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Superintendencia de Industria y Comercio
E. S. D.

Trámite : 011 **Evento : 378** **Actuación : 449**

Referencia	:	Expediente No. **09.088.171**
Asunto	:	Solicitud de patente para: **COMPOSICIÓN PARA INHALACIÓN**
Solicitante	:	**ALMIRALL, S.A.**
Fecha solicitud	:	21 de agosto de 2009
Opositor	:	**LABORATORIOS SYNTHESIS S.A.S.**

I. ALEGATOS CONTESTACIÓN OPOSICIÓN

Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, por el presente escrito doy respuesta a la oposición formulada por la sociedad **LABORATORIOS SYNTHESIS S.A.S.** opositora en el asunto de la referencia.

II. CONTESTACIÓN DENTRO DEL TÉRMINO LEGAL

El oficio No. **10867**, mediante el cual su Despacho dispuso otorgar a mi representada término para presentar sus argumentaciones a favor de la patentabilidad del invento de que trata el asunto en referencia, me fue notificado por fijación en lista el 14 de julio de 2011. Para atender a la mencionada providencia solicité una prorroga de 60 días, mediante memorial radicado el 4 de octubre de 2011. Por lo tanto, la presente defensa es presentada en tiempo oportuno.

III. RESPUESTA A LAS PETICIONES

Me opongo a las peticiones de la sociedad opositora y en consecuencia, solicito a ese Despacho que con fundamento en las consideraciones de hecho y de derecho que se presentan en este escrito se declare sin fundamento la oposición presentada por la sociedad **LABORATORIOS SYNTHESIS S.A.S.**

En consecuencia, se declare que el invento titulado **COMPOSICIÓN PARA INHALACIÓN** cumple con los requisitos exigidos en el artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina y demás normas concordantes.

IV. FUNDAMENTO FÁCTICO DE LA OPOSICIÓN

A continuación procederé a enumerar de manera resumida los argumentos que la opositora considera pertinentes para presentar oposición a la presente solicitud:

4.1. "En primera instancia, se debe advertir que en el capítulo reivindicatorio de la solicitud objeto de la presente oposición, se hace referencia a métodos terapéuticos, no sólo a la composición farmacológica o a un método de fabricación mediante la cual se obtenga un producto tangible [...]".

4.2. "[...] Bien, pues antes de la fecha de prioridad de esta solicitud se encuentran los siguientes documentos que afectan la patentabilidad de la presente solicitud:

- D1: MOLFINO NESTOR A: "DRUGS IN CLINICAL DEVELOPMENT FOR CHRONIC OBSTRUCTIVE PULMONARY DISEASE" RESPIRATION, KARGER, BASEL, CH, vol 72, no. 1, 2005, páginas 105-112.
- D2: WO2005115462, publicada el 8 de diciembre de 2005.

4.3. "Ahora bien, el documento D1, está centrado en los compuestos existentes mejorados y los compuestos descubiertos que se encuentran en ensayos clínicos, pero no son comercializados todavía. Los compuestos mejorados existentes incluyen: isómeros de los broncodilatadores de acción prolongada, los agonistas beta2 adrenérgicos una vez al día, anticolinérgicos y corticosteroides. Para el mismo documento D1 la gama de nuevos compuestos se encuentra en constante fluctuación y cuenta con medicamentos antiinflamatorios, antioxidantes, modificadores de leucotrienos y una serie de compuestos que tienen a tratar diferentes aspectos de la EPOC (COPD), como la hipertensión pulmonar y la hipofosfatemia. Dicho documento D1 revela de forma particular el uso del agonista selectivo de los receptores muscárnicos M3, bromuro de aclidinio en el tratamiento de la enfermedad pulmonar obstructiva crónica (EPOC) y el asma; la solicitud objeto de la presente oposición no es novedosa pues lo reclamado ya era parte del arte anterior".

4.4. "El documento D2 por su parte se refiere a una combinación que comprende (a) un agonista Beta2 y (b) un antagonista de los receptores muscarínicos M3 que es 3(R)-(2-hidroxi-2,2-ditien-2-ilacetoxi)-1-(3-fenoxipropil)-1-azoniabiciclo[2,2,2]octano, en forma de una sal que tiene un anión X, que es un anión farmacéuticamente aceptable de un ácido mono o polivalente para el tratamiento de trastornos respiratorios. Dicho documento D2 describe el uso combinado

del agonista selectivo de los receptores muscárnicos M3, bromuro de aclidinio e inhibidores específicos de POE4 y beta-2-miméticos o coriticosteroides en el tratamiento del enfisema, COPD y el asma, también revela composiciones inhalables en polvo que comprenden el bromuro de aclidinio y la lactosa".

4.5. "Tomando los documentos D1 y D2 como el estado del arte más cercano a la solicitud objeto de la presente oposición, no es posible reconocer altura inventiva, ya que sería obvio para un experto en la materia a partir de dicho documento D1 por sí solo, o en combinación con D2 que revela composiciones inhalables en polvo que comprenden el bromuro de aclidinio, concebir métodos para el tratamiento por inhalación de una enfermedad o estado respiratorio en un paciente que necesite dicho tratamiento sin producir en dicho paciente efectos antimuscárnicos sistémicos, comprendiendo administrar a dicho paciente una cantidad eficaz de aclidinio. Así entonces las reivindicaciones de la solicitud objeto de la presente oposición, carecen de altura inventiva".

4.6. Por consiguiente, respecto a la solicitud pretendida se tiene que carece de novedad y nivel inventivo.

V. RESPUESTA A LOS HECHOS Y ALEGATOS

A continuación presento a su Despacho las razones que demuestran que contrario a lo señalado por la apoderado de la sociedad opositora, el invento para el cual se solicita la protección a través de la concesión de la patente en el expediente de la referencia, cumple cabalmente con el requisitos del artículo 14, 16 y demás normas concordantes de la Decisión 486 de la Comisión de la Comunidad Andina.

Además, los argumentos que aquí se presentan demuestran que los fundamentos de la opositora carecen de fundamento válido y por tanto, no pueden ni deben ser tomados en cuenta para negar la patente solicitada.

5.1. <u>Modificación de la solicitud de acuerdo con el artículo 34 de la Decisión 486:</u>

En primer lugar y con el fin de presentar la materia reivindicada de conformidad con los lineamientos actuales, se ha procedido con la modificación del pliego reivindicatorio. Lo anterior, en virtud del artículo 34 de la Decisión 486, en donde se establece que la modificación de una solicitud puede realizarse en cualquier momento del trámite mientras no implique la ampliación del alcance inicialmente solicitado.

Consecuentemente, se presenta un nuevo pliego reivindicatorio conformado por 13 reivindicaciones, en donde se han excluido las reivindicaciones relacionadas con materia no patentable.

En vista de lo anterior, es posible establecer que la objeción comunicada por la sociedad opositora en cuanto a la presencia de materia no patentable de acuerdo con la legislación actual, no es más aplicable a la presente solicitud.

5.2. Argumentos a favor de la novedad y nivel inventivo de la presente solicitud

5.2.1. Señala la apoderada de la parte opositora que la presente solicitud incumple los requisitos de patentabilidad establecidos en la Decisión 486. A este respecto es relevante aclarar cuáles son los lineamientos establecidos por los artículos 16 y 18 de la Decisión 486, sobre estos requerimientos.

En primer lugar, el Artículo 16 establece que una solicitud es novedosa cuando no está comprendida en el estado de la técnica. De acuerdo con la práctica actual, la novedad de una solicitud se determina por medio de la comparación de las características técnicas esenciales de la invención con respecto al estado del arte más cercano. Si se encuentra un documento (anterioridad) **que revele todas las características técnicas de la invención entonces podrá considerarse que afecta su novedad.**

De la misma manera, de acuerdo con el artículo 18 de la Decisión 486 una solicitud tiene nivel inventivo si para una persona medianamente versada en la materia, el objeto no resulta obvio a partir del arte previo. La práctica actual establece una comparación de las características técnicas con el fin de determinar si las diferencias técnicas aportadas por la solicitud son obvias a la luz del arte previo. Igualmente, en algunos casos se evalúa el efecto técnico aportado por la invención.

Sin embargo, en el texto de la oposición no se realiza un análisis claro que permita determinar con facilidad que las dos anterioridades citadas afectarían la novedad y/o el nivel inventivo de la presente solicitud. En primer lugar no se indica cuál es el estado del arte más cercano y en segundo lugar, solamente se indican las anterioridades que de acuerdo con la opositora mencionan elementos de la invención acá reivindicada, pero no existe una evaluación o análisis de las características técnicas de la invención.

5.2.2. Así, en el presente caso, es posible establecer que los documentos D1 y D2 citados por la opositora no revelan todas y cada una de las características de la composición aquí reivindicada, tal como se define en la reivindicación 1 de la presente solicitud.

En efecto, la invención que se tramita en el presente expediente se refiere a antagonistas M3 como antimuscarínicos o anticolinérgicos y como ese Despacho puede apreciar, existe un amplio número de compuestos que se conocen en la técnica de compuestos anticolinérgicos.

La presente invención se basa en el sorprendente hallazgo de que un antagonista M3 específico es particularmente inestable en el plasma humano. El antagonista M3 específico en cuestión se especifica en las reivindicaciones de la presente solicitud mediante su denominación común internacional (INN) de aclidinio. La inestabilidad del aclidinio en plasma significa que éste se puede aplicar a un paciente mediante inhalación, para lograr un efecto local en los pulmones, y posteriormente se degradará rápidamente ya que se realiza fuera de los pulmones en la sangre. Por lo tanto se puede lograr un efecto local en los pulmones sin provocar actividad antimuscarínica sistémica significativa.

La evidencia para la inestabilidad de aclidinio en el plasma humano se puede encontrar en los Ejemplos de la presente solicitud, así:

- En el ejemplo 1 se compara la estabilidad del aclidinio en el plasma humano con aquel de otros tres ésteres muscarínicos. Dos de los fármacos de comparación probados, tiotropio e ipratropio, son fármacos anticolinérgicos líderes del mercado actual.

  Los resultados en el Ejemplo 1 demuestran que el aclidinio se hidroliza rápidamente en el plasma humano, con una vida útil de plasma de menos de 5 minutos. En contraste, los otros tres ésteres antimuscarínicos probados fueron bastante resistentes a la degradación mediante eserasas en el plasma. Sin embargo, la degradación del tiotropio, ipratropio y glicopirrolato en plasma no fue biológicamente significativa durante el estudio de 60 minutos reportado en el Ejemplo 1.

- Los Ejemplos 2 y 3 reportan resultados de estudios clínicos que muestran que el aclidinio puede lograr un efecto local terapéuticamente efectivo en el pulmón sin presencia detectable en el plasma de los pacientes tratados. Esto, por supuesto, es completamente consistente con los resultados del Ejemplo 1.

  Estas ventajas benéficas del aclidinio también son evidentes a partir de los resultados reportados en las referencias bibliográficas adjuntas. Adelante se discuten dichos documentos adicionales.

- En Artículos como *"Aclidinium bromide, a new, long-acting, inhaled muscarinic antagonist: In Vitro plasma inactivation and phamacological activity of its main metabolites" (Sonia Sentellas y otros, European Journal of Pharmaceutical Sciences 39 (2010) 283-290),* se evalúa la estabilidad del aclidinio, tiotropio e ipratropio en plasma humano y animal.

También investiga la actividad del ácido carboxílico y metabolitos de alcohol del aclidinio. Los resultados se presentan en la página 289, columna derecha, último párrafo, en los siguientes términos:

> *"En resumen, los datos reportados aquí muestran que el aclidinio se hidroliza rápidamente en el plasma, debido probablemente a la división enzimática de su éster, para producir su alcohol y derivados de ácido carboxílico en todas las especies estudiadas. Estos metabolitos principales de aclidinio carecen de cualquier afinidad significativa para los receptores muscarínicos".*

Como el Examinador apreciará, la rápida degradación del aclidinio para inactivar metabolitos impedirá cualquier actividad antimuscarínica sistémica significativa.

- De igual forma, en el Artículo *"Discovery of Novel Quaternary Ammonium Derivatives of (3R)-Quinuclidinol Esters as Potent and Long-Acting Muscarinic Antagonist with Potencial for Minimal Systemic Exposure alter Inhaled Administration: Identification of (3R)-3-{[Hydroxy(di-2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane Bromide (Aclidinium Bromide)" (María Prat y otros, Journal of Medicinal Chemistry, 08/04/2009, 5076-5092),* también se evalúa la estabilidad en el plasma de un número de diferentes antagonistas M3. Por ejemplo, la Tabla 6 en la página 5085, demuestra que el aclidinio (compuesto 54) es menos estable en el plasma que otros compuestos estructuralmente similares con diferentes grupos éster.

- Los dos artículos describen datos de animales que evalúan el efecto in vivo del aclidinio en la salivación, producción de heces y retención de orina. Los procesos fisiológicos que rigen la salivación, producción fecal y retención de orina se sabe que se afectan por la actividad antimuscarínica sistémica. Los resultados en los afiches indican que el aclidinio (a) no tiene efecto significativo en la función renal o urinaria en modelos con animales, (b) tiene menos potencial para inhibir la salivación en ratones anestesiados a diferencia de fármacos anticolinérgicos, y (c) no tienen efecto significativo en la motilidad colónica en conejillos de indias anestesiados.

Los resultados descritos anteriormente demuestran claramente que, de todos los diversos fármacos antimuscarínicos a disposición de un médico, **el aclidinio se puede utilizar ventajosamente en el tratamiento de los pacientes que son particularmente susceptibles a la actividad antimuscarínica sistémica**.

5.2.3. En consecuencia, ese Despacho puede apreciar que los documentos citados como anterioridades por la sociedad opositora, no describen la administración de aclidinio mediante inhalación a cualquiera de los grupos de pacientes especificados en el capítulo descriptivo y en el

reivindicatorio. Tampoco hay sugerencias en ninguno de los documentos citados de que el aclidinio se pueda administrar a dichos pacientes debido a su falta de actividad antimuscarínica sistémica. Por lo tanto la materia objeto de protección como patente de invención que se tramita en el presente expediente goza de los requisitos de novedad y nivel inventivo.

VI. PETICIONES

Con fundamento en lo anterior solicito a ese Despacho lo siguiente:

6.1 Declarar infundada la oposición presentada por **LABORATORIOS SYNTHESIS S.A.S.**

6.2 Declarar que el invento titulado "**COMPOSICIÓN PARA INHALACIÓN**" para el cual mi representada solicita el otorgamiento de la patente de invención cumple con los requisitos del artículo 14 y concordantes de la Decisión 486 de la Comisión de la Comunidad Andina.

VII. ANEXOS

Anexo a este escrito los siguientes documentos:

7.1. Copia del artículo *"Aclidinium bromide, a new, long-acting, inhaled muscarinic antagonist: In Vitro plasma inactivation and phamacological activity of its main metabolites" (Sonia Sentellas y otros, European Journal of Pharmaceutical Sciences 39 (2010) 283-290);*

7.2. Copia del artículo *"Discovery of Novel Quaternary Ammonium Derivatives of (3R)-Quinuclidinol Esters as Potent and Long-Acting Muscarinic Antagonist with Potencial for Minimal Systemic Exposure alter Inhaled Administration: Identification of (3R)-3-{[Hydroxy(di-2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane Bromide (Aclidinium Bromide)" (María Prat y otros, Journal of Medicinal Chemistry, 08/04/2009, 5076-5092);*

7.3. Formulario de Modificaciones y Correcciones;

7.4. Recibo por concepto de modificaciones de solicitud en trámite.

7.5. Nuevo capítulo reivindicatorio conformado por 13 reivindicaciones.

VIII. NOTIFICACIONES

Mi representada y yo recibiremos notificaciones en la Secretaría de su Despacho o en mi oficina de abogada ubicada en la Carrera 4ª No. 72-35 de la ciudad de Bogotá, D.C.

Señor Director,

LUZ CLEMENCIA DE PAEZ
C.C. 35.456.344 de Usaquén
T.P.A. No. 23.555

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No. M-2011-207659\JGG

Industria y Comercio
SUPERINTENDENCIA

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Act. 449 RESPUESOPOSICI Folios: 38

FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES
2000-10

1 SOLICITUD DE:

☐ Corrección de Error Material ☒ Modificación a una solicitud

2 SOLICITANTE	Nombre: **ALMIRALL S.A.** Sociedad constituida y existente bajo las leyes de España Dirección: Ronda del General Mitre 151, 08022 Barcelona, España Teléfono: 546 09 70 E-mail: clientes@cavelier.com Fax: 249 40 70 Domicilio: Barcelona, España	**IDENTIFICACION** C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Número: _____
3 REPRESENTANTE O APODERADO	Nombre: Luz Clemencia de PÁEZ Dirección: Carrera 4ª No. 72-35 Teléfono: 347 36 11 E-mail: cavelier@cavelier.com Fax: 217 92 11 Domicilio: Bogotá, Colombia	**IDENTIFICACION** C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Número: 35.456.344 TP 23.555

4 **Número de expediente:** 09.088.171 **Clase:** A61K 031/439

Título: COMPOSICIÓN PARA INHALACIÓN

5 **Descripción de la corrección o modificación solicitada**

Presento un nuevo capítulo reivindicatorio, conformado por 13 reivindicaciones, el cual solicito a ese Despacho tener en cuenta, al momento de efectuar el correspondiente examen de patentabilidad de la invención que se tramita en el expediente arriba indicado. (Ver hojas anexas).

6 **Comprobante de pago No.** 11-126453 **Fecha:** 28 de diciembre de 2011

7 **Anexos:**

☒ Comprobante de pago de la tasa por concepto de modificación de solicitud en trámite.

☐ Poderes, si fuere el caso

☐ Certificado de existencia y representación legal, cuando el solicitante sea persona jurídica

JGG/ID.207828

8 NOMBRE: LUZ CLEMENCIA DE PAEZ FIRMA:

C.C. 35.456.344 de Usaquén T.P.A. 23.555

REIVINDICACIONES

1. Aclidinio caracterizado porque no produce efectos antimuscarínicos sistémicos cuando se administra a un paciente que sufre o es susceptible de sufrir un estado que puede ser agravado por la actividad antimuscarínica sistémica.

2. Aclidinio, de conformidad con la reivindicación 1, en el que la enfermedad o el estado respiratorio se selecciona de bronquitis aguda o crónica, enfisema, asma y enfermedad pulmonar obstructiva crónica, especialmente asma y enfermedad pulmonar obstructiva crónica.

3. Aclidinio, de conformidad con las reivindicaciones precedentes, en donde el paciente sufre o es susceptible de sufrir uno o más estados seleccionados de:

i. esquizofrenia, disminución de la concentración, confusión, agitación, delirio, falta de atención, disminución de la memoria, depresión respiratoria,
ii. glaucoma, xeroftalmía, aumento del tamaño de las pupilas, visión borrosa, aumento de la presión intraocular,
iii. próstata dilatada u obstruida, dificultad urinaria,
iv. estrechamiento u obstrucción del intestino delgado, colon dilatado, estreñimiento crónico, esófago inferior dilatado, disminución de la motilidad gástrica, estreñimiento,
v. xerostomía, irritación de garganta, disminución de la sudación,
vi. enfermedad cardiovascular (incluyendo cualquiera de reestenosis, arterioesclerosis, estado previo a un ictus o infarto de miocardio, insuficiencia cardiaca congestiva), arritmia, taquicardia,
vii. enfermedad de Parkinson, enfermedad de Alzheimer, demencia, y
viii. miastenia grave.

4. Aclidinio, de conformidad con cualquiera de las reivindicaciones precedentes, en donde:

el paciente es un hombre; y/o
el paciente tiene más de sesenta años; y/o
el paciente pretende conducir o manejar maquinaria durante el tratamiento; y/o
el paciente está recibiendo un segundo fármaco que es un agente anticolinérgico sistémicamente activo o un agente que puede provocar o agravar cualquiera de los estados definidos en la reivindicación 3, en el que el segundo fármaco se selecciona de antisicóticos atípicos, antidepresivos tricíclicos y antihistaminas.

5. Aclidinio, de conformidad con la reivindicación 4, en donde el paciente está recibiendo un fármaco que está destinado a mejorar la función de la acetilcolina.

6. Aclidinio, de conformidad con cualquiera de las reivindicaciones precedentes, en el que el aclidinio está en forma de bromuro de aclidinio.

7. Aclidinio, de conformidad con cualquiera de las reivindicaciones precedentes, en el que el aclidinio está en forma de un polvo seco adecuado para inhalación.

8. Aclidinio, de conformidad con cualquiera de las reivindicaciones precedentes, en donde el paciente recibe uno o más medicamentos adicionales para el tratamiento de la enfermedad o el estado respiratorio.

9. Aclidinio, de conformidad con la reivindicación 8, en el que el medicamento adicional para el tratamiento de la enfermedad o el estado respiratorio se selecciona de agonistas β-adrenérgicos, corticoesteroides o glucocorticoides, inhibidores de PDE IV, antihistaminas, anticuerpos anti-IgE, inhibidores de leucotrieno D4, inhibidores de egfr-quinasa, inhibidores de la quinasa p38 y/o antagonistas del receptor NK1.

10. Aclidinio, de conformidad con la reivindicación 9, en el que el medicamento adicional se selecciona de corticoesteroides y/o agonistas β-adrenérgicos.

11. Aclidinio, de conformidad con cualquiera de las reivindicaciones 1, 6 y 7, para el tratamiento o prevención, por inhalación, de una enfermedad o estado respiratorio como se ha definido en una cualquiera de las reivindicaciones 1 o 2, en un paciente como se ha definido en una cualquiera de las reivindicaciones 1, 3 a 5 y 8 a 10.

12. Una composición farmacéutica, para el tratamiento o prevención, por inhalación, de una enfermedad o estado respiratorio como se ha definido en una cualquiera de las reivindicaciones 1 o 2, en un paciente como se ha definido en una cualquiera de las reivindicaciones 1, 3 a 5 y 8 a 10, la cual comprende un vehículo farmacéuticamente aceptable y, como principio activo, aclidinio, como se ha definido en una cualquiera de las reivindicaciones 1, 6 y 7.

13. Una composición farmacéutica de conformidad con la reivindicación 12, en la que el vehículo farmacéuticamente aceptable es seleccionado de mono-, di- o poli-sacáridos y azúcares alcoholes, preferiblemente lactosa.

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Article

Discovery of Novel Quaternary Ammonium Derivatives of (3*R*)-Quinuclidinol Esters as Potent and Long-Acting Muscarinic Antagonists with Potential for Minimal Systemic Exposure after Inhaled Administration: Identification of (3*R*)-3-{[Hydroxy(di-2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane Bromide (Aclidinium Bromide)

María Prat,* Dolors Fernández,[†] M. Antonia Buil, María I. Crespo, Gaspar Casals, Manuel Ferrer, Laia Tort, Jordi Castro, Juan M. Monleón, Amadeu Gavaldà, Montserrat Miralpeix, Israel Ramos, Teresa Doménech, Dolors Vilella, Francisca Antón,[‡] Josep M. Huerta, Sonia Espinosa, Manuel López, Sonia Sentellas, Marisa González, Joan Albertí, Victor Segarra, Alvaro Cárdenas,[§] Jorge Beleta, and Hamish Ryder[∥]

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The objective of this work was to discover a novel, long-acting muscarinic M_3 antagonist for the inhaled treatment of chronic obstructive pulmonary disease (COPD), with a potentially improved risk–benefit profile compared with current antimuscarinic agents. A series of novel quaternary ammonium derivatives of (3*R*)-quinuclidinol esters were synthesized and evaluated. On the basis of its overall profile, (3*R*)-3-{[hydroxy(di-2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (aclidinium bromide) emerged as a candidate for once-daily maintenance treatment of COPD. This compound is a potent muscarinic antagonist, with long duration of action in vivo, and was found to have a rapid hydrolysis in human plasma, minimizing the potential to induce class-related systemic side effects. Aclidinium bromide is currently in phase III development for maintenance treatment of patients with COPD.

Introduction

Chronic obstructive pulmonary disease (COPD[a]) is a major global health concern, which is characterized by airflow obstruction that deteriorates progressively and has a limited reversibility after bronchodilator therapy.[1] Projections from the World Health Organization predict that COPD will become the fourth most common cause of death by 2030 and the third most common cause of chronic disability by 2020.[2,3] Acetylcholine is involved in airway smooth muscle contraction and narrowing, and cholinergic tone appears to be the major reversible component of airway obstruction in COPD.[4–6]

Three subtypes of muscarinic receptor are expressed in the human airway: M_1, M_2, and M_3 receptors.[7] The M_3 receptor is the primary subtype responsible for bronchial and tracheal smooth muscle contraction.[8] M_2 receptors localized in cholinergic nerve endings act as negative feedback of acetylcholine release from the nerve.[9,10] Finally, M_1 receptors are localized to parasympathetic ganglia in the airways, facilitating ganglionic neurotransmission.[11]

Inhaled anticholinergics are an effective class of bronchodilators in the management of symptomatic patients with COPD: ipratropium is a short-acting agent requiring up to four doses per day, whereas tiotropium is a long-acting, once-daily treatment (Figure 1).[11–13] Certain systemic anticholinergic effects are associated with these compounds, including dry mouth (reported in 11.6% of patients treated with tiotropium bromide versus 3.5% of patients treated with placebo), glaucoma, constipation, increased heart rate, and urinary retention.[14,15] Therefore, a compound that has similar or improved bronchodilatory efficacy to these agents but causes fewer unwanted anticholinergic effects would be desirable as a monotherapy or as part of a combination therapy because of its lower potential risk of additive systemic pharmacology and drug–drug interactions.

The objective of our work was to discover a novel and potent inhaled muscarinic M_3 antagonist suitable for once-daily administration with an improved safety profile for the maintenance treatment of COPD.

Chemistry

3-Quinuclidinol esters are known to be potent muscarinic antagonists, and various examples of these compounds have been described in the literature.[16–22] Notably, the (3*R*)-quinuclidinol ester revatropate (Figure 1), an M_3, M_1 selective muscarinic antagonist, was developed as a potential treatment for COPD.[23–25] This compound entered phase II clinical trials, although the development of revatropate has since been discontinued, probably because of its short duration of action.[23,24]

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[a]Abbreviations: CHO-K1, Chinese hamster ovary K1; COPD, chronic obstructive pulmonary disease; QNB, quinuclidinyl benzylate; MQNB, quinuclidinyl benzylate methiodide; PET, positron emission tomography.

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Quaternization of the tertiary amino function is a common chemical manipulation in this class of compounds, widely used to minimize absorption across membranes, resulting in low oral bioavailability and blood−brain barrier permeability.[26,27] Antimuscarinic activity has been reported for certain quaternary ammonium derivatives of quinuclidinyl benzylate (QNB), with aliphatic chains of different length on the N^+ of the azoniabicyclo ring,[28−30] although the potency and duration of action of these agents in the lung are unknown. Radiolabeled QNB and its methiodide salt MQNB are also used as muscarinic ligands in positron emission tomography (PET) to investigate receptor distribution and expression changes in several pathologies.[31,32]

Here we describe the synthesis of a variety of new (3*R*)-quinuclidinol esters and their corresponding quaternary ammonium derivatives, which were prepared with the objective of studying the potential of this scaffold to deliver the desired activity profile. These compounds were synthesized as outlined in Schemes 1−4.

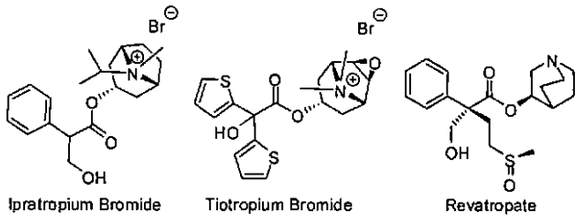


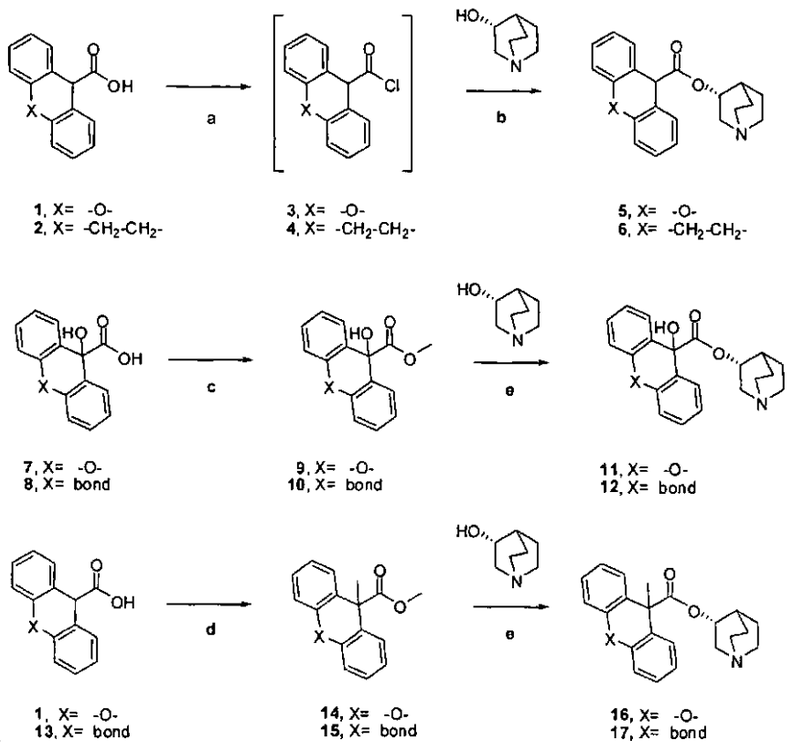
Figure 1

(3*R*)-Quinuclidinol esters **5**,[19,20] **6**, **11**,[33] **12**, **16**, and **17** were prepared from carboxylic acids **1**, **2**,[34] **7**,[35] **8**, and **13** (Scheme 1). Transformation of acids **1** and **2** into their acyl chloride derivatives (compounds **3** and **4**), followed by reaction with commercially available (3*R*)-quinuclidinol, led to the esters **5** and **6**. (3*R*)-Quinuclidinol esters **11** and **12** were obtained from their corresponding methyl ester derivatives, **9** and **10**, which were synthesized from α-hydroxy acids **7** and **8**, respectively. Acids **1** and **13** were methylated in the α position and esterified in a one-pot reaction to give methyl ester derivatives **14** and **15**. Transesterification of compounds **14** and **15** with (3*R*)-quinuclidinol yielded compounds **16** and **17**.

Scheme 2 shows the synthesis of (3*R*)-quinuclidinol esters with a dithienyl moiety (compounds **19**, **21**, **23**, and **26**). The reaction of dimethyl oxalate with 2-thienylmagnesium bromide and 3-thienylmagnesium bromide afforded methyl esters **18** and **22**, respectively.[36] Reduction of compound **18** by reaction with stannous chloride in acidic conditions[37] yielded the methyl ester **20**. The reaction of 2-oxopropanoic acid with thiophene in acidic media gave acid **24**,[38] which was transformed to its methyl ester **25** by standard methods. Reaction of synthesized methyl esters **18**, **20**, **22**, and **25** with (3*R*)-quinuclidinol by means of sodium hydride in refluxing toluene afforded the desired compounds **19**, **21**, **23**, and **26**, respectively.

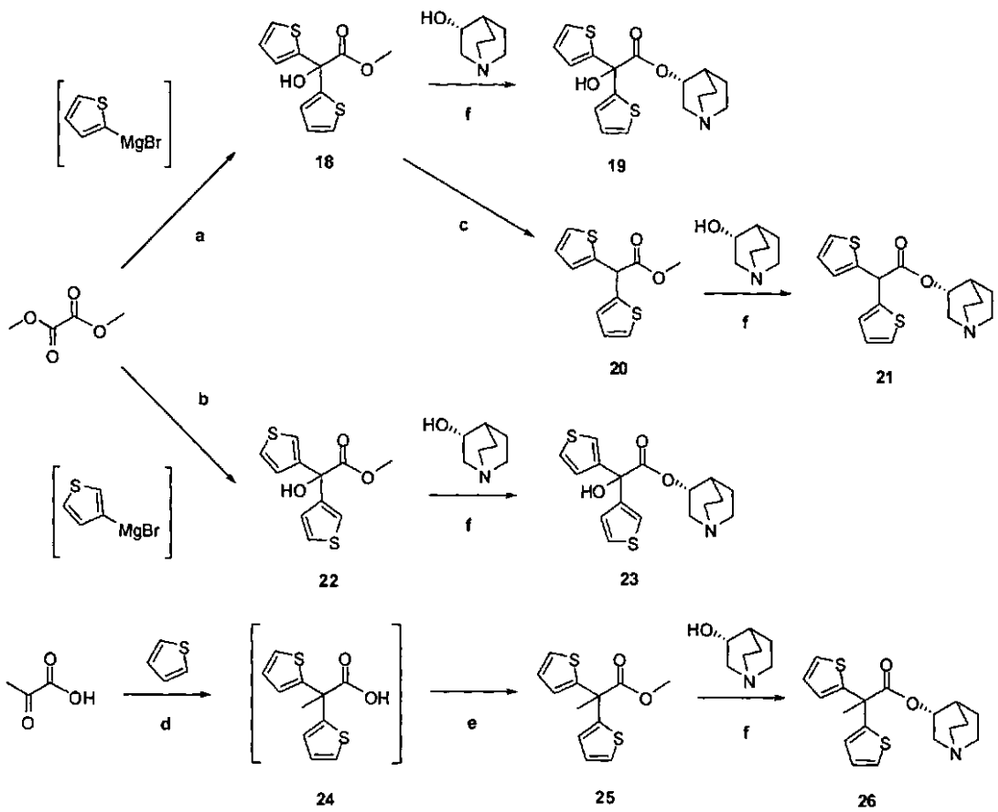
The synthesis of (3*R*)-quinuclidinol esters **33** ((*R*)-QNB[19]), **35**, **36**, **38**, and **39** are described in Scheme 3. Transformation of commercially available acids **27** and **28** to their corresponding acyl chloride derivatives **29** and **30**, followed by reaction with (3*R*)-quinuclidinol, afforded the glyoxalate derivatives

Scheme 1. Synthesis of (3*R*)-Quinuclidinol Esters **5**, **6**, **11**, **12**, **16**, and **17**[a]



[a] Reagents and conditions: (a) $CHCl_3$, oxalyl chloride, DMF (catalytic), room temp; (b) toluene, reflux, or $CHCl_3$, room temp; (c) MeOH, H_2SO_4 (catalytic), reflux, or acetone, K_2CO_3, $(CH_3O)_2SO_2$, reflux; (d) (i) THF, LDA (addition at 0 °C), reflux, (ii) CH_3I, room temp, (iii) MeOH, H_2SO_4, reflux; (e) toluene, HNa, reflux.

Scheme 2. Synthesis of (3*R*)-Quinuclidinol Esters **19**, **21**, **23**, and **26**[a]



[a] Reagents and conditions: (a) diethyl ether, reflux; (b) (i) diethyl ether, −30 °C, (ii) diethyl ether, 0−5 °C; (c) AcOH glacial, HCl gas, $Cl_2Sn \cdot 2H_2O$, 15 °C; (d) H_2O, H_2SO_4, 40−50 °C; (e) acetone, K_2CO_3, $(CH_3O)_2SO_2$, reflux; (f) toluene, HNa, reflux.

31[39] and **32**, respectively. Reaction of compounds **31** and **32** with commercially available solutions of different organometallic reagents yielded esters **33**, **34**, and **37**. Compounds **34** and **37** were both obtained as a mixture of diastereomers that were further separated by crystallization, yielding compounds **35**, **36**, **38**, and **39**, respectively.

The stereochemistry of the ester moiety in compounds **35**, **36**, **38**, and **39** was deduced from the configuration of acids **40**, **41**, and **42**, obtained by hydrolysis (Scheme 3). The configuration of these acids was elucidated by comparison of their optical properties (specific optical rotation [α], circular dichroism (CD) curve [Δε]) with published values of structurally related acids of known configuration.[40−42]

Quaternary ammonium derivatives **43**−**64** were obtained by reaction of selected (3*R*)-quinuclidinol esters with a variety of bromoalkyl or bromoalkylaryl derivatives (Scheme 4).

Results and Discussion

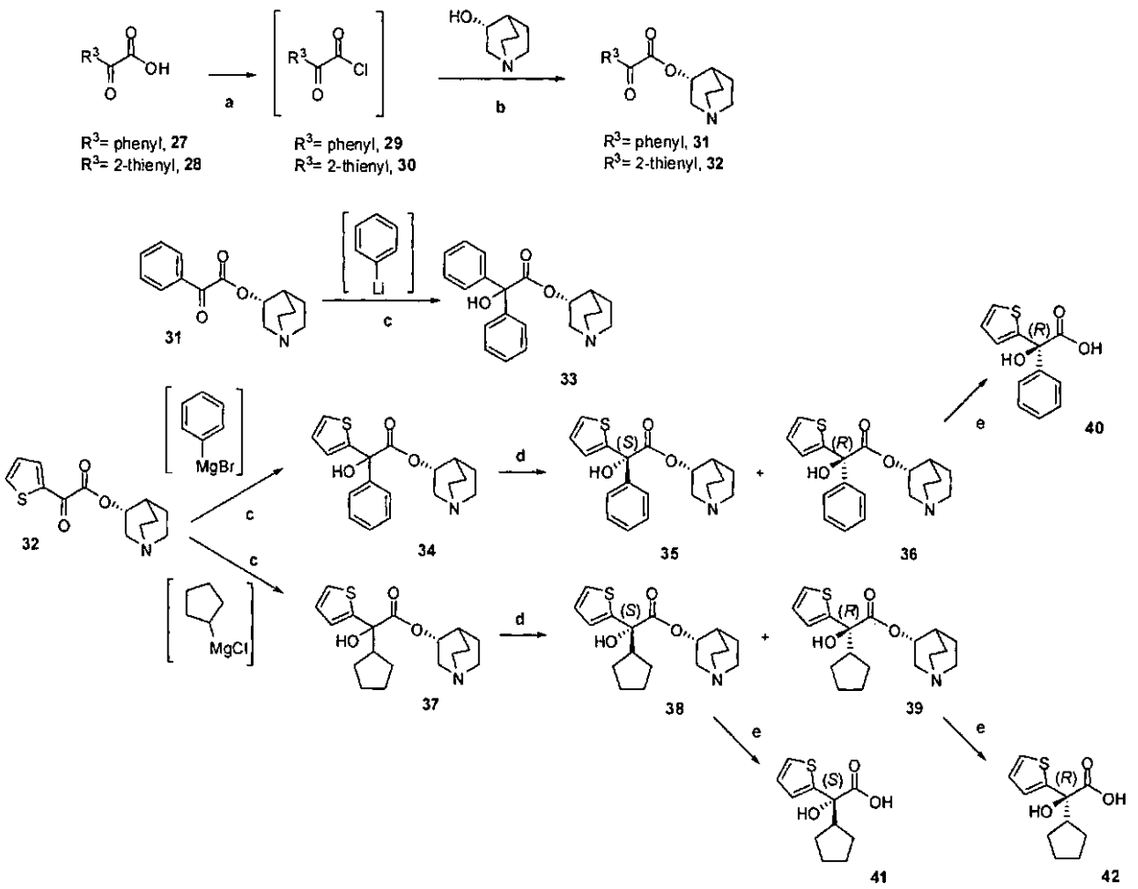
In Vitro Receptor-Binding Studies. We evaluated the muscarinic receptor-binding affinities of the synthesized compounds using membranes expressing the human M_1, M_2, and M_3 receptors transfected into Chinese hamster ovary-K1 (CHO-K1) cells. [^{3}H]-*N*-Methylscopolamine was used as a radioligand in displacement assays. Nonspecific binding was measured in the presence of atropine (1 μM).

The influence of R^1 in the binding affinity (IC_{50}, defined as the concentration required to induce 50% inhibition of muscarinic receptor activity) for some of the nonquaternized compounds previously described is shown in Table 1. The affinities of tiotropium bromide and ipratropium bromide were also determined for reference.[43] When R^1 was a noncyclized moiety, all tested derivatives were potent M_3 receptor ligands, with affinities at the low nanomolar range; however, like the reference compounds, they were not selective for M_3 receptors over M_1 and M_2 receptors. Among them, compound **19**, with two 2-thienyl substituents, had the highest affinity for the M_3 receptor, with an IC_{50} 1.5- and 9-fold lower than tiotropium bromide and ipratropium bromide, respectively. The tested R^1-cyclized subset of compounds maintained the low nanomolar muscarinic activity, with the exception of compound **6**.

To explore the SAR around R^2, we picked up two of the nonquaternized compounds previously described. We chose the most active derivative, compound **19**, and also compound **12** as a representative of the R^1-cyclized subset of compounds. Muscarinic receptor-binding affinities (IC_{50}) for their quaternized derivatives are shown in Table 2. Among the derivatives of compound **12** evaluated (compounds **46**−**48**), the highest binding affinity was obtained for compound **48**, with a phenoxypropyl chain. The binding affinity profile of compound **19** was retained for almost all of the quaternized derivatives tested (compounds **51**−**56**). The highest potencies were obtained for compound **54**, with a phenethyl chain, and compound **56**, with a phenoxypropyl chain also.

On the basis of the in vitro profile, compounds **12** and **19** and their active derivatives were selected for further evaluation.

Scheme 3. Synthesis of (3*R*)-Quinuclidinol Esters **33**, **35**, **36**, **38**, and **39**[a]



[a] Reagents and conditions: (a) $CHCl_3$, oxalyl chloride, DMF (catalytic), room temp; (b) $CHCl_3$, room temp; (c) THF, from $t \leq -40$ °C to room temp; (d) separation of diastereomers by crystallization; (e) MeOH, NaOH, room temp or EtOH, NaOH 2 N, room temp.

In Vivo Duration of Action Studies. The duration of action of selected compounds was evaluated by measuring the inhibition of bronchospasm induced by acetylcholine in anesthetized guinea pigs at different time points.[44] Duration of action results for compounds **12** and **19** and their corresponding quaternized derivatives are shown in Table 2, along with data for reference compounds, tiotropium bromide and ipratropium bromide.

Compound **12** had a short duration of action (3.9 h), comparable to that of ipratropium bromide (3.4 h). However, quaternization with a heptyl group (compound **46**), a phenethyl group (compound **47**), or a phenoxypropyl group (compound **48**) resulted in a longer duration of action (> 6 h), similar to that of tiotropium bromide. Most of the quaternized derivatives of compound **19** that were evaluated (compounds **51**, **52**, **54**, and **56**) retained the long duration of action observed for compound **19** (> 6 h). These results were comparable to those obtained for tiotropium bromide.

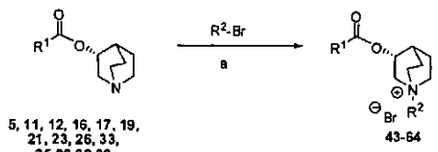
In conclusion, quaternization appeared to retain or improve the duration of action of these compounds relative to the corresponding unquaternized compounds.

On the basis of the results of M_3 receptor-binding affinity and duration of action studies, a series of compounds with phenethyl (compounds **43**, **44**, and **47**) or phenoxypropyl (compounds **45**, **48**, **60**, **63**, and **64**) as R^2 substituents and with diverse R^1 groups on the ester moiety were synthesized and evaluated. M_3 receptor-binding affinities and duration of action data for these compounds are presented in Table 3. All tested compounds showed high M_3 muscarinic affinity, ranging from 0.18 to 1.62 nM, and exhibited long durations of action (> 6 h). Compounds with the highest M_3 receptor-binding affinities (compounds **54**, **56**, and **63**) were selected for further evaluation.

In Vivo Characterization of Selected Compounds. Table 4 describes the duration of action and potency (IC_{50}) for compounds **54**, **56**, and **63**, reverting the bronchospasm induced by acetylcholine in guinea pigs. These compounds showed relevant potency in this in vivo model, comparable to tiotropium bromide and ipratropium bromide.

We further evaluated the preliminary in vivo safety profiles of compounds **54**, **56**, and **63** in mice using an observational test focusing on antimuscarinic side effects. Tiotropium bromide and ipratropium bromide were also evaluated for reference. Each compound was administered intraperitoneally at a dose of 30 mg/kg. Compound **56** did not produce mydriasis, a class-related adverse effect of muscarinic antagonists, and had the most favorable safety profile of the three compounds tested. For all of the other compounds tested in mice, including tiotropium bromide and ipratropium bromide, mydriasis was observed. At this dose, all the compounds tested showed no other adverse effects.

Scheme 4. Preparation of Quaternary Ammonium Derivatives **43** to **64**[a]



starting cmpd	quaternary ammonium derivative					
	R^2					
	CH_3-	$CH_3(CH_2)_6$-	$PhCH_2$-	$Ph(CH_2)_2$	$Ph(CH_2)_4$-	$PhO(CH_2)_3$-
5				**43**		
11				**44**		**45**
12		**46**		**47**		**48**
16						**49**
17						**50**
19	**51**	**52**	**53**	**54**	**55**	**56**
21						**57**
23						**58**
26						**59**
33						**60**
35 (3*R*, 2*S*)						**61**
36 (3*R*, 2*R*)						**62**
38 (3*R*, 2*S*)						**63**
39 (3*R*, 2*R*)						**64**

[a] Reagents and conditions: (a) THF reflux or $CHCl_3$ room temp or $CHCl_3$/acetonitrile room temp.

On the basis of its in vitro binding affinity, in vivo potency, duration of action, and preliminary safety profile, compound **56** was selected for further biological characterization.[45]

Human Plasma Stability Studies. To better understand the reasons for the favorable in vivo safety profile of compound **56** and to evaluate its potential for low systemic class-related side effects in humans, stability studies in human plasma for compounds **54**, **56**, and **63** were performed (Table 4). The plasma stability of tiotropium bromide and ipratropium bromide was also evaluated for reference. Each compound was incubated at 37 °C in human plasma, and the amount of remaining compound after 15 min were monitored. The degradation of this type of compound in human plasma would be expected to be mediated by ester cleavage. Surprisingly, compound **56** was almost fully degraded at the 15 min time point, providing a rationale for a wider safety margin of this compound. In order to elucidate the mechanism responsible of such a quick plasma degradation, we evaluated human plasma and chemical stability half-lives of these compounds at the same experimental conditions (37 °C and pH 7.4). In comparison with reference compounds, compounds **54** and **56** showed human plasma stability half-lives much shorter than the corresponding chemical stability half-lives, suggesting that plasma degradation of compounds **54** and **56** is mainly driven by enzymatic cleavage.

This interesting result led us to investigate the human plasma stability of several (3*R*)-quinuclidinol esters up to 60 min.

Table 5 shows the influence of R^2 substitution on plasma stability for analogues of compound **56**. All the quinuclidinol esters described in Table 5 were less stable in human plasma than tiotropium bromide and ipratropium bromide (scopine and tropine derivatives). Quaternized compounds **51**, **54**, and **56** were less stable than the unquaternized compound **19**. Thus, to achieve rapid hydrolysis in human plasma, quaternization of the (3*R*)-quinuclidinol ester **19** is required.

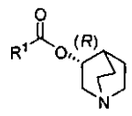
Tables 6 and 7 show the influence of the R^1 group on the rate of degradation in human plasma. The presence of two 2-thienyl substituents on the R^1 group (compound **56**) reduced stability in human plasma compared with the presence of two 3-thienyl substituents (compound **58**) or two phenyl substituents (compound **60**). Replacing one 2-thienyl group with a phenyl group (compounds **61** and **62**) or a cyclopentyl group (compounds **63** and **64**) increased plasma stability compared with compound **56**. For these compounds with a chiral α carbon, the 2*R*-enantiomers (compounds **62** and **64**) were less stable than the corresponding 2*S*-enantiomers (compounds **61** and **63**).

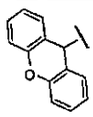
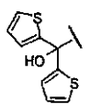
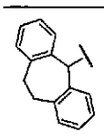
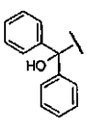
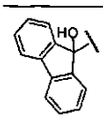
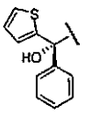
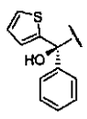
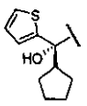
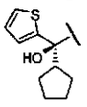
Bridging the two phenyl groups of compound **60** (compound **48**) resulted in lower stability. Substitution of the fluorenyl group (compounds **47** and **48**) by a xanthenyl group (compounds **44** and **45**) resulted in greater stability.

The human plasma stability was greater for compounds with a methyl substituent (compounds **49**, **50**, and **59**) than for compounds with a hydroxyl substituent (compounds **45**, **48**, and **56**), and compounds with a hydrogen substituent were the least stable (compound **43** vs compound **44**; and compound **57** vs compound **56**).

In summary, among the (3*R*)-quinuclidinol esters evaluated for human plasma stability, compounds **51**, **54**, **56**, and **57** were found to degrade most rapidly in human plasma.

Table 1. Effects of R^1 Substitution on M_1, M_2, and M_3 Muscarinic Receptor-Binding Affinities for (3*R*)-Quinuclidinol Ester Derivatives



R^1	cmpd	binding affinity[a] (IC_{50}, nM) M_3	M_2	M_1	R^1	cmpd	binding affinity[a] (IC_{50}, nM) M_3	M_2	M_1
	**5**	0.76 (0.01)	2.83 (0.37)	0.31 (0.04)		**19**	0.23 (0.01)	0.26 (0.001)	0.20 (0.02)
	**6**	94 (1)	79 (1)	15 (1)		**33**	0.90 (0.05)	0.40 (0.06)	0.36 (0.004)
	**12**	0.92 (0.13)	1.14 (0.18)	0.26 (0.02)		**35** (2*S*-isomer)	0.31 (0.03)	0.21 (0.02)	0.20 (0.04)
						**36** (2*R*-isomer)	0.42 (0.001)	0.26 (0.01)	0.23 (0.01)
						**38** (2*S*-isomer)	0.39 (0.05)	0.23 (0.04)	0.19 (0.02)
						**39** (2*R*-isomer)	0.70 (0.14)	0.54 (0.005)	0.24 (0.08)
	tiotropium bromide	0.35[b] (0.01)	0.21[b] (0.04)	0.22[b] (0.03)					
	ipratropium bromide	2.07[b] (0.03)	2.24[b] (0.16)	2.65[b] (0.13)					

[a] Values shown are the mean, $n = 2$ (SD). [b] Values shown are the mean, $n = 3$ (SD).

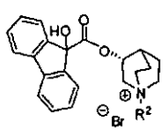
These results help us to understand the more favorable in vivo safety profile of compound **56** compared with the other compounds tested and reinforce the decision to take compound **56** forward as a candidate for further studies.

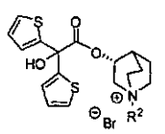
Conclusion

With the objective of identifying a novel, once-daily antimuscarinic agent for the maintenance treatment of COPD by inhalation, we synthesized a series of quaternized (3*R*)-quinuclidinol esters. In vitro and in vivo tests were then conducted to evaluate the potential beneficial characteristics of these compounds in terms of clinical efficacy (muscarinic receptor-binding affinity, duration of action) and low class-related side effects (preliminary in vivo safety, human plasma stability). On the basis of these preclinical results, compound **56** (aclidinium bromide) was selected as a candidate for a complete biological characterization. Aclidinium bromide showed high M_3 receptor-binding affinity and had duration of action longer than that of ipratropium bromide and comparable to that of tiotropium bromide. Aclidinium bromide also demonstrated a favorable in vivo safety profile likely to be due to its rapid human plasma hydrolysis, indicating low potential for systemic class-related side effects. The combination of high potency, long duration of action, low oral absorption, and rapid plasma degradation in the same molecule led us to a novel, effective, and long-acting anticholinergic compound with the potential for a wider safety margin than currently available inhaled anticholinergic therapies. Overall, this profile supports the potential for this compound as a safe and effective monotherapy or as part of a combination therapy for COPD.

Aclidinium bromide is currently in phase III development for maintenance treatment of patients with COPD and in phase II studies in combination with a long-acting β_2-agonist.

Table 2. Effects of R^2 Substitution on the M_1, M_2, and M_3 Muscarinic Receptor-Binding Affinities and in Vivo Duration of Action for Novel Quaternized (3*R*)-Quinuclidinol Ester Derivatives





		binding affinity[a] (IC_{50}, nM)			duration of action		binding affinity[a] (IC_{50}, nM)			duration of action
R^2	cmpd	M_3	M_2	M_1	*T* (h)	cmpd	M_3	M_2	M_1	*T* (h)
–	**12**	0.92 (0.13)	1.14 (0.18)	0.26 (0.02)	3.9[c]	**19**	0.23 (0.01)	0.26 (0.001)	0.20 (0.02)	> 6[c]
CH_3-						**51**	0.60 (0.01)	0.46 (0.003)	0.43 (0.12)	> 6[c]
$CH_3(CH_2)_6$-	**46**	1.68 (0.08)	4.50 (0.03)	3.10 (0.17)	> 6[d]	**52**	0.39 (0.003)	0.36 (0.09)	0.41 (0.04)	> 6[c]
$PhCH_2$-						**53**	200 (1)	12 (1)	100 (52)	NE[f]
$Ph(CH_2)_2$-	**47**	1.04 (0.12)	2.24 (0.28)	1.12 (0.02)	> 6[d]	**54**	0.20[b] (0.003)	0.18[b] (0.06)	0.14[b] (0.04)	> 6[c]
$Ph(CH_2)_4$-						**55**	0.45 (0.07)	0.38 (0.12)	0.29 (0.02)	6[d]
$PhO(CH_2)_3$-	**48**	0.71 (0.07)	1.08 (0.14)	0.63 (0.02)	> 6[c]	**56**	0.17[b] (0.05)	0.17[b] (0.05)	0.14[b] (0.03)	> 6[d]
						tiotropium bromide	0.35[b] (0.01)	0.21[b] (0.04)	0.22[b] (0.03)	> 6[d]
						ipratropium bromide	2.07[b] (0.03)	2.24[b] (0.16)	2.65[b] (0.13)	3.4[e]

[a] Values shown are the mean, $n = 2$ (SD). [b] Values shown are the mean, $n = 3$ (SD). [c] Administered concentration of 3 mg/mL, $n = 4$–6 animals. [d] Administered concentration of 1 mg/mL, $n = 4$–6 animals. [e] Administered concentration of 0.3 mg/mL, $n = 4$–6 animals. [f] NE = not evaluated. The duration of action for compound **53** was not evaluated because its muscarinic receptor binding affinity was too low.

Experimental Section

Biology: General. (3*R*)-Quinuclidinol ester compounds and tiotropium bromide were synthesized by the Department of Medicinal Chemistry (Laboratorios Almirall, Barcelona, Spain). Ipratropium bromide was purchased from Sigma Chemicals (Tres Cantos, Spain). All antagonists were dissolved in dimethyl sulfoxide.

In Vitro Receptor-Binding Studies. All binding assays were performed in 96-well plates (Nunc, Thermo Fischer Scientific, Roskilde, Denmark) with membrane preparations expressing human M_1, M_2, and M_3 receptors (transfected CHO-K1 cells; Membrane Target Systems, Perkin-Elmer Life and Analytical Sciences, Boston, MA). 1-[*N*-Methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS specific activity of 82 Ci mmol) from Perkin-Elmer Life and Analytical Sciences (Boston, MA) was used in all binding affinity studies.

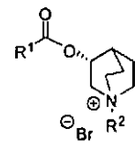
The membrane protein concentrations were 8.1, 5, and 4.9 μg/well for M_1, M_2, and M_3 receptors, respectively. The [^{3}H]NMS concentration was 0.3 nM for M_1 and 1 nM for both M_2 and M_3 assays. A range of antagonist concentrations (10^{-14}–10^{-5} M) was tested to generate competition curves. Nonspecific binding was determined in the presence of 1 μM atropine. Reagents were dissolved in assay binding buffer (phosphate buffered saline with calcium and magnesium) to a total volume of 200 μL. After a 4 h incubation period at room temperature in order to reach equilibrium, 150 μL samples of the reaction mixtures were transferred to GF/C filter plates pretreated for 1 h with wash buffer containing 0.05% polyethylenimine. Bound and free [^{3}H]NMS samples were then separated by rapid vacuum filtration and four washes with ice-cold wash buffer. Samples were dried for 30 min before addition of 30 μL of OptiPhase Supermix (Perkin-Elmer Life and Analytical Sciences, Boston, MA), and radioactivity was quantified using a MicroBeta microplate scintillation counter (Trilux, Perkin-Elmer Boston, MA). The binding affinities (IC_{50} values) for each antagonist were determined for the three muscarinic receptors using GraphPad Prism software (San Diego, CA).

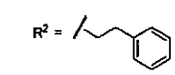
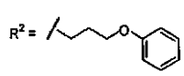
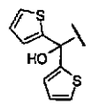
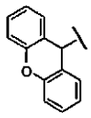
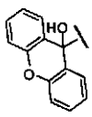
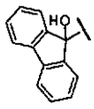
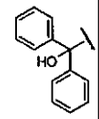
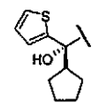
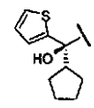
In Vitro Human Plasma Stability Studies. The stability of the test compounds was assessed using pooled human plasma samples from 10 healthy volunteers. Blood samples were collected in lithium heparin tubes, and the plasma was separated by centrifugation, pooled, and stored at −20 °C until use.

Stock solutions of test compounds (2 mM) were prepared by dissolving corresponding amounts of compounds in 0.4 mL of 0.1 N hydrochloric acid before adding acetonitrile to a final volume of 2 mL. Working solutions of test compounds were prepared by diluting from 2 mM stock solutions in water. All working solutions were prepared immediately before use. The final concentration of the test compounds was 1 μM.

Human plasma samples were incubated in triplicate for 5 min at 37 °C (Unitronic 320 OR, JPselecta, Spain) before the addition of the test compound to initiate the reaction (final concentration, 1 μM). At predetermined times during incubation (0, 15, and 60 min), aliquots of the incubation mixtures (100 μL) were transferred to another tube containing 300 μL of cold acetonitrile/1 N HCl (90/10, v/v) to stop the reaction. Samples were then centrifuged at 2800*g* for 10 min at 4 °C. Control incubations in the absence of the test compound were also performed. Additional incubations for the most unstable compounds (**51**, **56**, and **57**) were also performed at predetermined times (0, 5, 10, and 15 min) and assayed as indicated above.

Table 3. Effects of R^1 Substitution on the M_3 Muscarinic Receptor-Binding Affinities and the in Vivo Duration of Action for Novel Quaternized (3*R*)-Quinuclidinol Ester Derivatives (R^2 = Phenethyl; R^2 = Phenoxypropyl)



	R^2 = 					R^2 = 				
		binding affinity[a] (IC_{50}, nM)			duration of action		binding affinity[a] (IC_{50}, nM)			duration of action
R^1	cmpd	M_3	M_2	M_1	*T* (h)	cmpd	M_3	M_2	M_1	*T* (h)
	**54**	0.20[b] (0.003)	0.18[b] (0.06)	0.14[b] (0.04)	> 6[c]	**56**	0.17[b] (0.05)	0.17[b] (0.05)	0.14[b] (0.03)	> 6[d]
	**43**	0.31 (0.02)	1.82 (0.19)	0.44 (0.03)	> 6[d]					
	**44**	0.28 (0.12)	0.54 (0.15)	0.29 (0.10)	> 6[d]	**45**	0.42 (0.03)	0.36 (0.06)	0.23 (0.01)	> 6[d]
	**47**	1.04 (0.12)	2.24 (0.28)	1.12 (0.02)	> 6[d]	**48**	0.71 (0.07)	1.08 (0.14)	0.63 (0.02)	> 6[c]
						**60**	0.61 (0.03)	0.44 (0.01)	0.31 (0.05)	> 6[c]
						**63** (2*S*-isomer)	0.18[b] (0.02)	0.14[b] (0.04)	0.15[b] (0.01)	> 6[d]
						**64** (2*R*-isomer)	1.62 (0.21)	1.01 (0.29)	0.81 (0.17)	> 6[c]
	tiotropium bromide	0.35[b] (0.01)	0.21[b] (0.04)	0.22[b] (0.03)	> 6[d]					
	ipratropium bromide	2.07[b] (0.03)	2.24[b] (0.16)	2.65[b] (0.13)	3.4[c]					

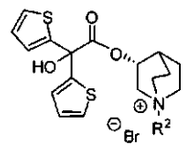
[a] Values shown are the mean, $n = 2$ (SD). [b] Values shown are the mean, $n = 3$ (SD). [c] Administered concentration of 3 mg/mL, $n = 4-6$ animals. [d] Administered concentration of 1 mg/mL, $n = 4-6$ animals. [e] Administered concentration of 0.3 mg/mL, $n = 4-6$ animals.

Table 4. Overall in Vitro and in Vivo Characterization of Selected Compounds

compd	binding affinity,[a] K_i (nM)			in vivo model		safety observational test	human plasma stability,[a,b]		chemical stability,[b,c]
	M_3	M_2	M_1	duration of action, T (h)	IC_{50} (μg/mL)	muscarinic-related side effects (30 mg/kg, ip)[d]	% disappearance (15 min)	$t_{1/2}$ (min)[e]	$t_{1/2}$ (min)[e]
54	0.08 (0.001)	0.08 (0.02)	0.08 (0.02)	>6[f]	54	mydriasis	68 (3)	7.9	56
56	0.07 (0.02)	0.08 (0.02)	0.08 (0.02)	>6[g]	140	none	99 (0)	2.2	60
63	0.07 (0.01)	0.06 (0.01)	0.09 (0.004)	>6[g]	120	mydriasis	<5	>60	>60
tiotropium bromide	0.14 (0.002)	0.10 (0.02)	0.12 (0.02)	>6[g]	45	mydriasis	5 (6)	>60	>60
ipratropium bromide	0.83 (0.01)	1.02 (0.07)	1.51 (0.08)	3.4[h]	68	mydriasis	<5	>60	>60

[a] Values shown are the mean, $n=3$ (SD). [b] Experimental conditions: pH 7.4, $T = 37$ °C. [c] Values shown are the mean, $n=2$ (SD). [d] ip=intraperitoneal. [e] Half-life calculated with at least three experimental time points. [f] Administered concentration of 3 mg/mL, $n = 4-6$ animals. [g] Administered concentration of 1 mg/mL, $n=4-6$ animals. [h] Administered concentration of 0.3 mg/mL, $n=4-6$ animals.

Table 5. Effect of R^2 Substitution on the in Vitro Human Plasma Stability of Compound **19** Derivatives



R^2	compd	binding affinity,[a] IC_{50} (nM)			human plasma stability % disappearance[b]			
		M_3	M_2	M_1	5 min	15 min	60 min	$t_{1/2}$ (min)[c]
	19	0.23 (0.01)	0.26 (0.001)	0.20 (0.02)	NE[d]	14 (5)	53 (6)	54.5
CH_3-	**51**	0.60 (0.01)	0.46 (0.003)	0.43 (0.12)	51 (1)	94 (0)	NE[d]	3.6
$Ph(CH_2)_2-$	**54**	0.20[b] (0.003)	0.18[b] (0.06)	0.14[b] (0.04)	NE[d]	68 (3)	99 (0)	7.9
$PhO(CH_2)_3-$	**56**	0.17[b] (0.05)	0.17[b] (0.05)	0.14[b] (0.03)	78 (2)	99 (0)	NE[d]	2.2
	tiotropium bromide	0.35[b] (0.01)	0.21[b] (0.04)	0.22[b] (0.03)	NE[d]	5 (6)	35 (3)	>60
	ipratropium bromide	2.07[b] (0.03)	2.24[b] (0.16)	2.65[b] (0.13)	NE[d]	<5	<5	>60

[a] Values shown are the mean, $n=2$ (SD). [b] Values shown are the mean, $n=3$ (SD). [c] Half-life calculated with at least three experimental time points. [d] NE = not evaluated.

The analyses of test compounds were performed by ultraperformance liquid chromatography (Acquity Ultra Performance LC, Waters, Milford, MA) with a Quattro Premier mass spectrometry detector operating in positive mode (Micromass Technologies, Waters, Milford, MA).

In all experiments, the stability of test compounds was determined as the percentage of remaining compound at the time point of interest (assuming 100% of the compound is present at time 0).

The test compound elimination half-life ($t_{1/2}$) in human plasma was estimated using the WinNonlin software, version 5.0.1. (Pharsight Corporation). The slope of the linear regression from log [S] versus time plot ($-k$) was determined, and the in vitro plasma half-life was determined as $t_{1/2} = -0.693/k$.

Chemical Stability Studies. The stability of the test compounds was assessed by monitoring for 24 h the 210 nm UV−UPLC chromatograms of 0.05 mg/mL compound solutions at 37 °C and pH 7.4.

An amount of 1 mg/mL test solution of each compound in DMSO (dimethyl sulfoxide) was prepared. At time zero, 50 μL of the test solution was added to 1 mL of pH 7.4 buffer solution (final concentration, 0.05 mg/mL). This solution was placed in the UPLC sample manager thermostabilized at 37 °C, and an amount of 5 μL was injected into the UPLC system (time zero). Final test solutions were prepared in duplicate. At predetermined times (0, 1, 4, 8, 12, 16, 20, and 24 h), an amount of 5 μL of each solution was injected in the UPLC system.

Control solutions for each compound were prepared using acetonitrile instead of pH 7.4 buffer solution. All compounds were stable in acetonitrile solutions.

The analyses of test compounds were performed by ultraperformance liquid chromatography (Acquity Ultra Performance LC, Waters, Milford, MA) with a PDA Acquity detector (Waters, Milford, MA).

In all experiments, the stability of test compounds was determined by integration of 210 nm extracted and blank subtracted chromatogram.

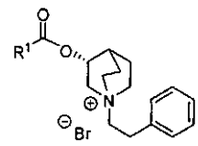
All obtained chromatograms were processed using Empower software (Waters, Milford, MA). The 210 nm chromatograms were extracted from 3D diode array signal, and a blank chromatogram was subtracted. Purity of compounds tested at each time point was determined by area normalization.

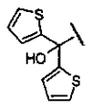
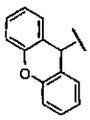
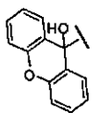
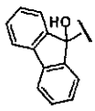
The test compound stability half-life ($t_{1/2}$) at 37 °C and pH 7.4 was estimated using the Kinetica software, version 4.2 (InnaPhase). The slope of the linear regression from log[S] versus time plot ($-k$) was determined, and the chemical stability half-life was determined as $t_{1/2} = -0.693/k$.

In Vivo Studies. Animals. Male Dunkin−Hartley guinea pigs (400−600 g) or male Swiss mice (20−25 g) were obtained from Harlan (Interfauna Ibérica, Spain). Animals were housed in groups of four or five at 20−24 °C under a 12 h light/dark cycle. Food and water were available ad libitum. All experiments were carried out with the approval of the Animal Ethical Committee of Almirall.

In Vivo Duration of Action Studies. Guinea pigs were anesthetized with an intraperitoneal injection of 1 g/kg urethane and 20 mg/kg sodium pentobarbital. Additional anesthetic was administered after 60 min, as required. The trachea was cannulated, and the lungs were artificially ventilated with a small rodent ventilator (Ugo Basile, Biological Research Apparatus, Comerio-Varese, Italy) at a rate of 60 strokes/min and a tidal volume of 10 mL/kg. Animals were maintained at 37 °C with a homeothermic blanket throughout the experiment. Blood pressure was measured from the carotid artery, and acetylcholine was administered via the jugular vein. Intrapulmonary pressure and blood pressure were measured by blood pressure transducers (MLT0699, ADInstruments-Panlab, Barcelona, Spain) connected to a bridge amplifier (PowerLab/8sp, ADInstruments-Panlab, Madrid, Spain).

Table 6. Effect of R^1 Substitution on the in Vitro Human Plasma Stability of Compound **54** Analogues



		binding affinity[a] (IC_{50}, nM)			human plasma stability (% disappearance)[b]			
R^1	cmpd	M_3	M_2	M_1	5 min	15 min	60 min	$t_{1/2}$ (min)[c]
	**54**	0.20[b] (0.003)	0.18[b] (0.06)	0.14[b] (0.04)	NE[d]	68 (3)	99 (0)	7.9
	**43**	0.31 (0.02)	1.82 (0.19)	0.44 (0.03)	NE[d]	22 (6)	53 (4)	56.1
	**44**	0.28 (0.12)	0.54 (0.15)	0.29 (0.10)	NE[d]	7 (11)	15 (3)	> 60
	**47**	1.04 (0.12)	2.24 (0.28)	1.12 (0.02)	NE[d]	35 (4)	90 (1)	17.6
	tiotropium bromide	0.35[b] (0.01)	0.21[b] (0.04)	0.22[b] (0.03)	NE[d]	5 (6)	35 (3)	> 60
	ipratropium bromide	2.07[b] (0.03)	2.24[b] (0.16)	2.65[b] (0.13)	NE[d]	< 5	< 5	> 60

[a] Values shown are the mean, $n = 2$ (SD). [b] Values shown are the mean, $n = 3$ (SD). [c] Half-life calculated with at least three experimental time points. [d] NE = not evaluated.

The data were recorded using Chart 5 software (ADInstruments-Panlab, Barcelona, Spain).

After induction of anesthesia and preparation, animals were allowed to stabilize for 10 min before bronchoconstriction was induced by an intravenous administration of acetylcholine. Acetylcholine (Sigma, Tres Cantos, Spain) was administered at a dose (10–60 μg/kg) that approximately doubled the basal intrapulmonary pressure. Repeated bolus injections of acetylcholine at the selected dose were administered until two reproducible responses were obtained; the mean of the two final responses before the addition of the test compound corresponded to the maximal response to acetylcholine and was used to evaluate the antibronchoconstrictor effect of the test compounds.

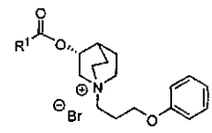
Five minutes after the last administration of acetylcholine that was used to calculate maximal bronchoconstriction, the test compound was administered via a nebulizer (5 s duration; Mumed ultrasonic nebulizer, Mumed Systems Ltd., London, U.K.) to investigate the reversal of the bronchoconstriction. Test compounds were delivered in the concentration range 0.3–3 mg/mL. Acetylcholine doses were then administered 5, 10, 20, 30, 60, 120, 180, 240, 300, and 360 min after the administration of the test compound to evaluate the antibronchoconstrictor effects of each compound. This was expressed as a percentage of the maximal response to acetylcholine. Duration of action (T, h) was defined as the time taken to recover 50% of the maximum inhibitory effect achieved by the test compound and was derived from a time-course bronchoconstriction curve and calculated with a one-phase exponential decay formula using GraphPad Prism software (San Diego, CA).

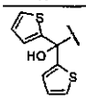
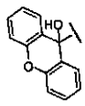
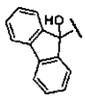
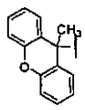
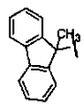
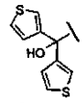
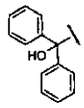
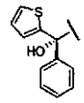
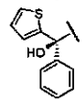
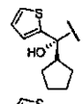
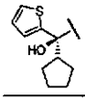
In Vivo Bronchospasm Inhibitory Activity. The in vivo potency (IC_{50}) of selected compounds was determined using the guinea pig model described above, at the t_{max} (data not shown), from a sigmoidal dose–response curve. T_{max} was defined as the time taken from the administration of the test compound to the time at which maximum inhibition of the acetylcholine-induced bronchoconstriction was achieved. The IC_{50} was calculated using GraphPad Prism software (San Diego CA).

In Vivo Safety Observational Test. Assessment of safety was performed for selected compounds administered intraperitoneally at a dose of 30 mg/kg, using an in vivo safety observational test focusing on antimuscarinic side effects (e.g., mydriasis, xerostomia) in Swiss mice. This assay comprises standardized observational tests that assess the neurobiological state of rodents and include measures of autonomic and sensorimotor functions, convulsive behavior, and excitability up to 24 h after drug administration.

Chemistry: General. Reagents, starting materials, and solvents were purchased from commercial suppliers and used as

Table 7. Effect of R^1 Substitution on the in Vitro Human Plasma Stability of Compound **56** Analogues



R^1	cmpd	binding affinity[a] (IC_{50}, nM) M_3	M_2	M_1	human plasma stability (% disappearance)[b] 5 min	15 min	60 min	$t_{1/2}$ (min)[c]
	**56**	0.17[b] (0.05)	0.17[b] (0.05)	0.14[b] (0.03)	78 (2)	99 (0)	NE[d]	2.2
	**45**	0.42 (0.03)	0.36 (0.06)	0.23 (0.01)	NE[d]	6 (1)	30 (4)	> 60
	**48**	0.71 (0.07)	1.08 (0.14)	0.63 (0.02)	NE[d]	68 (5)	98 (0)	10.9
	**49**	0.24 (0.01)	0.27 (0.05)	0.28 (0.01)	NE[d]	< 5	< 5	> 60
	**50**	0.96 (0.11)	2.10 (0.10)	1.26 (0.05)	NE[d]	15 (12)	29 (3)	> 60
	**57**	0.37 (0.01)	0.35 (0.001)	0.34 (0.02)	100 (0)	100 (0)	NE[d]	0.6
	**58**	0.24 (0.12)	0.18 (0.03)	0.23 (0.01)	NE[d]	50 (13)	71 (1)	15.2
	**59**	0.59 (0.05)	0.73 (0.03)	0.60 (0.05)	NE[d]	32 (2)	82 (2)	23.9
	**60**	0.61 (0.03)	0.44 (0.01)	0.31 (0.05)	NE[d]	25 (7)	39 (3)	> 60
	**61** (2*S*-isomer)	0.25 (0.02)	0.17 (0.01)	0.16 (0.02)	NE[d]	26 (3)	79 (0)	26.5
	**62** (2*R*-isomer)	0.48 (0.004)	0.31 (0.01)	0.38 (0.01)	NE[d]	78 (1)	99 (0)	8.9
	**63** (2*S*-isomer)	0.18[b] (0.02)	0.14[b] (0.04)	0.15[b] (0.01)	NE[d]	< 5	< 5	> 60
	**64** (2*R*-isomer)	1.62 (0.21)	1.01 (0.29)	0.81 (0.17)	NE[d]	11 (5)	10 (3)	> 60
	tiotropium bromide	0.35[b] (0.01)	0.21[b] (0.04)	0.22[b] (0.03)	NE[d]	5 (6)	35 (3)	> 60
	ipratropium bromide	2.07[b] (0.03)	2.24[b] (0.16)	2.65[b] (0.13)	NE[d]	< 5	< 5	> 60

[a] Values shown are the mean, $n = 2$ (SD). [b] Values shown are the mean, $n = 3$ (SD). [c] Half-life calculated with at least three experimental time points. [d] NE = not evaluated.

received. Concentration refers to evaporation under vacuum using a Büchi rotatory evaporator. Reaction products were purified, when necessary, by flash chromatography on silica gel (40–63 μm) with the solvent system indicated. Melting points were recorded on a Büchi B-540 instrument. ^{1}H NMR spectra were performed using a Varian Gemini 2000 spectrometer operating at a frequency of 200 or 300 MHz. Samples were dissolved in deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide (DMSO-d_6). Tetramethylsilane (TMS) was used as reference. The following abbreviations were used to assign spectra: (s) singlet, (d) doublet, (dd) double doublet, (ddd) double double doublet, (t) triplet, (dt) double triplet, (td) triple doublet, (m) multiplet, (br s) broad signal. HPLC–UV–MS chromatograms were acquired in a Waters Alliance 2695 chromatographer equipped with a Waters 2996 diode-array detector and a Waters ZQ mass spectrometer detector. HPLC analysis was conducted according to method A, B, or C, with the retention time (t_R) expressed in min. UV chromatograms were processed at 210 nm with blank subtraction. For HPLC method A, chromatography was performed on a Kromasil C18 column (100 mm × 4.6 mm, 5 μm). The mobile phase, at a flow of 1 mL/min, was a 30 min binary gradient (5–85%) of water (containing 0.01 M phosphoric acid at pH 3.0 with sodium hydroxide, 0.1 N) and acetonitrile. The total run time was 35 min. For HPLC method B, chromatography was performed on a Symmetry C18 column (100 mm × 2.16 mm, 3.5 μm). The mobile phase, at a flow of 0.4 mL/min, was a 20 min binary gradient of water (containing 0.01 M ammonic formiate at pH 3.0) and a mixture of acetonitrile–methanol 50:50 (containing 0.01 M ammonic formiate) (0–95%). The total run time was 26 min. For HPLC method C, chromatography was performed on a Symmetry C18 column (100 mm × 2.16 mm, 3.5 μm). The mobile phase, at a flow of 0.4 mL/min, was a 20 min binary gradient of water (containing 0.01 M ammonic formiate at pH 7.4) and a mixture of acetonitrile/methanol, 50:50 (containing 0.01 M ammonic formiate) (0–95%). The total run time was 26 min. Specific optical rotations were measured in a Perkin-Elmer 241 MC polarimeter at the wavelength of the D-line of sodium (λ = 589.3 nm) at 25 or 22 °C using a layer of 1 dm. Circular dichroism spectra were recorded on a Jasco J-720 spectropolarimeter controlled by J-700 software using the following acquisition conditions: optical path 0.1 cm, temperature 25 °C, purge gas nitrogen at 5 L/min, spectral width 1.0 nm, response time 4 s, wavelength 200–300 nm, scan speed 10 nm/min, number of scans 2. Samples were dissolved in methanol (concentration, 0.43 mM).

Tiotropium bromide was synthesized by the Department of Medicinal Chemistry (Laboratorios Almirall, Barcelona, Spain). Ipratropium bromide was purchased from Sigma Chemicals (Tres Cantos, Spain). The purity of these compounds was determined by HPLC–MS.

The methods used for the preparation of compounds **2**,[34] **7**,[35] **9**, **10**, **14**, and **15** (Scheme 1); **18**,[36] **20**, **22**,[36] and **25** (Scheme 2); and **31**[39] and **32** (Scheme 3) are available as Supporting Information.

Preparation of (3*R*)-Quinuclidinol Esters 5[19,20] **and 6 (Scheme 1). (3*R*)-1-Azabicyclo[2.2.2]oct-3-yl 9*H*-xanthene-9-carboxylate (5)**[19,20]**.** To a solution of the acid **1** (5 g, 22.1 mmol) in 65 mL of $CHCl_3$ (amylene stabilized), cooled to 0 °C, was added DMF (2 drops) followed by oxalyl chloride (3.1 mL, 36.6 mmol) dissolved in 5 mL of $CHCl_3$. After 1.5 h of stirring at room temperature, the reaction mixture was concentrated and the residue was coevaporated with $CHCl_3$ three times to give the acyl chloride **3** (100%). Compound **3**, dissolved in 30 mL of $CHCl_3$, was slowly added to a solution of (3*R*)-quinuclidinol (3.05 g, 23.9 mmol) in 50 mL of $CHCl_3$. The mixture was stirred at room temperature overnight. After this reaction time, solvent was evaporated and the residue was dissolved in toluene and extracted with 2 N HCl. The acid layers were combined and basified with solid K_2CO_3. The basic solution was extracted with $CHCl_3$. The organic layers were combined, washed with water, dried over $MgSO_4$, and concentrated. The obtained residue was purified by formation of the hydrochloride salt. The residue was dissolved in diethyl ether (150 mL), and a solution of EtOH/HCl (g) was slowly added. The solid obtained was filtered and washed with diethyl ether to give 5.6 g (68%) of the hydrochloride salt of compound **5**, mp 227 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.55–1.93 (m, 4 H), 2.06–2.17 (m, 1 H), 2.82–2.99 (m, 2 H), 2.98–3.15 (m, 2 H), 3.15–3.29 (m, 1 H), 3.48–3.63 (dd, J = 12.8, 9.2 Hz 1 H), 4.88–4.99 (m, 1 H), 5.27 (s,1 H), 7.11–7.28 (m, 4 H), 7.38 (dd, J = 8.2, 7.0 Hz, 2 H), 7.49 (dd, J = 7.0, 5.5 Hz, 2 H). HPLC (method B): 96.6%, t_R = 11.0 min. MS (ESI) 336 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl 10,11-dihydro-5*H*-dibenzo-[*a*,*d*][7]annulene-5-carboxylate (6) was prepared as described for compound **5** starting from the acid **2**[34] (2.15 g, 9.0 mmol) and (3*R*)-quinuclidinol (1.26 g, 9.9 mmol). The reaction conditions used in the second step were as follows: toluene, reflux, 2 h. Purification by column chromatography (silica gel, chloroform/methanol/NH_4OH, 95:5:0.5, as eluent) afforded 1.5 g (48%) of compound **6**, mp 112–113 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.07–1.36 (m, 2 H), 1.36–1.53 (m, 1 H), 1.54–1.69 (m, 1 H), 1.84–1.93 (m, 1 H), 2.47–2.58 (m, 2 H), 2.59–2.76 (m, 3 H), 2.80–3.00 (m, 2 H), 3.06–3.18 (ddd, J = 14.7, 7.9, 2.1 Hz, 1 H), 3.25–3.43 (m, 2 H), 4.75–4.83 (m, 1 H), 4.82 (s, 1 H), 7.09–7.33 (m, 8 H). HPLC (method B): 99.8%, t_R = 11.4 min. MS (ESI) 348 m/z $(M + H)^+$.

Preparation of (3*R*)-Quinuclidinol Esters 11,[33] **12, 16, and 17 (Scheme 1) and 19, 21, 23, and 26 (Scheme 2). (3*R*)-1-Azabicyclo[2.2.2]oct-3-yl 9-hydroxy-9*H*-xanthene-9-carboxylate (11)**[33]**.** To a stirred solution of the methyl ester **9** (2 g, 7.8 mmol) and (3*R*)-quinuclidinol (1.16 g, 9.1 mmol) in 50 mL of toluene was added NaH 60% in mineral oil (0.125 g, 3.12 mmol) in several portions. The mixture was refluxed with continuous removal of distillate and replacement with fresh toluene over 1.5 h. After this time, the reaction mixture was cooled to room temperature and extracted with 2 N HCl. The acid extracts were combined, washed with a small volume of AcOEt, basified by addition of solid K_2CO_3, and extracted with $CHCl_3$. The organic extracts were combined, washed with water, dried over Na_2SO_4, and concentrated. The obtained product was purified by dissolving in diethyl ether and repeating the acid/base extraction process to afford 1.86 g (68%) of compound **11**, mp 175 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 0.88–1.09 (m, 2 H), 1.22–1.49 (m, 2 H), 1.53–1.62 (m, 1 H), 1.88–1.98 (d, J = 14.3 Hz, 1 H), 1.99–2.14 (m, 1 H), 2.37–2.57 (m, 3 H), 2.81–2.93 (ddd, J = 14.6, 8.0, 2.4 Hz, 1 H), 4.49–4.58 (m, 1 H), 7.01–7.09 (br s, 1 H), 7.13–7.28 (m, 4 H), 7.33–7.45 (m, 2 H), 7.58–7.65 (dd, J = 7.6, 1.8 Hz, 2 H). HPLC (method A): 99.9%, t_R = 10.4 min. MS (ESI) 352 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl 9-hydroxy-9*H*-fluorene-9-carboxylate (12) was prepared as described for compound **11** starting from the methyl ester **10** (5 g, 20.8 mmol). The obtained product was purified by crystallization from $CHCl_3$/diisopropyl ether to afford 4.63 g (66%) of compound **12**, mp 217 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 0.90–1.08 (m, 2 H), 1.23–1.50 (m, 2 H), 1.54–1.67 (m, 1 H), 1.95–2.17 (m, 2 H), 2.35–2.58 (m, 3 H), 2.82–2.98 (dd, J = 14.3, 7.9 Hz, 1 H), 4.50–4.63 (m, 1 H), 6.74 (br s, 1 H), 7.27–7.38 (m, 2 H), 7.38–7.48 (m, 2 H), 7.51 (d, J = 6.41 Hz, 2 H), 7.81 (d, J = 7.32 Hz, 2 H). HPLC (method B): 99.7%, t_R = 8.0 min. MS (ESI) 336 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl 9-methyl-9*H*-xanthene-9-carboxylate (16) was prepared as described for compound **11** starting from the methyl ester **14** (2.65 g, 10.4 mmol). The yield was 1.91 g (53%) of **16** as an oil. Compound **16** (0.3 g, 0.86 mmol) was treated with oxalic acid (0.077 g, 0.86 mmol) in acetone/diethyl ether to give 0.25 g (66%) of the oxalate salt, mp 152 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.18–1.33 (m, 1 H), 1.39–1.56 (m, 1 H), 1.59–1.82 (m, 2 H), 1.90 (s, 3 H),

1.94−2.04 (m, 1 H), 2.53−2.67 (m, 1 H), 2.69−2.79 (d, J = 14.0 Hz, 1 H), 2.90−3.14 (m, 3 H), 3.42−3.55 (ddd, J = 14.2, 8.4, 2.7 Hz, 1 H), 4.84−4.96 (m, 1 H), 7.11−7.21 (m, 4 H), 7.30−7.40 (m, 2 H), 7.41−7.50 (m, 2 H), 7.80−10.80 (br s, 2 H). HPLC (method A): 100.0%, t_R = 15.1 min. MS (ESI) 350 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl 9-methyl-9*H*-fluorene-9-carboxylate (17) was prepared as described for compound **11** starting from the methyl ester **15** (3.15 g, 13.2 mmol). The obtained product was purified by column chromatography (silica gel, chloroform/isopropanol, 80:20, as eluent) to give 3.44 g (80%) of compound **17** as an oil. Compound **17** (0.3 g, 0.89 mmol) was treated with oxalic acid (0.080 g, 0.89 mmol) in acetone/diethyl ether to give 0.26 g (68%) of the oxalate salt, mp 186.3 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.34−1.61 (m, 2 H), 1.61−1.86 (m, 2 H), 1.75 (s, 3 H), 1.98−2.07 (m, 1 H), 2.72−2.87 (m, 1 H), 2.90−2.99 (d, J = 14.6 Hz, 1 H), 2.99−3.21 (m, 3 H), 3.47−3.59 (ddd, J = 14.0, 8.2, 2.4 Hz, 1 H), 4.82−4.95 (m, 1 H), 7.33−7.41 (m, 2 H), 7.42−7.50 (m, 2 H), 7.60−7.66 (d, J = 7.0 Hz, 1 H), 7.66−7.72 (d, J = 7.3 Hz, 1 H), 7.87−7.93 (d, J = 7.3 Hz, 2 H), 6.50−11.50 (br s, 2 H). HPLC (method A): 100.0%, t_R = 14.7 min. MS (ESI) 334 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl hydroxy(di-2-thienyl)acetate (19) was prepared as described for compound **11** starting from the methyl ester **18**[36] (6.0 g, 23.6 mmol). The obtained product was purified by treatment with diisopropyl ether to afford 4.11 g (50%) of compound **19**, mp 180 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.12−1.34 (m, 1 H), 1.37−1.68 (m, 3 H), 1.87−1.95 (m, 1 H), 2.34−2.75 (m, 5 H), 3.04−3.15 (ddd, J = 14.7, 8.1, 2.0 Hz 1 H), 4.76−4.87 (m, 1 H), 6.98−7.04 (m, 2 H), 7.09−7.12 (m, 2 H), 7.37 (br s, 1 H), 7.46 −7.54 (m, 2 H). HPLC (method B): 98.8%, t_R = 7.7 min. MS (ESI) 350 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl di-2-thienylacetate (21) was prepared as described for compound **11** starting from the methyl ester **20** (1.67 g, 7.0 mmol). The obtained product was purified by column chromatography (silica gel, chloroform/methanol/NH_4OH, 95:5:0.5, as eluent) to give 0.82 g (35%) of compound **21** as an oil. Compound **21** (0.82 g, 2.5 mmol) was treated with fumaric acid (0.144 g, 1.23 mmol) in acetone/diethyl ether to give 0.58 g (60%) of the fumarate salt, mp 122 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.34−1.52 (m, 1 H), 1.52−1.78 (m, 3 H), 1.98−2.07 (m, 1 H), 2.62−2.72 (d, J = 14.6 Hz, 1 H), 2.72−2.97 (m, 4 H), 3.27−3.37 (ddd, J = 14.5, 8.4, 2.1 Hz, 1 H), 4.84−4.96 (m, 1 H), 5.85 (s, 1 H), 6.55 (s, 2 H), 6.97−7.03 (dd, J = 5.2, 3.7 Hz, 2 H), 7.07−7.12 (m, 2 H), 7.47−7.52 (dd, J = 5.2, 1.2 Hz, 2 H). HPLC (method B): 98.7%, t_R = 10.7 min; MS(ESI) 334 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl hydroxy(di-3-thienyl)acetate (23) was prepared as described for compound **11** starting from the methyl ester **22**[36] (3.55 g, 14.0 mmol). The obtained product was purified by crystallization in acetonitrile. The resulting solid was filtered and washed with diethyl ether to afford 1.54 g (31.5%) of compound **23**, mp 151 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.10−1.31 (m, 1 H), 1.31−1.68 (m, 3 H), 1.82−1.94 (m, 1 H), 2.36−2.45 (d, J = 14.6 Hz, 1 H), 2.45−2.72 (m, 4 H), 3.02−3.15 (ddd, J = 14.6, 8.2, 2.3 Hz, 1 H), 4.73−4.83 (m, 1 H), 6.54−6.68 (br s, 1 H), 7.06−7.12 (dt, J = 5.2, 1.5 Hz, 2 H), 7.34−7.40 (td, J = 3.3, 1.4 Hz, 2 H), 7.46−7.54 (dt, J = 5.1, 3.4 Hz, 2 H). HPLC (method A): 99.0%, t_R = 10.0 min. MS (ESI) 350 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl 2,2-di-2-thienylpropanoate (26) was prepared as described for compound **11** starting from the methyl ester **25** (0.86 g, 3.4 mmol). The yield was 1.11 g (94.1%) of compound **26** as an oil. Compound **26** (0.25 g, 0.72 mmol) was treated with oxalic acid (65 mg, 0.72 mmol) in acetone/diethyl ether to give 0.25 g (79%) of the oxalate salt, mp 126.7−128.6 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.50−1.70 (m, 2 H), 1.69−1.93 (m, 2 H), 2.07 (s, 3 H), 2.14−2.24 (m, 1 H), 2.78−2.95 (m, 1 H), 2.95−3.24 (m, 4 H), 3.54−3.67 (ddd, J = 13.9, 8.4, 1.8 Hz, 1 H), 5.01−5.15 (m, 1 H), 6.97−7.04 (ddd, J = 5.2, 3.5, 1.7 Hz, 2 H), 7.04−7.10 (m, 2 H), 7.47−7.52 (m, 2 H), 7.75−10.75 (br s, 2 H). HPLC (method B): 99.3%, t_R = 11.1 min. MS (ESI) 348 m/z $(M + H)^+$.

Preparation of (3*R*)-Quinuclidinol Esters 33,[19] 34, 35, 36, 37, 38, and 39 (Scheme 3). (3*R*)-1-Azabicyclo[2.2.2]oct-3-yl hydroxy(diphenyl)acetate (33)[19]. To a solution of compound **31**[39] (1 g, 3.86 mmol) in 2 mL of THF, cooled to −40 °C, was slowly added phenyllithium (1.8 M solution in cyclohexane/diethyl ether, 2.4 mL, 4.32 mmol). The mixture was stirred for 10 min at −40 °C. Then it was warmed to room temperature and the stirring was continued for 1 h. The reaction mixture was poured over a saturated aqueous NH_4Cl solution and extracted with diethyl ether. The ether extracts were combined, washed with brine, dried over Na_2SO_4, and concentrated. The obtained residue was purified by treatment with diisopropyl ether to afford 0.46 g (35%) of compound **33**, mp 188 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.07−1.24 (m, 1 H), 1.24−1.40 (m, 1 H), 1.39−1.65 (m, 2 H), 1.82−1.90 (m, 1 H), 2.30−2.46 (m, 2 H), 2.52−2.67 (m, 3 H), 3.00−3.15 (ddd, J = 14.7, 8.1, 2.3 Hz, 1 H), 4.75−4.86 (m, 1 H), 6.59 (br s, 1 H), 7.16−7.47 (m, 10 H). HPLC (method B): 95.6%, t_R = 9.4 min. MS (ESI) 338 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl hydroxy(phenyl)2-thienylacetate (34). To a solution of compound **32** (16.6 g, 62.5 mmol) in 300 mL of THF, cooled to −70 °C, was added phenylmagnesium bromide (1.0 M solution in THF, 75 mL, 75 mmol). The mixture was stirred for 15 min at ≤−60 °C, and then it was warmed to room temperature and the stirring continued for 2 h. The reaction mixture was poured over a saturated aqueous NH_4Cl solution and extracted with ethyl acetate. The organic extracts were combined, washed with brine, dried over Na_2SO_4, and concentrated. The obtained solid was treated with isopropanol at room temperature, filtered, and washed with diethyl ether to yield 9.85 g (38%) of compound **34** as a mixture of diastereomers **35** and **36**, which were separated by crystallization. The diastereomeric resolution was monitored by ^{1}H NMR, and crystallizations were repeated until the signals of the minor diastereomer were negligible.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl (2*R*)-hydroxy(phenyl)2-thienylacetate (36). After five successive crystallizations of compound **34** from boiling isopropanol, an amount of 2.08 g (16%) of diastereomer **36** was obtained. Diastereomeric purity by ^{1}H NMR was ≥95%, mp 174−175 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.07−1.27 (m, 1 H), 1.27−1.41 (m, 1 H), 1.41−1.64 (m, 2 H), 1.80−1.87 (m, 1H), 2.36−2.71 (m, 5 H), 3.03−3.15 (ddd, J = 14.6, 8.2, 1.9 Hz, 1 H), 4.74−4.86 (m, 1 H), 7.00 (s, 1 H), 7.01−7.10 (m, 2 H), 7.24−7.53 (m, 6 H). HPLC (method B): 99.9%, t_R = 8.6 min. MS (ESI) 344 m/z $(M + H)^+$.

The absolute configuration of the ester moiety of compound **36** was determined by correlation with the absolute configuration of (+)-hydroxy(phenyl)2-thienylacetic acid (compound **40**). Diastereomer **36** was hydrolyzed (MeOH, NaOH, 30 min, at room temperature) to give compound **40**, $[\alpha]^{25}_D$ = +25.4° (c = 2, EtOH). The *R* configuration for the acid **40** was assigned by comparison of its [α] value with the data described in the literature for (2*S*)-hydroxy(phenyl)2-thienylacetic acid: $[\alpha]^{25}_D$ = −20° (c = 2, EtOH).[40]

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl (2*S*)-hydroxy(phenyl)2-thienylacetate (35). The mother liquors from the first crystallization of compound **34** were enriched with the diastereomer **35**. The solution was concentrated and cooled. The precipitated solid was filtered and discarded. The filtrate was concentrated and treated with diethyl ether, and the resulting solid was filtered to give 0.89 g (7%) of compound **35**. Diastereomeric purity by ^{1}H NMR was ≥95%, mp 151.8−152.6 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.11−1.32 (m, 1 H), 1.35−1.64 (m, 3 H), 1.87−194 (m, 1 H), 2.28−2.46 (m, 2 H), 2.46−2.69 (m, 3 H), 2.97−3.15 (ddd, J = 14.7, 8.1, 2.0 Hz, 1 H), 4.71−4.86 (m, 1 H), 6.98 − 7.10 (m, 3 H), 7.26−7.52 (m, 6 H). HPLC (method B): 94.5%, t_R = 8.3 min. MS (ESI) 344 m/z $(M + H)^+$.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl cyclopentyl(hydroxy)2-thienylacetate (37) was prepared as described for compound **34**

starting from a solution of compound **32** (39.0 g, 147 mmol) in THF (325 mL) and cyclopentyl magnesium chloride (2.0 M solution in diethyl ether, 86 mL, 172 mmol). Purification by column chromatography (silica gel, chloroform/methanol/NH_4OH, 97:3:0.5 to 97:4:0.5, as eluent) afforded 22.8 g (46%) of compound **37** as a mixture of diastereomers **38** and **39**, which were separated by crystallization. The diastereomeric resolution was monitored by ^1H NMR, and crystallizations were repeated until the signals of the minor diastereomer were negligible.

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl (2*S*)-cyclopentyl(hydroxy)2-thienylacetate (38). After successive macerations of the mixture **37** with diisopropyl ether (twice) and with diethyl ether (twice), an amount of 3.8 g (15%) of diastereomer **38** was obtained. Diastereomeric purity by ^1H NMR was ≥95%, mp 157.3−158.7 °C. ^1H NMR (300 MHz, $CDCl_3$) δ ppm 1.36−1.83 (m, 11 H), 1.83−1.97 (m, 1 H), 2.03−2.15 (m, 1 H), 2.50−2.64 (m, 1 H), 2.64−2.98 (m, 5 H), 3.09−3.23 (dd, J = 14.8, 8.1 Hz, 1 H), 4.08 (br s, 1 H), 4.87−4.98 (m, 1 H), 6.96−7.04 (m, 1 H), 7.12−7.19 (m, 1 H), 7.25−7.31 (m, 1 H). HPLC (method B): 99.2%, t_R = 9.8 min. MS (ESI) 336 m/z $(M + H)^+$.

The absolute configuration of the ester moiety of compound **38** was determined by correlation with the absolute configuration of (−)-cyclopentyl(hydroxy)2-thienylacetic acid[42] (compound **41**). Diastereomer **38** was hydrolyzed (EtOH/NaOH, 2 N, 2 h at room temperature and 2 h at 60 °C) to give compound **41**, $[\alpha]^{22}_D$ = −6.44° (c = 1, EtOH). CD curve: λ (nm) = 233, $\Delta\varepsilon$ (M^{-1} cm^{-1}) = −4.19 . Configuration *S* was assigned by comparison of the CD curve with that of the acid **68**[41] (see the Supporting Information for more details).

(3*R*)-1-Azabicyclo[2.2.2]oct-3-yl (2*R*)-cyclopentyl(hydroxy)2-thienylacetate (39). The mother liquors from the first treatment of compound **37** with diisopropyl ether were enriched with the diastereomer **39**. The solution was concentrated, and the residue was purified by column chromatography (silica gel, chloroform/methanol/NH_4OH, 97:3:0.5 to 97:4:0.5, as eluent). The obtained product was treated with diisopropyl ether (twice) and then with chloroform/diethyl ether to give 1.2 g (5%) of compound **39**. Diastereomeric purity by HPLC (method B) was 92.1%; mp was not determined. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 1.18−1.69 (m, 12 H), 1.80 −1.89 (m, 1 H), 2.40−2.89 (m, 6 H), 3.05−3.22 (dd, J = 14.5, 8.1 Hz, 1 H), 4.64−4.74 (m, 1 H), 5.99 (br s, 1 H), 6.95−7.00 (dd, J = 4.9, 3.7 Hz, 1 H), 7.10 (d, J = 3.7 Hz, 1 H), 7.40 (d, J = 4.9 Hz, 1 H). HPLC (method B): 95.7% (compound **39**, 88.1%; compound **38**, 7.5%), t_R = 9.5 min. MS (ESI) 336 m/z $(M + H)^+$.

The absolute configuration of the ester moiety of compound **39** was determined by correlation with the absolute configuration of (+)-cyclopentyl(hydroxy)2-thienylacetic acid[42] (compound **42**). Diastereomer **39** was hydrolyzed (EtOH/NaOH, 2 N, 2 h at room temperature and 2 h at 60 °C) to give compound **42**, $[\alpha]^{22}_D$ = +6.63° (c = 1, EtOH). CD curve: λ (nm) = 233, $\Delta\varepsilon$ (M^{-1} cm^{-1}) = +4.18. Configuration *R* was assigned by comparison of the CD curve with that of the acid **69**.[41] (see the Supporting Information section for more details).

Preparation of Quaternary Ammonium Derivatives 43−64 (Scheme 4). (3*R*)-1-(2-Phenylethyl)-3-[(9*H*-xanthen-9-ylcarbonyl)oxy]-1-azoniabicyclo[2.2.2]octane bromide (43). To a solution of compound **5** (5.05 g, 15.05 mmol) in 100 mL of THF was added (2-bromoethyl)benzene (6.88 g, 37.20 mmol). The mixture was refluxed for 9 h and then stirred at room temperature for 80 h. The precipitated solid was filtered and washed with diethyl ether to obtain 6.5 g (83%) of compound **43**, mp 258 °C. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 1.65−2.03 (m, 4 H), 2.14−2.24 (m, 1 H), 2.89−3.10 (m, 2 H), 3.18−3.34 (m, 1 H), 3.35−3.55 (m, 5 H), 3.55−3.70 (m, 1 H), 3.85−3.98 (ddd, J = 13.7, 8.2, 2.1 Hz, 1 H), 5.01−5.12 (m, 1 H), 5.33 (s, 1 H), 7.14−7.22 (m, 3 H), 7.22 (s, 1 H), 7.24−7.42 (m, 7 H), 7.46−7.60 (ddd, J = 17.0, 7.6, 1.5 Hz, 2 H). HPLC (method B): 100.0%, t_R = 12.3 min. MS (ESI) 440 m/z $(M - Br)^+$.

(3*R*)-3-{[(9-Hydroxy-9*H*-xanthen-9-yl)carbonyl]oxy}-1-(2-phenylethyl)-1-azoniabicyclo[2.2.2]octane bromide (44). To a solution of compound **11** (0.3 g, 0.85 mmol) in 4 mL of acetonitrile and 6 mL of chloroform was added (2-bromoethyl)benzene (0.79 g, 4.25 mmol). The mixture was stirred at room temperature for 6 days. The solvents were evaporated, and the obtained residue was treated with diethyl ether. The resulting solid was filtered and washed with diethyl ether to obtain 0.29 g (64%) of compound **44**, mp 221 °C. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 1.20−1.42 (m, 1 H), 1.51−1.73 (m, 1 H), 1.73−1.99 (m, 2 H), 2.01−2.14 (m, 1 H), 2.66−2.94 (m, 3 H), 2.98 (d, J = 13.7 Hz, 1 H), 3.23−3.54 (m, 5 H), 3.81 (dd, J = 12.1, 9.0 Hz, 1 H), 4.98−5.11 (m, 1 H), 7.13−7.39 (m, 10 H), 7.44 (t, J = 7.5 Hz, 2 H), 7.67 (t, J = 8.5 Hz, 2 H). HPLC (method B): 95.1%, t_R = 11.4 min. MS (ESI) 456 m/z $(M - Br)^+$.

(3*R*)-3-{[(9-Hydroxy-9*H*-xanthen-9-yl)carbonyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (45) was prepared as described for compound **43** starting from compound **11** (0.95 g, 2.70 mmol) and (3-bromopropoxy)benzene (0.87 g, 4.05 mmol). The reaction time was 20 h at reflux temperature. Trituration with diethyl ether gave 1.33 g (87%) of compound **45**, mp 112.7−114.9 °C. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 1.24−1.40 (m, 1 H), 1.56−1.71 (m, 1 H), 1.71−1.93 (m, 2 H), 1.91−2.13 (m, 3 H), 2.62−2.78 (m, 1 H), 2.86−2.98 (d, J = 13.7 Hz, 1 H), 3.19−3.48 (m, 5 H), 3.74−3.88 (ddd, J = 13.7, 7.9, 1.5 Hz, 1 H), 3.93−4.05 (t, J = 5.7 Hz, 2 H), 4.97−5.07 (m, 1 H), 6.91−7.02 (m, 3 H), 7.19 (s, 1 H), 7.19−7.37 (m, 6 H), 7.38−7.49 (m, 2 H), 7.64−7.71 (ddd, J = 6.4, 6.4, 1.2 Hz, 2 H). HPLC (method B): 98.7%, t_R = 12.1 min. MS (ESI) 486 m/z $(M - Br)^+$.

(3*R*)-1-Heptyl-3-{[(9-hydroxy-9*H*-fluoren-9-yl)carbonyl]oxy}-1-azoniabicyclo[2.2.2]octane bromide (46) was prepared as described for compound **44** starting from compound **12** (0.25 g, 0.75 mmol) and 1-bromoheptane (0.67 g, 3.75 mmol). The reaction time was 72 h. Trituration with diethyl ether gave 0.33 g (85.5%) of compound **46**, mp 217 °C. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 0.89 (t, J = 7.0 Hz, 3 H), 1.13−1.70 (m, 12 H), 1.70−1.93 (m, 2 H), 2.03−2.11 (m, 1 H), 2.68−2.84 (m, 1 H), 2.98−3.19 (m, 3 H), 3.18−3.43 (m, 4 H), 3.78 (ddd, J = 13.6, 7.8, 2.4 Hz, 1 H), 4.97−5.05 (m, 1 H), 7.36 (dd, J = 7.5, 1.1 Hz, 2 H), 7.45 (dd, J = 7.3, 7.3 Hz, 2 H), 7.59 (d, J = 7.0 Hz, 1 H), 7.64 (d, J = 7.6 Hz, 1 H), 7.84 (dd, J = 7.3, 1.1 Hz, 2 H). HPLC (method B): 99.6%, t_R = 12.6 min. MS (ESI) 434 m/z $(M - Br)^+$.

(3*R*)-3-{[(9-Hydroxy-9*H*-fluoren-9-yl)carbonyl]oxy}-1-(2-phenylethyl)-1-azoniabicyclo[2.2.2]octane bromide (47) was prepared as described for compound **43** starting from compound **12** (0.7 g, 2.1 mmol) and (2-bromoethyl)benzene (0.58 g, 3.15 mmol). The reaction time was 31 h at reflux and 6 days at room temperature. Trituration with diethyl ether/acetonitrile gave 0.89 g (81.5%) of compound **47**, mp 118 °C. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 1.34−1.54 (m, 1 H), 1.60−1.76 (m, 1 H), 1.76−2.03 (m, 2 H), 2.07−2.15 (m, 1 H), 2.78−3.06 (m, 3 H), 3.21 (d, J = 13.4 Hz, 1 H), 3.28−3.58 (m, 5 H), 3.88 (dd, J = 5.3, 2.6 Hz, 1 H), 5.01−5.13 (m, 1 H), 6.87 (s, 1 H), 7.27−7.41 (m, 7 H), 7.47 (dd, J = 7.2 Hz, 2 H), 7.60 (d, J = 7.6 Hz, 1 H), 7.66 (d, J = 7.2 Hz, 1 H), 7.85 (d, J = 7.6 Hz, 2 H). HPLC (method B): 95.5%, t_R = 10.2 min. MS (ESI) 440 m/z $(M - Br)^+$.

(3*R*)-3-{[(9-Hydroxy-9*H*-fluoren-9-yl)carbonyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (48) was prepared as described for compound **43** starting from compound **12** (2.2 g, 6.6 mmol) and (3-bromopropoxy)benzene (2.86 g, 13.3 mmol). The reaction time was 18 h at reflux temperature. Trituration with diethyl ether/acetonitrile gave 3.17 g (88%) of compound **48**, mp 108 °C. ^1H NMR (300 MHz, DMSO-d_6) δ ppm 1.35−1.54 (m, 1 H), 1.57−1.75 (m, 1 H), 1.75−1.96 (m, 2 H), 1.96−2.18 (m, 3 H), 2.77−2.96 (m, 1 H), 3.16 (d, J = 13.7 Hz, 1 H), 3.24−3.54 (m, 5 H), 3.86 (ddd, J = 8.1, 5.2, 2.0 Hz, 1 H), 4.01 (t, J = 5.8 Hz, 2 H), 4.96−5.08 (m, 1 H), 6.86 (s, 1 H), 6.90−7.03 (m, 3 H), 7.26−7.40 (m, 4 H), 7.45 (dd, J = 7.3 Hz, 2 H), 7.61 (d, J = 7.6 Hz, 1 H), 7.66 (d, J = 7.3 Hz, 1 H), 7.84 (d, J = 7.3 Hz, 2 H). HPLC (method A): 99.9%, t_R = 15.8 min. MS (ESI) 470 m/z $(M - Br)^+$.

(3*R*)-3-{[(9-Methyl-9*H*-xanthen-9-yl)carbonyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (49) was prepared as described for compound **44** starting from compound **16** (0.10 g, 0.28 mmol) and (3-bromopropoxy)benzene (0.30 g, 1.40 mmol). The reaction time was 72 h. Trituration with diethyl ether gave 0.11 g (69%) of compound **49**, mp 195 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.33−1.48 (m, 1 H), 1.59−1.74 (m, 1 H), 1.74−1.96 (m, 2 H), 1.92 (s, 3 H), 1.97−2.18 (m, 3 H), 2.74−2.91 (m, 1 H), 3.05−3.18 (d, J = 13.7 Hz, 2 H), 3.23−3.50 (m, 5 H), 3.79−3.92 (m, 1 H), 3.97−4.07 (t, J = 5.8 Hz, 2 H), 5.00−5.10 (m, 1 H), 6.91−7.02 (m, 3 H), 7.12−7.23 (m, 4 H), 7.27−7.42 (m, 4 H), 7.44−7.50 (dd, J = 8.1, 1.7 Hz, 1 H), 7.50−7.55 (dd, J = 8.1, 1.4 Hz, 1 H). HPLC (method A): 98.4%, t_R = 21.3 min. MS (ESI) 484 m/z (M − Br)$^+$.

(3*R*)-3-{[(9-Methyl-9*H*-fluoren-9-yl)carbonyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (50) was prepared as described for compound **44** starting from compound **17** (0.20 g, 0.6 mmol) and (3-bromopropoxy)benzene (0.64 g, 3.0 mmol). The reaction time was 72 h. Trituration with diethyl ether gave 0.20 g (61%) of compound **50**, mp 204 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.42−1.63 (m, 1 H), 1.63−1.97 (m, 3 H), 1.78 (s, 3 H), 1.97−2.25 (m, 3 H), 2.94−3.15 (m, 1 H), 3.20−3.60 (m, 6 H), 3.80−3.97 (m, 1 H), 3.96−4.18 (t, J = 5.5 Hz, 2 H), 4.91−5.05 (m, 1 H), 6.87−7.01 (m, 3 H), 7.24−7.50 (m, 6 H), 7.62−7.70 (d, J = 6.7 Hz, 1 H), 7.72−7.80 (d, J = 7.0 Hz, 1 H), 7.86−7.95 (d, J = 6.7 Hz, 2 H). HPLC (method A): 99.4%, t_R = 20.8 min. MS (ESI) 468 m/z (M − Br)$^+$.

(3*R*)-3-{[Hydroxy(di-2-thienyl)acetyl]oxy}-1-methyl-1-azoniabicyclo[2.2.2]octane bromide (51) was prepared as described for compound **44** starting from compound **19** (0.20 g, 0.57 mmol) and bromomethane (2.85 mL of a 1 M solution in acetonitile, 2.85 mmol). The reaction time was 18 h. Trituration with diethyl ether gave 0.20 g (79%) of compound **51**, mp 251 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.60−1.84 (m, 2 H), 1.82−2.07 (m, 2 H), 2.29 (m, 1 H), 3.02 (s, 3 H), 3.19−3.58 (m, 5 H), 3.90−4.03 (ddd, J = 13.3, 8.4, 2.1 Hz, 1 H), 5.15−5.27 (m, 1 H), 6.97−7.07 (dt, J = 5.0, 3.8 Hz, 2 H), 7.16−7.24 (ddd, J = 8.1, 3.5, 1.2 Hz, 2 H), 7.49 (s, 1 H) 7.50−7.55 (ddd, J = 5.1, 3.7, 1.2 Hz, 2 H). HPLC (method B): 99.7%, t_R = 7.7 min. MS (ESI) 364 m/z (M − Br)$^+$.

(3*R*)-1-Heptyl-3-{[hydroxy(di-2-thienyl)acetyl]oxy}-1-azoniabicyclo[2.2.2]octane bromide (52). To a solution of compound **19** (0.50 g, 1.43 mmol) in 10 mL of chloroform was added 1-bromoheptane (1.02 g, 5.52 mmol). The mixture was stirred at room temperature for 7 days. The solvent was evaporated, and the residue was treated with diethyl ether. The resulting solid was filtered and washed with diethyl ether to obtain 0.49 g (66%) of compound **52**, mp 134 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 0.88 (t, J = 6.7 Hz, 3 H), 1.16−1.39 (m, 8 H), 1.5−1.8 (m, 4 H), 1.83−2.06 (m, 2 H), 2.25−2.35 (m, 1 H), 3.03−3.54 (m, 7 H), 3.89 (ddd, J = 13.9, 8.2, 1.7 Hz, 1 H), 5.16−5.29 (m, 1 H), 7.02 (ddd, J = 5.1, 3.6, 2.3 Hz, 2 H), 7.17 (td, J = 3.6, 1.2 Hz, 2 H), 7.49 (s, 1 H), 7.52 (ddd, J = 5.1, 2.3, 1.2 Hz, 2 H). HPLC (method A): 99.5%, t_R = 17.9 min. MS (ESI) 448 m/z (M − Br)$^+$.

(3*R*)-1-Benzyl-3-{[hydroxy(di-2-thienyl)acetyl]oxy}-1-azoniabicyclo[2.2.2]octane bromide (53) was prepared as described for compound **44** starting from compound **19** (0.20 g, 0.57 mmol) and (bromomethyl)benzene (0.34 mL, 2.85 mmol). The reaction time was 18 h. Trituration with diethyl ether gave 0.23 g (77%) of compound **53**, mp 89.0−90.6 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.57−2.03 (m, 4 H), 2.26 (m, 1 H), 2.98−3.15 (m, 1 H), 3.22−3.54 (m, 4 H), 3.81−3.94 (m, 1 H), 4.47 (d, J = 12.8 Hz, 1 H), 4.54 (d, J = 12.8 Hz, 1 H), 5.21 (m, 1 H), 6.96 (dd, J = 8.6, 3.6 Hz, 2 H), 7.01−7.11 (d, J = 3.1 Hz, 2 H), 7.44 (s, 1H), 7.45−7.61 (m, 7 H). HPLC (method B): 95.6%, t_R = 10.3 min. MS (ESI) 440 m/z (M − Br)$^+$.

(3*R*)-3-{[Hydroxy(di-2-thienyl)acetyl]oxy}-1-(2-phenylethyl)-1-azoniabicyclo[2.2.2]octane bromide (54) was prepared as described for compound **44** starting from compound **19** (0.20 g, 0.57 mmol) and (2-bromoethyl)benzene (0.53 g, 2.86 mmol). The reaction time was 72 h. Crystallization from acetonitrile gave 0.18 g (59%) of compound **54**, mp 216 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.60−1.89 (m, 2 H), 1.89−2.13 (m, 2 H), 2.27−2.40 (m, 1 H), 2.89−3.12 (m, 2 H), 3.17−3.32 (m, 1 H), 3.37−3.67 (m, 6 H), 3.92−4.08 (m, 1 H), 5.22−5.34 (m, 1 H), 7.03 (dd, J = 3.4 Hz, 2 H), 7.19 (dd, J = 3.7 Hz, 2 H), 7.24−7.42 (m, 5 H), 7.49 (s, 1 H), 7.54 (dd, J = 3.4 Hz, 2 H). HPLC (method A): 99.6%, t_R = 15.5 min. MS (ESI) 454 m/z (M − Br)$^+$.

(3*R*)-3-{[Hydroxy(di-2-thienyl)acetyl]oxy}-1-(4-phenylbutyl)-1-azoniabicyclo[2.2.2]octane bromide (55) was prepared as described for compound **52** starting from compound **19** (0.50 g, 1.43 mmol) and (4-bromobutyl)benzene (1.22 g, 5.73 mmol). The reaction time was 7 days. Trituration with diethyl ether gave 0.32 g (41%) of compound **55**, mp 64 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.50−1.72 (m, 4 H), 1.72−2.12 (m, 5 H), 2.34−2.44 (m, 1 H), 2.61 (t, J = 6.6 Hz, 2 H), 3.34−3.59 (m, 5 H), 3.60−3.69 (m, 1 H), 3.74 (d, J = 13.7 Hz, 1 H), 4.20 (dd, J = 13.0, 8.4 Hz, 1 H), 5.18−5.32 (m, 1 H), 6.93 (dd, J = 5.0, 3.8 Hz, 2 H), 7.09−7.33 (m, 9 H). HPLC (method B): 98.6%, t_R = 12.5 min. MS (ESI) 482 m/z (M − Br)$^+$.

(3*R*)-3-{[Hydroxy(di-2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (56) was prepared as described for compound **44** starting from compound **19** (5.0 g, 14.33 mmol) and (3-bromopropoxy)benzene (15.97 g, 74.23 mmol). The reaction time was 18 h. Trituration with acetonitrile gave 7.20 g (89%) of compound **56**, mp 230 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.63−1.87 (m, 2 H), 1.85−2.06 (m, 2 H), 2.06−2.24 (m, 2 H), 2.26−2.38 (m, 1 H), 3.13−3.29 (m, 1 H), 3.32−3.64 (m, 6 H), 3.91−4.07 (m, 1 H), 4.03 (t, J = 5.8 Hz, 2 H), 5.19−5.31 (m, 1 H), 6.91−7.00 (m, 3 H), 7.00−7.06 (m, 2 H), 7.17−7.22 (m, 2 H), 7.28−7.36 (m, 2 H), 7.50 (s, 1H), 7.50−7.56 (m, 2 H). HPLC (method B): 99.8%, t_R = 11.9 min. MS (ESI) 484 m/z (M − Br)$^+$.

(3*R*)-3-{(Di-2-thienylacetyl)oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (57) was prepared as described for compound **44** starting from compound **21** (0.25 g, 0.75 mmol) and (3-bromopropoxy)benzene (0.81 g, 3.75 mmol). The reaction time was 24 h. Trituration with diethyl ether gave 0.34 g (83%) of compound **57**, mp 148 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.72−2.05 (m, 4 H), 2.05−2.23 (m, 2 H), 2.25−2.36 (m, 1 H), 3.20−3.34 (m, 1 H), 3.36−3.64 (m, 4 H), 3.40−3.48 (t, J = 7.9 Hz, 2 H), 3.90−4.03 (m, 1 H), 4.00−4.06 (t, J = 5.8 Hz, 2 H), 5.14−5.24 (m, 1 H), 5.91 (s, 1 H), 6.92−6.99 (m, 3 H), 6.99−7.04 (ddd, J = 5.0, 3.6, 1.2 Hz, 2 H), 7.12−7.18 (dd, J = 7.3, 3.4 Hz, 2 H), 7.26−7.36 (m, 2 H), 7.48−7.54 (d, J = 5.2 Hz, 2 H). HPLC (method B): 94.5%, t_R = 13.2 min. MS (ESI) 468 m/z (M − Br)$^+$.

(3*R*)-3-{[Hydroxy(di-3-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (58) was prepared as described for compound **43** starting from compound **23** (0.5 g, 1.43 mmol) and (3-bromopropoxy)benzene (0.46 g, 2.15 mmol). The reaction time was 6 h at reflux and 72 h at room temperature. Trituration with diethyl ether gave 0.68 g (86%) of compound **58**, mp 219 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.50−1.66 (m, 1 H), 1.66−1.82 (m, 1 H), 1.82−2.03 (m, 2 H), 2.03−2.24 (m, 2 H), 2.25−2.37 (m, 1 H), 3.08−3.25 (m, 1 H), 3.28−3.60 (m, 6 H), 3.86−4.02 (m, 1 H), 4.01−4.08 (t, J = 5.7 Hz, 2 H), 5.15−5.26 (m, 1 H), 6.78 (s, 1 H), 6.91−7.01 (m, 3 H), 7.11−7.17 (d, J = 5.2 Hz, 2 H), 7.32 (m, 2 H), 7.42−7.48 (m, 2 H), 7.48−7.55 (m, 2 H). HPLC (method B): 99.7%, t_R = 11.8 min. MS (ESI) 484 m/z (M − Br)$^+$.

(3*R*)-3-[(2,2-Di-2-thienylpropanoyl)oxy]-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (59) was prepared as described for compound **44** starting from compound **26** (0.25 g, 0.72 mmol) and (3-bromopropoxy)benzene (0.77 g, 3.60 mmol). The reaction time was 72 h. Trituration with diethyl ether gave 0.35 g (92%) of compound **59**, mp 170 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.60−2.25 (m, 6 H), 2.10 (s, 3 H), 2.25−2.34 (m, 1 H), 3.02−3.21 (m, 1 H), 3.30−3.63 (m, 6 H), 3.90−4.08 (m, 1 H), 4.01−4.09 (t, J = 5.5 Hz,

2 H), 5.14−5.26 (m, 1 H), 6.92−7.00 (m, 3 H), 7.00−7.06 (dd, J = 5.0, 3.6 Hz, 2 H), 7.08−7.14 (m, 2 H), 7.26−7.36 (m, 2 H), 7.48−7.54 (d, J = 5.19 Hz, 2 H). HPLC (method B): 97.9%, t_R = 14.0 min. MS (ESI) 482 m/z (M − Br)$^+$.

(3*R*)-3-{[Hydroxy(diphenyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (60) was prepared as described for compound **44** starting from compound **33** (0.40 g, 1.18 mmol) and (3-bromopropoxy)benzene (1.23 g, 5.69 mmol). The reaction time was 18 h. Trituration with diethyl ether gave 0.28 g (42%) of compound **60**, mp 199 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.44−1.62 (m, 1 H), 1.61−1.79 (m, 1 H), 1.80−2.01 (m, 2 H), 2.01−2.22 (m, 2 H), 2.22−2.34 (m, 1 H), 2.99−3.18 (m, 1 H), 3.19−3.57 (m, 6 H), 3.85−3.99 (m, 1 H), 3.99−4.09 (t, J = 5.5 Hz, 2 H), 5.14−5.33 (m, 1 H), 6.89−7.02 (m, 3 H), 7.20−7.51 (m, 13 H). HPLC (method B): 94.9%, t_R = 12.6 min. MS (ESI) 472 m/z (M − Br)$^+$.

(3*R*)-3-{[(2*S*)-2-Hydroxy-2-phenyl-2-(2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (61) was prepared as described for compound **43** starting from compound **35** (0.5 g, 1.50 mmol) and (3-bromopropoxy)benzene (0.62 g, 2.92 mmol). The reaction time was 3 h at reflux and overnight at room temperature. The precipitated solid was filtered and washed with diethyl ether to obtain 0.66 g (79%) of compound **61**, mp 206.4−207.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.57−1.82 (m, 2 H), 1.82−2.03 (m, 2 H), 2.03−2.20 (m, 2 H), 2.25−2.35 (m, 1 H), 3.03−3.19 (m, 1 H), 3.24−3.55 (m, 6 H), 3.87−3.99 (ddd, J = 13.6, 8.2, 1.7 Hz, 1 H), 3.99−4.07 (t, J = 6.0 Hz, 2 H), 5.18−5.28 (m, 1 H), 6.91−7.00 (m, 3 H), 7.00−7.04 (dd, J = 5.0, 3.5 Hz, 1 H), 7.1−7.14 (dd, J = 3.7, 1.2 Hz, 1 H), 7.19 (s, 1 H), 7.27−7.43 (m, 5 H), 7.43−7.55 (m, 3 H). HPLC (method B): 99.7%, t_R = 12.3 min. MS (ESI) 478 m/z (M − Br)$^+$.

(3*R*)-3-{[(2*R*)-2-Hydroxy-2-phenyl-2-(2-thienyl)acetyl]oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (62) was prepared as described for compound **43** starting from compound **36** (0.5 g, 1.50 mmol) and (3-bromopropoxy)benzene (0.62 g, 2.92 mmol). The reaction time was 3 h at reflux and overnight at room temperature. The precipitated solid was filtered and washed with diethyl ether to obtain 0.69 g (82%) of compound **62**, mp 240.8−241.4 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.45−1.61 (m, 1 H), 1.63−1.79 (m, 1 H), 1.81−2.03 (m, 2 H), 2.03−2.23 (m, 2 H), 2.22−2.33 (m, 1 H), 3.05−3.21 (m, 1 H), 3.30−3.57 (m, 6 H), 3.88−4.01 (m, 1 H), 3.99−4.07 (t, J = 5.8 Hz, 2 H), 5.19−5.29 (m, 1 H), 6.92−7.00 (m, 3 H), 7.01−7.06 (dd, J = 4.9, 3.7 Hz, 1 H), 7.14−7.17 (dd, J = 3.5, 1.1 Hz, 1 H), 7.20 (s, 1 H), 7.27−7.41 (m, 5 H), 7.45−7.57 (m, 3 H). HPLC (method B): 99.9%, t_R = 12.2 min. MS (ESI) 478 m/z (M − Br)$^+$.

(3*R*)-3-{[(2*S*)-2-Cyclopentyl-2-hydroxy-2-(2-thienyl)acetyl]-oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (63) was prepared as described for compound **43** starting from compound **38** (1.0 g, 2.98 mmol) and (3-bromopropoxy)benzene (0.96 g, 4.44 mmol). The reaction time was 8.5 h at reflux and 16 h at room temperature. The precipitated solid was filtered and washed with diethyl ether to obtain 1.53 g (93%) of compound **63**, mp 168−170 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.27−1.67 (m, 8 H), 1.79−2.05 (m, 4 H), 2.05−2.19 (m, 2 H), 2.26−2.36 (m, 1 H), 2.75−2.91 (m, 1 H), 3.17−3.27 (d, J = 13.4 Hz, 1 H), 3.27−3.62 (m, 6 H), 3.85−3.97 (ddd, J = 13.4, 8.7, 2.3 Hz, 1 H), 4.04 (t, J = 5.8 Hz, 2 H), 5.11−5.19 (m, 1 H), 6.19 (s, 1 H), 6.91−6.98 (m, 3 H), 6.98−7.03 (dd, J = 4.9, 3.7, 1 H), 7.15−7.18 (dd, J = 3.7, 1.2 Hz, 1 H), 7.28−7.35 (dd, J = 7.8 Hz, 2 H), 7.42−7.45 (dd, J = 4.7, 1.1 Hz, 1 H). HPLC (method B): 99.7%, t_R = 13.1 min. MS (ESI) 470 m/z (M − Br)$^+$.

(3*R*)-3-{[(2*R*)-2-Cyclopentyl-2-hydroxy-2-(2-thienyl)acetyl]-oxy}-1-(3-phenoxypropyl)-1-azoniabicyclo[2.2.2]octane bromide (64) was prepared as described for compound **43** starting from compound **39** (1.0 g, 2.98 mmol) and (3-bromopropoxy)benzene (0.96 g, 4.44 mmol). The reaction time was 6 h at reflux and 12 h at room temperature. The precipitated solid was filtered and washed with diethyl ether to obtain 1.20 g (73%) of compound **64**, mp 181 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.27−1.67 (m, 8 H), 1.67−2.05 (m, 4 H), 2.05−2.20 (m, 2 H), 2.20−2.28 (m, 1 H), 2.76−2.95 (m, 1 H), 3.18−3.27 (d, J = 13.7 Hz, 1 H), 3.28−3.62 (m, 6 H), 3.95−3.97 (ddd, J = 13.7, 8.9, 2.1 Hz, 1 H), 4.05 (t, J = 5.5 Hz, 2 H), 5.10−5.19 (m, 1 H), 6.18 (s, 1 H), 6.93−6.99 (m, 3 H), 6.98−7.03 (dd, J = 4.9, 3.7, 1 H), 7.17−7.20 (dd, J = 3.7, 1.2 Hz, 1 H), 7.28−7.73 (dd, J = 7.8 Hz, 2 H), 7.42−7.46 (dd, J = 4.9, 1.2 Hz, 1 H). HPLC (method B): 99.8%, t_R = 13.1 min. MS (ESI) 470 m/z (M − Br)$^+$.

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Supporting Information Available: (i) Purity of compounds (HPLC); (ii) preparation of the acids **68** and **69**; (iii) assignment of the absolute configuration of acids **41** and **42** and circular dichroism (CD) curves; (iv) preparation of compounds **2**, **7**, **9**, **10**, **14**, and **15** (Scheme 1); (v) preparation of compounds **18**, **20**, **22**, and **25** (Scheme 2); (vi) preparation of compounds **31** and **32** (Scheme 3); (vii) references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Review

Aclidinium bromide, a new, long-acting, inhaled muscarinic antagonist: *In vitro* plasma inactivation and pharmacological activity of its main metabolites

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ABSTRACT

Aclidinium bromide is a novel, long-acting inhaled muscarinic antagonist drug in Phase III clinical trials for chronic obstructive pulmonary disease (COPD). The aims of this study were to evaluate the *in vitro* stability of the ester drug aclidinium in plasma from various species, and the *in vitro* and *in vivo* pharmacological activity of its hydrolysis metabolites. Following incubation of aclidinium in pooled samples of human, rat, guinea pig or dog plasma, the rate of hydrolysis was quantified by reversed phase ultra performance liquid chromatography and mass spectrometry. Tiotropium and ipratropium were used as comparators. The *in vitro* biochemical profile of the hydrolysis metabolites of aclidinium was assessed in human M_1 to M_5 muscarinic receptors and in a standard selectivity panel (85 G protein-coupled receptors [GPCRs], ion channels and enzymes). The bronchodilator activity of the metabolites of aclidinium bromide was studied in guinea pigs after acetylcholine-induced bronchoconstriction.

Aclidinium was rapidly hydrolysed into carboxylic acid and alcohol derivatives in guinea pig, rat, human and dog plasma with half-lives of 38, 11.7, 2.4 and 1.8 min, respectively. In contrast, ≥70% of tiotropium and ipratropium remained unchanged in the plasma after 60 min of incubation. The carboxylic acid and alcohol metabolites had no significant affinity for any of the muscarinic receptors, other GPCRs, ion channels or enzymes studied and showed no relevant antibronchoconstrictory activity *in vivo*. These results suggest that aclidinium may have a reduced systemic exposure and therefore less propensity for class-related systemic side effects in the clinical setting.

Contents

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is a preventable and treatable lung disease characterised by airflow limitation that is not fully reversible (Rabe et al., 2007). It is estimated that COPD caused approximately 2.7 million deaths worldwide in 2002 (Lopez et al., 2006). In 2001, COPD represented the sixth most common cause of mortality and the disease is expected to be the third most common cause of mortality worldwide by 2020 (Lopez et al., 2006).

The major reversible component of airway obstruction in COPD is believed to be cholinergic (Gross and Skorodin, 1984a,b; Gross, 1988). Inhaled anti-cholinergics, which increase bronchodilation by blocking muscarinic receptors on airway smooth muscle, are established treatment options for COPD (Barnes, 2004). Currently, only two inhaled muscarinic antagonists are available for the management of symptomatic COPD patients: ipratropium, a short-acting agent requiring up to 4 doses per day and tiotropium, a long-acting, once-daily treatment (Barnes, 2004; Gross, 2006; Hanania and Donohue, 2007). Certain systemic anti-cholinergic effects are associated with these compounds, including dry mouth (reported in 11.6% of patients treated with tiotropium bromide), glaucoma, constipation, increased heart rate and urinary retention (Kesten et al., 2006; Lee et al., 2008; Singh et al., 2008). Therefore, a compound with similar or improved bronchodilatory effects and lower systemic availability resulting in fewer unwanted anti-cholinergic effects would be attractive.

Aclidinium bromide is a novel, long-acting muscarinic antagonist in Phase III clinical trials for the maintenance treatment of COPD. Aclidinium is a potent muscarinic receptor antagonist with a long residence half-life at human M_3 receptors and shorter residence time at human M_2 receptor, resulting in kinetic selectivity of the M_3 versus the M_2 receptor subtype (Gavaldà et al., 2009). These pharmacological characteristics of aclidinium result in its long-lasting antibronchoconstrictory effect with a fast onset of action in both *in vitro* and *in vivo* animal models (Gavaldà et al., 2009). Aclidinium undergoes rapid hydrolysis in human plasma, contributing to a low and transient systemic exposure and a reduced potential for class-related systemic side effects in the clinical setting (Prat et al., 2009).

This study evaluated the contribution of *in vitro* enzymatic and non-enzymatic hydrolysis to the cleavage of the ester bond of aclidinium, and characterised the *in vitro* and *in vivo* pharmacological activity of the two metabolites resulted from aclidinium hydrolysis.

2. Materials and methods

2.1. Materials

Aclidinium bromide, its alcohol metabolite (LAS34823 [3(*R*)-hydroxy-1-(3-phenoxy-propyl)-1-azonia-bicyclo[2.2.2]octane, bromide]), its carboxylic acid metabolite (LAS34850 [dithienylglycolic acid, sodium salt]) and tiotropium bromide were synthesised at the Department of Medicinal Chemistry, Laboratorios Almirall (Barcelona, Spain). Chemical structures of aclidinium bromide, LAS34823 and LAS34850 are depicted in Fig. 1. Ipratropium bromide, atropine sulphate, acetylcholine chloride, urethane and phosphate-buffered saline (PBS) with calcium and magnesium were purchased from Sigma Chemicals (Madrid, Spain) and sodium pentobarbital was obtained from Industrial Kern (Barcelona, Spain). Membrane preparations expressing human M_1 to M_5 receptors (obtained from transfected Chinese hamster ovary CHO-K1 cells) were purchased from Membrane Target Systems, PerkinElmer Life and Analytical Sciences (Boston, MA, USA). 1-[*N*-methyl-^{3}H] scopolamine methyl chloride (^{3}H-NMS) was obtained from PerkinElmer Life and Analytical Sciences.

Stock solutions of aclidinium, ipratropium and tiotropium for plasma stability were prepared at a final concentration of 1 mg/ml (expressed as free base) by dissolving each compound in a 0.1 N hydrochloric acid:acetonitrile (20:80, v/v) mixture. All equilibrium binding studies were performed in 96-well plates (Nunc, Thermo Fischer Scientific, Roskilde, Denmark), with all stock solutions of the compounds being prepared at 10^{-2} M in dimethyl sulphoxide (DMSO). In the anaesthetised guinea pig studies, aclidinium and its hydrolysis metabolites were dissolved in 0.2–1% (v/v) HCl in the presence of polyethylene glycol 300. Ipratropium and tiotropium were dissolved in distilled water.

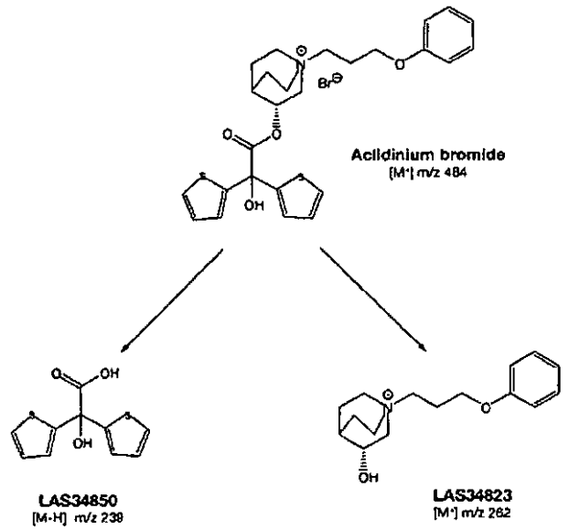

Fig. 1. Hydrolytic cleavage of the ester moiety of aclidinium bromide into its alcohol (LAS34823) and carboxylic acid (LAS34850) derivatives.

2.2. Animals

Male Dunkin-Hartley guinea pigs (400–600 g at the time of experimental procedures) were obtained from Harlan (Interfauna Ibérica, Sant Feliu de Codines, Spain or Horst, The Netherlands). Guinea pigs were housed in groups of four or five at 20–24 °C under a 12-h light/dark cycle. Food (maintenance diet for guinea pigs with vitamin C [SAFE 114, SAFE, France]) and water were available *ad libitum*. Male Beagle dogs (9–20 kg at the time of experimental procedures) were supplied by guaranteed commercial suppliers. Dogs were housed at 15–21 °C, 40–70% humidity, under a 12-h light/dark cycle and fed on a maintenance diet (Harlan TEKLAD 2021, Madison, WI, USA) with free access to water. All experiments were carried out with the approval of the Animal Ethical Committee of Almirall, Barcelona, Spain.

2.3. Animal and human plasma samples

Plasma samples from male Dunkin-Hartley guinea pigs ($n = 10$) and male Beagle dogs ($n = 4$) were obtained by centrifugation of blood samples taken into sodium heparin tubes (2000 × *g* at 4 °C) for 10 min. Plasma samples from six human volunteers (three male and three female) were similarly obtained. Male Wistar rat plasma was obtained from RCC Cida (Barcelona, Spain) using sodium heparin as an anticoagulant. The resulting plasma from each species was pooled and stored at −80 °C until use.

2.4. Methods

2.4.1. In vitro plasma stability

Plasma samples from each species were incubated in triplicate for 5 min at 37 °C before the addition of the test compound to initiate the reaction (final concentration: 40 ng/ml, expressed as free base). The final molar concentrations were 83 nM, 102 nM and 120 nM for aclidinium bromide, tiotropium bromide and ipratropium bromide, respectively. The final concentration of acetonitrile in the incubations was below 0.5%.

Aliquots of the incubation mixtures (100 μl) were taken at predetermined time points during the incubation, up to 90 min for aclidinium and up to 6 h for tiotropium and ipratropium. Each aliquot was transferred to a vessel containing 300 μl of cold acetonitrile:1N hydrochloric acid (90/10, v/v) to stop the reaction. Samples were then centrifuged at 2500 × *g* for 10 min at 4 °C. Blank incubations in the absence of the test compound were also performed.

The analyses of test compounds were performed by ultra performance liquid chromatography (UPLC) with mass spectrometry detection.

2.4.2. Non-enzymatic hydrolysis of aclidinium bromide

Incubation mixtures containing 50 mM sodium phosphate (pH 2.0, 4.0, 6.0, 7.0, 7.4, 8.0 and 9.0) and aclidinium (50 μM) in 0.5 ml (final volume) were pre-incubated at 37 °C for 3 min. Reactions were initiated by the addition of 25 μl of 1 mM aclidinium bromide prepared in 0.01N HCl:acetonitrile (80:20, v/v). The final concentration of acetonitrile in the incubation mixtures was 1%. At pre-defined times of 30, 60, 120 and 180 min, 100 μl aliquots of the incubation mixtures were taken and the reactions stopped by the addition of 100 μl of cold acetonitrile:0.2N hydrochloric acid (20:80, v/v) followed by centrifugation at 2500 × *g* for 10 min at 4 °C. Samples corresponding to time zero were prepared manually separately. All the incubations were carried out in duplicate. The amount of aclidinium and the acid and alcohol hydrolysis products in the clear supernatant fraction were determined. All samples were kept at +4 °C once obtained and during HPLC–UV analysis to prevent aclidinium ester cleavage.

2.4.3. HPLC–UV and UPLC–MS/MS analysis

The determination of aclidinium bromide and its hydrolysis metabolites in incubation samples from stability studies conducted in phosphate buffer at different pH values was performed using a Waters Alliance 2790 HPLC system (Waters, Milford, MA, USA) with UV detection (Waters 2996PDA) at 219 nm (LAS34823) and 238 nm (aclidinium and LAS34850). Aclidinium and its metabolites were separated on a Waters Symmetry C_{18} (250 mm × 4.6 mm, 5 μm) protected by a Tracer Spherisorb ODS-2CN (10 mm × 4.6 mm) and eluted using a gradient with (solvent A) 10 mM sodium phosphate containing 5 mM 1-octanesulphonic acid (pH 2.8) and (solvent B) acetonitrile. The initial mobile phase composition was 35% B and progressed linearly to 50% B in 12 min. The mobile phase was returned to the starting solvent mixture in 0.1 min and the system equilibrated for 8 min between runs. A constant flow rate of 1 ml/min was employed. The injection volume was 90 μl and the retention times were approximately 4.2, 6.8 and 13 min for LAS34823, LAS34850 and aclidinium, respectively (Fig. 2A).

The determination of aclidinium, tiotropium and ipratropium in incubation samples from plasma stability studies were conducted by using UPLC with mass spectrometry detection. Gradient chromatographic conditions (maximum 3.5 min run time) were employed using a Waters Acquity C_{18} (2.1 mm × 50 mm, 1.7 μm) on a UPLC Acquity system (Waters, Milford, MA, USA). The mobile phase used for analysis was (solvent A) 10 mM formic acid:2.5 mM ammonia (pH 3.0) and (solvent B) acetonitrile at a flow-rate of 0.4 ml/min (aclidinium and ipratropium) and 0.35 ml/min (tiotropium). Different linear gradient programmes were used: from 30% to 90% solvent B in 1 min (aclidinium), from 5% to 50% solvent B in 2 min (tiotropium), and from 5% to 50% solvent B in 1.2 min (ipratropium). The retention times were 0.9, 1.7 and 1.1 min for aclidinium, tiotropium and ipratropium, respectively. A final step of column washout with 90% solvent B for approximately 1 min was also conducted in all analysis before re-equilibration at starting eluent conditions.

Detection was performed on a Quattro Premier triple quadrupole mass spectrometer (Waters Micromass) with electrospray ion source operating in positive ion mode. The capillary voltage was set to 3.0 kV, the source block temperature was 120 °C, and the desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 550 l/h. The cone gas (nitrogen) was set to 50 l/h. Quantification was performed using multiple reaction monitoring (MRM) for the transitions 484 → 262 (aclidinium; cone voltage, 50 V; collision energy, 35 eV), 392 → 152 (tiotropium; cone voltage, 45 V; collision energy, 30 eV), and 332 → 166 (ipratropium; cone voltage, 25 V; collision energy, 50 eV). Data acquisition and processing were performed with MassLynx version 4.1 software (Waters). Fig. 2B shows the multiple reaction monitoring (MRM) chromatograms obtained from the UPLC–MS/MS analysis of 40 ng/ml aclidinium, 40 ng/ml tiotropium bromide and 40 ng/ml ipratropium bromide in human plasma.

2.4.4. Identification of hydrolysis metabolites of aclidinium bromide

The identification of the aclidinium bromide hydrolysis metabolites formed in incubations was conducted by comparison of the electrospray-ionisation mass spectra and the chromatographic retention times of the metabolites with those of authentic standards of LAS34823 (alcohol derivative) and LAS34850 (carboxylic acid derivative) using a Quattro Premier triple quadrupole mass spectrometer (Waters Micromass, Manchester, UK) with electrospray ion source operating in positive and negative ion mode.

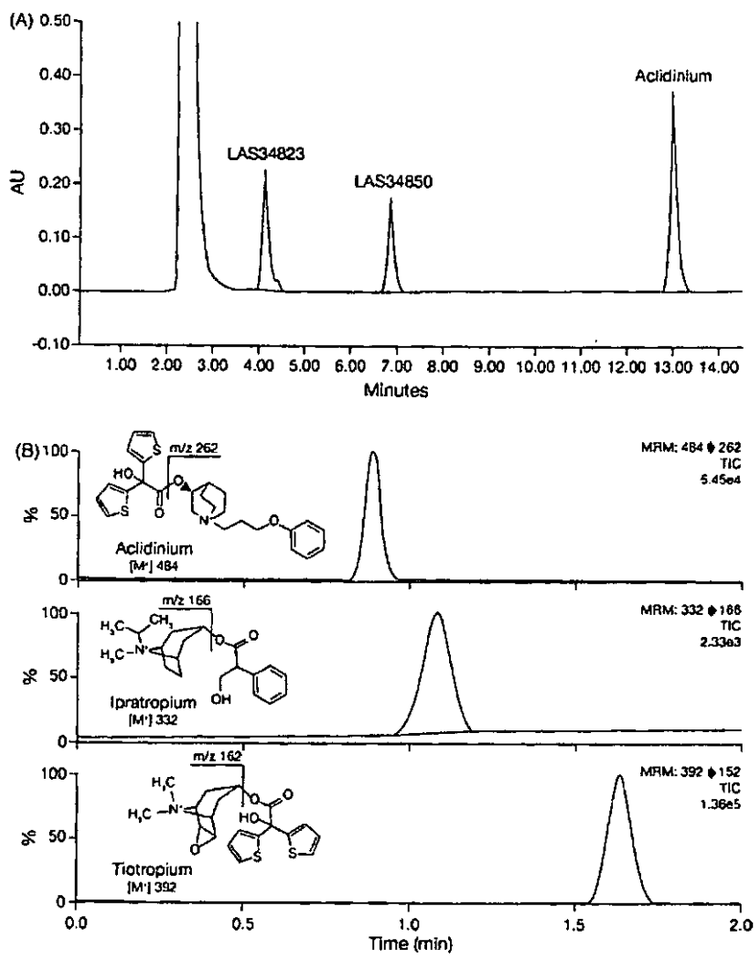

Fig. 2. (A) LC–UV (220 nm) chromatograms of a standard solution of 50 μM aclidinium, 50 μM LAS34823 and 50 μM LAS34850 in 50 mM phosphate buffer and (B) representative LC–MSMS MRM chromatograms of aclidinium, tiotropium and ipratropium bromide in human plasma. Plasma samples contained test substances at a concentration of 40 ng/ml.

2.4.5. Affinity for the human M_1 to M_5 muscarinic receptor subtypes

The affinity of aclidinium and its main metabolites for the different human muscarinic receptor subtypes was determined by measuring their ability to displace bound ^{3}H-NMS in cell membrane preparations expressing one of the human muscarinic receptor subtypes at equilibrium.

Protein concentrations were 8.1, 10.0, 4.9, 4.5 and 5.0 μg/well for M_1, M_2, M_3, M_4 and M_5 receptor membrane preparations, respectively. The ^{3}H-NMS concentration was 0.3 nM for the M_1 and M_4 assays and 1 nM for the M_2, M_3, and M_5 assays similar to the radioligand equilibrium dissociation constant (K_d) for the different muscarinic receptor subtypes. A range of compound concentrations (10^{-14} to 10^{-5} M) were tested in duplicate to generate competition curves. Non-specific binding was determined in the presence of atropine (1 μM). Assay reagents were dissolved in assay binding buffer (PBS with calcium and magnesium) to a total volume of 200 μl. After a 2- (M_1 to M_4) or 6- (M_5) h incubation period at room temperature in 96-well microtiter plates to ensure that equilibrium was achieved for all compounds tested, 150 μl aliquots of the reaction were transferred to GF/C filter plates (Millipore, Barcelona, Spain) pre-treated for 1 h with wash buffer (Tris 50 mM, NaCl 100 mM, pH 7.4) containing 0.05% polyethylenimine. Bound and free ^{3}H-NMS were then separated by rapid vacuum filtration followed by four washes with ice-cold wash buffer. Filters were then dried for 30 min before addition of 30 μl OptiPhase Supermix (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and radioactivity was quantified using a MicroBeta Trilux microplate scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA, USA).

2.4.6. Biochemical profile for the main aclidinium metabolites

The *in vitro* selectivity profile of the main metabolites of aclidinium bromide was assessed in a standard selectivity panel of 85 receptor and enzyme assays including these receptors: A_1, A_{2A}, A_3, α_1 (non-selective), α_2 (non-selective), β_1, β_2, AT_1, AT_2, BZD (central), B_1, B_2, CB_1, CB_2, CCK_A, CCK_B, CRF_1, D1, D2, D3, D4, ET_A, ET_B, GABA (non-selective), AMPA, kainate, NMDA, H_1 (central), H_2, H_3, I_1 (peripheral), I_2 (central), LTB_4, LTD_4, MC_4, M (non-selective), NK_1, NK_2, NK_3, Y (non-selective), N (neuronal),

opiate (non-selective), ORL1, PCP, P2X, P2Y, 5-HT (non-selective), σ (non-selective), glucocorticoid, oestrogen, progesterone, androgen, TRH, V_1, V_2, Ca_2^+ channel (L, DHP site), Ca_2^+ channel (L, diltiazem site), Ca_2^+ channel (L, verapamil site), K^+_{ATP} channel, K^+_V channel, SK^+_{Ca} channel, Na^+ channel (site2), Cl^- channel, NE transporter, DA transporter, GABA transporter, choline transporter, 5-HT transporter. Enzymes included in this study were: PDE1, PDE2, PDE3, PDE4, PDE5, adenylyl cyclase (basal), guanylyl cyclase (basal), PKC, COMT, GABA transaminase, glutamate decarboxilase, MAO-A, MAO-B, phenylethanolamine *N*-methyl transferase, tyrosine hydroxylase, ATPase (Na^+/K^+) and acetylcholinesterase.

The effects of the acid and alcohol metabolites of aclidinium (10 μM each) on the assay signal (specific binding or enzyme activity) were determined and tested in duplicate and the percentage inhibition calculated. A reference compound appropriate for each individual assay was also tested in duplicate at a minimum of seven different concentrations. The IC_{50} values obtained from the dose–response curves were validated by comparison with those in the literature. Assays were performed by CEREP (Celle L'Evescault, France).

2.4.7. *In vivo effects on acetylcholine inducing bronchoconstriction in anaesthetised guinea pigs*

Guinea pigs were anaesthetised with an intraperitoneal injection of 1 g/kg urethane and 20 mg/kg sodium pentobarbital. Additional anaesthetic was administered after 60 min as required. The trachea was cannulated and the lungs artificially ventilated with a small rodent ventilator (Ugo Basile, Biological Research Apparatus, Comerio-Varese, Italy) at a rate of 60 strokes/min and a tidal volume of 10 ml/kg. Animals were maintained at 37 °C with a homeothermic blanket throughout the experiment.

Blood pressure was measured in a cannulated carotid artery and acetylcholine was administered via a cannulated jugular vein. Intrapulmonary pressure and blood pressure were measured by blood pressure transducers (MLT0699, ADInstruments-Panlab, Barcelona, Spain) connected to a bridge amplifier (PowerLab/8sp, ADInstruments-Panlab, Barcelona, Spain). The data were recorded using Chart 5 software (ADInstruments-Panlab, Barcelona, Spain).

After induction of anaesthesia and preparation, animals were allowed to stabilise for 10 min before bronchoconstriction was induced by an intravenous (i.v.) bolus of acetylcholine. Acetylcholine was administered at a dose (10–60 μg/kg) that approximately doubled the basal intrapulmonary pressure. Repeated bolus injections of acetylcholine at the selected dose were administered until two reproducible responses were obtained; the mean of the two final responses before the addition of the antagonists corresponded to the baseline response to acetylcholine and was used to evaluate the antibronchoconstrictor effect of the antagonists.

Five minutes after the last administration of acetylcholine used to calculate maximal bronchoconstriction, the test antagonist was administered via a nebuliser (5 s duration; Mumed ultrasonic nebuliser, Mumed Systems Ltd., London, UK). Compounds were delivered at a concentration of 1 mg/ml. Acetylcholine doses were then administered 5, 10, 15, 20, 25, 30, 60 and 120 min after the administration of the compounds to evaluate their antibronchoconstrictor effects. The effect was expressed as a percentage of the baseline response to acetylcholine. Data were analysed using Microsoft Excel software.

2.5. *Data analysis*

In plasma stability studies, the percentage of the remaining unaltered compound (the sample concentration at time 0 was determined as 100%) was calculated for each time point. The constant k and the half-life ($t_{1/2} = -0.693/k$) were calculated from the slope of the linear regression line in the elimination phase of the semi logarithmic plot using WinNonlin software (version 5.0.1, Pharsight Corp., USA).

Affinity at equilibrium for muscarinic receptors subtypes was determined as IC_{50} for aclidinium. IC_{50} values were obtained from at least three independent curves of 10 antagonist concentrations run in duplicate. Affinities of LAS34823 and LAS34850 were expressed as % inhibition at the maximum concentration tested (10 μM). All adjustments were performed using Prism (GraphPad software, San Diego, CA, USA).

Table 1
Apparent half-lives of aclidinium, ipratropium and tiotropium in Wistar rat, Dunkin Hartley guinea pig, Beagle dog and human plasma (37 °C). Mean values ($n = 3$).

Compound	Half-life (h)			
	Rat	Guinea pig	Dog	Human
Aclidinium[a]	0.19 (11.7)	0.64 (38.3)	0.03 (1.8)	0.04 (2.4)
Tiotropium	1.2	1.9	1.2	1.6
Ipratropium	>6	>6	>6	>6

[a] In brackets, half-lives are expressed in minutes.

3. Results

3.1. *In vitro plasma stability*

The comparative hydrolytic cleavage of the ester compounds aclidinium, tiotropium and ipratropium in plasma samples from different species was evaluated at 37 °C. The plasma hydrolytic cleavage of aclidinium, tiotropium and ipratropium exhibited a mono-exponential decline in all conditions evaluated. Aclidinium was hydrolysed in plasma samples from all species studied, with apparent half-lives at 37 °C of 11.7, 38.3, 1.8 and 2.4 min in rat, guinea pig, dog and human plasma, respectively (Table 1, Fig. 3). In contrast, tiotropium and ipratropium were relatively resistant to ester hydrolysis in plasma. The plasma hydrolysis of tiotropium was similar in all species with an apparent plasma half-life between 1 and 2 h. No significant hydrolysis of ipratropium was observed in plasma at 37 °C for up to 6 h of incubation in all species evaluated.

3.2. *Identification of metabolites*

Hydrolysis of the ester compound aclidinium bromide occurred in aqueous solutions and plasma samples to yield the carboxylic acid derivative (LAS34850) and alcohol derivative (LAS34823). The identification of these two metabolites was confirmed by comparing the MS and MS/MS spectra with those of the authentic standards. The MS and the MS/MS spectra of both metabolites are shown in Fig. 4. LAS34823 yielded a molecular ion of m/z 262 in positive mode. The main fragments in the product ion mass

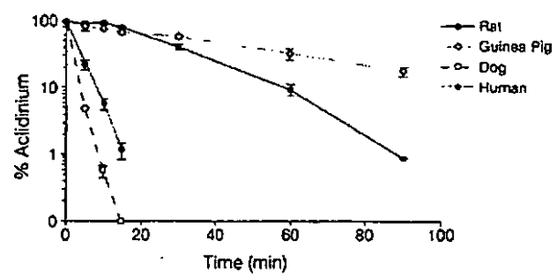

Fig. 3. Rat, guinea pig, dog and human plasma stability time course of aclidinium bromide (semi-logarithmic plot). Mean values ± SD ($n = 3$).

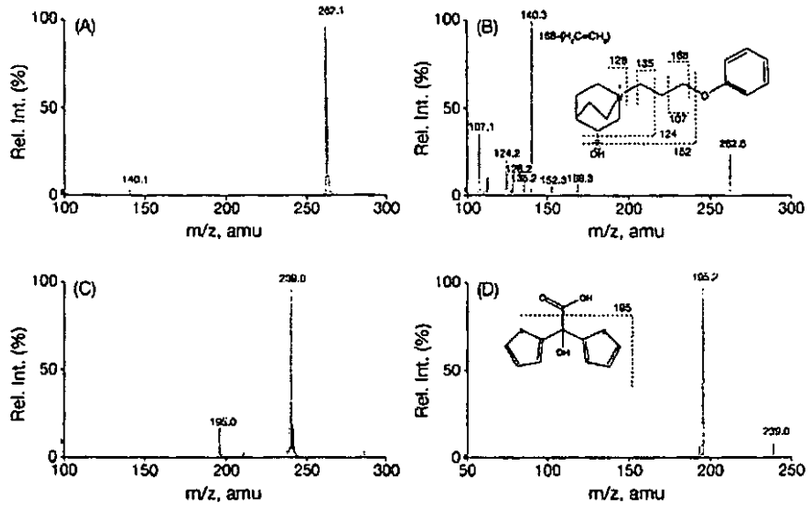

Fig. 4. MS and MS/MS spectra of the hydrolysis metabolites of aclidinium bromide: (A) (+)-ESI-MS spectrum of LAS34823 (alcohol metabolite); (B) (+)-ESI-MS/MS spectrum of LAS34823 (alcohol metabolite); (C) (−)-ESI-MS spectrum of LAS34850 (acid metabolite); (D) (−)-ESI-MS/MS spectrum of LAS34850 (acid metabolite).

spectrum were at *m/z* 168, 140, 135, 128, 124 and 107, which were consistent with the proposed structure. LAS34850 showed, in negative mode, a molecular ion at *m/z* 239 and a prominent fragment ion at *m/z* 195 in the MS and MS/MS spectra, respectively.

3.3. Non-enzymatic hydrolysis of aclidinium

The stability of aclidinium (50 μM) in 50 mM phosphate buffer was evaluated *in vitro* at different pHs at 37 °C. At basic and neutral pH, the concentration of compound decreased exponentially with time, indicating that the hydrolysis followed first order kinetics. The hydrolysis rate increased with pH, with a hydrolysis half-life of 1.2 h at physiological pH 7.4 (Fig. 5). The compound was essentially stable at pH ≤4.

3.4. Affinity studies for human muscarinic receptor subtypes, M_1 to M_5 at equilibrium

The affinity of the two aclidinium main metabolites for human muscarinic receptors was assessed in a radioligand binding displacement assay using ^{3}H-NMS and membranes of CHO-K1 cells expressing the human M_1 to M_5 receptor subtypes.

LAS34823 (alcohol metabolite) and LAS34850 (acid metabolite) at concentrations up to 10 μM, were devoid of any significant affinity for any of the muscarinic subtypes studied. In contrast, aclidinium, potently blocked the specific binding of ^{3}H-NMS to human M_1 to M_5 receptors in a concentration-dependent manner (Table 2).

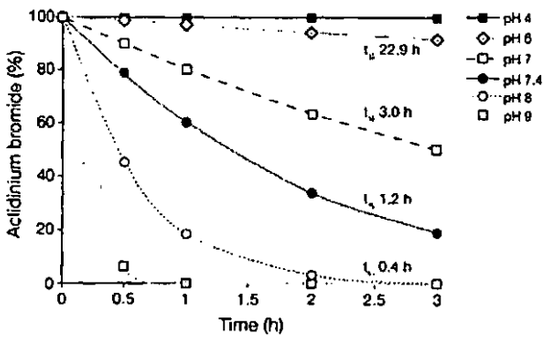

Fig. 5. Aclidinium stability and apparent half-lives ($t_{1/2}$) in 50 mM phosphate buffer (37 °C) at different pH values. Aclidinium bromide was also stable at pH 2.0 (results not shown). Each experimental point represents the mean of duplicate samples from independent experiments.

3.5. Selectivity profile

Neither of the two aclidinium main metabolites had any relevant affinity for the receptors or enzymes represented in the standard selectivity panel described in the methods section (data not shown). Maximal effect of aclidinium metabolites at all the receptors, enzymes, ion channels and transporters was below 50% at the 10 μM concentration tested. This implies a separation with respect to the activity of aclidinium for muscarinic receptors of more than 10,000-fold. It is therefore considered to be highly unlikely that the metabolites described could exert any relevant pharmacological activity through the panel of targets evaluated.

3.6. In vivo effects on the acetylcholine induced bronchoconstriction in anaesthetised guinea pigs

To evaluate the potential *in vivo* bronchoprotective effect of inhaled aclidinium and its main metabolites, 1000 μg/ml of each compound was administered to different groups of anaesthetised guinea pigs prior to acetylcholine-induced bronchoconstriction. Aclidinium produced a potent and sustained bronchoprotection (72.0–88.4%) over the 120-min study period (Fig. 6). In contrast, neither LAS34823 nor LAS34850 reversed the acetylcholine-induced bronchoconstriction to any relevant extent.

4. Discussion

Aclidinium bromide is a novel, long-acting, inhaled muscarinic antagonist currently in development for the maintenance treatment of COPD. It was selected from a series of quaternised (3*R*)-quinuclidinol esters as a potent muscarinic M_3 antagonist,

Table 2
Affinity of aclidinium and its main metabolites LAS34823 and LAS34850 for human M1, M2, M3, M4 and M5 receptors.

	M_1	M_2	M_3	M_4	M_5
Aclidinium IC_{50} (nM)	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.4 ± 0.0
LAS34823 (% at 10 μM)	−0.2 ± 2.0	10.6 ± 3.4	2.6 ± 1.7	5.6 ± 3.0	6.0 ± 3.2
LAS34850 (% at 10 μM)	9.3 ± 2.8	−0.4 ± 1.4	1.8 ± 1.8	9.2 ± 3.6	3.2 ± 2.4

Affinities for aclidinium bromide are reported as IC_{50} (nM) values for the displacement of ^{3}H-NMS from the corresponding receptor preparations. In the case of LAS34823 and LAS34850, the residual affinity is expressed as maximal percentage of binding displacement at 10 μM. Data are expressed as mean ± standard error of three independent experiments.

with long duration of action as a bronchodilator in guinea pigs and rapid human plasma hydrolysis (Prat et al., 2009).

This study characterised the *in vitro* biological and chemical stability of aclidinium bromide, and the *in vitro* and *in vivo* pharmacological profile of its two main metabolites. Plasma hydrolysis of aclidinium bromide was studied in samples from rat, guinea pig, dog and humans. Aclidinium was rapidly hydrolysed in dog and human plasma ($t_{1/2}$ <5 min) but slightly more stable in rat ($t_{1/2}$ = 11.7 min) and guinea pig ($t_{1/2}$ = 38.3 min). A similar result for aclidinium hydrolysis in human plasma was obtained in a previous study (Prat et al., 2009). These results suggest a rapid hydrolytic cleavage of the ester moiety of aclidinium bromide once it reaches systemic circulation, especially in dogs and humans. The contribution of enzymatic hydrolysis would be much more important in dog and human plasma compared with rat and guinea pig plasma.

Additionally, aclidinium showed non-enzymatic hydrolysis of its ester bond at neutral and basic pH. Acid-catalysed hydrolysis was not observed below pH 4 and changes in pH near 7.4 caused an important change in the rate of hydrolysis, confirming that aclidinium hydrolysis is very sensitive to pH in the physiological range. The marked difference in the rate of aclidinium hydrolysis observed in plasma across the species ($t_{1/2}$ = 0.03–0.64 h) and in phosphate buffer at pH 7.4 ($t_{1/2}$ = 1.2 h) suggests that enzymatic hydrolysis plays a major role in the overall hydrolysis of aclidinium. Under the experimental conditions used in this study, the tiotropium plasma half-lives were higher than that of aclidinium and were similar in all species evaluated (1–2 h).

In healthy subjects, pharmacokinetic studies of aclidinium up to 6000 μg by the inhaled route have shown rapid elimination from systemic circulation ($t_{1/2}$ <1.5 h), consistent with a rapid *in vivo* plasma hydrolysis of aclidinium (Ferrer et al., 2008; Jansat et al., 2009). Biological and chemical hydrolysis of the ester bond of aclidinium *in vitro* results in two major compounds, the alcohol (LAS34823) and the carboxylic acid (LAS34850) metabolites. Both metabolites have also been detected in human plasma after an inhaled dose of aclidinium (400 μg and 800 μg), likely as a result of a rapid hydrolysis in plasma, where the major circulating compound was the acid derivative. The systemic exposure of the acid

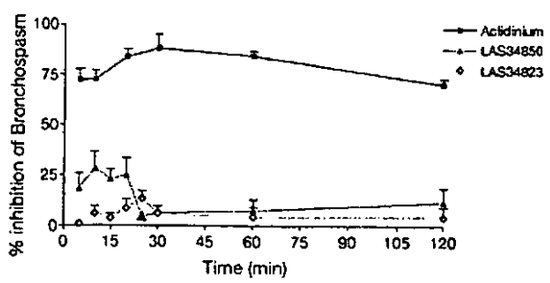

Fig. 6. Effect of aerosolised aclidinium and its metabolites on the acetylcholine-induced bronchoconstriction in anaesthetised guinea pigs. Reversal of bronchospasm values are represented as mean ± SEM. Data are calculated from an average of three or four animals.

metabolite was at least 100-fold higher than aclidinium (Jansat et al., 2009). In contrast, the systemic exposure of the alcohol metabolite was much lower and, between 2- and 5-fold that of aclidinium. The relatively high plasma concentration of the acid metabolite is mainly due to differences in oral absorption, which is higher with the acid metabolite than with aclidinium and the alcohol metabolite (Jansat et al., 2009). In addition, other minor oxidative metabolites of aclidinium have been observed *in vitro* and *in vivo* in animal species and humans (data not shown).

Systemic exposure of anti-cholinergics has been associated with unwanted physiological effects such as tachycardia, dry mouth, urinary retention and constipation (Lieberman III, 2004). Aclidinium undergoes rapid hydrolysis in plasma, resulting in low and transient systemic exposure to the active drug. The *in vitro* and *in vivo* characterisation of both metabolites was performed to determine the potential pharmacological contribution of these metabolites. In radioligand displacement studies carried out at equilibrium using ^{3}H-NMS, aclidinium demonstrated high subnanomolar affinity for all human muscarinic receptor subtypes analysed. In contrast, the two major metabolites showed no significant affinity for human muscarinic receptors up to 10 μM, with displacement being lower than 10% in all cases. The data obtained for aclidinium are consistent with those previously described (Gavaldà et al., 2009).

The *in vitro* pharmacological selectivity profile studies were performed with both aclidinium metabolites. The alcohol (LAS34823) and carboxylic acid metabolites (LAS34850) have no relevant affinity for any of the receptors and enzymes tested. This, together with the high selectivity for the muscarinic receptors reported previously for aclidinium (Gavaldà et al., 2009) and its low systemic exposure, suggests that aclidinium and its main metabolites may have reduced potential in triggering unwanted anti-cholinergic side effects. This is supported by the results from the safety studies of aclidinium in rats, guinea pigs and dogs, where aclidinium bromide has demonstrated reduced effects on the cardiovascular, gastric and renal systems compared with tiotropium and ipratropium (Gras et al., 2008a,b; Montero et al., 2008). Additionally, inhaled aclidinium in healthy subjects receiving up to 6000 μg also was shown to have a favourable safety and tolerability profile (Ferrer et al., 2008; Jansat et al., 2009).

The effect of LAS34823 and LAS34850 on the acetylcholine-induced bronchoconstriction model in guinea pigs was also assessed in comparison with aclidinium bromide. The two main aclidinium metabolites did not show any relevant bronchodilatory activity *in vivo*. In contrast, a potent bronchodilatory activity was observed after inhaled administration of aclidinium, in concordance with results reported previously (Gavaldà et al., 2009). These data demonstrate that the activity observed *in vivo* corresponds exclusively to aclidinium bromide without contribution from its hydrolysis products.

In summary, the data reported here show that aclidinium is rapidly hydrolysed in plasma, likely due to the enzymatic cleavage of its ester, to yield its alcohol and carboxylic acid derivatives in all species studied. These main metabolites of aclidinium are devoid of any significant affinity for the muscarinic receptors, or a wide panel of selected G-protein coupled receptors, ion channels or

enzymes studied, and did not show any relevant bronchodilatory activity in vivo. The rapid cleavage to inactive metabolites and the low systemic exposure of aclidinium, together with its long residence time at the muscarinic M_3 receptor, confer this molecule a unique mechanism of action profile that supports a favourable safety profile and benefit-to-risk ratio in the clinical practice.

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