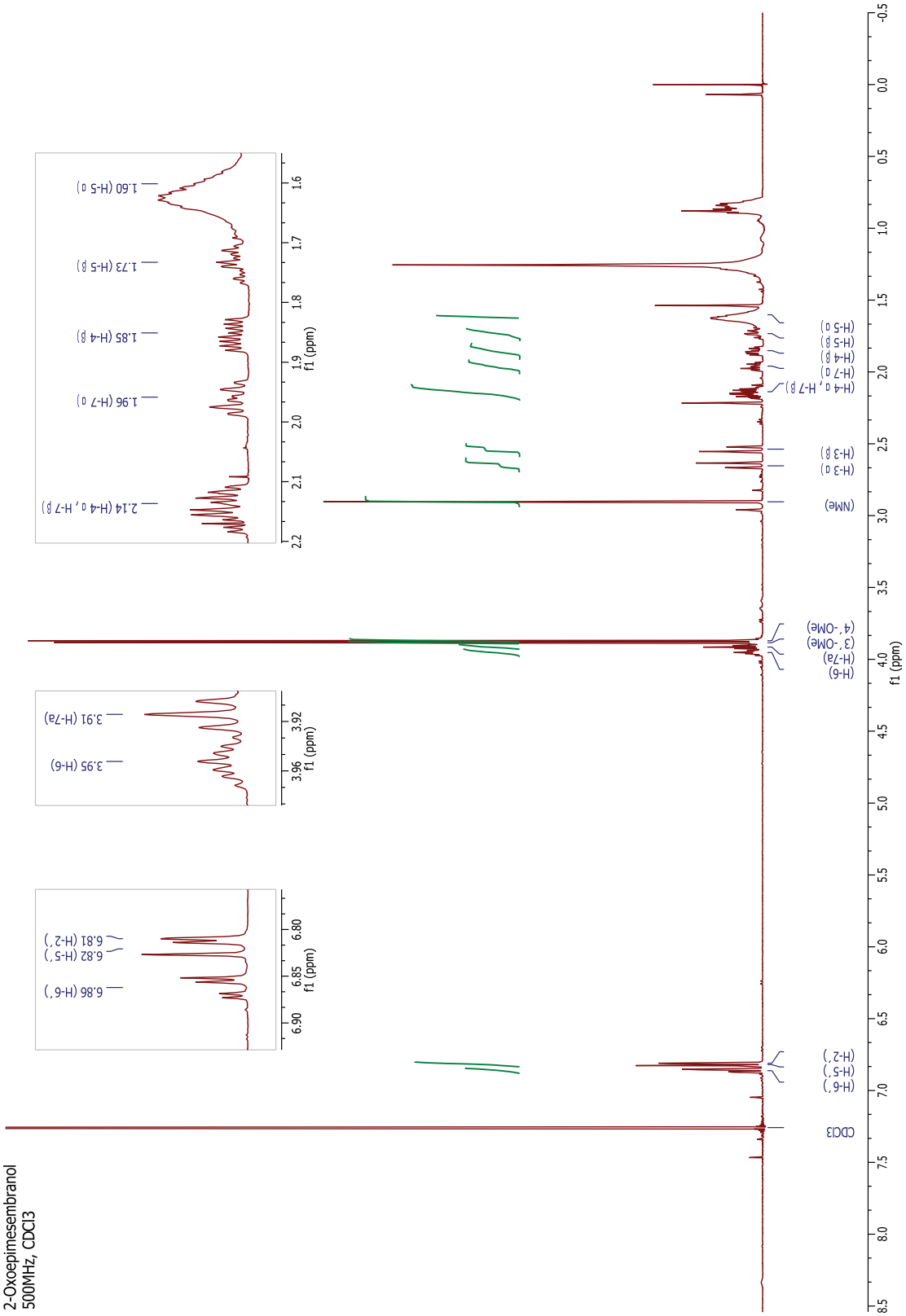
7.2.11. 2-Oxoepimesembranol

Posición	^{1}H δ (J in Hz)	COSY	NOESY	^{13}C δ	HMBC
2	-	-	-	173.8 *s*	-
3α	2.65 *d* (16.4)	H3β	H3β, H4α/β, H5α, H7α, H2´, H6´	45.7 *t*	C2, C3a, C4, C7a, C1´
3β	2.54 *d* (16.4)	H3α	H3α, H4α, H7a, H2´, H6´	45.7 *t*	C2, C3a, C4, C7a, C1´
3a	-	-	-	42.9 *s*	-
4α	2.14 *ddd* (15.0, 10.0, 3.6)	H4β, H5α/β	H3α/β, H4β, H5α/β, H2´, H6´	30.0 *t*	C3, C3a, C5, C6, C7a, C1´
4β	1.85 *ddd* (14.5, 7.4, 3.6)	H4α, H5α/β	H3α, H4α, H5α/β, H6, H2´, H6´	30.0 *t*	C3, C3a, C5, C6, C7a, C1´
5α	1.60 *m (solap.)*	H4α/β, H5β, H6	H3α, H4α/β, H5β, H6	30.1 *t*	C3a, C6, C7
5β	1.73 *ddtd* (14.0, 10.0, 3.6, 1.0)	H4α/β, H5α, H6	H4α/β, H5α, H6, H2´, H6´	30.1 *t*	C3a, C6, C7
6	3.95 *tt* (6.1, 3.6)	H5α/β, H7α/β	H4β, H5α/β, H7α/β	66.1 *d*	-
7α	1.96 *dddd* (14.6, 6.0, 5.5, 1.0)	H6, H7β, H7a	H3α, H6, H7β, H7a, NMe	32.8 *t*	C3a, C4, C5, C6, C7a
7β	2.14 *dddd* (14.7, 5.0, 3.6, 1.0)	H6, H7α, H7a	H6, H7α, H7a, H2´, H6´, NMe	32.8 *t*	C3a, C4, C5, C6, C7a
7a	3.91 *dd* (5.5, 5.0)	H7α/β	H3β, H7α/β, H2´, H6´, NMe	62.3 *d*	C2, C3a, C4, C6, C1´
1´	-	-	-	137.4 *s*	-
2´	6.81 *d* (2.4)	H6´	H3α/β, H4α/β, H5β, H7β, H7a	110.0 *d*	C3a, C4´, C6´
3´	-	-	-	149.2 *s*	-
4´	-	-	-	148.0 *s*	-
5´	6.82 *d* (8.4)	H6´	-	111.2 *d*	C1´, C3´
6´	6.86 *dd* (8.4, 2.3)	H2´, H5´	H3α/β, H4α/β, H5β, H7β, H7a	118.2 *d*	C3a, C2´, C4´
NMe	2.90 *s*	-	H7α/β, H7a	28.1 *q*	C2, C7a
OMe (3´)	3.88 *s*	-	-	56.2 *q*	C3´
OMe (4´)	3.87 *s*	-	-	56.1 *q*	C4´



Espectro de Masas




2-Oxoepimesembranol
500MHz, CDCl3
CDCl3
(H-6')
(H-5')
(H-2')
(H-6)
(H-7a)
(3'-OMe)
(4'-OMe)
(NMe)
(H-3 α)
(H-3 β)
(H-4 α, H-7 β)
(H-7 α)
(H-4 β)
(H-5 β)
(H-5 α)
f1 (ppm)
6.86 (H-6')
6.82 (H-5')
6.81 (H-2')
3.95 (H-6)
3.91 (H-7a)
2.14 (H-4 α, H-7 β)
1.96 (H-7 α)
1.85 (H-4 β)
1.73 (H-5 β)
1.60 (H-5 α)